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RESEARCH ARTICLE

The stability of different housekeeping genes in human periodontal ligament cells under inflammatory conditions

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ABSTRACT

Purpose: Stability of housekeeping genes as internal reference for RT-qPCR analyses is mandatory for a correct interpretation of results. As no normalization benchmark exists and reference gene validation is highly specific for individual experiments, it was the purpose of this study to identify stable candidates for investigations on periodontal inflammation.

Basic procedures: Human PDL cells from one cell line (Lonza) and three primary donors were challenged with IL-1ß (5 ng/ml) or centrifugation (170 × g) for 6 h under serum-free conditions. Unstimulated cells represented controls. qRT-PCR was performed with a TaqMan® array of 32 housekeeping genes (n = 3). Transcriptional stability was analyzed for (i) mean absolute CT values and (ii) relative fold changes. Finally, stability of mean CT values across specimens was evaluated for most stable candidates. Statistics were performed with one-way ANOVA and Bonferroni correction and one sample t-test, with 95% confidence level. Values represent mean ± SEM.

Main findings: 18S was constant in experimental groups and specimens for mean absolute CT values and relative fold changes, and *MT-APT6* for mean absolute CT values. Both genes exhibited low CT thresholds ranging from 20.2 ± 0.1 to 25.9 ± 0.2 for *18S,* and from 18.9 ± 0.0–23.7 ± 0.1 for *MT-APT6.* Likewise stable *YWHAZ* ranged between 32.6 ± 0.2 and 37.2 ± 0.2 cycles. However, candidates were unstable across specimes.

Principal conclusions: Reference validation is mandatory for RT-qPCR analyses in new experimental designs. Here, only three genes out of 32 turned out to be appropriate candidates. Due to low CT values and stability, *18S* and *MT-APT6* are most valid genes for data normalization in experiments with PDL cells under inflammatory conditions and are recommended as standards under these premises.

1. Introduction

Gene expression analysis via real time-quantitative reverse transcription polimerase chain reaction (RT-qPCR) is the most sensitive technique in molecular biology for detection of mRNA targets and investigation of transcriptional regulations (Gadkar and Filion, 2014; Slotte et al., 2012). This extremely versatile technology is based on the fluorescence based detection of targets in a three-step thermo-cycling amplification method under real-time conditions (Gadkar and Filion, 2014).

Definite data normalization is a prerequisite to achieve precise results in the analysis of transcriptional expression and alterations of target genes in order to overcome the impairments of this technique aroused by experimental error during RNA extraction and processing (Vandesompele et al., 2002).

The gold standard for normalizing the genes of interest in an experimental setup is the scaling against housekeeping genes (HKGs), whose synthesis is provided in all nucleated cell types as their existence is mandatory for cell survival (Cook et al., 2009; Eisenberg and Levanon, 2013). Coamplification of adequate HKGs as internal reference during transcriptional analyses facilitates the compensation for inhomogeneities among individual samples due to varying enzymatic efficiencies and preparation steps, which is mandatory for a correct interpretation of final results. A large variety of HKGs is available, usually from the field of basic cellular maintenance or cytoskeletal components. Thus, stable expression levels regardless of cell type, developmental stage, cell cycle state and environmental conditions are expected to be maintained through these genes (Eisenberg and Levanon, 2013; Turabelidze et al., 2010). Consequently, recorded variations of these reference genes measured in the same setup with the genes of interest repro-

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duce the cumulative error of the whole analysis (Huggett et al., 2005).

Even though a broad portfolio of HKGs exists, to date no particular gene could be defined as universal reference incorporating all the requirements of an ideal normalization benchmark (Vandesompele et al., 2002). Investigations revealed that most of the standard HKGs exhibit variations under certain experimental conditions, despite being expected to be absolutely stable (Guénin et al., 2009). Furthermore, accuracy also varies depending on the anatomical origin of cells and tissue types, which is of special importance for studies with material of different histological origin (Sadek et al., 2012; Warrington et al., 2000). These drawbacks are critical and compromise the quality of data analyses, especially when minor expression differences of target genes are studied and absolute stability of controls is a must.

Consequently, strategies to control and compensate for these variances have been established. These involve inter alia the measurement of a panel of different HKGs (Vandesompele et al., 2002).

Here, programs using algorithms for the determination of stability across samples exist, such as geNorm, NormFinder and BestKeeper tools (Lopa et al., 2016). Standard guidelines alternatively re-commend the validation of data by running at least two HKGs from different functional classes to avoid co-regulation, that should ideally feature constant coamplification ratios (Thellin et al., 1999). Another option is to pretest stability for the HKG of choice in each novel experiment to demonstrate its suitability for this investigative setup (Thellin et al., 1999; Turabelidze et al., 2010).

In molecular biology, *GAPDH* and *ACTB* represent the most frequently used HKGs (Li et al., 2018; Suzuki et al., 2000; Zheng et al., 2018). These two genes are well established references in qualitative analysis methods based on their high ubiquitous expression levels in all cells (de Jonge et al., 2007). However, *GAPDH* and *ACTB* feature distinct variations both in vitro and in vivo, namely among cell types, during proliferation and cell cycle as much as in response to experimental stimuli (Stürzenbaum and Kille, 2001; Suzuki et al., 2000; Zhong and Simons, 1999). This aspect is even more important when target gene variations are subtle and thus potentially smaller than HKG variations, leading to a concealment of effects. Nonetheless, numerous studies assume these control genes to be stable without determining their expression stability for the given experimental conditions and thus evoke avoidable drawbacks in the transcriptional analyses of candidate genes.

In periodontal research, most works focus on periodontal inflammation, which is either bacterially induced or a sterile process due to mechanical overload (Konermann et al., 2012a). These two different entities are representative for tissue destruction due to periodontitis and for the clinical side effects of orthodontic tooth movement, respectively. In the corresponding in vitro analyses, bacterial insults are typically mimicked by stimulation of cells with the cytokine IL-1ß, which is a key inflammatory mediator in the pathogenesis of periodontal disease (Konermann et al., 2012b; Page, 1991). Sterile inflammation aroused by orthodontic tooth movement is commonly simulated by application of centrifugal force on cultures, as this procedure equals the in vivo effect of mechanical loading (Redlich et al., 2004). By default, these experiments are performed on periodontal ligament (PDL) cells, the major cellular component of the periodontium (Konermann et al., 2012a). These fibroblastic cells are either primary cells obtained from healthy human donors undergoing tooth extraction or a commercially available cell line ((Konermann et al., 2013, 2017).

To the best of our knowledge, investigations on transcriptional alterations due to periodontal inflammation via RT-qPCR apparently failed to analyze the stability of HKG expression from a panel of 32 targets against the background of both the experimental setting and the origin of the specimens.

As reference gene validation is highly specific for each individual experimental model, the purpose of this study was to identify the most stable HKGs out of 32 potential candidates for investigations in the field of periodontal inflammation, either mechanically or bacterially induced. In particular, our investigations focused on the proper selection of HKGs for studies on expression profiling of selected genes for PDL cells. Thus, our study aimed at defining valid HKGs as internal standards for PDL cells under inflammatory conditions to evaluate biological significances of even subtle expression differences in the genes of interest. Consequently, the results of our work will impact the significance and informative value of studies on the clinical pictures and side effects in the field of both orthodontics and periodontics approached by molecular biology methods.

2. Material and methods

The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Institutional Review Board. Specimens were collected with patient informed consent.

2.1. Cell culture

Human PDL cells were purchased from Lonza (Basel, Switzerland) and prepared according to the manufacturer's instructions.

Furthermore, cultures of human PDL cells from three periodontal healthy donors were explanted from the middle third of the root surface of caries-free teeth removed during routine extraction for orthodontic reasons. The teeth were extracted from adolescent patients after examination of defined variables for clinically healthy periodontal tissues with absence of bleeding on probing, probing depth <4 mm and loss of attachment level <3 mm.

Cells were grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated FCS (Invitrogen) and 1 μg/ml penicillin/streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 atmosphere and passaged using trypsin/EDTA (Invitrogen) after reaching confluence. For experimental treatment, cells in the 5th passage were seeded in 6-well plates (Falcon, Thermo Fisher Scientific, Waltham, MA, USA) at n = 3 for each primary cell specimen and for the cell line. One day before experimentation, cells were provided with serum-free medium for 24 h to prevent any artificial changes of molecular activation levels and to eliminate nonspecific cell cycle effects by serum proteins. Cells were challenged with human recombinant IL-1ß (5 ng/ml; Biotrend Chemikalien, Cologne, Germany) to mimic bacterially induced inflammation or were centrifuged (Multifuge 3 S-R, Heraeus, Hanau, Germany) at 170 × g to simulate sterile inflammation, each for 6 h under serum-free conditions. Untreated cells in serum-free medium served as control.

2.2. RNA extraction, quality control and cDNA synthesis

Total mRNA was isolated and purified from cell lysates using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturerś protocol. Isolated mRNA was quantified spectrophotometrically (Nanodrop; Thermo-Fischer Scientific, Wilmington, DE, USA) and its purity was determined at 260/280 absorbance ratio.

mRNA was reverse-transcribed to cDNA employing the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The 20 μl cDNA synthesis reaction oligo(dT) primer mix was prepared according to the manufacturerś protocol with 1000 ng as the highest amount of starting RNA. Synthesis steps were the oligo(dT) primer cDNA reaction at 42 °C for 90 min and the reverse

transcriptase inactivation at 85 °C for 5 min performed on an iCycler (Bio-Rad Laboratories).

2.3. RT-qPCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was operated on a StepOnePlus™ Real-Time PCR System (Thermo-Fischer Scientific) with a TaqMan® Array Human Endogeneous Control Plate (Thermo-Fischer Scientific), a Housekeeping & Control Gene Panel containing 32 genes plated in triplicate for each sample (n = 3). The mix was prepared according to the manufacturer's protocol with 3 ng of the starting cDNA amount. Amplification and real-time data acquisition were operated using the following cycle conditions: two minutes at 50 °C, 20 s at 95 °C, followed by 40 cycles with denaturation for one minute at 95 °C and annealing plus extention for 20 s at 60 °C. Negative controls of nuclease-free water were included to obviate DNA contamination in the PCR mix. Melting curve analysis verified the specifity of the PCR products.

2.4. Data evaluation and statistical analysis

A candidate HKG for data normalization in experimental setups with PDL cells maintained under inflammatory conditions was defined as the gene with the most stable expression and absence of statistically significant changes upon challenge.

Therefore, three different sets of statistical analyses were performed for each candidate gene.

As first analytic test, the mean absolute CT values of control conditions, IL-1ß stimulation and mechanical challenge were tested for statistical significant differences for each gene in each specimen (n = 3 per condition). As stability across the conditions should also be given on the overall level, calculations were secondly performed for mean absolute CT values of the whole collective of specimens (n = 12 per condition).

The second analysis investigated the expression stability of selected genes with regard to the fold changes of stimulated compared to control cells for each of the four cell specimens (n = 3 per condition), and afterwards again for the whole collective (n = 12 per condition).

For each of these two analytic tests, a gene exhibiting stable expression in the cell line, the primary cells and in the whole collective taken together was categorized as high ranking with order 1. A gene with stability in the whole collective plus constancy in the cell line or the three primary cells was categorized as moderate ranking with order 2.

For genes with rank 1 or 2, stability of mean CT values across the four cell specimens was finally evaluated for the control condition, the IL-1ß challenge and for the centrifugation in a third analytic step.

Statistical evaluations were performed with one-way ANOVA for comparison between the conditions followed by Bonferroni correction. Gene induction upon stimulation compared to controls was analyzed with the one sample t-test after application of the $2^{(-\Delta\Delta C(T))}$ method.

Analytic tests were performed with the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All values are expressed as mean ± SEM and represent experimental groups with n = 3 versus n = 12 according to the indications in the text. The level for statistical significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

Study on transcriptional stability of HKGs for data normalization in PDL cells revealed potential candidate genes for the cells analyzed. However, most of the tested genes failed to successfully pass the standards set for the statistical analyses to identify the most stable HKGs.

Table 1
Stable HKGs in PDL cells itemized by cell source and statistical analysis.

	CT value analysis	ddCT analysis
All specimes (n = 12)	18S	18S
	MT-ATP6	MT-ATP6
	YWHAZ	YWHAZ
	RPL37A	
	RPL30	
Cell line (n = 3)	18S	18S
	MT-ATP6	
Primary donor 1 (n = 3)	18S	18S
	MT-ATP6	YWHAZ
	YWHAZ	
	HMBS	
	POLR2A	
	ABL1	
	RPL37A	
Primary donor 2 (n = 3)	18S	18S
	MT-ATP6	YWHAZ
	YWHAZ	
	HPRT1	
	UBC	
Primary donor 3 (n = 3)	18S	18S
	MT-ATP6	MT-ATP6
	YWHAZ	YWHAZ
	ACTB	PSMC4
	TBP	EIF2B1
		PES1
Ranked genes	18S – 1	18S – 1
	MT-ATP6 – 1	YWHAZ – 2
	YWHAZ – 2	

List of stable genes for data normalization in experimental designs with PDL cells under inflammatory conditions. Subdivisions display the different analytic tests as much as the cell sources tested. Ranked genes at the bottom of the table represent genes with the highest validity. Order 1 implies stable expression in the cell line, the primary cells and in the whole collective taken together. Order 2 implies stable expression in the whole collective plus constancy in the cell line or the three primary cells.

The first criterion for stable candidate HKG selection was homogeneity of CT values for controls, IL-1ß stimulated cells as much as centrifuged samples. Here, all specimens tested exhibited invariant expression levels for *18S* and for *MT-ATP6*. Except the cell line, primary cells were additionally constant for *YWHAZ*. The three primary cell lines moreover manifested some other genes to be invariant, but without any congruency. Invariant genes are listed for each specimen in Table 1. Interestingly, the statistically significant differences seen for the other 29 genes analyzed predominantly aroused from transcriptional changes due to centrifugation, resulting in variances related to controls and to IL-1ß stimulated cells. Only for some genes, significant regulations could be observed between controls and IL-ß stimulation, which was most frequently observable in the cell line. The CT values for each of the 32 genes analyzed are presented in Table 2 for the whole collective taken together.

Data from the second analytic step investigating the transcriptional stability for all specimens taken together featured *18S*, *MT-APT6* and also *YWHAZ* to reproduce stable CT values across all conditions. Information is available in Table 1.

Thirdly, analyses on the absence of gene induction upon stimulation compared to controls revealed one gene to be stable across all specimens without statistically significant fold changes, which was namely *18S*. Concordant with the previous analyses, *18S* featured a constant expression pattern for all specimens tested. Furthermore, the same applied for *YWHAZ* in all primary cells. However, this was not applicable for the cell line. One of the primary cell specimens showed additional invariances for the genes *PSMC4, EIF2B1, PES1* and again for *MT-ATP6,* but this was a singular trend only seen in this cell line. When analyzing the whole collective together for

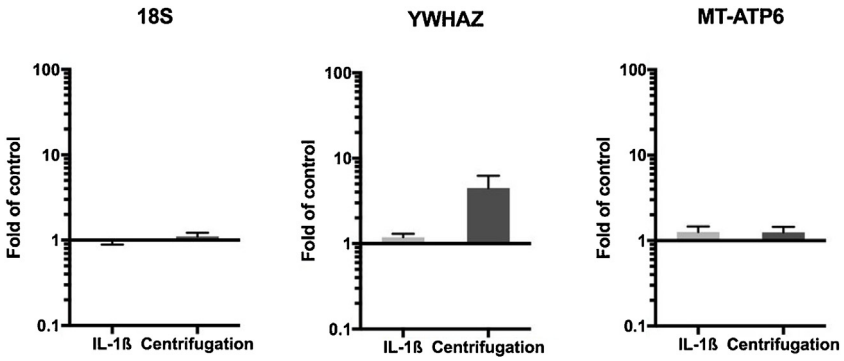

Fig. 1. Relative gene expression stability of *18S, MT-ATP6* and *YWHAZ* in PDL cells.
Transcriptional expression analysis (fold of control) of *18S, MT-ATP6* and *YWHAZ* in PDL cells exemplary shown for the whole collective of specimens taken together. PDL cells were challenged with or without human recombinant IL-1ß (5 ng/ml) to mimic bacterially induced inflammation or were maintained with or without centrifugation (170 × g) to simulate sterile inflammation, each for 6 h under serum-free conditions. Unstimulated cells in serum-free medium served as control.
Gene induction upon stimulation compared to controls was analyzed with the one sample t-test (95% confidence level) after application of the $2^{(-\Delta\Delta C(T))}$ method.
Values represent the mean ± SEM (n = 3) of the relative differential gene expression (fold of control). $P < 0.5$ was set statistically significant.

Table 2
CT values of the 32 HKGs analyzed in 4 different PDL cell specimens.

Gene	Control	IL-1ß	Centrifugation
18S	23.2 + 0.6	23.1 + 0.6	23.6 + 0.7
GAPDH	23.8 + 0.4	23.8 + 0.4	26.6 + 0.4
HPRT1	29.6 + 0.3	29.7 + 0.3	32.5 + 0.3
GUSB	28.8 + 0.4	28.9 + 0.4	31.2 + 0.5
ACTB	23.7 + 0.6	23.7 + 0.5	27.9 + 0.9
B2M	23.5 + 0.4	23.9 + 0.4	26.5 + 0.5
HMBS	32.2 + 0.5	31.9 + 0.5	35.0 + 0.6
IPO8	32.2 + 0.5	32.3 + 0.4	36.1 + 0.4
PGK1	28.6 + 0.4	28.7 + 0.3	32.0 + 0.5
RPLP0	23.6 + 0.4	24.1 + 0.2	27.1 + 0.6
TBP	32.9 + 0.4	32.7 + 0.3	35.6 + 0.4
TFRC	32.0 + 0.4	31.9 + 0.4	35.8 + 0.5
UBC	25.4 + 0.4	25.6 + 0.3	28.6 + 0.6
YWHAZ	35.3 + 0.4	35.4 + 0.3	36.6 + 0.2
PPIA	23.9 + 0.4	23.9 + 0.2	26.0 + 0.4
POLR2A	34.3 + 0.5	34.4 + 0.6	36.4 + 0.4
CASC3	31.6 + 0.5	31.3 + 0.4	35.2 + 0.5
CDKN1A	28.2 + 0.5	28.1 + 0.5	30.9 + 0.4
CDKN1B	31.6 + 0.5	31.8 + 0.4	34.5 + 0.3
GADD45A	29.8 + 0.4	29.9 + 0.4	32.2 + 0.6
PUM1	32.4 + 0.6	32.3 + 0.6	35.7 + 0.5
PSMC4	29.8 + 0.4	30.0 + 0.4	32.0 + 0.6
EIF2B1	30.7 + 0.4	30.8 + 0.4	33.7 + 0.4
PES1	30.5 + 0.5	30.4 + 0.4	32.4 + 0.5
ABL1	33.8 + 0.5	33.7 + 0.6	35.8 + 0.5
ELF1	31.8 + 0.5	31.6 + 0.5	34.3 + 0.4
MT-ATP6	21.5 + 0.5	21.7 + 0.3	21.7 + 0.4
MRLP19	30.8 + 0.4	30.9 + 0.4	33.2 + 0.5
POP4	29.8 + 0.4	29.6 + 0.3	32.7 + 0.4
RPL37A	22.9 + 0.4	22.9 + 0.4	24.0 + 0.4
RPL30	23.5 + 0.3	23.6 + 0.3	24.8 + 0.4
RPS17	23.4 + 0.4	23.7 + 0.3	24.9 + 0.4

The CT values for each of the 32 genes analyzed for the whole collective of specimens (n = 4) taken together. Data are presented as mean ± SEM.

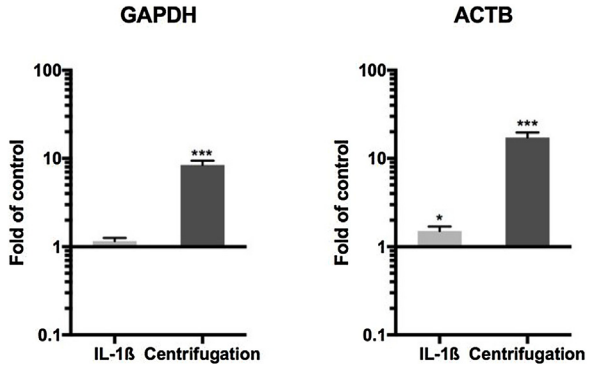

Fig. 2. Relative gene expression of *GAPDH* and *ACTB* in PDL cells.
Transcriptional expression analysis (fold of control) of *1 GAPDH* and *ACTB* in PDL cells exemplary shown for the whole collective of specimens taken together. PDL cells were challenged with or without human recombinant IL-1ß (5 ng/ml) to mimic bacterially induced inflammation or were maintained with or without centrifugation (170 × g) to simulate sterile inflammation, each for 6 h under serum-free conditions. Unstimulated cells in serum-free medium served as control.
Gene induction upon stimulation compared to controls was analyzed with the one sample t-test (95% confidence level) after application of the $2^{(-\Delta\Delta C(T))}$ method.
Values represent the mean ± SEM (n = 3) of the relative differential gene expression (fold of control). $^{*}P < 0.5$, $^{**}P < 0.1$ and $^{***}P < 0.001$

fold changes in stimulated samples compared to controls, *18S*, *MT-APT6* and also *YWHAZ* were held steady. In agreement with the first analyses, significant variances mainly aroused from mechanically stimulated cells and only random for IL-1ß stimulation.The results are exemplary shown for *18S*, *MT-APT6* and *YWHAZ* in Fig. 1, and for *GAPDH* and *ACTB* in Fig. 2, displaying graphics of the results for the whole collective taken together.

According to the evaluation criteria for these two analytic tests applied, a gene exhibiting stable expression in the cell line, the primary cells and in the whole collective taken together was categorized as high ranking with order 1, and a gene with stability in the whole collective plus constancy in the cell line or in the three primary cells was categorized as moderate ranking with order 2. As seen in Table 1, *18S* was ranked as 1 in both analytic sets. *MT-APT6* was ranked as 1 in one analytic test, and *YWHAZ* was ranked as 2 in both evaluations. Data are again summarized in Table 1. Thus, these three genes were further analyzed for stable CT values across all four specimens, each in the control, the IL-1ß and the centrifugation group. The results for *18S, MT-APT6* and *YWHAZ* are presented in Fig. 3. As an overall trend of the evaluation for these three genes, statistically significant differences were principally evoked by divergent CT values from the cell line seen for all experimental groups. Evaluation of *YWHAZ* exhibited most homogeneous results, as significances only occurred between the primary donors and the cell line in the control group and the IL-1ß group, whereas no significant differences could be observed for the centrifugation group. Both genes ranked with a grade 1 featured slightly more variances within groups. *MT-APT6* showed discrepancies almost exclusively related to the cell line as well, but here also in the centrifugation group. Here, statistical significance was also reached among two primary donors. For beforehand most constant *18S,* dicrepancies between primary donor 1 and 2 were observable in all conditions tested, in addition to the divergent CT values from the cell line. Regarding the individual CT values of these three genes, the number of cycles to reach the defined fluorescence intensity were highest

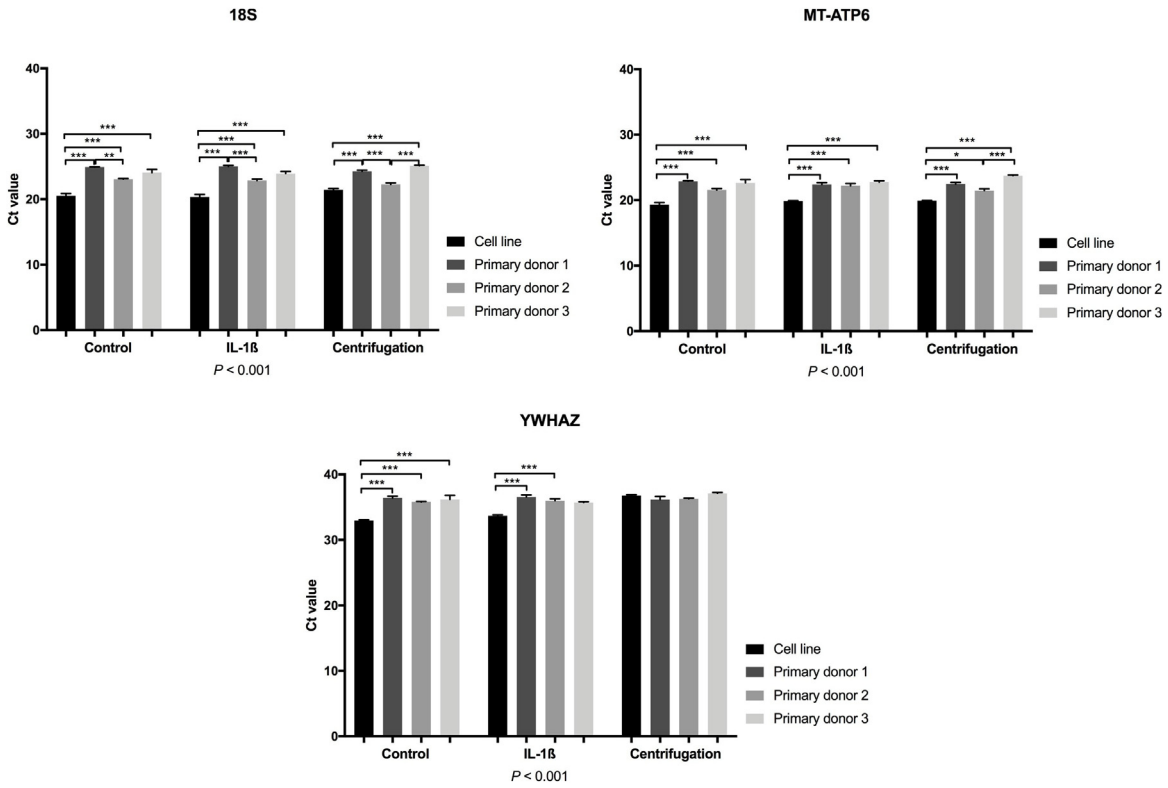

Fig. 3. CT value stability of the most stable HKGs *18S, MT-ATP6* and *YWHAZ* in PDL cells across specimens.
PDL cells were challenged with or without human recombinant IL-1ß (5 ng/ml) to mimic bacterially induced inflammation or were maintained with or without centrifugation (170 × g) to simulate sterile inflammation, each for 6 h under serum-free conditions. Unstimulated cells in serum-free medium served as control.
Analyses were performed with one-way ANOVA followed by Bonferroni *post hoc* analysis with 95% confidence level. Values represent the mean CT values ± SEM (n = 3). $^{*}P < 0.5$, $^{**}P < 0.1$ and $^{***}P < 0.001$

for Y*WHAZ* with 32.6 ± 0.2 as lowest number seen in the control cell line and with 37.2 ± 0.2 as maximum value recorded for a centrifuged primary donor. On the contrary, *18S* and *MT-APT6* exhibited distincly lower CT values. *18S* ranged from 20.2 ± 0.1–25.9 ± 0.2, and *MT-APT6* from 18.9 ± 0.0–23.7 ± 0.1. Interestingly, the highest and the lowest values seen for each of the three genes were derived from exactly the same specimens.

4. Discussion

The RT-qPCR method emerged as a benchmark technology in research for quantitative analyses of transcriptional expression patterns (Bustin, 2010). As both cells and tissues are highly versatile and dynamic regimes featuring pronounced heterogeneity due to environmental factors or cell cycle states, it is obligatory to provide every gene expression study with validated normalization standards.

Our investigations revealed that only three candidate genes out of the 32 targets analyzed exhibited stable expression in the analytic tests performed, which were *18S*, *MT-APT6* and *YWHAZ*.

Here, *18S* exhibited the highest stability, as it was constant in each experimental group and in each specimen tested, namely the cell line, the primary cells and in the whole collective taken together. This is in concordance with other investigations where *18S* is proposed as normalization standard due to its insignificant variations (Thellin et al., 1999). As only shortcoming, *18S* expression was not absolutely constant across the different primary cells as much as the cell lines within each of the conditions tested in our work. For the two other genes of highest ranking, that trend was also observable, but to a lesser extent as discrepancies were primarily seen for the cell line. This observation was also made in a study on keratinocytes from multiple sources, underlining that different origins of cells impact their genetic profile due to overall odds in transcriptional activity between tissues (Guénin et al., 2009). In consequence, it is of major importance to characterize HKG expression profiles in each specific cell type before establishing expression studies, even though it is no obligation so far and underetimated in many studies (Allen et al., 2008). This observation was also made by the second work on gene expression normalization in PDL cells besides this one, which propagated varying gene stability rankings to be expected depending on the cell source (Kirschneck et al., 2017). They analyzed reference reliability in PDL cells exposed to periodontal inflammation only in a very specific primary specimen group and with experimental setups for simulating orthodontic force and periodontitis very different from the ones in this study. By the use of four different algorithms, they identified *PPIB* and *RPL22* or *TBP* as most suitable for their orthodontic force setup, and *PPIB* plus *TBP* for their periodontitis simulation. However, they exclusively investigated 13 potential candidate genes which did not comprise *18S* and *MT-APT6,* and moreover no cell line in addition to primary cells as in this study. The second work of this group focused on transcriptional analyses in rats and again found substantial differences in HKG expression stability between investigated groups comprising rat dental, periodontal and alveolar bone tissues in the context of orthodontic tooth movement and periodontitis (Kirschneck et al., 2016). In our study, another advantage of *18S* beside its high transcriptional stability is its low CT threshold ranging from 20.2 ± 0.1 to 25.9 ± 0.2. This is an important benefit especially when analyzing genes of interest with high expression levels within samples. *MT-APT6* exhibited similarly low CT values from 18.9 ± 0.0 to 23.7 ± 0.1, whereas Y*WHAZ* ranged between 32.6 ± 0.2 and 37.2 ± 0.2 cycles.

The low CT values of *MT-APT6* together with its stable expression in the cell line, the primary cells and in the whole collective taken together in one of the analytic tests categorized as high ranking with order 1 makes this gene the second most valid HKG for data normalization.

Besides the three genes described above, all other potential HKGs investigated were highly variable in PDL cells under the given conditions. Amongst these, commonly used *GAPDH* and *ACTB* also did not fulfill the criteria of valid internal standards as their expression lacked stability in the different experimental conditions, underlining the limits of these genes as HKGs previously described in the literature (Stürzenbaum and Kille, 2001; Suzuki et al., 2000; Zhong and Simons, 1999). These pronounced variabilities might be explained by the fact that the essential functions of these molecules are various as they are additionally involved in other cellular processes (Thellin et al., 1999). Hence, the use of these putative standards for gene normalization should be reconsidered and in either case never be applied without any proper validation in the specific experimental setup of interest. Also, the internal standards proposed in our work that are constant for PDL cells under the conditions examined have to be considered to vary in other experimental settings. Thus, it is important that publications avoid limiting the provision of technical information and instead provide sufficient data on their methodology applied in order to retrace the standards applied and to assess the validity of the experiments.

In order to standardize the methodology of the transcriptional analyses, literature recommends to run analyses with at least two HKGs from different functional fields or to focus on one internal standard if experimental behavior of this gene is previously tested under the same experimental conditions (Dent et al., 1997; Lemay et al., 1996). However, there are no universally accepted guidelines for data handling so far and thus each working group has to assess its own standards.

In summary, systematic validation of HKGs is mandatory for ensuring accuracy and reliability of results obtained by RT-qPCR and have to be an integral component of every new experimental design. The reference genes can be very secure and stable under some circumstances, but on the contrary very fluctuating in other settings. In our investigations, only three genes out of 32 turned out to be appropriate candidates for an accurate data normalization. According to their low CT values and their hoomogeneously stable expression patterns regardless of experimental condition or cell source, the HKGs *18S* and *MT-APT6* represent the most valid genes for data normalization in experimental designs with PDL cells under inflammatory conditions and can be recommended as standards under these premises. Thus, these three candidates are reliable references for transcriptional analyses of clinical pathologies in periodontal tissues due to periodntitis and orthodontic tooth movement. As consideration for future research, these candidates are recommended to be validated in preliminary studies along with internal standards previously applied for investigations when establishing new experimental setups. By following this approach, the ideal HKG can be identified for each clinical problem and its underlying molecular specificity as a basis for transcriptional studies.

5. Conclusions

Reference gene validation is mandatory for obtaining precise RT-qPCR analyses in new experimental designs. For transcriptional evaluation of PDL cells maintained in an inflammatory environment, only three genes out of 32 turned out to be appropriate candidates. Due to low CT values and stability, *18S* and *MT-APT6* are considered the most valid genes for data normalization in experiments with PDL cells under inflammatory conditions and are recommended as standards under these premises.

CRediT authorship contribution statement

M. Setiawan: Investigation, Data curation, Formal analysis. **A. Jäger:** Supervision, Validation, Writing - review & editing. **A. Konermann:** Writing - original draft, Conceptualization, Project administration, Methodology.

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