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EF1α is a suitable housekeeping gene for RT-qPCR analysis during osteogenic differentiation of mouse bone marrow-derived mesenchymal stem cells

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The expression of predominant housekeeping genes used in RT-qPCR can vary during development and differentiation. The frequently used housekeeping genes (ACTB, GAPDH, 18S rRNA, EF1α and RPL 13a) were evaluated during an early stage of the osteogenic differentiation of mouse bone marrow-derived mesenchymal stem cells (mMSCs) (under normal conditions or treated with CCG-4986) to identify housekeeping genes whose expression remained constant during osteogenic differentiation. When we used RGS4 mRNA, which was determined as copy number per μg of total RNA, to normalize gene expression, we observed that the relative EF1α expression profile was consistent with RGS4 expression after treatment with CCG-4986. All the relative expression profiles of the EF1α, 18S rRNA, and RPL13a housekeeping genes were consistent with RGS4 profiles determined by measuring mRNA copies under normal osteogenic differentiation conditions. The expression profiles calibrated by ACTB and GAPDH were not consistent with those determined using mRNA copy number in untreated cells or cells treated with CCG-4986 under osteogenic differentiation conditions. Under normal osteogenic differentiation conditions, EF1α, 18S rRNA, and RPL 13a are suitable housekeeping genes for RT-qPCR analysis. However, EF1α is the only suitable gene upon CCG-4986 treatment.

Key words: EF1α; RGS4; 18S rRNA; RT-qPCR; RPL 13a; CCG-1986

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INTRODUCTION

Housekeeping genes are widely used as internal controls for quantitative real-time PCR (RT-qPCR) analysis of target gene mRNA. The predominant housekeeping genes used include ACTB (Stathopoulou *et al.*, 2002), GAPDH (Barber *et al.*, 2005), 18S rRNA (Goidin *et al.*, 2001; Filby & Tyler, 2007; Bas *et al.*, 2004), EF1α and RPL 13a (Curtis *et al.*, 2010). An inherent assumption in the use of housekeeping genes is that their expression remains constant in the cells or tissues under investigation. Although some housekeeping genes are expressed at relatively constant levels, the expression of other housekeeping genes may vary depending on experimental conditions. Recently, the expression of several housekeeping genes was shown to vary in different cells and diseases (Filby & Tyler, 2007; Curtis *et al.*, 2010; Selvey *et al.*, 2001; Zhong & Simons, 1999; Thellin *et al.*, 1999; Van Hiel *et al.*, 2009; Glare *et al.*, 2002). Validating housekeeping gene expression levels is crucial for accurate quantification of mRNA with RT-qPCR.

To date, commonly used housekeeping genes have not been validated during the osteogenic differentiation of marrow-derived mesenchymal stem cells. 18S rRNA is the most frequently used housekeeping gene (Kha *et al.*, 2004; de Boer *et al.*, 2004). However, EF1α and RPL 13a may also represent suitable housekeeping genes for RT-qPCR analysis in marrow-derived mesenchymal stem cells (Curtis *et al.*, 2010). Whether the expression of housekeeping genes remains constant during differentiation of stem cells treated with specific reagents is unclear.

Regulator of G protein signaling (RGS) proteins are GTPase-activating proteins (GAP) for various Gα subunits of heterotrimeric G proteins. The heterotrimeric G proteins and RGS proteins participate in differentiation of many cells (Cheng *et al.*, 2008; Murai *et al.*, 2010; Appleton *et al.*, 2006). RGS4, an RGS protein, may participate in the chondrogenic differentiation of the ATDC5 cell line (Wu *et al.*, 2011). CCG-4986, an inhibitor of RGS4, can inhibit the GAP activities of RGS4, which could lead to the regulation of Giα and Gqα signaling involved in the differentiation of stem cells (Tu *et al.*, 2007; Su *et al.*, 1993; Wang & Malbon, 1996).

We aimed to validate the potential use of different housekeeping genes as internal controls to normalize the expression of genes of interest at an early differentiation stage of marrow-derived mesenchymal stem cells. Five housekeeping genes, ACTB, GAPDH, 18S RNA, EF1α and RPL 13a, were selected for validation. These genes were chosen based on their different cellular functions and their prior use as housekeeping genes in many gene expression studies.

In this study, the osteogenic differentiation of mouse marrow-derived mesenchymal stem cells was induced with or without CCG-4986 treatment (inhibitor of RGS4). The expression profile of RGS4 was examined

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Abbreviations: mMSCs, mouse bone marrow-derived mesenchymal stem cells; RGS4, regulator of G-protein signaling 4; CCG-4986, methyl-N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate; RT-qPCR: quantitative real-time RT-PCR; EF1α, elongation factor 1 alpha; 18S rRNA, 18S ribosomal RNA; ACTB, beta-actin; RPL 13a, 60S ribosomal protein L13a; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 1. Primer pairs used for Real Time RT-qPCR analysis

18s rRNA (Fleig *et al.*, 2007; Yang *et al.*, 2006)	Sense primer: 5′-TTGACGGAAGGGCACCACCAG-3′	Antisense primer: 5′-GCACCACCACCCACGGAATCG-3′	GenBank Number: NR_003278.
ACTB (Reitinger *et al.*, 2007; Sage *et al.*, 2011)	Sense primer: 5′-GGCTGTATTCCCCTCCATCG-3′	Antisense primer: 5′-CCAGTTGGTAACAATGCCATGT-3′	GenBank Number: NM_007393.
EF1α (Van Itallie *et al.*, 2006)	Sense primer: 5′-CTGAACCATCCAGGCCAAAT-3′	Antisense primer: 5′-GGCTGTGTGACAATCCAG-3′	GenBank Number: NM_010106.
GAPDH	Sense primer: 5′-AGGTTGTCTCCTGCGACTTCA-3′	Antisense primer: 5′-TGGTCCAGGGTT TCTTACTCC-3′	GenBank Number: NM_008084.
RGS4	Sense primer: 5′-GAAATGGGCTGAATCGTTGG-3′	Antisense primer: 5′-GTTGCTTGCACTGAGATG-3′	GenBank Number: NM_009062.
RPL 13a (Murray *et al.*, 2010; Leystra *et al.*, 2012)	Sense primer:: 5′-TTCGGCTGAAGCCTACCAGAAAGT-3′	Antisense primer: 5′-GCATCTTGGCCTTTTTCCGTT -3′	GenBank Number: NM_009438.

during an early stage of osteogenic differentiation by measuring RGS4 mRNA copy number. The chosen housekeeping genes were used as internal controls to generate the relative expression profile of RGS4. The most suitable housekeeping genes were determined by comparing their relative expression profiles with the expression profile of RGS4, which was generated by measuring the mRNA copy number.

METHODS & MATERIALS

Culture and osteogenic differentiation of mMSCs. mMSCs (WeiKai Biotech, Tianjin, China) were cultured and passaged in MSC culture medium (WeiKai Biotech) according to the manufacturer's instructions (Nadri & Soleimani, 2009). mMSCs between 3 and 10 passages were passaged in 24-well plates and used for osteogenic differentiation experiments. For osteogenic differentiation, mMSCs were cultured in a medium (WeiKai Biotech) containing 10^{-7} M dexamethasone, 10 mM [beta]-glycerol phosphate and 50 μM ascorbate-2-phosphate (Heng *et al.*, 2010). In the case of methyl-N-[(4-chlorophenyl)sulfonyl]-4-nitrobenzenesulfinimidoate (CCG-4986, ChemBridge, San Diego, CA, USA) treatment groups, 10 μM CCG-4986 was added to the osteogenic differentiation medium. mMSCs in which osteogenic differentiation was induced with or without CCG-4986 treatment were harvested at days 0, 1, 2 and 3.

RNA extraction. RNA from mMSCs was extracted with the simply P total RNA extraction kit (BioFlux Cat#BSC52 M1, Hangzhou, China) according to the manufacturer's instructions. Briefly, cells were cultured in 24-well plates and collected and resuspended in 100 μl of R1 solution. Next, 600 μl of R2 solution was added and mixed thoroughly. The lysates were transferred to spin columns and centrifuged for 30 s. The spin columns were washed twice with wash buffer and transferred to sterile 1.5 ml microcentrifuge tubes, and the total RNA was eluted from the spin columns with 30 μl of elution buffer. RNA aliquots of 4 μ1 were quantified with GeneQuant pro (GE Healthcare, USA). Samples with (A260–A320)/(A280–A320) ratios less than 1.7 were excluded from the subsequent analysis.

Real time RT-qPCR. All primer pairs used in this experiment were synthesized by Invitrogen Inc. (Shanghai, China). The primer pair sequences are listed in Table 1.

The primer pairs were reconstituted in nuclease-free water. A 2 μM stock solution containing both forward and reverse primers was mixed and stored at –20°C. Primer pairs were used at a final concentration of 100 nM in each RT-qPCR assay. Aliquots of 100 ng of total RNA were reverse transcribed to cDNA using the PrimeScript RT reagent kit with gDNA Eraser (DR-

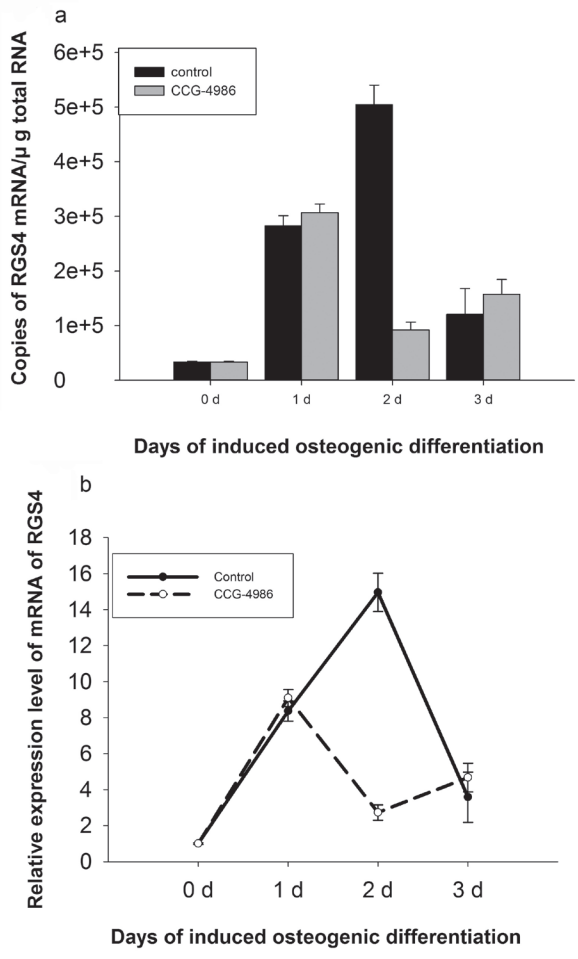

Figure 1. Expression profiles of RGS4 determined by mRNA copy number.
Cells in which osteogenic differentiation was induced without CCG-4986 treatment are the control group. The results are represented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression relative to the control at day 0, which was arbitrarily set at 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA copy numbers. 1b, The expression profiles of RGS4 mRNA normalized to control at day 0.

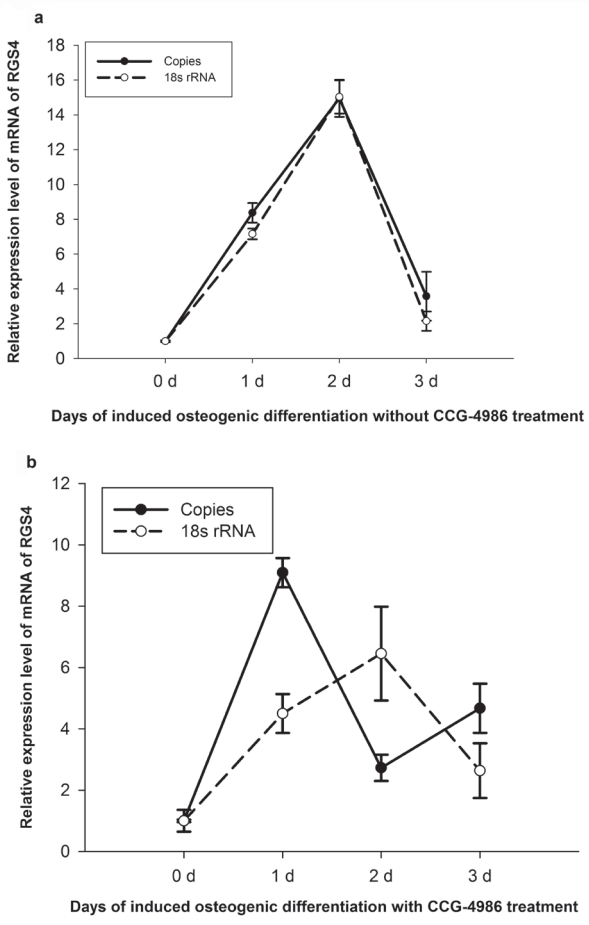

Figure 2. Expression profiles of RGS4 mRNA normalized to 18S rRNA.
The expression profiles of RGS4 mRNA during osteogenic differentiation measured by copy number and normalized to control are the copies group. The expression profiles normalized to 18S rRNA are the 18S RNA group. The results are presented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression relative to day 0, which was arbitrarily set as 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA without CCG-4986 treatment. 1b, The expression profiles of RGS4 mRNA with CCG-4986 (10 μM) treatment.

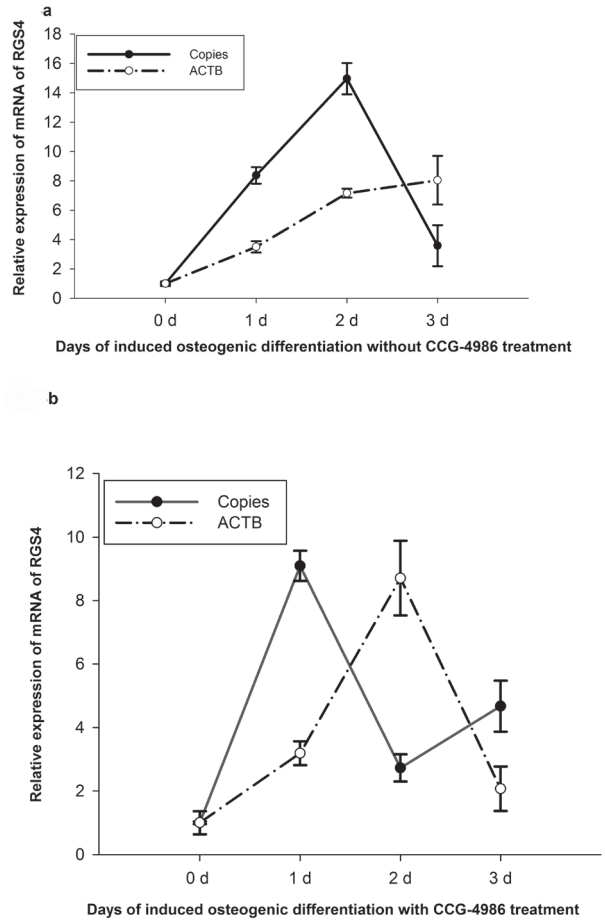

Figure 3. Expression profiles of RGS4 mRNA normalized to ACTB.
The expression profiles of RGS4 mRNA during osteogenic differentiation measured by copy number and normalized to control are the mRNA copy group. The expression profiles normalized to ACTB are the ACTB group. The results are presented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression relative to day 0, which was arbitrarily set as 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA without CCG-4986 treatment. 1b, The expression profiles of RGS4 mRNA with CCG-4986 (10 μM) treatment.

R047A, TaKaRa). RT-qPCR was performed using 1 μl of cDNA using the Mx3000P Multiplex Quantitative PCR System (Stratagene, USA) with the SYBR Premix Ex Taq Kit(DRR820A, TaKaRa). For RGS4 copy number analysis, the pCMV6-Kan/Neo RGS4 expression vector (MC200200, Origene, Beijing, China) was employed as a standard at concentrations of 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 copies per μl. The cycling conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 sec, 57°C for 15 sec and 72°C for 15 sec. Mx3000P v2.00 software was used to analyze the number of copies and relative levels of RGS4 mRNA.

Statistical Analysis. Data sets containing N= 4 independent experiments (4 samples per condition per experiment) were used for statistical analysis. A one-way ANOVA (Tukey's post-hoc analysis) was used to calculate statistical significance using SigmaPlot version 12.00 for Windows, Systat Software, Inc. (San Jose, CA, http://www.sigmaplot.com). Error bars represent the S.E.M.

RESULTS AND DISCUSSION

The expression profile of RGS4 under early-stage osteogenic differentiation conditions with or without CCG-4986 treatment

We first determined the expression profile of RGS4 at days 0, 1, 2 and 3 during osteogenic differentiation. The RGS4 mRNA copy numbers were $3.37\pm0.13\times10^4$ (day 0), $2.82\pm0.19\times10^5$ (day 1), $5.04\pm0.36\times10^5$ (day 2) and $1.21\pm0.47\times10^5$ (day 3) (Fig. 1a, control) per μg of total RNA without CCG-4986 treatment and $3.37\pm0.13\times10^4$ (day 0), $3.07\pm0.16\times10^5$ (day 1), $9.19\pm1.45\times10^4$ (day 2) and $1.58\pm0.27\times10^5$ (day 3) (Fig. 1a, CCG-4986) per μg of total RNA with CCG-4986 (10 μM) treatment. The relative RGS4 mRNA levels normalized to day 0 are illustrated in Fig. 1b. Under osteogenic differentiation conditions without CCG-4986 (Fig. 1b, control), the RGS4 mRNA levels

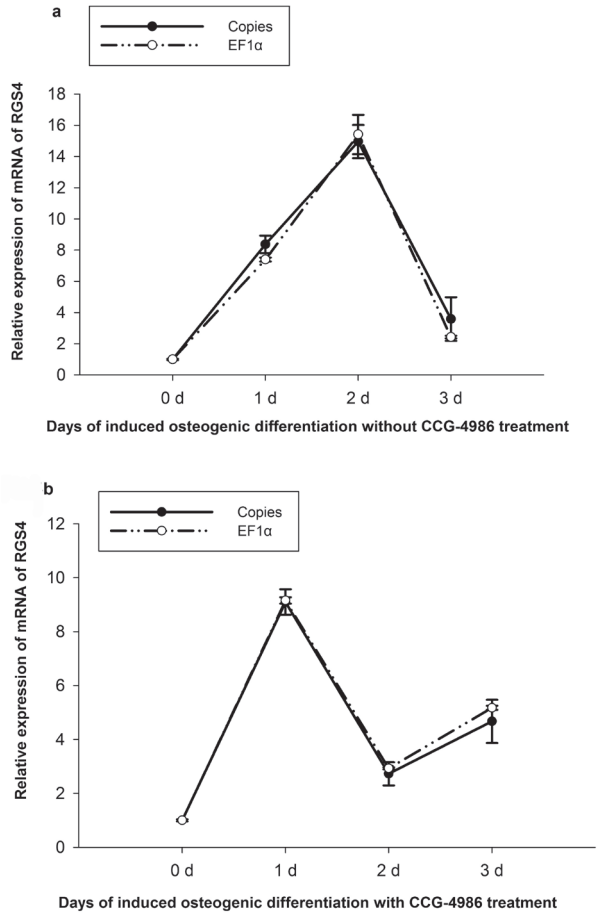

Figure 4. Expression profiles of RGS4 mRNA normalized to EF1α.
The expression profiles of RGS4 mRNA during osteogenic differentiation measured by copy number and normalized to control are the mRNA copy group. The expression profiles normalized to EF1α are the EF1α group. The results are presented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression relative to day 0, which was arbitrarily set as 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA without CCG-4986 treatment. 1b, The expression profiles of RGS4 mRNA with CCG-4986 (10 μM) treatment.

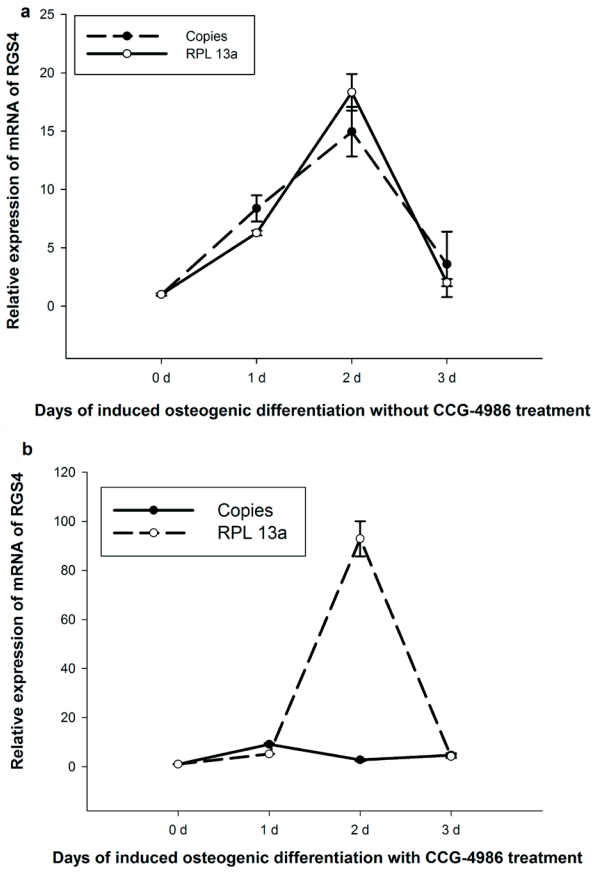

Figure 5. Expression profiles of RGS4 mRNA normalized to RPL 13a.
The expression profiles of RGS4 mRNA during osteogenic differentiation measured by copy number and normalized to control are the mRNA copy group. The expression profiles normalized to RPL 13a are the RPL 13a group. The results are presented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression from day 0, which was arbitrarily set as 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA without CCG-4986 treatment. 1b, The expression profiles of RGS4 mRNA with CCG-4986 (10 μM) treatment.

increased 8.37-, 14.96- and 3.58-fold at days 1, 2 and 3, respectively. In cells treated with CCG-4986 (Fig. 1b, CCG-4986, 10 μM), the RGS4 mRNA levels increased 9.10-, 2.72- and 4.67-fold at days 1, 2 and 3, respectively. These results indicate that RGS4 mRNA was upregulated during the osteogenic differentiation of mMSCs and suggest that RGS proteins and G-protein signaling systems (Murai *et al.*, 2010; Appleton *et al.*, 2006; Wu *et al.*, 2011; Sharma *et al.*, 2011) may participate in mMSC osteogenic differentiation. CCG-4986 was the first non-peptide compound discovered that selectively inhibited the RGS4 subtype of the regulator of G-protein signaling (Roman *et al.*, 2007). CCG-4986 has been shown to inhibit RGS4 function through covalent modification of two spatially distinct cysteine residues (Cys132 and Cys148) in RGS4 (Roman *et al.*, 2010; Kimple *et al.*, 2007). Our results revealed that CCG-4986 also inhibited the expression of RGS4 mRNA in osteogenically differentiated mMSCs at day 2, but the mechanism remains unclear.

The expression profile of RGS4 normalized to 18S rRNA, ACTB, EF1α, RPL 13a and GAPDH under early-stage osteogenic differentiation conditions with or without CCG-4986 treatment

The expression profiles of RGS4 normalized to 18S rRNA, EF1α and RPL 13a were consistent with those determined with RGS4 mRNA copy number under osteogenic differentiation conditions without CCG-4986 treatment (Fig. 2a, Fig. 4a, and Fig. 5a). However, when cells were treated with CCG-4986 (10 μM), only the expression profile normalized to EF1α was consistent with RGS4 mRNA copy number (Fig. 2b, Fig. 4b, and Fig. 5b). These results suggest that 18S rRNA, EF1α and RPL 13a could be used as housekeeping genes during osteogenic differentiation without CCG-4986 treatment. Only EF1α was a suitable housekeeping gene for RT-qPCR analysis under osteogenic differentiation conditions upon CCG-4986 treatment. These results also indicate that CCG-4986 can affect the expression of 18S rRNA and RPL13a genes.

During osteogenic differentiation with or without CCG-4986 (10 μM) treatment, the expression pro-

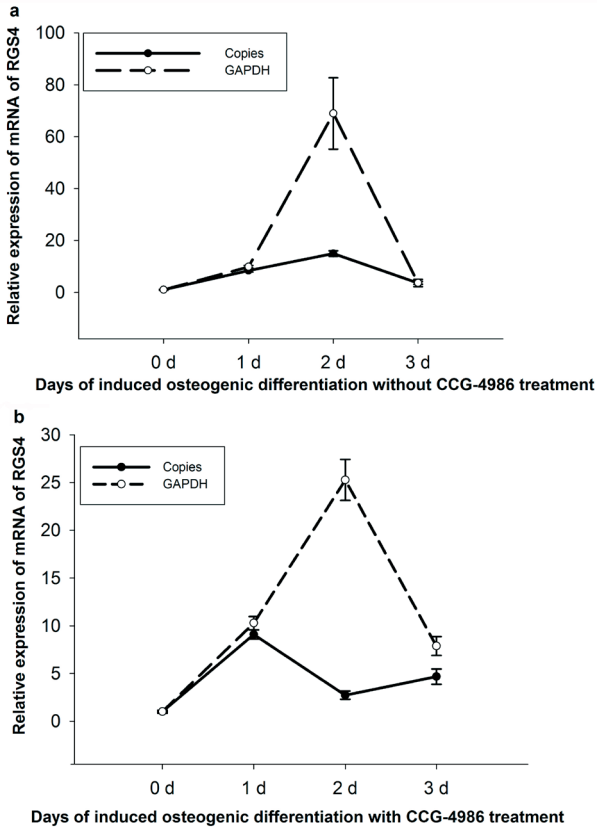

Figure 6. Expression profiles of RGS4 mRNA normalized to GAPDH.
The expression profiles of RGS4 mRNA during osteogenic differentiation measured by copy number and normalized to control are the mRNA copy group. The expression profiles normalized to GAPDH are the GAPDH group. The results are presented as the means ± S.E.M (one-way ANOVA test) and expressed as the fold increase in expression relative to day 0, which was arbitrarily set as 1× RGS4 mRNA expression. Each sample was analyzed in duplicate. 1a, The expression profiles of RGS4 mRNA without CCG-4986 treatment. 1b, The expression profiles of RGS4 mRNA with CCG-4986 (10 μM) treatment.

files normalized to ACTB (Figs. 3a, 3b) and GAPDH (Figs. 6a, 6b) were not consistent with RGS4 mRNA copy number. These results indicate that ACTB and GAPDH expression is not constant during osteogenic differentiation.

The expression of many housekeeping genes has been shown to vary under different experimental conditions [2, 6–8, 11]. Our results suggest that the expression of 18S rRNA and EF1α could be constant during the early stage of osteogenic differentiation without CCG-4986 treatment. However, the expression of ACTB and GAPDH varied during the early stage of osteogenic differentiation with or without CCG-4986 treatment. RGS4 and G-proteins may regulate the expression of 18S rRNA, ACTB, RPL 13a and GAPDH in response to CCG-4986 treatment during the early stage of osteogenic differentiation because the expression profiles normalized to these housekeeping genes were not consistent with RGS4 mRNA copy number.

Taken together, our results showed that EF1α, 18S rRNA, and RPL 13a are suitable housekeeping genes (in descending order of preference) for RT-qPCR analysis during the early stage of mMSC osteogenic differentiation without CCG-4986 treatment. EF1α is the most suitable housekeeping gene in early mMSC osteogenic differentiation with CCG-4986 treatment. Neither ACTB nor GAPDH is suitable for RT-qPCR analysis of early mMSC osteogenic differentiation under normal conditions or upon treatment with CCG-4986. Taken together, EF1α is a suitable housekeeping gene for RT-qPCR analysis during mMSC osteogenic differentiation.

Acknowledgments

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