Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v46i1.68465

Alteration of housekeeping genes and proteins through senescence of human skin fibroblast cells

Beyza Goncu[1*], Omer Faruk Duzenli[2] and Emrah Yucesan[3,4]

[1]Department of Medical Services and Techniques, Vocational School of Health Services, Bezmialem Vakif University, Sultangazi Ilhan Varank Yerleskesi, 34265, Istanbul, Turkey. [2]Experimental Research Center, Bezmialem Vakif University, Istanbul, Turkey. [3]Department of Medical Biology, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey. [4]Department of Neurogenetics, Institute of Neurological Sciences, Istanbul University-Cerrahpasa, Istanbul, Turkey. *Author for correspondence. E-mail: bgoncu@bezmialem.edu.tr

ABSTRACT. The expression levels of certain genes or proteins may exhibit various features regardless of the tissue type. The housekeeping genes and reference proteins are often used during the normalization of expression studies. Herein, we aim to determine and validate the most stable reference gene and protein for a healthy human fibroblast cell line. This study used a finite fibroblast cell line derived from healthy human skin (CCD1079Sk). The samples between 25 and until senescence occurs at passage 55 were collected. Morphological changes during aging by cultivation were observed in each passage. Seven genes (ACTB, SDHA, GAPDH, RPLP0, VCL, TBP, 18S) were evaluated during cultivation and ranked according to their stability via the RefFinder interface. This study reports strong variations for both gene and protein expression studies and the morphology of the cells. Notably, during cultivation, the cell morphology showed an irreversible change starting from passage 43, before the known replicative lifespan of CCD1079Sk. The lack of an RPLP0 subunit after passage 39 may limit the cultivation range when protein expression is required. GAPDH is also an ideal reference gene and protein when studies include gene and protein expression. Or, as a second option, Vinculin may be the first choice when mRNA expression is required, and β-Actin is the best choice for reference protein when the studies include only protein expression. Plus, such as SDHA, RPLP0, and TBP should be avoided. These results will benefit further studies, including normalization for both gene, protein, and cell type.

Keywords: Housekeeping gene; Housekeeping protein; CCD1079Sk; Vinculin; GAPDH; β-actin.

Received on June 04, 2023
Accepted on March 15, 2024

Introduction

Housekeeping gene selection is always challenging for humans and other life forms (Sinha, Saxena, Singh, Krishnamurthy, & Varshney, 2015). This challenge must be overcome and is fundamental in mRNA and protein expression studies. Housekeeping genes, which have been examined in various studies, particularly the delta-delta Ct analysis proposed by Livak (Livak & Schmittgen, 2001) for the quantitation of mRNA expression studies and used in many other studies, directly affect the results of the analysis (Riedel et al., 2014). Although it is adopted that housekeeping genes and their proteins are expressed in all cells by definition, the levels of housekeeping genes/proteins expressed in different tissues can be variable. These genes are involved in the essential maintenance of cells and, thus, are expected to occur in all cells (Eisenberg & Levanon, 2013). The optimal housekeeping gene for studying gene and protein expression in a specific organism should be optimized for detection and be stability within the same cells undergoing varied treatments (Caracausi et al., 2017). Therefore, selecting the appropriate housekeeping gene and/or protein to be used in an expression study is crucial for the accuracy and reliability of the study.

Direct access to the human sample is sometimes only possible in human mRNA expression studies. In these cases, different cell lines that exhibit the characteristics of human tissues have generally been used with high accuracy (Dello Russo et al., 2018; Uphoff, Pommerenke, Denkmann, & Drexler, 2019). Choosing the suitable cell line is just as important as choosing the wrong one. The literature has argued this issue regarding the fallacy of cell line selection and its consequences (Drexler, Dirks, Matsuo, & MacLeod, 2003; Lacroix, 2008). Several cell lines are used in biomedical studies due to their similarity potential. Among them, fibroblasts are remarkable models because of their specific features; for instance, fibroblasts in patients will

maintain all genetic backgrounds during reprogramming induced pluripotent stem cells. They are also the primary cells of stromal tissue and play a crucial function in repairing and healing damaged organs (Fernandes et al., 2016; Rieske, Krynska, & Azizi, 2005). Studies with the help of human fibroblasts are interpreted in a wide range, from morphological studies to specific diseases, e.g., corneal epithelial regeneration studies (Buken et al., 2019; Lu et al., 2012). Despite this broad field of study, the cultivation processes of human fibroblasts sometimes require optimization (Villegas & McPhaul, 2005). Occasionally, patients may not allow their tissues to be taken by punch biopsy. In these cases, healthy human fibroblast cell lines can be used instead of human fibroblasts (Yu et al., 2007). CCD1079Sk, a healthy fibroblast cell line, is one of the cell lines that can be used in such cases. Previous studies reported in the literature use this cell line (Chowjarean, Phiboonchaiyanan, Harikarnpakdee, & Tengamnuay, 2019; Luo et al., 2012).

Herein, we aimed to determine the optimal housekeeping gene and protein candidate for using mRNA and protein expression studies. Therefore, we used the CCD1079Sk finite cell line to evaluate the genes and proteins efficacy and to detect the most proper one. For this purpose, firstly, we selected commonly used seven housekeeping genes (ACTB, SDHA, GAPDH, RPLP0, VCL, TBP, 18S) and evaluated their stability with the Delta CT method, NormFinder (Andersen, Jensen, & Orntoft, 2004), GeNorm (Vandesompele et al., 2002), and BestKeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) via RefFinder (De Spiegelaere et al., 2015). We also conducted Western blot experiments in parallel to detect the possible correlation between gene expression and protein levels. During the following time intervals, cytoskeletal staining also evaluates cell morphology to visualize the effect of senescence. The findings describe the assessment of the most stable housekeeping gene and reference protein through passages for CCD1079Sk fibroblast cells.

Material and methods

Cell culture

CCD1079Sk (CRL-2097, ATCC®CRL-2097™), a finite fibroblast cell line derived from healthy human skin, was used. It was cultured by using DMEM/F12 (1:1) (1X) (Gibco, MA, USA) (with 1% Penicillin-streptomycin, 0.1% Primocin, 10% FBS) medium at 37 °C with humidified atmosphere incubator containing 5% CO_2. The passaging procedure was performed every 2-3 days. In each passage, cells were divided into two groups for total RNA and protein isolation in a 75mm petri dish. The trypsinization process was performed using trypsin 0.25%, EDTA 0.02% (in PBS w/o Ca and Mg, w: Phenol red) (PAN-Biotech, Bavaria, Germany). Samples were collected in each passage between 25 and until senescence occurs at passage 56, which is reported by Expasy (Expasy, 2021; Gasteiger et al., 2003). Total RNA and protein samples were obtained from each passage between 25 and 45 and passages 50 and 54 (in total n=23 for each RNA and protein sample group). The cell count and viability status were quantified in Muse Cell Analyzer (Merck Millipore, MA, USA) with a Muse Count & Viability assay kit (Merck Millipore, MA, USA).

Cytoskeleton staining

The cytoskeleton of the CCD1079Sk cell line from passages including 25, 30, 35, 40, 45, 50, and 55 were stained with Flash Phalloidin™ Red 594 (Cat no.424203 from BioLegend). Nuclei were counterstained with DAPI, and the images were acquired using a laser scanning confocal microscope (Leica TCS-SPE, Leica Microsystems, Wetzlar, Germany) from Aziz Sancar Institute of Experimental Medicine, Istanbul University as a service purchase.

Total RNA Isolation and cDNA Synthesis

Cultivated cells were collected with a cell scraper with 1X PBS. Then, total RNA was isolated using the EcoPURE Total RNA Kit (Ecotech, Erzurum, Turkey). The isolated RNA's concentration and quality were determined using Multiskan GO (Thermo Fisher Scientific, MA, USA). The 260nm and 280nm ratios between 1.8 and 2.1 were assumed to be acceptable. Then, the RNA concentration of each sample was equalized to 100 ng μL^{-1}. Afterwards, cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, MA, USA). Before performing q/RT-PCR, the cDNA samples were diluted for one-fifth with ultrapure water.

Primer design and q/RT-PCR

Seven housekeeping genes (ACTB, SDHA, GAPDH, RPLP0, VCL, TBP, 18S, respectively) (Table 1) from the literature were selected as candidates. For these genes, primer design was performed by using Primer3Plus (Untergasser et al., 2007). The list of primers and amplicon sizes was compiled in Table 1. The primer specificity was confirmed in silico by using UCSC, and then BLAST (NCBI) was performed. The ideal annealing temperature was determined with gradient PCR in T100 Thermal Cycler (Bio-Rad, CA, USA), and it was quantitated in agarose gel electrophoresis. After that, the expression levels were analyzed with SensiFAST SYBR® No-ROX Kit (Meridian Bioscience Inc., Ohio, USA) in the CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). q/RT-PCR was performed with a two-step method including 95 °C for 2 minutes, cDNA was amplified for 45 cycles consisting of denaturing at 95 °C for 5 seconds and annealing at 61.8 °C (ACTB, GAPDH, RPLP0) or 61.3 °C (SDHA) or 62 °C (VCL) or 60.2 °C (TBP) or 58 °C (18S) for 15 seconds, and extension at 72 °C for 10 seconds, with a final extension step at 72 °C for 2 minutes.

Table 1. The list of seven candidate housekeeping genes, primer sequences, amplicon sizes and their features. (kDa: Kilodalton).

Gene Symbol	mRNA Accession Number	Uniprot ID	Gene Name	Function	Molecular Weight (kDa)	Amplicon Size
ACTB	NM_001101	P60709	Actin beta	A component of cytoskeletal protein	41.7	250 bp
	F':CATGTACGTTGCTATCCAGGC			R': CTCCTTAATGTCACGCACGAT		
SDHA	NM_004168	P31040	Succinate dehydrogenase complex flavoprotein subunit A	Critical catalytic subunit of succinate-ubiquinone oxidoreductase	72.7	225 bp
	F': AGGAACCCGAGGTTTTCACT			R': GCTGCAACAGTGTGTGACCT		
GAPDH	NM_001256799	P04406	Glyceraldehyde-3-phosphate dehydrogenase	Catalyzes Glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate	36	81 bp
	F': TCGACAGTCAGCCGCATCT			R': ACTCCGACCTTCACCTTCCC		
RPLP0	NM_001002	P05388	Ribosomal protein lateral stalk subunit P0	A component of the large subunit of ribosomal protein	34.2	97 bp
	F': AGCCCAGAACACTGGTCTC			R': ACTCAGGATTTCAATGGTGCC		
VCL	NM_014000	P18206	Vinculin	Cytoskeletal protein related to cell-cell and cell-matrix junctions	123.8	152 bp
	F': GCCAAGCAGTGCACAGATAA			R': GCCTGCTCAGACTCCTCATC		
TBP	NM_003194	P20226	TATA-box binding protein	A transcription factor	37.7	170 bp
	F': TATAATCCCAAGCGGTTTGC			R': GCTGGAAAACCCAACTTCTG		
RNA 18S	X03205	-	Human 18S ribosomal RNA	Ribosomal subunit	-	68 bp
	F': GCAATTATTCCCCATGAACG			R': GGGACTTAATCAACGCAAGC		

Preparation of Total Protein Extraction

Whole protein samples of each cell passage between 25 and 55 were collected from cultivated cells and lysed with a cell scraper while using 300 µL RIPA lysis buffer (containing 5 µL of phenylmethylsulfonyl fluoride, 5 µL of sodium orthovanadate solution, and 5 µL of protease inhibitor cocktail) (Santa Cruz, CA, USA). The mixture was transferred to the tube on ice and vortexed five times each between ice incubation, then stored at -80 °C until all the samples from each passage were retrieved. Afterward, samples were centrifuged for 15 minutes at 13000 g at 4 °C. The supernatant was collected in a fresh tube, then stored at -80 °C until use, and the pellet was discarded. Total protein concentrations were determined using the Qubit Protein Assay Kit (Thermo Fisher Scientific, MA, USA) in Qubit Fluorometer 2.0 (Thermo Fisher Scientific, MA, USA).

SDS-PAGE and Immunoblotting

Protein samples were prepared with 2x Laemmli Buffer (Bio-Rad, CA, USA) before Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Thirty µg protein samples were used for each lane for each sample obtained from each passage of the CCD1079Sk cells. The protein samples were separated on 4-12% SDS-PAGE. The groups consisting of a total of 23 samples were divided into three groups for SDS-PAGE; the first group was included with the first eight samples (gel 1, passages 25 to 32), and the second group was

included with seven samples (gel 2, passage 33 to 39) and the third group was included with eight samples (passages 40 to 45, 50 and 55 respectively). The polyacrylamide gels were run at 50V for 25 minutes, 130V for one hour, and 40 minutes. The separated proteins on polyacrylamide gels were transferred to 0.2 µm polyvinyl difluoride membranes (PVDF) (Bio-Rad, CA, USA) in 1x transfer buffer while containing ice-cold 20% MeOH with the semi-dry hot transfer system at 2.5A and 25V for 8 minutes (Trans-Blot Turbo Transfer System Bio-Rad, CA, USA). The membrane was blocked with 1X tris buffered saline (TBS) containing 0.2% Tween-20 (TBST) and 5% non-fat milk at 4 °C overnight.

The primary antibodies were rabbit monoclonal β-Actin (1:3000 dilution; Cell Signaling Technology, MA, USA), rabbit monoclonal SDHA (1:2000 dilution; Abclonal, MA, USA), mouse HRP-conjugated GAPDH (1:5000 dilution; Abclonal, MA, USA), rabbit polyclonal RPLP0 (1:4000 dilution; Abclonal, MA, USA), rabbit monoclonal Vinculin (VCL) (1:4000 dilution; Abclonal, MA, USA), rabbit polyclonal TBP (1:3000 dilution; Abclonal, MA, USA). The secondary antibody was HRP-conjugated anti-rabbit IgG (1:2000 dilution; Cell Signaling Technology, MA, USA). Membrane washing steps were performed with TBST for 5 minutes 5 times. Chemiluminescence detection was carried out with ClearBand ECL Western Blotting Substrate (Ecotech, Erzurum, Turkey) using Fusion Fx7 (Vilber Lourmat, Collégien, France). In all the experimental phases carried out in three groups, all conditions were applied the same. The membranes were evaluated with the exact exposure times for each variable, particularly in chemiluminescence imaging.

Statistics

The most and least stable housekeeping genes and proteins were determined using the RefFinder interface (De Spiegelaere et al., 2015). RefFinder is an interface that utilizes four different housekeeping gene detection algorithms. These are Delta CT, NormFinder, geNorm, and BestKeeper. The standard deviation of Ct values of candidate housekeeping genes is calculated to detect the stability of genes in the Delta CT method (Livak & Schmittgen, 2001). NormFinder uses the model-based approach for the detection stability of gene expression (Vandesompele et al., 2002). geNorm was used based on a pairwise comparison approach to evalute the best stable housekeeping gene (Andersen et al., 2004). BestKeeper determines the stability of genes using pair-wise correlation (Pfaffl et al., 2004). In addition, RefFinder ranks the candidate housekeeping genes from best stable to least stable according to the comparison of the results of four algorithms. Default parameters were used for all software unless otherwise noted. In addition, Western blot images were analyzed using ImageJ (Schneider, Rasband, & Eliceiri, 2012).

Results

Cellular morphology during the cultivation of CCD1079Sk cells was evaluated by cytoskeleton staining with F-actin. In Figure 1, the significant changes in the cell structure are visible. Through time, more elongated cell morphology was visual while F-actin accumulation increased to the edges and density decreased around the nucleus compared with early passages. In our observation, the predominant morphology was an elongated cell shape. Moreover, viability and population profile before the cultivation process for each passage were determined. Mean cell viability was found at 96.6%, and the cell viability data from each passage was compiled in Supplementary Figure 1. The morphology of cells was also followed during cultivation. As shown in the light microscopy images in Supplementary Figure 2, the cell morphology changed around passage 40 and continued until passage 55 of the cellular senescence limit.

RefFinder platform outputs Delta Ct, geNorm, BestKeeper, and NormFinder packages, assuming all the variables have 100% efficiency. According to Delta CT, BestKeeper, and NormFinder, VCL was the most stable candidate. Additionally, geNorm found the most stable genes VCL and 18S. All methods found SDHA the least stable gene (Figure 2a, upper left). The stability of the candidate housekeeping genes was indicated from a more stable to a minor stable gene with a comprehensive ranking of all methods as VCL, GAPDH, 18S, TBP, RPLP0, ACTB, and SDHA, respectively (Figure 2a, low left). Furthermore, the candidate reference protein was determined; BestKeeper found GAPDH; NormFinder reported β-Actin; geNorm suggested GAPDH and β-Actin as the most stable protein reference.

All methods estimated RPLP0 Isoforms as the least stable protein references (Figure 2b, upper right). In the comprehensive ranking of all methods, the stability order of the reference proteins from the most to the least stable protein candidate was determined with the order of β-Actin> GAPDH> VCL> SDHA> TBP> RPLP0 Isoform 1> RPLP0 Isoform 2 (Figure 2b, low right). The stability of the candidate housekeeping gene for each

variable and the cycle of threshold (Ct) values were obtained by q/RT-PCR (Supplemental data file, Table 1a)[1]. In addition, all candidate housekeeping genes were evaluated at the protein level except 18S. The immunoblot images (Figure 2c obtained with Western blotting) were analyzed using ImageJ (Raw immunoblot images provided in the Supplemental data file). The density of each reference protein was estimated using area values by ImageJ analysis (Supplemental data file, Table 1b). Then, the stability ranking was determined via RefFinder. Detailed outputs of RefFinder were compiled in the Supplemental data file.

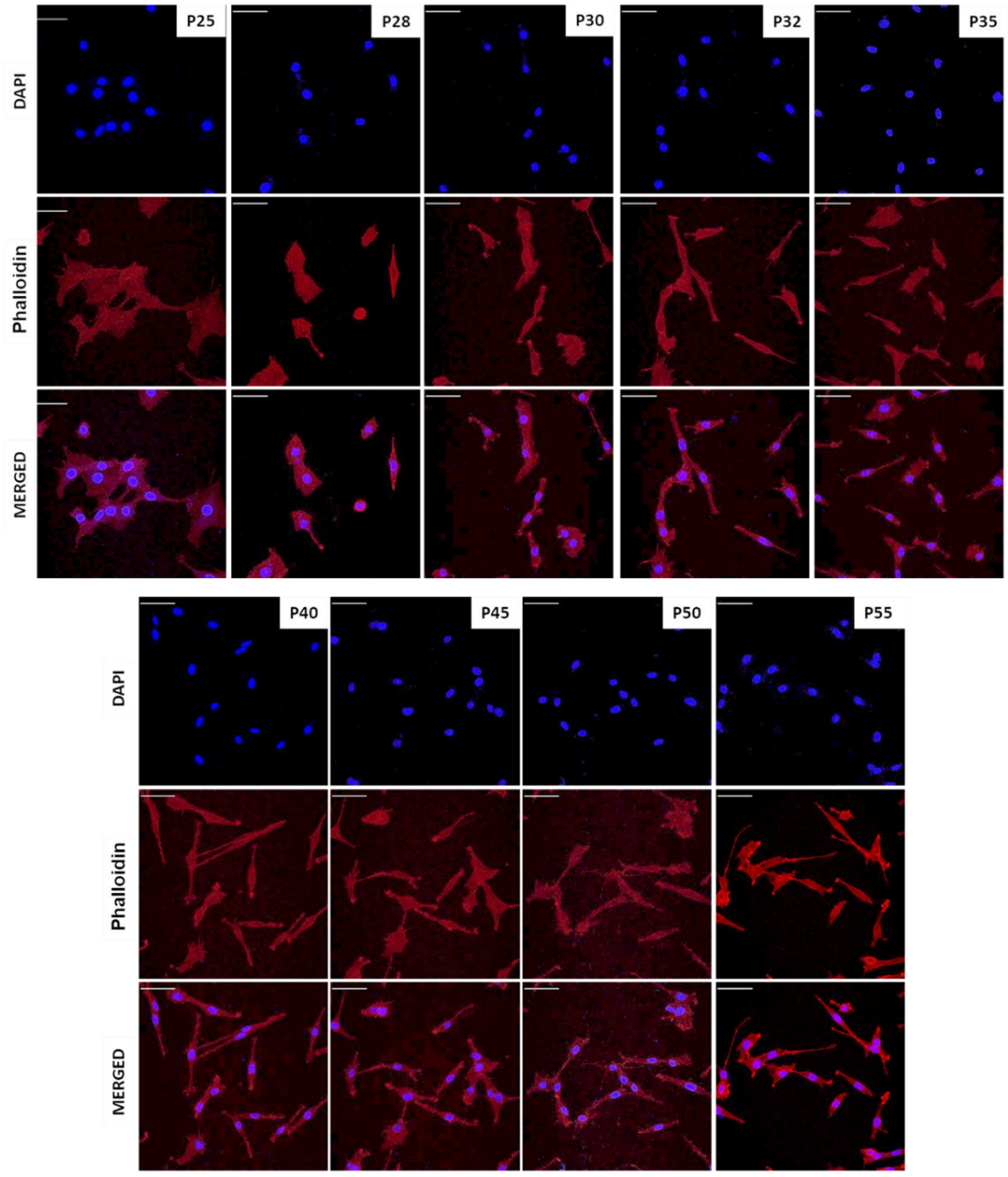


Figure 1. Confocal microscopy of the cytoskeleton staining of CCD1079Sk cell line. The F-actin (Phalloidin Red) staining is shown in red. The nucleus stained with DAPI is shown in blue. Scale bar 50 µm. P: passage.

Furthermore, in line with the ImageJ density data, we used each variable to evaluate relative adjusted density (Supplemental data file, Table 2). When comparing relative adjusted densities between β-Actin, GAPDH, and VCL, the standard deviations were found between 0.17 – 0.48, which increased only for SDHA, RPLP0 isoforms, and TBP when used for normalization as a reference protein. Accordingly, the differences

[1] Supplemental data file were provided via link: https://drive.google.com/file/d/1KF_1dc3SeG4iy3ILhMPnNn_SlJBnSCq2/view?usp=sharing

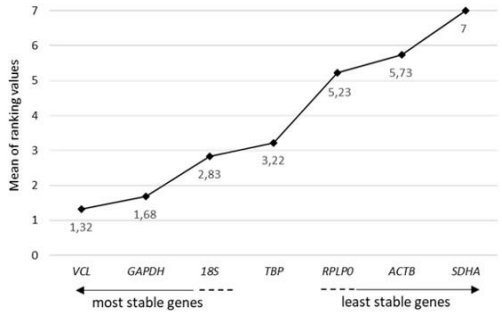
between standard deviation values were 2.59 – 4.07 (min-max) for SDHA, 1.07 – 1.94 (min-max) for isoform-1, 1.27 – 2.08 (min-max) for isoform-2 and 1.1 – 2.0 (min-max) for TBP. Interestingly, β-Actin was found to be the most stable with the lowest standard deviation in protein expression, which is also the least stable gene we detected in gene expression during cultivation.

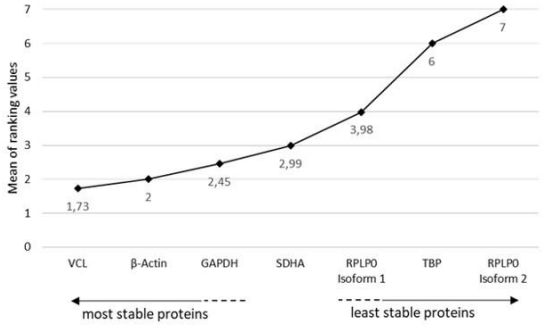
A.

Method	1	2	3	4	5	6	7
Delta CT	VCL	GAPDH	TBP	18S	RPLP0	ACTB	SDHA
BestKeeper	VCL	GAPDH	TBP	18S	ACTB	RPLP0	SDHA
Normfinder	VCL	GAPDH	TBP	18S	RPLP0	ACTB	SDHA
Genorm	VCL | 18S		VCL	TBP	RPLP0	ACTB	SDHA
Recommended Comprehensive Ranking	VCL	GAPDH	18S	TBP	RPLP0	ACTB	SDHA

B.

Method	1	2	3	4	5	6	7
BestKeeper	β-Actin	RPLP0 Isoform 1	VCL	GAPDH	SDHA	TBP	RPLP0 Isoform 2
Normfinder	VCL	β-Actin	GAPDH	SDHA	RPLP0 Isoform 1	TBP	RPLP0 Isoform 2
Genorm	GAPDH | SDHA		VCL	β-Actin	RPLP0 Isoform 1	TBP	RPLP0 Isoform 2
Recommended Comprehensive Ranking	VCL	β-Actin	GAPDH	SDHA	RPLP0 Isoform 1	TBP	RPLP0 Isoform 2





C.

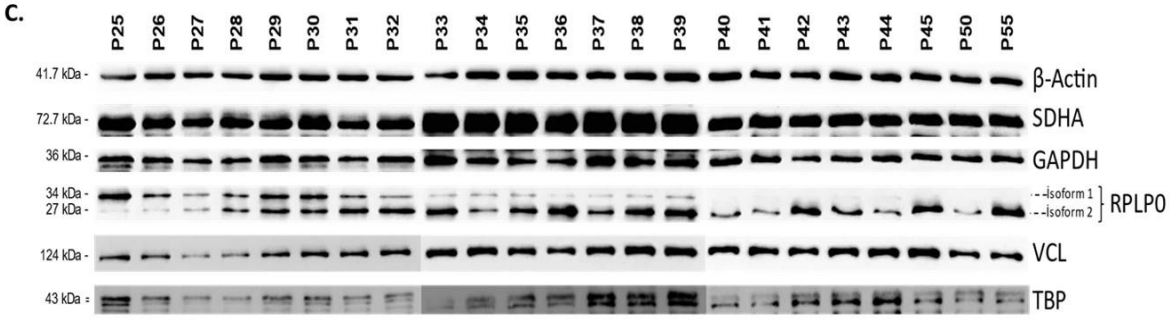


Figure 2. A. Table indicates RefFinder output from q/RT-PCR results. The ranking order is from most-stable (1) to least-stable (7) (upper left). The graph indicates the ranking of candidate housekeeping genes according to the comprehensive gene stability analysis of RefFinder (low left). **B.** Table indicates RefFinder output of the stability of protein expression levels (upper right). The ranking order is from most-stable (1) to least-stable (7). The graph indicates the ranking of candidate reference proteins according to the comprehensive gene stability analysis of RefFinder (low right). **C.** Immunoblot images of β-Actin, SDHA, GAPDH, RPLP0 Isoform 1 and Isoform 2, VCL, and TBP protein expression levels during each passage of the CCD1079Sk cell line. kDa: Kilodaltons. P: Passage. CT: Cycle of threshold.

Discussion

The proper choice is a must to define an accurate normalization method while evaluating the gene and/or protein expression levels of a molecule of interest. The definition of housekeeping (-genes/-proteins) is to classify raw data obtained from housekeeping gene/protein and variables to normalize gene/protein. These genes or proteins are assumed to be commonly expressed in all. However, the expression levels of housekeeping genes/proteins may exhibit different or similar patterns depending on the cell type.

There is an increase in the determination of the stability of housekeeping genes and limited reference protein expressions. Recently, Xie et al. (2021) reported the validated 15 housekeeping genes for other cell lines originated from pigs. Their results show different housekeeping genes for different cell lines; 3D4/21 and IPEC-J2 cells, ALDOA and SDHA, and PK-15 cells TOP2B, TBP, and PPIA were the most stable housekeeping genes. In another report, Köhsler, Leitsch, Muller, and Walochnik (2020) reported the first validated housekeeping gene among 11 candidates for Acanthamoeba species, a pathogenic organism, and according to their report, the most stable housekeeping gene was 18S and HPRT1, respectively. Based on the

current literature, the reference/housekeeping gene or a protein candidate is now defined as the reference for normalization according to the species, disease, specimens, or conditions, including heat shock and oxidative stress (Köhsler et al., 2020; Maltseva et al., 2013; Szczepkowska, Harazin, Barna, Deli, & Skipor, 2021; Torres, Lama, Mantecon, Flemetakis, & Infante, 2021; Xie et al., 2021). Unfortunately, contrary to all housekeeping gene studies, protein normalization is still underestimated. A couple of appropriate reference protein recommendations are reported in the current literature. Protein selection for proper cell type is as essential as gene selection. To support this information, Feng et al. (2021) identified protein reference standards for sperm specimens and reported that only β-tubulin was found stable. Another approach found in the literature is determining the total protein amount, which may serve as a more proper normalization method than individual protein. For this purpose, Hu et al. (2016) evaluated seven commonly used reference proteins (β-actin, GAPDH, TUBB, HIST1H2BC, RPL19, NONO, and RPS18) in colorectal adenocarcinoma and hepatocellular carcinoma cells. They performed western blotting by preparing protein concentrations with serial dilutions and reported that protein levels were not as stable as expected, and the amount of total protein may serve as a suitable reference control (Hu et al., 2016). According to their feedback, the apparent discrepancy between western blot and total protein staining must be addressed. Notably, the suggested total protein staining with the fluorescent dyes requires more advanced laboratory equipment. Still, chemiluminescent detection is a more reachable way to visualize by Western blots; researchers should consider that the chemiluminescent signals will easily reach saturating intensities and may lose linearity. Additionally, Hu et al. suggested mass spectrometry screening to confirm the outcome precisely (Hu et al., 2016).

A current study determined the stability of reference gene-protein and cytoskeletal changes of the CCD1079Sk cell line that exhibits irreversible cell cycle arrest (cellular senescence) after 56 passages in vitro (Expasy, 2021). Based on our findings, there is a limitation to using this cell line as a healthy control group. The starting point for the morphological changes during the standard cultivation process is approximately passage 40. Among the most commonly used housekeeping genes/proteins were assessed and found that VCL is the most stable gene and β-Actin is the most stable reference protein specifically for this healthy fibroblast cell line. These differences between stable gene and protein expressions of the housekeeping genes suggest using VCL if the mRNA expression is required or using β-Actin if the protein expression level is necessary. GAPDH is second "the most stable" for both mRNA and protein expressions.

Regarding our findings, we strongly recommend using GAPDH when looking for mRNA and protein expression levels in normalization studies. The seven candidates'two "least stable" reference genes and/or proteins were also determined. These were SDHA for both mRNA and protein and RPLP0 for only mRNA among the seven candidate housekeeping genes. SDHA is a part of mitochondrial complex II of the electron transport chain. There are only four succinate dehydrogenase complex subunits. Besides, Bowman and Birch-Machin (2016) used primary skin fibroblast cells from 27 individuals (aged 6 to 72 years) and reported that mRNA and protein expression levels of SDHA decreased significantly with aging.

RPLP0 is a large ribosomal subunit P0 for rRNA binding, and this molecule has two isoforms (Rich & Steitz, 1987). Nazet et al. (2019) reported that RPLP0 is the ideal reference gene for fibroblast cells derived from synovial fluids from healthy and osteoarthritis patients. As is known, the diverse role of the fibroblast cells depends on the tissue type and shows a higher degree of heterogeneity among its origin. The fundamental aspects of fibroblast biology are still emerging (Plikus et al., 2021). Strikingly, RPLP0 mRNA expression level was found to be the fifth stable reference gene; also, the protein expression for both isoforms of RPLP0 was found to be the least stable reference protein according to the comprehensive ranking. In addition, cell senescence is mainly associated with nucleolar stress, DNA scars, growth arrest, transcriptional stress, or lysosomal expansion (Payea, Anerillas, Tharakan, & Gorospe, 2021). The replicative exhaustion might indirectly affect ribosomal subunits, including this cell line. When examining the rank differences between the two isoforms, we showed that isoform-1 protein expression significantly decreased after passage 39, and both isoforms showed an unstable profile for each passage of CCD1079Sk cells. Hence, using the RPLP0 gene/protein as housekeeping may be misleading.

Moreover, the distribution of filamentous actin (F-actin) through specified passages of CCD1079Sk cells was visualized by phalloidin staining. Results showed different variations in both morphology and altered organization of the cytoskeleton after late passages in a time-dependent manner. These changes through senescence in morphology can be attributed to several factors. A particular study about quantitative proteomic analysis shows the importance of the chromosome condensin complex in this cell line. They

showed that the subculture of this cell line reveals decreased telomere length during cultivation. Therefore, researchers suggest that this senescent cell line is a suitable model for skin aging, diseases, and even cancer studies (Meng et al., 2018). Also, another study established a clonal stem cell line using CCD1079Sk cells (Dejosez & Zwaka, 2018). Based on our experience and the limitation of skin-originated cell lines needed during functional characterization studies, a healthy skin fibroblast cell line is required as a model and/or to compare with a specific disease model (Aslanger et al., 2022). Most functional characterization studies use cancer-originated cell lines like MCF7 or embryonic-originated epithelial cells such as 293T because of their higher capacity as a transfection host. However, even cell type requires normalization to reflect equal demonstration between, e.g., healthy fibroblast cells to mutation-carrying primary fibroblast cells. The main suggestion to impede cellular normalization or demonstration of a mutation carrying disease model in vitro is that the CCD1079Sk cell is a candidate. The difference between using primary fibroblast and immortalized healthy fibroblast cells might cause a negative correlation between mRNA and protein expression. Researchers should take into account this variance before studying. Future studies will provide accurate expression comparisons between primary fibroblasts and fibroblast cell lines. Based on the morphological changes, passages 1 to 43 are the proper cultivation range, and also, by the lack of an RPLP0 subunit after passage 39, this limit should limit the cultivation range when protein expression is required. After this limit, cells continue to show irreversible morphological changes. This study also validates that GAPDH should be the first normalization choice for gene and protein expression level studies. This outcome is valid even if the cellular senescence limit is reached when necessary. Alternatively, as a second option, VCL may be used rather than the others when only aimed at mRNA, and β-Actin should be used other than the others when protein expression studies are required.

Conclusion

In conclusion, we showed and strongly recommended that GAPDH will be the most suitable and stable housekeeping gene and protein for healthy CCD1079Sk skin fibroblast cells. Future studies should evaluate the possible changes in housekeeping genes/proteins with different cultivation conditions, such as starvation or stimulation with growth factors.

Acknowledgments

The authors thank Dilek Ozturk, Ph.D., for providing the CCD1079Sk cell line and Prof. Mukaddes Esrefoglu and Prof. Fahri Akbas for the equipment support. This article contains no studies with human or animal subjects performed by authors.

This study was financially supported by the Bezmialem Vakif University Scientific Research Funding Unit (Project number 20210232).

References

Andersen, C. L., Jensen, J. L., & Orntoft, T. F. (2004). 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 Research, 64*(15), 5245-5250. DOI: https://doi.org/10.1158/0008-5472.can-04-0496

Aslanger, A. D., Goncu, B., Duzenli, O. F., Yucesan, E., Sengenc, E., & Yesil, G. (2022). Biallelic loss of TRAPPC9 function links vesicle trafficking pathway to autosomal recessive intellectual disability. *Journal of Human Genetics, 67*(5), 279–284. DOI: https://doi.org/10.1038/s10038-021-01007-8

Bowman, A., & Birch-Machin, M. A. (2016). Age-dependent decrease of mitochondrial Complex II activity in human skin fibroblasts. *The Journal of Investigative Dermatology, 136*(5), 912-919. DOI: https://doi.org/10.1016/j.jid.2016.01.017

Buken, C., Sahana, J., Corydon, T. J., Melnik, D., Bauer, J., Wehland, M., ... Grimm, D. (2019). Morphological and molecular changes in juvenile normal human fibroblasts exposed to simulated microgravity. *Scientific Reports, 9*(1), 11882. DOI: https://doi.org/10.1038/s41598-019-48378-9

Caracausi, M., Piovesan, A., Antonaros, F., Strippoli, P., Vitale, L., & Pelleri, M. C. (2017). Systematic identification of human housekeeping genes possibly useful as references in gene expression studies. *Molecular Medicine Reports, 16*(3), 2397-2410. DOI: https://doi.org/10.3892/mmr.2017.6944

Chowjarean, V., Phiboonchaiyanan, P. P., Harikarnpakdee, S., & Tengamnuay, P. (2019). A natural skin anti-ageing serum containing pseudobulb ethanolic extract of *Grammatophyllum speciosum*: a randomized double-blind, placebo-controlled trial. *International Journal of Cosmetic Science, 41*(6), 548-557. DOI: https://doi.org/10.1111/ics.12571

De Spiegelaere, W., Dern-Wieloch, J., Weigel, R., Schumacher, V., Schorle, H., Nettersheim, D., ... Fink, C. (2015). Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One, 10*(3). DOI: https://doi.org/10.1371/journal.pone.0122515

Dejosez, M., & Zwaka, T. P. (2018). Generation of hiPSTZ16 (ISMMSi003-A) c Generation of hiPSTZ16 (ISMMSi003-A) cell line from normal human foreskin fibroblasts. *Stem Cell Research, 26*, 44-46. DOI: https://doi.org/10.1016/j.scr.2017.11.019

Dello Russo, C., Cappoli, N., Coletta, I., Mezzogori, D., Paciello, F., Pozzoli, G., ... Battaglia, A. (2018). The human microglial HMC3 cell line: where do we stand? A systematic literature review. *Journal of Neuroinflammation, 15*(1), 259. DOI: https://doi.org/10.1186/s12974-018-1288-0

Drexler, H. G., Dirks, W. G., Matsuo, Y., & MacLeod, R. A. (2003). False leukemia-lymphoma cell lines: an update on over 500 cell lines. *Leukemia, 17*(2), 416-426. DOI: https://doi.org/10.1038/sj.leu.2402799

Eisenberg, E., & Levanon, E. Y. (2013). Human housekeeping genes, revisited. *Trends Genet, 29*(10), 569-574. DOI: https://doi.org/10.1016/j.tig.2013.05.010

Expasy. (2021). *Cellosaurus, CCD1079Sk cell line* [online]. (access date September 27, 2021). Retrieved from https://web.expasy.org/cellosaurus/CVCL_2337

Feng, Y., Wang, R., Su, D., Zhai, Y., Wang, L., Yu, L., ... Ma, F. (2021). Identifying new sperm Western blot loading controls. *Andrologia, 53*(11), e14226. DOI: https://doi.org/10.1111/and.14226

Fernandes, I. R., Russo, F. B., Pignatari, G. C., Evangelinellis, M. M., Tavolari, S., Muotri, A. R., & Beltrao-Braga, P. C. (2016). Fibroblast sources: Where can we get them? *Cytotechnology, 68*(2), 223-228. DOI: https://doi.org/10.1007/s10616-014-9771-7

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., & Bairoch, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research, 31*(13), 3784-3788. DOI: https://doi.org/10.1093/nar/gkg563

Hu, X., Du, S., Yu, J., Yang, X., Yang, C., Zhou, D., ... Wan, C. (2016). Common housekeeping proteins are upregulated in colorectal adenocarcinoma and hepatocellular carcinoma, making the total protein a better "housekeeper". *Oncotarget, 7*(41), 66679-66688. DOI: https://doi.org/10.18632/oncotarget.11439

Kohsler, M., Leitsch, D., Muller, N., & Walochnik, J. (2020). Validation of reference genes for the normalization of RT-qPCR gene expression in *Acanthamoeba* spp. *Scientific Reports, 10*(1), 10362. DOI: https://doi.org/10.1038/s41598-020-67035-0

Lacroix, M. (2008). Persistent use of "false" cell lines. *International Journal of Cancer, 122*(1), 1-4. DOI: https://doi.org/10.1002/ijc.23233

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method. *Methods, 25*(4), 402-408. DOI: https://doi.org/10.1006/meth.2001.1262

Lu, R., Bian, F., Lin, J., Su, Z., Qu, Y., Pflugfelder, S. C., & Li, D. Q. (2012). Identification of human fibroblast cell lines as a feeder layer for human corneal epithelial regeneration. *PLoS One, 7*(6), e38825. DOI: https://doi.org/10.1371/journal.pone.0038825

Luo, L. Z., Gopalakrishna-Pillai, S., Nay, S. L., Park, S. W., Bates, S. E., Zeng, X., ... O'Connor, T. R. (2012). DNA repair in human pluripotent stem cells is distinct from that in non-pluripotent human cells. *PLoS One, 7*(3), e30541. DOI: https://doi.org/10.1371/journal.pone.0030541

Maltseva, D. V., Khaustova, N. A., Fedotov, N. N., Matveeva, E. O., Lebedev, A. E., Shkurnikov, M. U., ... Tonevitsky, A. G. (2013). High-throughput identification of reference genes for research and clinical RT-qPCR analysis of breast cancer samples. *Journal of Clinical Bioinformatics, 3*(1), 13. DOI: https://doi.org/10.1186/2043-9113-3-13

Meng, Q., Gao, J., Zhu, H., He, H., Lu, Z., Hong, M., & Zhou, H. (2018). The proteomic study of serially passaged human skin fibroblast cells uncovers down-regulation of the chromosome condensin complex proteins involved in replicative senescence. *Biochemical and Biophysical Research Communications, 505*(4), 1112-1120. DOI: https://doi.org/10.1016/j.bbrc.2018.10.065

Nazet, U., Schröder, A., Grässel, S., Muschter, D., Proff, P., & Kirschneck, C. (2019). Housekeeping gene validation for RT-qPCR studies on synovial fibroblasts derived from healthy and osteoarthritic patients with focus on mechanical loading. *PLoS One, 14*(12). DOI: https://doi.org/10.1371/journal.pone.0225790

Payea, M. J., Anerillas, C., Tharakan, R., & Gorospe, M. (2021). Translational Control during Cellular Senescence. *Molecular and Cellular Biology, 41*(2). DOI: https://doi.org/10.1128/mcb.00512-20

Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters, 26*(6), 509-515. DOI: https://doi.org/10.1023/b:bile.0000019559.84305.47

Plikus, M. V., Wang, X., Sinha, S., Forte, E., Thompson, S. M., Herzog, E. L., ... Horsley, V. (2021). Fibroblasts: Origins, definitions, and functions in health and disease. *Cell, 184*(15), 3852-3872. DOI: https://doi.org/10.1016/j.cell.2021.06.024

Rich, B. E., & Steitz, J. A. (1987). Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. *Molecular and Cellular Biology, 7*(11), 4065-4074. DOI: https://doi.org/10.1128/mcb.7.11.4065-4074.1987

Riedel, G., Rudrich, U., Fekete-Drimusz, N., Manns, M. P., Vondran, F. W., & Bock, M. (2014). An extended ΔCT-method facilitating normalisation with multiple reference genes suited for quantitative RT-PCR analyses of human hepatocyte-like cells. *PLoS One, 9*(3), e93031. DOI: https://doi.org/10.1371/journal.pone.0093031

Rieske, P., Krynska, B., & Azizi, S. A. (2005). Human fibroblast-derived cell lines have characteristics of embryonic stem cells and cells of neuro-ectodermal origin. *Differentiation, 73*(10), 474-483. DOI: https://doi.org/10.1111/j.1432-0436.2005.00050.x

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671-675. DOI: https://doi.org/10.1038/nmeth.2089

Sinha, P., Saxena, R. K., Singh, V. K., Krishnamurthy, L., & Varshney, R. K. (2015). Selection and validation of housekeeping genes as reference for gene expression studies in pigeonpea (*Cajanus cajan*) under heat and salt stress conditions. *Frontiers in Plant Science, 6*, 1071. DOI: https://doi.org/10.3389/fpls.2015.01071

Szczepkowska, A., Harazin, A., Barna, L., Deli, M. A., & Skipor, J. (2021). Identification of reference genes for circadian studies on brain microvessels and choroid plexus samples isolated from rats. *Biomolecules, 11*(8), 1-18. DOI: https://doi.org/10.3390/biom11081227

Torres, S., Lama, C., Mantecon, L., Flemetakis, E., & Infante, C. (2021). Selection and validation of reference genes for quantitative real-time PCR in the green microalgae *Tetraselmis chui*. *PLoS One, 16*(1), e0245495. DOI: https://doi.org/10.1371/journal.pone.0245495

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research, 35*(Suppl. 2), W71-W74. DOI: https://doi.org/10.1093/nar/gkm306

Uphoff, C. C., Pommerenke, C., Denkmann, S. A., & Drexler, H. G. (2019). Screening human cell lines for viral infections applying RNA-Seq data analysis. *PloS One, 14*(1), e0210404. DOI: https://doi.org/10.1371/journal.pone.0210404

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*(7), Research0034. DOI: https://doi.org/10.1186/gb-2002-3-7-research0034

Villegas, J., & McPhaul, M. (2005). Establishment and culture of human skin fibroblasts. *Current Protocols in Molecular Biology, 71*: 28.3.1-28.3.9. DOI: https://doi.org/10.1002/0471142727.mb2803s71

Xie, C. D., Wang, B., Shen, Z. J., Yao, W. Y., Ao, H., Li, B., ... Zhou, R. (2021). Validation of the Reference Genes for the gene expression studies in different cell lines of pig. *BioMed Research International, 2021*, 5364190. DOI: https://doi.org/10.1155/2021/5364190

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., ... Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science, 318*(5858), 1917-1920. DOI: https://doi.org/10.1126/science.1151526