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Corresponding Author: Kyoung Seob Song

Authors: Jin-Woo Jeong[1], Cheol Park[2], Hee-Jae Cha[3], Su Hyun Hong[4,5], Shin-Hyung Park[6], Gi-Young Kim[7], Woo Jean Kim[8], Cheol Hong Kim[9], Kyoung Seob Song[8,*,#], Yung Hyun Choi[4,5,#]

Institution: [1]Freshwater Bioresources Utilization Bureau, Nakdonggang National Institute of Biological Resources,
[2]Department of Molecular Biology, Dongeui University,
[3]Department of Parasitology and Genetics, Kosin University College of Medicine,
[4]Department of Biochemistry, Dong-Eui University College of Korean Medicine,
[5]Anti-Aging Research Center, Dong-Eui University,
[6]Department of Pathology, Dong-Eui University College of Korean Medicine,
[7]Department of Marine Life Sciences, Jeju National University,
[8]Department of Anatomy, Kosin University College of Medicine,
[9]Department of Pediatrics, Sungkyunkwan University Samsung Changwon Hospital,

Cordycepin inhibits lipopolysaccharide-induced cell migration and invasion in human colorectal carcinoma HCT-116 cells through down-regulation of prostaglandin E_2 receptor EP4

Jin-Woo Jeong[1], Cheol Park[2], Hee-Jae Cha[3], Su Hyun Hong[4,5], Shin-Hyung Park[6], Gi-Young Kim[7], Woo Jean Kim[8], Cheol Hong Kim[9], Kyoung Seob Song[10,*] & Yung Hyun Choi[4,5,*]

[1]Freshwater Bioresources Utilization Bureau, Nakdonggang National Institute of Biological Resources, Sangju 17104, [2]Department of Molecular Biology, College of Natural Sciences, Dongeui University, Busan 47340, [3]Department of Parasitology and Genetics, Kosin University College of Medicine, Busan 49267, [4]Department of Biochemistry, Dong-Eui University College of Korean Medicine, Busan 47227, [5]Anti-Aging Research Center, Dong-Eui University, Busan 47340, [6]Department of Pathology, Dong-Eui University College of Korean Medicine, Busan 47227, [7]Department of Marine Life Sciences, Jeju National University, Jeju 63243, [8]Department of Anatomy, Kosin University College of Medicine, Busan 49267, [9]Department of Pediatrics, Sungkyunkwan University Samsung Changwon Hospital, Changwon 51353, [10]Department of Physiology, Kosin University College of Medicine, Busan 49267, Korea

*Corresponding authors.

Kyoung Seob Song.

Tel.: +82-51-990-6236; Fax.: +82-51-990-3081; E-mail: kssong@kosin.ac.kr

Yung Hyun Choi.

Tel: +82-51-850-7413; Fax: +82-51-853-4036; E-mail: choiyh@deu.ac.kr

Running title: The effect of Cordycepin on LPS-induced cells.

ABSTRACT

Prostaglandin E_2 (PGE_2), a major product of cyclooxygenase-2 (COX-2), plays an important role in the carcinogenesis of many solid tumors including colorectal cancer. Because PGE_2 functions by signaling through PGE_2 receptors (EPs), which regulate tumor cell growth, invasion, and migration, there is growing interest in the therapeutic potential of targeting EPs. In the present study, we investigated the role of EP4 on the effectiveness of cordycepin in inhibiting migration and invasion of HCT116 human colorectal carcinoma cells. Our data indicate that cordycepin suppressed lipopolysaccharide (LPS)-enhanced cell migration and invasion through inactivation of matrix metalloproteinase (MMP)-9 and down-regulation of COX-2 expression and PGE_2 production. These events were associated with inactivation of EP4 and activation of AMP-activated protein kinase (AMPK). Moreover, the EP4 antagonist AH23848 prevented LPS-induced MMP-9 expression and cell invasion in HCT116 cells. However, the AMPK inhibitor, compound C, and knockdown of AMPK activity *via* siRNA attenuated the cordycepin-induced inhibition of EP4 expression. Cordycepin treatment also reduced the activation of CREB. These findings provide evidence that cordycepin suppresses the migration and invasion of HCT116 cells through modulating EP4 expression and the AMPK-CREB signaling pathway. Therefore, cordycepin has the potential to be a potent anti-cancer agent in therapeutic strategies against colorectal cancer metastasis.

Key words: Cordycepin, Cell migration/invasion, EP4, CREB, AMPK

1. INTRODUCTION

Cordyceps species is as a rare herb that is occasionally used in traditional medicine (1, 2). It has been utilized for the treatment of various diseases, such as cancer, and is also known to have anti-tumor properties, the ability to scavenge free radicals, and immune-stimulating abilities (3-5). Cordycepin is the main bioactive component in Cordyceps species such as *Cordyceps militaris* and *C. sinensis* (2, 4). Cordycepin has many biological properties including inhibition of inflammation (6), inhibition of platelet aggregation (7), and anti-tumor effects (8). However, the molecular mechanism by which cordycepin inhibits cancer cell migration and invasion remains unclear.

The physiological functions and signaling of prostaglandin E_2 (PGE_2) are related to the activation of the EP receptors (EP1-4), which are G-protein-coupled receptors (GPCRs) (9). PGE_2, regulated by cyclooxygenase-2 (COX-2), promotes cell proliferation and the invasion of colorectal tumors (10). Signaling through the EP2 receptor activates the protein kinase A (PKA) pathway, which induces phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) in the gastrointestinal tract (11). However, the molecular mechanism by which this intracellular mediator is related to cell invasion and migration, as well as the anti-inflammatory effects of PGE_2, in colorectal cancer is still unclear. Identification of the intracellular signaling mechanism that mediates cell invasion and movement, which in turn mediate the effects of PGE_2, is critical to understanding the main properties of colorectal cancer and developing effective therapies.

AMP-activated protein kinase (AMPK) is a well-conserved serine/threonine protein kinase containing a catalytic subunit (α) and two regulatory subunits (β and γ) and is expressed in many tissues (12). Some research suggests that AMPK can function as a tumor suppressor by altering

inflammation and causing cell-cycle arrest during tumorigenesis (13, 14). In addition, when activated by 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) or phenformin, AMPK induces cell death through the mitogen-activated protein kinases pathway (15, 16). Taken together, these findings suggest that AMPK activation may be useful in controlling cell death in colorectal cancer cells.

Because cordycepin can greatly affect the pathogenesis of colorectal disease, we hypothesized that cordycepin down-regulates EP4 expression and its downstream signaling functions in human colorectal cancer cells. To examine the signaling pathway involved, we performed *in vitro* human cell-based assays. Our results suggest that cordycepin inhibits cell invasion and migration in lipopolysaccharide (LPS)-treated HCT-116 cells *via* the EP4-AMPK-CREB axis. These pathways give new insights into the molecular mechanism of cell invasion and may reveal novel targets for therapeutic medications.

2. RESULTS

2.1. Cordycepin inhibits LPS-induced cell migration and invasion in HCT116 human colorectal carcinoma cells

In order to investigate the pharmacological potential of cordycepin on LPS-induced cell migration and invasion, we first determined the dose dependence of the cytotoxic effects of cordycepin in the absence or presence of LPS for 48 h in HCT116 cells using an MTT assay. Cordycepin at 25–50 μg/ml did not have a cytotoxic effect on HCT-116 cells with or without 2.5 μg/ml LPS (Fig. 1A). Therefore, a concentration of cordycepin within this range was applied in the remaining experiments. We next used gelatin zymography and Western blot analyses to investigate the inhibitory effects of cordycepin on the activation and expression of matrix

metalloproteinase (MMP) proteins. Interestingly, cotreatment with both cordycepin and LPS inhibited the activation and expression of MMP-9 compared to LPS alone, but it had no effect on MMP-2 activity or expression (Fig. 1B). *In vitro* invasion and migration assays were used to investigate the inhibitory effects of cordycepin on the invasive potency of LPS-treated HCT116 cells. As shown in Fig. 1C and D, LPS-stimulated cell migration and cell invasion were significantly inhibited by cordycepin. These results suggest that cordycepin has an inhibitory effect on the invasiveness of LPS-treated HCT-116 cells at nontoxic concentrations.

2.2. Cordycepin inhibits the expression of COX-2 and EP4 and the production of PGE2 in LPS-treated HCT116 cells

Previous reports have shown that cell migration and invasion increase the activation of the EPs and the expression of the COX-2 product PGE_2 in several cancer cell lines (16-18). Thus, we investigated whether the EP4-COX-2-PGE_2 cascade affects LPS-induced cell migration and invasion in HCT116 cells. Whereas LPS did not alter *COX-2 mRNA* level, it slightly increased COX2 protein level (Fig. 2A). But, cordycepin dramatically inhibited LPS-induced *COX-2 mRNA* and protein expression. Cordycepin also exhibited an inhibitory effect on LPS-induced PGE_2 production at a concentration of 50 μg/ml as demonstrated by enzyme immunoassays (Fig. 2B), suggesting that cordycepin's inhibitory effects on LPS-induced PGE_2 production are under the control of COX-2 gene expression. In addition, we attempted to identify which of the PGE_2 receptor subtypes is responsible for LPS-mediated production of PGE_2 in HCT-116 cells. Interestingly, only EP4 mRNA expression was affected by cordycepin but not EP2, EP3, or EP4 (Fig. 2C and D; Supplement Table 1). These results suggest that cordycepin inhibits EP4 and COX-2 expression to regulate PGE_2 production in HCT116 cells.

2.3. Cordycepin inhibits MMP9-mediated cell migration and invasion.

To determine whether cordycepin could inhibit LPS-mediated MMP9 expression via EP4, we used EP4 antagonist, AH23848. It dramatically inhibited LPS-induced MMP-9 expression, but not MMP-2 expression (Fig. 3A and B). In addition, cell migration and invasion assays were performed to examine whether cordycepin could inhibit cell invasion and migration in LPS-treated cells through inhibiting EP4 receptor. Whereas AH23848 inhibited LPS-mediated cell migration and invasion (Figs. 3C and D), overexpressed EP4 partially increased them (supplement Fig.1). These results show that cordycepin inhibited LPS-mediated cell migration and invasion via MMP and EP4.

2.4. Cordycepin-activated AMPK inhibits EP4 gene expression in LPS-treated cells and Cordycepin decreases CREB activation by inhibiting EP4 gene expression

AMPK is related to many physiological phenomena including cell death (19, 20), autophagy (21), and metabolic control (22). In particular, AMPK can control cell polarity, migration, and cytoskeletal dynamics (23). As shown in Fig. 4A, the phosphorylation level of AMPK and acetyl-CoA carboxylase (ACC), a downstream target kinase of AMPK, was increased by cordycepin treatment in a time-dependent manner, suggesting that AMPK signaling was activated. To determine whether cordycepin-activated AMPK can affect EP4 expression, we utilized the AMPK inhibitor, compound C. Cordycepin strongly inhibited EP4 gene expression (Fig. 4C), and compound C restored EP4 gene expression in LPS-treated cells. We carried out COX-2 activity assay and PGE2 ELISA assay to know whether compound C could partially restore COX-2 activity and PGE2 production. Whereas cordycepin decreased LPS-mediated COX-2 activity and PGE2 production, compound C slightly increase them in LPS-treated cells (supplement Fig. 2). The results from a genetic study using AMPK-specific siRNA (Fig. 4D) were the same as the

pharmaceutical study (Fig. 4C), suggesting that cordycepin inhibits EP4 gene expression *via* the AMPK pathway in LPS-treated cells. In addition, many studies have reported that CREB may be a down-stream protein of the AMPK pathway (24, 25). Thus, we determined CREB activation in LPS-treated cells. Cordycepin dramatically inhibited CREB activation in LPS-treated cells in a time-dependent manner (Fig. 4E). H89, an inhibitor of PKA and MSK, was used to inhibit CREB activation (26). As expected, H89 strongly inhibited EP4 gene expression by inhibiting CREB activation in LPS-treated cells (Fig. 4F), demonstrating that CREB may be critical for EP4 gene expression. To determine whether cordycepin could inhibit LPS-mediated cell migration and invasion via CREB activation, we performed these assay. Cotreatment with cordycepin and H89 dramatically inhibited cell migration and invasion compared to cordycepin alone in LPS-treated cells (Fig. 4G). These results suggest that cordycepin strongly decreases CREB-mediated cell migration and invasion through altered EP4 gene expression in LPS-treated cells.

3. DISCUSSION

In this study, we examined the anti-cell migration and -invasion effects of cordycepin in LPS-treated human colorectal carcinoma HCT-116 cells. Cordycepin had no cytotoxic effects on HCT-116 cells at the concentrations tested (Fig. 1), but it did show anti-inflammatory activity. Previous studies have shown that cordycepin has anti-cancer effects in human breast cancer cells (27), human lung cancer cells (28), oral squamous cell carcinoma (29), and human bladder cancer cells (30). In addition, cordycepin has anti-cancer effects in human colorectal cancer cells, but there are few reports on cordycepin's role in altering early events (i.e., migration and invasion) to prevent the progression into cell transformation. Because most of the literature on cordycepin's anti-tumor effects has focused on cell apoptosis, the effects of cordycepin on early events, like

cell migration and invasion during tumorigenesis, remains a matter of speculation. In particular, there have been no reports that cordycepin inhibits LPS-induced cell migration and invasion in human colorectal cancer cells until now, although cordycepin was found to decrease tumor necrosis factor-α-induced cell migration and invasion in human bladder cancer cells (31). The major difference in these pathways is the function of the EP4 receptor versus the mediator of intracellular signaling nuclear factor-κB. This is a critical finding because it is the first stage of signal transduction at the receptor as an early event.

Understanding the physiological characteristics of the EP4 receptor could give extra insight into the intracellular signaling mechanism that leads to protein complex formation of G-protein coupled receptors (32). Cordycepin decreased gene expression of the EP4 receptor, but not EP1, -2, or -3, and reduced PGE_2 production (Fig. 2C and D). This is not surprising because EP4 is preferentially expressed in the heart and intestines and expressed to a lesser degree in the lungs, kidneys, and brain (33). EP4 is $G\alpha_s$ protein that stimulates cAMP production, and cordycepin dramatically increased AMPK activation (Fig. 4B) and significantly inhibited EP4 gene expression, suggesting that AMPK can specifically inhibit EP4 gene expression. Funahashi *et al*. (34) reported that EP-induced PGE_2 expression abolished AMPK activity through protein kinase A signaling transmission. Reciprocally, increased AMPK activity by cordycepin could inhibit EP4 activity to prevent its physiological signaling. Taken together, these data suggest that LPS increases EP4 activation to produce PGE_2, which induces cell migration and invasion by inhibiting AMPK and that cordycepin inhibits this signaling mechanism, giving it anti-cancer properties affecting the early tumorigenesis stages.

Cordycepin inhibits CREB signaling to regulate its physiological functions. CREB-related gene expression is important for microenvironmental homeostasis under pathological conditions, and CREB has been considered a major target of novel drugs. As seen in Fig. 4E, cordycepin inhibited

LPS-induced CREB activation, and both H89 and forskolin induced CRE activity, suggesting that cordycepin decreased CREB activation *via* the EP4-AMPK pathway to prevent cell migration and invasion in HCT-116 cells.

In conclusion, our results demonstrate that the levels of CREB activation were dramatically decreased by the negative regulation of AMPK expression through the deactivation of EP4 in cordycepin-treated HCT116 cells. This study gives novel insights into the molecular mechanisms of cell migration in HCT-116 cells. Controlling expression of the EP4 receptor may be a new strategy to prevent cell migration and invasion of HCT-116 cells. The study also highlights the potential for therapeutic use of cordycepin in the treatment of colorectal cancer.

4. MATERIALS AND METHODS

4.1. Reagents

Cordycepin (MW, 251.2; Product No. C3394) from *C. militaris*, LPS (*Escherichia coli* 026:B6), Griess reagent, Tween-20, bovine serum albumin (BSA), H89, forskolin, compound C, and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Antibodies against COX-2, MMP-2, MMP-9, AMPK, pAMPK, ACC, pACC, CREB, pCREB, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The peroxidase-labeled donkey anti-rabbit immunoglobulin, peroxidase-labeled sheep anti-mouse immunoglobulin, and enhanced chemiluminescence (ECL) detection kit were purchased from Amersham Corp. (Arlington Heights, IL, USA). The enzyme-linked immunosorbent assay (ELISA) kit for PGE_2 was obtained from R&D Systems (Minneapolis, MN, USA). COX-2, EP1-4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were purchased from Bioneer (Seoul, Korea).

All other chemicals were purchased from Sigma-Aldrich Chemical Co.

4.2. Cell culture and MTT assay

HCT-116 human colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 1% penicillin-streptomycin at 37°C in a humid environment containing 5% CO_2. For the cell viability assay, HCT-116 cells were seeded in 6-well plates at a density of 2×10^5 cells per well. After 24 h of stabilization, the cells were treated with various concentrations of cordycepin for 1 h then stimulated with LPS (2.5 μg/ml) for an additional 48 h. Cell viability was determined using the MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes.

4.3. In vitro wound-healing assay

HCT-116 cells were seeded into 6-well plates and grown overnight to confluence. The monolayers were then scratched with a 200-μl pipette tip to create a wound and washed twice with serum-free RPMI-1640 to remove floating cells; the medium was then replaced with serum-free medium supplemented with cordycepin baicalein in the presence or absent of LPS. The rate of wound closure was assessed and imaged 48 h later. Each value is derived from three randomly selected fields.

4.4. Matrigel invasion assay

HCT-116 cells were incubated in RPMI-1640 with 10% FBS and collected *via* trypsinization. Cells (2×10^5 cells/well) in serum-free medium were added to the inner cup of a 24-well transwell chamber (Corning Life Sciences, Oneonta, NY, USA) that had been coated with 50

ml of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA; 1:10 dilution in serum-free medium). Medium supplemented with 10% serum or the indicated agent was then added to the outer cup. After 48 h, those cells that had migrated through the Matrigel and the 8-mm pore size membrane were fixed, stained with hematoxylin and eosin (H&E, Sigma-Aldrich Chemical Co.), and then photographed under an inverted microscope. Each experiment was performed in triplicate.

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Figure legends

Fig. 1. Inhibitory effects of cordycepin on the migration and invasion of human colorectal carcinoma HCT116 cells. (A) Cells were incubated with varying concentrations of cordycepin in the absence or presence of LPS for 48 h in serum-free medium, and proliferation was determined using an MTT assay. The data are expressed as the mean ±SD of triplicate experiments. *$p<0.05$ compared to control. (B) Cells at 80% confluence were treated with various concentrations of cordycepin in serum-free medium. The conditioned media was collected after 48 h, and gelatin zymography was performed in triplicate. Representative blots are shown. The equal amounts of cellular proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with specific antibodies and were visualized using an ECL. Actin was used as an internal control. (C) Cells were scratched with a pipette tip and treated with cordycepin or LPS for 48 h. Migrated cells were imaged using phase-contrast microscopy. (D) The cells were plated onto the apical side of Matrigel-coated filters in serum-free medium. Medium containing 20% FBS was placed in the basolateral chamber to act as a chemoattractant. After 48 h, cells on the apical side were removed using a Q-tip. The cells on the bottom of the filter were then stained using H&E, and then photographed.

Fig. 2. Inhibitory effects of cordycepin on PGE_2 production and expression of COX-2 and EPs in LPS-stimulated HCT-116 cells. Cells were pre-treated with 50 μg/ml of cordycepin for 1 h before incubation with LPS for 48 h. Total RNA (A; upper panel) and proteins (A; lower panel) was prepared and used for RT-PCR analysis of COX-2 gene expression with GAPDH as an internal control. Actin was used as an internal control. (B) The culture supernatants were isolated, and the amount of PGE_2 production was determined. The data are expressed as the mean ± SD of three

independent experiments. **p<0.01 compared to LPS alone. (C) Total RNA was prepared and used for RT-PCR analysis of EP1-4 gene expression. (D) The data represent the EP1-4 mRNA levels in the LPS-treated cultures compared with control cultures from three independent experiments (**p<0.01, ***p<0.001).

Fig. 3. Increase in cordycepin-induced anti-invasiveness by inhibition of EP4 signaling in HCT-116 cells. (A and B) Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with AH23848 or cordycepin (50 μg/ml) for 48 h. The cells were lysed, and equal amounts of protein were subjected to Western blotting. (C and D) Cells were pretreated with LPS (2.5 μg/ml) for 1 h before being challenged with AH23848 or cordycepin (50 μg/ml) for 6 h. The cell migration (C) and Matrigel invasion (D) assays were performed. The experiment was repeated three times, and similar results were obtained.

Fig. 4. Involvement of the AMPK pathway in the reduction of EP4 expression by cordycepin. (A; upper panel) Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with cordycepin (50 μg/ml) for the indicated times. (A; lower panel) Cells were treated with LPS (2.5 μg/ml) for the indicated times. Immunoblotting analyses were performed with anti-pAMPK, anti-AMPK, anti-pACC and anti-ACC antibodies. (B) The effect of cordycepin and compound C on AMPK activation in LPS-stimulated HCT-116 cells. Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with either cordycepin (50 μg/ml) only or both cordycepin and compound C (10 μM) for 12 h. (C) The effect of cordycepin and compound C on EP4 expression in LPS-stimulated cells. Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with either cordycepin (50 μg/ml) only or both cordycepin and compound C (10 μM) for 12 h. (D) The cells were transfected with AMPK small interfering (siRNA) or control siRNA for 24 h. The transfected cells were treated

with 50 μg/ml cordycepin for 48 h. (E; upper panel) Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with cordycepin (50 μg/ml) for the indicated times. (E; lower panel) Cells were treated with LPS (2.5 μg/ml) for the indicated times. Cells were pretreated with LPS (2.5 μg/ml) for 1 h before treatment with either cordycepin (50 μg/ml) only or both cordycepin and H89 (10 μM) for 12 h. Then, we performed Western blot and RT-PCR (F) and cell migration and invasion assay (G).

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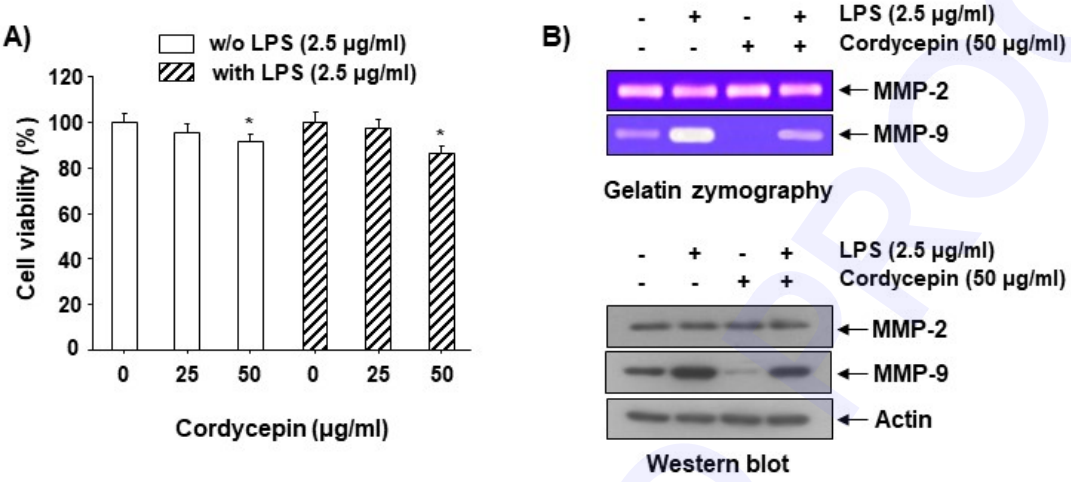


Fig. 1. Fig. 1-1

Fig. 1. Jeong *et. al.*

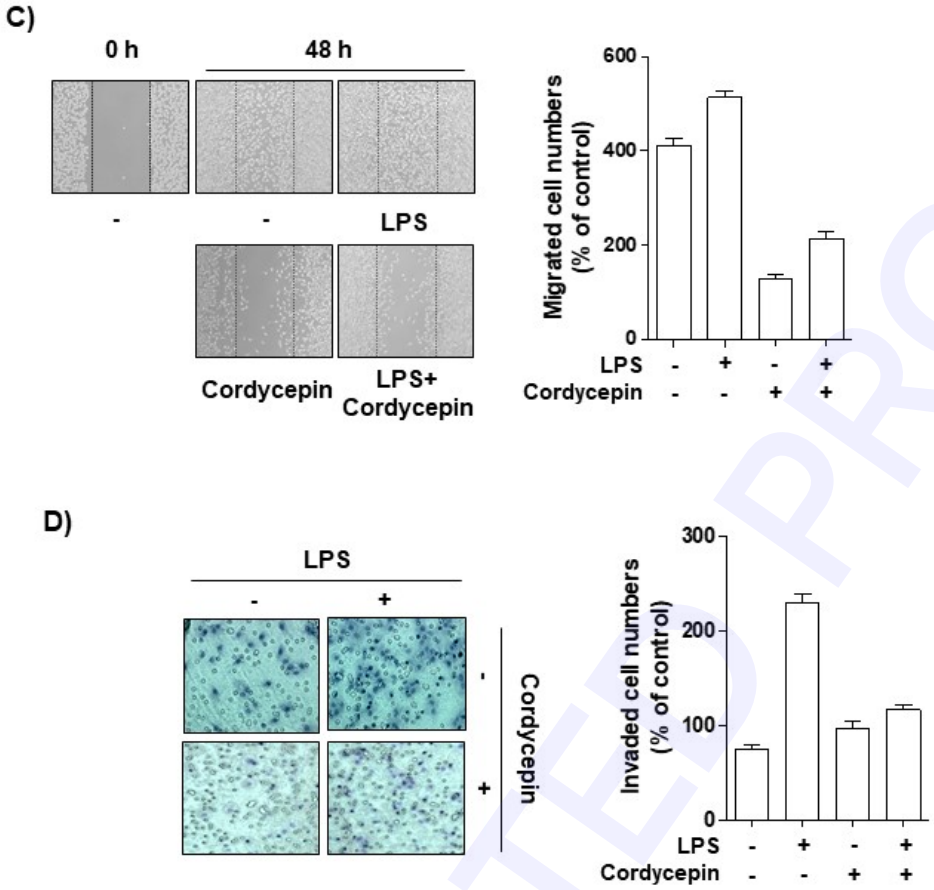


Fig. 2. Fig. 1-2

Fig. 2. Jeong *et. al.*

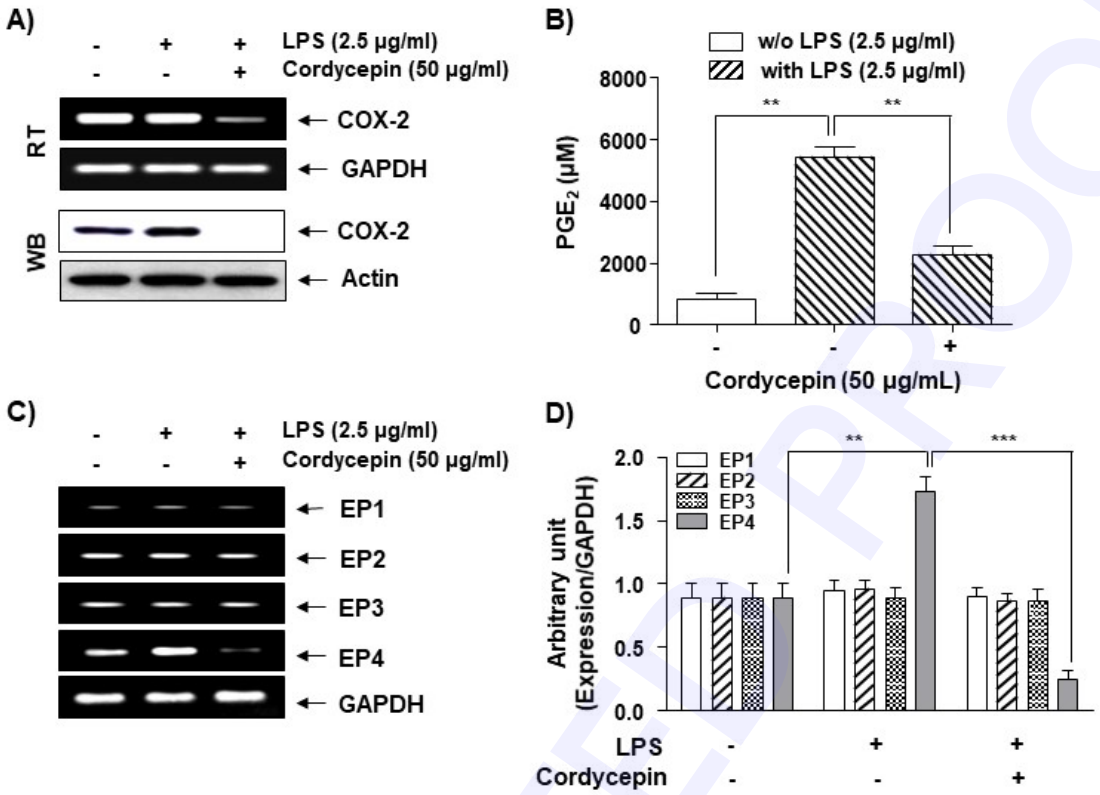


Fig. 3. Fig. 2

Fig. 3. Jeong *et. al.*

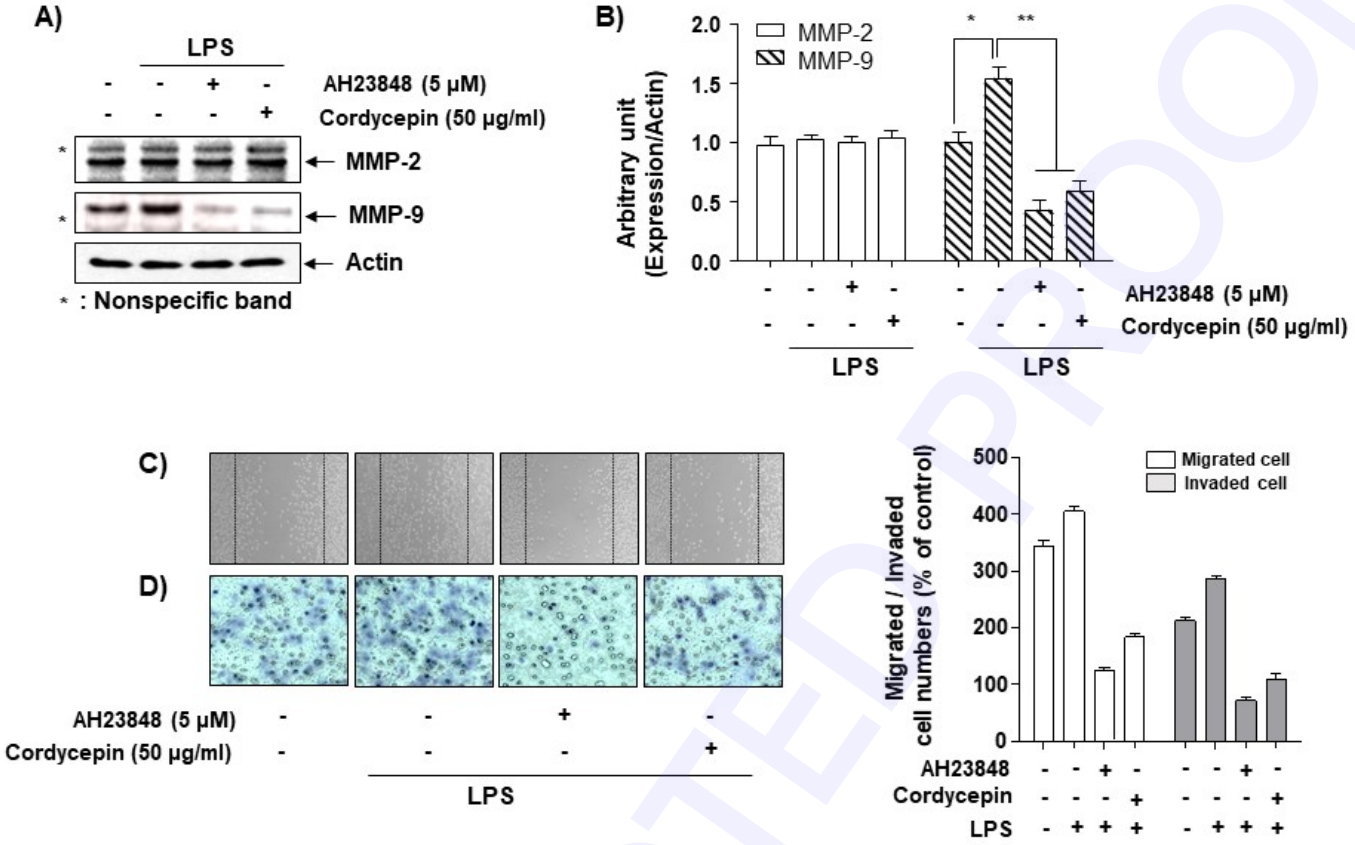


Fig. 4. Fig. 3

Fig. 4. Jeong *et. al.*

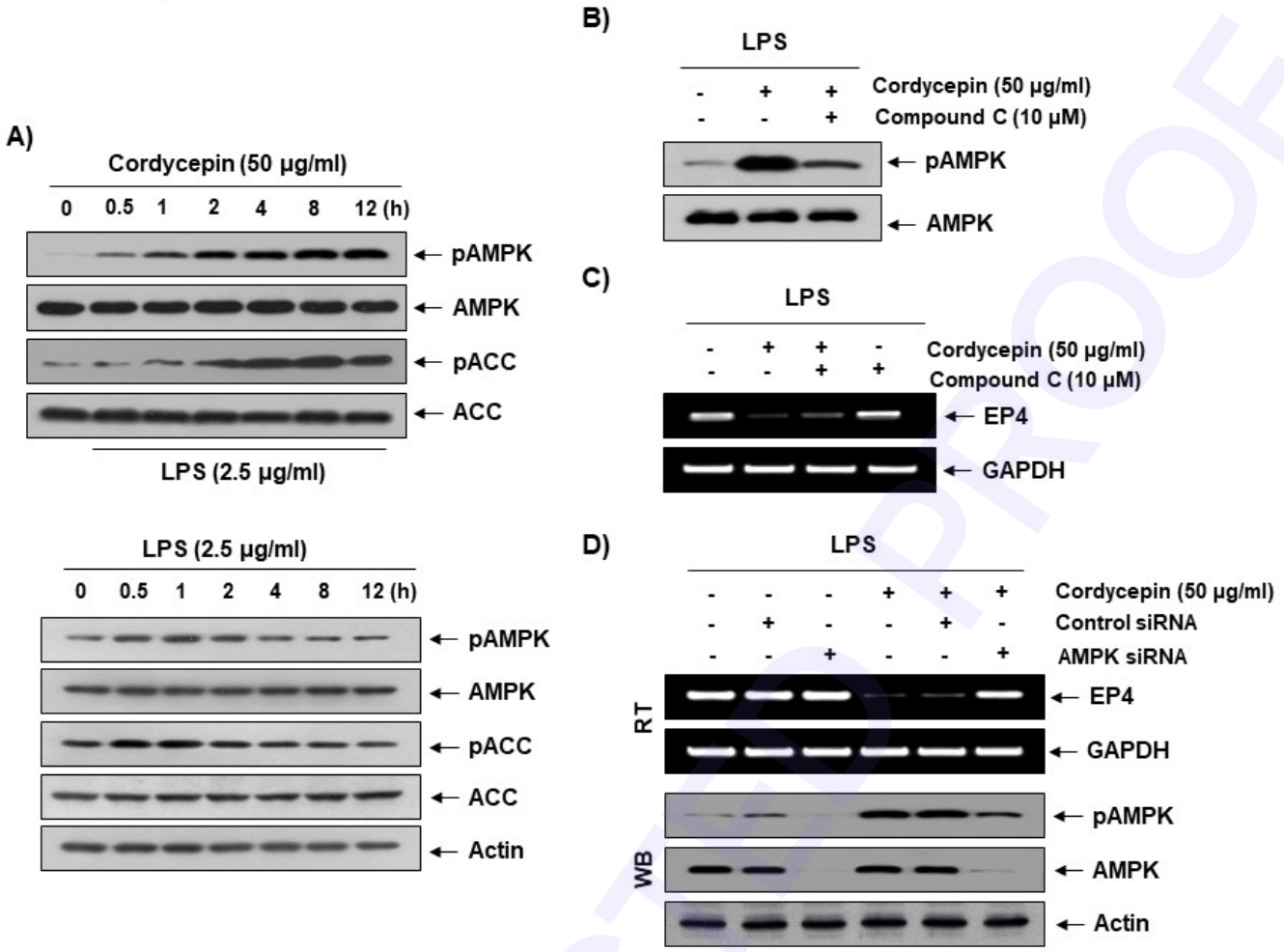


Fig. 5. Fig. 4-1

Fig. 4. Jeong *et. al.*

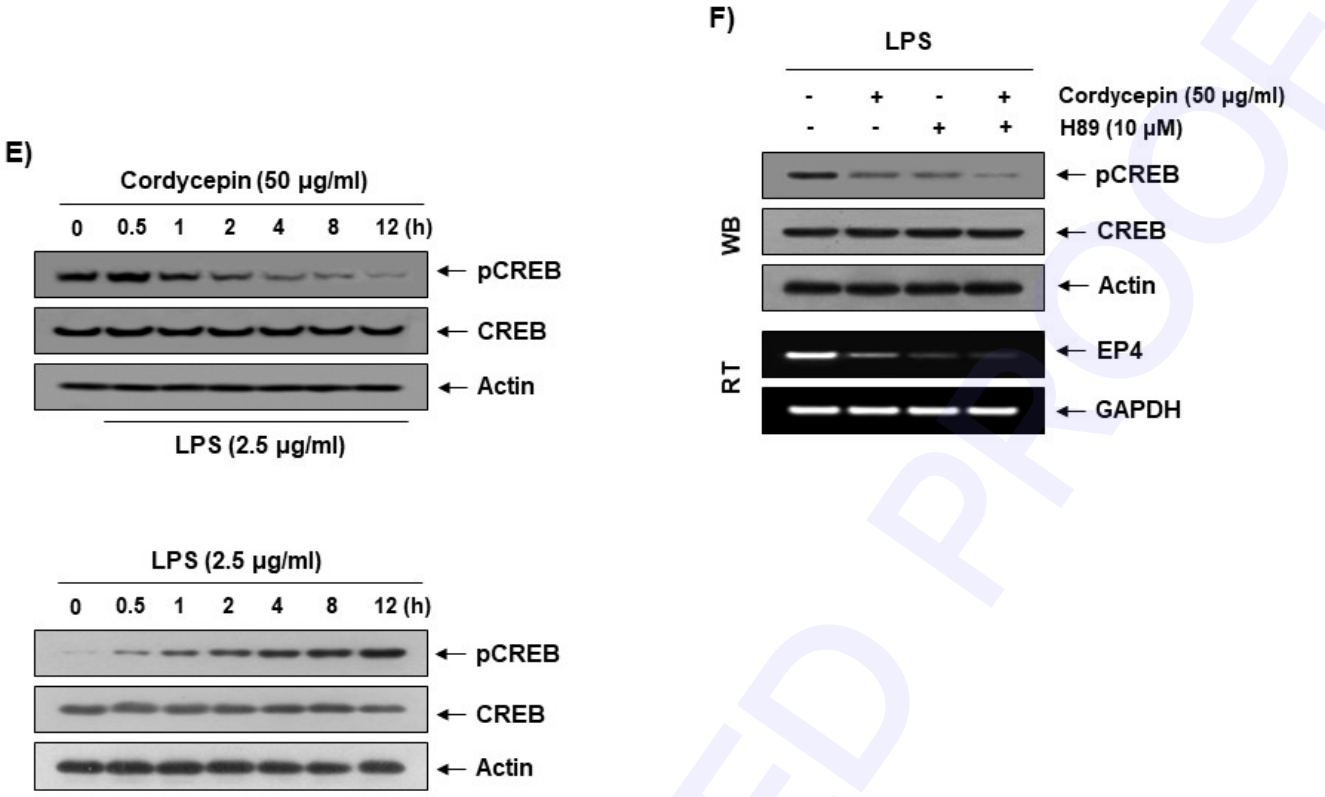


Fig. 6. Fig. 4-2

Fig. 4. Jeong *et. al.*

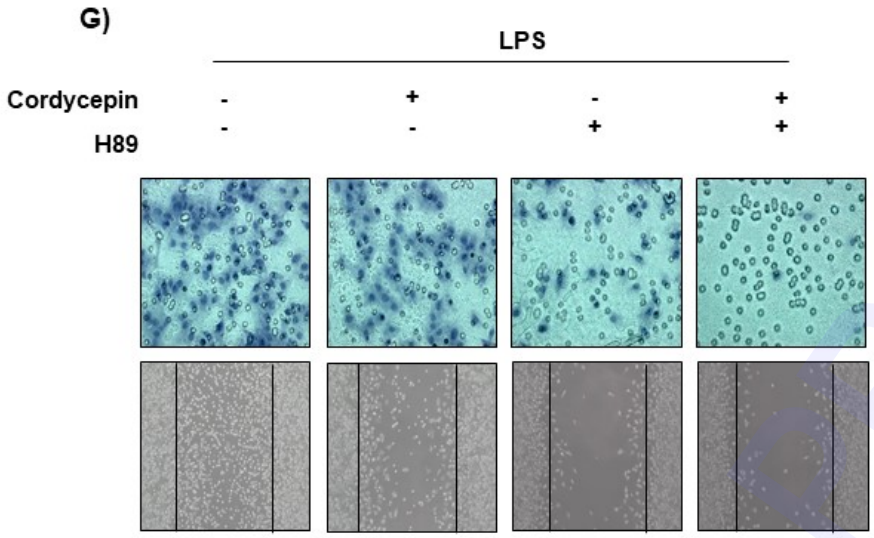
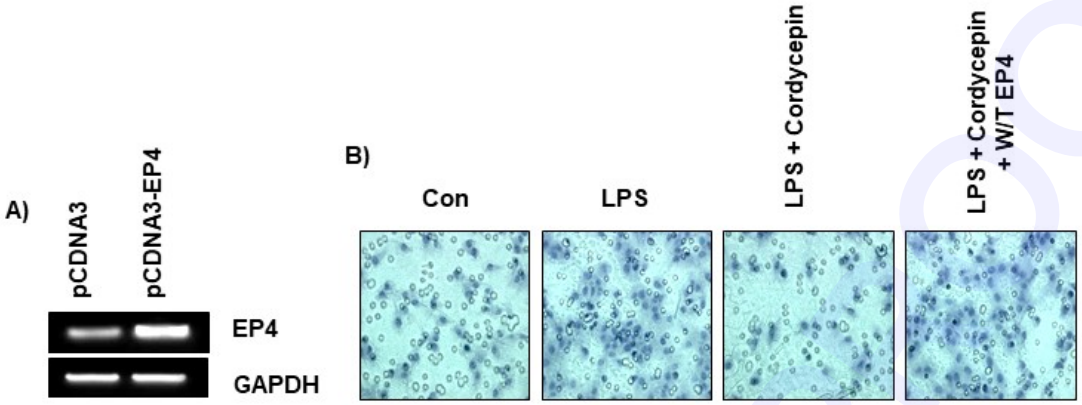


Fig. 7. Fig. 4-3

Supplement. Table 1. Sequences of the primers used in RT-PCR analysis.

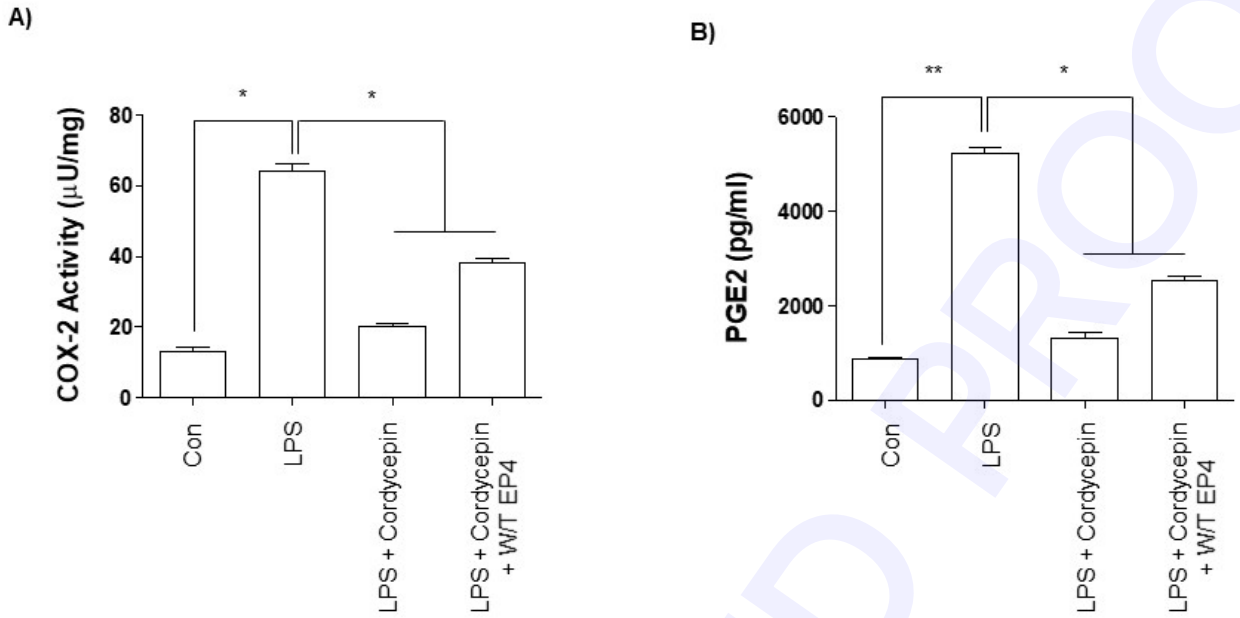
Gene	Primer	Sequence	Accession number
COX-2	Sense primer	CAGCAAATCCTTGCTGTTCC	XM-004028064.1
	Anti-sense primer	TGGGCAAAGAATGCAAACATC	
EP1	Sense primer	ATGTCCGAAGCAAACATCAC	NM-010927.3
	Anti-sense primer	TAATGTCCAGGAAGTAGGTG	
EP2	Sense primer	ATGAGCACAGAAAGCATGATC	NM-013693.1
	Anti-sense primer	TACAGGCTTGTCACTCGAATT	
EP3	Sense primer	CTCGTGCTGTCGGACCCATAT	NM-008361.3
	Anti-sense primer	TTGAAGACAAACCGCTTTTCCA	
EP4	Sense primer	TGGTATGTGGGCTGGCTG	NM-000958
	Anti-sense primer	GAGGACGGTGGCGAGAAT	
GAPDH	Sense primer	TTCACCACCATGGAGAAGGC	XR031141.1
	Anti-sense primer	GGCATGGACTGTGGTCATGA	

sFig. 1. Jeong *et. al.*



Sup. 2.

sFig. 2. Jeong *et. al.*



Sup. 3.