Selection and validation of reference gene for RT-qPCR studies in co-culture system of mouse cementoblasts and periodontal ligament cells

Jiawen Yong1*,†, Sabine Groeger2, Gisela Ruiz-Heiland1† and Sabine Ruf1†

Abstract
Objective: RT-qPCR is a reliable method for gene expression analysis, but the accuracy of the quantitative data depends on the appropriate selection of reference genes. A Co-culture system consisting of periodontal ligament cells (SV-PDL) and cementoblasts (OCCM-30) to investigate the crosstalk between these two cell lines under orthodontic condition is essential for experimental orthodontic setups in-vitro. Therefore, we aimed to identify a set of reliable reference genes suitable for RT-qPCR studies for prospective co-culture systems of OCCM-30 and SV-PDL cells.

Results: The results demonstrated that PPIB, GUSB and RPLP0 turned out to be the three most stable reference genes for OCCM-30 in the co-culture system, while PPIB, POLR2A and RPLP0 have the three highest rankings for SV-PDL cells in the co-culture system. The most stable gene combination were PPIB and POLR2A in the co-culture system. In conclusion, PPIB is overall the most stably expressed reference gene for OCCM-30 or SV-PDL cell line in the system. The combination of PPIB and POLR2A as reference genes are indicated to be the potential and mandatory to obtain accurate quantification results for normalizing RT-qPCR data in genes of interest expression in these two cell lines co-culture systems.

Keywords: Reference genes, Cementoblasts, Periodontal ligament cells, RT-qPCR, Co-culture

Introduction
Cementoblasts are located on the cementum covered root surface and have the lifelong capability to produce cementum [1]. Periodontal ligament cells are fibroblast-like cells characterized by collagen production [2]. Reverse Transcriptase-quantitative polymerase chain reaction (RT-qPCR) [3] is a versatile molecular technique for quantification of the expression of genes of interest due to the method's merits concerning its high sensitivity, simplicity and specificity as well as its accuracy [4, 5]. Reference genes are considered to be consistently expressed in various tissues and treatments [6], which guarantee precise gene expression quantification by accurate and valid data normalization [7].

Our laboratory will deliver a co-culture system of periodontal ligament cells with cementoblasts which in-vitro is used to mimic the biological conditions to explore the interaction between these two-cell lines [8–11]. Therefore, a commonly stable reference gene selection is of vital role for the co-culture system RT-qPCR experiment set.

Together from previous studies [12–15], the ribosomal 60S protein L22 (RPL22), Peptidylprolyl isomerase B (PPIB), polymerase RNA II polypeptide A (POLR2A),
Ribosomal protein, large, P0 (RPLP0), glucuronidase, beta (GUSB), Actin-beta (β-actin), TATA-binding protein (TBP), ubiquitin C (UBC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activate protein, zeta (YWHAZ), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), Ribosomal protein L18 (RPL) and beta-5 class I (TUBB) were identified as reliable reference genes.

Since co-culture system could be a promising method to analyze the biological effect response to mimic orthodontically induced tooth movement in-vitro. The identification of the most reliable reference genes for RT-qPCR analysis on OCCM-30 and SV-PDL, is an essential step to facilitate further research in this area.

**Main text**

**Materials and methods**

**Mono cell culture**

Immortalized murine mouse cementoblast (OCCM-30) cell line [16] and immortalized mouse murine periodontal ligament (SV-PDL) cells [17] were provided by Prof. Martha J. Somerman (Laboratory of Oral Connective Tissue Biology, NIH, Bethesda, USA). The optimal density of OCCM-30 [18] and SV-PDL cells [17] were previously determined, thus 60—100% confluence status of cells were used for cell confluence experiments [15].

Both cells were cultured at a density of 1*10^6 cell/well in D-MEM (31885-023, Gibco) containing 10% FBS (10270-106, Gibco), 1% Penicillin/Streptomycin (15140-122, Gibco) and 1% HEPES (15630-056, Gibco) in a humidified atmosphere of 5% CO₂ at 37°C.

**Direct cell–cell contact culture**

The direct cell–cell contact system was established by seeding each cell line by same number of 1*10^6 cells/well in the same 6-well plate. After incubation overnight to allow for firm adherence to the bottom, the cell–cell contact system was established [19]. For the experiment, control group (as 0 h) was set when cells reached approximately 60% confluence. The mRNA was harvested at 0, 12 and 24 h at the same day.

**Co-culture system**

The OCCM-30/SV-PDL co-culture system was established through 6-well plate and ThinCert® Cell Culture Inserts (pore size 0.4 µm, porosity/transparent membrane) (657,641, Greiner Bio-One) enabling the cells to exchange soluble factors [8] as previous described [11]. Briefly, the SV-PDL cells (1*10^6 cell/well) were seeded into 6-well plate and OCCM-30 cells (1*10^6 cell/well) were seeded into the ThinCert® inserts. After 6 h cultivation to allow for adherence, the inserts containing OCCM-30 cells are placed on top in the 6-well plate containing SV-PDL cells (Fig. 2A) on bottom. Then, the co-culture system was established after co-incubation for another 10 h. For the experiment, control group (as 0 h) was set when cells reached approximately 60% confluence. The mRNA was harvested at 0, 12, 24 h at the same day.

**RT-qPCR analysis**

Cells were harvested with 350 µL buffer RLT (Qiagen, Germany). Afterwards, RNA was isolated with RNase Mini Kit (Qiagen, Germany) following an on-column DNA digestion (RNase-Free DNase, Qiagen, Germany) including DNase step for removal of genomic DNA. After isolation, the eluted RNA purity and quantity of each sample was verified photometrically by OD readings of the A260/280 nm ratio (Nanodrop 2000, Thermo Fisher Scientific, USA).

For every RT-qPCR 20 µL volume reaction, we used 8 µL DNase-free water (Sigma-Aldrich), 10 µL SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad), 1.0 µL cDNA and 1.0 µL primer [20]. Primers are designed by Bio-Rad (Additional file 1: Table S1). For analysis, a C_q cut-off of 40 was applied.

**Stability assessment and statistical analysis**

Statistical analysis regarding reference genes stability was performed by using four different mathematical procedures: geNorm (qBase+, Biogazelle) [21], NormFinder (version 0.953) [22], BestKeeper (version 1) [23] and Comparative ΔC_q method [24]. The values of cycle threshold (C_q) were inputted to all programs (Additional file 2: Table S2). For evaluation, the selected reference genes were listed based on their stability values (geNorm: M value; NormFinder: V_n/V_n+1; BestKeeper: Pearson’s r value; and comparative ΔC_q: mean of SD value). Graphic were produced by GraphPad software (version 8.0). Descriptive statistics are shown as arithmetic mean values ± standard deviation (SD). The ranking sum for each gene is calculated by the summation of four respective rankings (Table 1).
Table 1: The rank of candidate reference gene stability for monocultured OCCM-30 or SV-PDL, direct cell-cell culture, co-cultured OCCM-30 cells and co-cultured SV-PDL cells, overall ranks were calculated by the four algorithms application (geNorm, NormFinder, comparative ΔCq and BestKeeper, respectively)

| Rank | Methods | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
|------|---------|--------|------------|----------------|------------|
|      |         | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
|      |         | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |
| 1.0  | geNorm  | geNorm | NormFinder | Comparative ΔCq | BestKeeper |

Monocultured cementoblasts (OCCM-30)

(1) RPL22 13 GUSB 0.032 TBP 0.065 RPL22 0.22 PPIB 0.750 0.470 2.278
(2) PPIB 17 POLR2A 0.037 PPIB 0.065 RPLP0 0.26 YWHAZ 0.685 1.541 7.321
(3) POLR2A 21 RPL22 0.042 β-actin 0.092 POLR2A 0.40 GAPDH 0.679 0.390 2.282
(4) RPLP0 23 RPLP0 0.058 RPL22 0.136 GUSB 0.40 TBP 0.620 0.503 2.121
(5) GUSB 23 UBC 0.063 RPL 0.212 UBC 0.53 RPL22 0.612 0.662 2.679
(6) β-actin 24 β-actin 0.107 RPL 0.212 UBC 0.53 RPL22 0.612 0.662 2.679
(7) TBP 24 GAPDH 0.083 TBP 0.212 UBC 0.53 RPL22 0.612 0.662 2.679
(8) UBC 27 PPIB 0.090 GUSB 0.831 TBP 0.59 UBC -0.038 0.489 1.756
(9) GAPDH 32 YWHAZ 0.095 UBC 0.859 RPL 0.64 POLR2A -0.115 0.197 0.875
(10) YWHAZ 35 EEF1A1 0.113 EEF1A1 1.005 RPL 0.81 GUSB -0.282 0.331 1.393
(11) EEF1A1 39 TBP 0.183 GAPDH 2.421 GAPDH 2.12 RPLP0 -0.428 0.152 0.988
(12) RPL - UBC 0.124 RPL 0.26 β-actin 0.64 POLR2A -0.115 0.197 0.875
(13) TUBB - - RPLP0 0.125 TUBB 0.51 TUBB 0.440 0.126 0.818

Monocultured periodontal ligament cells (SV-PDL)

(1) GUSB 14 GUSB 0.003 EEF1A1 0.112 GUSB 0.18 TBP 0.928 0.447 1.766
(2) RPLP0 19 GAPDH 0.004 GUSB 0.112 RPLP0 0.19 RPL22 0.921 0.762 2.951
(3) RPL22 22 RPLP0 0.007 POLR2A 0.123 EEF1A1 0.19 UBC 0.916 0.660 2.360
(4) TBP 22 β-actin 0.021 β-actin 0.124 POLR2A 0.24 YWHAZ 0.910 0.455 2.103
(5) β-actin 22 PPIB 0.031 TBP 0.124 RPL22 0.26 β-actin 0.905 0.537 3.093
(6) PPIB 26 RPL22 0.040 RPLP0 0.125 TUBB 0.31 GAPDH 0.868 0.518 2.894
(7) POLR2A 27 UBC 0.045 PPIB 0.206 PPIB 0.51 PPIB 0.739 0.446 2.094
(8) GAPDH 30 TBP 0.050 TUBB 0.282 TBP 0.53 RPLP0 0.440 0.126 0.818
(9) TUBB 36 POLR2A 0.075 RPL22 0.475 β-actin 0.66 RPL 0.355 0.250 0.996
(10) EEF1A1 38 TUBB 0.100 RPL 0.526 RPL 0.92 GUSB 0.328 0.138 0.561
(11) YWHAZ 49 EEF1A1 0.127 GAPDH 5.241 GAPDH 4.98 POLR2A 0.169 0.226 0.989
(12) UBC - YWHAZ 0.850 YWHAZ 6.351 YWHAZ 5.99 TUBB 0.125 0.268 0.930
(13) RPL - - UBC 0.124 TUBB 0.51 TUBB 0.440 0.126 0.818

Direct cell-cell contact cultured of OCCM-30 and SV-PDL

(1) GUSB 7 POLR2A 0.086 GUSB 0.005 RPLP0 0.29 POLR2A 0.912 0.57 2.27
(2) POLR2A 8 GUSB 0.089 POLR2A 0.007 GUSB 0.48 GUSB 0.889 0.62 2.36
(3) RPLP0 14 RPL22 0.11 RPL22 0.019 PPIB 0.48 PPIB 0.872 1.07 4.83
Table 1 (continued)

| Rank | Methods                      | geNorm   | NormFinder | Comparative ΔCq | BestKeeper  |
|------|------------------------------|----------|------------|-----------------|-------------|
|      |                              | Ranking  | Stability   | Ranking        | Stability   |
|      |                              | order    | value (M)   | order          | value       |
|      |                              | sum      |             |                 |             |
| (4)  | RPL22                        | 15       | RPLP0 0.212 | RPLP0 0.029    | POLR2A 0.59 |
| (5)  | PPIB                         | 16       | PPIB 0.399  | PPIB 0.058     | RPL22 1.09  |

Co-cultured OCCM-30-control

| Rank | Methods | Ranking | Stability | Rank | Stability | SD (± Cq) | CV (% Cq) |
|------|---------|---------|-----------|------|-----------|-----------|-----------|
| (1)  | PPIB    | 8       | PPIB 0.211| GUSB 0.006 | RPLP0 0.31 | 1.5       | 5.31      |
| (2)  | GUSB    | 10      | POLR2A 0.224 | PPIB 0.013 | GUSB 1.00 | 0.977     | 4.34      |
| (3)  | RPLP0   | 14      | RPLP0 0.325 | RPL22 0.028 | PPIB 1.02 | 0.977     | 3.46      |
| (4)  | POLR2A  | 14      | GUSB 0.347  | POLR2A 0.040 | POLR2A 1.03 | 0.658     | 1.01      |
| (5)  | RPL22   | 14      | RPL22 0.704  | RPLP0 0.045 | RPL22 1.59 | 0.220     | 1.65      |

Co-cultured OCCM-30 with 3 indicated time (n = 18)

| Rank | Methods | Ranking | Stability | Rank | Stability | SD (± Cq) | CV (% Cq) |
|------|---------|---------|-----------|------|-----------|-----------|-----------|
| (1)  | PPIB    | 9       | RPLP0 0.409 | PPIB 0.016 | RPLP0 0.63 | 0.949     | 0.72      |
| (2)  | GUSB    | 11      | GUSB 0.478  | GUSB 0.021 | POLR2A 0.74 | 0.767     | 0.6       |
| (3)  | RPLP0   | 11      | PPIB 0.515  | POLR2A 0.027 | GUSB 0.78 | 0.977     | 0.94      |
| (4)  | POLR2A  | 14      | POLR2A 0.565 | RPL22 0.031 | PPIB 0.78 | 0.977     | 0.94      |
| (5)  | RPL22   | 17      | RPL22 0.840  | RPLP0 0.035 | RPL22 1.27 | 0.427     | 0.64      |

Co-cultured SV-PDL control

| Rank | Methods | Ranking | Stability | Rank | Stability | SD (± Cq) | CV (% Cq) |
|------|---------|---------|-----------|------|-----------|-----------|-----------|
| (1)  | PPIB    | 9       | RPLP0 0.308 | PPIB 0.004 | RPLP0 0.11 | 0.998     | 0.65      |
| (2)  | POLR2A  | 11      | PPIB 0.309  | POLR22 0.004 | PPIB 0.52 | 0.983     | 0.68      |
| (3)  | RPLP0   | 12      | POLR2A 0.378 | POLR2A 0.009 | RPL22 0.62 | 0.980     | 0.53      |
| (4)  | RPL22   | 12      | GUSB 0.390  | GUSB 0.011 | POLR2A 0.74 | 0.973     | 0.47      |
| (5)  | GUSB    | 16      | RPL22 0.732  | RPLP0 0.023 | GUSB 0.78 | 0.700     | 0.09      |

Co-cultured SV-PDL with 3 indicated time (n = 18)

| Rank | Methods | Ranking | Stability | Rank | Stability | SD (± Cq) | CV (% Cq) |
|------|---------|---------|-----------|------|-----------|-----------|-----------|
| (1)  | PPIB    | 8       | POLR2A 0.161 | PPIB 0.005 | RPLP0 0.22 | 0.980     | 0.31      |
| (2)  | POLR2A  | 10      | GUSB 0.165  | RPLP0 0.015 | PPIB 0.42 | 0.823     | 0.5       |
| (3)  | RPLP0   | 11      | RPLP0 0.214 | GUSB 0.016 | POLR2A 0.60 | 0.774     | 0.54      |
| (4)  | GUSB    | 12      | PPIB 0.218  | POLR2A 0.017 | GUSB 0.60 | 0.746     | 0.57      |
| (5)  | RPL22   | 19      | RPL22 0.422  | RPL22 0.019 | RPL22 0.74 | 0.646     | 0.19      |

A higher rank denotes lower expression stability. Individual primer efficiency was taken in to account by the abbreviations of Cq (threshold cycle), SD (standard deviation), CV (coefficient of variation) and r (Pearson's correlation coefficient). The genes are ordered form the highest to the lowest ranking.
Results

1. Expression levels of candidate reference genes in mono cell culture

For OCCM-30 cells, as showed in Fig. 1A, the β-actin, GAPDH, EEF1A1 and RPLP0 are the candidate reference genes most abundantly expressed with Cq values below 2. The genes TBP, RPL22, PPFB, YWHAZ, POLR2A, GLUSB, UBC and RPL are all moderately expressed with Cq values ranging from 20 to 30. Due to the cut-off applied, 10 out of 12 measurements for TUBB (Cq values 37.17) in the mono-cultured OCCM-30 dataset are removed from Fig. 1A.

For SV-PDL cells, Fig. 1B shows that the β-actin, GAPDH, EEF1A1 and RPLP0 are the candidate reference genes most abundantly expressed with Cq values below 20. The genes TBP, RPL22, PPFB, YWHAZ, POLR2A, TUBB, GLUSB, UBC and RPL are all moderately expressed with Cq values ranging from 20 to 30 in Fig. 1B.

2. Stability analysis of candidate reference genes in mono cell culture system

For studies with monocultured cementoblasts, total ranking results in Table 1 show that RPL22 is the least regulated reference gene in the preselected panel on OCCM-30 cells. Similarly, although PPFB is not as stable as RPL22, it ranks higher than POLR2A in all calculation in cementoblasts as showed in Fig. 1C.

It is revealed that GLUSB reached the best stability values on monocultured SV-PDL cells. The geNorm and the ΔCq method in Fig. 1E show the same results.

In this case, GLUSB ranked highest in the comparison, but GAPDH was less stable compared to RPLP0. The geNorm analysis revealed that the use of two reference genes in this case GLUSB and GAPDH for normalization in RT-qPCR is adequate for studies in monoculture of OCCM-30 cells (Fig. 1D) and SV-PDL cells (Fig. 1F). Notably, the output results of geNorm in the selection study showed that the M values of TUBB and RPL in OCCM-30 cells and RPL in SV-PDL cells were missing, indicating their exclusion for further analysis. The ranking order and the stability values calculated with the geNorm and NormFinder programs did not change when TUBB was excluded from the database.

In concordance with the above given results, PPFB, GLUSB, RPLP0, POLR2A and RPL22 were selected as the most five stable reference genes based on ranking sum for further analysis in the direct cell–cell contact and co-culture system.

3. Stability analysis of 5 chosen reference genes in direct cell–cell contact culture and co-culture system

According to the single cell-culture results, the three highest ranking genes were selected for each cell line, respectively. These were GLUSB, POLR2A, RPLP0, RPL22 and PPFB. The entire ranking shows that GLUSB, POLR2A and RPLP0 were the least regulated reference genes when both cell lines were cultivated with direct contact. The suitable number of reference targets in the direct cell–cell contact experimental situation was 2. As such, the suitable normalization factor can be calculated as the geometric mean [21] of reference targets GLUSB and POLR2A for the direct cell–cell contact culture system (Table 1, Fig. 2B, C).

(See figure on next page.)

Fig. 1 A Cq values are presented as quantification cycle (Cq, n = 3) as second derivate maximum of the fluorescence curve and are inversely proportional to the amount of target mRNA within 1 µg of total RNA retrieved from the cementoblasts. Cq expression values of candidate reference genes, overall are for specimens without treatment (n = 13*3 duplication) in cementoblasts. B Expression levels of candidate reference genes in periodontal ligament cells (n = 13*3 duplication) without treatment. Cq values exported with identical threshold setting (mean of three technical replicates). Boxplots represent the median (central horizontal line), the interquartile range (IQR, 25/75 quartile, box) and the data range (whiskers) without outliers and extreme values. Outliers and extreme values are defined as Cq values more than 1.5 and 3 times the IQR apart from the upper/lower quartile and are denoted as circles and asterisms respectively. C On OCCM-30 cells, the geNorm analysis of the expression stability values (M value) of the 13 candidate reference genes, for which specific primers could be constructed. Average expression stability values of overall (pooled) specimens derived by stepwise exclusion of the least stable reference gene across all specimens and experiment conditions (n = 13*3 duplication). A smaller M value indicates a more stable expression. The most stable genes are on the right and the least stable genes are on the left. D Pairwise variation (V) of the 13 candidate reference genes calculated by geNorm to determine the suitable number of reference genes for OCCM-30 cells for RT-qPCR data normalization in overall studies (n = 13*3 duplication). The threshold used was 0.15. E On periodontal ligament cells, the geNorm analysis of the expression of the 13 candidate reference genes tested. Overall average expression stability values (M) derived by stepwise exclusion of the least stable reference gene across all specimens and experiment conditions. A higher M indicates a less gene expression. F Determination of the suitable number of reference genes for RT-qPCR data normalization on periodontal ligament cells. The geNorm calculation by the pairwise variation (V) indicates that V values lower than 0.15 indicated a sufficient normalization can be achieved.
Fig. 1 (See legend on previous page.)
As analyzed using different incubation times during the same day in the indirect co-culture systems, results showed that across three different time period, PPIB, GUSB and RPLP0 have the best stability values for co-cultured OCCM-30 cells. The PPIB, POLR2A and RPLP0 reached the best stability values for co-cultured SV-PDL cells. geNorm analysis shows that the suitable number of reference targets in this experimental situation was 2 and can be calculated as the geometric mean of reference targets PPIB and POLR2A (Table 1; Fig. 2D–G). Altogether, PPIB was the most stable reference gene for the co-culture system.

Discussion

The in-vitro co-culture model is based on the location of OCCM-30 and SV-PDL cells which was subjected to mimicked specific conditions during OTM. The porosity membrane allows the cells to exchange soluble factors in the co-culture setup (Fig. 2A). Thus, the co-culture system seems reasonable to investigate the intercellular communication between these two cell lines of the periodontal compartment which become closer when orthodontic force is applied [10, 25]. However, reference gene selection is depending on the exact research questions and thus applies to both the experimental condition and the corresponding control, in this sense, the suitable reference genes in hypoxic- or orthodontic force-induced conditions need to be further investigated [25].

In the present work, from our analysis PPIB achieved the most stable results in all four algorithms methods for the use as a reference gene according to the comparison of all potential reference genes in co-culture system at different time point in the same day. This matches with previous research's publication analyzing the combined dental, periodontal and alveolar bone tissue of rat which showed that PPIB and YWHAZ were the most stable reference genes for RT-qPCR analysis in untreated rats with additional periodontitis [26]. PPIB is also reported to have the highest expression stability values and reliability on hPDLF subjected to static mechanical strain [27]. Besides PPIB, GUSB and RPLP0 were ranked as the most stable reference genes for co-cultured OCCM-30 cells at different time periods. This is in concordance with the ranking in the control group, indicating that these three highest ranking genes are stably for the co-cultured OCCM-30 cells. Similarly, PPIB, POLR2A and RPLP0 were recommended as the three most stable reference genes for co-cultured SV-PDL cells at different time points within the same day.

Direct cell–cell contact culture is more closely to the in-vivo condition that both cell types are cultivated together. However, it would be difficult to compare the gene expression in the direct cell–cell contact culture compared to the mono-cultured cells. Thus, for the purpose, discrimination of two types of cells using special surface markers assessed by flow cytometry would be more accurate to provide separate results of the different reference genes. Besides, for the indirect co-culture system, the Cq values expression of the reference genes may be changed dependent upon the specific placement of cells within the experimental setup. Although this study did not specifically refer to this issue, one might speculate that each cell type exerts different on the other when the position of two types of cells changed as shown in Fig. 2A. Furthermore, in the present proposed setup, the cells of two types in the insert and on the bottom of the wells are < 1 mm apart. Therefore, it might be necessary to investigate if the magnitude of gravity interferes with diffusion.

We concluded that PPIB, GUSB and RPLP0 are the most stable reference genes for normalization in RT-qPCR studies using OCCM-30 cells in a co-culture system. PPIB, POLR2A and RPLP0 were demonstrated to be the most reliable normalizers for SV-PDL cells used for RT-qPCR gene expression analysis in the co-culture system. The PPIB is an ideal reference and combination of PPIB and POLR2A for RT-qPCR experiments can improve the normalization in co-culture systems.

Limitations

In direct cell–cell contact culture, the gene expression results should be considered as a mean of both cell types, thus it's necessary to separate these two types of cells using FACS by their special membrane marker to provide separate results of reference genes. Different orthodontic induced conditions such as hypoxia and

(See figure on next page.)

**Fig. 2** A Schematic representation of the co-culture system used in the experiment with SV-PDL cells plated on the upper insert and OCCM-30 cells in the lower well. B, D, F The stability of top four reference genes was assessed by geNorm (n = 15) for both the direct cell–cell contact culture system (B) and the co-culture system (D,F). Lower M value predicts higher stability. C, E, G The suitable number of reference genes was determined by geNorm for both direct cell–cell contact culture system (C) and the co-culture system (E,G). The value of V less than the recommended cut-off of 0.15 is attained with two reference genes.
Fig. 2 (See legend on previous page.)
mechanical forces are not included in the stimulation, which needs to be further investigated.

Abbreviations
Cq: Cycle threshold; FACS: Fluorescence-activated cell sorting; hPDLF: Human periodontal ligament fibroblasts; OCCM-30: Mouse cementoblast; OTM: Orthodontic tooth movement; PBS: Phosphate-buffer saline; RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction; SD: Standard deviation; SV-PDL: Periodontal ligament.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13104-022-05948-x.

Acknowledgements
We sincerely thank Prof. J. Deschner and Dr. M. Nokhbehserm (Department of Periodontology, University of Bonn, Germany) to facilitate the sending of OCCM-30 cells and Prof. M. Somerman, (Laboratory of Oral Connective Tissue Biology, NIH, Bethesda, MD, USA) to kindly provide the SV-PDL cells. We also thank the China Scholarship Council (CSC) for Ph.D. financial support.

Authors’ contributions
JWY acquired and analyzed the data. JWY, GR-H and SG interpreted the data and wrote the manuscript. JWY, GR-H, SR conceived, designed and supervised the study. All authors read and approved the final manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Department of Orthodontics, Faculty of Medicine, Justus Liebig University of Giessen, Schlangenzahl 14, 35392 Giessen, Germany. 2Department of Periodontology, Faculty of Medicine, Justus Liebig University of Giessen, Giessen, Germany.

Received: 15 October 2021 Accepted: 2 February 2022 Published online: 15 February 2022

References
1. Yong J, von Bremen J, Ruiz-Heiland G, Ruf S. Adiponectin interacts in vitro with cementoblasts influencing cell migration, proliferation and cemen
togenesis partly through the MAPK signaling pathway. Front Pharmacol. 2020;11:585346.
2. Jonsson D, Nebel D, Bratthall G, Nilsson BO. The human periodontal ligament cell: a fibroblast-like cell acting as an immune cell. J Periodontal. 2011;46(2):153–7.
3. Bustin SA, Benes V, Garson JA, Hellmанс J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaff M, Shipley GL, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611–22.
4. Adamski MG, Gumann P, Baird AE. A method for quantitative analysis of standard and high-throughput qPCR expression data based on input sample quantity. PloS ONE. 2014;9(8):e103917.
5. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol. 2002;29(1):23–39.
6. Ma R, Xu S, Zhao Y, Xia B, Wang R. Selection and validation of appropriate reference genes for quantitative real-time PCR analysis of gene expression in Lycoris aurea. Front Plant Sci. 2016;7:536.
7. Pfaff M, Hagele T. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-qPCR. Biotechnol Lett. 2001;23(4):275–82.
8. Kats A, Gerasimcik N, Nareoja T, Niederberg J, Grenlov S, Lagnohed E, Desai S, Andersson G, Yucel-Lindberg T. Aminothiazoles inhibit osteoclastogenesis and PGE2 production in LPS-stimulated co-cultures of periodontal ligament and RAW 264.7 cells, and RANKL-mediated osteoclastogenesis and bone resorption in PBMCs. J Cell Mol Med. 2019;23(2):1152–63.
9. Shi J, Baumert U, Folwaczny M, Wichelhaus A. Influence of static forces on the expression of selected parameters of inflammation in periodontal ligament cells and alveolar bone cells in a co-culture in vitro model. Clin Oral Investig. 2019;23(6):2617–28.
10. Shi J, Folwaczny M, Wichelhaus A, Baumert U. Differences in RUNX2 and P2RX7 gene expression between mono- and coculture of human periodontal ligament cells and human osteoclasts under compressive force application. Orthod Craniofac Res. 2019;22(3):168–76.
11. Steller D, Scheibert A, Sturmheit T, Hakim SG. Establishment and validation of an in vitro co-culture model for oral cell lines using human PBMC-derived osteoclasts, osteoblasts, fibroblasts and keratinocytes. Sci Rep. 2020;10(1):16861.
12. Yong J, von Bremen J, Groeger S, Ruiz-Heiland G, Ruf S. Hypoxia-inducible factor 1-alpha acts as a bridge factor for crosstalk between ERK1/2 and caspases in hypoxia-induced apoptosis of cementoblasts. J Cell Mol Med. 2021;25:9710–23.
13. Yong J, von Bremen J, Ruiz-Heiland G, Ruf S. Adiponectin as well as compressive forces regulate in vitro beta-catenin expression on cementoblasts via mitogen-activated protein kinase signaling activation. Front Cell Dev Biol. 2021;9:645005.
14. Kirschneck C, Batschkus S, Pfof P, Kostler J, Spanier G, Schroder A. Valid gene expression normalization by RT-qPCR in studies on hPDL fibroblasts with focus on orthodontic tooth movement and periodontitis. Sci Rep. 2017;7(1):14751.
15. Niedere A, Craveiro RB, Azaraj I, Brockhaus J, Bastian A, Kirschneck C, Wolf M. Selection and validation of reference genes by RT-qPCR for murine cementoblasts in mechanical loading experiments simulating orthodontic forces in vitro. Sci Rep. 2020;10(1):10893.
16. D’Errico JA, Berry JE, Ouyang H, Strayhorn CL, Windle JJ, Somerman MJ. Employment of a transgenic animal model to obtain cementoblasts in vitro. J Periodontol. 2000;71(1):63–72.
17. Hakki SS, Wang D, Franchischi RT, Somerman MJ. Bone sialoprotein gene transfer to periodontal ligament cells may not be sufficient to promote mineralization in vitro or in vivo. J Periodontol. 2006;77(2):167–73.
18. Mercado KP, Helguera M, Hocking DC, Dalecki D. Estimating cell concentration in three-dimensional engineered tissues using high frequency quantitative ultrasound. Ann Biomed Eng. 2014;42(6):1292–304.
19. Bloemen V, Schoenmaker T, de Vries Tj, Everts V. Direct cell-cell contact between periodontal ligament fibroblasts and osteoblast precursors synergistically increases the expression of genes related to osteoclas
togenes. J Cell Physiol. 2010;222(3):565–73.
20. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Ribenge FS, Olsvik PA, Penning LC, Toegel S. MIQE precis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol. 2010;11:74.
by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):RESEARCH0034.

22. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64(15):5245–50.

23. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett. 2004;26(6):509–15.

24. Silver N, Cotroneo E, Proctor G, Osaaln S, Paterson KL, Carpenter GH. Selection of housekeeping genes for gene expression studies in the adult rat submandibular gland under normal, inflamed, atrophic and regenerative states. BMC Mol Biol. 2008;9:64.

25. Yong J, Elisabeth Groeger S, Ruf S, Ruiz-Heiland G. Influence of leptin and compression in GAS-6 mediated homeostasis of periodontal ligament cell. Oral Dis. 2021. https://doi.org/10.1111/odi.14092.

26. Kirschneck C, Proff P, Fanghanel J, Wolf M, Roldan JC, Romer P. Reference genes for valid gene expression studies on rat dental, periodontal and alveolar bone tissue by means of RT-qPCR with a focus on orthodontic tooth movement and periodontitis. Ann Anat. 2016;204:93–105.

27. Nazet U, Schroder A, Spanier G, Wolf M, Proff P, Kirschneck C. Simplified method for applying static isotropic tensile strain in cell culture experiments with identification of valid RT-qPCR reference genes for PDL fibroblasts. Eur J Orthod. 2020;42(4):359–70.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.