Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere-derived cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors

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Abstract Infarction irreversibly damages the heart, with formation of an akinetic scar that may lead to heart failure. Endogenous cardiac stem cells (CSCs) are a promising candidate cell source for restoring lost tissue and thereby preventing heart failure. CSCs may be isolated in vitro, via the formation of cardiospheres, to give cardiosphere-derived cells (CDCs). Although qRT-PCR analyses of CDCs have been performed, no justification for the selection of the housekeeping gene has been published. Here, we evaluated the most suitable housekeeping gene for RNA expression analysis in CDCs cultured under normoxia, hypoxia, hypoxia or with prolyl-4-hydroxylase inhibitors (PHDIs), from both neonatal and adult rats, to determine the effects of ageing and different culture conditions on the stability of the housekeeping gene for CDCs. Six candidate housekeeping genes, [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (Actb), hypoxanthine phosphoribosyltransferase 1 (HPRT-1), beta-2-microtubulin (b2M), 60S acidic ribosomal protein large P1 (RPLP-1) and TATA box binding protein (Tbp)] were evaluated in this study. Analysis using geNorm and NormFinder revealed that GAPDH was the most constant housekeeping gene among all genes tested under normoxia for both neonatal and adult CDCs, whereas Actb was the most stable housekeeping gene under hypoxia. For the PHDI-treated CDCs, overall, GADPH, Actb and b2M were more consistently expressed, whereas HPRT-1, RPLP-1 and Tbp showed unstable expression. The ranking for b2M, HPRT-1 and RPLP-1 stability was different for neonatal and adult cells, indicating that expression of these genes was age-dependent. Lastly, independent of age or culture conditions, Tbp was the least stable housekeeping gene. In conclusion, a combination of Actb and GADPH gave the most reliable normalization for comparative analyses of gene transcription in neonatal and adult rat CDCs preconditioned by hypoxia or PHDIs.

Keywords Housekeeping genes · Cardiosphere-derived cells · Hypoxia · Prolyl-4-hydroxylase inhibitors

Introduction

Stem cells were first identified within the heart in 2003 [1], challenging the generally accepted paradigm that the heart is a post mitotic organ [2, 3]. In 2004, Messina et al. [4] developed a method to isolate stem cells from human and murine heart, expanding them as cardiospheres (CSP).
Such CSp were clonogenic, expressed stem and endothelial progenitor cell antigens/markers, and appeared to have the properties of adult cardiac stem cells. The CSp were expanded to obtain a reasonable number of cardiogenic-derived cells (CDC) for transplantation [5]. CSp and CDCs derived from heart explant culture in vitro caused myocardial regeneration and functional improvement when injected into the infarcted mouse heart in vivo [4, 5].

Preconditioning of stem cells to enable survival in the hypoxic environment has been postulated to improve cell production efficiency and potency for myocardial repair [6]. The implantation of hypoxic (5% O2) CDCs into infarcted hearts of mice in vivo resulted in greater cell engraftment and better functional recovery than with conventionally cultured (normoxic) CDCs [6]. Hypoxia triggers a number of physiological and cellular adaptations to reduced oxygen, with many processes involved in oxygen homeostasis being mediated by the hypoxia-inducible factor (HIF) transcriptional complex, which is negatively regulated by the prolyl-4-hydroxylase (PHD) enzyme. The PHD enzyme is a conserved subfamily of dioxygenases that uses oxygen and 2-oxoglutarate as a co-substrate and iron as a co-factor to catalyse the post-translational hydroxylation of specific prolyl residues of HIF-α subunits [7–9]. Upon hydroxylation, the HIF-1α subunit is recognized by the von Hippel–Lindau protein which targets the subunit to proteosomal degradation [9]. The sensitivity of PHD hydroxylase capacity depends on its co-substrate and co-factor. Thus, limited oxygen supply or inhibition of the 2-oxoglutarate or iron could inhibit PHD activity and thus potently activate the HIF response [10–13].

Here, we cultured CDCs under hypoxia (2% oxygen) or used three different prolyl-4-hydroxylase inhibitors (PHDI s) for CDC HIF stabilization. The PHDI s used in this study included dimethyloxaloylglycine (DMOG) – a cell permeable, competitive inhibitor of 2-oxoglutarate [11], ethyl 2-(2,3-dihydroxybenzamido) acetate (EDBA) – an aspirin metabolite that activates the HIF system via generic 2-oxoglutarate oxygenase inhibition [14], and 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetic acid (BIC) – a specific PHD inhibitor patented by FibroGen Inc. (FG2216), which has been used in clinical trials as a pro-angiogenic compound acting via the HIF-1α system [12].

Gene expression analyses are useful to investigate changes in the CDC phenotype after hypoxic and PHDI treatment. The most commonly used technique for gene expression analysis is quantitative (real-time) reverse transcriptase polymerase chain reaction (qRT-PCR), in which expression magnitude is normalized to a reference gene. This gene, referred to as a housekeeping gene, is typically a constitutive gene that is expressed at relatively constant levels in all cells independent of experimental conditions. Selection of the housekeeping gene is crucial because it directly influences the interpretation of qRT-PCR data. However, no one housekeeping gene is universal under all experimental conditions. Many papers have been published using qRT-PCR analysis of CDCs [15–17], hypoxic CDCs [6] or other cells treated with PHDI s [18–21], but none have described the background for selecting a suitable housekeeping gene for quantification. Therefore, in this study, we evaluated the most suitable housekeeping gene for RNA expression analysis in normoxic, hypoxic and PHDI-preconditioned CDCs isolated from both neonatal and adult rat hearts, using a panel of 6 housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (Actb), hypoxanthine phosphoribosyltransferase 1 (HPRT-1), beta-2-microtubulin (β2M), 60S acidic ribosomal protein large P1 (RPLP-1) and TATA box binding protein (Tbp) (Table 1). These genes were chosen as they are most commonly used as reference genes in rat qRT-PCR studies [16, 22–32] (Additional file 1).

Results

Hypoxic response

Western blot analysis showed that CDCs cultured under hypoxia or treated with PHDI s had significantly increased HIF-1α protein levels, compared to normoxic CDCs (Fig. 1). This indicated that the hypoxic challenge to the cells and the PHDI treatments successfully elevated HIF-1α.

RNA and cDNA quality

RNA was isolated from neonatal and adult CDCs subjected to five different culture conditions (Table 2). The RNA purity was measured using a NanoDrop® Spectrophotometer (NanoDrop Technologies). On average, the A260/280 ratio of RNA samples was 2.01 ± 0.04, indicating protein-free pure RNA, and the ratio of A260/230 was 1.98 ± 0.21, indicating that the RNA was phenol and ethanol free. cDNA was synthesized from the pure RNA template and cDNA purity was verified with the mean ratio of A260/280 = 1.81 ± 0.01.

Primer efficiency

Primers for GAPDH, Actb, HPRT-1, β2M, RPLP-1, Tbp and HIF-1α were designed using Primer3 software. A primer efficiency test using serial dilution curves gave efficiencies for all primers from 92 to 99% with a correlation coefficient (R2) ranging from 0.92 to 0.99 (Table 3), indicating that all the designed primers worked successfully and gave consistent results throughout efficiency testing.
Table 1 List of reference genes and target gene used in the present study

| No. | Code | Primer | Gene name | Forward primer 5’ to 3’ | Reverse primer 5’ to 3’ | Gene accession number | Function of the protein coded by this gene |
|-----|------|--------|-----------|-------------------------|-------------------------|-----------------------|------------------------------------------|
| 1   | HKG  | GAPDH  | Glyceraldehyde-3-phosphate dehydrogenase | GGGTGTGAACCACGAGAAAT | ACTGTGGTCATGAGCCTTC | NM_017008 | Oxidoreductase in glycolysis and gluconeogenesis |
| 2   | HKG  | Actb   | Beta-actin | CTAAGGCAACCGTGAAAAG | AACACAGCCTGGATGCTAC | NM_031144 | Cytoskeletal structure protein |
| 3   | HKG  | HPRT-1 | Hypoxanthine phosphoribosyltransferase 1 | CTCATGGACTGATTATGGACAGAC | GCAGGTCAGCAAGAACATTTATAGCC | NM_012583 | Recycle purines in cells |
| 4   | HKG  | β2M    | Beta-2-microglobulin | CCGTGATCTTTCTGGTGCTT | ATTTGAGGGTGGTGAACGTG | NM_012512 | Highly conserved proteins that are involved in cell motility, structure and integrity |
| 5   | HKG  | RPLP-1 | 60S acidic ribosomal protein large P1 | AAAGCAGCTGGTGTCAATGTT | GCAGATGAGGCTTCAATGTT | ENSRNOG00000013874* | Protein synthesis |
| 6   | HKG  | Tbp    | TATA box binding protein | TTCTGGGAAATGGTGCTC | CCCACCATGTCTGGATCTT | NM_001004198 | Transcription factor that binds to TATA box located at upstream of the transcription start site in eukaryotic gene promoters |
| 7   | TG   | HIF-1α | Hypoxia induced factor-1 alpha | GGTGGATATGTCTGGGTGAG | TTCAACTGTTTGGAGGACAGA | NM_024359 | Transcription factors that respond to reduced oxygen changes in the cellular environment |

Gene symbol, name, primer sequences, gene accession and function are shown.
* Exon sequences browsed using Ensembl Genome Browser; others were browsed using GenBank Genome Browser. HKG housekeeping gene, TG target gene.
The expression profiles of candidate housekeeping genes were determined using qRT-PCR. The cycle threshold (Ct) values were plotted directly, assuming the same threshold for all genes. Ct is defined as the number of cycles needed for fluorescence to reach a specific threshold level of detection and is inversely related to the amount of initial RNA template present in the sample. The six candidate housekeeping genes showed a wide Ct expression range, with values between 14 and 28 cycles (Fig. 2). These genes had different expression levels, with RPLP-1 being the most abundantly expressed gene, presenting average Ct values below 16 cycles. Actb, β2M, and GAPDH were moderately expressed in these samples, with average Ct values between 16 and 22 cycles, while Tbp and HPRT-1 were the least expressed housekeeping genes, with Ct values above 24 cycles. For qRT-PCR normalization, a moderately expressed housekeeping gene is preferred because extremely high or low expression of a housekeeping gene could introduce variability to the data analysis.

HKGs stability test using geNorm and NormFinder

To identify the housekeeping gene with the most stable expression in the CDC samples, two software-based methods were used: geNorm and NormFinder.
**geNorm analysis**

geNorm is a well published Microsoft Excel based program designed to validate stability of housekeeping gene expression. The underlying principles and calculations are described in Vandesompele et al. [33]. In brief, geNorm ranks gene stability by an average expression stability value (M), which is the average pairwise variation of a single candidate housekeeping gene with all other candidate housekeeping genes. More stable gene expression is indicated by lower M values.

To investigate the effects of ageing on the ranking of candidate genes, housekeeping gene stability was determined in neonatal and adult CDCs (Fig. 3a, b). In the neonatal cells, both Actb and GAPDH genes had the lowest M value, followed by RPLP-1, β2M, HPRT-1 and Tbp. This indicated that in neonatal CDCs preconditioned with either hypoxia or PHDI, Actb and GAPDH were most stable genes and Tbp was the least stable gene (Fig. 3a). In the adult cells, a combination of Actb and GAPDH genes was found to be the most stable housekeeping genes, compared to other candidate genes. However, the ranking for β2M, HPRT-1 and RPLP-1 was different from that found in the neonatal group (Fig. 3b), indicating that expression of β2M, HPRT-1 and RPLP-1 was age-dependent. Independent of age, Tbp was the least stable housekeeping gene. When neonatal and adult CDCs were analyzed in combination, a similar ranking of candidate genes was observed (Fig. 3c).

In addition, geNorm analysis revealed the optimal number of housekeeping genes required for reliable and accurate normalization of qRT-PCR expression data, based on the average pairwise variation \( V_{n/n+1} \) calculated between \( n \) and \( n + 1 \) housekeeping genes (recommended cut-off threshold of \( V_{n/n+1} = 0.15 \)) [33]. In both neonatal and adult groups, the pairwise variation \( V_{2/3} < 0.15 \) indicated there was no need to include a third gene to calculate the normalization factor. Therefore, the two best performing housekeeping genes, GAPDH and Actb, could accurately normalize qRT-PCR expression (Fig. 4).

However, a limitation of this software is that the M value measured is the expression of a single gene as a ratio of the other genes tested. Thus, we could not resolve the best gene out of the two best performing genes, GADPH and Actb. In addition, geNorm analysis relies on the principle that the two best performing genes are identical in all samples, regardless of the experimental conditions or treatments, which is not necessarily true. To address this problem, NormFinder software was also used.

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**Fig. 3** geNorm analysis of the candidate housekeeping genes average expression stability, M. Genes with highest M value represents the least stable gene. Samples were analyzed separately according to the age group. a neonatal, b adult or c were analyzed in a combined set (results obtained from three sets of biological replicates)

**Fig. 4** geNorm analysis of the optimal number of housekeeping genes required for data normalization. A variation coefficient, V, value below 0.15 was accepted as indicating the optimal number of genes required for the data normalization. V2/3 < 0.15 indicated that 2 genes were required for the data normalization (results obtained from three sets of biological replicates)
NormFinder analysis

NormFinder, introduced by Andersen et al. [34], assesses the expression stability of a gene by evaluating the variation of expression within groups or between groups. Genes with the lowest variation values (V) have the most stable expression and are therefore ideal reference genes. This program allows not only the estimation of overall expression variation of the candidate housekeeping genes across a group of samples, but also of the variation between subgroups of a sample, e.g., normoxic CDCs versus hypoxic CDCs or non-treated CDCs versus PHDI-treated CDCs. Using NormFinder, we found that GADPH was the most stable gene expressed in both neonatal and adult normoxic cells, while Actb was the most stable gene expressed in both neonatal and adult hypoxic cell groups. For DMOG and EDBA-treated samples, GADPH was the best housekeeping gene for neonatal cells and Actb was best for adult cells. For BIC-treated samples, GADPH was the best housekeeping gene for neonatal cells and β2M was the best for adult cells (Table 4a, b). Overall, GADPH, Actb and β2M were the most consistently expressed housekeeping genes, always ranked in the first three places, while HPRT-1, RPLP-1 and Tbp were unstable, ranked in the last three places (Table 4c). Intergroup stability showed that a combination of GAPDH and Actb were the best housekeeping genes (Fig. 5) (Additional information as given in Additional file 2).

HIF-1α mRNA expression normalized by different HKGs

We used normalization to different housekeeping genes to compare the gene expression of HIF-1α in CDCs preconditioned under normoxia or hypoxia or with PHDIs, for CDCs from both neonatal and adult rat hearts. Normalization was performed using GADPH, Actb, HPRT-1, RPLP-1, β2M, Tbp and a combination of GAPDH and Actb as reference genes. As shown in Fig. 6, when expression levels were normalized to the non-stable housekeeping genes, HPRT-1, RPLP-1 or Tbp, HIF-1α expression was the same for the normoxic, hypoxic and PHDI-treated samples for either neonatal or adult groups. Normalization to GADPH, Actb or β2M gave significantly different results in some, but not all, samples. All hypoxic and PHDI-treated samples from both neonatal and adult groups showed significant increases in HIF-1α expression, compared with the normoxic control, only when the qRT-PCR data were normalized to the combination of GAPDH and Actb, the optimal HKGs combination validated using geNorm and NormFinder, demonstrating that the simultaneous use of two optimal housekeeping genes (GAPDH and Actb) is required to generate valid normalized qRT-PCR data.

Discussion

This study clearly illustrates the differences in stability of housekeeping genes in rat CDCs cultured under normoxia (21% O2), hypoxia (2% O2) or with the PHDIs: DMOG, EDBA or BIC.

Preconditioning of cells under hypoxia or in the presence of an iron chelator (EDBA), a 2-oxoglutarate analogue (DMOG) or a specific prolyl hydroxylase inhibitor (BIC), inhibits PHD enzyme activity and subsequently activates the HIF transcriptional complex [10–13]. Analysis of protein levels in hypoxic and PHDI-treated CDCs revealed a significant increase in HIF-α expression, compared to normoxic CDCs, in both the neonatal and adult group, indicating that the hypoxic challenge to the cells was successful. Analysis of mRNA levels in hypoxic and PHDI-treated CDCs using qRT-PCR is, however, not as straightforward as the protein analysis. The analysis of qRT-PCR data required normalization using a consistently expressed housekeeping gene.

RPLP-1, which plays a role in the elongation step of protein synthesis [35], was the most abundantly expressed gene in the rat CDCs, whereas HPRT-1 (an enzyme which recycles purine in cells) [36] and Tbp (a transcription factor which binds to the TATA-box DNA sequence) [37], were the least expressed genes. Genes with extensive or low expression are not suitable reference genes for qRT-PCR due to their extreme transcription expression. Our conclusions were validated by the geNorm and Normfinder analysis, which showed that these three genes were relatively unstable, compared to GAPDH, β2M and Actb, which showed moderate expression in rat CDC.

To evaluate the effect of ageing on HKG stability, we grouped the samples into neonatal and adult groups and analyzed the data either separately or as a combined set using geNorm. In both approaches, we found that a combination of GAPDH and Actb were best to be used as housekeeping gene, while the Tbp was the least stable among all the candidate genes. The instability of β2M, HPRT-1 and RPLP-1 at different stages of heart development is a drawback in their use as housekeeping genes.

Normalization with multiple reference genes is becoming more prevalent, but studies that apply this normalization approach are limited. Based on geNorm analysis, we found that an optimum of two housekeeping genes was needed to perform a valid normalization. To confirm this, we evaluated the expression of HIF-1α mRNA in the study samples normalized to only one housekeeping gene or to a combination of the two best housekeeping genes as selected by geNorm analysis. Interestingly, only the data set normalized to the geometric mean of GADPH and Actb genes showed a significant increase in HIF-1α expression in every sample. Thus, two housekeeping genes seemed optimal for accurate normalization in this study.
Table 4 Ranking of candidate HKGs and their expression stability, V in the different sample groups of the study evaluated using NormFinder

| Ranking | Stability value | Ranking | Stability value | Ranking | Stability value | Ranking | Stability value |
|---------|-----------------|---------|-----------------|---------|-----------------|---------|-----------------|
| (a) Neonatal CDCs | | | | | | | |
| 1) GAPDH | 0.053 | 1) Actb | 0.139 | 1) GAPDH | 0.087 | 1) GAPDH | 0.051 | 1) GAPDH | 0.092 |
| 2) Actb | 0.113 | 2) GAPDH | 0.160 | 2) Actb | 0.362 | 2) Actb | 0.121 | 2) Actb | 0.192 |
| 3) β2M | 0.158 | 3) β2M | 0.189 | 3) β2M | 2.224 | 3) β2M | 0.152 | 3) β2M | 0.591 |
| 4) RPLP–1 | 4.606 | 4) Tbp | 1.910 | 4) HPRT–1 | 3.215 | 4) HPRT–1 | 2.951 | 4) RPLP–1 | 1.757 |
| 5) Tbp | 14.744 | 5) RPLP–1 | 3.068 | 5) RPLP–1 | 4.935 | 5) RPLP–1 | 5.014 | 5) HPRT–1 | 3.055 |
| 6) HPRT–1 | 15.586 | 6) HPRT–1 | 17.241 | 6) Tbp | 20.389 | 6) Tbp | 11.328 | 6) Tbp | 14.794 |
| (b) Adult CDCs | | | | | | | |
| 1) GAPDH | 0.233 | 1) Actb | 0.200 | 1) Actb | 0.216 | 1) Actb | 0.333 | 1) β2M | 0.040 |
| 2) Actb | 0.747 | 2) GAPDH | 0.388 | 2) GAPDH | 0.414 | 2) GAPDH | 0.447 | 2) Actb | 0.156 |
| 3) β2M | 1.077 | 3) GAPDH | 0.419 | 3) β2M | 1.498 | 3) β2M | 0.542 | 3) GAPDH | 0.275 |
| 4) RPLP–1 | 2.502 | 4) RPLP–1 | 4.592 | 4) HPRT–1 | 4.380 | 4) Tbp | 1.482 | 4) RPLP–1 | 1.495 |
| 5) HPRT–1 | 10.524 | 5) HPRT–1 | 8.784 | 5) RPLP–1 | 4.682 | 5) RPLP–1 | 2.106 | 5) Tbp | 1.608 |
| 6) Tbp | 13.181 | 6) Tbp | 15.939 | 6) Tbp | 6.490 | 6) HPRT–1 | 10.410 | 6) HPRT–1 | 3.381 |
| (c) Combined (neonatal and adult CDCs) | | | | | | | |
| 1) GAPDH | 0.002 | 1) GAPDH | 0.315 | 1) Actb | 0.359 | 1) Actb | 0.263 | 1) β2M | 0.072 |
| 2) Actb | 0.161 | 2) Actb | 1.059 | 2) GAPDH | 1.215 | 2) β2M | 0.718 | 2) Actb | 0.248 |
| 3) β2M | 0.298 | 3) β2M | 1.793 | 3) β2M | 2.201 | 3) GAPDH | 1.491 | 3) GAPDH | 1.105 |
| 4) RPLP–1 | 3.981 | 4) RPLP–1 | 2.188 | 4) HPRT–1 | 3.160 | 4) RPLP–1 | 2.519 | 4) RPLP–1 | 1.263 |
| 5) HPRT–1 | 12.482 | 5) Tbp | 4.208 | 5) RPLP–1 | 3.844 | 5) Tbp | 4.834 | 5) HPRT–1 | 2.520 |
| 6) Tbp | 25.530 | 6) HPRT–1 | 6.658 | 6) Tbp | 16.492 | 6) HPRT–1 | 6.447 | 6) Tbp | 9.298 |

Bold type indicates the reference genes that were consistently ranked the top places, and thus can be categorized as stable HKGs in this study as compared to other three candidate genes.

Samples were analyzed separately according to age group, (a) neonatal and (b) adult or were analyzed in a (c) combined set of neonatal and adult. Gene with the lowest stability value has the most stable expression. Stability values are listed from most stable to least stable.

In our experiments, the sample sets included different stages of heart development (neonatal versus adult rat hearts), different oxygen tensions (normoxic versus hypoxic) and three different PHDI drug treatments (DMOG, EDBA or BIC). For NormFinder analysis, we grouped the samples within each experiment, taking into account the genes associated with cardiac progenitor cells in normoxic and hypoxic CDCs or between physiologically-induced hypoxia using hypoxic culture and pharmacologically-induced hypoxia using PHDIs. We found that GADPH was the most stable gene expressed in the normoxic cell group. However, the expression of this gene was unstable in hypoxic CDCs where Actb was the most stable gene expressed. For the PHDI-treated samples, there were different best housekeeping genes selected for each inhibitor, yet overall, GADPH, Actb and β2M were the most stable reference genes. Although different housekeeping genes were selected for the different experimental conditions, when the stability of housekeeping genes were considered over all groups, the best housekeeping genes revealed by NormFinder analysis was GADPH and Actb combined.

Conclusions

In conclusion, both geNorm and NormFinder analysis revealed that GADPH and Actb were the best housekeeping genes for this study. geNorm also indicated that two housekeeping genes were required for valid qRT-PCR data normalization. In most cases, using multiple housekeeping genes resulted in more accurate and reliable normalization compared with the use of only one reference gene. Thus, we suggest that Actb and GADPH genes should be both used as the housekeeping genes in qRTP-PCR involving normoxia, hypoxic and PHDI-treated neonatal and adult rat CDCs.

Methods

Animals

Sprague–Dawley (SD) rats were used for all experiments. All experiments were performed with approval from The
University of Oxford Animal Ethics Review Committees and the Home Office (London, UK).

Primary cardiac stem cell culture

CDCs were isolated and expanded according to a protocol described previously [5]. In brief, neonatal (1–3 days) and adult (4 months) SD rat hearts were isolated, and minced into small explants in 0.05% trypsin–EDTA (Invitrogen). Explants were plated on fibronectin-coated petri dishes with 1.5 ml of complete explant medium (CEM, Additional file 3) to generate explant-derived cells (EDCs). EDC, once confluent, were isolated and seeded onto poly-d-lysine coated 24-well plates with cardiosphere growth medium (CGM, Additional file 3) to form spherical cell clusters, known as Csp. Loosely attached Csp were harvested and plated onto fibronectin-coated T75 flasks with CEM, at a density of $1 \times 10^4$ cells/flask for expansion into cardiosphere derived cells (CDCs). All the cells were cultured in incubator under 21% O$_2$ and 5% CO$_2$, at 37°C, unless otherwise stated.
CDC preconditioned with hypoxia and PHDIs

For hypoxic-preconditioned cells, CDCs were cultured under 2% O2 (hypoxia) and 5% CO2 at 37°C using an incubator (Wolf Laboratories, UK) adjustable to different oxygen concentrations by infusion of nitrogen (N2). The O2 concentration was monitored continuously using an O2 sensor. For PHDI-preconditioning, normoxic CDCs (cultured under 21% O2 and 5% CO2, at 37°C) were treated with 1 mM of DMOG, 0.5 mM of EDBA or 30 μM of BIC for 24 h at 80% cell confluency.

Western blot analysis for HIF-1α protein

Normoxic, hypoxic and PHDI-preconditioned CDCs were lysed in cell lysis buffer containing protease inhibitor. The homogenate was boiled and centrifuged for 5 min, the supernatant frozen and stored at −80°C. The lysate (50 μg/μl) was subjected to SDS–polyacrylamide gel (10%) electrophoresis, and protein was subsequently transferred to a nitrocellulose membrane (Pall Life Science, Mexico). The membrane was incubated overnight with rat HIF-1α primary antibody diluted (1:2000) in 5% milk in TBS-Tween (Novus, NB100–479). The secondary antibody used was goat anti-rabbit conjugated to horseradish peroxidase (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive protein signal was visualized by an ECL plus detection kit (Perkin Elmer). Protein bands were quantified using Un-Scan-It, Version 6.1 (Silk Scientific, USA).

Primer design and optimization

Primers were designed using Primer3 software based on interpretation of GenBank or Ensembl Genome Browser search results (Table 2). Primer specificity was enhanced by designing a primer pair that flanked the exon–exon border of the gene of interest, to avoid the primers amplifying genomic DNA that may be contained in samples. Primer specificity was confirmed by blasting the primer sequence against genomic databases available at NCBI. No significant homology sequence should be found, to ensure gene specific amplification rather than amplification of several transcripts with one primer pair.

RNA extraction and DNase treatment

Total RNA was extracted from cultured CDCs using Trizol reagent (Sigma) according to the manufacturer’s instructions. DNase treatment was performed using Turbo DNA-free (Ambion) to degrade any DNA present. The concentration and purity of RNA was determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., USA). A ratio of A260/A280 ≈ 2.0 is generally accepted as pure RNA.

cDNA synthesis

cDNA was synthesized from the RNA template using AB high capacity transcriptase kit (Applied Biosystem). Every 1 μg RNA sample was reverse transcribed using 1 μl reverse transcriptase, 2 μl random primer, 0.8 μl dNTPs (10 mM each), 2 μl buffer and topped up by RNase free water to a total volume of 20 μl. The reaction mixture was subjected to incubation for 10 min at 25°C, 120 min at 37°C and 5 s at 85°C.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR amplification was performed using Applied Biosystems StepOnePlus Real-Time PCR System (AB International, CA). The qRT-PCR mastermix was prepared by adding 10 μl AB Sybrgreen PCR mastermix (AB International, CA), 1 μl forward primer, 1 μl reverse primer, 1 μl cDNA and 7 μl distilled water. The total volume for each real-time PCR reaction was 20 μl. The PCR program was set up with an initial heat activation step at 95°C for 10 min. Then, 40 cycles of thermocycling were performed with a denaturation step at 95°C for 15 s, an annealing step at 60°C for 30 s and an extension step at 72°C for 30 s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 4°C/s to 95°C, cooling it at 4°C/s to 70°C, keeping it at 70°C for 20 s, and then slowly heating it at 4°C/s to 95°C. Fluorescence was measured through the slow heating phase. Melting curves were used to determine the specificity of PCR products.

Analysis of housekeeping gene expression stability

Raw Ct values of housekeeping genes were converted to linear values compatible with the geNorm and NormFinder programs. For comparison of housekeeping gene stability between groups, the software geNorm, version 3.4 (Visual Basic application tool for Microsoft Excel) [33] was used, while for housekeeping genes stability comparison within group, NormFinder (a Microsoft Excel Add-in) [34] was used. Both programs were used according to developer’s recommendations as published.

Analysis of target gene expression

Raw Ct values of the target gene (HIF-1α) were analyzed using two different methods to determine the importance of normalization to different housekeeping gene(s) in qRT-PCR data analysis.
Data analysis using $2^{-\Delta\Delta Ct}$ method

Simple relative quantification of target gene expression normalized to one housekeeping gene was performed using the $2^{-\Delta\Delta Ct}$ method. Derivation of the equation, including assumptions, experimental design and validation test, have been described in Applied Biosystem User Bulletin No. 2 (P/N 4303859). Analyses of gene expression data using this method have appeared in the literature [38–40].

Data analysis using geNorm method

Relative quantification of target gene expression normalized to more than one housekeeping gene was performed using the geNorm method. In this method, target gene expression was normalized to the geometric mean expression of the best combination of housekeeping genes subjected to expression stability analysis using geNorm software. Analyses of gene expression data using this method have appeared in the literature [23, 41–43].

Statistical analysis

The software program, SPSS version 18 was used for further statistical analysis of gene and protein expression among experimental groups. In all cases expression data were compared by one-way analysis of variance (ANOVA) (statistical significance < 0.05). The significance level (α) was set at 0.05.

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