Luteolin decreases IGF-II production and downregulates insulin-like growth factor-I receptor signaling in HT-29 human colon cancer cells

Do Young Lim¹, Han Jin Cho¹, Jongdai Kim²,³, Chu Won Nho⁴, Ki Won Lee⁵ and Jung Han Yoon Park¹,²*

Abstract

Background: Luteolin is a 3',4',5,7-tetrahydroxyflavone found in various fruits and vegetables. We have shown previously that luteolin reduces HT-29 cell growth by inducing apoptosis and cell cycle arrest. The objective of this study was to examine whether luteolin downregulates the insulin-like growth factor-I receptor (IGF-IR) signaling pathway in HT-29 cells.

Methods: In order to assess the effects of luteolin and/or IGF-I on the IGF-IR signaling pathway, cells were cultured with or without 60 μmol/L luteolin and/or 10 nmol/L IGF-I. Cell proliferation, DNA synthesis, and IGF-IR mRNA levels were evaluated by a cell viability assay, [³H]thymidine incorporation assays, and real-time polymerase chain reaction, respectively. Western blot analyses, immunoprecipitation, and in vitro kinase assays were conducted to evaluate the secretion of IGF-II, the protein expression and activation of IGF-IR, and the association of the p85 subunit of phosphatidylinositol-3 kinase (PI3K) with IGF-IR, the phosphorylation of Akt and extracellular signal-regulated kinase (ERK)1/2, and cell division cycle 25c (CDC25c), and PI3K activity.

Results: Luteolin (0 - 60 μmol/L) dose-dependently reduced the IGF-II secretion of HT-29 cells. IGF-I stimulated HT-29 cell growth but did not abrogate luteolin-induced growth inhibition. Luteolin reduced the levels of the IGF-IR precursor protein and IGF-IR transcripts. Luteolin reduced the IGF-I-induced tyrosine phosphorylation of IGF-IR and the association of p85 with IGF-IR. Additionally, luteolin inhibited the activity of PI3K activity as well as the phosphorylation of Akt, ERK1/2, and CDC25c in the presence and absence of IGF-I stimulation.

Conclusions: The present results demonstrate that luteolin downregulates the activation of the PI3K/Akt and ERK1/2 pathways via a reduction in IGF-IR signaling in HT-29 cells; this may be one of the mechanisms responsible for the observed luteolin-induced apoptosis and cell cycle arrest.

Background

Colon cancer is the second most frequent cause of cancer-related death in the Western world [1]. Dietary patterns and lifestyle are the principal determining factors for colorectal cancer risk. The results of epidemiological studies have shown that the consumption of fruits and vegetables can reduce or prevent the risk of colon cancer [2]. Flavonoids are polyphenols, which are abundantly present in fruits and vegetables, and have been shown to have a variety of biological effects, including cancer prevention.

Insulin-like growth factors (IGFs) are polypeptides that stimulate the growth of a variety of mammalian cells [3]. These effects are mediated through the insulin-like growth factor I receptor (IGF-IR), and IGF-I and IGF-II are well-known ligands of IGF-IR. The binding of these ligands to IGF-IR results in the autophosphorylation of the receptor at the intracellular domain of β-subunits, resulting in the activation of the intrinsic tyrosine kinase of the IGF-IR. Subsequently, several adaptor molecules

* Correspondence: jyoon@hallym.ac.kr
1Department of Food Science and Nutrition, Hallym University, Chuncheon, 200-702, Korea
Full list of author information is available at the end of the article
are treated with DMSO at a final concentration of 0.1%.

MTT assays. Luteolin was dissolved in DMSO and all cells were subjected to 24 h of serum starvation with DMEM/F12 supplemented with 5 mg/L transferrin, 1 g/L BSA, and 5 μg/L selenium (serum-free medium). The cells were plated in 100 mm dishes at a concentration of 2 × 10^6 cells/dish and after 24 h, the monolayers were serum-starved and treated with various concentrations of luteolin (0 - 60 μmol/L) for 24 h. Conditioned media were collected and concentrated 20-fold, and immunoblot analysis was conducted using anti-IGF-II clone S1F2 (Upstate Biotechnology, Inc., Lake Placid, NY, USA) as previously described [22].

**Immunoprecipitation and immunoblot analyses**

Cells were incubated for 2 h with 0 or 60 μmol/L of luteolin, and 10 nmol/L of IGF-I was added. At 0, 1, or 30 min after the addition of IGF-I, the cell lysates were prepared and immunoprecipitated with indicated antibodies. Immunoblot analyses were conducted as described previously [23]. Signals were detected via the enhanced chemiluminescence method using SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). The relative abundance of each protein band was analyzed via densitometric scanning of the exposed films. Immunoblots were probed with an antibody for β-actin as a protein loading control. The following antibodies were purchased from the indicated suppliers: anti-IGF-IRβ (C-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-phospho-tyrosine-RCK20 antibody (PY20) linked to horseradish peroxidase (Transduction Laboratories, Palo Alto, CA, USA); anti-PI3K p85 antibody (Upstate Biotechnology, Inc.); anti-phospho-IGF-IR (P-IGF-IR, Abcam, Cambridge, MA, USA); and anti-ERK-1/2, anti-P-ERK-1/2 (Thr202/Tyr203), anti-cell division cycle 25c (CDC25c), anti-P-CDC25c, anti-Akt, and anti-P-Akt Ser473 (Cell Signaling Technology, Inc., Beverly, MA, USA).

**Real-time-polymerase chain reaction (RT-PCR)**

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using 3 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real time-PCR was conducted as described previously [24]. Sequences used for primer sets were as follows: IGF-IR;
forward-TGG AGT GCT GTA TGC CTC TG, back-
ward-TGA TGA CCA GTG TTG GCT GG, β-actin;
forward-GTT TGA GAC CTT CAA CAC CCC, back-
ward-GTG GCC ATC TCC TGC TCG AAG TC. The
levels of mRNA were normalized to β-actin and the
control (0 μmol/L luteolin) levels were set to 100%.

PI3K assay
PI3K activity was estimated as described previously [25].
Cell lysates (1 mg protein) were immunoprecipitated
with a polyclonal antibody against IGF-IRβ followed by
incubation with protein A-Sepharose beads. After wash-
ing, the beads were resuspended in 20 μL of kinase buffer
containing 4 μg of phosphatidylinositol (Sigma, St. Louis,
MO, USA), 10 μmol/L of ATP, 5 mmol/L of MnCl2, and
10 μCi of [γ-32P]ATP and incubated for 20 min at 30°C.
In order to determine whether luteolin directly inhibits
the kinase activity of PI3K, active PI3Kα (100 ng, Milli-
pore, Billerica, MA, USA) was incubated for 10 min in
the absence or presence of 20 μmol/L of luteolin at 30°C
in 20 μL of kinase buffer. Phosphatidylinositol (25 μg)
was added and the incubation was continued for another
5 min at room temperature. 10 μCi of [γ-32P]ATP was
then added and reactions were incubated for 10 min at
30°C. The resultant 32P-labeled phosphatidylinositol 3-
phosphate (PIP) lipids were separated from reaction pro-
ducts by thin layer chromatography (TLC) and visualized
by autoradiography. The radioactive PIP signals were
quantitated via densitometry using the Bio-profile Bio-1D
application (Vilber-Lourmat, France) [23].

Statistical analyses
Data were expressed as means ± SEM values and ana-
yzed via analysis of variance. Differences between treat-
ment groups were analyzed by Duncan’s multiple range
test or Student’s t-test. The means were considered sig-
ificantly different at P < 0.05. All statistical analyses
were conducted using the SAS system for Windows,
version 8.12 (SAS, Inc., Cary, NC, USA).

Results
Luteolin reduces IGF-II secretion in HT-29 cells
In the previous study, we observed that luteolin inhib-
ited HT-29 human colon cancer cell proliferation by
inducing cell cycle arrest and apoptosis [21]. However,
the treatment of IEC-6 rat intestinal epithelial cells at
the same concentrations (20 - 60 μmol/L) of luteolin for
24 h did not alter the viability of these cells (data not
shown). In order to assess the effect of luteolin on IGF-
II secretion of HT-29 cells, cells were treated with 20 -
60 μmol/L of luteolin for 24 h and conditioned media
were assayed via immunoblot analysis. Luteolin reduced
the secretion of pro- and mature-IGF-II in a dose-
dependent manner (Figure 1).

Luteolin abrogates the growth stimulatory effects of
exogenous IGF-I on HT-29 cells
In order to determine whether luteolin inhibits the
growth-stimulatory effects of exogenous IGF-I, cells
were treated with 0 or 60 μmol/L of luteolin in the
absence or presence of 10 nmol/L IGF-I for 24, 48, or
72 h. IGF-I increased but luteolin significantly reduced the numbers of viable cells. The treatment of cells with IGF-I did not alleviate the growth-inhibitory effects of luteolin (Figure 2A). To explore the effect of luteolin on DNA synthesis in HT-29 cells, an [3H]thymidine incorporation assay was conducted. DNA synthesis was induced by IGF-I treatment, and luteolin significantly inhibited the stimulatory effect of IGF-I (Figure 2B).

**Luteolin reduces the levels of the IGF-IR precursor protein and IGF-IR transcripts in HT-29 cells**

Because luteolin reduced IGF-II secretion but exogenous IGF-I did not abrogate the growth-inhibitory effect of luteolin, we attempted to determine whether luteolin inhibits the IGF-I signaling pathway. Western blot analysis revealed that the levels of the IGF-IR precursor protein were reduced 2 h after the addition of luteolin, whereas the levels of the IGF-IR β-subunit were unaltered (Figure 3A). Additionally, IGF-IR transcript levels were reduced dose-dependently in cells treated with luteolin for 2 h (Figure 3B). Furthermore, the levels of IGF-IR mRNA were reduced further at 24 h of luteolin treatment as compared to those at 2 h (Figure 3C).

**Luteolin inhibits IGF-I-induced activation of IGF-IR, Akt, and ERK1/2 in HT-29 cells**

In order to determine whether luteolin down-regulates IGF-I-induced tyrosine phosphorylation of the IGF-IR, cells were treated for 2 h with 0 or 60 μmol/L of luteolin, and IGF-I was stimulated with 10 nmol/L IGF-I for 0, 1, or 30 minutes. Total cell lysates were prepared and immunoprecipitated using an IGF-IRβ antibody. The immune complexes were used for Western blot analysis with an anti-P-tyrosine antibody (PY20). IGF-I induced tyrosine phosphorylation of IGF-IR at 1 min; tyrosine phosphorylation levels were slightly reduced at 30 min. Luteolin significantly inhibited the phosphorylation of IGF-IRβ at 1 min after IGF-I treatment. At 30 min, the phosphorylation status of IGF-IR did not differ between the control and luteolin-treated cells.

To evaluate the association of the p85 subunit of PI3K with IGF-IR, we conducted immunoprecipitation of cell lysates with an IGF-IRβ antibody and subsequent immunoblotting with a p85 antibody. IGF-I stimulated the association of the p85 regulatory subunit of PI3K with IGF-IR within 1 min, which was significantly inhibited by luteolin treatment (Figure 4A). The association of p85 with IGF-I was reduced at 30 min, and no difference was observed in the association of these two molecules between the control and luteolin-treated cells at this time period. Western blot analysis of total cell lysates revealed that IGF-I markedly increased P-IGF-IR levels and luteolin reduced those in both HT-29 and Caco-2 cells (Figure 4B). For the determination of PI3K activity, the immune complex was incubated with [32P]ATP and phosphatidylinositol. Luteolin reduced both basal and IGF-I-induced PI3K activity in HT-29 cells at 1 min of IGF-I treatment. However, this difference disappeared at 30 min after IGF-I treatment (Figure 4C). In order to determine whether the luteolin-induced inhibition of PI3K is the result of direct interaction with this kinase, active PI3K was incubated with 20 μmol/L of luteolin in the kinase reaction. Luteolin inhibited PI3K activity in a cell-free system (Figure 4D). The activation of PI3K leads to the activation of Akt [7]. Akt phosphorylation was induced by IGF-I treatment at 1 min without any changes in total Akt expression.
luteolin significantly reduced the level of Akt activation (Figure 5).

IGF-I stimulated ERK1/2 activation in HT-29 cells was detected at 30 min of IGF-I treatment, and luteolin inhibited the phosphorylation of ERK1/2 in the absence or presence of IGF-I treatment (Figure 6). Because ERK1/2 activation was reported to lead to the activation of the protein phosphatase CDC25c during the G2/M transition of cell cycle progression [26], we subsequently attempted to determine whether luteolin treatment results in a reduction in the phosphorylation of CDC25c. The levels of P-CDC25c were increased at 30 min after IGF-I addition and significantly reduced in cells treated with luteolin, regardless of whether or not the HT-29 cells were treated with IGF-I (Figure 6C).

Discussion

The IGF system (IGF-I, IGF-II, IGF-binding protein, and IGF-IR) performs an important role in the growth of various cancer cells, including colon cancer cells [8,27]. We have reported previously that luteolin inhibited the proliferation of HT-29 human colon cancer cells by inducing cell cycle arrest and apoptosis [21]. The results of a previous study revealed that luteolin reduced the expression of cyclin D1 and cyclin B1 and inhibited the activities of CDKs, thereby suppressing HT-29 cell cycle progression. Additionally, luteolin induced the activation of caspases and reduced the levels of proteins involved in the suppression of apoptosis, including Bcl-xL and Mdm-2 [21]. Thus, in the present study, we explored the upstream signals that are important for the regulation of cell cycle progression and apoptosis in HT-29 cells. Our previous data demonstrated that HT-29 cells synthesized and secreted IGF-II and expressed IGF-IR, and that IGF-II stimulated HT-29 cell growth via an autocrine mechanism [10,28]. Kim et al. also reported that the reduction of IGF-II secretion in Caco-2 colon cancer cells inhibited cell growth [11]. Using PC-3 and DU145 human prostate cancer cells, Fang et al. [29] have demonstrated that luteolin inhibits the IGF-I-induced activation of IGF-IR and AKT as well as the downstream targets of AKT, p70S6K1, GSK-3β, and FKHR/FKHRL1. In the present study, we demonstrate that, in HT-29 human colon carcinoma cells, luteolin 1) reduces IGF-II secretion; 2) inhibits the growth-stimulatory effects of IGF-I; 3) reduces the levels of IGF-IR transcripts and the IGF-IR precursor protein; 4) reduces the IGF-I-induced tyrosine phosphorylation of IGF-IRβ and the association of p85 with IGF-IRβ; 5) inhibits IGF-I-induced PI3K activity; 6) inhibits IGF-I-induced Akt activation; and 7) inhibits the IGF-I-induced phosphorylation of ERK1/2 and CDC25c. These results indicate that the reduction in IGF-II secretion and changes in IGF-IR signaling by luteolin may be important factors underlying the growth-inhibitory effects of HT-29 cells. Additionally, we have demonstrated that luteolin directly inhibits the activity of PI3K in a cell-free system.

Figure 3 Luteolin reduces the levels of the IGF-IR protein and mRNA in HT-29 cells (A) HT-29 cells were plated and treated with luteolin as described in Figure 1 for 2 h. Total cell lysates were prepared and immunoblot analyses were conducted. Photographs of the chemiluminescent detection of the blots, which were representative of three independent experiments, are shown. The relative abundance of IGF-IR to their own β-actin was quantified via densitometric scanning of the exposed films, and the control levels were set at 100%. (B) HT-29 cells were plated and treated with luteolin as described in Figure 1 for 2 h. (C) Total RNA was isolated and real-time PCR was conducted. Each bar represents mean ± SEM (n = 3). (A, B) Means without a common letter differ, P < 0.05. (C) *Different from 0 μmol/L of luteolin at each treatment time, P < 0.05.
Figure 4  Effects of luteolin on IGF-I-induced tyrosine phosphorylation of IGF-IR, the association of p85 with IGF-IR, and PI3K activity in human colon cancer cells. Cells were plated and cultured as described in Figure 1. (A) HT-29 cells were treated for 2 h with 0 or 60 μmol/L of luteolin and lysed with or without stimulation of 10 nmol/L IGF-I for 0, 1, or 30 minutes. Total cell lysates were incubated with anti-IGF-IR antibody and the immune complexes were precipitated with protein A-Sepharose. The immunoprecipitated proteins were analyzed via Western blotting with antibodies raised against phosphotyrosine (PY20), IGF-IR, or p85. (B) HT-29 and Caco-2 cells were plated and treated as described above. Total cell lysates were analyzed via Western blotting with an antibody raised against P-IGF-IR. Photographs of the chemiluminescent detection of the blots, which were representative of three independent experiments, were shown. (C) The immune complexes obtained from HT-29 cells were incubated with phosphatidylinositol and [γ-32P]ATP. (D) Active PI3K and luteolin were incubated with phosphatidylinositol and [γ-32P]ATP as described in the Materials and Methods section. Phosphatidylinositol 3-phosphate (PIP) generated by immunoprecipitated PI3K (C) or active PI3Kα (D) was separated via thin-layer chromatography (TLC). An autoradiograph of the TLC plate, which was representative of three independent experiments, is shown. (A, B, C) The relative abundance of each blot was quantified via densitometric scanning of the exposed films and the control levels (0 μmol/L luteolin, without IGF-I stimulation) were set at 100%. Each bar represents the mean ± SEM (n = 3). *Different from 0 μmol/L of luteolin at a stimulation time, P < 0.05.
When HT-29 cells were treated with exogenous IGF-I, IGF-I did not abrogate the growth-inhibitory effects of luteolin (Figure 2), although luteolin reduced IGF-II secretion (Figure 1). These results indicated that luteolin inhibits IGF-IR signaling in HT-29 cells. IGF-IR consists of two extracellular α-subunits and two transmembrane β-subunits, and IGF-I and IGF-II bind to the α-subunits of IGF-IR, thus resulting in the activation of the intrinsic tyrosine kinase in the intracellular domain of the β-subunits [28]. In this study, luteolin reduced the levels of the IGF-IR precursor but did not reduce the levels of IGF-IR α-subunits; this suggests that the levels of IGF-IR α-subunits may have been reduced by luteolin treatment. The finding that IGF-IR mRNA levels were continuously decreased during 24 h of luteolin treatment (Figure 3C) indicates that the expression of IGF-IR protein is regulated by luteolin, at least in part, at an RNA level. The effects of luteolin on IGF-IR mRNA and protein stability will require further study in the future.

Fang et al. demonstrated that prostate cancer cells in which the IGF-IR gene is knocked down grew at a slower rate relative to that in control cells, and the inhibition of cell growth by luteolin treatment was similar to that observed in IGF-IR-depleted cells [29]. In this study, we demonstrate that luteolin inhibits IGF-II secretion, and that IGF-I-stimulated HT-29 cell proliferation was inhibited by luteolin (Figure 2). These results suggest that the inhibition of the IGF/IGF-IR signaling pathway by luteolin might be one of the mechanisms for the suppression of proliferation and apoptosis in HT-29 cells. In 1994, Lahm et al. demonstrated that
Alpha IR3, a neutralizing monoclonal antibody directed against human IGF-IR, inhibited proliferation in HT-29 cells [30]. It has also been demonstrated that the blockade of IGF-IR with IGF-IR monoclonal antibodies inhibited proliferation, arresting the cell cycle and inducing the apoptosis of HT-29 cells [31]. Additionally, an antihuman/mouse IGF-II-neutralizing antibody effectively inhibited the hepatic metastasis of HT-29 cells [32]. In vitro experiments have also demonstrated that IGF-II-neutralizing antibody treatment completely blocked IGF-IR phosphorylation in serum-starved HT-29 cells [33]. These results indicate that IGF-II is an autocrine growth factor of HT-29 cells and that the inhibition of IGF-II secretion and/or IGF-IR signaling inhibits HT-29 cell proliferation.

In our HT-29 cells, it is possible that the luteolin-induced downregulation of the IGF-IR a-subunit results in reduced phosphorylation of the b-subunit. This is also possible that luteolin directly interferes with the binding of IGF-I to IGF-IR, which would consequently inhibit the phosphorylation of the b-subunit. This reduced IGF-I-induced tyrosine phosphorylation of IGF-IRp by luteolin led to the reduced association of p85 with IGF-IRb and the subsequent activation of PI3K/Akt and ERK1/2 (Figures 4, 5 and 6). Additionally, luteolin inhibited PI3K activity in a cell-free system (Figure 4D), thereby indicating that luteolin can also modulate the activity of this enzyme via direct interaction with this kinase. As the activation of Akt and ERK1/2 induces cell proliferation and inhibits apoptosis in various cancers [34,35], the PI3K/Akt and ERK1/2 pathways may be important targets in cancer therapies involving natural bioactive compounds [6,23,28,29,36-38]. Akt regulates the expression and activity of proteins involved in the regulation of apoptosis and cell cycle progression, including Bad, p21, cyclin D1, and Mdm-2 (Reviewed in [37]).

Previously, we have demonstrated that luteolin downregulates the expression of Mdm-2 and cyclin D1 [21]. Fang et al. also reported that luteolin treatment induced a reduction in the levels of P-IGF-IR, P-Akt, and cyclin D1 in PC3 prostate cancer cells [29]. The results of previous studies and of the present study indicate that the inhibition of Akt activation by luteolin may result in the downregulation of Mdm-2 and cyclin D1, which may contribute to the induction of apoptosis and cell cycle arrest in colon and prostate cancer cells. Collectively, these results indicate that the downregulation of IGF-IR/PI3K/Akt by luteolin is one of the principal signaling pathways for the induction of cell cycle arrest and apoptosis in HT-29 cells.

ERK-MAP kinases also regulate cell cycle- and apoptosis-related proteins. ERK1/2 activation leads to the phosphorylation of the protein phosphatase CDC25c during the G2/M transition of cell cycle progression [26]. Phosphorylated CDC25c dephosphorylates CDC2, which results in the activation of the CDC2/cyclin B1 complex. Luteolin has been reported to reduce the levels of the CDC25c, CDC2, and cyclin B1 proteins and induces G2/M phase arrest in human gastric cancer cells [39]. In our previous study, luteolin reduced cyclin B1 levels, markedly inhibited CDC2 activity, and promoted G2/M phase arrest in HT-29 cells [21]. In the present study, we determined that luteolin reduced the levels of P-CDC25c in HT-29 cells (Figure 6C). Together, these results indicate that the attenuated ERK1/2 activation contributed to the reduction of P-CDC25c levels in luteolin-treated cells. The reduction in CDC25c activation may have contributed to the induction of G2/M arrest in HT-29 cells.

Conclusions
We demonstrated that luteolin reduced the secretion of pro- and mature-IGF-II and reduced the levels of the IGF-IR precursor protein in HT-29 cells, and subsequently reduced the activation of the Akt and ERK1/2 pathways. The inhibition of the IGF-IR signaling pathway may be one of the mechanisms by which luteolin inhibits Akt and ERK1/2 signaling in HT-29 cells, thereby inhibiting cell growth and inducing apoptosis (Figure 7). The present results help delineate the

Figure 7 A tentative scheme for luteolin regulation of the IGF-IR signaling pathway in HT-29 human colon cancer cells
Luteolin reduces the secretion of IGF-II and levels of IGF-IR mRNA and protein, which leads to a reduction in IGF-IR phosphorylation and a subsequent inhibition of PI3K/Akt and ERK1/2/CDC25c activation. Additionally, luteolin directly inhibits PI3K activity. These changes in IGF-1 signaling contribute to luteolin-induced apoptosis and cell cycle arrest in HT-29 cells. *From our previously published results [21].
mechanisms of luteolin actions for future animal studies with colon cancer models. Such studies will determine whether luteolin can be developed into a chemopreventive agent for use against colon cancer.

Abbreviations
IGF-IR: insulin-like growth factor-1 receptor; IGF-II: insulin-like growth factor-II; ERK-1/2: extracellular signal-regulated kinase-1/2; PI3K: phosphatidylinositol-3 kinase; PIP: phosphatidylinositol 3-phosphate; TLC: thin layer chromatography; CDK: cyclin-dependent kinase; CDC: cell division cycle.

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Author details
1Department of Food Science and Nutrition, Hallym University, Chuncheon, 200-702, Korea. 2Department of Food Science and Biotechnology, Kangwon National University, Chuncheon, 200-701, Korea. 3Department of Agriculural and Food Science, Gangneung Institute, Gangneung, 210-340, Korea. 4Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul, 151-921, Korea.

Authors’ contributions
DYL, JK, KWL, and JHP designed and planned this research; DYL and HJC performed the assays and analyzed the data. DYL wrote the first draft and JHP, DYL, HJC, and CWN revised the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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