Housekeeping gene validation for RT-qPCR studies on synovial fibroblasts derived from healthy and osteoarthritic patients with focus on mechanical loading

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Abstract

Selection of appropriate housekeeping genes is essential for the validity of data normalization in reverse transcription quantitative PCR (RT-qPCR). Synovial fibroblasts (SF) play a mediating role in the development and progression of osteoarthritis (OA) pathogenesis, but there is no information on reliable housekeeping genes available. Therefore the goal of this study was to identify a set of reliable housekeeping genes suitable for studies of mechanical loading on SF from healthy and OA patients. Nine genes were evaluated towards expression stability and ranked according their relative stability determined by four different mathematical procedures (geNorm, NormFinder, BestKeeper and comparative ΔCq). We observed that RPLP0 (ribosomal protein, large, P0) and EEF1A1 (eukaryotic translation elongation factor 1 alpha 1) turned out to be the genes with the most stable expression in SF from non-OA or OA patients treated with or without mechanical loading. According to geNorm two genes are sufficient for normalization throughout. Expression of one tested target gene varied considerably, if normalized to different candidate housekeeping genes. Our study provides a tool for accurate and valid housekeeping gene selection in gene expression experiments on SF from healthy and OA patients with and without mechanical loading in consistent with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines and additionally demonstrates the impact of proper housekeeping gene selection on the expression of the gene of interest.

Introduction

For gene expression analysis there are currently three methods available: RNA-Seq, microarray analysis and RT-qPCR. RNA-Seq and microarray analysis were used to analyse many different gene expression profiles [1–5]. In contrast to these both methods reverse transcriptase quantitative PCR (RT-qPCR) allows to analyse the impact of various experimental conditions on the
expression of one single gene [6–9]. Therefore RT-qPCR is still the method of choice for gene expression evaluation in most areas of molecular biology. Carefully chosen housekeeping genes guarantee precise gene expression quantification by accurate and valid data normalization [6,8,10]. One major feature of an optimal housekeeping gene is a small variation in expression across various experimental setups and cell types [8,11]. There are many studies dealing with the variation of expression stability of housekeeping genes across different experimental conditions or cell types [11,12]. The usage of not validated housekeeping genes in RT-qPCR studies can lead to potential bias and misinterpretation of experimental outcomes. To achieve conformity in RT-qPCR data evaluation, MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) were established several years ago [10]. Usage of these guidelines is proposed to enhance reproducibility of RT-qPCR results [10]. The choice of housekeeping genes has a direct impact on the results of target gene analysis by RT-qPCR. To investigate the stability of different housekeeping genes, some mathematical algorithms like geNorm [12], NormFinder [13], BestKeeper [14] and the comparative $\Delta C_q$ method [15,16] were developed.

The geNorm [12] algorithm calculates the mean pairwise variation of the $C_q$ values of one tested housekeeping gene compared with all other tested genes and specifies that value as stability M. Housekeeping genes with elevated M values are suggested to have a higher pairwise variation. As enhanced pairwise variation correlates with unstable expression ratios, housekeeping genes with higher M values are not ideal for normalization [12]. With geNorm it is also possible to define the minimum required amount of housekeeping genes for target gene normalization, as it computes the gene stability by average pairwise variation among internal control genes [12].

The NormFinder algorithm [13] calculates intra- and intergroup variation between the tested housekeeping genes. Furthermore it designates a conjoint stability value for each tested housekeeping gene applying a model-based approach [13]. In this mathematical algorithm increased gene expression stability is associated with decreasing stability values.

The comparative $\Delta C_q$ method evaluates housekeeping genes on the basis of the standard deviation of the average $\Delta C_q$ aberrations of each tested housekeeping gene to all other tested housekeeping genes. Therefore it collates relative housekeeping gene expression within groups of biological replicates and from all other tested housekeeping genes [15,16].

The BestKeeper [14] algorithm makes use of the standard deviation of mean $C_q$ of each tested housekeeping gene and evaluates gene stability by pairwise bivariate correlations of $C_q$ values of each gene using a "BestKeeper Index". Stably expressed housekeeping genes are reported to have higher r values. Until now various studies were performed to assess appropriate housekeeping genes for different experimental setups and cell types, but there are still no reliable housekeeping genes for studies on human synovial fibroblasts published.

Synovial fibroblasts play a leading part in the maintenance of a healthy joint. The synovium encases articular joints throughout the human body and maintains the integrity of articular cartilage by regulating synovial fluid volume and composition producing lubricin and hyaluronic acid [17]. Among others, synovial fibroblasts make up the major cell population in the synovium of joints and play a critical mediating role in the development and progression of osteoarthritis (OA), since they are able to secrete proinflammatory cytokines [18] and express immune-receptors like toll-like receptors (TLR) [19,20]. The degenerative disease OA is associated with several afflictions such as chronic pain, articular cartilage degradation and subchondral bone remodelling as well as induced synovitis [21]. Aging, obesity, sport injuries, genetic predisposition [22] and mechanical overload [23,24] are reported to be risk factors for OA development. Also excessive mechanical loading on normal articular cartilage may lead to the development of OA by disruption of cartilage matrix homeostasis [24,25]. For several
years, interest in the stromal-cell-like synovial fibroblasts has increased, as they were identified as key players in the innate immune-system response, inflammation-related processes and intercellular actions and as principal performers involved in OA development and progression.

The study was designed to 1) identify a set of stably expressed housekeeping genes for human synovial fibroblasts derived from OA and non-OA patients, particularly in experiments with mechanical loading simulating OA pathogenesis and progression, 2) to assess the effects of different housekeeping genes used for normalization on target gene expression and 3) to compare various mathematical procedures used regarding their conformity. As mechanical stress loading is one major risk factor for the development of OA, we aimed to assess stable housekeeping genes for pressure application in both tested synovial fibroblast cell lines.

**Materials and methods**

**In vitro cell culture experiment setup**

Synovial fibroblasts from a healthy, non-OA patient were obtained directly from BioIVT (PCD-90-0645). Synovial fibroblasts from an OA patient were obtained and cultured from tissue to be discarded during knee surgery in the Department of Orthopedics at the University of Regensburg. The study was approved by the Ethics Committee of the Faculty of Medicine Regensburg (approval ID 12-170-0150) and written informed consent was obtained from the tissue donor.

Approximately 70,000 synovial fibroblasts per well, either derived from a non-OA or an OA patient, were seeded on a 6-well plate and preincubated under cell culture conditions for 24 h. Afterwards incubation was continued for another 48 h with or without mechanical loading according to an established and published model for inducing compressive force on adherently growing fibroblasts [8,26,27]:

- 1st group: synovial fibroblasts from a healthy, non-OA patient (N-SF), incubated under cell culture conditions for a total of 72 h (n = 6);
- 2nd group: synovial fibroblasts from a healthy non-OA patient (N-SF) exposed to static compressive force (2 g/cm² pressure, Fig 1) for 48 h after 24 h of preincubation (n = 6);

![Image](https://doi.org/10.1371/journal.pone.0225790.g001)

**Fig 1. In vitro application of mechanical loading to synovial fibroblasts.** Application of a sterile glass disc of defined size and weight to the cell layer, exerting a compressive force of 2g/cm².

Table 1. Design of the experiment setup.

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition</th>
<th>Duration (h)</th>
<th>Condition Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st group</td>
<td>1st group</td>
<td>72</td>
<td>Incubated under cell culture conditions for a total of 72 h (n = 6)</td>
</tr>
<tr>
<td>2nd group</td>
<td>2nd group</td>
<td>48</td>
<td>Incubated under cell culture conditions for the first 24 h followed by mechanical loading for 24 h (n = 6)</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0225790.g001
• 3rd group: synovial fibroblasts from an OA patient (OA-SF), incubated under cell culture conditions for a total of 72 h (n = 6);
• 4th group: synovial fibroblasts from an OA patient (OA-SF) exposed to static compressive force (2 g/cm² pressure, Fig 1) for 48 h after 24 h of preincubation (n = 6).

RNA isolation and purity determination
In order to remove residual cell culture media, synovial fibroblasts were washed two times with phosphate-buffered saline (PBS). RNA was isolated using peqGOLD TriFast™ (PEQLAB, 1 ml/well conforming with the manufacturer’s instructions [9,28,29]. The resulting RNA pellet was reconstituted in 25 μl nuclease-free water (Carl-Roth) and immediately cooled on ice. Photometrical adsorption measurements at 280nm and 260nm revealed purity and quantity of the eluted RNA (NanoDrop, Implen). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies) based on the provided protocol of the manufacturer (S1 File). At least, one sample per group had to be excluded from further analysis due to poor or not measurable RNA integrity values (S2 File).

Reverse transcription (cDNA synthesis)
For cDNA synthesis we transcribed 100 ng RNA per sample using a combination of 0.1 nmol random hexamer primer (Life Technologies), 0.1 nmol oligo-dT18 primer (Life Technologies) mixed with 1×M-MLV-buffer (Promega), 40 nmol dNTP mix (Carl-Roth), 40 U RNase inhibitor (Life Technologies) and M-MLV reverse transcriptase (Promega) and added nuclease-free H₂O (Carl-Roth) to a final volume of 20 μl. We then incubated the samples at 37˚C for 1 h and finally inactivated the reverse transcriptase at 95˚C for 2 min. Experimental variation was reduced by simultaneous synthesis of cDNA for all samples.

Real-time quantitative RT-PCR
The used oligonucleotides were designed based on the gene sequences achieved from the Nucleotide database NCBI (GeneBank, National Centre for Biotechnology Information) and validated for absence of secondary structures, self and cross dimers as well as primer efficiency and specificity, as already described [8] (Table 1). Eurofins MWG Operon LLC (Huntsville; High Purity Salt Free Purification HPSF®) was assigned for primer synthesis and purification.

For each RT-qPCR reaction we used 7.5 μl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 10 pmol/μl of the respective forward and reverse primer and 1.5 μl of the diluted cDNA (1:10). RNase-free H₂O (Carl-Roth) was added to a total volume of 15 μl. All cDNA samples were tested as three replicates per housekeeping gene and on the same 96 well PCR plate per biological replicate in 45 cycles (95˚C for 5 min, per cycle 95˚C for 10 s, 60˚C for 8 s, 72˚C for 8 s) to reduce possible inter-run variations on relative housekeeping gene stability assessment. Non-template controls and reverse transcription controls were additionally performed. For qPCR a Mastercycler® ep realplex-S thermocycler (Eppendorf AG, Hamburg, Germany) was used in conjunction with 96-well PCR plates (Biozym Scientific) covered with BZO Seal Filmcover sheets (Biozym Scientific) [8,30,31].

Assessment of reference gene stability
We calculated C_q values with the realplex software (version 2.2, Eppendorf AG) using the CalqPlex algorithm. The arithmetic mean of each C_q triplet per tested housekeeping gene and
<table>
<thead>
<tr>
<th>Gene acronym</th>
<th>Gene name (Homo sapiens)</th>
<th>Gene function</th>
<th>Accession Number (NCBI GeneBank)</th>
<th>Chromosomal location (length)</th>
<th>5´-forward primer-3´ (length / %GC / max. Tm / SSAT)</th>
<th>5´-reverse primer-3´ (length / %GC / max. Tm / SSAT)</th>
<th>Primer Location (max. AG Cross Dimer)</th>
<th>Amplicon (length, %GC, Tm, SSAT)</th>
<th>Amplicon location (bp of Start/Stop)</th>
<th>Intron- flanking (length)</th>
<th>In silico qPCR specificity</th>
<th>Variants targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEF1A1</td>
<td>eukaryotic translation elongation factor 1 alpha 1</td>
<td>enzymatic delivery of aminoacyl-tRNAs to ribosomes</td>
<td>NM_001402.5 (3528bp)</td>
<td>CCTGCCTCTCCAGGATGTCTAC (280bp / 59.4% / 59.1% / -3.0 / 5)</td>
<td>GGAGACAAAGGTACACCATAC (280bp / 62.1% / 54.6% / -3.2 / 2)</td>
<td>exon 5 / 6 (2.9)</td>
<td>105bp, 52.8%, 86.5°C, no SSAT</td>
<td>804/908</td>
<td>Yes (87bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
</tr>
<tr>
<td>GABPD</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>enzyme in glycolysis and gluconeogenesis</td>
<td>NM_002046.5 (1421bp)</td>
<td>TGCTGCTCTCCAGGATGTCTAC (220bp / 59.4% / 59.1% / -0.7 / 3)</td>
<td>GGAGCAAAGGTACACCATAC (280bp / 62.1% / 54.6% / -3.2 / 2)</td>
<td>exon 8 / 9 (2.4)</td>
<td>74bp, 50.0%, 86.0°C, no SSAT</td>
<td>1091/1104</td>
<td>Yes (104bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
</tr>
<tr>
<td>POLR2A</td>
<td>polymerase (RNA) II (DNA directed)</td>
<td>transcription of DNA into mRNA</td>
<td>NM_000937.4 (220bp)</td>
<td>TCCTCAAACTCCAAAAGACCAGTG (21bp / 61.9% / 52.4% / -3.3 / 4)</td>
<td>AGTGCTTGATGATCTGCTG (280bp / 59.8% / 55.0% / -3.0 / 2)</td>
<td>exon 23 / 24 (2.1)</td>
<td>108bp, 53.7%, 87.8°C, no SSAT</td>
<td>3798/3905</td>
<td>Yes (468bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<td>PPB</td>
<td>peptidylprolyl isomerase A (cyclophilin B)</td>
<td>ER, cyclosporine-binding protein in eukaryotic cells</td>
<td>NM_000942.4 (1869bp)</td>
<td>AAGAAGATGATGATCTGCTG (21bp / 61.9% / 52.4% / -3.3 / 4)</td>
<td>AGTGCTTGATGATCTGCTG (280bp / 59.8% / 55.0% / -3.0 / 2)</td>
<td>exon 3 / 4 (2.1)</td>
<td>88bp, 53.4%, 86.1°C, no SSAT</td>
<td>416/533</td>
<td>Yes (3194bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>RNA18S</td>
<td>18S ribosomal RNA, translation of mRNA in protein</td>
<td></td>
<td>NR_003286.2 (1869bp)</td>
<td>CCGCCCGTCGGCATGTATTAG (19bp / 57.1% / 39.1% / -1.7 / 6)</td>
<td>GCCCGTCGGCATGTATTAG (19bp / 57.1% / 39.1% / -1.7 / 6)</td>
<td>exon 1 / 2 (1.5)</td>
<td>98bp, 44.9%, 83.8°C, no SSAT</td>
<td>115/212</td>
<td>Yes (4597bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>RPL22</td>
<td>ribosomal protein L22</td>
<td>translation of mRNA in protein</td>
<td>NM_000983.3 (2099bp)</td>
<td>GTGACACCATACCCTGTA (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>GGTTCACACCTTTCCGCTCT (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>exon 2 / 3 (1.5)</td>
<td>98bp, 44.9%, 83.8°C, no SSAT</td>
<td>115/212</td>
<td>Yes (4597bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
</tr>
<tr>
<td>RPLP0</td>
<td>ribosomal protein L0p, P0</td>
<td>translation of mRNA in protein</td>
<td>NM_003002.3 (1229bp)</td>
<td>GAACACTTGCATCTGCGTTC (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>GGTTCACACCTTTCCGCTCT (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>exon 6 / 7 (1.8)</td>
<td>120bp, 50.8%, 86.5°C, no SSAT</td>
<td>803/921</td>
<td>Yes (1091bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-box binding protein</td>
<td>general transcription factor</td>
<td>NM_003944.4 (2191bp)</td>
<td>GGGCGGTTACAAGCGGCTTC (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>GGTTCACACCTTTCCGCTCT (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>exon 12 / 13 (2.4)</td>
<td>88bp, 51.2%, 86.5°C, no SSAT</td>
<td>79/164</td>
<td>Yes (2483bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>YWHAZ</td>
<td>tryptophan 5-monooxygenase activation protein, zeta</td>
<td>signal transduction</td>
<td>NM_003406.3 (3003bp)</td>
<td>AAGGAGATTACATTGACCTTGG (248bp / 66.0% / 48.0% / 0.0 / 4)</td>
<td>AGTTTCTGGATGATCTGCTG (280bp / 59.8% / 48.0% / 0.0 / 4)</td>
<td>exon 4 / 5 (2.2)</td>
<td>98bp, 47.3%, 84.0°C, no SSAT</td>
<td>304/572</td>
<td>Yes (617bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>COX2</td>
<td>cyclooxygenase 2</td>
<td>prostaglandin synthase</td>
<td>NM_000963.3 (2515bp)</td>
<td>GAGCAGGACGAAATATAGCCATG (280bp / 64.0% / 50.0% / 0.0 / 2)</td>
<td>GTGACACCATACCCTGTA (280bp / 59.4% / 59.1% / -3.0 / 4)</td>
<td>exon 4 / 5 (3.2)</td>
<td>131bp, 42.0%, 82.9°C, no SSAT</td>
<td>1417/1587</td>
<td>Yes (486bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>IL6</td>
<td>interleukin 6</td>
<td>proinflammatory cytokine</td>
<td>NM_000600.3 (1201bp)</td>
<td>TGGCAAAACACCTGAAGCAAC (280bp / 64.0% / 48.7% / -1.3 / 3)</td>
<td>CCGCAAACTCCCAAGACCCAGT (280bp / 64.0% / 48.7% / -1.3 / 3)</td>
<td>exon 2 / 3 (1.5)</td>
<td>117bp, 43.6%, 83.7°C, no SSAT</td>
<td>370/486</td>
<td>Yes (704bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<td>COL1A2</td>
<td>collagen, type 1, alpha 2</td>
<td>extracellular matrix protein</td>
<td>NM_000889.3 (1541bp)</td>
<td>AGAAAGACACCTGCGTACAG (280bp / 64.0% / 52.4% / -3.4 / 4)</td>
<td>GGTTCACACCTTTCCGCTCT (280bp / 59.4% / 55.0% / -3.0 / 4)</td>
<td>exon 5 / 6 (1.7)</td>
<td>108bp, 44.8%, 83.3°C, no SSAT</td>
<td>4139/4248</td>
<td>Yes (730bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
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<tr>
<td>P4HA1</td>
<td>prolyl 4-hydroxylase, alpha polypeptide 1</td>
<td>key enzyme in collagen synthesis</td>
<td>NM_001973 (2800bp)</td>
<td>GGTTCCTGGATGCGATTTGG (235bp / 66.0% / 47.8% / 0.0 / 2)</td>
<td>GTGACACCATACCCTGTA (280bp / 59.4% / 55.0% / -3.0 / 4)</td>
<td>exon 13 / 14 (0.9)</td>
<td>140bp, 41.9%, 82.2°C, no SSAT</td>
<td>1306/1541</td>
<td>Yes (1337bp)</td>
<td>Yes</td>
<td>(BLAST/UCSC)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

ER = endoplasmic reticulum; Tm = melting temperature of primer/specific qPCR product (amplicon); %GC = guanine/cytosine content; bp = base pairs; Comp. = Complementarity; SSAT = secondary structure at annealing temperature.

https://doi.org/10.1371/journal.pone.0225790.t001
sample was used for analysis. The stability for each tested potential housekeeping gene was assessed by four different mathematical procedures: geNorm [12], NormFinder [13], BestKeeper [14] and the comparative $\Delta C_q$ method [16]. We performed stability calculations with the official corresponding Microsoft-Excel-based software applets according to the developers’ instructions. The comparative $\Delta C_q$ method was executed by manual calculations [16]. The application of the geNorm and NormFinder algorithms requires a transformation of native $C_q$ data to the linear scale expression quantities $Q = E^{(C_{qmin} - C_{qsample})}$ computing the qPCR efficiency (E) of each gene [8,9,16]. For evaluation, the tested housekeeping genes were listed based on their stability values (geNorm: M, NormFinder: $\rho_{ig}/\sigma_i$, deltaCT: mean SD of $\Delta C_q$; BestKeeper: Pearson’s r), determined by the chosen algorithms and set up. To assess the optimal number of housekeeping genes for solid RT-qPCR normalization, we used the geNorm [12] algorithm. A gene was considered superfluous for normalization, if the pairwise variation ($V_n/V_{n+1}$) of two pairs of housekeeping genes with one pair including an accessory gene was $\leq 0.15$. By compilation of a bivariate correlation matrix (Pearson’s correlation coefficient r, two-sided, normality confirmed by Shapiro-Wilk tests and histogram evaluation) including the assessed stability values achieved by two respective algorithms, the variations between the tested mathematical procedures were ranked.

**Normalization of target gene expression**

To assess the impact of housekeeping gene stability on relative expression of the target gene prolyl-4-hydroxylase-alpha-1 (P4HA1), collagen-1-alpha-2 (COL1A), cyclooxygenase-2 (COX2) and interleukin-6 (IL6) we determined the relative gene expression as $2^{-\Delta C_q}$ [32] with $\Delta C_q = C_q$ (target gene) – $C_q$ (housekeeping gene), divided by the respective arithmetic $2^{-\Delta C_q}$ mean of the untreated synovial fibroblast controls derived from a healthy subject with their relative gene expression normalized as 1. Using the software application SPSS Statistics 24 (IBM, Armonk, NY, USA), data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene’s test). Experimental groups were compared by Welch-corrected one-way ANOVAs. We used Games–Howell post hoc tests for pairwise comparisons. All differences were considered statistically significant at $p \leq 0.05$. Descriptive statistics are given as arithmetic mean ± standard deviation.

**Results**

**Quality and integrity of RNA samples**

The mean concentration of harvested RNA (n = 20) was assessed by its optical density (260nm) as 25.5 ng/$\mu$l (standard deviation SD 10.8 / Min. 12.1 / Max. 46.4) with a mean $OD_{260nm/280nm}$ ratio of 1.91 (SD 0.14 / Min. 1.82 / Max. 2.21) indicating a negligible contamination with protein (S1 Table). The RNA integrity number (RIN) algorithm allocates a RIN number score from 1 to 10 with a value of 10 representing completely intact RNA and a value of 1 degraded RNA [33]. Three samples displayed poor RIN values and one sample concentration was too low for RIN measurement. These samples were excluded from housekeeping gene analysis. For the other samples RIN values ranged from 9.3 to 9.8 (mean 9.2, SD 0.4), indicating negligible RNA degradation (S1 File). We also confirmed integrity of total RNA by assessing the ratio of 28S/18S ribosomal RNA, which ranged from 1.1 to 2.4 (S1 File). The negative controls (reverse transcription negative control, negative NTC reactions) did not show the presence of interfering genomic DNA and contamination, as the observed $C_q$ values were substantially higher than those of of wells containing samples or reverse transcriptase (S2 Table).
Primer specificity and Cq expression levels

We confirmed primer specificity by agarose gel electrophoresis and melting curve analysis (S2 File). The range of observed Cq values of the tested genes was between 9.0 to 29.4 cycles (Fig 2, S3 Table). RNA18S showed the lowest values and TBP the highest.

Refinement of housekeeping gene number for normalization

For studies with synovial fibroblasts, two housekeeping genes in RT-qPCR in the tested experimental conditions were adequate for normalization according to the geNorm algorithm using their geometric mean (Fig 3a).

Relative performance as housekeeping gene

By using the geNorm algorithm, we found RPLP0 and GAPDH to be the most stably expressed housekeeping genes overall (Table 2, Fig 3b). Similarly, comparing N-SF and OA-SF fibroblasts without mechanical loading with the geNorm algorithm, we determined RPLP0, GAPDH and PPIB to have the lowest expression variation (Table 2). Additionally, we also analysed untreated N-SF and compressive-force-treated N-SF with this algorithm and revealed EEF1A1 and RPLP0 to be the most stably expressed housekeeping genes under these conditions (Table 2).

The NormFinder algorithm identified RPLP0 and EEF1A1 as most stable genes overall (Table 2). NormFinder confirmed the geNorm findings for N-SF and OA-SF fibroblasts without compressive force application and for untreated and loaded N-SF (Table 2), as this algorithm also revealed RPLP0 and PPIB or EEF1A1 and RPLP0 as most stably expressed housekeeping genes.

The comparative ΔCq method [16] was in line with NormFinder results (Table 2) regarding the combined conditions, as it also defined RPLP0 and EEF1A1 to be the most stably expressed
genes. For the other tested conditions, the comparative $\Delta C_q$ method confirmed the findings of NormFinder and geNorm algorithms (Table 2).

The BestKeeper algorithm [14] was also in accordance with NormFinder and the comparative $\Delta C_q$ method suggesting EEF1A1 as the best housekeeping gene overall (Table 2). For experiments regarding N-SF and OA-SF experiments BestKeeper defined RNA18S and TBP as reliable housekeeping genes (Table 2). For N-SF treated with or without compressive force BestKeeper proposed EEF1A1 and TBP as suitable housekeeping genes (Table 2).

Conformity of used algorithms for housekeeping gene stability analysis
We performed bivariate correlations of gene rankings between the used algorithms to assess their conformity (Fig 4, n = 20). geNorm, NormFinder and the comparative $\Delta C_q$ method display significant correlations of the ranking of the tested housekeeping genes. Only BestKeeper algorithm showed discrepancies, as it did not correlate with the other algorithms used (Fig 4).

Effect of housekeeping gene stability on relative target gene expression
To assess the importance of the correct choice of housekeeping genes for experiments on N-SF and OA-SF with and without mechanical loading, we calculated relative gene expression of the target gene prolyl-4-hydroxylase-alpha-1 (P4HA1, Fig 5), collagen-1-alpha-2 (COL1A2), cyclooxygenase-2 (COX2) and interleukin-6 (IL-6; all in S3 File) using the candidate housekeeping genes tested in this study, which differ in expression stability. P4HA1 is responsible for the
Table 2. Housekeeping gene stability ranking for human synovial fibroblast experiments with compressive force application (compressive force vs. untreated control), experiments on osteoarthritis (osteoarthritis vs. normal fibroblasts) and pooled/overall experimental conditions. Calculations based on the algorithms geNorm, NormFinder, comparative ΔCq and BestKeeper. A higher rank indicates reduced expression stability.

<table>
<thead>
<tr>
<th>Rank</th>
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Cq = quantification cycle; SD = standard deviation; CV = coefficient of variation; r = Pearson’s correlation coefficient.

https://doi.org/10.1371/journal.pone.0225790.1002

Proper three-dimensional folding of newly synthesized procollagen chains. The expression levels of P4HA1 varied in a wide range depending on the housekeeping gene used for normalization (Fig 5). We observed significant pressure effects in N-SF with EEF1A1, GAPDH, POLR2A, RNA18S, RPL22, RPLP0 and YWHAZ used for normalization, but using PPIB as housekeeping gene, we determined no significant induction of P4HA1 (Fig 5). Differences between N-SF and OA-SF under physiological conditions seemed to be significant with GAPDH, PPIB and YWHAZ. With all other tested housekeeping genes there were no significant differences in P4HA1 expression between N-SF and OA-SF detectable (Fig 5). Variations between N-SF and OA-SF after compressive force treatment only appeared significant with EEF1A1, GAPDH,
RPLP0, TBP and YWHAZ as housekeeping genes (Fig 5), but not with POLR2A, PPIB, RNA18S or RPL22.

Furthermore, we calculated the statistical differences between results of target gene analysis for P4HA1, COL1A2, COX-2 and IL-6 normalized to the least stable housekeeping gene PPIB and the geometric mean of EEF1A1/RPLP0 as most stable housekeeping genes using an unpaired t test for the different conditions and groups (intragroup comparisons, S4 Table). We determined a significant difference only for IL-6 target gene expression in one experimental condition. Nonetheless, there are differences in significance levels due to normalization with different reference genes (Fig 5, S3 File).

Discussion

We identified RPLP0, EEF1A1, TBP and PPIB to be reliable housekeeping genes for normalization of target gene expression in RT-qPCR studies on human synovial fibroblasts derived from non-OA or OA patients by analysing the expression stability of at least nine potentially suitable housekeeping genes with four different mathematical algorithms. In general RPLP0 and EEF1A1 proved to be most stable throughout all tested experimental conditions and algorithms. As required by the MIQE guidelines, these genes have different functions in cell metabolism, which indicates that they are not co-regulated and may be used in conjunction for normalization of gene expression [10]. RPLP0 is involved in protein synthesis, as this gene encodes for one large 60S acidic ribosomal protein subunit [34]. EEF1A1 encodes for an isoform of the alpha subunit of the elongation factor-1 complex [35], which acts as GTPase and actin-bundling protein [36]. RPLP0, TBP and PPIB seem to be ideal for studies focusing on differences between non-OA and OA synovial fibroblasts. In contrast, PPIB encodes for a protein-binding cyclosporine in the endoplasmic reticulum, which is important in collagen type I folding [37]. PPIB is also associated with pathological conditions potentially affecting osteoarthritis, such as osteogenesis imperfecta [38]. TBP encodes for a TATA-box-binding protein, which is involved in transcription processes by the regulation of the RNA polymerase I [39]. A
Prolyl-4-hydroxylase-alpha-1 mRNA expression relative to

**Geometric mean of EEF1A1/RPLP0**

![Graph showing influence of housekeeping gene choice on fold change expression of P4HA1.](https://doi.org/10.1371/journal.pone.0225790.g005)

Fig 5. Influence of the choice of housekeeping gene used for normalization and its stability on the fold-change expression of P4HA1 in N-SF and OA-SF without and with additional static pressure application. Distinct differences in relative gene expression are evident with significance of pairwise comparisons varying across the individual housekeeping genes used for normalization (n = 6 per group). Statistics: Welch-corrected ANOVA with Games-Howell post-hoc tests.

https://doi.org/10.1371/journal.pone.0225790.g005
set of two internal control genes were determined to be adequate for reliable reference normalization using their geometric mean.

We confirmed high intraassay reliability and precision of the obtained data [40], based on the satisfactory quality of the obtained RNA samples and RT-qPCR analysis. We confirmed protein-free and qualitatively adequate RNA by assessment of the purity and integrity of total isolated RNA, as protein contamination can inhibit cDNA synthesis and qPCR reaction and therefore lead to biased Cq values [41]. We also confirmed primer specificity in silico and in vitro.

Various studies on other tissues and experimental setups exist, which have determined stable housekeeping genes in the used conditions and tissues [42–44]. But most of them used only two algorithms for housekeeping gene stability assessment [11,45]. In this study we used four mathematical procedures and evaluated their conformity to assess, whether a combined usage of these algorithms adds reliability in housekeeping gene stability calculations. NormFinder, geNorm and comparative ΔCq algorithms correlated to a high degree with each other, which was mirrored by similar gene stability rankings. The BestKeeper algorithm on the other hand differed in its assessment from the other procedures. Originally, it was designed to assess general suitability of a housekeeping gene for RT-qPCR in a consecutive two-step assessment based on mean Cq standard deviation and correlation analysis and not to compare possible housekeeping genes. The other algorithms like geNorm or the comparative ΔCq method implement either pairwise comparisons of housekeeping genes with linear quantities or apply an approach of linear quantity models, as it can be seen in the NormFinder algorithm [13].

To assess the impact of choosing appropriate housekeeping genes for normalization, we calculated P4HA1 gene expression normalized to the different candidate housekeeping genes. We observed distinct differences in the significance levels attributable to the relative stability of the respective housekeeping gene used for normalization. These results confirm the importance of housekeeping gene validation and of proper selection of stably expressed housekeeping genes in experiments on N-SF and OA-SF synovial fibroblasts.

**Conclusions**

We identified RPLP0, EEF1A1, TBP and PPIB to be reliable housekeeping genes for normalization of target gene expression in RT-qPCR studies on human synovial fibroblasts derived from non-OA or OA patients by analysing the expression stability of at least nine potentially suitable housekeeping genes with four different mathematical procedures. RPLP0 and EEF1A1 proved to be the most stably expressed housekeeping genes regarding studies on synovial fibroblasts focusing on experimental compressive force loading, whereas RPLP0, TBP and PPIB seem to be ideal for studies focusing on differences between non-OA and OA synovial fibroblasts. For accurate normalization, a set of two housekeeping genes was determined as sufficient, when using their geometric mean.

**Supporting information**

S1 Table. Yield (quantity) and quality of extracted total RNA per biological replicate (well).

(DOCX)

S2 Table. MIQE checklist for authors, reviewers and editors.

(DOCX)
S3 Table. Raw Cq values of RT-qPCR for the two experimental groups and nine potentially suitable housekeeping genes.

S4 Table. Statistical differences given as p values in each experimental group (intragroup comparisons) between results of target gene analysis for P4HA1, COLIA2, COX-2 and IL-6 normalized to a single reference gene and results normalized to the geometric mean of EEF1A1/RPLP0. Statistics: unpaired t-test using GraphPad Prism version 8.0.

S1 File. RNA integrity analysis.

S2 File. Primer specificity evaluation via (a) agarose gel and (b) melting curve analysis of all used samples (left) and a single sample (right).

S3 File. Influence of the choice of housekeeping gene used for normalization on the fold-change expression of COLIA2, COX2 and IL6 in N-SF and OA-SF without and with additional static pressure application.

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Author Contributions

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Investigation: Ute Nazet.

Supervision: Peter Proff, Christian Kirschneck.

Writing – original draft: Ute Nazet, Agnes Schröder, Christian Kirschneck.

Writing – review & editing: Susanne Grässel, Dominique Muschter, Peter Proff.

References


