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Validation of reference genes for real-time PCR of cord blood mononuclear cells, differentiating endothelial progenitor cells, and mature endothelial cells

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ABSTRACT
In the last ten years, endothelial progenitor cells (EPCs) have gained interest as an attractive cell population in regenerative medicine for vascular applications. This population is defined as the precursor of endothelial mature cells (ECs) through a process of differentiation. To our knowledge, no single marker can be used to discriminate them from mature ECs. To effectively study their differentiation kinetics, gene expression must be assessed. Quantitative real-time PCR (RT-qPCR) is widely used to analyze gene expression. To minimize the impact of variances from RT-qPCR, a rigorous selection of reference genes must be performed prior to any experiments due to variations in experimental conditions. In this study, CD34+ mononuclear cells were extracted from human cord blood and differentiated into EPCs after seeding for a maximum period of 21 days. To choose the best combinations of reference genes, we compared the results of EPCs, CD34+ mononuclear cells, and mature endothelial cells to ensure that the differentiation kinetics did not affect the expression of our selected reference genes. The expression levels of seven genes, namely, YWHAZ, GAPDH, HPRT1, RPLP0, UBC, B2M, and TBP were thus compared. The algorithms geNorm, NormFinder, BestKeeper, and the Comparative ΔCt method were employed to assess the expression of each candidate gene. Overall results reveal that the expression stability of reference genes may differ depending on the statistical program used. YWHAZ, GAPDH, and UBC composed the optimal set of reference genes for the gene expression studies performed by RT-qPCR in our experimental conditions. This work can thus serve as a starting point for the selection of candidate reference genes to normalize the levels of gene expression in endothelial progenitor cell populations.

KEYWORDS
Vascular applications, Endothelial progenitor cells, RT-qPCR, Reference genes

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1 INTRODUCTION

Within the last decade, endothelial progenitor cells (EPCs) have become a promising source of primary cells for vascular tissue engineering [1]. These cells can be isolated from peripheral blood, cord blood, bone marrow, or fetal liver [2]. In adults, EPCs are responsible for postnatal vasculogenesis phenomena and vessel repairs [3]. Initially, the differentiation of mesodermal cells to angioblasts then to endothelial cells (ECs) was thought to be restricted during embryonic development [4]. It is only in 1997 that Asahara and colleagues contradicted this concept by isolating a new type of primitive circulating cell in peripheral blood, referred to in this state as endothelial progenitor cells (EPCs). This specific cell type is responsible for the processes of vasculogenesis in embryos [3,5]. Postnatal neovascularization, also called angiogenesis, is known as the mechanism by which endothelial mature cells can sprout from a pre-existing structure [6,7], while vasculogenesis describes a new vessel formed with no existing structure, due to a primitive and circulating cell type [8]. In the embryo, vasculogenesis occurs to form to the heart, the first primitive plexus and its network [9], while angiogenesis refers to the remodeling and expansion of this network [10]. In adult bone marrow and peripheral blood, the discovery of circulating endothelial progenitors implied that the vasculogenesis process could occur either to repair vessel damage or to regenerate endothelium following injury or synthetic vessel grafting. Before the discovery of EPCs, the re-endothelialization process was thought to occur only in the anastomosis area of the graft from the ingrowth of pre-existing vessels. Shi et al. [11] observed isolated endothelial cells lining the lumen of a grafted prosthesis but at the center of the graft, away from the anastomosis. This event has been referred to as “fallout endothelialization” and is described as a distinct re-endothelialization process of a vascular prosthesis. However, the complete endothelialization of a graft in humans has never been reported, even after decades of implantation. The emergence of endothelial progenitor cells (EPCs) may provide a promising approach to solve this problem [12]. This type of progenitor arises from a heterogeneous population of stem cells called hemangioblasts, which can lead to the differentiation of hematopoietic stem cells (HSCs) or endothelial cells, depending on the environment. *In vitro*, the common precursors between hematopoietic stem cells or endothelial cells are mononuclear cells extracted from peripheral blood or cord blood. Depending on the cell culture media, mononuclear cells can proceed toward one or the other type of cells. Following their initial step toward endothelial lineage, EPCs appear to differentiate rapidly, and their phenotype and morphology can be difficult to distinguish from those of endothelial mature cells. Nowadays, no single marker exists to discriminate EPCs from endothelial mature cells, yet with several markers and functional testing, they can be distinguished from mature cells. In the early stage of differentiation, EPCs and hematopoietic stem cells (HSCs) share some antigenic determinants, such as CD34, CD133, Tie-2, c-Kit, Sca-1, and CD45 [3,13]. When differentiating to mature endothelial cells, they lose stem cell markers while gaining the expression of endothelial markers, including CD31, KDR (VEGFR-2), and vWF (Von Willebrand Factor). They are also positive for ac-LDL and have the potential to produce nitric oxide [14]. It was then discovered that EPCs define two subsets of cell types, namely, early and late EPCs [15]. Early EPCs appear before 10 days and display a spindle-shaped morphology, with a low rate of proliferation and an inability to form new vessels. Early EPCs were shown to lose the expression of endothelial characteristic markers after three weeks of culture [16]. In contrast, the expression of endothelial markers of late EPCs tends to become stronger after passages, leading to a fully mature endothelial cell phenotype [16,17]. However, there remains no clear definition of the EPC phenotype, and even the term EPCs is used incorrectly. In 2004, Ingram et al. proposed a new way to identify and designate late EPCs, as they display high proliferative potential. Cord blood-derived EPCs can achieve at least 100 populations doubling [18]. These cells are called HPP-ECFCs, meaning high proliferative potential endothelial cell-forming colonies due to their ability to form a colony with only one cell [19]. Their high proliferative potential can be explained by their remaining high telomerase activity with successive passages in culture [20]. In cord blood-extracted cells, the length of the telomeres is longer compared to that of telomeres in healthy adults [21].

Moreover, peripheral blood EPCs show high proliferative activity but lower than that by cord blood cells [18]. In an attempt to define EPCs, Yoder et al. worked on redefining endothelial progenitor cells via clonal analysis by comparing the function of Colony Forming Unit Endothelial cells (CFU-ECs) and ECFCs. From these two cell populations, only ECFCs are able to form secondary colonies and are the vessels forming EPCs [22].

EPCs able to form secondary colonies and repair damaged vessels are found in the circulation in relatively low numbers. Cells expressing CD34, VEGFR-2, and CD133 represent 0.002% of the total number of mononuclear cells in peripheral blood and 0.01% in cord blood [23]. Although the process of recruitment at the vascular damaged site is well described in the literature, the differentiation of CD34+ cells into EPCs and then into mature endothelial cells is not well characterized. Furthermore, to our knowledge, there is no clear definition of what exactly are EPCs.

To assess and follow the differentiation of EPCs in different chemical and physical environments, the gene expression using quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) was investigated. This powerful method, which can quickly and reliably measure mRNA levels, can detect low-expression genes, as it is sensitive to a very small number of mRNA copies [24]. However, when RT-qPCR is used incorrectly, this can lead to misinterpretation and misleading conclusions. The present study makes use of the minimum information for publication of Quantitative Real-Time PCR experiments, also referred to as the MIQE guidelines [25]. Many researches have reported experiments performed with only one gene for normalization, such as GAPDH, β-actin, or 18S, which represent the three most used genes in RT-qPCR; however, their expression can be unstable, depending on the treatment, tissue, cell type, or differentiation state of the cells involved [26,27]. Moreover, employing a single reference gene without validation can lead to unreliable gene expression data. Thus, many experimental results cannot be reproduced, sometimes because of insufficient data normalization and quality. Normalization can overcome issues with mRNA sample extraction and handling as well as limit the effect of having different amounts of starting materials from one sample to another [25]. The stability of seven candidate reference genes was therefore tested with three types of cells: mononuclear CD34+ cells (extracted from cord blood), EPCs (treated or untreated), and HSVECs (human saphenous vein endothelial cells). In the present study, treated ECs refers to cells that were seeded on functionalized biomaterials while the untreated ones were seeded on collagen. The reference genes were chosen among several genes displaying minimal variability in their level of expression under different experimental conditions. The critical point here is that cells were investigated in their differentiation state, meaning that the expression of several genes could change during this process [28]. To ensure that the expression of the candidate reference genes remained stable during the process, mononuclear cells enriched CD34+ population were used as undifferentiated cells and human saphenous vein endothelial cells were deployed as mature cells. EPCs emerging from the mononuclear cells and cultured on collagen were likely to possess clonal endothelial colony-forming cell (ECFC) ability and were thus named ECFCs following their appearance under these conditions [22]. The obtained ECFCs were seeded onto modified biomaterials used for vascular applications [29]. To obtain reliable results, every condition had to be tested to ensure that both the cells and consequently their gene expression undergo all of the environmental changes.

2 MATERIALS AND METHODS

2.1 Experimental design

Seven potential candidate reference genes (see **Erreur! Source du renvoi introuvable.** and Table) were evaluated in mononuclear cells enriched CD34+, endothelial cell-forming colonies (ECFCs) and human saphenous vein endothelial cells. HSVECs served as the control for the endothelial mature cells in our experiments. ECFCs were cultured onto collagen-coated wells or modified biomaterials for vascular applications. Three biological replicates were used for each

condition since we have limited numbers of blood donations. cDNA from three independent samples of treated and untreated ECFCs, mononuclear cells enriched CD34+ population cells, and human saphenous vein endothelial cells (HSVECs) were then pooled to provide the most stable reference gene. Using the pooled sample approach to determine temperature annealing and efficiency made it possible to cover the entire sample spectrum.

Table 1. Evaluated Reference Genes

Symbol	Gene name	Function
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Regulates the signal transduction pathway by binding to phosphoserine protein on a variety of signaling molecules
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Responsible for carbohydrate metabolism
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Crucial to the generation of purine through the purine salvage model
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Encodes a ribosomal protein that is a component of the 60S subunit
UBC	Ubiquitin C	Essential in maintaining the ubiquitin level under stress conditions
B2M	β-2-microglobulin	Component of major histocompatibility complex class I molecules
TBP	Tata box binding protein	Transcription initiation from RNA polymerase II promoter

2.2 Isolation of mononuclear CD34+ cells and expansion of EPCs

As there are more mononuclear cells in cord blood compared to peripheral blood, and because cord blood derived colonies appeared larger than peripheral blood colonies, EPCs were isolated from cord blood to ensure collecting sufficient amount of cells at low passage (less differentiated state) [18,30]. EPCs (or ECFCs, as referred to here) were obtained from cord blood donations and each procedure was approved by the CHU de Québec - Université Laval Ethics Committee following the informed consent of donors at the Saint-François d'Assise Hospital, Quebec City, Canada. In agreement with the CHU de Québec - Université Laval Ethics Committee, all sample donations were carried out anonymously and limited in number.

The samples were collected in cord blood collection bags (Pall, USA). Mononuclear cells were first isolated from total cord blood by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, Canada). The cells were then rinsed out twice with 2 mM of PBS EDTA and once with EBM-2 (Lonza, Switzerland). The cells were enriched thereafter with CD34+ cells by magnetically activated cell sorting (CD34 Microbead Kit Ultrapure, Miltenyi Biotec, Germany) onto MS MACS columns, following the manufacturer's instructions. The cells were finally plated on collagen-coated (collagen type I solution, rat tail from Sigma, USA) 6-well plates (Corning, NY, USA). The medium, namely, EGM2-MV from Lonza supplemented with 10% FBS (5% from the Lonza kit and 5% from Fetal Bovine Serum, HyClone™, USA), was changed 4 days after plating and every 2 days

thereafter. Colonies normally appear between 7 and 21 days after plating. After 17 days, colonies were harvested and seeded onto collagen-coated 6-well plates for 2 days of expansion. Prior the seeding of cells on functionalized biomaterials, the surfaces were sterilized overnight with 70% ethanol and subsequently rinsed 3 times with PBS. The cells were then seeded at a density of 30,000 cells per cm^2. After 2 days on the surfaces, the cells were lysed in TRIzol™ for subsequent RT-qPCR. The cells were lysed directly in the plate and all of the samples were frozen and stored at -80°C prior to RNA purification. "Untreated ECFCs" refers to ECFCs seeded on collagen type-1 well plate, while "treated ECFCs" refers to cells seeded on functionalized biomaterials.

2.3 Human saphenous vein cells extraction and culture

HSVECs were isolated from healthy human saphenous vein segments removed during varicose vein stripping surgeries, as previously described [31]. All of the procedures were approved by the CHU de Québec - Université Laval Ethics Committee following the informed consent of donors at the Saint-François d'Assise Hospital in Quebec City. Following isolation, the cells were frozen in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO, Sigma, Canada). In each experiment, HSVECs were thawed at passage 4 and maintained in 6-well tissue culture plates in complete EGM2-MV culture medium. Cell harvesting was performed using TRIzol™ for RT-qPCR two days after seeding. The cells were lysed directly in the plate and all of the samples were frozen and stored at -80°C prior to RNA purification.

2.4 RNA isolation and cDNA synthesis

Total RNA isolated in TriZol reagent was purified by means of the Aurum total mini kit (BioRad, USA), following the manufacturer's protocol. To ensure reproducibility, all the samples were treated identically. For each sample, three biological replicates were processed. Following isolation, RNA purity was assessed by measuring absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} using a Nanodrop spectrophotometer (Nanodrop 2000, ThermoScientific, USA), while RNA integrity was determined by means of the RNA6000 Pico Kit (Agilent Technologies, USA). Briefly, 1 µL of each sample RNA solution at a concentration of 5 ng/µL was charged into a chip. Each RNA sample was tested. Once the RNA integrity was ascertained, genomic DNA (gDNA) was removed, as gDNA can shift the Cq values, particularly for low-expression genes. The Cq value is defined by the cycle at which the fluorescence of a sample first increases and crosses the threshold line and when the curvature of the amplification curve is at its maximum. Finally, RNA was reversed into cDNA by means of the Quantitect Reverse Transcription Kit (Qiagen, Germany).

2.5 Quantitative real-time procedure

Real-time PCR was performed using a 96-well plate (96 fast PCR plates, Starstedt, Germany) and the TaqMan® gene expression assay (X20) (FAM™ dye-labeled MGB probes, Table 1) and TaqMan® Fast Advanced Master Mix (X2) (Applied Biosystems, USA). PCR reactions were achieved, and the data were acquired with the 7500 Fast Real-Time PCR System (Applied Biosystems, USA). The PCR conditions were then established (Table , see the mean Cq values of three technical triplicates were used for data analysis).

2.6 PCR product assessment

The size of the seven amplicons was assessed by electrophoresis on agarose gel. 25 ng of each PCR product were charged onto 3% agarose gel (Low Electroendosmosis (EEO) Ultrapure Bioreagent, J. T. Baker, USA). The PCR products were then mixed with a loading buffer (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol, Sigma and Fisher, Canada). The

running buffer used for the electrophoresis was Tris-acetate-EDTA buffer (TAE). To analyze the size of our PCR product, we used a ladder (Quick-Load® Purple 50 bp DNA Ladder, New England BioLabs, USA). Band detection was performed using a SYBR™ Safe DNA Gel Stain (Life Technologies, USA). The gels were run using the wide mini-sub ® Cell GT Horizontal Electrophoresis System with a PowerPac™ Basic Power Supply (BioRad, USA), and images of the gels were acquired by means of G:BOX F3 (Syngene, USA).

Table 2. Candidate Reference Genes for Validation

Symbol	Cat No.	Amplicon size (bp)	UniGene No.	Gene Bank Accession No.
YWHAZ	Hs03044281_g1	106	Hs.492407	NM_003406.3
GAPDH	Hs02786624_g1	157	Hs.544577	NM_002046.4
HPRT1	Hs02800695_m1	82	Hs.412707	NM_000194.2
RPLP0	Hs00420895_gH	76	Hs.546285	NM_001002.3
UBC	Hs00824723_m1	71	Hs.520348	NM_021009.5
B2M	Hs99999907_m1	75	Hs.534255	NM_004048.2
TBP	Hs00427620_m1	91	Hs.590872	NM_003194.4

Table 3. Thermal Cycling Profile

Parameters	UNG incubation	Polymerase activation	PCR (50 cycles)	
	Hold	Hold	Denature	Anneal/extend
Temp. (°C)	50	95	95	60
Time (mm:ss)	02:00	00 :20	00 :03	00 :30

2.7 PCR efficiency

To calculate the reference genes, two-fold dilutions were prepared from pooled cDNA samples, with each sample composed of an equal cDNA quantity for each experimental condition. The dilution factor was determined following verification of the annealing temperature. When the Cq is too low, it is better to use two-fold dilutions to dilute the cDNA and acquire a good signal after the amplification. The PCR reactions were achieved as previously described. PCR efficiency (E) and

the correlation coefficient (R^2) were determined with the calibration curve of each probe using the following formula: E (%)= $(10^{(-1/slope)-}1)$ x100 [32] with the slopes determined from a standard curve obtained by converting, into a logarithm, the initial cDNA template concentration on the x axis and the Cq on the y axis.

Table 4. RT-qPCR Amplification Efficiency

Symbol	**Efficiency (%)**
YWHAZ	99.2
GAPDH	101.4
HPRT1	112.7
RPLP0	113.1
UBC	106.2
B2M	111.5
TBP	106.4

2.8 Data analysis

The stability of the standard gene expression analysis is necessary to standardize the gene of interest. It is now recognized that the standardization of a gene with a constant expression is the key to obtaining accurate results. In this study, in order to choose the ideal housekeeping genes or reference genes, several candidates were tested to identify the two or three most stable genes for the proposed experimental conditions. In this regard, three software platforms are commonly used to determine the stability of reference genes for RT-qPCR experiments.

2.8.1 BestKeeper analysis.

BestKeeper, an Excel-based application, determines the optimal housekeeping gene to use for a specific experiment. This program computes the Cq values of each candidate based on the standard deviation (SD), which represents the stability of the gene, the coefficient of correlation (R^2), and the percentage covariance. The lower the SD value, the better the stability. If the SD > 1, the gene is deemed unreliable [33].

2.8.1.1 NormFinder analysis.

NormFinder, another Excel-based algorithm, computes expression stability values to identify the most stable genes from a series of candidates. Normfinder provides a stability value for each gene, with a high stability value representing a high gene expression variance. Therefore, in NormFinder analysis, the best gene presents the lowest stability value [34]. This program enabled us to study the variation between sample subgroups of the sample set (treated/untreated).

2.8.2 GeNorm analysis.

GeNorm is used to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given set of samples. The genes are ranked based on the parameter M, which is the gene expression normalization factor calculated for each sample based on the geometric mean of a user-defined number of reference genes. This program makes it possible to identify an adequate number of reference genes from the Cq values of the set of samples and housekeeping gene candidates. Reference genes with low M values are considered to be reliably stable, contrary to genes with M > 1.5, which cannot be used as reliable and stable references [41].

2.8.3 ΔCq method.

The ΔCq program compares the relative expression of pairs of genes among all of the candidates within each tested sample. If the relative expression between the pairs of genes remains constant for each sample, it means that the pairs of genes can be used as reference genes. In the same way, if the Cq is variable, it means that the combination of these two genes is not reliable enough to normalize the gene expression in a given experimental condition. In this study, the ΔCq program compared pair-to-pair variations, allowing for the identification of two reference genes to normalize our data [42].

2.9 Final ranking

All of the candidate genes were ranked by the four methods. As the ranking could be slightly different among these programs, the means of the three ranking numbers for BestKeeper, NormFinder, and geNorm were calculated.

3 RESULTS

3.1 Annealing temperature, amplification efficiency

When employed correctly, RT-qPCR can be a powerful method to study gene expression. One of the first things to confirm is the annealing temperature. There is no exact temperature for each primer. An annealing temperature that is too low can lead to non-specific amplification, while a too high temperature can lead to poor yield and purity due to the poor annealing of primers [43]. In this study, three temperatures were tested: 58, 60 and 62°C. The ΔRn were plotted against PCR cycle number (see graphs in supplementary data). At 60°C, the ΔRn values are lower than the two other temperatures, which means that the number of cycles to reach a plateau value is lower and thereby the efficiency is higher. Moreover, the quantity of fluorescent signal emits by the probe hybridized to the target sequence is maximized, so, we choose this temperature to proceed to the PCR and study the PCR products obtained at the end of the procedure. At 60°C, the amplification efficiency was determined for each of the 7 selected candidate reference genes based on the slope of the standard curve. Table lists the obtained efficiencies established at between 99.2 and 113.1%. Each primer allowed for the identification of cDNA amplification products and each candidate showed one specific band at the expected length (see Table and Figure 1). To study the PCR product after the reaction on the agarose gel, we used the complete PCR product obtained with the commercial mix of the primers and the TaqMan fluorescent probe (FAM) instead of PCR product obtained with only primers (without fluorescent probe). As the absorption and emission wavelengths of the FAM TaqMan probe (495 and 520 nm) is very close to the SYBR safe (502 and 530 nm), it is possible that the FAM released from the TaqMan probe following the PCR hung on the proteins and/or on the DNA, which would have caused these fluorescent traces at higher molecular weights. Despite these traces, no clear band was observed for all the probes at smaller or higher molecular weights, which means that there is no degradation product and no contaminant in our samples.

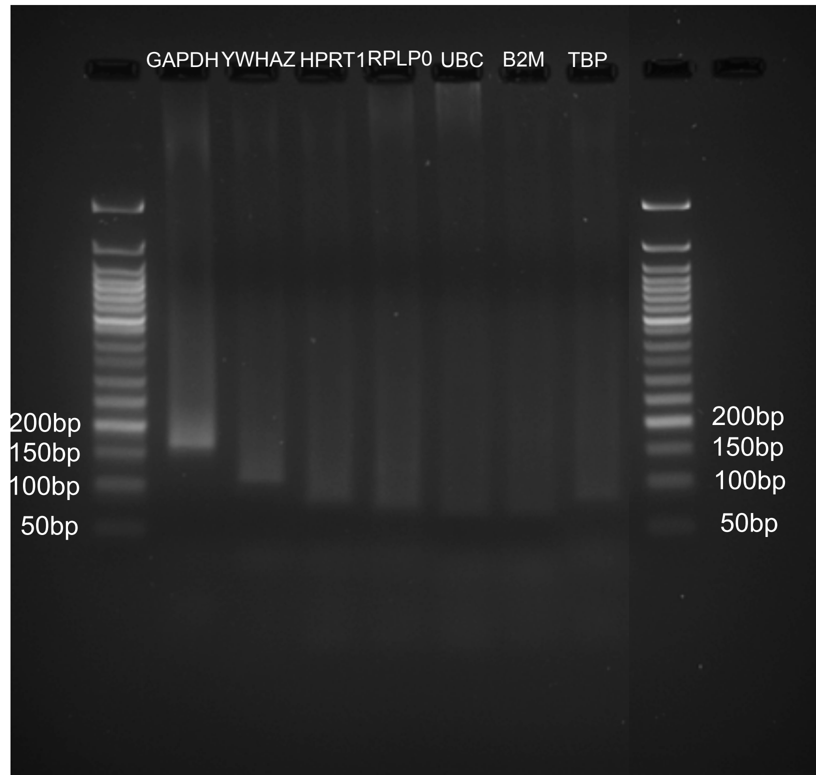

Figure 1. PCR-amplified products, 25 ng of total amplified cDNA of each target were loaded per lane. Each primer gave rise to a single band of the expected length.

3.2 Cq values

The expression level of each candidate was evaluated as quantification cycle (Cq) values, with three biological and three technical replicates for each type of sample (mononuclear cells enriched CD34+ population, ECFC-treated or untreated and HSVECs). The box plot showed differences in Cq values for all seven candidates (see Fig.2). The levels of the candidates varied slightly from 23.21 to 32.75 Cq. As shown in Figure 2, RPLP0 was the most abundant gene, with a mean Cq of 24.35, while TBP showed the lowest expression level of all of our samples with a Cq mean value of 31,91. Among all of the candidates analyzed, RPLP0 showed the largest variation in terms of standard deviation, with a recorded expression variate of 2,72 Cq between EPCs at passage 0 and HSVECs, while UBC and HPRT1 showed the lowest standard deviations with a Cq variation below 0.5. The ideal reference gene should have a low standard deviation among samples and replicates and a high gene expression, defined by low Cq value. A Grubb's test, also called the extreme studentized deviate, was performed to determine whether the extreme values obtained were significant outliers (significant level set at $P < 0.01$). No outliers were detected in our results; consequently, as the Cq values were in the same range, no sample was excluded from the remainder of the analysis [44,45].

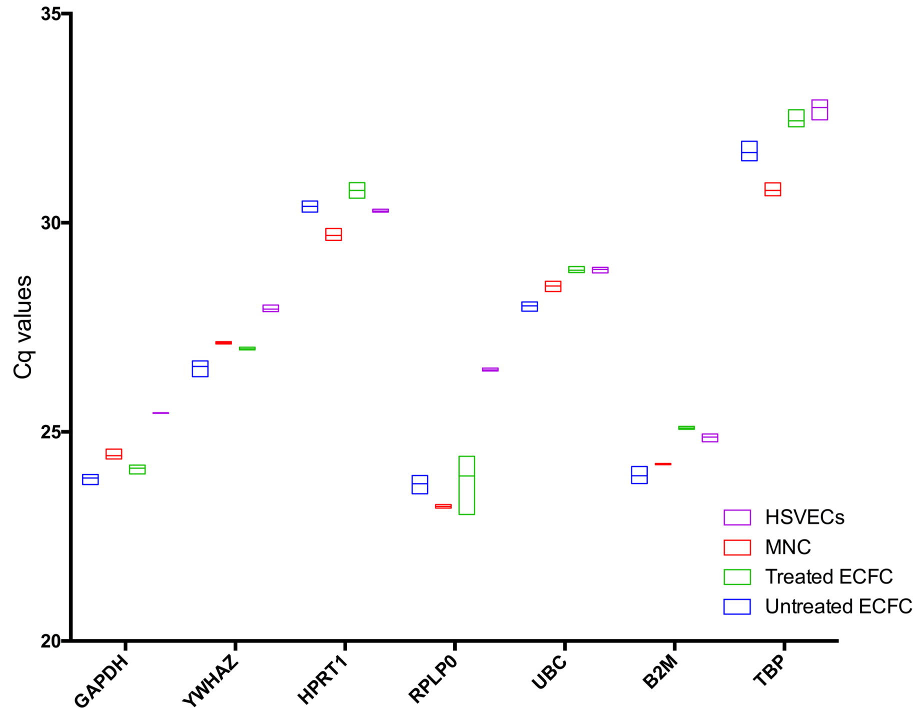

Figure 2. Distribution of the Cq values for the seven candidate reference genes. The black horizontal line within the box plot represents the median Cq value. The box indicates the first quartile Q1 and the third quartile Q3.

3.3 Analysis of candidate housekeeping gene stability

BestKeeper produces different statistical values describing the geometric and arithmetic mean of the crossing point value (CP), also called Cq, which also describes the minimal and maximal Cq values for each candidate. The standard deviation is calculated, providing information on the variability of the expression. To consider a gene reliable, the SD value should be below 1; any gene with a SD higher than 1 must be excluded from the candidate reference genes [39]. In this study, RPLP0 had a standard deviation of 1.07 and was thus considered unsuitable for our experiment. The second important value computed by this program is the Pearson correlation coefficient, which can be between +1 and -1. A value of +1 defines a total positive linear correlation, signifying that the higher the value, the higher the stability will be. In this experiment, the least stable gene was HPRT1 with a value of 0.344, rendering this gene ineligible as a housekeeping gene. TBP, GAPDH, and YWHAZ produced the highest values, followed by UBC and B2M. Considering the three important values obtained by BestKeeper, the best candidates among the seven genes tested were therefore YWHAZ, TBP, and GAPDH, followed by UBC (see Table).

Normfinder is an Excel-based visual tool that can compute the Cq value to estimate a stability value for a set of candidate genes. This program can calculate gene stability through quantitative methods, by either PCR or microarrays, from a sample set containing any number of samples organized in groups. It therefore ranks genes by their stability values; the lowest stability value is given to the most stable candidate reference gene, which in our case was YWHAZ. As shown with BestKeeper, RPLP0 was determined as the least stable gene. Compared to BestKeeper, however, Normfinder determined that the second most stable gene was GAPDH, which is frequently used as a reference for RT-qPCR normalization as shown in Table .

Table 5. Expression Stability Evaluated by BestKeeper

	YWHAZ	GAPDH	HPRT1	RPLP0	B2M	TBP	UBC
n	12	12	12	12	12	12	12
Geo Mean [CP]	27.15	24.47	30.28	24.32	24.54	31.90	28.56
Ar Mean [CP]	27.15	24.48	30.29	24.35	24.54	31.91	28.56
min [CP]	26.56	23.90	29.70	23.22	23.95	30.77	28.01
max [CP]	27.93	25.45	30.77	26.48	25.09	32.76	28.88
std dev [± CP]	0.39	0.49	0.30	1.07	0.45	0.69	0.31
CV [% CP]	1.44	1.99	0.98	4.37	1.82	2.15	1.09
coeff. of corr. [r]	0.834	0.86	0.344	0.94	0.771	0.864	0.78

Table 6. Reference Gene Stability Evaluations by NormFinder

Gene	Stability value	Standard error	Ranking
GAPDH	0.264	0.271	2
YWHAZ	0.184	0.303	1
HPRT1	0.725	0.355	6
RPLP0	1.832	0.755	7
UBC	0.409	0.275	3
B2M	0.410	0.275	4
TBP	0.415	0.276	5

With the geNorm algorithm, the average stability (M) is measured and provides a more stable expression across a panel of different samples. This program calculates the pairwise variation to determine the optimal number of reference genes to be used in a given experiment. In this study, the analyzed genes were ranked according to their stability value M, from the least stable to the most stable (see Figure 3). RPLP0 was ranked as the least stable gene and YWHAZ as the most stable gene. For the normalization of RT-qPCR data, it is recommended to use at least two reference genes for accurate gene expression results. As seen in Figure 3, GAPDH was identified as the second most stable gene. The calculated pairwise variation indicates the optimal number of reference genes to use. A value below the threshold of 0.15 means that this is the optimal number of genes to use. In our experiment, when two genes versus three genes were used, the pairwise

variation was higher than 0.15, whereas the value obtained when we compared three genes with four genes was below 0.15. To confirm previous results, when all of the genes were used to calculate the pairwise variation, the value increased, meaning that at least one of our candidates had a negative effect on this value, which in this case was RPLP0. The threshold value was attained for V3/4, signifying that the inclusion of an additional reference gene was required for this study (see

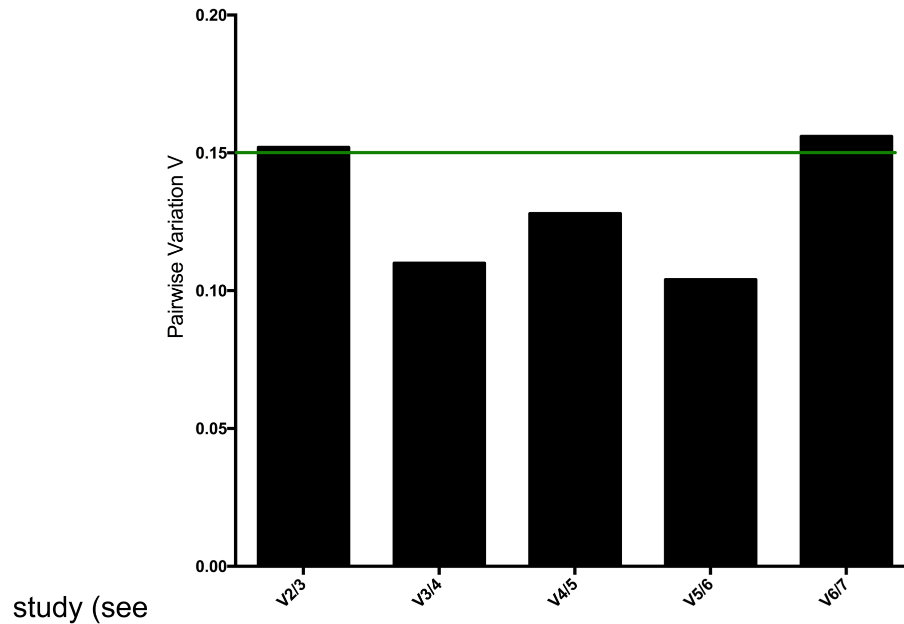

Figure 4). The geNorm program thus recommended using the three most stable genes (YWHAZ, GAPDH, and UBC) so as to normalize our data with low expression variations.

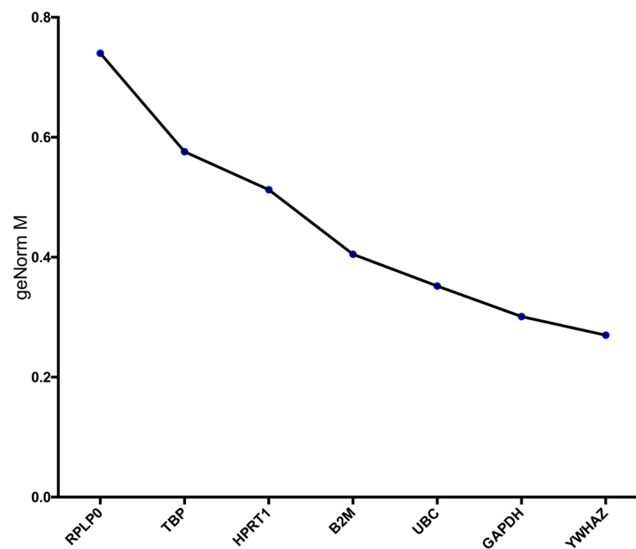

Figure 3. Expression stability value M of seven RG candidates as calculated by geNorm (qbase+). The lowest M value indicates the most stable expression.

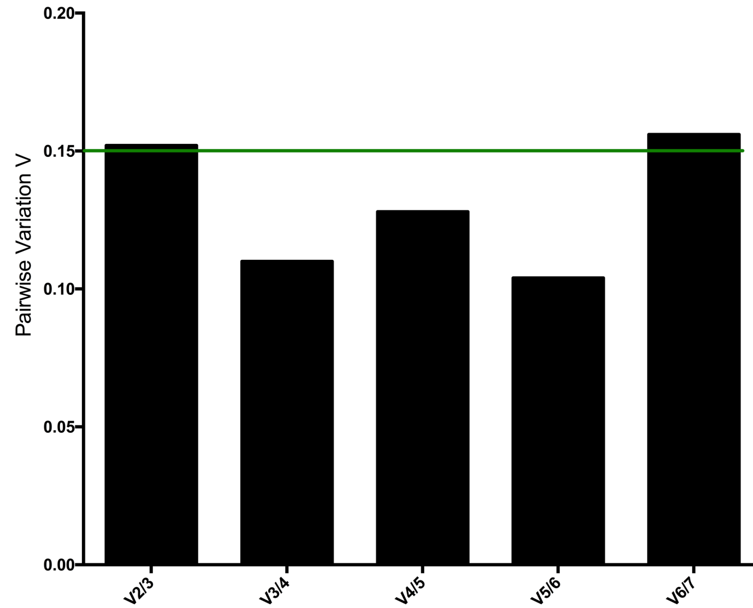

Figure 4. Pairwise variation to determine the optimal number of RGs for RT-qPCR by geNorm. V3/4 exhibited value below 0.15, thus indicating that 3 reference genes were necessary for the experiment.

The ΔCq method determines the most stable candidates by comparing the relative expression of pairs of genes for each sample and treatment. In this study, all of the combinations of gene pairs were tested. The conclusion was that tendencies exist when certain genes are associated with a second one and that this can be detected by the standard deviation of the ΔCq following the variability in gene expression. The ΔCq value is calculated, and if the value between pairs of genes remains constant in the sample being tested, it means that these two reference genes are accurate for use in the experiment. If the ΔCq value fluctuates, it means that one or two of the

genes is variably expressed. As shown in

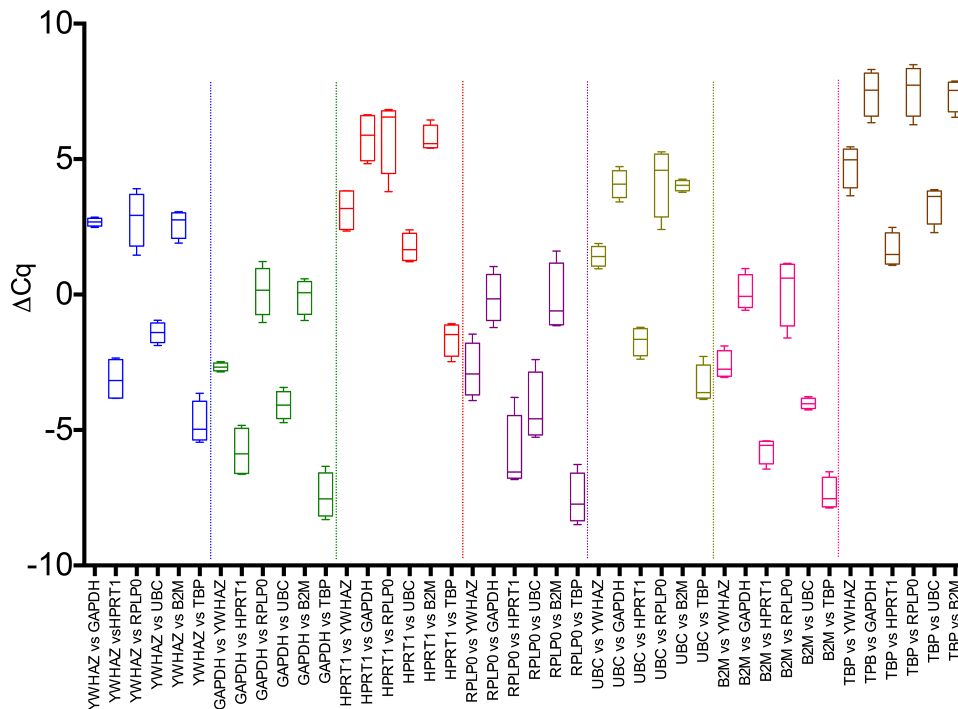

Figure 5, the lowest observed variation between two genes was for YWHAZ and GAPDH. Therefore, as the geNorm program suggested, the addition of a third reference gene was recommended for our type of study, which is UBC as geNorm and NormFinder ranked this gene in the third position. With the ΔCq method, the variation between YWHAZ and GAPDH was 0.153, which was in the same range as the variation determined by NormFinder. In addition, the variation between YWHAZ and UBC was 0.381, which was close to the value of 0.408 given by NormFinder.

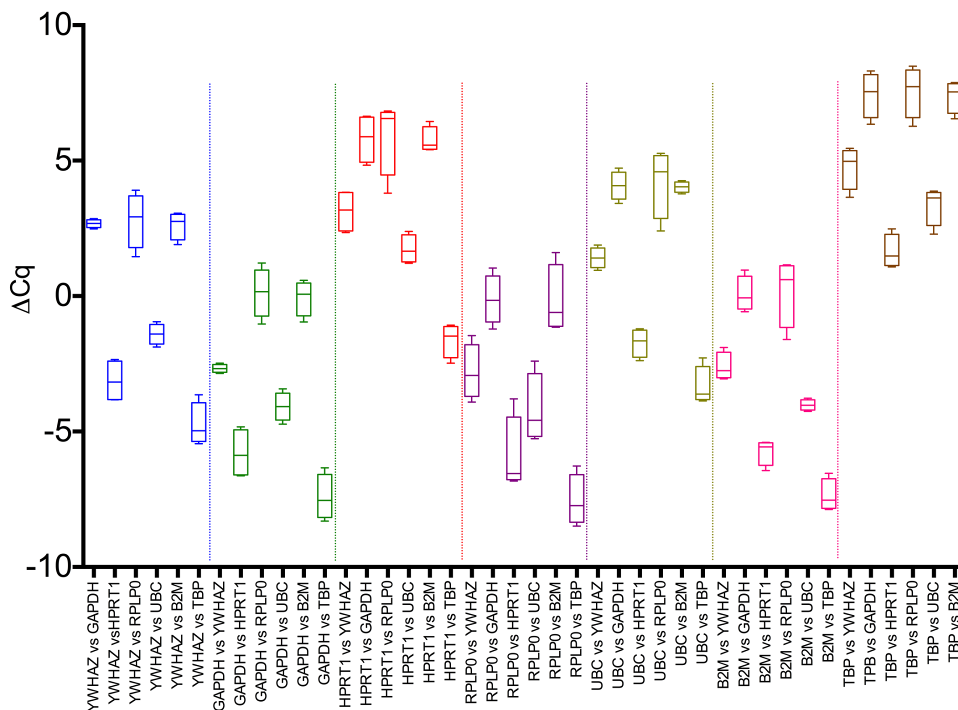

Figure 5. Reference gene evaluation stability by the ΔCq approach.

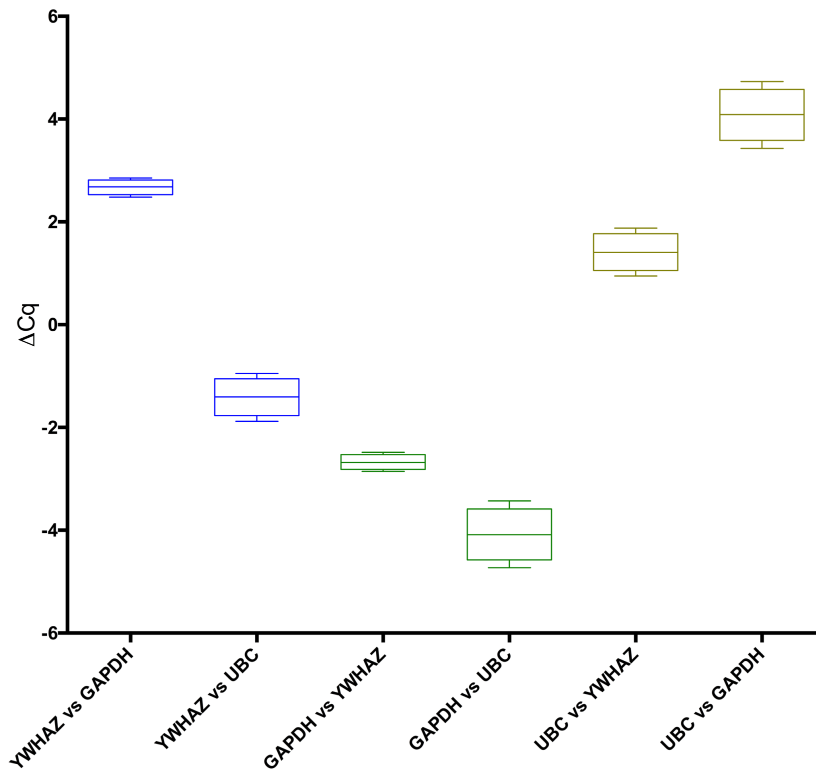

Figure 6. Reference gene stability of the three best candidates, as determined by ΔCq.

As shown in Figure 6, the recorded variation between GAPDH and UBC was slightly higher, yet lower than the variation in the other pair of genes, showing a value of 0.531, which is a magnification of that presented in Figure 5, with the best three candidates designated by geNorm. When YWHAZ was compared to the six other genes, this candidate showed the lowest average standard deviation at 0.606. Similar findings were recorded for GAPDH, with an average standard deviation of 0.664, while UBC recorded a standard deviation of 0.607, meaning that these three genes were associated with the least amount of variability (see Table).

To confirm the results obtained with BestKeeper, NormFinder, and geNorm, RPLP0 was compared to the other six genes, revealing a higher deviation in this gene, with a value of 1.142. This was also the case for TBP and HPRT1; hence, these three genes were considered as being variable and therefore not adequately reliable for normalization in our proposed experimental conditions. Interestingly, the standard deviation obtained for B2M was 0.618, which was lower than that recorded for GAPDH; however, B2M was ranked fourth with NormFinder and geNorm and in fifth position with BestKeeper. For our study, it was clear that YWHAZ was the first candidate to use, followed by GAPDH and UBC (see Table).

Table 7. Reference Gene Evaluation Stability by the ΔCq Method

Sample	SD*	Mean SD
YWHAZ vs GAPDH	0.152	0.606
YWHAZ vsHPRT1	0.785	
YWHAZ vs RPLP0	1.018	
YWHAZ vs UBC	0.381	
YWHAZ vs B2M	0.514	
YWHAZ vs TBP	0.785	
GAPDH vs YWHAZ	0.152	0.665
GAPDH vs HPRT1	0.899	
GAPDH vs RPLP0	0.918	
GAPDH vs UBC	0.532	
GAPDH vs B2M	0.653	
GAPDH vs TBP	0.835	
HPRT1 vs YWHAZ	0.785	0.790
HPRT1 vs GAPDH	0.899	
HPRT1 vs HPRT1	1.432	
HPRT1vs RPLP0	0.529	
HPRT1 vs B2M	0.477	
HPRT1 vs TBP	0.617	
RPLP0 vs YWHAZ	1.018	1.143
RPLP0 vs GAPDH	0.918	
RPLP0 vs HPRT1	1.432	
RPLP0 vs UBC	1.279	
RPLP0 vs B2M	1.268	
RPLP0 vs TBP	0.941	
UBC vs YWHAZ	0.381	0.607
UBC vs GAPDH	0.532	
UBC vs HPRT1	0.529	
UBC vs RPLP0	1.279	
UBC vs B2M	0.199	
UBC vs TBP	0.721	
B2M vs YWHAZ	0.514	0.618
B2M vs GAPDH	0.653	
B2M vs HPRT1	0.477	
B2M vs RPLP0	1.268	
B2M vs UBC	0.199	
B2M vs TBP	0.599	
TBP vs YWHAZ	0.785	0.750
TPB vs GAPDH	0.835	
TBP vs HPRT1	0.617	
TBP vs RPLP0	0.941	
TBP vs UBC	0.721	
TBP vs B2M	0.599	

* Standard deviation (SD) is given for the variation in Cq values over the 12 samples.

3.4 Final ranking

Table 8. Ranking of Reference Gene Stability with the Three Software Programs

Ranking	BestKeeper	NormFinder	geNorm	Final Ranking
1	YWHAZ	YWHAZ	YWHAZ	**YWHAZ**
2	GAPDH	GAPDH	GAPDH	**GAPDH**
3	TBP	UBC	UBC	**UBC**
4	UBC	B2M	B2M	B2M
5	B2M	TBP	HPRT1	TBP
6	HPRT1	HPRT1	TBP	HPRT1
7	RPLP0	RPLP0	RPLP0	RPLP0

4 DISCUSSION

Recruiting endothelial progenitor cells (EPCs) that can differentiate into endothelial mature cells appears to be a promising strategy to overcome the lack of spontaneous *in situ* endothelialization in vascular substitutes. The endothelial cell monolayer is necessary to render the inner surface of a graft compatible with blood, as the endothelium is the ultimate hemocompatible surface [46]. EPCs arise from the CD34+ cell population [27,47–49], [5]. [50]. In the case of vessel damage (ischemia) or the grafting of substitutes, endothelial progenitor cells are mobilized from bone marrow through cytokine signalization, such as SDF-1 [51], VEGF [52], or G-SCF [46,47]. Although the process of EPC recruitment has been widely studied, the differentiation of CD34+ cells into EPCs and EPCs into mature endothelial cells continues to elude researchers.

Mononuclear cells extracted from cord blood were enriched with CD34+ and then seeded onto a collagen-coated surface to produce endothelial progenitor cells colonies. Both of these distinct types of cells express common immaturity markers such as CD34 and CD133 (prominin), but in the case of EPCs, the expression of these two markers decreases as they differentiate into a more mature phenotype. During this process, mature markers such as CD31, vWF, and eNOS are upregulated over time under *in vitro* culture [18]; a sensitive method of detection is thus required to detect these subtle changes. In this regard, RT-qPCR gene expression analysis is a powerful and sensitive tool to obtain optimal quantitative results.

Numerous studies have demonstrated that using reliable and non-variable genes is key to normalize data and obtain accurate results. The expression of endogenous genes can vary depending on the treatment, the growth factor, or even the environmental conditions involved. In the study of biomaterials, the chemical composition but also the stiffness [53] and the roughness [54,55] of the materials on which the cells are seeded have an influence on gene expression. The ideal reference gene must be expressed at the same level in every sample and under all of the experimental conditions tested.

As we are led to work with EPCs at different stages of differentiation, employing housekeeping genes that can remain stable under these conditions is of the utmost importance. Despite the fact that EPCs were discovered in 1997 and have been used extensively since then in research, their differentiation process in mature cells is not yet fully understood. Generally, the literature regarding this cell type describes them as being part stem cells and part mature cells.

We evaluated the potential of seven housekeeping genes to use with mononuclear cells enriched CD34+ population, endothelial progenitors, and human saphenous vein cells to study the differentiation of CD34+ cells from cord blood into endothelial cell-forming colonies. These three types of cells are totally different. Mononuclear cells represent a non-adherent heterogeneous population of cells composed of primary vascular and hematopoietic stem cells, monocytes, lymphocytes, platelets, and granulocytes, whereas endothelial progenitors and human saphenous vein cells are adhesive cells that form a monolayer. However, following magnetic sorting, the subset of CD34+ mononuclear cells was led toward primary vascular cells with endothelial differentiation media, and other types of cells were removed to obtain a homogenous cell population. No other studies have validated reference genes to use with an endothelial progenitor cell subset. Furthermore, very few works relate the validation of housekeeping genes for mononuclear cells, as existing studies exclusively concern cells extracted from peripheral blood.

First and foremost, the validation procedure of RT-qPCR to determine gene efficiency, primer length, and housekeeping genes was performed prior to initiating RT-qPCR experiments. We thus compared the relative expression of seven candidate reference genes with three types of cells, namely mononuclear cells enriched CD34+ population, treated and untreated endothelial progenitor cells, and human saphenous vein endothelial cells. The amplification efficiency of all of the studied candidates was found to be between 99.2 and 113.1% at the optimized annealing temperature of 60°C, meaning that all of the primers were correctly amplified with our experimental conditions. Following the amplification reaction, the PCR products were analyzed to assess the length of the primers used for our study. Only one band on agarose gel was observed for each of the seven candidates, thus concluding that there was no contamination of our samples with gDNA, which thereby confirms that the gDNA wipeout step was successful.

Four methods were used to determine the best combination of housekeeping genes to use for the three cell populations. All four programs ranked YWHAZ and GAPDH as the two most stable genes for our experimental conditions. Of interest is that geNorm provided an indication of how many housekeeping genes could be used to achieve reliable data normalization. Here, as the cut-off value was attained for V3/4, geNorm indicated that three rather than two reference genes should be used (see Fig. 4).

The ΔCq method compares the relative expression of two genes to assess the level of variation between two housekeeping genes. We observed that the combination of YWHAZ and GAPDH produced the lowest standard variation value; in comparison, for YWHAZ and UBC, this value increased slightly, but remained lower than in other combinations. For GAPDH and UBC, the value increased, but remained acceptable. Thus, UBC was ranked third by two out of the three programs (BestKeeper, NormFinder, and geNorm) for our experiments. Three software programs are commonly used to identify the best housekeeping gene or the best combination to use for a given experiment, yet only a few studies compare the results of more than one program. Because comparisons between programs can produce variable results, the use of more than one type of algorithm in the validation of reference genes is highly recommended.

For each cell or tissue type and under specific experimental conditions, a validation step is required to ensure that no significant change in gene expression occurs [39]. The relative expression of the seven candidate genes varied between the samples. In analyzing three cell sources, the most significant variability occurred in mature cells, proving that differentiating blood stem cells possess a unique gene signature. Mononuclear cells are composed of a subset of several types of cells; this subset is a heterogeneous mix of stem cells and mature cells that can form vascular structure, blood cells, and immune cells. Considering the overall diversity of cells contained in mononuclear cells, identifying a reliable reference gene is an obvious prerequisite.

Oturai and colleagues compared the stability of eight reference genes to select the best combination to use with mononuclear cells (from peripheral blood) and the different subset of cells contained in these mononuclear cells. The cells were then compared with cells isolated from patients suffering from multiple sclerosis and patients treated with interferon-β. In general, these authors

observed that GAPDH was not a suitable candidate for all experimental conditions. However, the combination of UBC and YWHAZ showed the best stability when used with all of the cell sources (mononuclear cells, patient cells, and treated patient cells) [57]. GAPDH is frequently used as housekeeping gene, but because it displays variable stability with tissues, cell types, and experimental conditions, validating the use of this gene is mandatory before tackling any RT-qPCR experiments. For Oturai and al., GAPDH was found to be unsuitable for their experimental conditions.

In comparison, in our study, mononuclear cells were obtained from cord blood and not peripheral blood and were magnetically sorted to render a homogeneous population rather than multiple types of cells capable of generating variability in gene expression. Even within a same subset of cells, the variability of expression between experiments was remarkable.

To validate a reference gene for human umbilical vein endothelial cells (HUVECs) seeded onto substrates of different stiffness, Chen et al. concluded that GAPDH was among the least stable genes and that B2M and HPRT1 were the two most stable genes, which is inconsistent with our findings [53]. As we used cells directly extracted from cord blood or saphenous veins, the variability between samples was such that at least three biological replicates were required to address and overcome the variability issues.

Żyżyńska et al. compared the relative expression results of eight candidates with HUVECs extracted from three different donors. Their findings regard one cell type with different biological replicates, and with the variability of expression, the ranking by three programs produced different results [58], thus proving that in terms of reference gene identification, using three programs is fully justified. Similarly, in identifying housekeeping genes in HUVECs treated with hydrogen peroxide, RPLP0 was shown to be among the most stable genes, however in our study, this candidate was found to be the least stable gene [59].

Our findings highlight that the use of a non-stable reference gene presenting variances may lead to incorrect results that can limit the possibility of demonstrating relevant biological differences. An optimal setup for reference gene validation must be carried out for every experiment and every type of sample, whether treated or not. In this regard, this study shows that the greater the number of samples used for gene validation, the better the chances of overcoming variability issues among samples or biological replicates.

5 CONCLUSIONS

Our investigation demonstrates that the combination of three housekeeping genes, namely, YWHAZ, GAPDH, and UBC was necessary to obtain reliable normalization data by RT-qPCR for the assessment of ECFCs for the proposed experimental conditions. Gene expression was indeed variable among the samples and experimental conditions. This work will thus be useful to compare the relative gene expression of stem cell markers and mature endothelial cell markers of mononuclear cells enriched CD34+ population, treated and untreated EPCs, and endothelial mature cells.

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REFERENCES

[1] H.-S. Hung, W.-C. Shyu, C.-H. Tsai, S.-H. Hsu, S.-Z. Lin, Transplantation of Endothelial Progenitor Cells as Therapeutics for Cardiovascular Diseases, Cell Transplant. 18 (2009) 1003–1012. doi:10.3727/096368909X12483162196683.

[2] C. Urbich, Endothelial Progenitor Cells: Characterization and Role in Vascular Biology, Circ. Res. 95 (2004) 343–353. doi:10.1161/01.RES.0000137877.89448.78.

[3] T. Asahara, A. Kawamoto, Endothelial progenitor cells for postnatal vasculogenesis, AJP Cell Physiol. 287 (2004) C572–C579. doi:10.1152/ajpcell.00330.2003.

[4] W. Risau, I. Flamme, Vasculogenesis, Annu. Rev. Cell Dev. Biol. 11 (1995) 73–91.

[5] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, Science. 275 (1997) 964–966.

[6] P. Carmeliet, Angiogenesis in life, disease and medicine, Nature. 438 (2005) 932–936. doi:10.1038/nature04478.

[7] Peter Carmeliet, Mechanisms of angiogenesis and arteriogenesis, Nat. Med. 6 (2000) 389. doi:10.1038/74651.

[8] C.J. Drake, Embryonic and adult vasculogenesis, Birth Defects Res. Part C Embryo Today Rev. 69 (2003) 73–82. doi:10.1002/bdrc.10003.

[9] S. Patan, Vasculogenesis and Angiogenesis as Mechanisms of Vascular Network Formation, Growth and Remodeling, J. Neurooncol. 50 (2000) 1–15. doi:10.1023/A:1006493130855.

[10] J.A. Nagy, L. Benjamin, H. Zeng, A.M. Dvorak, H.F. Dvorak, Vascular permeability, vascular hyperpermeability and angiogenesis, Angiogenesis. 11 (2008) 109–119. doi:10.1007/s10456-008-9099-z.

[11] Q. Shi, M.H.-D. Wu, N. Hayashida, A.R. Wechezak, A.W. Clowes, L.R. Sauvage, Proof of fallout endothelialization of impervious dacron grafts in the aorta and inferior vena cava of the dog, J. Vasc. Surg. 20 (1994) 546–557. doi:10.1016/0741-5214(94)90279-8.

[12] P. Zilla, D. Bezuidenhout, P. Human, Prosthetic vascular grafts: Wrong models, wrong questions and no healing, Biomaterials. 28 (2007) 5009–5027. doi:10.1016/j.biomaterials.2007.07.017.

[13] P.F. Davies, J.A. Spaan, R. Krams, Shear Stress Biology of the Endothelium, Ann. Biomed. Eng. 33 (2005) 1714–1718. doi:10.1007/s10439-005-8774-0.

[14] A. de Mel, G. Jell, M.M. Stevens, A.M. Seifalian, Biofunctionalization of Biomaterials for Accelerated in Situ Endothelialization: A Review, Biomacromolecules. 9 (2008) 2969–2979. doi:10.1021/bm800681k.

[15] L. Bordenave, P. Menu, C. Baquey, Developments towards tissue-engineered, small-diameter arterial substitutes, Expert Rev. Med. Devices. 5 (2008) 337–347. doi:10.1586/17434440.5.3.337.

[16] T. Liu, S. Liu, K. Zhang, J. Chen, N. Huang, Endothelialization of implanted cardiovascular biomaterial surfaces: The development from in vitro to in vivo: Endothelialization of Implanted Cardiovascular Biomaterial Surfaces, J. Biomed. Mater. Res. A. 102 (2014) 3754–3772. doi:10.1002/jbm.a.35025.

[17] A. Kawamoto, D.W. Losordo, Endothelial Progenitor Cells for Cardiovascular Regeneration, Trends Cardiovasc. Med. 18 (2008) 33–37. doi:10.1016/j.tcm.2007.11.004.

[18] M.C. Yoder, Human Endothelial Progenitor Cells, Cold Spring Harb. Perspect. Med. 2 (2012) a006692–a006692. doi:10.1101/cshperspect.a006692.

[19] F. Timmermans, J. Plum, M.C. Yöder, D.A. Ingram, B. Vandekerckhove, J. Case, Endothelial progenitor cells: identity defined?, J. Cell. Mol. Med. 13 (2008) 87–102. doi:10.1111/j.1582-4934.2008.00598.x.

[20] C.-C. Cheng, S.-J. Chang, Y.-N. Chueh, T.-S. Huang, P.-H. Huang, S.-M. Cheng, T.-N. Tsai, J.-W. Chen, H.-W. Wang, Distinct angiogenesis roles and surface markers of early and late endothelial progenitor cells revealed by functional group analyses, BMC Genomics. 14 (2013) 182.

[21] J. Hur, C.-H. Yoon, H.-S. Kim, J.-H. Choi, H.-J. Kang, K.-K. Hwang, B.-H. Oh, M.-M. Lee, Y.-B. Park, Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasculogenesis, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 288–293. doi:10.1161/01.ATV.0000114236.77009.06.

[22] D.A. Ingram, Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood, Blood. 104 (2004) 2752–2760. doi:10.1182/blood-2004-04-1396.

[23] N. Prasain, M.R. Lee, S. Vemula, J.L. Meador, M. Yoshimoto, M.J. Ferkowicz, A. Fett, M. Gupta, B.M. Rapp, M.R. Saadatzadeh, M. Ginsberg, O. Elemento, Y. Lee, S.L. Voytik-Harbin, H.M. Chung, K.S. Hong, E. Reid, C.L. O'Neill, R.J. Medina, A.W. Stitt, M.P. Murphy, S. Rafii, H.E. Broxmeyer, M.C. Yoder, Differentiation of human pluripotent stem cells to cells similar to cord-blood endothelial colony–forming cells, Nat. Biotechnol. 32 (2014) 1151–1157. doi:10.1038/nbt.3048.

[24] E.J. Kushner, G.P. Van Guilder, O.J. MacEneaney, J.N. Cech, B.L. Stauffer, C.A. DeSouza, Aging and endothelial progenitor cell telomere length in healthy men, Clin. Chem. Lab. Med. 47 (2009). doi:10.1515/CCLM.2009.016.

[25] T. Murohara, H. Ikeda, J. Duan, S. Shintani, K. Sasaki, H. Eguchi, I. Onitsuka, K. Matsui, T. Imaizumi, Transplanted cord blood–derived endothelial precursor cells augment postnatal neovascularization, J. Clin. Invest. 105 (2000) 1527.

[26] M.C. Yoder, L.E. Mead, D. Prater, T.R. Krier, K.N. Mroueh, F. Li, R. Krasich, C.J. Temm, J.T. Prchal, D.A. Ingram, Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals, Blood. 109 (2007) 1801–1809.

[27] M. Peichev, A.J. Naiyer, D. Pereira, Z. Zhu, W.J. Lane, M. Williams, M.C. Oz, D.J. Hicklin, L. Witte, M.A. Moore, others, Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors, Blood. 95 (2000) 952–958.

[28] L.M. Pelus, H. Bian, S. Fukuda, D. Wong, A. Merzouk, H. Salari, The CXCR4 agonist peptide, CTCE-0021, rapidly mobilizes polymorphonuclear neutrophils and hematopoietic progenitor cells into peripheral blood and synergizes with granulocyte colony-stimulating factor, Exp. Hematol. 33 (2005) 295–307.

[29] I. Petit, D. Jin, S. Rafii, The SDF-1–CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis, Trends Immunol. 28 (2007) 299–307.

[30] S.A. Bustin, Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. 29 (2002) 23–39.

[31] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments, Clin. Chem. 55 (2009) 611–622. doi:10.1373/clinchem.2008.112797.

[32] E. Deindl, K. Boengler, N. Van Royen, W. Schaper, Differential expression of GAPDH and β-actin in growing collateral arteries, Mol. Cell. Biochem. 236 (2002) 139–146.

[33] A. Radonić, S. Thulke, I.M. Mackay, O. Landt, W. Siegert, A. Nitsche, Guideline to reference gene selection for quantitative real-time PCR, Biochem. Biophys. Res. Commun. 313 (2004) 856–862. doi:10.1016/j.bbrc.2003.11.177.

[34] J. Kim, Y.-J. Jeon, H.E. Kim, J.M. Shin, H.M. Chung, J.-I. Chae, Comparative proteomic analysis of endothelial cells progenitor cells derived from cord blood- and peripheral blood for cell therapy, Biomaterials. 34 (2013) 1669–1685. doi:10.1016/j.biomaterials.2012.11.017.

[35] J. Synnergren, T.L. Giesler, S. Adak, R. Tandon, K. Noaksson, A. Lindahl, P. Nilsson, D. Nelson, B. Olsson, M.C. Englund, Differentiating human embryonic stem cells express a unique housekeeping gene signature, Stem Cells. 25 (2007) 473–480.

[36] N.B. Thébaud, R. Bareille, R. Daculsi, C. Bourget, M. Rémy, H. Kerdjoudj, P. Menu, L. Bordenave, Polyelectrolyte multilayer films allow seeded human progenitor-derived endothelial cells to remain functional under shear stress in vitro, Acta Biomater. 6 (2010) 1437–1445. doi:10.1016/j.actbio.2009.11.012.

[37] M.-C. Boivin, P. Chevallier, C.A. Hoesli, J. Lagueux, R. Bareille, M. Rémy, L. Bordenave, M.-C. Durrieu, G. Laroche, Human saphenous vein endothelial cell adhesion and expansion on micropatterned polytetrafluoroethylene, J. Biomed. Mater. Res. A. 101A (2013) 694–703. doi:10.1002/jbm.a.34367.

[38] A. Tichopad, Standardized determination of real-time PCR efficiency from a single reaction set-up, Nucleic Acids Res. 31 (2003) 122e – 122. doi:10.1093/nar/gng122.

[39] M.W. Pfaffl, A. Tichopad, C. Prgomet, T.P. Neuvians, Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations, Biotechnol. Lett. 26 (2004) 509–515.

[40] C.L. Andersen, J.L. Jensen, T.F. Ørntoft, 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. 64 (2004) 5245–5250.

[41] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol. 3 (2002) research0034–1.

[42] N. Silver, S. Best, J. Jiang, S.L. Thein, Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR, BMC Mol. Biol. 7 (2006) 33.

[43] S. Taylor, M. Wakem, G. Dijkman, M. Alsarraj, M. Nguyen, A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines, Methods. 50 (2010) S1–S5. doi:10.1016/j.ymeth.2010.01.005.

[44] A. Forootan, R. Sjöback, J. Björkman, B. Sjögreen, L. Linz, M. Kubista, Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR), Biomol. Detect. Quantif. 12 (2017) 1–6. doi:10.1016/j.bdq.2017.04.001.

[45] E. Ragni, M. Viganò, P. Rebulla, R. Giordano, L. Lazzari, What is beyond a qRT-PCR study on mesenchymal stem cell differentiation properties: how to choose the most reliable housekeeping genes, J. Cell. Mol. Med. 17 (2013) 168–180. doi:10.1111/j.1582-4934.2012.01660.x.

[46] P. Rajendran, T. Rengarajan, J. Thangavel, Y. Nishigaki, D. Sakthisekaran, G. Sethi, I. Nishigaki, The Vascular Endothelium and Human Diseases, Int. J. Biol. Sci. 9 (2013) 1057–1069. doi:10.7150/ijbs.7502.

[47] I. Ahrens, H. Domeij, D. Topcic, I. Haviv, R.-M. Merivirta, A. Agrotis, E. Leitner, J.B. Jowett, C. Bode, M. Lappas, K. Peter, Successful In Vitro Expansion and Differentiation of Cord Blood Derived CD34+ Cells into Early Endothelial Progenitor Cells Reveals Highly Differential Gene Expression, PLoS ONE. 6 (2011) e23210. doi:10.1371/journal.pone.0023210.

[48] C.-L. Fan, Y. Li, P.-J. Gao, J.-J. Liu, X.-J. Zhang, D.-L. Zhu, Differentiation of endothelial progenitor cells from human umbilical cord blood CD34^+ cells in vitro, Acta Pharmacol. Sin. 24 (2003) 212–218.

[49] M. Qin, X. Guan, H. Wang, Y. Zhang, B. Shen, Q. Zhang, W. Dai, Y. Ma, Y. Jiang, An effective ex-vivo approach for inducing endothelial progenitor cells from umbilical cord blood CD34+ cells, Stem Cell Res. Ther. 8 (2017). doi:10.1186/s13287-017-0482-9.

[50] S. Balaji, A. King, T.M. Crombleholme, S.G. Keswani, The Role of Endothelial Progenitor Cells in Postnatal Vasculogenesis: Implications for Therapeutic Neovascularization and Wound Healing, Adv. Wound Care. 2 (2013) 283–295. doi:10.1089/wound.2012.0398.

[51] K. Stellos, H. Langer, K. Daub, T. Schoenberger, A. Gauss, T. Geisler, B. Bigalke, I. Mueller, M. Schumm, I. Schaefer, P. Seizer, B.F. Kraemer, D. Siegel-Axel, A.E. May, S. Lindemann, M. Gawaz, Platelet-Derived Stromal Cell-Derived Factor-1 Regulates Adhesion and Promotes Differentiation of Human CD34+ Cells to Endothelial Progenitor Cells, Circulation. 117 (2008) 206–215. doi:10.1161/CIRCULATIONAHA.107.714691.

[52] T. Takahashi, C. Kalka, H. Masuda, D. Chen, M. Silver, M. Kearney, M. Magner, J.M. Isner, T. Asahara, Ischemia-and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization, Nat. Med. 5 (1999) 434–438.

[53] G. Chen, L. Zhao, J. Feng, G. You, Q. Sun, P. Li, D. Han, H. Zhou, Validation of Reliable Reference Genes for Real-Time PCR in Human Umbilical Vein Endothelial Cells on Substrates with Different Stiffness, PLoS ONE. 8 (2013) e67360. doi:10.1371/journal.pone.0067360.

[54] P.. Brett, J. Harle, V. Salih, R. Mihoc, I. Olsen, F.. Jones, M. Tonetti, Roughness response genes in osteoblasts, Bone. 35 (2004) 124–133. doi:10.1016/j.bone.2004.03.009.

[55] W.-C. Chen, Y.-S. Chen, C.-L. Ko, Y. Lin, T.-H. Kuo, H.-N. Kuo, Interaction of progenitor bone cells with different surface modifications of titanium implant, Mater. Sci. Eng. C. 37 (2014) 305–313. doi:10.1016/j.msec.2014.01.022.

[56] B. Kozera, M. Rapacz, Reference genes in real-time PCR, J. Appl. Genet. 54 (2013) 391–406. doi:10.1007/s13353-013-0173-x.

[57] D.B. Oturai, H.B. Søndergaard, L. Börnsen, F. Sellebjerg, J. Romme Christensen, Identification of Suitable Reference Genes for Peripheral Blood Mononuclear Cell Subset Studies in Multiple Sclerosis, Scand. J. Immunol. 83 (2016) 72–80. doi:10.1111/sji.12391.

[58] B. Żyżyńska-Granica, K. Koziak, Identification of Suitable Reference Genes for Real-Time PCR Analysis of Statin-Treated Human Umbilical Vein Endothelial Cells, PLoS ONE. 7 (2012) e51547. doi:10.1371/journal.pone.0051547.

[59] T. Li, H. Diao, L. Zhao, Y. Xing, J. Zhang, N. Liu, Y. Yan, X. Tian, W. Sun, B. Liu, Identification of suitable reference genes for real-time quantitative PCR analysis of hydrogen peroxide-treated human umbilical vein endothelial cells, BMC Mol. Biol. 18 (2017). doi:10.1186/s12867-017-0086-z.