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ABOUT COVER

AIMS AND SCOPE

The primary aim of *World Journal of Stem Cells* (*WJSC*, *World J Stem Cells*) is to provide scholars and readers from various fields of stem cells with a platform to publish high-quality basic and clinical research articles and communicate their research findings online. *WJSC* publishes articles reporting research results obtained in the field of stem cell biology and regenerative medicine, related to the wide range of stem cells including embryonic stem cells, germline stem cells, tissue-specific stem cells, adult stem cells, mesenchymal stromal cells, induced pluripotent stem cells, embryonal carcinoma stem cells, hemangioblasts, lymphoid progenitor cells, *etc*.

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ORIGINAL ARTICLE

Basic Study

RPLP0/TBP are the most stable reference genes for human dental pulp stem cells under osteogenic differentiation

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Abstract

BACKGROUND

Validation of the reference gene (RG) stability during experimental analyses is essential for correct quantitative real-time polymerase chain reaction (RT-qPCR) data normalisation. Commonly, in an unreliable way, several studies use genes involved in essential cellular functions [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S rRNA, and β-actin] without paying attention to whether they are suitable for such experimental conditions or the reason for choosing such genes. Furthermore, such studies use only one gene when Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines recommend two or more genes. It impacts the credibility of these studies and causes distortions in the gene expression findings. For tissue engineering, the accuracy of gene expression drives the best experimental or therapeutical approaches.

AIM

To verify the most stable RG during osteogenic differentiation of human dental pulp stem cells (DPSCs) by RT-qPCR.

METHODS

We cultivated DPSCs under two conditions: Undifferentiated and osteogenic differentiation, both for 35 d. We evaluated the gene expression of 10 candidates for RGs [ribosomal protein, large, P0 (*RPLP0*), TATA-binding protein (*TBP*), *GAPDH*, actin beta (*ACTB*), tubulin (*TUB*), aminolevulinic acid synthase 1 (*ALAS1*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), eukaryotic translational elongation factor 1 alpha (*EF1a*), succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*), and beta-2-microglobulin (*B2M*)] every 7 d (1, 7, 14, 21, 28, and 35 d) by RT-qPCR. The data were analysed by the four main algorithms, ΔCt method, geNorm, NormFinder, and

BestKeeper and ranked by the RefFinder method. We subdivided the samples into eight subgroups.

RESULTS

All of the data sets from clonogenic and osteogenic samples were analysed using the RefFinder algorithm. The final ranking showed RPLP0/TBP as the two most stable RGs and TUB/B2M as the two least stable RGs. Either the ΔCt method or NormFinder analysis showed TBP/RPLP0 as the two most stable genes. However, geNorm analysis showed RPLP0/EF1α in the first place. These algorithms' two least stable RGs were B2M/GAPDH. For BestKeeper, ALAS1 was ranked as the most stable RG, and SDHA as the least stable RG. The pair RPLP0/TBP was detected in most subgroups as the most stable RGs, following the RefFinfer ranking.

CONCLUSION

For the first time, we show that RPLP0/TBP are the most stable RGs, whereas TUB/B2M are unstable RGs for long-term osteogenic differentiation of human DPSCs in traditional monolayers.

Key Words: Dental pulp stem cells; Reference gene; Housekeeping gene; Endogenous gene; Osteogenic differentiation; RefFinder

Core Tip: Detecting the best reference genes (RGs) under specific conditions is a good practice to improve the understanding of gene expression. Stem cells have been largely studied during commitment to particular cell lineages for many applications, such as tissue engineering. In this way, dental pulp stem cells (DPSCs) are promising for craniofacial reconstruction. For the first time, we show that the best pair of RGs for the osteogenic differentiation of human DPSCs are ribosomal protein, large, P0/TATA-binding protein by quantitative real-time polymerase chain reaction through the four algorithms (ΔCt comparative method, geNorm, BestKeeper, and NormFinder) and ranked by RefFinder.

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INTRODUCTION

Quantitative real-time polymerase chain reaction (RT-qPCR) is a powerful messenger RNA expression analysis technique. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline describes good practices for publishing reliable gene expression data. One critical step is choosing the best reference gene (RG) (widely used as a housekeeping, control, or endogenous gene) for data normalisation. This gene or a set of genes must have a constitutive and stable expression during the experimental conditions evaluated, abundant levels, and be involved in essential cellular functions. However, cell type and metabolic status significantly influence gene stability, and different candidates can arise. Using a non-ideal RG leads to misinterpretations of the results, and consequently, incorrect scientific information[1].

A systematic review highlighted that between 2010 and 2015, the most used RGs were actin beta (*ACTB*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), even though only 15% of the papers have shown evidence that they have checked the stability condition of the genes used[2]. Currently, these genes are still the most used, and in particular, GAPDH has established itself in many fields as the standard gene used for normalisation. It is essential to highlight that there is no absolute RG, and it is always necessary to test the stability of these genes among all tested conditions to choose the gene with the best normalising potential. The stability can be evaluated by algorithms named ΔCt comparative method[3], geNorm[4], NormFinder[5], and BestKeeper[6]. These algorithms are accessible through the web-based RefFinder, an initiative that calculates these four algorithms and has its ranking method[7].

Stem cells from many sources have been studied extensively for regenerative medicine approaches. In tissue bone engineering, it is suitable for stem cells to be committed to the osteoblastic lineage to create a new bone. Among them, mesenchymal stem cells (MSCs) are largely investigated because they can be found in all tissues and organs, have many pro-regenerative properties (differentiation multipotential, immunomodulation, secretion of trophic factors, *etc.*), but can display different potentials for osteoblastic differentiation, according to their embryonic/tissue origin. MSC populations from dental tissues have the same embryonic origin as craniofacial bones, making them promising candidates for craniofacial reconstruction[8]. Dental pulp stem cells (DPSCs) were first isolated and characterised by Gronthos *et al*[9]. DPSCs are obtained in a non-invasive way from the permanent tooth and have a high proliferative rate, immunomodulatory properties, and multilineage differentiation capacity, especially for osteogenic ones. Together, they are an attractive source of cells for Bone Tissue Engineering and Regenerative Medicine applications[10].

However, few studies have addressed the most stable RG during osteogenic differentiation and no one in DPSCs. Thus, our study aimed to verify the most stable RG during osteogenic differentiation of human DPSCs by RT-qPCR. To validate, we chose the ten most used RG found during osteogenic differentiation in other MSCs [ribosomal protein, large, P0 (*RPLP0*), TATA-binding protein (*TBP*), *GAPDH*, ACTB, tubulin (*TUB*), aminolevulinic acid synthase 1 (*ALAS1*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), eukaryotic translational elongation factor 1 alpha (*EF1a*), succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*), and beta-2-microglobulin (*B2M*)] through the most common and reliable methods (algorithms) ΔCt comparative method, geNorm, BestKeeper, and NormFinder and ranked by RefFinder.

MATERIALS AND METHODS

DPSC isolation and expansion

Three human third molar teeth were extracted from 15- to 23-year-old healthy donors (University Hospital, University of São Paulo, Brazil). The analysis will designate them as donors #1, #2, and #3. Informed consent was obtained from donors (approval from the Human Ethics Committee - CAAE: 51097315.7.0000.5467 and CAAE: 51097315.7.3001.0076). An incision on the enamel-dentin junction was made, and the dental pulps were harvested. Then they were mechanically and enzymatically disaggregated (collagenase type I - 6 mg/mL and dispase - 8 mg/mL for 1 h at 37 °C), and single cells were obtained by filtration through a 70-μm mesh filter. Cells were seeded in T25 flasks and maintained in a clonogenic medium (α-MEM supplement with 2 mM glutamine + 10% BFS + 50 μg/mL ascorbic acid + 100 U/mL ampicillin + 100 + 100 μg/mL streptomycin). Cells were trypsinized when they reached 80%-90% subconfluence. The medium was refreshed every 2-3 d, and cells were incubated under a humidity atmosphere at 37 °C and 5% CO_2.

DPSC differentiation in vitro

Undifferentiated DPSCs (#4) were seeded (5000 cells/cm^2) in P35 dishes and induced for osteogenic (α-MEM supplement with 2 mM glutamine + 10% BFS + 50 μg/mL ascorbic acid + 1 μM dexamethasone + 10 mM β-glycerophosphate + 100 U/mL ampicillin + 100 μg/mL streptomycin) differentiation for 1, 7, 14, 21, 28, and 35 d. The medium was refreshed every 2-3 d, and cells were incubated under a humidity atmosphere at 37 °C and 5% CO_2. The validation of osteogenic differentiation was performed by alizarin red staining.

Total RNA extraction, cDNA synthesis, and RT-qPCR

Cells were seeded on P100 plates (5000 cells/cm^2). Samples were collected and lysed in 1 mL TRIzol reagent (15596-026; Life Technologies, Carlsbad, CA, United States), following the manufacturer's protocol. The purified total RNA was resuspended in 50 μL DEPC water. An aliquot of each sample was quantified in Nanodrop (2000c Spectrophotometer; Thermo Fisher Scientific, Waltham, MA, United States), and only RNA with an optical density $A_{260/280}$ ratio between 1.9 and 2.1 was used for RT-qPCR analysis. The purified mRNA was stored at -80 °C until further use. Then total RNA (1 μg or 500 ng) was treated with 1 μL DNAse I (18068-015; Invitrogen), 1 μL buffer, and DEPC H_2O to a final volume of 10 μL. It was left for 15 min at room temperature and 10 min at 65 °C. The 1 μL EDTA (25 mM) was added to inactivate the enzyme. Subsequently, complementary DNAs were synthesised by RT-PCR (18080-093; SuperScript™ III Reverse Transcriptase, Invitrogen). RT-PCR was performed in two steps: (1) Alignment (65 °C for 5 min): For a final volume of 13 μL, 11 μL RNA treated with DNase I, 1 μL oligo(dT) (18418-012; Invitrogen) and 1 μL of 10 mM dNTP (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 2.5 mM dTTP) (100 mM dNTP Set, PCR grade, 10297-018; Invitrogen); and (2) Reverse transcription (50 °C for 60 min and 70 °C for 15 min): After alignment, 4 μL of 5X Buffer, 1 μL DTT (0.1 M), 1 μL RNaseOUTTM (40 U/μL) (10777-019; Invitrogen) and 1 μL SuperScript III (200 U/μL), for a final volume of 20 μL. At the end of the second stage, the cDNA was obtained.

The synthesised cDNA was the template used for the reaction using the SYBR Green Dye I method and the evaluation of relative gene expression using Pfaffl[11]. Samples in osteogenic or clonogenic media in 1 d were used as calibrator samples. Candidate RGs and Runt-related transcription factor 2 (RUNX2) were used for osteogenic differentiation validation, which is described in Table 1. Samples synthesised from 1 μg RNA were diluted 1:1, and samples synthesised from 500 ng RNA were used neat. Reactions were performed in a total volume of 10 μL, containing 1 μL of the sample, 10 pM of each primer (400 nM), 5 μL SYBR Green Master Mix® (Applied Biosystems, Waltham, MA, United States) and water q.s.p. The reactions were performed on Applied Biosystems equipment (7500; Real-Time PCR System) at 60 °C in 40 cycles.

Evaluation of gene stability - algorithms

BestKeeper: BestKeeper algorithm performs for each candidate gene data analysis; the results are interpreted mainly by evaluating standard deviation (SD) coefficient of variation (CV) values and Pearson's correlation coefficient - the smaller the SD and CV, the more stable the gene. Typically, genes are considered stable with an SD < 1. For Pearson's correlation test, the higher the correlation value, the better, and $P < 0.05$. RefFinder, however, only uses SD for its ranking. It differs from other algorithms because it considers the intragene variation, not only intergene.

GeNorm: GeNorm considers that since a set of potentially stable genes was already selected, the Ct variation throughout the conditions must be similar for most genes, so it determines the two most stable genes that share a similar expression profile throughout all samples. It does so in a pairwise variation system where it calculates and compares the stability for all possible gene pair combinations, thus obtaining the gene stability value (*M*), defined as the arithmetic mean of the

Table 1 Candidate reference genes for quantitative real-time polymerase chain reaction

Gene symbol	Function	NCBI access	Primers sequences (5'-3')	Temperature (°C)
RPLP0	Component of the 60S subunit	NM_001002	F: AGCCCAGAACACTGGTCTC	60
			R: ACTCAGGATTTCAATGGTGCC	
TBP	Transcription, metabolic pathways	NM_003194.5	F: CACGAACCACGGCACTGATT	62
			R: TTTTCTTGCTGCCAGTCTGGA	
YWHAZ	Signal transduction	NM_001135702	F: TGATCCCCAATGCTTCACAAG	61
			R: GCCAAGTAACGGTAGTAATCTCC	
RUNX2	Master transcription factor for osteogenesis	NM_001015051	F: TGGTTACTGTCATGGCGGGTA	62
			R: TCTCAGATCGTTGAACCTTGCTA	
EF1α	Translation	NM_001402	F: GAAGCTGGTATCTCCAAGAATGG	61
			R: CGACAATTAGTTGTTTCACACCC	
GAPDH	Mitochondrial metabolism	NM_002046	F: GCATCCTGGGCTACACTGA	60
			R: CCACCACCCTGTTGCTGTA	
ACTB	Cytoskeleton	NM_001101	F: CGACAGGATGCAGAAGGAG	60
			R: TCCTGCTTGCTGATCCACAT	
B2M	Immune response	NM_004048	F: GAGGCTATCCAGCGTACTCCA	62
			R: CGGCAGGCATACTCATCTTTT	
ALAS1	Mitochondrial metabolism	NM_199166	F: AAATGAATGCCGTGAGGAAAGA	60
			R: CCCTCCATCGGTTTTCACACTA	
TUB	Cytoskeleton	NM_001293212	F: TCAACACCTTCTTCAGTGAAACG	60
			R: AGTGCCAGTGCGAACTTCATC	
SDHA	Mitochondrial metabolism	NM_004168	F: CAAACAGGAACCCGAGGTTTT	60
			R: CAGCTTGGTAACACATGCTGTAT	

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NCBI: National Cancer for Biotechnology Information: RPLP0: Ribosomal protein, large, P0; RUNX2: Runt-related transcription factor 2; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

standard deviations of a gene. The smaller, the more stable. The geNorm performs several "cycles" of calculating *M*, always excluding the least stable one and recalculating it with the remaining data, thus finding the most stable pair. In the final result, as a criterion (threshold), only genes with M less than 1.5 are considered stable for this algorithm. In addition, we must calculate the value of V_n/V_{n+1} when its value is less than 0.15, using the *n* RGs with the lowest *M* value; the contribution of 1 or more genes will not significantly improve the data normalisation. We run geNorm using the RefFinder value and R ctrlGene library package version 1.0.1 (https://rdrr.io/cran/ctrlGene/) for V_n/V_{n+1} calculation.

NormFinder: It uses a pairwise variation system similar to the geNorm; its differential analyses are of intra- and inter-group variation. In this way, it is not as significantly influenced by mutually co-regulated genes. The lower its value, the more stable the genes. Furthermore, the criterion (threshold) is the same as geNorm; only genes with *M* less than 1.5 are considered stable for the algorithm. The number of samples affects the calculation and is more accurate as n increases ($n \geq 8$).

ΔCt comparative method: It is based on mathematical principles similar to the pairwise comparison of the NormFinder and geNorm, with a more accessible calculation for researchers less articulated in mathematics. To do so, it performs the ΔCt method for each pair of genes within each sample, then evaluates the ΔCt mean for each pair and between every two groups. RGs are ranked by their associated arithmetic mean of standard deviation values, as the smaller the number, the more stable the gene. We will consider the two best-ranked genes as the best RG pair. This method bypasses the need to quantify the RNA, using ΔCt comparisons between genes accurately.

RefFinder: It is a user-friendly web-based tool containing the four main statistical gene stability algorithms (geNorm, NormFinder, BestKeeper, and the comparative ΔCt method). After calculation, it addresses a weight for each gene by their ranking position in each algorithm and calculates the geometric mean of their weights for the comprehensive final

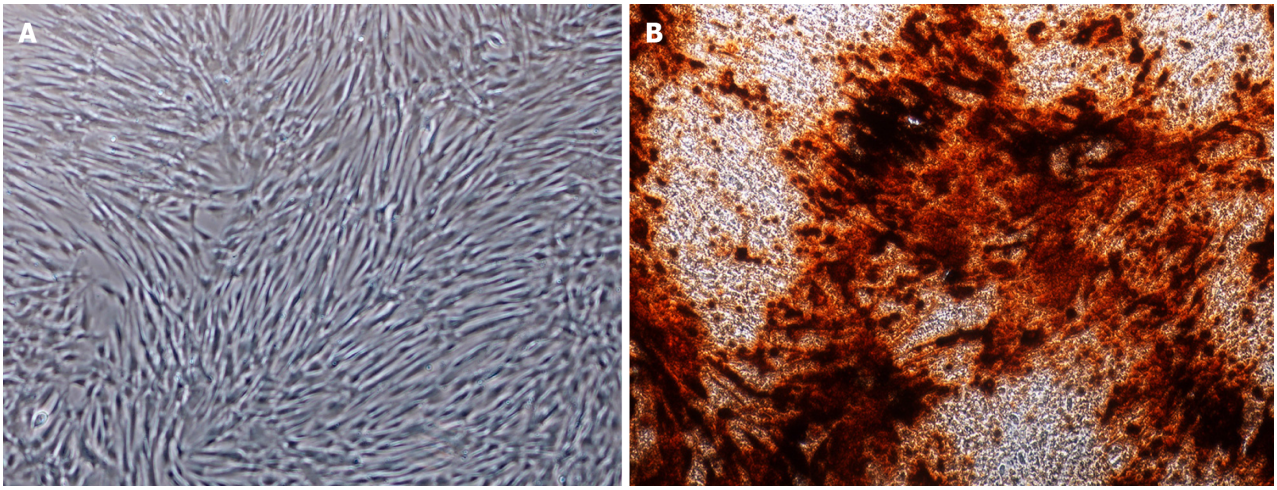

Figure 1 Isolated dental pulp stem cells and validation of osteogenic differentiation by alizarin red staining.

ranking. To date, it is available at https://blooge.cn/RefFinder/. A table for each group was made, with each column representing a gene and each row the original Ct value (not Ct mean) from a sample's qRT-PCR. All three patients' data were on all tables; thus, we calculated simultaneously.

Subsets: To better identify endogenous genes in the initial and final phases of differentiation, we divided the data into the early phase (from 1 to 21 d) and late phase (from 21 to 35 d) after induction of osteogenic differentiation.

RESULTS

DPSC validation of differentiation in vitro

The DPSCs display a fibroblast-like morphology (Figure 1A) and differentiate into osteoblast-like cells under osteogenic induction, confirmed by the alizarin red staining (Figure 1B).

Gene stability - overall analysis

All of the data sets from clonogenic and osteogenic samples were analysed by the RefFinder algorithm; since V_n/V_{n+1} were lower than 1.5, we will consider the top two ranked genes. The final ranking showed RPLP0/TBP as the two most stable RGs and TUB/B2M as the two least stable RGs (Figure 2A).

For the ΔCt method, we will consider the top two ranked genes. NormFinder and geNorm, the best RGs were those with stability values less than 1.5. Either ΔCt method and NormFinder analysis showed TBP/RPLP0 as the two most stable genes. However, geNorm analysis showed RPLP0/EF1α in the first place. These algorithms' two least stable RG were B2M/GAPDH (Table 2). For BestKeeper, the best-ranked RGs must be SD < 1. ALAS1 was ranked as the most stable RG, and SDHA as the least stable RG.

Gene stability - Subsets 1 (clonogenic medium under 35 d) and 2 (osteogenic medium under 35 d)

We analysed all samples from each group separately to identify differences between RG stability between clonogenic and osteogenic groups for long-time cultivation. By RefFinder ranking, TBP/RPLP0 were the most stable for Subset 1 and RPLP0/TBP for Subset 2, respectively. The least stable RG for Subset 1 were TUB/SDHA and GAPDH/B2M for Subset 2 (Figure 2B and C).

In both subsets, the two most stable genes by ΔCt method and NormFinder were equal to those by RefFinder (TBP/RPLP0). However, other genes demonstrated stability values less than 1.5 in NormFinder, such as YWAHZ (Subset 1) and EF1α/ACTB (Subset 2). For geNorm, while TBP/YWAHZ/B2M/SDHA are the most stable genes in Subset 1, the RPL0/EF1α/TBP were considered the best for Subset 2. Bestkeeper showed ALAS1 as the most stable in both subsets and SDHA and B2M as the least stable genes, respectively (Table 3).

Gene stability - Subsets 3 (clonogenic and osteogenic media from 1 to 21 d) and 4 (clonogenic and osteogenic media from 21 to 35 d)

Here, we analysed the early and late differentiation steps groups, considering clonogenic and osteogenic samples in the same group. By RefFinder ranking, RPLP0/TBP were again the two most stable for Subsets 3 and 4. The least stable RG for Subset 3 were TUB/GAPDH and B2M/GAPDH for Subset 4 (Figure 2D and E).

The most stable genes by ΔCt method were TBP/RPLP0 and TBP/ACTB for Subset 3 and 4, respectively. By NormFinder, it was ranked TBP/RPL0/ALAS1 for Subset 3 and TBP/ACTB/ALAS1/TUB for Subset 4. By geNorm, the RPLP0/EF1α were the most stable in Subset 3 and 4. Bestkeeper showed ALAS1 as the most stable in both Subsets,

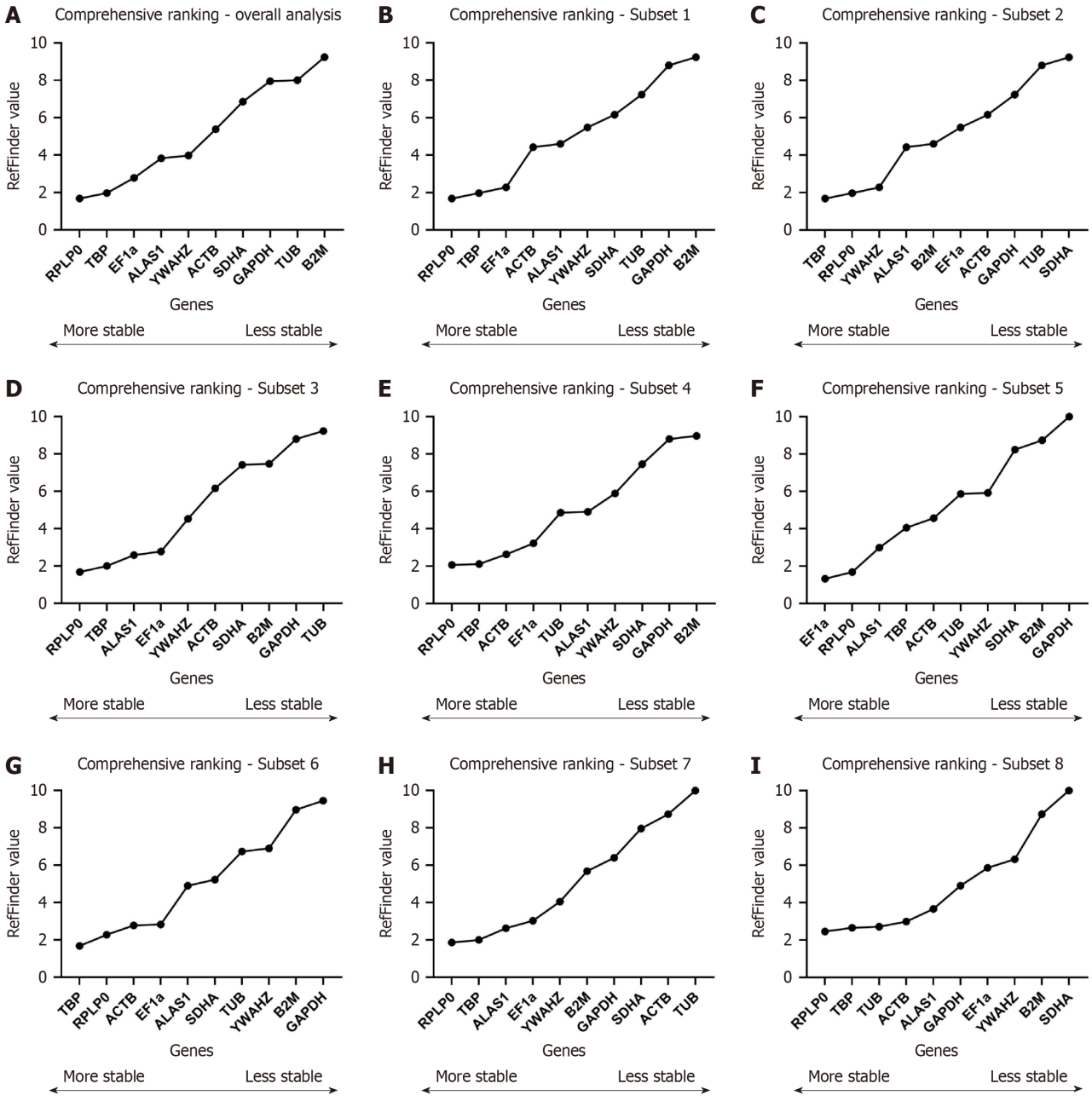

Figure 2 Gene expression stability by comprehensive ranking (RefFinder). A: Overall analysis (all samples); B: Subset 1 (clonogenic medium under 35 d); C: Subset 2 (osteogenic medium under 35 d); D: Subset 3 (clonogenic and osteogenic media from 1 to 21 d); E: Subset 4 (clonogenic and osteogenic media from 21 to 35 d); F: Subset 5 (only osteogenic medium from 1 to 21 d); G: Subset 6 (only osteogenic medium from 21 to 35 d); H: Subset 7 (only clonogenic medium from 1 to 21 d); I: Subset 8 (only clonogenic medium from 21 to 35 d). ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

similar to those found by Subsets 1 and 2, and SDHA and B2M as the least stable genes, respectively (Table 4).

Gene stability - Subsets 5 (only osteogenic medium from 1 to 21 d) and 6 (only osteogenic medium from 21 to 35 d)

We now analysed only early and late osteogenic differentiation step groups under osteogenic differentiation. By RefFinder ranking, the two most stable genes for Subset 5 (the early stages) were EF1α/RPLP0, whereas TBP/RPLP0 for Subset 6 (the late stages). The two least stable RG for Subsets 5 and 6 were GAPDH/B2M (Figure 2F and G).

The most stable genes by ΔCt method were EF1α/RPLP0 for Subset 5 and TBP/ACTB for Subset 6. By NormFinder, the most stable genes were EF1α/RPLP0/TBP/ALAS1 for Subset 5 and TBP/ACTB/RPL0/EF1α/SDHA for Subset 6. By geNorm, the RPLP0/EF1α were the most stable in Subset 5 and RPLP0/EF1α/ACTB/TBP/SDHA for Subset 6. Even though those genes have been ranked differently between Subsets 5 and 6, they coincide. Bestkeeper showed ALAS1 as the most stable in both Subsets, similar to those found by Subsets 1, 2, 3, and 4, and GAPDH and B2M as the least stable genes, respectively (Table 5).

Table 2 Gene stability of overall analysis from clonogenic and osteogenic samples

Main group	Rank	Delta Ct		NormFinder		geNorm		BestKeeper			
		Gene	Stability value	Gene	Stability value	Gene	M value	Gene	Std Dev	R	*P* value
1 to 35 d (clo + ost)	1	TBP	2.13	TBP	1.078	RPLP0/EFα1	0.889	ALAS1	0.70	0.335	0.003
	2	RPLP0	2.27	RPLP0	1.451	-	-	RPLP0	1.33	0.726	0.001
	3	YWAHZ	2.37	YWAHZ	1.62	TBP	1.712	EFα1	1.41	0.668	0.001
	4	EFα1	2.43	ACTB	1.659	YWAHZ	1.923	GAPDH	1.78	0.571	0.001
	5	ACTB	2.43	EFα1	1.707	SDHA	2.037	TBP	1.87	0.857	0.001
	6	ALAS1	2.55	ALAS1	1.84	ALAS1	2.152	ACTB	2.00	0.785	0.001
	7	SDHA	2.57	SDHA	1.948	ACTB	2.261	YWAHZ	2.40	0.876	
	8	TUB	2.68	TUB	2.06	B2M	2.337	TUB	2.41	0.777	
	9	B2M	2.72	B2M	2.122	TUB	2.402	SDHA	2.59	0.847	
	10	GAPDH	2.93	GAPDH	2.385	GAPDH	2.508	B2M	2.63	0.799	0.001

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; clo: Clonogenic; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ost: Osteogenic; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; Std Dev: Standard deviation; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

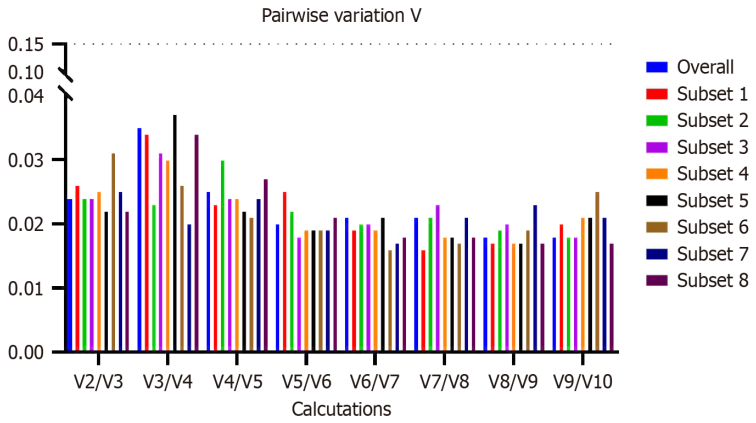

Figure 3 Pairwise variation calculated by geNorm.

Gene stability - Subsets 7 (only clonogenic medium from 1 to 21 d) and 8 (only clonogenic medium from 21 to 35 d)

We now analysed only early and late osteogenic differentiation step groups under clonogenic differentiation. By RefFinder ranking, the two most stable genes were RPLP0/TBP for Subsets 7 and 8. The least stable RG for Subset 5 were ACTB/TUB and B2M/SDHA for Subset 8 (Figure 2H and I).

The most stable genes by ΔCt method were TBP/RPLP0 for both Subsets 7 and 8. By and NormFinder, TBP/RPLP0/YWAHZ/ALAS1/B2M for Subset 7 and TBP/RPLP0/TUB/ACTB for Subset 8. By geNorm, the RPLP0/EF1-α/ALAS1/TBP were the most stable in Subset 7 and ACTB/TUB/RPLP0 for Subset 8. Bestkeeper shows ALAS1/EF1α/RPLP0 and ALAS1 as the most stable genes in Subsets 5 and 6, respectively, and TUB and SDHA as the least stable genes, respectively (Table 6).

Comparing the results obtained for the two most stable genes from the overall ranking with each group, by RefFinder, the pair RPLP0/TBP or TBP/RPLP0 were observed in 7 over 8. By the ΔCt method, the pair RPLP0/TBP or TBP/RPLP0 was detected in 5 over 8. NormFinder detected RPLP0/TBP or TBP/RPLP0 in 7 over 8. By geNorm, RPLP0/EF1α was detected in 6 over 8. Finally, ALAS1 was the most stable gene in all subsets.

Number of genes necessary for normalisation (V_n/V_{n+1})

MIQE guideline highlights that more than one gene is preferable for data normalisation[1]. The geNorm algorithm can evaluate the recommended number of genes for normalisation by V_n/V_{n+1} calculation. This means that the optimal number of RGs is determined by calculating the pairwise variation (V) between a given number of RGs and including an additional gene. A cut-off value of 0.15 has been suggested, where the inclusion of an extra gene has little effect on the

Table 3 Gene stability from Subset 1 (clonogenic medium under 35 d) and Subset 2 (osteogenic medium under 35 d)

Subset	Rank	Delta Ct		NormFinder		geNorm		BestKeeper			
		Gene	Stability value	Gene	Stability value	Gene	M value	Gene	Std Dev	R	*P* value
1: 1 to 35 d (clo)	1	TBP	2.040	TBP	1.024	TBP/YWAHZ	0.996	ALAS1	0.660	0.326	0.043
	2	RPLP0	2.220	RPLP0	1.423	-	-	RPLP0	1.200	0.700	0.001
	3	YWAHZ	2.220	YWAHZ	1.466	B2M	1.319	EFα1	1.250	0.537	0.001
	4	B2M	2.430	B2M	1.790	SDHA	1.449	GAPDH	1.660	0.647	0.001
	5	ACTB	2.460	ACTB	1.818	RPLP0	1.880	TBP	1.900	0.866	0.001
	6	ALAS1	2.480	ALAS1	1.821	EFα1	2.068	ACTB	2.140	0.799	0.001
	7	GAPDH	2.500	GAPDH	1.824	ALAS1	2.159	YWAHZ	2.240	0.850	0.001
	8	EFα1	2.560	EFα1	1.989	GAPDH	2.272	TUB	2.510	0.800	0.001
	9	SDHA	2.620	SDHA	2.099	ACTB	2.356	B2M	2.560	0.855	0.001
	10	TUB	2.680	TUB	2.161	TUB	2.421	SDHA	2.710	0.875	0.001
2: 1 to 35 d (ost)	1	TBP	2.140	TBP	1.087	RPLP0/EFα1	0.515	ALAS1	0.740	0.324	0.044
	2	RPLP0	2.170	RPLP0	1.276	-	-	RPLP0	1.460	0.786	0.001
	3	EFα1	2.180	EFα1	1.291	TBP	1.450	EFα1	1.570	0.812	0.001
	4	ACTB	2.320	ACTB	1.467	YWAHZ	1.777	ACTB	1.720	0.767	0.001
	5	YWAHZ	2.450	YWAHZ	1.758	SDHA	1.860	TBP	1.850	0.846	0.001
	6	SDHA	2.480	SDHA	1.789	ACTB	2.010	GAPDH	1.920	0.519	0.001
	7	TUB	2.570	ALAS1	1.853	TUB	2.108	TUB	2.190	0.744	0.001
	8	ALAS1	2.570	TUB	1.882	ALAS1	2.184	SDHA	2.460	0.818	0.001
	9	B2M	2.970	B2M	2.456	B2M	2.323	YWAHZ	2.530	0.901	0.001
	10	GAPDH	3.270	GAPDH	2.833	GAPDH	2.512	B2M	2.640	0.739	0.001

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; clo: Clonogenic; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ost: Osteogenic; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; Std Dev: Standard deviation; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

normalisation. For the first n value that shows a number less than 0.15 after V_n/V_{n+1} calculation, it is recommended to use the n most stable genes calculated. We evaluated the V_n/V_{n+1} for the leading group (all samples). The result was that V2/V3 was already lower than 0.15; therefore, we found that only the two most stable genes were necessary for data normalisation, TBP/RPLP0 (Figure 3). To do this normalisation, as Vandesompele *et al*[4] recommended, we simply take the geometric mean of the Ct of the two most stable genes and use it as a normalisation factor.

Validation of gene expression of RUNX2

We demonstrated the individual gene expression of each donor and the results together. As we used DPSC primary cell cultures, we expected differences in gene expression between the donors because of individual factors. The alizarin red staining (Figure 1) from donor #1 to donor #3 increased at 7, 14, and 21 d, respectively. When we normalise RUNX2 with TBP/RPLP0, the gene expression profile is similar to those seen for alizarin red staining (Figure 4F-H). When the RUNX2 normalisation with GAPDH occurs, the gene expression is overestimated or underestimated in both clonogenic and osteogenic media (Figure 4C-E). When we compared the relative expression of RUNX2 normalised by GAPDH or TBP/RPLP0, we observed that GAPDH tends to super estimate or underestimated in both clonogenic or osteogenic media gene expression than TBP/RPLP0 (Figure 4A and B).

DISCUSSION

For the first time, we show that the best pair of RGs for osteogenic differentiation of human DPSCs are RPLP0/TBP and TUB/B2M are the two least stable RGs through the four algorithms (ΔCt method, geNorm, BestKeeper, and NormFinder) and ranked by RefFinder. The RT-qPCR technique is an excellent tool for detecting gene transcription levels. Good

Table 4 Gene stability from Subset 3 (clonogenic and osteogenic media from 1 to 21 d) and Subset 4 (clonogenic and osteogenic media from 21 to 35 d)

Subset	Rank	Delta Ct		Normfinder		geNorm		BestKeeper			
		Gene	Stability value	Gene	Stability value	Gene	M value	Gene	Std Dev	R	*P* value
3: 1 to 21 d (clo + ost)	1	TBP	2.160	TBP	1.202	RPLP0/EFα1	0.594	ALAS1	0.750	0.433	0.001
	2	RPLP0	2.230	RPLP0	1.419	-	-	RPLP0	1.100	0.614	0.001
	3	YWAHZ	2.320	ALAS1	1.492	ALAS1	1.207	EFα1	1.100	0.535	0.001
	4	EFα1	2.320	YWAHZ	1.550	TBP	1.639	TBP	1.770	0.801	0.001
	5	ALAS1	2.330	EEF1α1	1.591	YWAHZ	1.814	ACTB	1.940	0.703	0.001
	6	ACTB	2.580	ACTB	1.938	SDHA	1.989	GAPDH	2.010	0.683	0.001
	7	SDHA	2.640	B2M	1.979	B2M	2.104	YWAHZ	2.080	0.819	0.001
	8	B2M	2.640	SDHA	2.048	ACTB	2.275	B2M	2.210	0.709	0.001
	9	TUB	2.790	TUB	2.252	TUB	2.383	SDHA	2.290	0.785	0.001
	10	GAPDH	2.970	GAPDH	2.411	GAPDH	2.499	TUB	2.350	0.738	0.001
4: 21 to 35 d (clo + ost)	1	TBP	1.980	TBP	0.749	RPLP0/EFα1	1.039	ALAS1	0.630	0.345	0.039
	2	ACTB	2.090	ACTB	1.075	-	-	RPLP0	1.740	0.860	0.001
	3	RPLP0	2.120	RPLP0	1.155	ACTB	1.427	EFα1	1.890	0.815	0.001
	4	TUB	2.280	TUB	1.447	TBP	1.636	ACTB	1.920	0.893	0.001
	5	YWAHZ	2.300	YWAHZ	1.604	TUB	1.738	TBP	1.940	0.915	0.001
	6	EFα1	2.360	EEF1α1	1.605	YWAHZ	1.866	GAPDH	1.970	0.456	0.005
	7	SDHA	2.540	SDHA	1.979	SDHA	1.980	TUB	2.120	0.829	0.001
	8	ALAS1	2.670	ALAS1	2.044	B2M	2.087	YWAHZ	2.620	0.899	0.001
	9	B2M	2.760	B2M	2.283	ALAS1	2.210	SDHA	2.810	0.838	0.001
	10	GAPDH	3.420	GAPDH	3.075	GAPDH	2.452	B2M	2.930	0.830	0.001

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; clo: Clonogenic; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ost: Osteogenic; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; Std Dev: Standard deviation; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

practices must be adopted to generate solid results supporting eventual interpretations and statements. An essential step in good RT-qPCR practices is checking the stability of the RGs adapted to normalise the results[1].

During induction of differentiation in MSCs *in vitro*, an intense change in gene expression profile is expected. For osteogenic differentiation, the first step is the cell commitment to the osteoblastic lineage. It generally happens around 1 to 7 d after the MSC induction with the osteogenic medium. The second step is the proliferation and differentiation of osteoprogenitor cells (7 to 14 d). The third step is differentiating these cells towards mature osteoblasts (14 to 21 d). Finally, from 21 d, matrix maturation and mineralisation are observed, as well as the early stages of osteoblast-osteocyte transition[12]. We cultivated DPSCs under clonogenic (negative control for differentiation) and osteogenic media for 35 d, and we analysed the gene expression every 7 d to understand the influence of cell status on RGs. Since we are evaluating long-term osteogenic differentiation, we separated the analyses into early (1 to 21 d) and late (21 to 35 d) stages of differentiation in DPSCs.

Quiroz *et al*[13] conducted the first study, which addressed the study of RGs during osteogenic differentiation in human bone marrow stem cells (BMSCs) from 14 to 20 d. They evaluated only ACTB, GAPDH, and RPL13a by two different mathematical approaches based on the ΔCt method[14], and RPL13a was the most stable gene, confirming that the most commonly employed RGs are not stable during differentiation. Even one decade later, many basic studies still ignore the stability of RG for specific MSC origins and differentiation conditions, such as cell culture methods (traditional monolayer or tridimensional techniques) and oxygen tension (normoxia and hypoxia), which will impact clinical studies. To explore the RGs in DPSCs, we selected ten putative genes previously explored to normalise gene expression data of osteogenic differentiation in traditional monolayer culture in different origins of human MSCs, such as fatty tissue[15-17], Wharton's Jelly[16], cord blood[15], umbilical cord[18], bone marrow[13,15,16,18-22], gingiva[23], fetal tissue[21], and from induced pluripotent stem cells (iPSCs)[24]. Other human cell lineages have also been analysed[25].

Table 5 Gene stability from Subset 5 (only osteogenic medium from 1 to 21 d) and Subset 6 (only osteogenic medium from 21 to 35 d)

Subset	Rank	Delta Ct		NormFinder		geNorm		BestKeeper			
		Gene	Stability value	Gene	Stability value	Gene	M value	Gene	Std Dev	R	*P* value
5: 1 to 21 d (ost)	1	EFα1	2.200	EFα1	1.255	RPLP0/EFα1	0.463	ALAS1	0.810	0.440	0.022
	2	RPLP0	2.210	RPLP0	1.278	-	-	RPLP0	1.330	0.731	0.001
	3	TBP	2.280	TBP	1.325	ACTB	1.514	EFα1	1.330	0.755	0.001
	4	ALAS1	2.380	ALAS1	1.485	TUB	1.629	ACTB	1.540	0.555	0.003
	5	YWAHZ	2.450	YWAHZ	1.706	ALAS1	1.742	TBP	1.860	0.787	0.001
	6	ACTB	2.480	ACTB	1.757	TBP	1.978	TUB	1.880	0.620	0.001
	7	TUB	2.600	TUB	1.948	YWAHZ	2.115	YWAHZ	2.170	0.845	0.001
	8	SDHA	2.660	SDHA	2.043	SDHA	2.222	B2M	2.240	0.609	0.001
	9	B2M	2.950	B2M	2.369	B2M	2.345	SDHA	2.290	0.752	0.001
	10	GAPDH	3.460	GAPDH	3.031	GAPDH	2.568	GAPDH	2.310	0.647	0.001
6: 21 to 35 d (ost)	1	TBP	1.820	TBP	0.578	RPLP0/EFα1	0.495	ALAS1	0.560	0.257	0.304
	2	ACTB	1.900	ACTB	0.584	-	-	TBP	1.750	0.963	0.001
	3	RPLP0	2.050	RPLP0	1.096	ACTB	1.024	RPLP0	1.810	0.878	0.001
	4	EFα1	2.110	EFα1	1.224	TBP	1.134	EFα1	2.040	0.883	0.001
	5	SDHA	2.160	SDHA	1.368	SDHA	1.440	ACTB	2.070	0.963	0.001
	6	YWAHZ	2.300	TUB	1.551	YWAHZ	1.557	SDHA	2.250	0.843	0.001
	7	TUB	2.310	YWAHZ	1.648	TUB	1.655	TUB	2.400	0.856	0.001
	8	ALAS1	2.830	ALAS1	2.254	B2M	1.842	GAPDH	2.510	0.492	0.038
	9	B2M	2.840	B2M	2.401	ALAS1	2.035	YWAHZ	2.760	0.927	0.001
	10	GAPDH	4.040	GAPDH	3.776	GAPDH	2.435	B2M	2.800	0.810	0.001

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ost: Osteogenic; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; Std Dev: Standard deviation; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

For the overall group, we noticed that by RefFinder ranking, the pair TBP/RPLP0 is recommended for normalising undifferentiated DPSCs and undergoing osteogenic differentiation by 35 d. RPLP0 is a gene member of the ribosomal protein family. TBP is a transcription factor that binds to the TATA-box sequences in the DNA promoter region, which belongs to the RNA-polymerase II pre-initiation complex (transcription of all protein-encoded genes). Jacobi *et al*[20] showed RPLP0 as the most stable gene during BMSC osteogenic differentiation for 14 d and B2M as the most unstable RG calculated by geNorm. Ragni *et al*[15] showed TBP/YWHAZ/GUSB as the most stable gene of bone marrow, adipose and cord blood MSCs under mesodermal differentiation (osteogenic, chondrogenic, and adipogenic for 21 d) and B2M as the most unstable RG ranked by geNorm and NormFinder. Specifically by geNorm, RPLP0/EEF1α were the best genes for osteogenic differentiation. Ayanoğlu *et al*[17] showed EF1α/RPLP13a/RPLP0 as the most stable gene of adipose-derived stem cell (ADSC) under osteogenic differentiation for 21 d and B2M as the most unstable RG ranked by ΔCt method and NormFinder. Recently, Okamura *et al*[24] also showed that TBP/RPLP0 are the most stable genes during human iPSC cell line into osteogenic differentiation for 28 d and B2M as the most unstable RG calculated by ΔCt method, geNorm, NormFinder, and BestKeeper. However, these authors did not recommend the REfFinder ranking because the ranking method is unclear. Even though the ranking method is not explicit, it is still a user-friendly tool for researchers to verify RGs. It is easy to format and input data and gives a well-determined ranking, suitable for those unfamiliar with interpreting the isolated algorithms. Since BestKeeper parameters do not consider pair analysis, most groups had similar rankings. It showed ALAS1 as the most stable, the only gene with a standard deviation of less than 1 (ideal for this algorithm). Using the four algorithms and ranking by RefFinder, these reported studies support our findings for the most and least stable genes analysed. Other studies have been reported inverted results, being RPLP0 or TBP unstable RG and B2M as a stable RG, in BMSC and fetal tissue-derived MSC[21], and BMSC[22], ADSC[17].

When we analysed the overall data and Subgroups, EF1α is ranked in the third or fourth place as a stable gene by RefFinder. In all subgroups, except subgroup 8, it composes a second position with RPLP0, which is calculated by geNorm. Specifically, in Subgroup 5 (only osteogenic medium from 1 to 21 d - early stage), it was classified in the first

Table 6 Gene stability from Subset 7 (only clonogenic medium from 1 to 21 d) and Subset 8 (only clonogenic medium from 21 to 35 d)

Subset	Rank	Delta Ct		NormFinder		geNorm		BestKeeper			
		Gene	Stability value	Gene	Stability value	Gene	M value	Gene	Std Dev	R	*P* value
7: 1 to 21 d (clo)	1	TBP	2.010	TBP	1.091	RPLP0/EFα1	0.661	ALAS1	0.690	0.379	0.051
	2	RPLP0	2.120	RPLP0	1.379	-	-	EEF1α1	0.820	0.144	0.473
	3	YWAHZ	2.150	YWAHZ	1.398	ALAS1	1.033	RPLP0	0.870	0.516	0.006
	4	ALAS1	2.150	ALAS1	1.404	TBP	1.430	TBP	1.600	0.778	0.001
	5	B2M	2.210	B2M	1.442	YWAHZ	1.617	GAPDH	1.630	0.722	0.001
	6	EFα1	2.370	GAPDH	1.615	B2M	1.718	YWAHZ	1.950	0.759	0.001
	7	GAPDH	2.370	EFα1	1.839	SDHA	1.890	B2M	1.980	0.762	0.001
	8	SDHA	2.610	SDHA	2.089	GAPDH	2.032	ACTB	2.180	0.789	0.001
	9	ACTB	2.630	ACTB	2.133	ACTB	2.212	SDHA	2.320	0.800	0.001
	10	TUB	2.920	TUB	2.546	TUB	2.354	TUB	2.660	0.815	0.001
8: 21 to 35 d (clo)	1	TBP	2.070	TBP	1.063	ACTB/TUB	0.766	ALAS1	0.670	0.480	0.044
	2	RPLP0	2.130	RPLP0	1.247	-	-	GAPDH	1.470	0.455	0.058
	3	TUB	2.200	TUB	1.378	RPLP0	1.417	RPLP0	1.680	0.841	0.001
	4	ACTB	2.210	ACTB	1.430	GAPDH	1.621	EEF1α1	1.780	0.752	0.001
	5	YWAHZ	2.280	YWAHZ	1.601	ALAS1	1.757	ACTB	1.780	0.801	0.001
	6	ALAS1	2.480	ALAS1	1.813	EEF1α1	1.932	TUB	1.850	0.799	0.001
	7	EFα1	2.580	EFα1	1.953	TBP	2.055	TBP	2.170	0.901	0.001
	8	GAPDH	2.680	B2M	2.231	YWAHZ	2.172	YWAHZ	2.520	0.862	0.001
	9	B2M	2.700	GAPDH	2.232	B2M	2.315	B2M	3.070	0.857	0.001
	10	SDHA	2.820	SDHA	2.388	SDHA	2.415	SDHA	3.240	0.884	0.001

ACTB: Actin beta; ALAS1: Aminolevulinic acid synthase 1; B2M: Beta-2-microglobulin; clo: Clonogenic EF1α: Eukaryotic translational elongation factor 1 alpha; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RPLP0: Ribosomal protein, large, P0; SDHA: Succinate dehydrogenase complex, subunit A, flavoprotein; Std Dev: Standard deviation; TBP: TATA-binding protein; TUB: Tubulin; YWHAZ: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta.

position: EEF1α/RPLP0 (RefFinder), EEF1/RPLP0 (ΔCt method), EEF1/RPLP0/TBP/ALAS1 (NormFinder), and RPLP0/EEF1α (geNorm). Previous works have shown EF1α is a suitable RG[17] or fluctuates in the first positions in the algorithms ranking[15,19]. EF1α is a gene involved in protein synthesis.

GAPDH and ACTB are widely used for the normalisation of RT-qPCR data. However, almost all studies to find the most stable RGs have identified that these genes are unsuitable for that, especially during osteogenic differentiation. In the overall analysis, B2M (RefFinder), B2M/GAPDH (ΔCt method and NormFinder), TUB/GAPDH (geNorm), and SDHA (BestKeeper) were the most unstable RGs. Our results show that B2M and GAPDH are the two least stable RGs in half of the Subgroups, calculated by ΔCt method, NormFinder and geNorm. SDHA and TUB also appear in combination with B2M and GAPDH. By BestKeeper, SDHA, B2M, and TUB are ranked as the least stable RGs in most of the Subgroups. β2-microglobulin protein is encoded by the B2M gene and is expressed in all nucleated cells. The SDH complex is located on the inner membrane of the mitochondria and participates in the citric acid cycle and the respiratory chain. The SDHA gene encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase, a mitochondrial respiratory chain complex. TUB proteins polymerise into microtubules, a significant component of the eukaryotic cytoskeleton.

When we compared the relative expression of RUNX2 normalised by GAPDH or TBP/RPLP0, we observe that GAPDH tends to overestimate gene expression more than TBP/RPLP0. This was reported in most of the studies cited before and generates a misinterpretation of gene expression during osteogenic differentiation.

CONCLUSION

For the first time, we show that RPLP0/TBP are the most stable RGs, while TUB/B2M are unstable RGs for long-term osteogenic differentiation of human DPSCs in traditional monolayers.

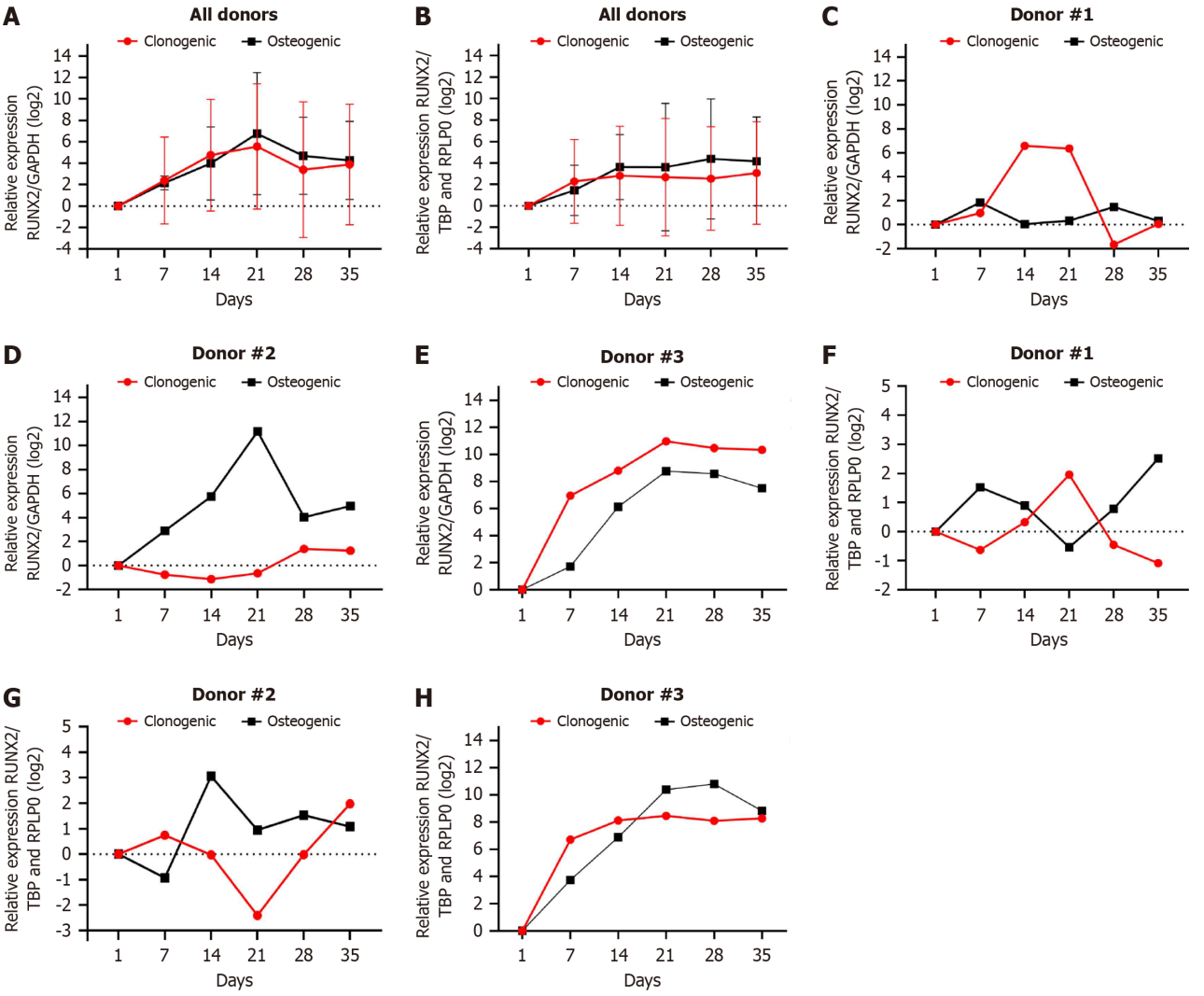

Figure 4 Effect of the reference gene (glyceraldehyde-3-phosphate dehydrogenase or TATA-binding protein/ribosomal protein, large, P0) on the gene expression of RUNX2. A: Normalization data of all samples (from the three donors) under clonogenic or osteogenic media by glyceraldehyde-3-phosphate dehydrogenase (GAPDH); B: Normalization data of all samples (from the three donors) under clonogenic or osteogenic media by TATA-binding protein (TBP)/ribosomal protein, large, P0 (RPLP0); C: Normalization data of only donor#1 under clonogenic or osteogenic media by GAPDH; D: Normalization data of only donor #1 under clonogenic or osteogenic media by TBP/RPLP0; E: Normalization data of only donor #2 under clonogenic or osteogenic media by GAPDH; F: Normalization data of only donor #2 under clonogenic or osteogenic media by TBP/RPLP0; G: Normalization data of only donor #2 under clonogenic or osteogenic media by GAPDH; H: Normalization data of only donor #3 under clonogenic or osteogenic media by TBP/RPLP0. RPLP0: Ribosomal protein, large, P0; TBP: TATA box binding protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RUNX2: Runt-related transcription factor 2.

FOOTNOTES

Author contributions: Gasparoni LM and Bronzeri CF performed the experiments and acquired the data; Ferreira DB and Paiva KBS analysed the data and wrote the manuscript; Paiva KBS designed and coordinated the study; and all authors approved the final version of the article.

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Institutional review board statement: The study was reviewed and approved by the Human Research Ethics Committee of the Institute of Biomedical Sciences and University Hospital, both University of São Paulo (CAAE: 51097315.7.0000.5467 and CAAE: 51097315.7.3001.0076).

Conflict-of-interest statement: All authors have no conflicts of interest to declare.

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