Amino Acids (2012) 42:2513–2519
DOI 10.1007/s00726-011-1050-8

SHORT COMMUNICATION

Epigallocatechin-3-*O*-gallate inhibits the production of thymic stromal lymphopoietin by the blockade of caspase-1/NF-κB pathway in mast cells

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Received: 25 April 2011 / Accepted: 23 July 2011 / Published online: 11 August 2011

Abstract The cytokine thymic stromal lymphopoietin (TSLP) has been implicated in the development and progression of allergic diseases such as atopic dermatitis, asthma, and chronic obstructive pulmonary disease. However, it has not yet been clarified the effect of epigallocatechin-3-*O*-gallate (EGCG) on the production of TSLP. Thus, we investigated how EGCG inhibits the production of TSLP in the human mast cell line (HMC-1) cells. Enzyme-linked immunosorbent assay, reverse transcription-polymerase chain reaction, luciferase assay, and Western blot analysis were used to investigate the effects of EGCG. EGCG inhibited the production and mRNA expression of TSLP in HMC-1 cells. EGCG also inhibited the nuclear factor-κB luciferase activity induced by phorbol myristate acetate plus A23187. Furthermore, EGCG inhibited the activation of caspase-1 in HMC-1 cells. These results provide evidence that EGCG can help us to treat inflammatory and atopic diseases through the inhibition of TSLP.

Keywords Thymic stromal lymphopoietin · Epigallocatechin-3-*O*-gallate · Nuclear factor-κB · Caspase-1

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Abbreviations

AD	Atopic dermatitis
EGCG	Epigallocatechin-3-*O*-gallate
NF-κB	Nuclear factor-κB
PMA	Phorbol myristate acetate
TSLP	Thymic stromal lymphopoietin

Introduction

Atopic dermatitis (AD) is one of the most common skin diseases, and its incidence is increasing in industrialized countries. AD, characterized by pruritic and eczematoid skin lesions, affects up to 20% of children and 1–3% of adults in most countries of the world (Zhang et al. 2009; Plötz and Ring 2010). The most common form of AD, accounting for 70–80% of cases, is allergic AD with elevated concentrations of total and allergen-specific immunoglobulin (Ig)E in serum and skin. The remaining 20–30% patients have nonallergic AD, with normal total IgE levels and negative serum allergen-specific IgE, reflecting multiple different pathogenic mechanisms and indicating that AD represents a collection of heterogeneous groups (Wu et al. 2010). As a result of the increasing prevalence of atopic disorders in developed countries, the burden of healthcare cost increases, and the quality of life of affected patients is significantly lowered by chronic eczematous lesions, pruritus, sleep loss, dietary restrictions and psychosocial affections (Plötz and Ring 2010).

Thymic stromal lymphopoietin (TSLP) was originally identified as a growth factor in culture supernatants of a thymic stromal cell line to support the development of murine B cells (Friend et al. 1994). In humans, TSLP is an interleukin (IL)-7-like cytokine molecule that was first

cloned (Quentmeier et al. 2001). TSLP plays a pivotal role in allergic diseases such as AD, asthma, and chronic obstructive pulmonary disease (Shan et al. 2010). Initially, TSLP was found to potently enhance the maturation of $CD11c^+$ dendritic cells, and TSLP-primed and activated dendritic cells promoted differentiation of naive $CD4^+$ T cells into proinflammatory T_H2 cells (Liu 2006). High expression of TSLP is a feature of keratinocytes in AD skin lesions, and TSLP-priming of dendritic cells in situ may serve to induce or enhance T_H2 responses within the skin, as well as systemically. Consistent with this viewpoint, TSLP was originally reported to exert its T_H2-promoting properties through a dendritic cell-mediated pathway in human beings that involved induction of OX40 ligand on dendritic cells (Reefer et al. 2010). In atopic diseases such as asthma, AD, and allergic rhinitis, not only epithelial cells, dendritic cells, eosinophils, and T cells but also mast cells are important. A number of studies reported that mast cells are activated and infiltrated in the skin lesion of AD animal model, suggesting the contribution of mast cells in AD (Kang et al. 2008; Oiwa et al. 2008; Morioka et al. 2009; Schneider et al. 2009; Zheng et al. 2009; Dumortier et al. 2010).

Caspase-1 is a member of the caspase family (Gordon et al. 1990). Quite unlike the role that most caspases have in apoptosis, caspase-1 mainly serves to cleave IL-1β and IL-18 from their inactive precursors to their active forms (Boost et al. 2007; Lamkanfi et al. 2007). In addition to the well-established role of caspase 1 in the maturation of IL-1β and IL-18, caspase 1 is also capable of activating the nuclear factor (NF)-κB (Lamkanfi et al. 2004). Activated NF-κB mediates the induction of TSLP gene expression in airway epithelial cells (Lee and Ziegler 2007).

Catechins are the major constituents of the polyphenols in green tea, and the most abundant catechin in green tea is epigallocatechin-3-*O*-gallate (EGCG, Fig. 1) (Zheng et al. 2011). EGCG has various effects such as anti-arthritic, anti-inflammatory, and anti-oxidant effects (Ahemd et al. 2008; Wheeler et al. 2004a; Rah et al. 2005). However, it has not yet been clarified the effect of EGCG on the production of TSLP. Thus, we investigated how EGCG inhibits the production of TSLP in mast cells.

Materials and methods

Reagents

Phorbol myristate acetate (PMA), A23187, and EGCG were purchased from Sigma Chemical Co. (St. Louis, MO, USA). We purchased IMDM from Gibco BRL (Grand Island, NY, USA), TSLP antibodies from R&D Systems (Minneapolis, MN, USA), TMB substrate from Pharmingen (Sandiego, CA, USA), caspase-1 antibody from Santa Cruz Biotechnology (Santacruz, CA, USA), GAPDH antibody from Assay Designs Inc. (Ann arbor, MI, USA).

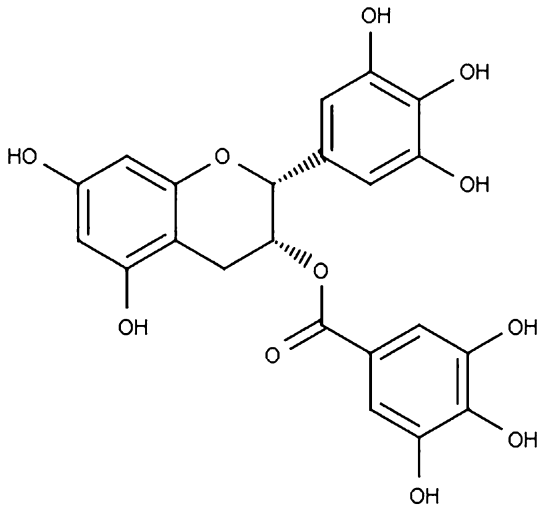

Fig. 1 Structure of EGCG

Preparation of EGCG

EGCG was prepared by dissolving with distilled water. Dilutions were made in phosphate buffered saline (PBS) and filtered through 0.22-μm syringe filter.

Cell culture

Human mast cell line, HMC-1 cells were grown in IMDM and supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum at 37°C in 5% CO_2 with 95% humidity.

Cytokine assay

We used enzyme-linked immunosorbent assay (ELISA) method to assay the culture supernatant for TSLP (Moon and Kim 2011). Sandwich ELISA for TSLP was carried out in duplicate in 96 well ELISA plate. First, we coated the plate with 100 μl aliquots of mouse anti-human TSLP monoclonal antibody at 1.0 μg/ml in PBS at pH 7.4 and incubated the plate overnight at 4°C. The plate was washed in PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for 1 h. After additional washes, the culture supernatant and TSLP standards were added and incubated at 37°C for 2 h. After 2 h incubation at 37°C, the wells were washed and then each of 0.2 μg/ml of biotinylated anti-human TSLP was added and again incubated at 37°C for 2 h. After washing the wells, streptavidin–peroxidase was added and plate was incubated for 20 min at 37°C. Wells

were again washed and TMB substrate (Pharmingen) was added. Color development was measured at 450 nm using an automated microplate ELISA reader. A standard curve was run on plate using recombinant human TSLP in serial dilutions.

Reverse transcription-polymerase chain reaction analysis

We used the method of Moon and Kim (2011) using an easy-BLUE™ RNA extraction kit (iNtRON Biotech, Republic of Korea) and isolated the total RNA from HMC-1 cells in accordance with the manufacturer's specification. The concentration of total RNA in the final elutes was determined by spectrophotometer. Total RNA (1 μg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with following primer for human TSLP (5′TAT GAG TGG GAC CAA AAG TAC CG3′; 5′GGG ATT GAA GGT TAG GCT CTG G3′). GAPDH (5′CAA AAG GGT CAT CAT CTC TG3′; 5′CCT GCT TCA CCA CCT TCT TG3′) was used to verify if equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 62°C for TSLP and GAPDH. Amplified fragment sizes for TSLP and GAPDH were 97 and 446 bp, respectively. Products were electrophoresed on 1.5% agarose gel and visualized by staining with ethidium bromide.

Transient transfection and luciferase assay

For the transfection, we seeded HMC-1 cells (1×10^7) in a 100-mm culture dish. We then used Lipofectamine™2000 purchased from Invitrogen (Carlsbad, CA, USA) to transiently transfect pNF-κB luciferase (LUC) and pSV40-LUC reporter gene constructs into HMC-1 cells. To measure the luciferase activity, we used a luminometer 1420 luminescence counter purchased from Perkin Elmer (Waltham, MA, USA) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of *firefly* luciferase activity to *renilla* luciferase activity.

Preparation of nuclear extracts

Nuclear extracts were prepared as described earlier (Schoonbroodt et al. 1997). Briefly, after activating the cells for the times indicated, we washed 5×10^6 cells in ice-cold PBS and centrifuged them at 15,000*g* for 1 min. We then resuspended them in 40 μl of a cold hypotonic buffer (10 mM Hepes/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, and 0.5 mM PMSF, pH 7.9). Subsequently, we allowed the cells to swell on ice for 15 min and lysed them gently with 2.5 μl of 10% Nonide P (NP)-40 and centrifuged them at 15,000*g* for 3 min at 4°C. The supernatant was discarded and the pellets were gently resuspended in 40 μl of cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 0.5 mM PMSF, pH 7.9) and then left on ice for 20 min. After conducting the centrifugation (15,000*g* for 15 min at 4°C), we froze the aliquots of supernatant containing the nuclear proteins in liquid nitrogen and stored them at −70°C until ready for analysis. Finally, we used the Bicinchoninic acid protein assay (Sigma, St. Louis, MO, USA) to measure the protein concentrations.

Western blot analysis

The cell lysates were prepared in a sample buffer containing sodium dodecyl sulfate (SDS). The samples were heated at 95°C for 5 min and briefly cooled on ice. Following the centrifugation at 15,000*g* for 5 min, the proteins in the cell lysates were then separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk in PBS–tween-20 for 1 h at room temperature and then incubated with primary and secondary antibodies. Finally, the protein bands were visualized by an enhanced chemiluminescence assay purchased from Amersham Co. (Newark, NJ, USA) following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). All results are expressed as the mean ± SEM. The statistical evaluation of the results was performed by an independent *t* test and an ANOVA with a Tukey post hoc test. The results were significant with a value of $P < 0.05$.

Results

Effect of EGCG on the production of TSLP in HMC-1 cells

To assess the inhibitory effect of EGCG on the production of TSLP, we stimulated the HMC-1 cells with PMA plus A23187 for 7 h considering the report of Moon and Kim (2011), and then used ELISA to analyze the supernatants for TSLP. The stimulation with PMA plus A23187 increased the TSLP production from HMC-1 cells (Fig. 2a). The level

of TSLP that increased due to PMA plus A23187 was significantly decreased by EGCG (50 μM) (Fig. 2a; $P < 0.05$). Maximal inhibition rate of TSLP production by EGCG (50 μM) was 32.97 ± 4.69%. When EGCG was given as pretreatment at various concentrations ranging from 0.5 to 50 μM, the cytotoxicity by EGCG was not shown (data not shown).

Effect of EGCG on the mRNA expression of TSLP in HMC-1 cells

To determine whether EGCG can modulate PMA plus A23187-induced mRNA expression of TSLP, we pretreated the cells with EGCG for 2 h before the PMA plus A23187 stimulation. We stimulated the cells with PMA plus A23187 for 5 h and then performed the RT-PCR analysis. The mRNA expression of TSLP was up-regulated by PMA plus A23187, but the up-regulated TSLP mRNA expression was decreased by treatment with EGCG (Fig. 2b). The inhibitory effect of 50 μM of EGCG was >0.5 and 5 μM; thus, we evaluated the effect of 50 μM of EGCG on next experiments, i.e. luciferase assay and Western blot analysis.

Effect of EGCG on the activation of NF-κB in HMC-1 cells

Following, we examined whether EGCG could modulate the luciferase expression specifically via NF-κB activation. As shown in Fig. 3a, the PMA plus A23187 stimulation increased the reporter gene activity. However, this increased NF-κB luciferase activity was significantly decreased by EGCG (50 μM, $P < 0.05$). The relative luciferase activity at the dose of 50 μM was 21.43 ± 2.65. The control and spontaneous values were 32.86 ± 3.12 and 1.35 ± 0.12, respectively.

Effect of EGCG on the activation of caspase-1 in HMC-1 cells

Finally, to examine the effect of EGCG on the activation of caspase-1, we performed a Western blot analysis for caspase-1 in cells stimulated by PMA plus A23187. In the control group that was stimulated by PMA plus A23187, the level of active caspase-1 was increased in HMC-1 cells. However, the level of active caspase-1 was decreased by treatment with EGCG (Fig. 3b).

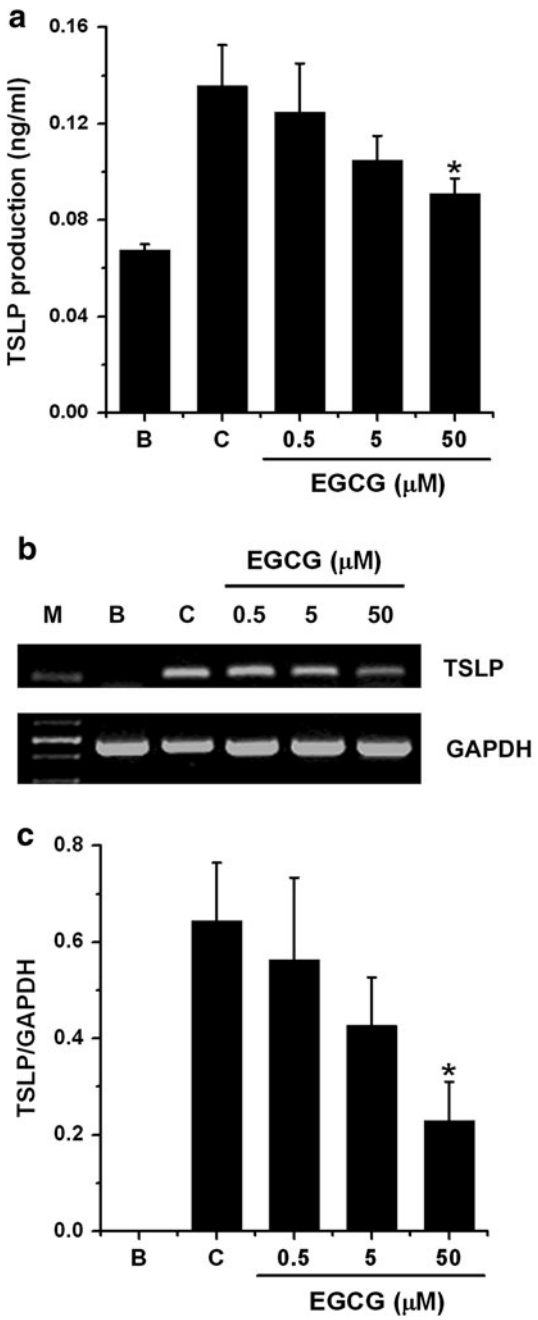

Fig. 2 Effects of EGCG on the production and mRNA expression of TSLP in HMC-1 cells. **a** HMC-1 cells (4×10^5) were treated with various concentrations (0.01–10 μM) of EGCG for 2 h and then stimulated with PMA plus A23187 for 7 h. The levels of TSLP in the supernatant were measured with the ELISA method. **b** HMC-1 cells (1×10^6) were stimulated with PMA plus A23187. The mRNA was measured with the RT-PCR method. **c** The TSLP mRNA expression levels were quantitated by densitometry. *M* marker, *B* unstimulated cells, *C* PMA plus A23187-stimulated cells

Discussion

As negative side effects of steroidal and nonsteroidal anti-inflammatory drugs increase, there is a need to develop new drugs with novel modes of action that do not produce considerable side effects. Natural product-based anti-inflammatory agents with a transcriptional mode of action, good efficacy, and lower risk of side effects offer

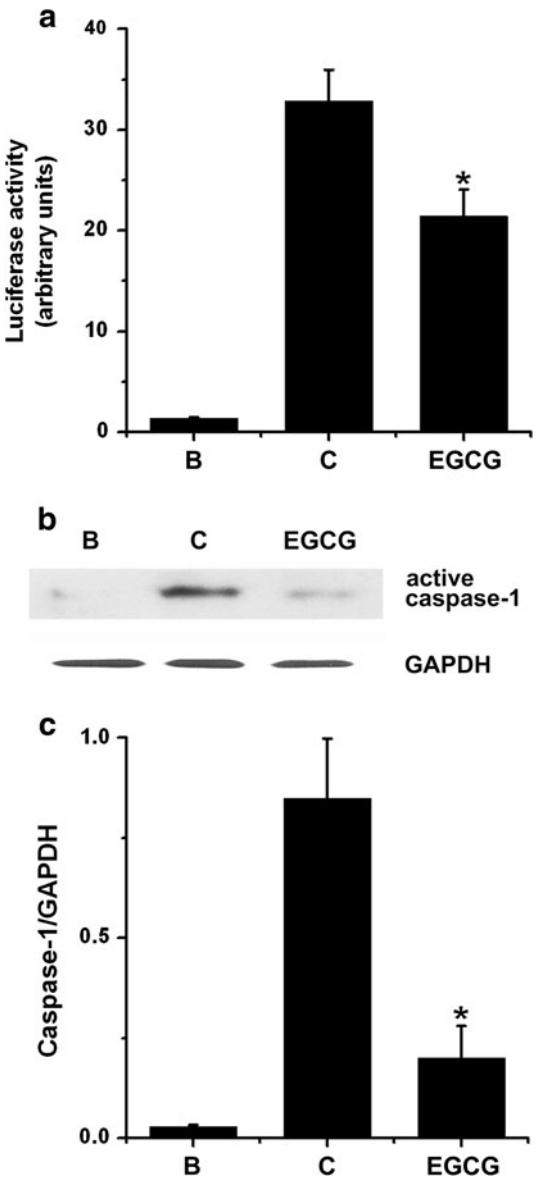

Fig. 3 Effects of EGCG on the activation of NF-κB and caspase-1 in HMC-1 cells. **a** HMC-1 cells (1×10^7) were transiently transfected pNF-κB-LUC and pSV40-LUC into and treated with EGCG (10 μM) for 2 h and then stimulated with PMA plus A23187 for 48 h. The NF-κB activity was assessed with a luciferase assay. **b** HMC-1 cells (5×10^6) were preincubated for 2 h with EGCG (10 μM) and treated with PMA plus A23187 for 1 h for caspase-1 activation. The total proteins were determined by Western blot analysis. **c** Each protein level was quantitated by densitometry. *B* unstimulated cells, *C* PMA plus A23187-stimulated cells, EGCG, 10 μM of EGCG-treated cells. Each datum represents the mean ± SEM of three independent experiments. $*P < 0.05$; significantly different from the control (PMA plus A23187-stimulated cells) value

promising treatment and prevention of inflammation-related conditions. EGCG is the most abundant catechin in green tea (Zheng et al. 2011). We therefore investigated the natural product-based compound EGCG as a natural product-based anti-inflammatory agent.

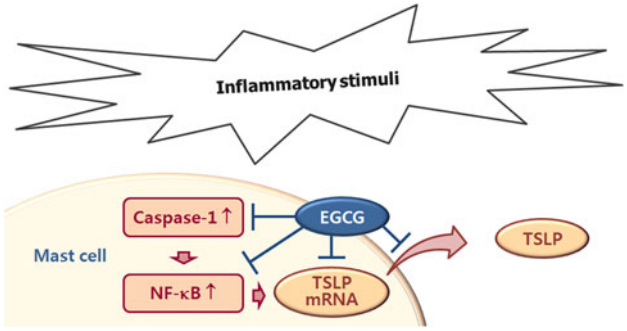

Fig. 4 Schematic diagram of proposed regulation of TSLP by EGCG. Upon receipt of pro-inflammatory stimuli, caspase-1 is activated and then the activated caspase-1 activates NF-κB in mast cells. Finally, the activated NF-κB induces expression of TSLP mRNA, followed by production of TSLP in mast cells. In this study, EGCG inhibited the activation of caspase-1 and NF-κB as well as the mRNA expression and production of TSLP in mast cells

In general, the PKC activator PMA is a substitute for diacylglycerol and A23187 is a widely used ionophore. Our previous study showed that TSLP is produced by PMA plus A23187 stimulation (Moon and Kim 2011). Lesional, but not unaffected, skin from patients with AD expresses high levels of TSLP (Ziegler 2010). In this study, EGCG inhibited the production and mRNA expression of TSLP (Fig. 2a, b). To our knowledge, this is the first study showing an inhibition of TSLP by EGCG in mast cells. Thus, we can assume that EGCG can help us to treat inflammation and AD.

Many studies reported that the expression of human TSLP mRNA was controlled by NF-κB in various cells such as fibroblasts and epithelial cells (Lee and Ziegler 2007; Ozawa et al. 2007; Lee et al. 2008). Indeed, the expression and production of TSLP was controlled by NF-κB in mast cells (Moon and Kim 2011). Several studies have reported that EGCG inhibits the NF-κB activation in trinitrobenzene sulfonic acid-administered mice and the phosphorylation of NF-κB in respiratory epithelial cells (Abboud et al. 2008; Wheeler et al. 2004b). Our result also showed that EGCG inhibited the NF-κB luciferase activity in mast cells (Fig. 3a). From previous reports and our result, we could confirm that NF-κB is a general transcription factor in fibroblasts, epithelial cells, mesangial cells, and mast cells.

Upon receipt of a pro-inflammatory stimulus, caspase-1 is activated (Humke et al. 2000). Our results also showed that caspase-1 was activated by pro-inflammatory stimulus in HMC-1 cells (Fig. 3b). Pretreatment of EGCG inhibited PMA plus A23187-induced caspase-1 activation (Fig. 3b). To our knowledge, this is also the first study showing an inhibition of caspase-1 activation by EGCG in mast cells. Thus, we can speculate that EGCG inhibits the expression and production of TSLP through the blockade of caspase-1/NF-κB signal cascade in mast cells.

In conclusion, we have shown that EGCG can regulate the inflammatory responses induced by PMA plus A23187 in mast cells. EGCG inhibited the expression and production of TSLP through the inhibition of caspase-1/NF-κB pathway. Overall, this study, summarized in Fig. 4, provides evidence that EGCG can help us to treat inflammatory and atopic diseases through the inhibition of TSLP.

Acknowledgments This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0005591) and by a grant from the Kyung Hee University in 2011 (KHU-20110092).

Conflict of interest The authors declare that they have no conflict of interest.

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