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Importance of reference gene selection for articular cartilage mechanobiology studies

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SUMMARY

Objective: Identification of genes differentially expressed in mechano-biological pathways in articular cartilage provides insight into the molecular mechanisms behind initiation and/or progression of osteoarthritis (OA). Quantitative PCR (qPCR) is commonly used to measure gene expression, and is reliant on the use of reference genes for normalisation. Appropriate validation of reference gene stability is imperative for accurate data analysis and interpretation. This study determined *in vitro* reference gene stability in articular cartilage explants and primary chondrocytes subjected to different compressive loads and tensile strain, respectively.

Design: The expression of eight commonly used reference genes (*18s*, *ACTB*, *GAPDH*, *HPRT1*, *PPIA*, *RPL4*, *SDHA* and *YWHAZ*) was determined by qPCR and data compared using four software packages (comparative delta-C_t method, geNorm, NormFinder and BestKeeper). Calculation of geometric means of the ranked weightings was carried out using RefFinder.

Results: Appropriate reference gene(s) for normalisation of mechanically-regulated transcript levels in articular cartilage tissue or isolated chondrocytes were dependent on experimental set-up. SDHA, YWHAZ and RPL4 were the most stable genes whilst glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and to a lesser extent Hypoxanthine-guanine phosphoribosyltransferase (HPRT), showed variable expression in response to load, demonstrating their unsuitability in such *in vitro* studies. The effect of using unstable reference genes to normalise the expression of aggrecan (ACAN) and matrix metalloproteinase 3 (MMP3) resulted in inaccurate quantification of these mechano-sensitive genes and erroneous interpretation/conclusions.

Conclusion: This study demonstrates that commonly used 'reference genes' may be unsuitable for *in vitro* cartilage chondrocyte mechanobiology studies, reinforcing the principle that careful validation of reference genes is essential prior to each experiment to obtain robust and reproducible qPCR data for analysis/interpretation.

Introduction

Articular cartilage has unique mechanical and physicochemical properties which are responsible for its load-bearing capabilities and near-frictionless movement; this is essential for dissipating mechanical loads applied to the joint[1]. The mechanical properties of articular cartilage are dependent on the composition, structural organisation and integrity of the tissue's extracellular matrix (ECM), which in turn are dependent on, and regulated by mechanical load. Abnormal mechanical load is a primary risk factor for the development of osteoarthritis (OA). Identification of differential gene expression patterns, either as targets or involved in novel mechano-biological pathways in articular cartilage, could be pivotal in providing new insights into molecular mechanisms behind the initiation and/or progression of OA.

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Quantitative PCR (qPCR) is the most utilised mRNA quantification method due to its sensitivity in measuring transcript levels[2]. Historically, most mRNA quantification has been performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18s or ACTB (β-actin) as the reference gene for analysing the response of articular cartilage to mechanical load. However, their suitability as reference genes is questionable, especially due to their potential regulation in a wide variety of physiological states. Gene expression profiling has become increasingly important in our understanding of biological mechanisms, therefore it is surprising that, to date, only one study has previously been performed to identify the reference gene(s) most suitable for normalisation of transcript levels in articular chondrocytes subjected to load[3]. Of the reference genes assessed (18s, ACTB, GAPDH and β2-microglobulin), 18s was deemed to have the most stable expression under the experimental conditions tested[3]. Most 'reference' genes have significant roles in cell survival, and as a consequence are expressed in all cell types. However, this does not eliminate the possibility that their expression levels might be modulated in response to specific stimuli e.g., load. Hence, not all 'reference' genes should be considered universally suitable for use as qPCR reference genes[2]. Therefore, there is a necessity to validate expression levels of potential reference genes to ensure stability of expression under the experimental conditions of the study, and to prevent misinterpretation of data.

In 2009, the MIQE guidelines (*M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments) was published describing essential criteria required for publication of qPCR data e.g., information on sample acquisition, RNA quality/integrity, qPCR validation and data analysis[4]. Furthermore, the MIQE guidelines indicated that when selecting the most stable reference gene(s), normalisation against one such gene is generally considered unacceptable, and that no fewer than three reference genes is advisable[5].

A number of software packages including the comparative delta-C_t method[6], geNorm[7], NormFinder[8] and BestKeeper[9] are commonly used to identify and validate stable expression of appropriate reference genes. In the current study, we utilised these software packages to determine the most stable reference genes in articular cartilage subjected to different loading regimens. Calculations of the geometric means of the ranked weightings obtained from the four software packages, using RefFinder (http://www.leonxie.com/referencegene.php) facilitated the identification of suitable reference genes for this tissue type and experimental design. Comparing the outcomes of these different approaches illustrated the impact that reference gene selection can have on experimental results.

In the present study we determined the stability in expression of eight reference genes in chondrocytes from both articular cartilage explants and isolated primary cells, under the influence of load *in vitro*. The data consistently demonstrated that GAPDH and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) showed the highest variability in expression of all reference genes tested. However, the most appropriate reference gene(s) for normalisation of mechanically-regulated transcript levels in articular cartilage tissue or isolated chondrocytes were dependent on individual experimental set-up, reinforcing the necessity to assess reference gene suitability for each study performed.

Materials and methods

Reagents were purchased from Sigma (Poole, UK) and were of analytical grade or above. All plasticware was certified DNase and RNase-free. Culture medium consisted of Dulbecco's Modified Eagle's Medium/Hams F12-glutamax™ (DMEM/F12(1:1)-glutamax™; Life Technologies, Paisley, UK) supplemented with 100 μg/ml penicillin, 100 U/ml streptomycin, 50 μg/ml ascorbate-2-phosphate and 1× insulin–transferrin–selenium–ethanolamine (1× ITS-X) to maintain the chondrocyte phenotype[10].

Cartilage explant and chondrocyte preparation

Full depth articular cartilage explants (5 mm diameter) were taken using a biopsy punch (Selles Medical Limited, Hull, UK) from the *metacarpophalyngeal* joint of 7-day old bovine calves within 6 h of slaughter[11]. Cartilage explants were stabilised in culture medium for 3 days prior to mechanical load. Primary chondrocytes were isolated from full depth articular cartilage slivers from the same tissue and subjected to an enzymatic digestion as previously described[12]; ethical approval was not required for bovine tissue collection. Chondrocytes were plated at high density (4×10^6 cells per well) in 6-well, flat-bottomed pronectin-coated plates (Bio-Flex culture plates; Dunn Laborotechnik, Asbach, Germany). Following isolation, cells were stabilised for 48 h prior to mechanical stimulation. All cultures were maintained at 37°C, 5% CO_2, 20% O_2.

Application of mechanical load

Cartilage explants, immersed in culture media, were subjected to a range of loading regimes (2.5 MPa, 5 MPa or 8 MPa at 1 or 4 Hz, 15 min) using the ElectroForce® 3200 (TA Instruments, Delaware, USA), and gene expression either analysed directly post-cessation of load or 24 h post-load. Chondrocytes were subjected to a physiological tensile strain (7.5% elongation, 1 Hz) for 30 min using the Flexcell FX-3000 system (Flexcell International Corp, Hillsborough, NC, USA)[12–14], and cells processed four hours post-cessation of load to analyse gene expression. Duplicate cultures of explants or cells, devoid of mechanical stimulation, were set up as controls. Cartilage explants were snap frozen and remained in liquid nitrogen until the RNA extraction. Isolated chondrocytes were lysed directly in TRIzol® (1 ml per well) and stored at −80°C until processed for RNA extraction.

RNA extraction and cDNA synthesis

Cartilage explants were homogenised in TRIzol® (1 ml per 50 mg wet weight tissue: Invitrogen, Paisley, UK) in liquid nitrogen using a dismembrator (Sartorious, Epsom, UK), and RNA extracted as previously described[15], except for the purification step which was completed using an RNeasy mini kit (Qiagen, Manchester, UK) according to manufacturer's instructions. RNA integrity was assessed using the 2100 Bioanalyzer (Agilent Technologies, Stockport, UK) and RIN scores >8.5 were observed. Complementary DNA (20 μl total volume) was generated from 300 ng total RNA using SuperScript® III reverse transcriptase (Invitrogen, Paisley, UK) and 0.5 μg random primers (Promega, Southampton, UK) according to manufacturer's instructions, and 1 μl utilised in each qPCR assay.

qPCR analysis

Real-time PCR (polymerase chain reaction) was performed using a MxPro3000 QPCR system (Agilent Technologies, Stockport, UK). A real-time qPCR assay based on SYBR green detection, using Brilliant III Ultra-Fast SYBR® QPCR mix (Agilent Technologies, Stockport, UK) was used for the transcriptional profiling of eight reference genes including 18s[16], GAPDH[17], ACTB, HPRT, SDHA (Succinate dehydrogenase complex, subunit A), RPL4 (Ribosomal Protein L4), PPIA and YWHAZ (tyrosine 3-monooxygenase/