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Compound K, a novel ginsenoside metabolite, inhibits adipocyte differentiation in 3T3-L1 cells: Involvement of angiogenesis and MMPs

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ABSTRACT

Compound K, a novel ginsenoside metabolite formed by intestinal bacteria, is shown to inhibit angiogenesis and matrix metalloproteinase (MMP) activities. Since growth and development of adipose tissue are thought to require adipogenesis, angiogenesis, and extracellular matrix remodeling, we investigated whether compound K inhibits adipocyte differentiation and its potential mechanisms. Treatment of 3T3-L1 adipocytes with compound K inhibited lipid accumulation and expression of adipocyte-specific genes (i.e., PPARγ, leptin, aP2, and C/EBPα). Compound K decreased mRNA levels of angiogenic factors (i.e., VEGF-A and FGF-2) and MMPs (i.e., MMP-2 and MMP-9), whereas it increased mRNA levels of angiogenic inhibitors (TSP-1, TIMP-1, and TIMP-2) in 3T3-L1 cells. MMP-2 and MMP-9 activities were also decreased in compound K-treated cells. These results demonstrate that compound K effectively inhibited adipogenesis and that this process may be mediated in part through changes in the expression of genes involved in angiogenesis and MMP system. Thus, by suppressing adipogenesis, compound K likely has therapeutic potential for the treatment of obesity and related disorders.

1. Introduction

Ginseng is widely used in oriental societies as a valuable medicine. There are extensive reports that ginseng has many pharmacological effects on the central nervous system, and the endocrine, immune, and cardiovascular systems [1–3]. Ginseng has also been reported to inhibit tumor growth by modulating angiogenesis, the formation of new blood vessels from pre-existing vessels [4,5]. Ginsenosides, the major active components of ginseng, exhibit potential as potent cancer chemopreventive agents partly via their anti-angiogenic activity [6–8].

Among the ginsenosides, compound K is one of the major metabolites formed by intestinal bacteria after oral administration of ginseng extract in humans and rats [9]. It was reported that compound K inhibits fibroblast growth factor (FGF)-induced angiogenesis in human umbilical vein endothelial cells (HUVEC) and induces apoptosis by down-regulating FGF receptor 3 in myeloma cells [10,11]. It also suppresses phorbol ester-induced expression of matrix metalloproteinase 9 (MMP-9), which is implicated in the process of invasion and angiogenesis of malignant tumors, in human astroglial cells [12].

Similar to neoplastic tissues, angiogenesis occurs in the growing adipose tissues of adults. Most tissues normally do not grow throughout adulthood and the supporting vasculature is quiescent, whereas adipose tissue can grow and regress throughout life. Adipose tissue is highly vascularized, and each adipocyte is nourished by an extensive capillary network [13–15]. It is therefore suggested that growth and differentiation of adipocytes are angiogenesis-dependent and may be blocked by angiogenesis inhibitors.

Several lines of evidence suggest that endogenous and exogenous MMPs also regulate adipogenesis [16–18]. During obesity, MMP expression is modulated in adipose tissue, and MMPs (e.g., MMP-2 and MMP-9) potentially affect adipocyte differentiation [16,19,20]. Based on the well-documented regulation of adipocyte growth and differentiation by angiogenesis and MMPs, we hypothesized that compound K, with its anti-angiogenic and MMP-inhibiting properties, can inhibit adipogenesis in 3T3-L1 adipocytes.

We treated 3T3-L1 adipocytes with compound K. Lipid accumulation and the expression of adipocyte-specific genes were significantly reduced in treated cells compared with controls. The mRNA expression levels of angiogenic factors, MMPs, and their inhibitors were also modulated by compound K in 3T3-L1 cells. These studies suggest that compound K may inhibit adipogenesis by regulating angiogenesis and MMPs, leading to the prevention of obesity and related disorders.

2. Materials and methods

2.1. Induction of preadipocyte differentiation

Mouse 3T3-L1 cells (ATCC, Manassas, VA, USA) were proliferated in Dulbecco's modified Eagle's medium (DMEM) containing

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10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells grown to confluency (day −2) were kept for two more days before agents were added (day 0). Cells were then incubated in a differentiation medium containing 0.5 mM 1-methyl-3-isobutyl-xanthine, 1 μM dexamethasone, and 1 μg/ml insulin (MDI) in DMEM with 10% fetal bovine serum. The cultures were continued for 2 days to induce adipocyte differentiation. Thereafter, cells were cultured in DMEM with 10% FBS for the rest of the differentiation process. Compound K (ChromaDex, Laguna Hills, CA, USA) and TNP-470 (Sigma–Aldrich, St. Louis, MO, USA) were administered on days 0 through 2 only, and the medium was changed every other day. Cells were stained on day 8 with Oil red O and photographed.

2.2. Zymography

MMP activity in 3T3-L1 adipocytes was determined by gelatin zymography. Proteins from 3T3-L1 cells were extracted with 10 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 0.2% NaN_3 (250 mg wet weight tissue per 1 ml buffer) at 4 °C. Cell extracts were mixed with zymography sample buffer (63 mM Tris–HCl, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue, pH 6.8) without heat denaturation. The HT1080 cell culture medium was used for the molecular weight markers for MMP. Electrophoresis was performed at 125 V in 10% SDS–polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were incubated in renaturing buffer containing 0.25% Triton X-100 for 30 min at room temperature and equilibrated in developing buffer (50 mM Tris-base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Brij-35) for 30 min at room temperature. The gels were then incubated in developing buffer overnight at 37 °C. The gels were stained with 0.1% Coomassie Brilliant Blue R-250 and destained with 10% acetic acid in 40% methanol.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA from 3T3-L1 cells was prepared using the Trizol reagent (Gibco-BRL, Grand Island, NY, USA). After 2 μg total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and an antisense primer cDNA was generated. The RNA was denatured for 5 min at 72 °C and then immediately placed on ice for 5 min. Denatured RNA was mixed with MMLV-RT, MMLV-RT buffer, and a deoxyribonucleotide triphosphate (dNTP) mixture, and incubated for 1 h at 42 °C. Synthesized cDNA fragments were amplified by PCR in an MJ Research Thermocycler (Waltham, MA, USA). The cDNA was mixed with PCR primers, *Taq* DNA polymerase (Nanohelix, Daejon, Korea), and a dNTP mixture. The PCR primers and conditions used for gene expression analysis are shown in Table S1. The PCR products were analyzed by electrophoresis in a 1% agarose gel. Relative expression levels are presented as the ratio of target gene cDNA to β-actin cDNA. PCR products were quantified from agarose gels using the GeneGenius (Syngene, Cambridge, UK).

2.4. Statistics

Unless otherwise indicated, all values are expressed as the mean ± standard deviation (SD). All data were analyzed using the

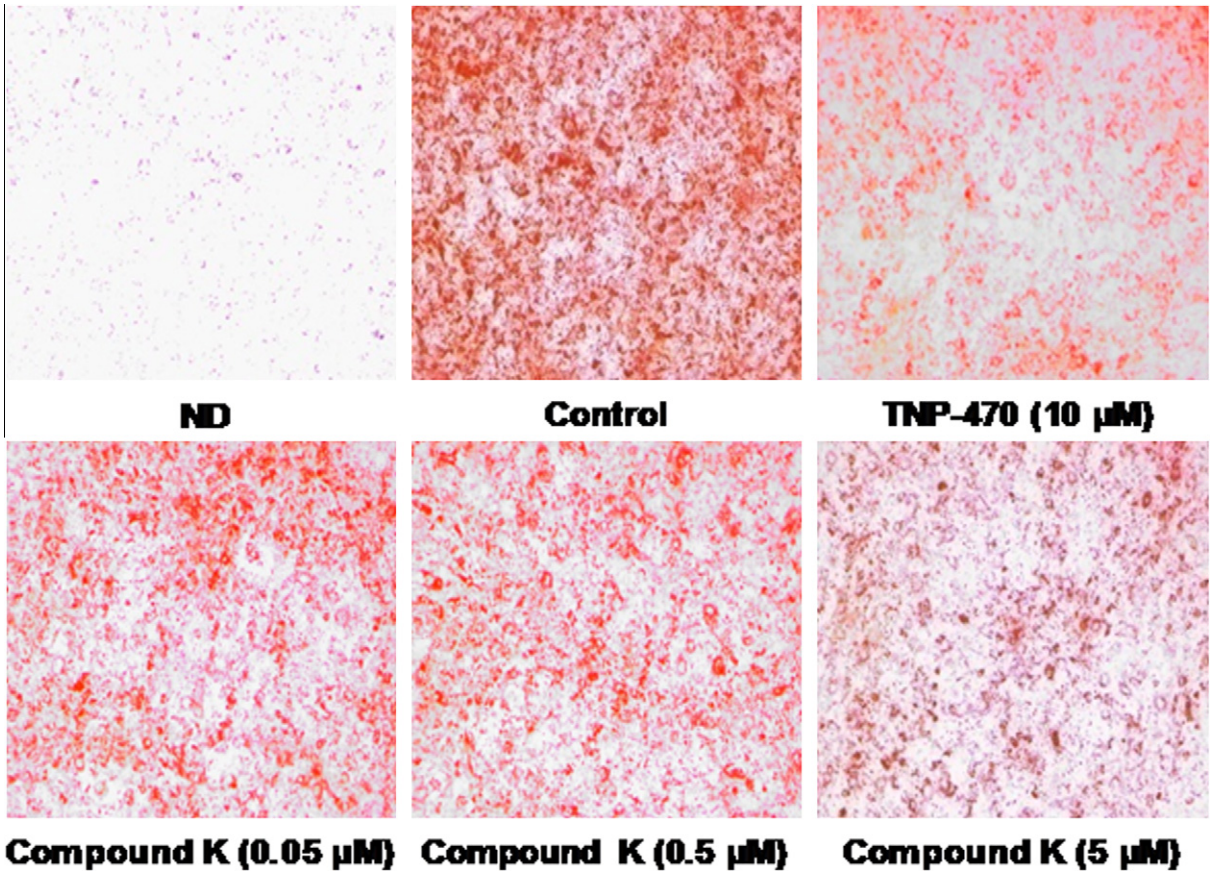

Fig. 1. Effects of compound K on lipid accumulation in 3T3-L1 cells. 3T3-L1 cells were treated with MDI (control), compound K, or TNP-470. At day 8 post-induction, cells were fixed, and neutral lipids were stained with Oil red O. ND, non-differentiated.

unpaired Student's *t*-test for statistically significant differences between groups. Statistical significance was defined as a value of $p < 0.05$.

3. Results

3.1. Inhibition of lipid accumulation by compound K in 3T3-L1 adipocytes

We examined the ability of the ginsenoside metabolite compound K to prevent adipogenesis in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells (control) after treatment with an MDI mixture accumulated intracellular lipid droplets compared with non-differentiated cells (ND), as shown by the increase in Oil red O staining (Fig. 1). However, incubation of differentiated cells with compound K decreased MDI-induced lipid accumulation in a dose-dependent manner, and lipid accumulation was significantly inhibited in the presence of 5 μM compound K. Similarly, the selective angiogenesis inhibitor TNP-470 (10 μM) also reduced triglyceride accumulation. These results suggest that compound K inhibits adipogenesis due in part to inhibition of angiogenesis.

3.2. Inhibition of adipocyte-specific gene expression by compound K in 3T3-L1 adipocytes

To quantify changes in adipocyte differentiation by compound K, we analyzed adipocyte marker gene expression. Compound K (5 μM) decreased peroxisome proliferator-activated receptor γ (PPARγ), adipocyte fatty acid-binding protein (aP2), leptin, and CCAAT/enhancer binding protein α (C/EBPα) mRNA levels compared with control (Fig. 2). Compound K decreased mRNA levels of PPARγ, aP2, leptin, and C/EBPα by 41%, 38%, 7%, and 79%, respectively. Thus, compound K inhibits MDI-induced expression of genes related to adipogenesis.

3.3. Changes in mRNA expression of angiogenic factors, MMPs, and their inhibitors by compound K in 3T3-L1 adipocytes

Treatment with compound K down-regulated and up-regulated the mRNA levels of angiogenic and anti-angiogenic factors, respectively, compared with untreated adipocytes. Compound K significantly decreased the mRNA levels of angiogenic factors vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2) by 39% and 53%, respectively (Fig. 3). In contrast, compound K increased the mRNA levels of anti-angiogenic molecule thrombospondin-1 (TSP-1) by 109%.

Similarly, compound K decreased MMP-2 and MMP-9 mRNA levels by 30% and 9%, respectively, whereas compound K increased the mRNA levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 by 184% and 83%, respectively (Fig. 3). Thus, these results indicate that compound K regulates mRNA expression of genes involved in angiogenesis and MMP system.

3.4. Inhibitory effects of compound K on MMP activity in 3T3-L1 adipocytes

MMP activity in 3T3-L1 adipocytes was examined using zymography on gelatin-containing gels. Gelatin zymography revealed that the activities of proMMP-9 (92 kDa), proMMP-2 (68 kDa), and active MMP-2 (58 kDa) in control adipocytes were substantially increased compared with those of ND. However, these activities were significantly reduced by treatment with compound K compared with untreated adipocytes. Compound K decreased proMMP-2 and proMMP-9 activities by 14% and 20%, respectively

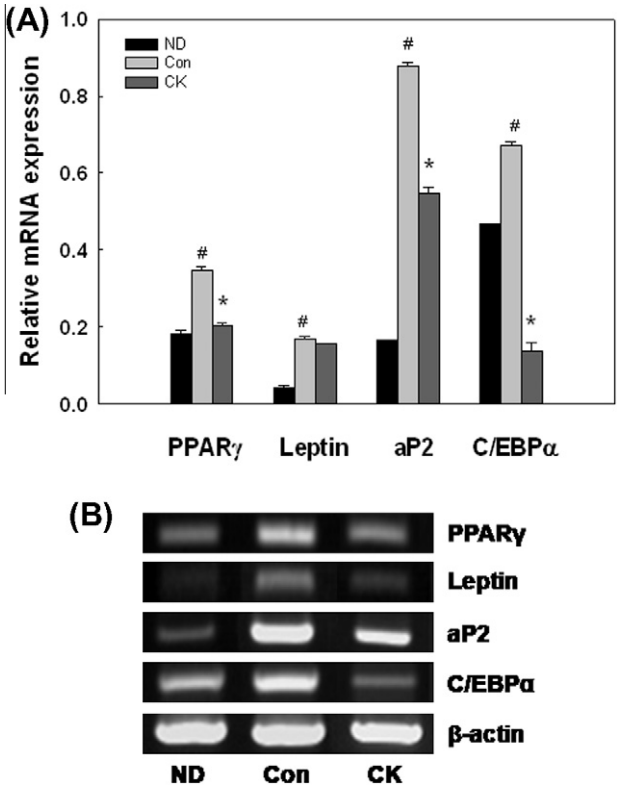

Fig. 2. Effects of compound K on adipocyte-specific gene expression in 3T3-L1 cells. (A) 3T3-L1 cells were treated with MDI (control) or compound K (5 μM). All values are expressed as the mean ± SD of R.D.U. (relative density units) using β-actin as a reference. (B) Representative PCR bands from one of three independent experiments are shown. $^{\#}p < 0.05$ compared with the ND group, $^{*}p < 0.05$ compared with the Con group. CK, compound K; Con, differentiated control; ND, non-differentiated.

(Fig. 4). Moreover, compound K reduced active MMP-2 levels by 12%.

4. Discussion

Obesity is characterized by increased adipose tissue mass that results from both increased fat cell number (hyperplasia) and increased fat cell size (hypertrophy) [21]. Fat mass can be regulated by various factors including adipogenesis, angiogenesis, and remodeling of the extracellular matrix [22]. Because our previous study suggested that ginseng reduces adipose tissue mass in part through inhibiting angiogenesis and MMP activities (not published), this study was undertaken to investigate whether the active ginsenoside metabolite compound K inhibits adipogenesis and to determine the expression patterns of genes involved in lipogenesis, angiogenesis, and MMPs.

Adipogenesis includes morphological changes, cessation of cell growth, expression of many lipogenic enzymes, and extensive lipid accumulation [23], contributing to the growth and expansion of adipose tissue. Our results demonstrate that compound K inhibits intracellular lipid accumulation and adipocyte-specific gene expression in 3T3-L1 adipocytes. As expected, MDI-treated differentiated control cells had an increased accumulation of triglyceride droplets compared with ND. However, compound K markedly prevented this MDI-induced lipid accumulation. Similar inhibition was observed in cells treated with the well-known angiogenesis inhibitor, TNP-470. Adipogenesis is initiated by the production of the key transcription factor PPARγ, which is responsible for inducing the expression of adipocyte-specific genes [24]. PPARγ is involved in the regulation of adipose angiogenesis. Interestingly,

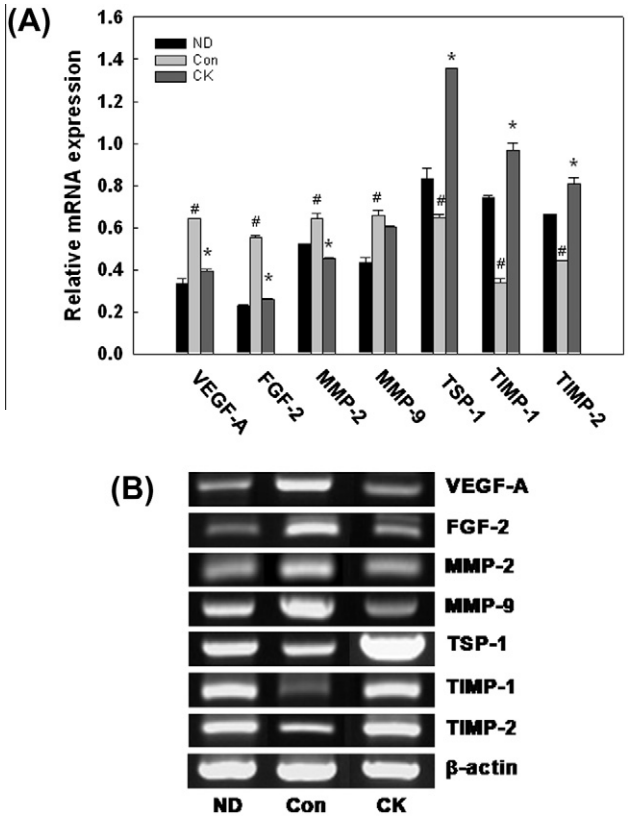

Fig. 3. Effects of compound K on mRNA expression of angiogenic factors, MMPs, and their inhibitors in 3T3-L1 cells. (A) 3T3-L1 cells were treated with MDI (control) or compound K (5 μM). All values are expressed as the mean ± SD of R.D.U. (relative density units) using β-actin as a reference. (B) Representative PCR bands from one of three independent experiments are shown. $^{\#}p < 0.05$ compared with the ND group, $^{*}p < 0.05$ compared with the Con group. CK, compound K; Con, differentiated control; ND, non-differentiated.

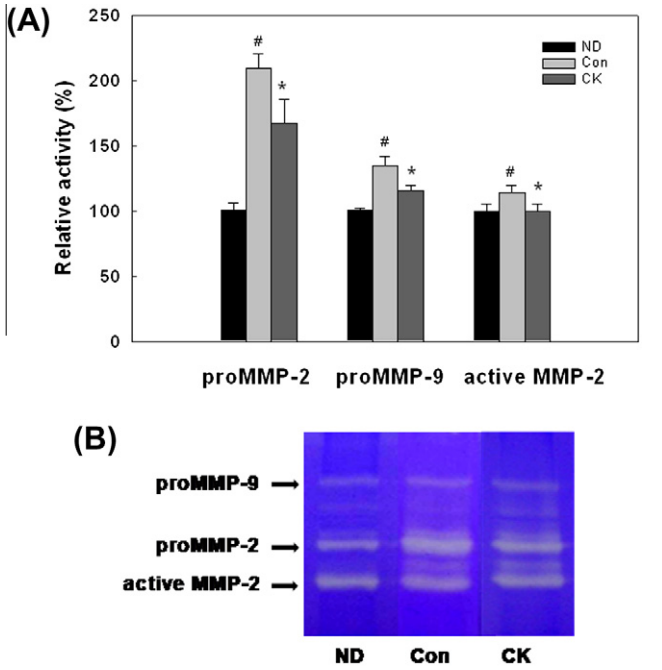

Fig. 4. Zymographic analysis of 3T3-L1 cells. (A) 3T3-L1 cells were treated with MDI (control) or compound K (5 μM). Protein extracts from 3T3-L1 cells were applied to a gelatin-containing gel. Gelatinolytic activity was measured by zymography. (B) Quantitative analyses of gelatinolytic activities. All values are expressed as the mean ± SD. $^{\#}p < 0.05$ compared with the ND group, $^{*}p < 0.05$ compared with the Con group. CK, compound K; Con, differentiated control; ND, non-differentiated.

the inhibition of adipocyte differentiation by the overexpression of a dominant-negative PPARγ construct impaired the development of adipose tissue and angiogenesis [25]. Consistent with the effects of compound K on lipid accumulation, compound K decreased the expression of PPARγ and the adipose-specific genes aP2, leptin, and C/EBPα which are directly implicated in the lipogenic pathways of 3T3-L1 adipocytes. These results indicate that compound K has an inhibitory effect on adipogenesis.

In contrast to our results, Brakenhielm et al. reported that exposure of 3T3-L1 cells to TNP-470 did not decrease intracellular lipid droplets even in the presence of TNP-470 concentrations up to 10 μM and did not prevent preadipocyte differentiation, suggesting that inhibition of angiogenesis can play a critical and indirect role in suppression of adipogenesis [26]. This difference may be due to differences in time and duration of compound K and TNP-470 treatment. While we treated 3T3-L1 cells with compound K and TNP-470 on days 0 through 2 after differentiation, Brakenhielm et al. treated the same cells with TNP-470 for 5–10 min after 16th day of differentiation. Accordingly, anti-angiogenic agents may be able to inhibit adipogenesis in our system.

Adipocytes produce multiple angiogenic factors and inhibitors that regulate adipose angiogenesis. Angiogenic factors, such as VEGF-A and FGF-2, promote the proliferation and differentiation of endothelial cells within fat tissue [17,27], whereas TSP-1 inhibits angiogenesis *in vivo* and impairs the migration and proliferation of cultured microvascular endothelial cells [28]. Adipocytes also produce MMPs and MMP inhibitors that are differentially expressed in adipose tissue in murine obesity models [19,20]. Recently, it was reported that compound K inhibits FGF-induced angiogenesis in HUVECs and suppresses phorbol ester-induced MMP-9 expression in human astroglial cells [10,12]. Similar to these effects, compound K decreased the mRNA levels of VEGF-A and FGF-2 in differentiated 3T3-L1 adipocytes compared with their levels in undifferentiated cells. Moreover, compound K increased the mRNA levels of the anti-angiogenic agent TSP-1 in 3T3-L1 cells. Similarly, compound K decreased MMP-2 and MMP-9 mRNA levels, whereas they increased TIMP-1 and TIMP-2 levels in 3T3-L1 adipocytes. MMP-2 and MMP-9 activities were also suppressed by compound K in these cells. Our data indicate that compound K exerts a specific regulatory effect on genes involved in both angiogenesis and the MMP system in adipocytes. Our observations support the hypothesis that compound K can inhibit adipogenesis due to its inhibition of angiogenesis and MMP activity.

These studies demonstrate that compound K suppresses adipogenesis in 3T3-L1 adipocytes. These events may be mediated in part through changes in the expression of genes responsible for angiogenesis and the MMP system. Thus, by reducing adipogenesis, compound K provides a possible therapeutic approach for the prevention and treatment of human obesity and its related disorders.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.142.

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