miR-142-3p restricts cAMP production in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells by targeting AC9 mRNA

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Cyclic AMP (cAMP) is a ubiquitous second messenger that regulates diverse cellular functions. It has been found that CD4⁺CD25⁺ regulatory T (T_{REG}) cells exert their suppressor function by transferring cAMP to responder T cells. Here, we show that miR-142-3p regulates the production of cAMP by targeting adenylyl cyclase (AC) 9 messenger RNA in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells. miR-142-3p limits the level of cAMP in CD4⁺CD25⁻ T cells by inhibiting AC9 production, whereas forkhead box P3 (FOXP3) downregulates miR-142-3p to keep the AC9/cAMP pathway active in CD4⁺CD25⁺ T_{REG} cells. These findings reveal a new molecular mechanism through which CD4⁺CD25⁺ T_{REG} cells contain a high level of cAMP for their suppressor function, and also suggest that the microRNA controlling AC expression might restrict the final level of cAMP in various types of cells.

Keywords: adenylyl cyclase 9; cAMP; FOXP3; miR-142-3p; T cells

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INTRODUCTION

As a ubiquitous second messenger, cyclic AMP (cAMP) can be generated by intracellular adenylyl cyclases (ACs) or delivered from donor cells to recipient cells through the gap junction channels that selectively transfer cAMP. The intercellular delivery of cAMP is not only crucial for regulating the function and

responses of the recipient cells (Vander Molen *et al*, 1996; Nathanson *et al*, 1999; Webb *et al*, 2002; Bopp *et al*, 2007), but also indicates that the self-generated cAMP in recipient cells does not meet the requirements for the rapid regulation of function or a rapid response to extracellular signals. Recent studies have revealed that the structure and function of channels for cAMP delivery are important for this type of intercellular communication (Bedner *et al*, 2006; Hernandez *et al*, 2007; Kanaporis *et al*, 2008). However, how the donor and recipient cells maintain various levels of cAMP remains unclear. More generally, the mechanisms involved in determining the upper level of intracellular cAMP in various types of cells are not fully understood.

In immune systems, a low level of intracellular cAMP is needed for the activation of conventional CD4⁺ T cells, whereas naturally occurring regulatory T (T_{REG}) cells contain high levels of cAMP and exert their suppressor function by transferring cAMP into conventional T cells through gap junctions (Bopp *et al*, 2007). It is known that phosphodiesterases, the enzymes that accelerate the turnover of cAMP, are upregulated in activated helper T cells (Li *et al*, 1999; Glavas *et al*, 2001) and downregulated in T_{REG} cells (Marson *et al*, 2007; Zheng *et al*, 2007). However, the ability of phosphodiesterases in activated T cells seems limited, as they are unable to digest further the exogenous cAMP from T_{REG} cells. Therefore, we reasoned that ACs might have a dominant effect on the differential production of cAMP in T-cell subsets. Here, we show that microRNA miR-142-3p controls the differential activities of AC9 by targeting AC9 messenger RNA (mRNA), and that the differential expression of miR-142-3p determines the production of cAMP in conventional CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells, which, through the delivery of cAMP, is the molecular basis for intercellular communication between these two T-cell subsets.

RESULTS AND DISCUSSION

Expression of AC9 and miR-142-3p in T cell subsets

To explore the molecular basis of the various levels of cAMP in CD4⁺CD25⁻ conventional T cells and CD4⁺CD25⁺ T_{REG} cells,

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Fig 1 | Expression of adenylyl cyclases and miR-142-3p in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ regulatory T cells. (A) The expression of AC9 in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells. The total RNA and the cell lysate ($n=5$ per group) were prepared from the sorted CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells. AC9 messenger RNA was analysed by using real-time PCR (left), and AC9 protein was quantitated by densitometric analysis after Western blot (right). (B,C) The expression of miR-142-3p in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells. The expression of miR-142-3p was detected by (B) RT-PCR and (C) real-time RT-PCR. The miR-142-3p level in the CD4⁺CD25⁺ T_{REG} cells was designated as 1 (C). AC9, adenylyl cyclase 9; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_{REG}, regulatory T cells.

we investigated the expression of ACs at the mRNA level. The results show that AC9 is differentially expressed in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells (supplementary Fig 1 online). The expression of AC9 was low in CD4⁺CD25⁻ T cells but high in CD4⁺CD25⁺ T_{REG} cells. Significantly, the AC9 mRNA level and protein level in CD4⁺CD25⁺ T_{REG} cells were about one-fold and 50-fold, respectively, higher than those in CD4⁺CD25⁻ T cells (Fig 1A), suggesting that different regulatory machineries exist at the translation level and control the difference of AC9 expression in these two T-cell subsets.

MicroRNAs, the endogenous non-coding small RNAs, are well known as regulators of gene expression at the translational level (Ambros, 2004; Brennecke *et al*, 2005) and are involved in many cellular processes, especially development and differentiation (Carrington & Ambros, 2003; Alvarez-Garcia & Miska, 2005; Carthew, 2006). Thus, we compared microRNA profiles in CD4⁺CD25⁻ T cells and natural T_{REG} cells (see supplementary Fig 2 online for the differential microRNA profiles). We then predicted the candidate target genes of the top five differentially

expressed microRNAs in each subset (supplementary Fig 2 online) by the combinatorial utilization of three algorithms, including TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.bio.nyu.edu/>) and Sanger microRNA target (<http://microrna.sanger.ac.uk/>). On the basis of the information obtained from the microarrays and the prediction of target genes, we focused our attention on miR-142-3p. miR-142-3p is the most striking gene to be differentially expressed in CD4⁺CD25⁻ and CD4⁺CD25⁺ cells, and has been found to be related to cell development and differentiation (Lagos-Quintana *et al*, 2002; Houbaviy *et al*, 2003). Furthermore, *Adcy9*, the gene encoding AC9, is one of its top seven candidate target genes (*Adcy9*, *Ctla*, *Rab2*, *Tf*, *Cfl2*, *Cpeb2*, and *Atf7ip*). To validate the differential expression of miR-142-3p, a 51-nucleotide primer with stem-loop structure (supplementary Fig 3 online) and specific primers for reverse transcription PCR (RT-PCR) were used. The result showed the marked differential expression of miR-142-3p in two subpopulations (Fig 1B). Consistently, the results from quantitative RT-PCR showed that the expression of miR-142-3p in CD4⁺CD25⁺ T_{REG} cells was 128-fold lower than that in CD4⁺CD25⁻ T cells (Fig 1C).

miR-142-3p regulates production of AC9 and cAMP

By using a reporter vector system, we confirmed that miR-142-3p targets the 3'-UTR (untranslated region) of AC9 mRNA (Fig 2A). Furthermore, transfection with the miR-142-3p inhibitor increased the levels of AC9 protein and cAMP in CD4⁺CD25⁻ T cells, and transfection with miR-142-3p decreased the levels of AC9 protein and cAMP in CD4⁺CD25⁺ T_{REG} cells (Fig 2B,C). In both cases the mRNA levels of AC9 were not significantly influenced (Fig 2B), indicating that miR-142-3p regulates AC9 expression at the translational level and the production of cAMP in these two T-cell subsets. In line with this, transfection with AC9 small interfering RNA (siRNA) also significantly decreased AC9 expression and cAMP level in CD4⁺CD25⁺ T_{REG} cells (supplementary Fig 4 online). These data indicate that the different production of cAMP in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ T_{REG} cells is controlled by differentially expressed miR-142-3p.

Different effects of miR-142-3p on T cell subsets

High and low levels of intracellular cAMP are required for the suppressor function of T_{REG} cells and the activation of conventional CD4⁺ T cells, respectively. Thus, the expression level of miR-142-3p might be related to the function of these two T-cell subsets. To verify this, we transfected CD4⁺CD25⁺ T_{REG} cells with miR-142-3p and analysed their suppressor function. The results show that miR-142-3p markedly impaired the inhibitory effect of T_{REG} cells on the proliferative response and cytokine production of CD4⁺CD25⁻ T cells (Fig 3A, supplementary Fig 5 online). Consistently, the suppressor function of CD4⁺CD25⁺ T_{REG} cells was also inhibited by AC9 siRNA (supplementary Fig 6 online). These data indicate that the downregulation of miR-142-3p confers a suppressor function on T_{REG} cells by increasing the levels of AC9 and cAMP.

In line with the increase of cAMP in CD4⁺CD25⁻ T cells by blockade of miR-142-3p with inhibitor, transfection with the miR-142-3p inhibitor significantly decreased the proliferation of CD4⁺CD25⁻ T cells in response to stimulation with anti-CD3 and APCs (antigen-presenting cells; Fig 3B). Consistently, the production of interleukin (IL)-2 and interferon (IFN)- γ was decreased by the blockade of miR-142-3p in CD4⁺CD25⁻

Fig 2 | miR-142-3p regulates AC9 expression and cAMP production in CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ regulatory T cells. (A) miR-142-3p targets 3'-UTR of AC9 messenger RNA (mRNA). miR-142-3p inhibited the expression of the luciferase gene containing AC9 3'-UTR but not mutated AC9 3'-UTR; **P* < 0.05, compared with AC9 3'-UTR group. (B) miR-142-3p regulates AC9 at the protein level. miR-142-3p inhibitor-transfected CD4⁺CD25⁻ T cells and miR-142-3p-transfected CD4⁺CD25⁺ T cells were used for mRNA analysis 48 h after transfection and for protein analysis 72 h after transfection. AC9 mRNA was detected by using real-time PCR (left). The relative level of AC9 protein was detected by Western blot (middle) and densitometric analysis (right). **P* < 0.05, compared with CD4⁺CD25⁻ T-cell control group (*n* = 5 per group); #*P* < 0.05, compared with CD4⁺CD25⁺ T-cell control group (*n* = 5 per group). (C) miR-142-3p regulates the level of intracellular cAMP. cAMP was prepared from miR-142-3p inhibitor-transfected CD4⁺CD25⁻ T cells or miR-142-3p-transfected CD4⁺CD25⁺ T_{REG} cells 48 h after transfection, and measured using a cAMP-specific ELISA kit; **P* < 0.05, compared with control groups. The data in this figure are the representatives of two independent experiments (*n* = 6 per group in each experiment). AC9, adenyl cyclase 9; cAMP, cyclic AMP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_{REG}, regulatory T cells; UTR, untranslated region.

T cells (Fig 3B). We then co-cultured the inhibitor-transfected cells with naive T cells in the presence of anti-CD3 and APCs. The results show that the activated naive T cells proliferated as normally as the control group (Fig 3C). Thus, the blockade of miR-142-3p effectively suppresses the activation and proliferation of CD4⁺CD25⁻ T cells, but cannot induce their differentiation into regulatory T cells.

FOXP3 regulates cAMP via the miR-142-3p pathway

Forkhead box P3 (FOXP3) is a master regulator of T_{REG} cell development (Hori *et al*, 2003; O'Garra & Vieira, 2004; Ziegler, 2006). The forced expression of FOXP3 converts CD4⁺CD25⁻ T cells into T_{REG} cells (Hori *et al*, 2003). Given that the downregulation of miR-142-3p is required for T_{REG} cell function but does not convert CD4⁺CD25⁻ T cells into T_{REG} cells, we reasoned that the sequential regulatory effects of FOXP3, miR-142-3p and AC9 are probably the mechanisms underlying the regulation of cAMP production in T cells. To address this, we first investigated the relationship of FOXP3 and miR-142-3p. The results show that the expression of FOXP3 in CD4⁺CD25⁺ T_{REG} cells was not altered by miR-142-3p transfection (supplementary Fig 7 online). Similarly, transfection of the miR-142-3p inhibitor into CD4⁺CD25⁻ T cells did not induce the expression of FOXP3

(supplementary Fig 7 online). By contrast, the transfection of CD4⁺CD25⁺ T_{REG} cells with FOXP3 siRNA resulted in not only the downregulation of FOXP3 at both the mRNA and protein levels but also the increase of miR-142-3p (Fig 4A, supplementary Fig 8 online). Furthermore, the expression of FOXP3 in CD4⁺CD25⁻ T cells by the transfection of FOXP3-expressing vector resulted in the decrease of miR-142-3p (Fig 4B). These data indicate that miR-142-3p does not influence the expression of FOXP3, but that FOXP3 regulates, directly or indirectly, the expression of miR-142-3p. Recently, Ono *et al* (2007) and Li *et al* (2007) showed that FOXP3 mediates transcriptional repression by interaction with AML1 (acute myeloid leukaemia 1)/RUNX1 (Runt-related transcription factor 1), histone acetyltransferase and class II histone deacetylases. FOXP3 probably inhibits certain transcription factors to downregulate the expression of miR-142-3p.

In line with the effect of FOXP3 on miR-142-3p expression, the forced expression of FOXP3 increased the AC9 expression and cAMP level in CD4⁺CD25⁻ T cells, and the downregulation of FOXP3 decreased the AC9 expression and cAMP level in CD4⁺CD25⁺ T_{REG} cells (Fig 4C,D). Consistently, AC9 expression was mainly changed at the translational level (Fig 4C). Therefore, the high concentration of cAMP in CD4⁺CD25⁺ T_{REG} cells is actually maintained by FOXP3, the master molecule that is

Fig 3 | miR-142-3p has different effects on CD4⁺CD25⁺ regulatory T cells and CD4⁺CD25⁻ T cells. (A) miR-142-3p inhibits the suppressor function of T_{REG} cells. CD4⁺CD25⁻ T cells (responder cells) were cultured alone, or co-cultured with T_{REG} cells, control oligo- or miR-142-3p- transfected T_{REG} cells in the presence of anti-CD3 and APCs. The proliferation (left) was determined by ³H-thymidine incorporation, and cytokines in the supernatants (right) were determined by ELISA. (B) Blockade of miR-142-3p impairs the activation of CD4⁺CD25⁻ T cells. Control CD4⁺CD25⁻ T cells, inhibitor control oligo- or miR-142-3p inhibitor-transfected CD4⁺CD25⁻ T cells were stimulated with anti-CD3 and irradiated APCs. The proliferation of cells (left) and the cytokines in the supernatants (right) were determined as above. (C) The blockade of miR-142-3p does not confer a suppressor phenotype on CD4⁺CD25⁻ T cells. CFSE-labelled native CD4⁺CD25⁻ T cells (responder) were cultured alone (group 'none'), or co-cultured with T_{REG} cells, miR-142-3p inhibitor- or inhibitor control oligo-transfected CD4⁺CD25⁻ T cells at a ratio of 1:1, and stimulated with anti-CD3 and irradiated APCs. The proliferation of responder cells was analysed by flow cytometry. The results are shown as the averages of proliferation index (left) and the representative dot plot of flow cytometric analysis (right). The data in this figure are the representatives of two independent experiments (*n* = 6 per group in each experiment); **P* < 0.05 compared with T_{REG} or controls. APC, antigen-presenting cell; ELISA, enzyme linked immunosorbent assay; IL-2, interleukin 2; IFN-γ, interferon-γ; T_{REG}, regulatory T cells.

required throughout the life of a T_{REG} cell (Lopes *et al*, 2007). Recent studies have shown that FOXP3 downregulates phosphodiesterase PDE3b, the enzyme that accelerates cAMP turnover, in T_{REG} cells (Marson *et al*, 2007; Zheng *et al*, 2007). This finding and ours indicate that FOXP3 efficiently keeps the AC9/cAMP pathway active by downregulating the expression of PDE3b and miR-142-3p. However, AC9/cAMP is not the only pathway regulated by the downregulation of miR-142-3p, as *Adcy9* is not the only target gene of miR-142-3p. The downregulation of miR-142-3p might be one of the means by which FOXP3 upregulates the expression of T_{REG}-associated genes in the cells. A closer examination of the candidate target genes of miR-142-3p might be useful for a deeper understanding of the development and function of CD4⁺CD25⁺ T_{REG} cells.

Conclusion

Our data show that a single microRNA, miR-142-3p, by virtue of its differential expression in conventional CD4⁺ T cells and CD4⁺CD25⁺ T_{REG} cells, can control the functions of both effector and suppressor cells. In addition, our data might reveal a general biological pathway for cAMP regulation. Besides the classic pathway of G protein/ACs, microRNAs might control the final level of intracellular cAMP, especially the upper limit, in various cells. Thus, this study provides a new insight into the regulation of cellular functions.

METHODS

Cell sorting. The splenocytes from naive BALB/c mice were labelled with fluorescein isothiocyanate-conjugated CD3, phycoerythrin-conjugated CD4 and APC-conjugated CD25 antibodies (eBioscience, San Diego, CA, USA). The CD3⁺CD4⁺CD25⁻ and CD3⁺CD4⁺CD25⁺ cells were sorted with stringent gating conditions (BD FACS Aria™ cell sorter); the sorted cells used for the experiments were 97–98% in purity, which was checked by using flow cytometry.

Analysis of miR-142-3p by RT-PCR and quantitative RT-PCR. A primer (supplementary Fig 3 online), designed using the RNA mfold version 2.3 server (Zuker, 2003), was used for reverse transcription after the identification of its specificity (supplementary Fig 9 online). 100 ng of enriched microRNA was used for the cDNA synthesis. A 67-bp cDNA product was amplified by PCR with the primers 5'-CTCCTGTAGTGTTCCTAC-3' (sense) and 5'-GACTGTTCTCTCTTCCTC-3' (antisense). For real-time PCR, the above primers and the Taqman probe [6-FAM]TTGCGACTA CACACACACACACA[BHQ1a-6FAM] were mixed with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a Stratagene quantitative RT-PCR thermal cycler.

Identification of the specificity of miR-142-3p to AC9 mRNA. A 235 bp fragment of 3'-UTR of AC9 mRNA containing the target

Fig 4 | FOXP3 regulates cAMP production by regulating miR-142-3p and AC9. (A,B) FOXP3 negatively regulates miR-142-3p. FOXP3 messenger RNA (mRNA) and miR-142-3p in CD4⁺CD25⁺ T_{REG} cells transfected with (A) FOXP3 small interfering RNA (siRNA) or (B) in CD4⁺CD25⁻ T cells transfected with FOXP3-expressing vector, were detected by real-time RT-PCR. The miR-142-3p level in the control group was designated as 1. (C) FOXP3 increases AC9 expression. pFOXP3-transfected CD4⁺CD25⁻ T cells and FOXP3 siRNA-transfected CD4⁺CD25⁺ T cells were used for mRNA analysis 48 h after transfection, and for protein analysis 72 h after transfection. AC9 mRNA was determined by real-time RT-PCR (upper), and AC9 protein was determined by Western blot (lower). (D) FOXP3 regulates the level of cytosolic cAMP. cAMP was prepared from FOXP3-expressing vector-transfected CD4⁺CD25⁻ T cells (left) or FOXP3 siRNA-transfected CD4⁺CD25⁺ T_{REG} cells (right) 48 h after transfection, and measured using the cAMP-specific ELISA kit; **P* < 0.05, compared with control. The data in this figure are the representatives of two independent experiments (*n* = 6 per group in each experiment). AC9, adenyl cyclase 9; cAMP, cyclic AMP; ELISA, enzyme linked immunosorbent assay; FOXP3, forkhead box P3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription PCR; T_{REG}, regulatory T cells.

The fragment was designated as AC9 3'-UTR and inserted into the pMIR-REPORTTM luciferase reporter vector (*SacI* and *HindIII* restriction enzyme sites; Ambion, Austin, TX, USA). Another expressing vector was also constructed by the insertion of a mutated AC9 3'-UTR in which the target sequence of miR-142-3p was mutated into ACAATAC using the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene). The recombinant reporter vectors with normal and mutated AC9 3'-UTR were then cotransfected with miR-142-3p into CHO cells, respectively, using the TransMessengerTM Transfection Reagent (Qiagen). The luciferase assay was performed according to the manufacturer's instructions.

Assay for cAMP. T cells were washed three times with HBSS buffer at 4 °C and resuspended in lysis buffer (10⁷/ml). After two freeze/thaw cycles, cAMP in the supernatant (100 μl) was assayed using the cAMP ELISA kit (R&D Systems) according to the manufacturer's protocol. The results were expressed as the concentration of cAMP in the supernatants.

Transient transfection. Stability-enhanced miR-142-3p (Dharmacon, Lafayette, CO, USA), miR-142-3p inhibitor (Ambion), FOXP3 siRNA (Invitrogen, Carlsbad, CA, USA), AC9 siRNA (Invitrogen) and the corresponding control oligonucleotides were purchased. FOXP3 cDNA was amplified from CD4⁺CD25⁺ T_{REG} cells and inserted into the pcDNA3.1 vector. For transient transfection, 200 pmol of synthesized oligonucleotide or 2 μg of plasmid was mixed with 100 μl of T-cell Nucleofector solution (Amaxa, Gaithersburg, MD, USA) and transfected into 3 × 10⁶ cells by electroporation using a Nucleofector II instrument (Yin *et al*, 2006). The transfection efficiency was around 70%, evaluated by flow cytometric analysis after a fluorescein isothiocyanate-labelled luciferase siRNA control (Dharmacon). After transfection, the cells were allowed to recover by incubating for 4 h at 37 °C, and then used for the following assays.

Cytokine assay by ELISA. The levels of IL-2 and IFN-γ in the supernatants were assessed using murine IL-2 and IFN-γ ELISA

sequence (ACACTAC) of miR-142-3p was amplified by RT-PCR (sense primer 5'-CTTTGAGCTCCUGUUGGUCUCCAAC-3', antisense primer 5'-CCTCAAGCTTAGCTCCTCCAAGTGATG-3').

kits, respectively (eBioscience) according to the manufacturer's protocol.

Proliferation assay. miR-142-3p inhibitor-transfected CD4⁺CD25⁻ T cells and control cells (2×10^4) were cultured in the presence of 5×10^4 APCs (splenocytes, 25 Gy irradiated) and anti-CD3 (1 µg/ml). [³H]-thymidine (1 µCi/well, Amersham Biosciences) was added during the last 10 h of a 72-h culture, and then the incorporation of [³H]-thymidine was measured to determine T-cell proliferation. The background c.p.m. (lower than 523 in all tests) from corresponding cells without anti-CD3 stimulation was subtracted from the c.p.m. value of each sample.

In vitro assessment of regulatory capacity. Naive CD4⁺CD25⁻ T cells were used as responder cells. A total of 2×10^4 responder cells were co-cultured with miR-142-3p- or control oligonucleotide-transfected CD4⁺CD25⁺ T_{REG} cells for 3 days in the presence of 5×10^4 irradiated APCs (splenocytes) and anti-CD3 (1 µg/ml). The proliferation of T cells and cytokine production was determined as above; alternatively, the responder cells were labelled with CFSE, and the proliferation of CFSE-labelled responder cells was analysed by using flow cytometry.

For the assessment of the regulatory capacity of CD4⁺CD25⁻ T cells transfected with miR-142-3p inhibitor, 2×10^4 of responder cells were labelled with CFSE, and then stimulated for 3 days as above, in the absence or presence of the transfected cells or control T_{REG} cells. The proliferation of CFSE-labelled responder cells was analysed by flow cytometry. The proliferation index was calculated with the software *ModFit LT 3.0* based on the data from flow cytometric analysis.

Statistics. Results were expressed as mean value $\pm$ s.d. and interpreted by ANOVA-repeated measure test. Differences were considered to be statistically significant when $P < 0.05$.

Other methods. The methods for real-time RT-PCR and Western blot are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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