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Selection of an inadequate housekeeping gene leads to misinterpretation of target gene expression in zinc deficiency and zinc supplementation models

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ABSTRACT

Background: Numerous studies try to find the most stable housekeeping gene for a specific experimental setup. However, there is no universal housekeeping gene described so far and, therefore, new testing of housekeeping genes at the beginning of a new experiment is of high importance.

Methods: In the present study, target gene expression of mitochondrial serine/threonine-protein phosphatase (PGAM)5, nuclear factor erythroid 2-related factor (Nrf)2, dynamin related protein (Drp)1 and kelch like ECH associated protein (Keap)1 was tested in zinc-deficient and zinc-supplemented THP-1 cells and compared to control cells. Normalization of results obtained by quantitative real-time polymerase chain reaction (qRT-PCR) was performed by using the housekeeping genes porphobilinogen deaminase (PBGD), β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Additionally, this 3 housekeeping genes were tested in Jurkat cells under these conditions.

Results: Surprisingly, analyses of one and the same target gene revealed opposite results depending on the used housekeeping gene. This was caused by significant altered housekeeping gene expressions due to zinc availability.

Conclusion: Therefore, this study highlights the importance of choosing adequate housekeeping genes, which might comprise the use of more than one housekeeping gene when different conditions are tested, such as zinc deficiency and zinc supplementation. Related to the herein used experimental setup, the use of*GAPDH* to study gene expression upon zinc deficiency and *PBGD* to study gene expression after zinc supplementation is recommended.

1. Introduction

Study of gene expression is often the first step in identifying potential target molecules, followed by a more detailed analysis on protein levels. To avoid misinterpretation of obtained results, which in turn leads to the support of false hypotheses, proper normalization of gene expression levels is indispensable.

The method of quantitative real-time polymerase chain reaction (qRT-PCR) has developed to an established and powerful tool in the study of gene expression. For adequate interpretation of the obtained results, target gene expression levels are normalized to a stably expressed internal reference gene, which is determined in the same biological sample at the same time. This highlights the importance of carefully identifying one or more suitable reference genes [1]. An appropriate internal standard should meet different criteria, such as constant expression among different tissues of one organism at all developmental stages. Moreover, it should stay unaffected by the experimental treatment [2]. Housekeeping genes were initially described to maintain basic cellular functions and therefore, expected to show constant expression levels in all cells and conditions. However, in reality those expectations are rarely met, since expression of potential reference genes is influenced by different factors, such as cell types and experimental treatments [3–5].

It seems likely that all genes are regulated under some conditions, excluding the existence of one universal reference gene, which shows constant expression in all tissues [6]. This emphasizes the importance of individual testing of housekeeping genes before each new experimental setup. The use of different housekeeping genes to perform appropriate normalization of target gene expression in the analysis of zinc-affected gene expression is claimed, *i.e.* analyzing the effect of either zinc deficiency or supplementation on target gene expression. Here, we show for the first time that results of gene expression vary by using different housekeeping genes under zinc deficiency vs. zinc supplementation conditions.

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Table 1
Primer sequences for qRT-PCR.

Gene	Primer sequences (5'→3')	Amplicon length (bp)
ACTB [21]	F-CGCCCCAGGCACCAGGGC R-GCTGGGGTGTTGAAGGT	284
dynamin related protein (*Drp*) *1*	F-AGAAAATGGGGTGGAAGCAGA R-AGGCACCTTGGTCATTCCTG	220
glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [22]	F-GAAGGTGAAGGTCGGAGTC R-GAAGATGGTGATGGGATTTC	225
kelch like ECH associated protein (Keap)1 [23]	F-GCTGATGAGGGTCACCAGTT R-CCAACTTCGCTGAGCAGATT	137
nuclear factor erythroid 2-related factor (*Nrf*)*2* [23]	F-GCTCATACTCTTTCCGTCGC R-ATCATGATGGACTTGGAGCTG	145
porphobilinogen deaminase (*PBGD*) [24]	F-ACGATCCCGAGACTCTGCTTC R-GCACGGCTACTGGCACACT	87
mitochondrial serine/threonine-protein phosphatase (*PGAM5*)	F-GCCGGAAGCTGTGCAGTATT R-GGTGGGTGATGCTGCCATTA	212

2. Materials and methods

2.1. Cell culture

The human leukemia monocytic cell line THP-1 and the acute T cell leukemia cell line Jurkat were cultured in RPMI 1640 (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA, Germany), 2 mM L-glutamine, 100 U/mL potassium penicillin, 100 U/mL streptomycin sulfate (all from Sigma-Aldrich, Germany). In addition THP-1 medium was supplemented with 2.5 μL β-mercaptoethanol (Merck, Germany)/500 ml and Jurkat medium were supplemented with 1% 100x non-essential amino acids and 1 mM sodium pyruvate. Cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Induction of zinc deficiency and zinc supplementation

To induce zinc deficiency in THP-1 or Jurkat cells, cell culture medium was treated with recycled divalent cation chelating resin Chelex 100 beads (Sigma-Aldrich, Germany) to remove zinc from the medium. The Chelex-treated medium was reconstituted with 500 μM $CaCl_2$ and 400 μM $MgCl_2$ (all from Merck, Germany) [7,8]. Moreover, the pH was adjusted to 7.4, resembling the pH value of the control medium. For zinc supplementation experiments 50 μM $ZnSO_4$ (Merck, Germany) was added to the medium. 1×10^5 cells/mL were cultured in either control, zinc-deficient or zinc-supplemented medium for three days. Subsequently, RNA isolation and flow cytometry measurements were performed as described.

2.3. qRT-PCR

RNA was isolated from 3×10^6 cells per sample using the extract me total RNA kit (Blirt, Poland) according to the manufacturer's instructions. Subsequently, 1 μg total RNA was used for cDNA synthesis by using the qScript cDNA synthesis kit (VWR/QuantaBioscience, Germany) according to the manufacturer's instructions. Quantitative analysis was performed using the fluorescent SYBR green reagent on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Germany) at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The used primers are listed in Table 1. Target gene expression was normalized to expression of the housekeeping genes *ACTB*, *GAPDH* and *PBGD*. All samples were run in duplicates and target gene expression was quantified by use of the $\Delta\Delta C_T$-method as previously described [9]. Comparison of housekeeping genes was performed by using C_T-values with analysis of n = 5 independent experiments in duplicates.

2.4. Flow cytometry measurement of intracellular free Zn^{2+} with FluoZin-3 AM

1×10^6 cells per sample were loaded with 1 mL measurement buffer [10], containing 1 μM FluoZin-3 AM (Thermo Fisher, Germany), gently shaken for 30 min at 37 °C in the dark. Cells were washed and resuspended in measurement buffer followed by another incubation step for 10 min at 37 °C with either 50 μM N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) to obtain minimal fluorescence, with $ZnSO_4$/pyrithione (100 μM/50 μM) (all Sigma-Aldrich, Germany) to obtain maximal fluorescence or samples were left untreated. Flow cytometry measurements were performed using FACSCalibur (BD, Germany). Calculation of intracellular labile zinc was performed as described before [11] using the dissociation constant K_D of 8.9 nM for the FluoZin-3/Zn^{2+} complex [12].

2.5. Statistical analysis

Experiments analyzing target gene expression and flow cytometry measurements were performed in n = 5 independent experiments. Analysis of housekeeping gene expression using C_T-values was performed with n = 5 independent experiments in duplicates. Statistical significance of experimental results was calculated by Student's *t*-test using Excel and GraphPad software. *P*-values of < 0.05 were considered significant.

3. Results

3.1. Altered intracellular zinc levels upon zinc deficiency and zinc supplementation

After incubating THP-1 or Jurkat cells for three days in either control, zinc-deficient or zinc-supplemented medium, the intracellular zinc status was assessed by flow cytometry measurements using the fluorescence probe FluoZin-3 AM (Fig. 1). Cells cultivated in Chelex-treated medium showed significant reduced intracellular zinc levels compared to control cells, whereas cells cultivated in zinc-

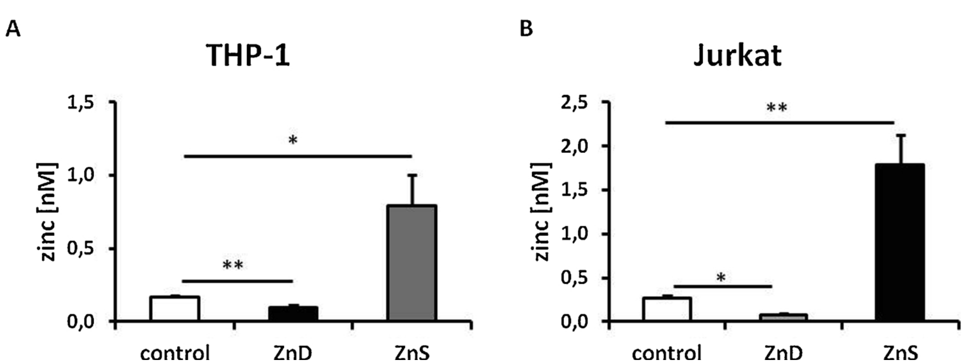

Fig. 1. Differences in intracellular zinc levels after cell incubation in control, zinc-deficient (ZnD) and zinc-supplemented (ZnS) medium. After incubating THP-1 (A) or Jurkat (B) cells for three days in either control, Chelex-treated (ZnD) or zinc-supplemented (ZnS, 50 μM $ZnSO_4$) medium, intracellular zinc levels were assessed by flow cytometry measurements using FluoZin-3 AM. Data are shown as mean + SEM from n = 5 independent experiments (* $p < 0.05$, ** $p < 0.01$; Student's *t*-test).

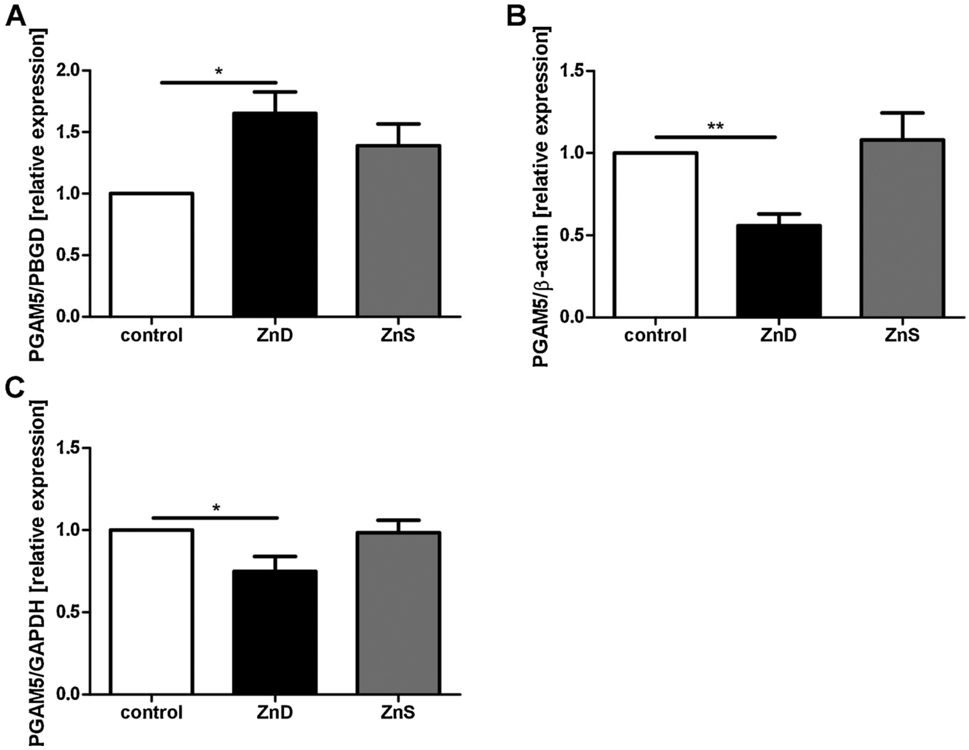

Fig. 2. PGAM5 expression shows up- or downregulation depending on the used housekeeping gene. THP-1 cells were incubated for three days in control, zinc deficiency (ZnD) or zinc supplementation (ZnS, 50 μM $ZnSO_4$) medium. PGAM5 expression was determined by qRT-PCR using *PBGD* (A), *ACTB* (B) and *GAPDH* (C) as housekeeping genes. Expression levels were normalized to untreated control cells. Data are shown as mean + SEM from n = 5 independent experiments (* $p < 0.05$, ** $p < 0.01$; Student's *t*-test).

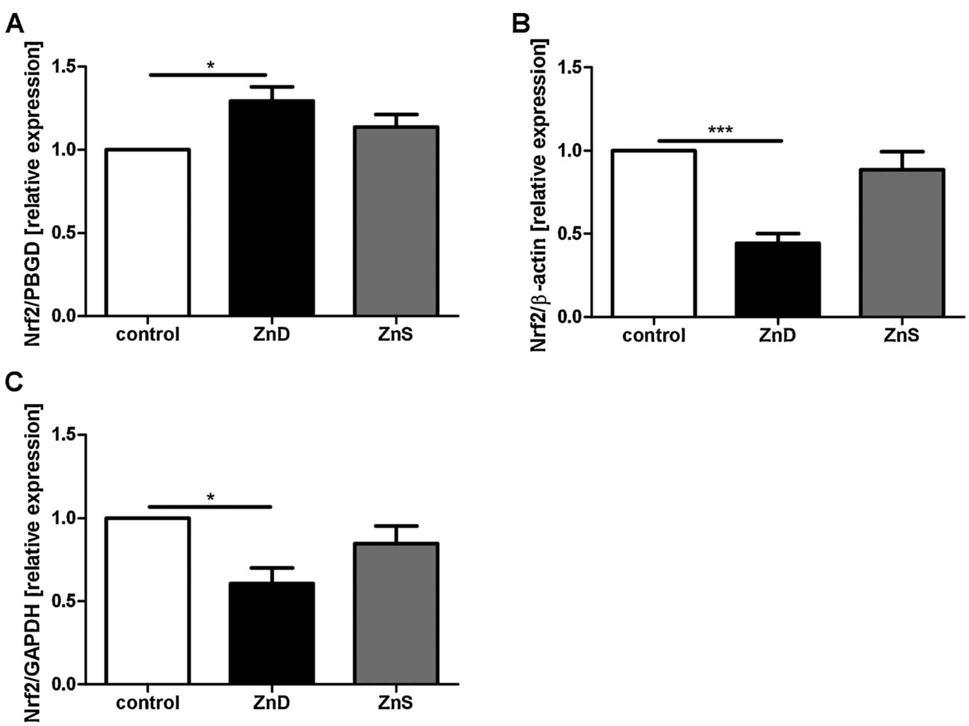

Fig. 3. Nrf2 expression shows up- or downregulation depending on the used housekeeping gene. THP-1 cells were incubated for three days in control, zinc deficiency (ZnD) or zinc supplementation (ZnS, 50 μM $ZnSO_4$) medium. Nrf2 expression was determined by qRT-PCR using *PBGD* (A), *ACTB* (B) and *GAPDH* (C) as housekeeping genes. Expression levels were normalized to untreated control cells. Data are shown as mean + SEM from n = 5 independent experiments (* $p < 0.05$, *** $p < 0.001$; Student's *t*-test).

supplemented medium (50 μM $ZnSO_4$) showed significantly increased intracellular zinc levels.

3.2. Zinc effects on target gene expression using different housekeeping genes

The influence of zinc on expression of the different potential target genes *PGAM5*, *Nrf2*, *Drp1* and *Keap1* was analyzed in the present study. To normalize the results obtained by qRT-PCR, the housekeeping genes *PBGD*, *ACTB* and *GAPDH* were used. Surprisingly, the expression of the same target gene showed significantly increased or *vice versa* significantly decreased expression depending on the used housekeeping gene. Analysis of PGAM5 (Fig. 2) and Nrf2 expression (Fig. 3) revealed significantly increased expression levels in zinc-deficient cells compared to control cells by using *PBGD* as housekeeping gene (Fig. 2A for PGAM5 and Fig. 3A for Nrf2), whereas expression levels in those samples were significantly decreased after using *ACTB* (Fig. 2B for PGAM5 and Fig. 3B for Nrf2) or *GAPDH* (Fig. 2C for PGAM5 and Fig. 3C for Nrf2) as housekeeping genes. The same trend was observed by analyzing Drp1 (Fig. 4) and Keap1 expression (Fig. 5). Related to Drp1

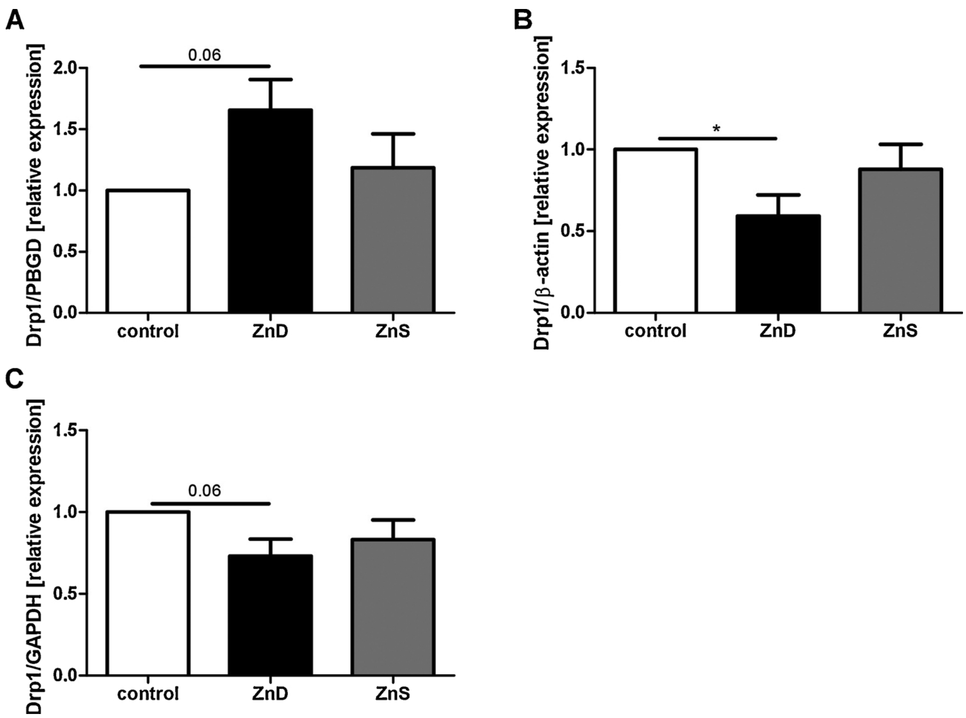

Fig. 4. Drp1 expression shows up- or down-regulation depending on the used housekeeping gene. THP-1 cells were incubated for three days in control, zinc deficiency (ZnD) or zinc supplementation (ZnS, 50 μM $ZnSO_4$) medium. Drp1 expression was determined by qRT-PCR using *PBGD* (A), *ACTB* (B) and *GAPDH* (C) as housekeeping genes. Expression levels were normalized to untreated control cells. Data are shown as mean + SEM from n = 5 independent experiments (* $p < 0.05$; Student's *t*-test).

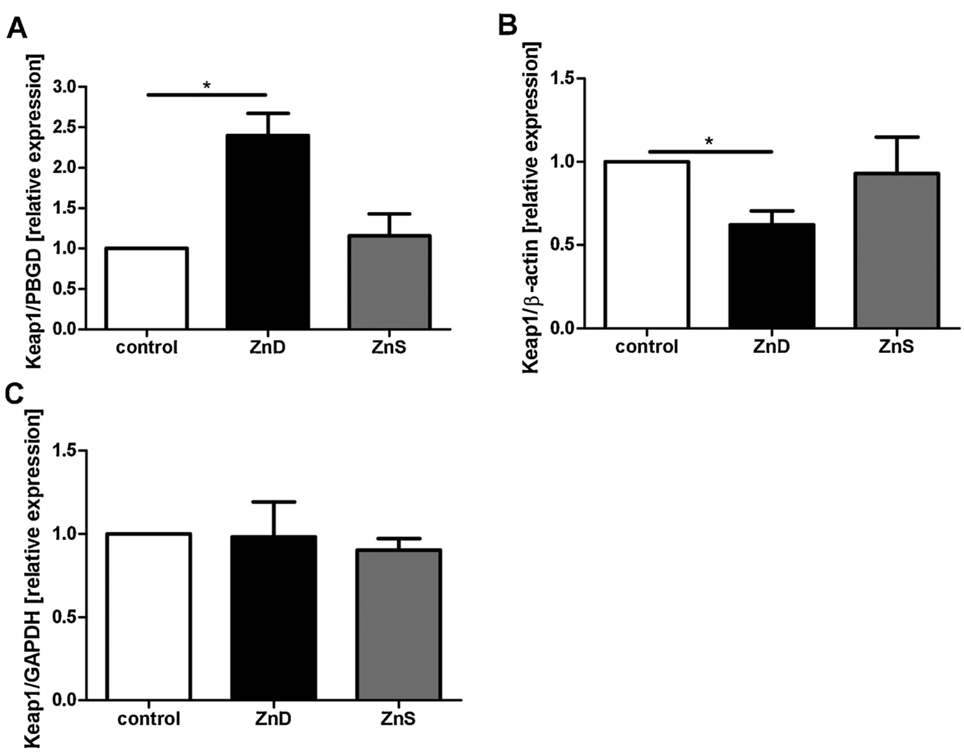

Fig. 5. Keap1 expression shows up- or down-regulation depending on the used housekeeping gene. THP-1 cells were incubated for three days in control, zinc deficiency (ZnD) or zinc supplementation (ZnS, 50 μM $ZnSO_4$) medium. Keap1 expression was determined by qRT-PCR using *PBGD* (A), *ACTB* (B) and *GAPDH* (C) as housekeeping genes. Expression levels were normalized to untreated control cells. Data are shown as mean + SEM from n = 3 independent experiments (* $p < 0.05$; Student's *t*-test).

expression, normalization to PBGD expression showed increased expression in zinc-deficient compared to control cells, however, missing significance ($p = 0.06$, Fig. 4A). Normalization to β-actin expression revealed significant reduced Drp1 expression (Fig. 4B) and normalization to GAPDH expression resulted in decreased Drp1 expression, missing significance ($p = 0.06$, Fig. 4C). The fourth tested potentially zinc-affected target gene *Keap1* normalized to PBGD expression showed significant upregulation after zinc deficiency compared to control cells (Fig. 5A), whereas Keap1 expression normalized to β-actin expression was significantly decreased (Fig. 5B). Use of GAPDH expression for normalization remained unaffected, excluding any zinc effect on Keap1 expression (Fig. 5C). Zinc supplementation of THP-1 cells showed no significant differences in target gene expression independent of the used housekeeping gene (Figs. 2–5).

3.3. Zinc significantly influences CT-values of different housekeeping genes

The obtained opposing results, being dependent on the used housekeeping gene (Figs. 2–5), led to the comparison of housekeeping gene C_T-values in THP-1 cells. Significant differences in expression

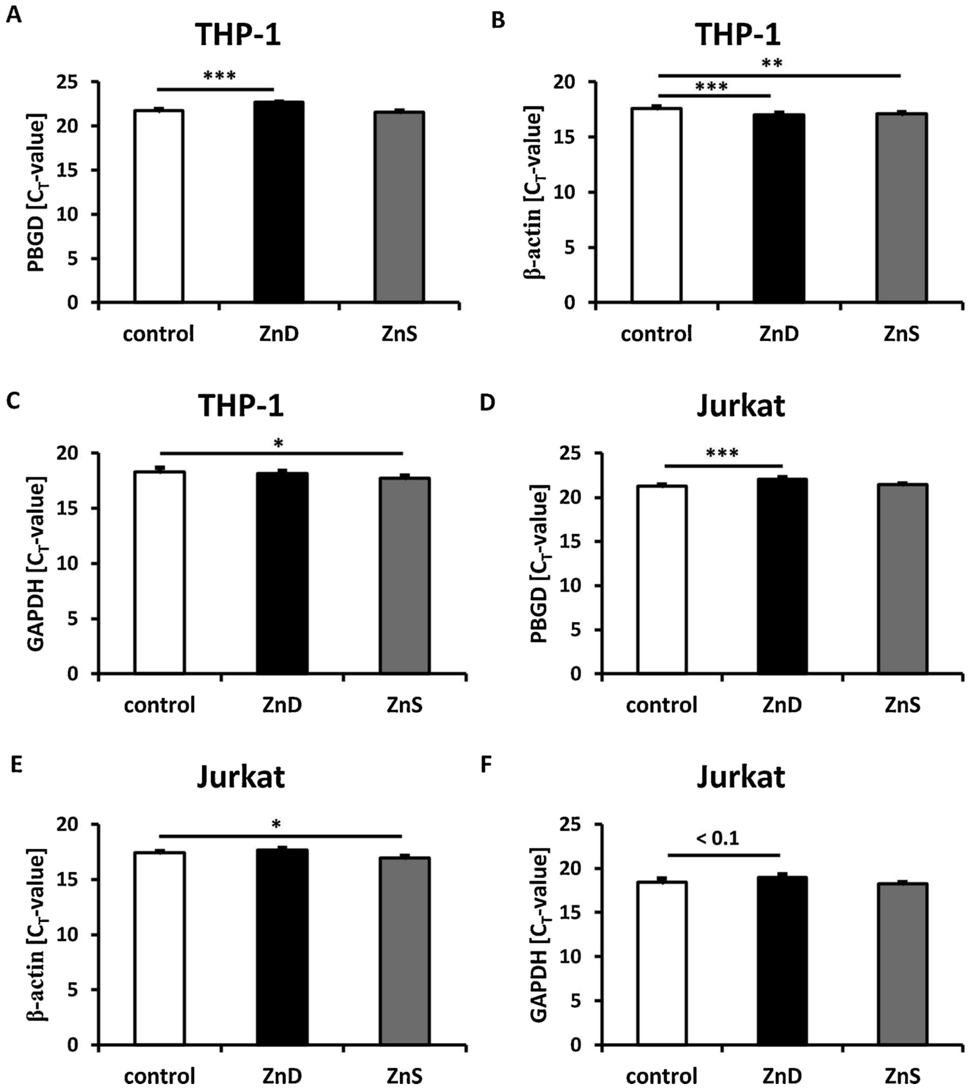

Fig. 6. C_T-values of different housekeeping genes are influenced by the intracellular zinc status. C_T-values of PBGD (A, D), β-actin (B, E) and GAPDH (C, F) were compared after THP-1 (A–C) or Jurkat (D–E) cells incubation for three days in either control, zinc deficiency (ZnD) or zinc supplementation (ZnS, 50 μM $ZnSO_4$) medium. Data are shown as mean + SEM from independent experiments (n = 5 in duplicates) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's *t*-test).

levels could be observed for PBGD (Fig. 6A), β-actin (Fig. 6B) and GAPDH (Fig. 6C) due to the availability of zinc, *i.e.* in the presence of zinc deficiency or zinc supplementation. In detail, C_T-values of PBGD showed significant increased values after zinc deficiency compared to values obtained from control cells (Fig. 6A). In contrast, C_T-values of β-actin were significantly reduced after zinc deficiency as well as after zinc supplementation compared to control cells (Fig. 6B), excluding the use of β-actin as housekeeping gene in zinc-dependent experiments. GAPDH expression revealed significantly reduced C_T-values after zinc supplementation compared to C_T-values obtained from control cells (Fig. 6C). To exclude a cell type specific effect, the experiments were reproduced with the T cell line Jurkat and showing similar results (Fig. 6D–F), and thereby supporting the idea of a general effect. Those results emphasize the use of two different housekeeping genes depending on the condition studied, either zinc deficiency, supporting the use of *GAPDH*, or zinc supplementation, supporting the use of *PBGD* as suitable housekeeping genes.

4. Discussion

The results obtained in the present study highlight the importance of accurate evaluation and choice of housekeeping genes. Depending on whether zinc deficiency or zinc supplementation is induced within cells, the use of different housekeeping genes might be advised due to significant regulation of housekeeping gene expression by Zn^{2+}. Otherwise, results obtained after normalization might lead to wrong hypotheses and interpretations. In this study the complete opposite outcome was observed, *i.e.* although gene expression was downregulated, the use of an inadequate housekeeping gene led to the opposite assumption of significant upregulated gene expression.

Del Pozo et al. analyzed the influence of low and high zinc and copper supplementation on expression of different housekeeping genes in human cell lines [13]. They compared the stability of different housekeeping genes and their results showed variations in gene stability related to the used cell type and metal supplementation. In detail, they observed changes in gene expression of *GAPDH* and *ACTB* after metal supplementation, which could be confirmed in the present study as well. Taken together, these findings support the common view of a missing universal candidate gene for all studied conditions. Metal deficiency was not studied by del Pozo et al., which constitutes an important difference. Regarding the use of an appropriate housekeeping gene, one needs to be careful in generally rejecting the use of one housekeeping gene until not all experimental conditions are tested.

More studies tested the effect of other metals than zinc on housekeeping gene expression as well. Manganese, for example, was shown to induce GAPDH expression in astrocytes [14] and the use of an iron-chelating agent revealed induction of GAPDH expression as confirmed by Northern blot analysis as well [15]. Bas et al. determined the expression levels of different housekeeping genes (18S rRNA, β-actin and GAPDH) and mRNA for different cytokines in human peripheral blood mononuclear cells (PBMC) [16]. Depending on which of the three housekeeping genes was used for normalization after cell stimulation, variations in the peak of cytokine gene expression were observed. This leads to false assumptions of the time of maximum gene expression and might cause misinterpretations of cellular responses in immune reactions [16]. In general, the use of *GAPDH* and *ACTB* as housekeeping genes is controversially discussed. However, if properly tested before, there is no reason of excluding those genes as reference genes [17]. An unsolved problem is, if the trace element status of primary cells like PBMC will influence the expression of housekeeping genes as well. If this is the case, gene expression studies in malnutritioned or oversupplemented people must be reanalyzed.

Apart from the already mentioned study by del Pozo et al. [13] using human cell lines, there are some existing studies performed in experimental plant models, trying to identify adequate housekeeping genes, analyzing metal stress and homeostasis [18,19]. Interestingly, results obtained from experiments with *Sedum alfredii* showed more stable gene expression under different heavy metal stresses (Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}) of traditional housekeeping genes than of novel genes [19]. This emphasizes again that use of some controversially discussed housekeeping genes, such as *ACTB*, should not be excluded from the beginning, but should be tested carefully if applicable for the designed experimental setup.

PBGD seems to represent an appropriate housekeeping gene, being expressed in all tissues by using of a housekeeping promoter [20]. Nevertheless, as observed in the present study, zinc deficiency significantly changes its expression, recommending its use as housekeeping gene only for the study of zinc supplementation. In contrast, *GAPDH* is advised to be used as an adequate reference gene in studying the effect of zinc deficiency as already mentioned. The use of *ACTB* as housekeeping gene in this experimental setup is not advisable at all, since it shows significant altered expression under zinc deficiency and zinc supplementation.

The herein obtained results indicate that it might not be sufficient to test housekeeping genes for one experiment in general, but to validate it for all planned conditions. Thus, single testing of the housekeeping gene under either zinc deficiency or zinc supplementation conditions is not sufficient since gene expression is influenced by zinc homeostasis. This might finally result in the use of different housekeeping genes using one housekeeping gene to study the effect of zinc deficiency and another to study the effect of zinc supplementation. The main message received from the obtained results, *i.e.* use of different housekeeping genes dependent on the treatment, might additionally be adapted to experiments in which the effect of other metals than zinc are tested on target gene expression.

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Declaration of Competing Interest

None.

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