STABILITY OF HOUSEKEEPING GENES IN HUMAN INTERVERTEBRAL DISC, ENDPLATE AND ARTICULAR CARTILAGE CELLS IN MULTIPLE CONDITIONS FOR RELIABLE TRANSCRIPTIONAL ANALYSIS

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Abstract

Quantitative gene expression analysis is widely used to evaluate the expression of specific tissue markers. To obtain reliable data it is essential to select stable housekeeping genes whose expression is not influenced by the anatomical origin of cells or by the culture conditions. No studies have evaluated housekeeping gene stability in intervertebral disc (IVD) cells and only few studies using cartilaginous endplate (CEP) and articular cartilage (AC) cells are present in the literature. We analysed the stability of four candidate housekeeping genes (*GAPDH*, *TBP*, *YWHAZ* and *RPL13A*) in human cells isolated from nucleus pulposus (NP) and annulus fibrosus (AF), CEP and AC. Cell isolation, expansion, cryoconservation, and differentiation in 3D pellets were tested. GeNorm, NormFinder, BestKeeper tools and the comparative ΔCt method were used to evaluate housekeeping gene stability. In each cell population, *TBP* alone or combined with *YWHAZ* was identified as the best normaliser in both monolayer and 3D pellets. *GAPDH* was the best performer only for AC cells in monolayer. In most culture conditions considering groups of two or more cell types, *TBP* was the most stable and *YWHAZ* was the second choice. *GAPDH* was the best performer only in 3D pellets with factors for AC and AF combined with CEP cells. *RPL13A* was the most stable only for AF with CEP cells at isolation. Our findings will be useful to properly design the experimental set-up of studies involving IVD, CEP or AC cells in different culture conditions, in order to obtain accurate and high quality data from quantitative gene expression analysis.

Keywords: Housekeeping genes, nucleus pulposus, annulus fibrosus, cartilaginous endplate, articular cartilage, hip, lumbar disc.

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Background

The characterisation of cells obtained from intervertebral disc (IVD), cartilaginous endplate (CEP) and articular cartilage (AC), and the establishment of proper conditions for the maintenance of their original phenotype, is needed to deepen the knowledge of the pathophysiological processes involving these anatomical sites and to develop novel tissue engineering strategies for their restoration (Demoor *et al.*, 2014; Rodrigues-Pinto *et al.*, 2014).

A reliable evaluation of the expression of specific tissue markers can be obtained by relative quantification of cell gene expression (Huggett *et al.*, 2005). In this context, to obtain accurate data, it is essential to select stable housekeeping genes (Dheda *et al.*, 2005), whose expression is not influenced by the different anatomical origin of the analysed cell types or by the different experimental culture conditions (Jacob *et al.*, 2013; Lee *et al.*, 2002; Suzuki *et al.*, 2000). Indeed, the use of a single housekeeping gene, whose stability has not been validated for specific experimental conditions, makes quantitative gene expression data unreliable (Huggett *et al.*, 2005; Wong and Medrano, 2005). In this context, standard guidelines for gene expression analysis recommend either the use of a single housekeeping gene that has been validated for each specific study or, alternatively, the use of at least two housekeeping genes (Thellin *et al.*, 1999; Vandesompele *et al.*, 2002).

To our knowledge, a comparative analysis of the stability of housekeeping genes in the different populations of human IVD cells has not been performed yet, and only a single study has analysed the stability of different housekeeping genes in CEP cells (Zhou *et al.*, 2014). Furthermore, only few studies have assessed housekeeping gene stability in human articular chondrocytes (ACs) (Foldager *et al.*, 2009; Ito *et al.*, 2014; Pombo-Suarez *et al.*, 2008; Toegel *et al.*, 2007). Results from these studies confirmed that the stability of housekeeping genes is

influenced by the different anatomical origin of cells (*e.g.* joint cartilage in comparison with lumbar CEP), or by cell exposition to different culture conditions. However, a systematic and complete analysis of the stability of housekeeping genes in human IVD, CEP cells and ACs, covering all the combinations of heterogeneous populations and all the *in vitro* culture steps from cell isolation, expansion, cryoconservation to cell differentiation in three-dimensional pellets with chondro-inductive factors has not been performed yet.

Hence, the aim of this study was to evaluate the stability of four different housekeeping genes in cells isolated from human IVD nucleus pulposus (NP) and annulus fibrosus (AF), CEP and AC. The stability of the analysed genes was not only evaluated on the basis of the different anatomical region of origin, but also considering the different steps involved in their *in vitro* culture and chondrogenic differentiation. The housekeeping genes selected for this study belong to different functional families so as to reduce the chances of co-regulation and, thus, the identification of a false-positive reference gene. They include genes coding for factors involved in cell metabolism, transcriptional factors and ribosomal proteins, and have been proved to be the most stable housekeeping genes in human bone marrow stem cells (BMSC) and adipose stem cells (ASC) stimulated toward chondrogenic differentiation in monolayer (both cell types) (Ragni *et al.*, 2013) or in pellet (BMSC) (Studer *et al.*, 2012).

In our study, to enhance the experimental design we followed the MIQE (Minimum Information for Publication of Quantitative Real Time PCR Experiments) guidelines (Bustin *et al.*, 2009). Finally, to assess the stability of candidate housekeeping genes, we applied the three different statistical algorithms, geNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004) and BestKeeper (Pfaffl *et al.*, 2004), and the comparative ΔCt method (Chen *et al.*, 2011), tools which are able to provide complementary information.

Materials and Methods

Cell isolation and expansion

The study was approved by the Institutional Review Board and specimens were collected with patient informed consent. Tissues from lumbar intervertebral disc of 4 patients, mean age 47 ± 10.4, affected by spine disorders were harvested during discectomy. Articular cartilage was collected during surgical procedures from the femoral head of 4 patients undergoing total hip arthroplasty, mean age 65.3 ± 12.7, affected by osteoarthritis (OA).

AC, NP, AF and CEP cells were isolated by enzymatic digestion (37 °C, 22 h) using type II collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) at the concentrations of 336 U/mL for articular cartilage (Lopa *et al.*, 2013), 224 U/mL for NP, 560 U/mL for AF (Colombini *et al.*, 2015), and 336 U/mL for CEP samples (Yuan *et al.*, 2013). After digestion, samples were filtered through a cell strainer and centrifuged (1000 ×*g*, 5 min). NP, AF, CEP cells and AC cells were counted and plated at 10^5 cells/cm^2 and 10^4 cells/cm^2 respectively, (37 °C, 5 % CO_2) in 4.5 mg/mL high glucose Dulbecco's modified Eagle medium (HG-DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % foetal bovine serum (FBS, Lonza, Basel, Switzerland), 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM Hepes, 1 mM sodium pyruvate (all reagents from Life Technologies), 5 ng/mL fibroblast growth factor-2 (FGF-2, Peprotech, Rocky Hill, NJ, USA) and 1 ng/mL transforming growth factor-β1 (TGF-β1, Peprotech). During culture, medium was replaced twice a week. At confluence, cells were detached using 0.05 % trypsin/0.053 mM EDTA (Life Technologies) and plated at 5 × 10^3 cells/cm^2 for the following passages. Cells were expanded for one passage (about 7 d, indicated as P1) and three passages (about 21 d, indicated as P3). To prepare pellet cultures, cells were used fresh or thawed at P3.

Pellet cultures

To obtain pellet cultures, 4 × 10^5 cells were centrifuged for 2 min at 232 ×*g* in 1.5 mL polypropylene conical tubes and maintained in culture for 21 d in chondrogenic differentiation medium: HG-DMEM supplemented with 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, 1.25 mg/mL human serum albumin (HSA) (Sigma-Aldrich, St. Louis, MO, USA), 1 % ITS+1 (containing 1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, 0.5 µg/mL sodium selenite, 50 mg/mL bovine serum albumin and 470 µg/mL linoleic acid, Sigma-Aldrich), 0.1 mM L-ascorbic acid-2-phosphate with (+F) or without (-F) 0.1 µM dexamethasone and 10 ng/mL of TGF-β1. Culture medium was changed twice a week (Colombini *et al.*, 2012; Lopa *et al.*, 2014). Pellet cultures were performed both in static and dynamic conditions. For dynamic pellet cultures, after centrifugation, tubes were placed and maintained on a 3D orbital shaker (20 rpm, Rotamax 120, Heidolph, Schwabach, Germany) for 21 d to provide continuous agitation of the medium (Candrian *et al.*, 2010).

Histological analysis

Pellets were fixed in 10 % neutral buffered formalin, embedded in paraffin and sectioned at 4 µm. Serial sections were stained with haematoxylin and eosin (HE) (Sigma-Aldrich) for morphological examination and Alcian blue (AB) (pH 2.5, Sigma-Aldrich) for glycosaminoglycan (GAG) detection.

RNA extraction, quality control and cDNA synthesis

Total RNA was isolated and purified from cell and pellet lysates using the PureLink® RNA Mini Kit (Life Technologies). RNase-Free DNase Set (Qiagen, Venlo, Netherlands) was used for residual genomic DNA digestion, the isolated RNA was quantified in triplicate by RiboGreen® RNA quantitation assay (Life Technologies) or spectrophotometrically (NanoDrop, Thermo Scientific, Waltham, MA USA) and its purity was estimated (260/280 nm absorbance ratio).

35 ng of the isolated RNA were reverse-transcribed to cDNA employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The final volume

of 20 µL included a 5× reaction mix containing oligo(dT), random hexamer primers and reverse transcriptase pre-blended with RNase inhibitor. The reaction mix was incubated for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C.

Gene expression analysis

Gene expression was evaluated by real time PCR (StepOne Plus, Life Technologies). 5 ng of cDNA were used as template and were incubated with a PCR mixture including TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays (Life Technologies) in a final volume of 20 µL. The following assays were used: *GAPDH*, Hs99999905_m1; *TBP*, Hs00427620_m1; YWHAZ, Hs03044281_g1; *RPL13A*, Hs04194366_g1; *ACAN*, Hs00153936_m1 and *COL2A1*, Hs00264051_m1. Amplification and real-time data acquisition were performed using the following cycle conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each assay, a series of four dilutions of cDNA (0.1-1-10-50 ng) were made to generate a standard curve. The PCR amplification efficiency (E) was determined by the slope of the standard curve:

$E(\%) = (10^{[-1/slope]} - 1) \times 100\ \%$.

2 replicates were analysed for each experimental group. Data were expressed according to the ΔCt method.

Bioinformatic tools and statistical analysis

Three statistical algorithms, geNorm (ver. 3.5) (Vandesompele *et al.*, 2002), NormFinder (ver. 0.953) (Andersen *et al.*, 2004) and BestKeeper (ver. 1) (Pfaffl *et al.*, 2004), and the comparative ΔCt method described by Chen *et al.* (2011) were used to evaluate the stability of the candidate housekeeping genes. The rank of stability was calculated starting from the M stability value after stepwise exclusion (the two most stable genes were reported with rank "1") with geNorm, the smallest stability value with NormFinder, a stability value called "standard deviation value" with Bestkeeper and the mean standard deviation value with the ΔCt Method. The overall ranking of all the housekeeping genes was determined by using the geometric mean of the ranking values calculated with each method, in accordance with the method proposed by the RefFinder algorithm (Xie *et al.*, 2012).

Statistical analysis was performed using (GraphPad Prism v5.00, GraphPad Software). Normal distribution of gene expression values was assessed by Kolmogorov-Smirnov normality test. Friedman test with Dunn's post-test and Spearman non-parametric correlation were used to compare data non-normally distributed. Level of significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Establishment of the experimental setup for 3D pellet culture

Macroscopically, the pellets cultured in dynamic conditions appeared larger than the ones maintained in static culture, with the exception of the AC pellets. In the absence of chondro-inductive factors, pellets of all the different cell types, either fresh or thawed, in static condition were smaller than their counterparts cultured with factors. GAGs deposition was superior in pellets cultured in static conditions and in the presence of chondro-inductive factors compared to samples cultured in dynamic conditions without chondro-inductive factors. Only ACs cultured in dynamic condition with factors showed an appreciable GAGs deposition, but, even in this case, lower than the one observed in static conditions. Similar results were obtained when using thawed cells. In particular, a higher amount of necrotic cells was observed in pellets cultured without chondro-inductive factors and in dynamic condition in comparison with their counterparts supplemented with factors and maintained in static culture. Fig. 1 reports the histological staining of pellets obtained from AC, NP, AF and CEP cells.

Taken together, these results allowed us to choose the more suitable model of pellet culture for our experiments, which consists in the use of thawed cells cultured in static conditions in presence of chondro-inductive factors.

RNA quality control and curves of efficiency of the analysed genes

A significantly higher RNA concentration (mean values of all samples are 19.52 ± 1.2 *versus* 17.16 ± 1.0 ng/µL, $p < 0.001$; mean ng/cell are 0.016 ± 0.004 *versus* 0.018 ± 0.005, $p < 0.01$) was determined using a fluorescence-based method (Ribogreen®) in comparison with absorbance measurement (Nanodrop), suggesting a higher sensitivity of the fluorescence method. However, the results obtained with these two methods showed a strong correlation ($R^2 = 0.82$, $p < 0.001$), as showed in Fig. 2. RNA purity was evaluated by absorbance measurement. The 260/280 ratios showed mean values of 1.95 ± 0.03 for RNA extracted from cells in monolayer and of 1.94 ± 0.03 for RNA isolated from cell pellets, demonstrating that we isolated high-quality RNA. In Table 1, standard curve parameters for *GAPDH*, *TBP*, *YWHAZ*, *RPL13A*, *ACAN* and *COL2A1* are reported. The efficiency of each assay ranged from 90.3 % to 100.9 % with linear R^2 from 0.992 to 0.998, indicating that all the tested assays are suitable for quantitative analysis.

Ranking of the housekeeping genes and validation of the optimal gene for normalisation in different cells and conditions

The validated housekeeping genes were obtained from the rank calculated starting from the output of the three bioinformatic tools and from the comparative ΔCt Method. The M stability values after stepwise exclusion of geNorm allowed identification of the two most stable genes, reported with rank '1'. Of note, all the tested housekeeping genes were stable because their M values were below the reference M value of 1.5. The stability values were considered for NormFinder, the most stable gene had the smallest stability value. The standard deviation values of Bestkeeper showed that all the tested housekeeping genes were stable because their standard deviation values were below the cutoff value of 1. The mean standard deviation values were considered for the comparative ΔCt Method.

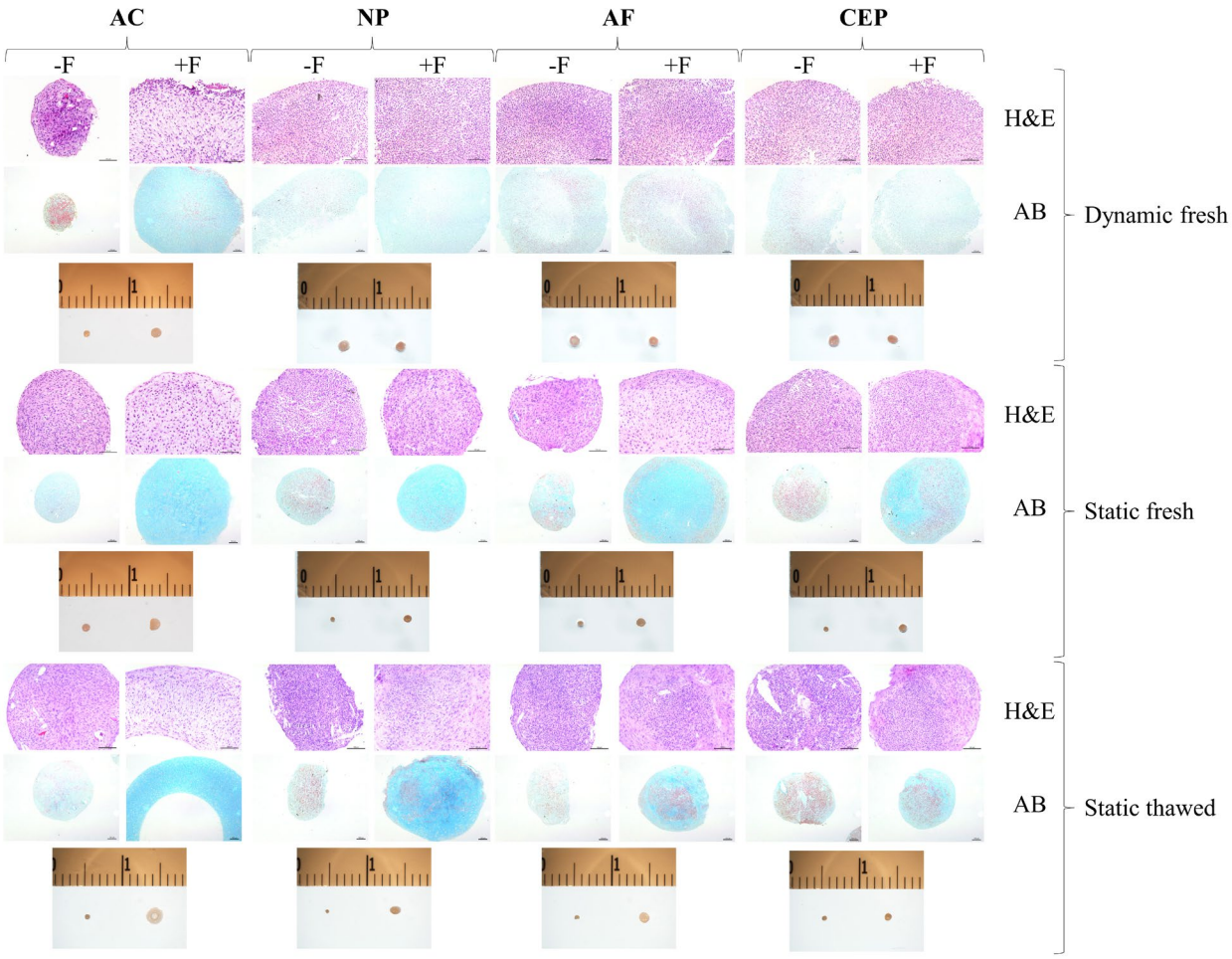

Fig. 1. Macroscopic appearance of pellets. Histological staining for haematoxylin-eosin (H&E) and Alcian blue (AB), magnification 20× and 10×, respectively; scale bar 100 µm. Cells have been expanded for 3 passages (about 21 d) and pellets have been prepared with fresh and thawed cells. Dynamic or static culture of pellets has been performed for 21 d in chondrogenic differentiation medium without (-F) or with (+F) chondro-inductive factors. Articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells.

Table 1. Candidate reference genes and selected markers to evaluate chondro-induction used for qRT-PCR experiments with correspondent standard curve parameters.

Abbreviation	Gene	Slope	Efficiency (E%)	Coefficient (R^2)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	-3.373	100.9	0.998
TBP	TATA box binding protein	-3.396	97.0	0.995
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	-3.339	99.3	0.996
RPL13A	Ribosomal protein L13a	-3.360	98.4	0.992
ACAN	Aggrecan	-3.577	90.3	0.997
COL2A1	Collagen, type II, alpha 1	-3.357	98.5	0.992

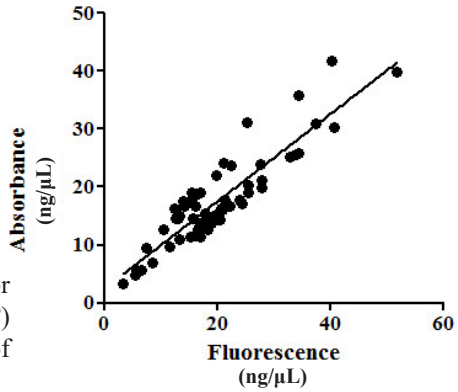

Fig. 2. Correlation of the two methods used for RNA quantification. Fluorescence (Ribogreen®) *vs*. absorbance (Nanodrop). The mean values of all the quantified samples are reported.

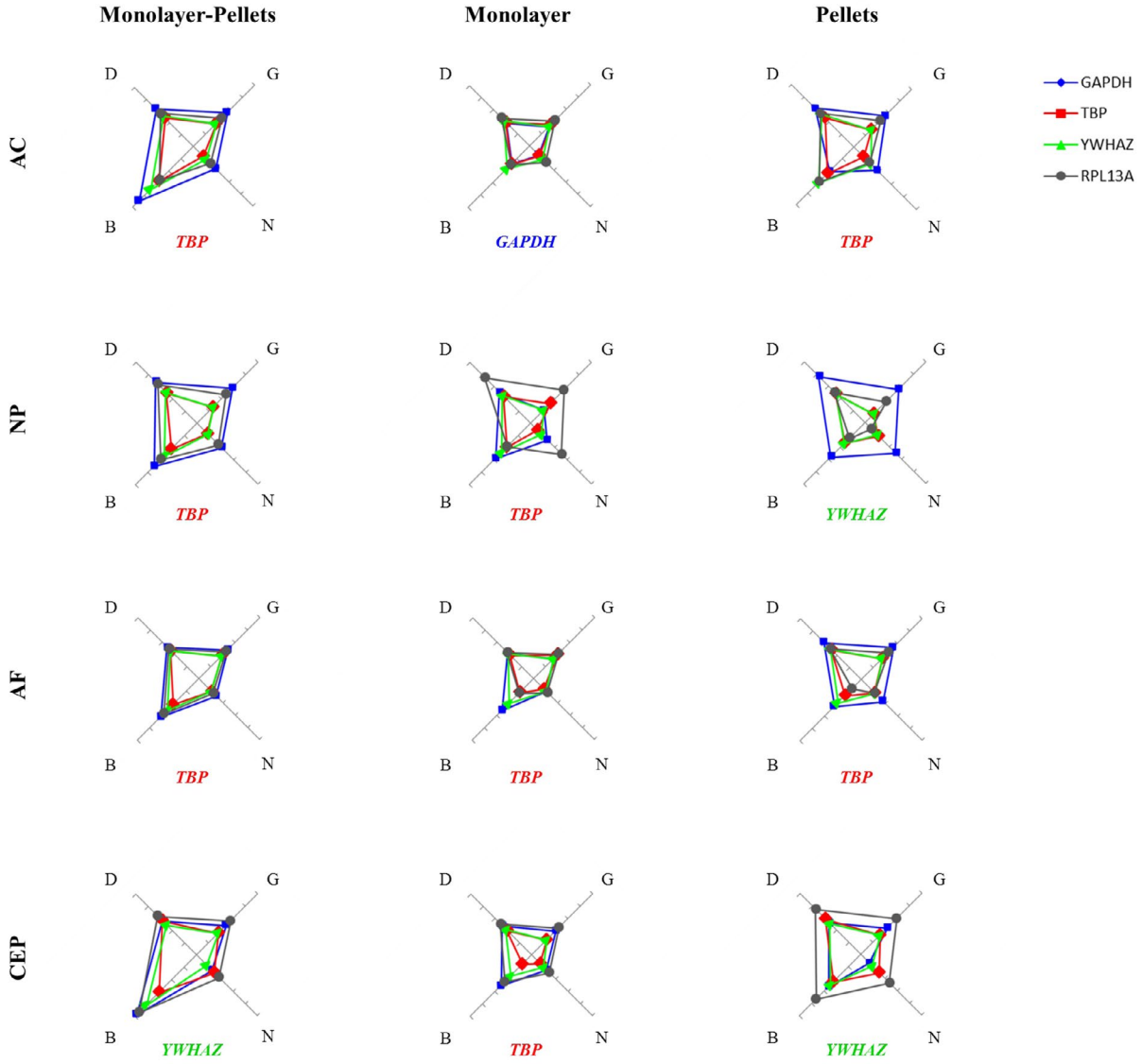

Fig. 3. Stability of the four tested housekeeping genes in single cell populations. Articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells have been analysed in monolayer and/or pellet (with or without chondro-inductive factors) culture. Each graph reports on axis numbers between 0 and 1, tick intervals 0.2, of M stability values after stepwise exclusion obtained from geNorm (G), stability values obtained from NormFinder (N), standard deviation values obtained from Bestkeeper (B), and mean standard deviation values obtained from the comparative ΔCt Method (D). The validated housekeeping gene based on the ranking obtained from the values of three algorithms (geNorm, NormFinder, BestKeeper) and the comparative ΔCt method is evidenced under each graph.

The results of the systematic analysis of the four tested housekeeping genes for AC, NP, AF and CEP cells analysed in common experimental conditions, such as cell expansion in monolayer, cell thawing or three-dimensional culture and maintenance with or without chondro-inductive factors, are provided in Figs. 3, 4 and 5.

Each graph reports on axis numbers between 0 and 1, tick intervals 0.2, of M stability values after stepwise exclusion obtained from geNorm (G), stability values obtained from NormFinder (N), standard deviation values obtained from Bestkeeper (B) and mean standard deviation values obtained from the comparative ΔCt Method (D). Each graph represents the stability of the four single housekeeping genes determined with the different algorithms in a specific condition. The smallest stability values and the smallest area of the graph indicate the more stable gene. In Fig. 3 we report on the stability of the housekeeping genes in each single population of cells cultured in monolayer or pellets. *TBP* alone or combined with *YWHAZ* were promising as normalisers for all cell types in both culture conditions. *GAPDH* was the best performer only for ACs cultured in monolayer. Figs. 4 and 5 compared the housekeeping genes stability in all the culture conditions (monolayer, expansion, thawing, pellets with or without chondro-inductive factors) considering pairs of cell types (Fig. 4), groups of three or all the populations of cells

Table 2. Stability ranking of the four tested housekeeping genes (from the most to the least stable) for the articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells analysed in monolayer and in pellet culture.

	Monolayer - Pellets	Monolayer	Pellets
AC	*TBP*	*GAPDH*	*TBP*
	YWHAZ	*TBP*	*YWHAZ*
	RPL13A	*YWHAZ*	*RPL13A*
	GAPDH	*RPL13A*	*GAPDH*
NP	*TBP*	*TBP*	*YWHAZ*
	YWHAZ	*YWHAZ*	*RPL13A*
	RPL13A	*RPL13A*	*TBP*
	GAPDH	*GAPDH*	*GAPDH*
AF	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*RPL13A*
	RPL13A	*GAPDH*	*YWHAZ*
	GAPDH	*RPL13A*	*GAPDH*
CEP	*YWHAZ*	*TBP*	*YWHAZ*
	TBP	*YWHAZ*	*GAPDH*
	GAPDH	*GAPDH*	*TBP*
	RPL13A	*RPL13A*	*RPL13A*

(Fig. 5). As observed for single cell types, *TBP* was the most stable housekeeping gene in the great majority of cases and *YWHAZ* the second most stable gene in the majority of the tested conditions. *GAPDH* was the best performer only in pellets with factors for ACs and for AF with CEP cells. Only for AF with CEP cells cultured in monolayer at passage 1, *RPL13A* was the most stable housekeeping gene. The rank for the stability of the housekeeping genes in all the culture conditions is reported in Tables 2 and 3. In summary, all the tested housekeeping genes were stable in all the analysed conditions. Particularly, *TBP* either alone (Fig. 6**a**) or coupled with *YWHAZ* (Fig. 6**b**) was the most stable gene for all types of cells in almost all the tested conditions.

To verify that the analysed housekeeping genes were suitable for normalisation purposes without causing altered results, we compared *ACAN* and *COL2A1* expression in all the analysed cells cultured in monolayer and pellets. The normalisation was performed using the two best housekeeping genes, *TBP-YWHAZ*, the best housekeeping gene, *TBP*, or the worst performer in our study, *GAPDH* (Table 4). *ACAN* expression normalised on either *GAPDH*, *TBP* or *TBP-YWHAZ* was significantly higher in pellets cultured with chondro-inductive factors in comparison with cells cultured in monolayer ($p < 0.05$) or in pellets without factors ($p < 0.001$). However, a greater significance was found in pellets with factors in comparison with cells in monolayer at passage 1 when data were normalised using *TBP* ($p < 0.01$) than with *GAPDH* or *TBP-YWHAZ* ($p < 0.05$). Differences were observed in the comparison of *ACAN* expression between monolayer and pellets without factors when different genes were used for normalisation. There was a significant increase ($p < 0.05$) in *ACAN* expression in cells at passage 3 or passage 1 in comparison with cells cultured in pellets without factors after normalisation with *TBP* and *TBP-YWHAZ* or *GAPDH*, respectively. The normalisation on different housekeeping genes led to a significant increase ($p < 0.001$) of *COL2A1* expression in cells cultured in pellets with chondro-inductive factors in comparison with cells in monolayer at passage 3 and cultured in pellets without factors. Another significant increase ($p < 0.05$) was observed normalising with *TBP* and *TBP-YWHAZ*, but not when normalising with *GAPDH,* in cells at passage 1 in comparison with cells in pellets without factors.

Discussion

In this study we assessed the stability of four different housekeeping genes in human cells, isolated from different anatomical part of lumbar IVD (NP, AF), CEP and AC, during different steps of *in vitro* culture and in three-dimensional chondrogenic differentiation. The three-dimensional pellet culture in static conditions with a chondro-inductive environment was chosen since it represents a common and well consolidated protocol to promote chondrogenic differentiation of progenitors (Indrawattana *et al.*, 2004). As expected (Lee *et al.*, 2001; Tallheden *et al.*, 2003), in our study this condition promoted phenotype maintenance. The *in vitro* culture models (*i.e.* monolayer and pellet culture) selected for this study represent standard and well-consolidated systems to achieve primary cell expansion and differentiation, albeit they cannot be considered as fully representative of the physiological cell condition *in vivo*. Indeed, the expansion of cells in monolayer for several passages is often required to obtain a suitable cell number to perform *in vitro* experiments, but it correlates with a progressive loss of physiological cell phenotype. On the other side, the three-dimensional pellet culture in the presence of specific growth factors promotes the reacquisition of a differentiated cell phenotype and self-organisation of cells within neo-synthetised matrix, even though the formation of a necrotic hypoxic core represents a limitation to the use of this culture model for a very extended period of time.

By an experimental point of view, we selected independent housekeeping genes known to be stable during chondro-induction of progenitor cells (Ragni *et al.*, 2013; Studer *et al.*, 2012) and minimised the sources of experimental variation by choosing the more suitable model of pellet culture, treating our RNA samples with DNAse digestion, analysing RNA purity, using two-step reverse transcription polymerase chain reaction (RT-PCR) and performing curves to assess the efficiency of the assays used for each gene. Free statistical complementary programs, based on different algorithms, were used to establish the candidate housekeeping genes stability to prove that our results were independent from the method of analysis.

Our results apply to IVD, CEP or AC cells collected from surgical samples of pathological subjects. This mimics most of the studies analysing the pathophysiology of these anatomical sites and the tissue engineering approaches addressed to treat these pathological states.

Table 3. Stability ranking of the four tested housekeeping genes (from the most to the least stable) in groups of two/three/four kind of cells for the articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells analysed in monolayer at first passage (P1), after thawing and expansion in monolayer (P3), and in pellet culture without (-F) or with (+F) chondro-inductive factors.

	Monolayer - Pellets	Monolayer	Pellets	P1	P3	-F	+F
AC-NP	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*GAPDH*	*YWHAZ*	*RPL13A*	*RPL13A*	*YWHAZ*	*YWHAZ*
	RPL13A	*YWHAZ*	*RPL13A*	*GAPDH*	*GAPDH*	*GAPDH*	*RPL13A*
	GAPDH	*RPL13A*	*GAPDH*	*YWHAZ*	*YWHAZ*	*RPL13A*	*GAPDH*
AC-AF	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*
	RPL13A	*RPL13A*	*RPL13A*	*RPL13A*	*GAPDH*	*GAPDH*	*GAPDH*
	GAPDH	*GAPDH*	*GAPDH*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*
AC-CEP	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*YWHAZ*	*GAPDH*
	YWHAZ	*GAPDH*	*YWHAZ*	*YWHAZ*	*GAPDH*	*TBP*	*TBP*
	GAPDH	*YWHAZ*	*GAPDH*	*GAPDH*	*YWHAZ*	*GAPDH*	*YWHAZ*
	RPL13A	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*
NP-AF	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*RPL13A*	*YWHAZ*	*YWHAZ*	*GAPDH*	*YWHAZ*
	RPL13A	*RPL13A*	*YWHAZ*	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*
	GAPDH	*GAPDH*	*GAPDH*	*GAPDH*	*RPL13A*	*YWHAZ*	*GAPDH*
NP-CEP	*YWHAZ*	*TBP*	*YWHAZ*	*TBP*	*TBP*	*TBP*	*TBP*
	TBP	*YWHAZ*	*TBP*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*GAPDH*
	GAPDH	*GAPDH*	*RPL13A*	*GAPDH*	*GAPDH*	*GAPDH*	*YWHAZ*
	RPL13A	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*
AF-CEP	*YWHAZ*	*TBP*	*YWHAZ*	*YWHAZ*	*TBP*	*GAPDH*	*GAPDH*
	TBP	*YWHAZ*	*TBP*	*RPL13A*	*GAPDH*	*TBP*	*TBP*
	GAPDH	*RPL13A*	*GAPDH*	*TBP*	*YWHAZ*	*YWHAZ*	*YWHAZ*
	RPL13A	*GAPDH*	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*
AC-NP-AF	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*YWHAZ*	*GAPDH*	*YWHAZ*	*GAPDH*	*YWHAZ*
	RPL13A	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*	*YWHAZ*	*GAPDH*
	GAPDH	*GAPDH*	*GAPDH*	*YWHAZ*	*GAPDH*	*RPL13A*	*RPL13A*
AC-NP-CEP	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*GAPDH*
	GAPDH	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	*GAPDH*	*YWHAZ*
	RPL13A	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*
AC-AF-CEP	*YWHAZ*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	TBP	*YWHAZ*	*YWHAZ*	*YWHAZ*	*GAPDH*	*GAPDH*	*GAPDH*
	GAPDH	*RPL13A*	*GAPDH*	*RPL13A*	*YWHAZ*	*YWHAZ*	*YWHAZ*
	RPL13A	*GAPDH*	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*
NP-AF-CEP	*YWHAZ*	*TBP*	*YWHAZ*	*TBP*	*TBP*	*TBP*	*TBP*
	TBP	*YWHAZ*	*TBP*	*YWHAZ*	*YWHAZ*	*GAPDH*	*YWHAZ*
	RPL13A	*GAPDH*	*RPL13A*	*GAPDH*	*GAPDH*	*YWHAZ*	*GAPDH*
	GAPDH	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*	*RPL13A*
AC-NP-AF-CEP	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*	*TBP*
	YWHAZ	*YWHAZ*	*YWHAZ*	*YWHAZ*	*YWHAZ*	*GAPDH*	*GAPDH*
	GAPDH	*GAPDH*	*GAPDH*	*RPL13A*	*GAPDH*	*YWHAZ*	*YWHAZ*
	RPL13A	*RPL13A*	*RPL13A*	*GAPDH*	*RPL13A*	*RPL13A*	*RPL13A*

All the tested housekeeping genes used in this study were stable enough to satisfy the minimal requirements for a housekeeping gene in all the analysed conditions. Remarkably, the best reference genes in IVD, CEP and AC were *TBP* and *YWHAZ*, which are not commonly used as reference genes. Nevertheless, differences in the best performer reference gene were found when considering the culture step/method (fresh/expanded cells, monolayer/three-dimensional culture) and one or more (combined) population of cells. The need to identify the best housekeeping genes in each specific experimental setting led us to design summary figures and a table to

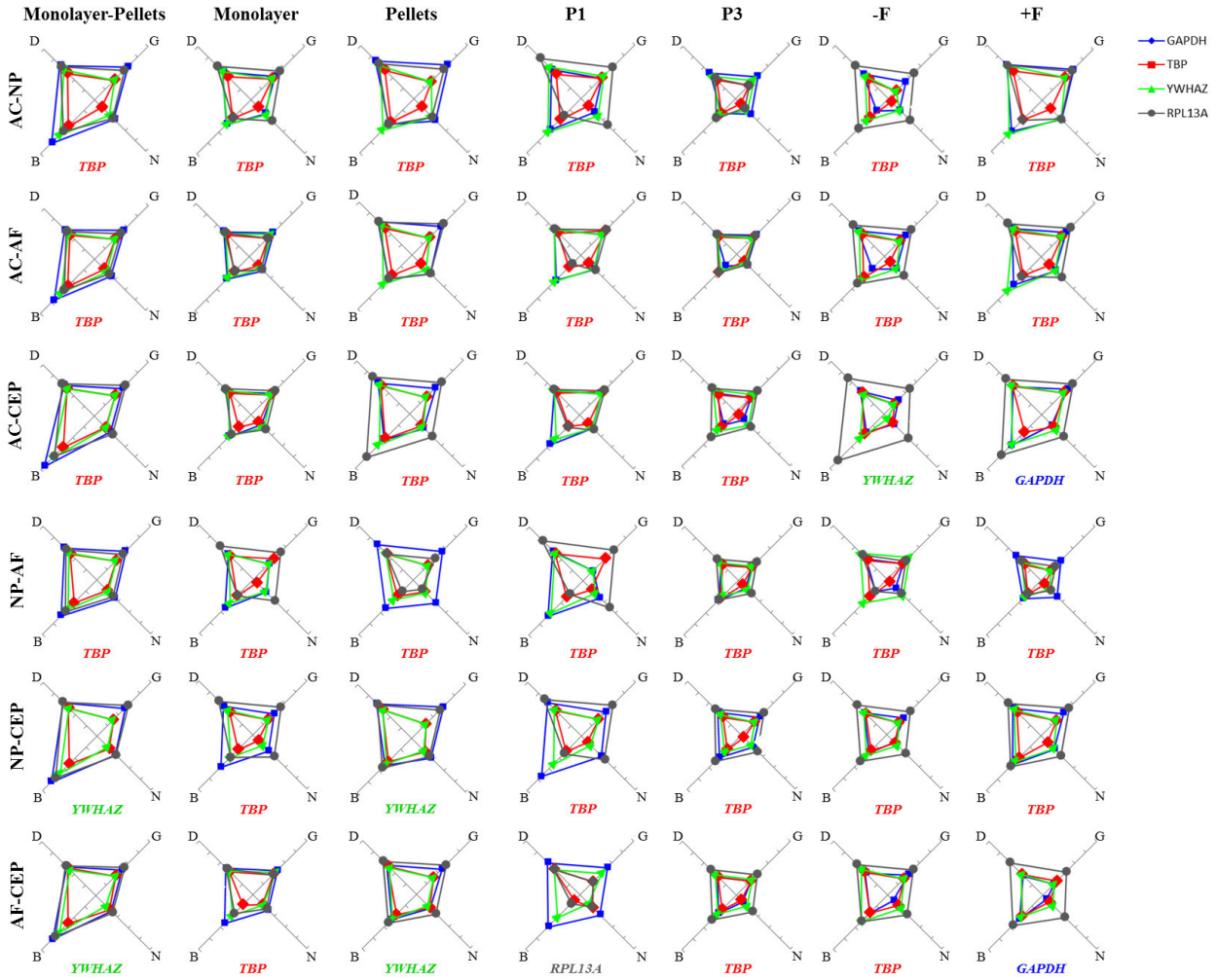

Fig. 4. Stability of the four tested housekeeping genes in groups of two types of cells. Articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells have been analysed in monolayer at first passage (P1), after thawing and expansion in monolayer (P3) and in pellet culture without (-F) or with (+F) chondro-inductive factors. Each graph reports on axis numbers between 0 and 1, tick intervals 0.2, of M stability values after stepwise exclusion obtained from geNorm (G), stability values obtained from NormFinder (N), standard deviation values obtained from Bestkeeper (B), and mean standard deviation values obtained from the comparative ΔCt Method (D). The validated housekeeping gene based on the ranking obtained from the values of three algorithms (geNorm, NormFinder, BestKeeper) and the comparative ΔCt method is evidenced under each graph.

Table 4. Differences in the *ACAN* and *COL2A1* expression in all the analysed cells cultured in monolayer at first passage (P1), after thawing and expansion in monolayer (P3), and in pellet culture without (-F) or with (+F) chondro-inductive factors. The normalisation was performed using the two best housekeeping genes, *TBP-YWHAZ*, the best housekeeping gene, *TBP*, or the worst performer in our study, *GAPDH*.

ACAN						
	P1 *vs.* P3	**P1 *vs.* F-**	**P1 *vs.* F+**	**P3 *vs.* F-**	**P3 *vs.* F+**	**F- *vs.* F+**
TBP-YWHAZ	ns	ns	*	*	*	***
TBP	ns	ns	**	*	*	***
GAPDH	ns	*	*	ns	*	***
COL2A1						
	P1 vs. P3	**P1 vs. F-**	**P1 vs. F+**	**P3 vs. F-**	**P3 vs. F+**	**F- vs. F+**
TBP-YWHAZ	***	*	ns	ns	***	***
TBP	***	*	ns	ns	***	***
GAPDH	***	ns	ns	ns	***	***

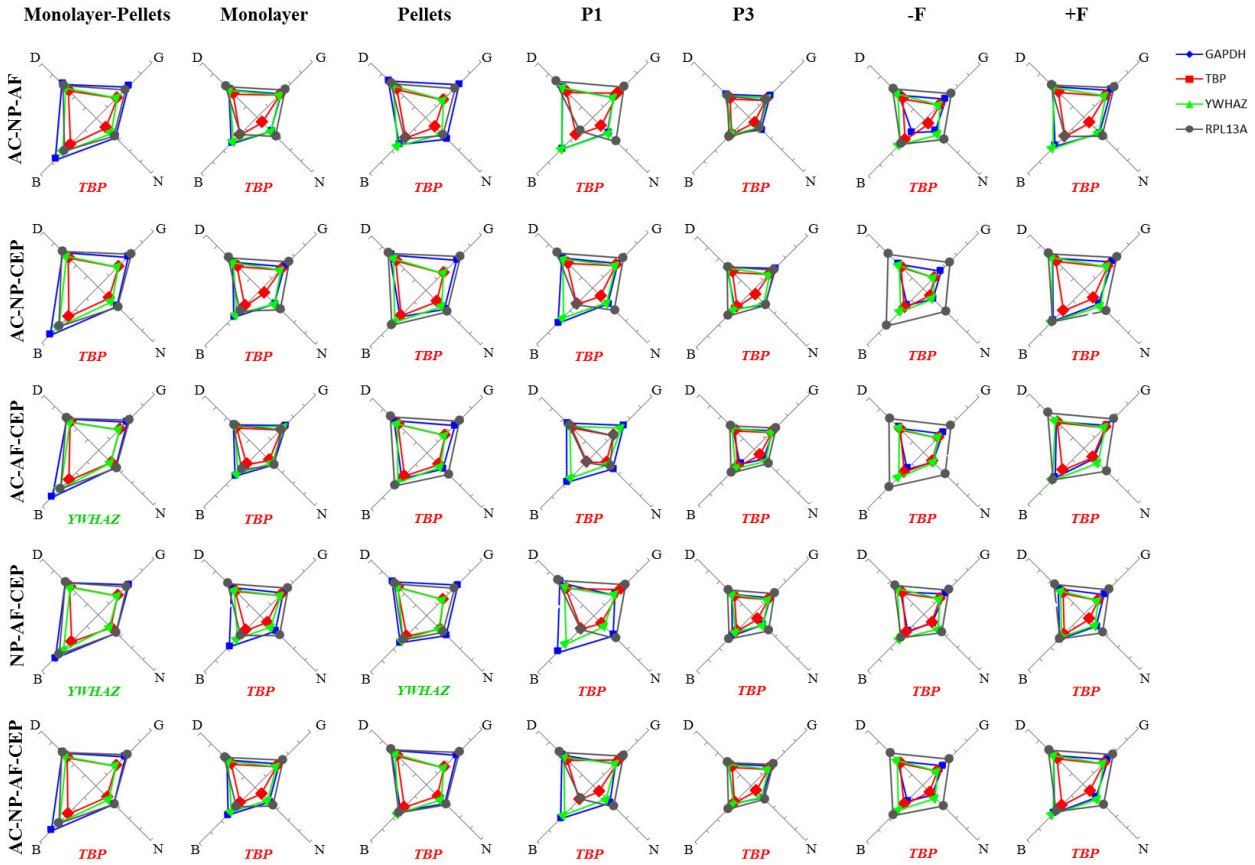

Fig. 5. Stability of the four tested housekeeping genes in groups of three/four types of cells. Articular cartilage (AC), nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP) cells have been analysed in monolayer at first passage (P1), after thawing and expansion in monolayer (P3), and in pellet culture without (-F) or with (+F) chondro-inductive factors. Each graph reports on axis numbers between 0 and 1, tick intervals 0.2, of M stability values after stepwise exclusion obtained from geNorm (G), stability values obtained from NormFinder (N), standard deviation values obtained from Bestkeeper (B), and mean standard deviation values obtained from the comparative ΔCt Method (D). The validated housekeeping gene based on the ranking obtained from the values of three algorithms (geNorm, NormFinder, BestKeeper) and the comparative ΔCt method is evidenced under each graph.

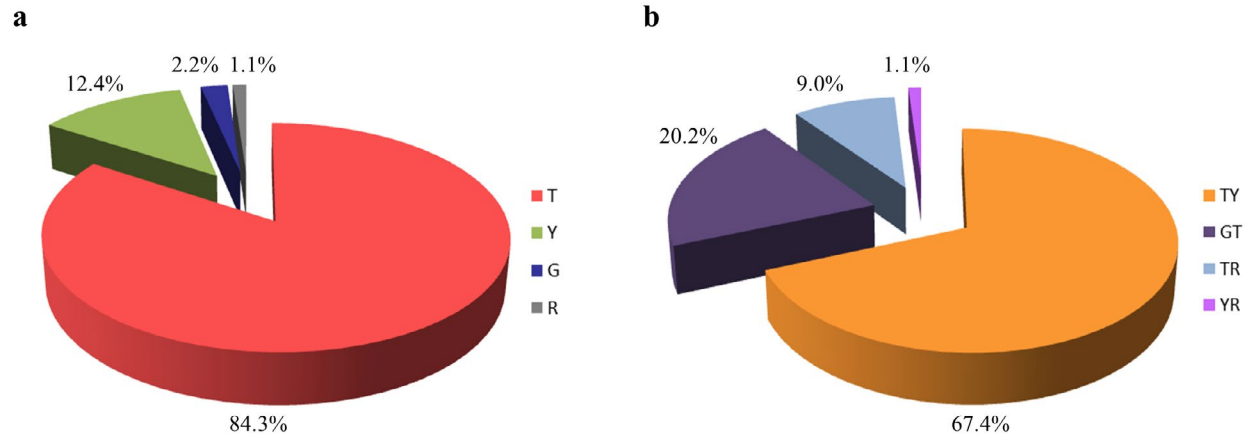

Fig. 6. Summary of the stability of the tested housekeeping genes. The most stable housekeeping gene (**a**) or the two most stable housekeeping genes (**b**) for all kind of cells in all the tested conditions. *TBP* (T), *YWHAZ* (Y), *GAPDH* (G), *RPL13A* (R).

provide detailed guidelines to planning templates for studies involving these culture conditions/cell types. In addition, the identification of *TBP* and *YWHAZ* as the most stable reference genes in IVD, CEP and AC will be useful in future co-culture studies of these cells with adipose- or bone marrow-derived stem cells, since these genes proved to be the most stable in progenitors induced towards the chondrogenic lineage (Ragni *et al.*, 2013).

Despite the good scores showed by all the tested housekeeping genes, we observed differences in the fold increase and in the significance of the expression of the two target genes when we tested the suitability for

normalisation purposes of the most stable gene (*TBP*), the pair of most stable genes (*TBP-YWHAZ*), and one of the worst performers (*GAPDH*). In particular, consistent results were obtained by using a combination of the two best housekeeping genes (*TBP-YWHAZ*), good practice emphasised by several authors (Andersen *et al.*, 2004; Vandesompele *et al.*, 2002), and the best housekeeping gene (*TBP*). On the contrary, the commonly and widely used *GAPDH* (Suzuki *et al.*, 2000; Vandesompele *et al.*, 2002) was not among the best housekeeping genes in most experimental conditions due to its variability (Bustin and Nolan, 2004; Huggett *et al.*, 2005). This is confirmed also in our analysis where, despite its stability, *GAPDH* showed a less accurate normalisation in comparison with that obtained with the other above mentioned genes.

To our knowledge, in literature there are no studies evaluating the stability of housekeeping genes in IVD cells and, thus, our findings will significantly improve the experimental set-up and data analysis in studies using these cells and culture conditions.

A comparison with previously published data can be done only between our results concerning AC and CEP cells and two studies performed by using fresh tissue, to be considered as a starting point. Pombo-Suarez *et al.* (2008) evaluated the suitability of housekeeping genes in human articular cartilage (hip and knee) obtained from elderly healthy subjects and patients with osteoarthritis. They observed that *TBP*, *RPL13A*, and *B2M* were the most stable genes, while *GAPDH* levels varied between samples. These results are partially in accordance with our findings. We found *TBP* gene as the most stable in hip articular chondrocytes cultured in both monolayer-pellets or in pellets alone. On the contrary, *GAPDH* was the most stable in monolayer culture and *RPL13A* was not between the best housekeeping genes.

Concerning human lumbar CEP, we found a recently published paper where appropriate housekeeping genes have been identified for this tissue (Zhou *et al.*, 2014). *SDHA* was the most stable gene and a combination of *SDHA*, *B2M* and *LDHA*, was the most suitable for normalisation, while *RPL13A* was the most unstable and the widely used *GAPDH*, *ACTB* and 18S rRNA were all inappropriate. The authors evaluated also if there were changes in the ranking of housekeeping genes when considering the presence of signal intensity changes in the vertebral endplate and subchondral bone marrow, three types of Modic changes (MC), in the samples. Although *SDHA* and *RPL13A* ranked again in the first and last position, respectively, *IPO8* was one of the most stably expressed genes in samples with type I MC, *TBP* was relatively stable in samples from types II and III MC. In accordance with these results, we found that *TBP* was the most stable housekeeping gene in CEP cells cultured in monolayer and that *RPL13A* was the most unstable gene in all the tested conditions. In addition, we identified *YWHAZ*, not investigated in the aforementioned study, as the best for gene expression normalisation of CEP cells maintained in chondro-inductive three-dimensional pellet culture.

In summary, our study evidenced the need to investigate the expression stability of housekeeping genes in cells obtained from specific anatomical regions and cultured in different experimental conditions to obtain accurate and high quality data from the quantitative evaluation of gene expression. The results obtained from this work will be useful as starting point when planning basic science or tissue engineering studies involving NP, AF and CEP cells and ACs together or alone, maintained in standard culture condition or in a chondro-inductive environment.

Conclusions

To our knowledge this is the first study to compare the stability of housekeeping genes in cells isolated from pathological IVD, CEP and AC cultured in monolayer or in a three-dimensional chondrogenic environment. This provides detailed guidelines to plan basic science or tissue engineering studies using this rationale.

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Discussion with Reviewers

Reviewer I: In the reviewer's opinion all candidate genes have a rather good efficiency in qRT-PCR and the differences are not very large. Therefore, it would be important to discuss whether the differences that are shown in this study by statistical evaluations with regard to stability of the different reference genes are of any biological relevance.

The study shows that stability of different housekeeping genes varies with different cells/conditions, and it is of course important to check suitability of housekeeping gene stability to decide for the best suitable ones. A further conclusion could be that it is better to consider multiple housekeeping genes in the quantitative evaluation of gene expression. However, in experimental practice it is often not possible to do such detailed evaluation of so many different reference genes - and the key question is if it is really important for the outcome of a study if the efficiency of all the reference genes is rather good.

Authors: The authors introduced Table 4 as an example of how the statistical interpretation of data can change when gene expression values are normalised using different housekeeping genes. Even if all the housekeeping genes selected for this study are rather stable, differences in the fold increase and in the significance of the expression of the target genes *ACAN* and *COL2A1* were found, showing a less accurate normalisation when using *GAPDH* in comparison with that obtained with *TBP*. This can assume important implications when analysing gene expression data characterised by high biological variability, such as for instance in studies performed with primary cells obtained from a high number of donors. In this study, to validate the selected reference genes we analysed two genes (*i.e. ACAN* and *COL2A1*) which are known to be strongly upregulated upon exposure to chondrogenic factors. The effect of the normalisation would be even more relevant when normalising the expression of genes that do not undergo such a strong induction. In this case, using a housekeeping gene with suboptimal stability may likely result in the overestimation or underestimation of transcriptional variations, leading to incorrect claims.

In the examples reported in Table 4, concerning the expression of both the target genes, *ACAN* and *COL2A1*, in monolayer cells at passage 1 (P1) in comparison with cells cultured in pellets for 21 d without growth factors (F-), two opposite messages were obtained when the normalisation was performed using *TBP* or *GAPDH*. A significant decrease in *COL2A1* expression was observed in cells cultured in pellets without growth factors in comparison with passage 1 monolayer cells when *TBP* was used to normalise the data, but this was not observed when normalising with *GAPDH*. On the contrary, a significant decrease in *ACAN* expression was observed in the same conditions when *GAPDH* was used to normalise the data, but this was not observed when normalising with *TBP*. Hence, from a biological point of view, the normalisation with the most stable housekeeping gene (*TBP*) compared to the normalisation with *GAPDH* leads to different claims regarding the effect of the three-dimensional culture on cell transcriptional profile.

Finally, standard guidelines for gene expression analysis recommend either the use of a single housekeeping gene that has been validated for each specific study or, alternatively, the use of at least two housekeeping genes (Thellin *et al*., 1999; Vandesompele *et al*., 2002). Considering this, in our opinion, the normalisation using a single housekeeping gene, thoroughly validated in a pilot study for each specific experimental condition, is sufficient to obtain a reliable and accurate relative quantification of the gene expression.

Editor's Note: Scientific Editor in charge of the paper: Mauro Alini