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Research paper

Identification of suitable reference genes for quantitative gene expression analysis in rat adipose stromal cells induced to trilineage differentiation

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ABSTRACT

This study was designed to (i) identify stable reference genes for the analysis of gene expression during in vitro differentiation of rat adipose stromal cells (rASCs), (ii) recommend stable genes for individual treatment conditions, and (iii) validate these genes by comparison with normalization results from stable and unstable reference genes. On the basis of a literature review, eight genes were selected: *Actb*, *B2m*, *Hprt1*, *Ppia*, *Rplp0*, *Rpl13a*, *Rpl5*, and *Ywhaz*. Genes were ranked according to their stability under different culture conditions as assessed using GenNorm, NormFinder, and RefFinder algorithms. Although the employed algorithms returned different rankings, the most frequently top-ranked genes were: *B2m* and/or *Ppia* for all 28 day treatments (ALL28); *Ppia* and *Hprt1* (adipogenic differentiation; A28), *B2m* (chondrogenic differentiation; C28), *Rpl5* (controls maintained in complete culture medium; CCM), *Rplp0* (osteogenic differentiation for 3 days; O3), *Rpl13a* and *Actb* (osteogenic differentiation for 7 days; O7), *Rplp0* and *Ppia* (osteogenic differentiation for 14 days; O14), *Hprt1* and *Ppia* (osteogenic differentiation for 28 days; O28), as well as *Actb* (all osteogenesis time points combined; ALLOSTEO). The obtained results indicate that the performance of reference genes depends on the differentiation protocol and on the analysis time, thus providing valuable information for the design of RT-PCR experiments.

1. Introduction

As a result of different extracellular signals, mesenchymal stem cells (MSCs) undergo a complex process of differentiation and phenotypic changes during development or tissue regeneration (da Silva Meirelles et al., 2016). As part of these phenotypic changes, some genes have their expression level enhanced according to lineage commitment, such as *Lpl* (for adipogenesis), *Sox9* (for chondrogenesis) and *Col1* and *Sp7* (for osteogenesis. (Zhong et al. 2015; Chen et al. 2016). Adipose stem cells (ASC) are relatively easy to isolate and are promising cell type to be used in clinical trials (Markarian et al. 2014; Bajek et al. 2016; Zhan et al. 2016). In order to unlock the full potential of these cells in tissue engineering, it is necessary to understand the processes that control the differentiation of MSCs. Quantitative real-time polymerase chain reactions (RT-qPCRs) represent a widely used approach to detect changes in gene expression. However, the accurate determination of the relative levels of transcripts of interest requires normalization using a reference gene, whose expression is constant under the experimental conditions.

Reference genes are often chosen on the basis of information reported in the literature. They are used for a wide variety of experimental conditions, which may affect their own expression. Most reference genes are involved in basic processes that are concerned with cell maintenance and function. Their expression should ideally be constant, stable, unregulated, and unaffected by experimental conditions and treatments. Various reports have shown that levels of reference gene expression are sensitive to the employed experimental conditions, as well as to the origin of the tissue sample and its heterogeneity, which may thus influence the recommended quantity of reference genes to be used (Schmittgen and Zakrajsek 2000; Warrington et al. 2000; Bustin 2002; Radonic et al. 2004; Farrokhi et al. 2012; Ragni et al. 2013; Li et al. 2015a).

Abbreviations: A28, 28 days of adipogenic induction; ALL28, all 28 days condictions combined; ALLOSTEO, all osteogenic condictions combined; C28, 28 days of chondrogenic induction; CCM, complete culture medium; MSCs, mesenchymal stromal cell; O14, 14 days of osteogenic induction; O28, 28 days of osteogenic induction; O3, 3 days of osteogenic induction; O7, 7 days of osteogenic induction; rASCs, rat adipose stromal cell; RT-qPCR, real time quantitative polymerase chain reaction.

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While it would be ideal to find a reference gene with a constant expression under different experimental conditions and for all cell types, such a generally applicable reference gene still remains elusive. However, suitable reference genes have been identified for specific experimental conditions and specific cell types (Willems et al. 2006; Guenin et al. 2009; Quiroz et al. 2010; Cai et al. 2014; Li et al. 2014; Tratwal et al. 2014; De Spiegelaere et al. 2015; Li et al. 2015b). Therefore, the aims of this study were: (i) to assess the stability of expression for the commonly used reference genes *Actb*, *B2m*, *Hprt1*, *Ppia*, *Rplp0*, *Rpl13a*, *Rpl5*, and *Ywhaz* in rat adipose stem cells (rASCs) after 28 days in adipogenic (A28), chondrogenic (C28), or complete culture medium (CCM; control), as well as after 3 (O3), 7 (O7), 14 (O14), and 28 (O28) days in osteogenic culture medium, using GeNorm, NormFinder and RefFinder algorithms; (ii) to identify suitable reference genes; and (iii) to validate these genes by normalization using stable and unstable reference genes.

2. Methodology

Indomethacin was purchased from Merck (Rio de Janeiro, Brazil), while rosiglitazone was ordered from GlaxoSmithKline (Middlesex, UK), and TRIzol reagent from Invitrogen. Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich Co LLC, while all plastic ware was purchased from BD Falcon (São Paulo, Brazil).

Complete culture medium (CCM) consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 U penicillin mL^{-1}, and 10 mg streptomycin mL^{-1} solution (Gibco Invitrogen). Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution was used to wash tissues and cells. All experiments were carried out using cells between passages 4 and 6 in technical and experimental triplicates.

2.1. Isolation and immunophenotyping of rASCs

Three female Lewis rats were obtained from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) and the use of these animals was approved by the FEPPS Ethics Committee (MEMO no. 02/201, August 7, 2013). Adipose tissue was collected from the inguinal region and digested in type I collagenase solution (250 U mL^{-1}) for approximately 30 min at 37 °C. The same volume of CCM was added to neutralize the collagenase. ASC cultures were established as previously described (Beyer Nardi and da Silva Meirelles 2006). Briefly, the digested tissue was centrifuged (400 × *g*) and the isolated cells were resuspended in CCM and cultured in 5% CO_2 at 37 °C, with medium change every 2–3 days. Cells between passages 3 and 6 were used for all experiments.

For the characterization by flow cytometry, cells were incubated with antibodies against CD44, CD29, CD11b, and CD45 (PharMingen BD, USA) conjugated with PE (Phycoerythrin) or FITC (Fluorescein Isothiocyanate) for 30 min at 4 °C. Control samples were incubated in the absence of antibodies. Cells were analyzed in a FACS Calibur (Becton Dickinson, USA) using the CellQuest software, and a minimum of 10,000 events was collected.

2.2. In vitro differentiation

In 6-well plates, cells were seeded at 10^4 cells cm^{-2} and maintained for 28 days in adipogenic medium, chondrogenic medium, or CCM. In the osteogenic differentiation medium, cells were maintained for 3, 7, 14, or 28 days. All media were changed twice a week. Differentiation media consisted of CCM supplemented with 10^{-8} M dexamethasone, 5 μg ascorbic acid 2-phosphate mL^{-1}, and 10 mM β-glycerol phosphate (osteogenic); 10^{-8} M dexamethasone, 2.5 μg insulin mL^{-1}, 100 μM indomethacin, and 3.5 μM rosiglitazone (adipogenic; Nora et al. 2012); 6.25 μg insulin mL^{-1}, 10 ng TGF-β1 mL^{-1}, and 50 nM ascorbic acid 2-phosphate (chondrogenic). To assess calcium deposition, which is indicative of osteogenic differentiation, wells were stained for 5 min with Alizarin Red S (pH 4.1). Lipid vacuoles in cells subjected to adipogenic differentiation were stained with Oil Red O for 5 min and observed using a phase-contrast microscope. The cartilaginous matrix was stained for 5 min with Alcian Blue (pH 2.5). Photomicrographs were taken with a digital camera (AxioCam MRC, Carl Zeiss), using AxioVision software 4.8 (Carl Zeiss). Each treatment was performed with cells derived from three different rats, in biological triplicate.

2.3. RNA extraction and cDNA synthesis

RNA was extracted using TRIzol reagent following the instructions of the manufacturer. RNA was quantified in a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). One microgram of RNA was used as a template for single-strand cDNA synthesis with the M-MLV Reverse Transcriptase Kit (Invitrogen) following the instructions

Table 1
Reference genes evaluated in this study: primer sequences, primer annealing positions, fragment sizes, and primer efficiencies.

Gene	Gene ID	Primers sequence	Annealing exon	Frag size	Efficiency	R^2
Actb	81822	F: CCCGCGAGTACAACCTTCTTG R: GTCATCCATGGCGAACTGGTG	1 and 1–2	71	109.19	0.9902
B2m	24223	F: TCGTGCTTGCCATTCAGAAAAC R: GCAGTTGAGGAAGTTGGGCT	1–2 and 2	86	96.23	0.9833
Hprt1	24465	F: TCCTCCTCAGACCGCTTTTC R: ATCACTAATCACGACGCTGGG	1 and 1–2	78	106.47	0.9965
Ppia	25518	F: CAGACAAAGTTCCAAAGACAGCA R: CACCCTGGCACATGAATCCT	2 and 3–4	117	98.90	0.9706
Rplp0	64205	F: CACTGGCTGAAAAGGTCAAGG R: GTGTGAGGGGCTTAGTCGAA	6–7 and 7	187	100.03	0.9987
Rpl5	81763	F: GCTTGCGTCCTGGAGGTG R: TCACGAACCCCATCCTGC	1 and 1–2	134	97.47	0.9835
Rpl13a	317646	F: ATGAACACCAACCCGTCTCG R: ACCACCATCCGCTTTTTCTTG	4 and 5–6	176	106.2	0.9981
Ywhaz	25578	F: CCCACTCCGGACACAGAATA R: TGTCATCGTATCGCTCTGCC	1–2 and 2	91	102.95	0.9938
Lpl	24539	F: TCCCAATGGAGGCACTTTCC R: CAGCTGGTCCACATCTCCAA	5 and 5–6	91	105.67	0.9837
Sox9	140586	F: AGTCGGTGAAGAATGGGCAA R: CTGAGATTGCCCGGAGTGC	2 and 2–3	158	98.04	0.9769
Col1	29393	R: GTACATCAGCCCAAACCCCA F: TCGCTTCCATACTCGAACTGG	49 and 49–50	87	107.73	0.9942
Sp7	300260	F: CTGCTTGAGGAAGAAGCTCACTA R: GCTGTTGAGTCTCGCAGAGG	1–2 and 2	101	96.99	0.9748