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Title:

Nanotopographical features influence derivation and selection of reference genes for stem cell differentiation protocols

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INTRODUCTION: Mechanotransduction pathways convert mechanical stimuli such as substrate rigidity, which can lead to changes in gene expression profiles [1]. Similarly, responses to different nanopattern surface-mediated biophysical cues may in fact alter the stability of 'housekeeping' or reference genes (RefG) (eg GAPDH, B-actin) – widely used in normalization for quantitative Real Time PCR experiments (qRT-PCR). Adherence to MIQE guidelines are now essential for publication of meaningful quantitiative gene expression data. This includes use of RefG(s) that are stable across all experimental conditions. Here we describe an approach to determining the most appropriate RefG(s) across different nano-topographies or cell culture settings, utilizing human stem cells. Our aim was to provide a rational approach for determination of the most appropriate RefG, under different culture conditions (cell type/ surface engineering/ cell differentiation) from a set of tested candidate reference genes, using PrimerDesign and geNorm software coupled with qRT-PCR.
METHODS: HepaRG101 bipotential progenitor cells [Biopredic Inc: BPI] were seeded (>95% viable; 30,000 cm^2) and cultured for ≤28 days [BPI Growth Medium] under 4 conditions: On prototype 2D-nanopatterned polymer substrates (NPS: 2 nanopatterns [each well 0.32cm^2]; including 1x planar control; fabricated using high-resolution electron beam lithography); or standard Corning plastic culture dishes (SCPs). For comparative analyses, 2 human iPS cell lines (reprogrammed with episomal vectors or Sendai virus systems) were selected for . HiPS cells following hepatic differentiation, so using different cell cultures settings. RNA extraction was undertaken using Life Technologies RNAqueous kit and RNA purity and quantity was measured using Thermo Fisher nanodrop and Agilent chip technology. cDNA was created using PrimerDesign nanoScript to RT kit and qRTPCR was performed using PrimerDesign-validated primers and master mix. Up to 12 candidate genes were assessed using geNorm.
RESULTS: HepaRG extracted total RNA samples showed both high quality (260/280 ratios: 1.8-2.0) and RNA integrity number (RIN 9-10). geNorm analysis of the initial 6 candidate genes did not provide a suitable RefG for data normalization (M>1 indicated low expression stability; where M = expression stability). Next, we assessed the expression stability levels of 12 candidate genes' and identified an optimal single reference gene (*CYC1*) as the most suitable RefG with the lowest M value for HepaRG transcriptional profiling. These data sets revealed that the widely used reference genes, GAPDH, 18s and β-Actin, lacking expression stability as ranked by the geNorm algorithm.. GeNorm analyses of two different iPS cell lines showed transcriptomic differences with respect to RefGs and ranked different candidate RefGs according to their M value. These results clearly indicate that the expression profiles of RefGs can both vary across different cell types, but also within a given cell type subject to a different experimental treatment/ conditions.
DISCUSSION & CONCLUSIONS: Adherence to MIQE guidelines is crucial when selecting reference genes to ensure results for TERM applications are reliable and reproducible. Our data for validation of RefGs for HepaRG progenitor cells concurs with recent studies1 in which application of standard RefGs (GAPDH, 18s or β-Actin) were sub-optimal for transcriptional profiling of HepaRG cells. Thus reliance on coventional 'housekeeping' genes, often under diverse test conditions, introduces potentially high variability of quantitative transciptomic expression data. In conclusion, stringent, objective selection/ validation of RefG(s), that are stable across all test conditions including cell type and biophysical substrate, is critical for meaningful, biologically relevant quantative gene expression results.
REFERENCES: 1. Hart et al., 2010 & Ceelen et al., 2011
[1] Shoetz et al Appreciating force and shape — the rise of mechanotransduction in cell biology