

# *Helicobacter pylori* stimulates urokinase plasminogen activator receptor expression and cell invasiveness through reactive oxygen species and NF- $\kappa$ B signaling in human gastric carcinoma cells

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Received December 21, 2006; Accepted January 29, 2007

**Abstract.** The gastric pathogen, *helicobacter pylori* (*H. pylori*), has been associated with the progression of gastric cancer. It was previously reported that *H. pylori* induced urokinase plasminogen activator receptor (uPAR) expression and stimulated cell invasiveness in human gastric cancer AGS cells. However, the precise mechanisms for how *H. pylori* upregulates uPAR are unclear. This study investigated the underlying signal pathways in *H. pylori*-induced uPAR in human gastric cancer AGS cells. The intracellular H<sub>2</sub>O<sub>2</sub> content, as determined using H<sub>2</sub>O<sub>2</sub>-sensitive probe 2',7'-dichlorodihydrofluorescein, increased after the *H. pylori* treatment. N-acetyl cysteine (NAC), an antioxidant, prevented the *H. pylori*-induced production of H<sub>2</sub>O<sub>2</sub> and uPAR expression. In addition, exogenous H<sub>2</sub>O<sub>2</sub> was found to increase uPAR mRNA expression and its promoter activity. Site-directed mutagenesis of the potential NF- $\kappa$ B element in the uPAR promoter showed that the redox-sensitive transcription factor NF- $\kappa$ B was essential for *H. pylori*-induced uPAR expression. The expression of vectors encoding a mutated-type NF- $\kappa$ B-inducing kinase and I- $\kappa$ B, and a specific inhibitor of NF- $\kappa$ B (BAY11-7082) decreased the *H. pylori*-induced uPAR promoter activity. Chromatin immunoprecipitation and the electrophoretic mobility shift assay confirmed that *H. pylori* increased the DNA binding activity of NF- $\kappa$ B. With the aid of NAC and H<sub>2</sub>O<sub>2</sub>, it was determined that reactive oxygen species (ROS) is an upstream signaling molecule for activating the NF- $\kappa$ B induced by *H. pylori*. The enhanced AGS cell invasiveness by *H. pylori* was partially abrogated by an NAC and BAY11-7082 treatment. These

results suggest that the ROS and NF- $\kappa$ B signaling pathway is important in *H. pylori*-induced uPAR expression and the increased cell invasiveness of human gastric cancer AGS cells.

## Introduction

Despite the improved prognosis of gastric cancer resulting from the early diagnosis and development of adjuvant therapy, gastric cancer remains the second most common cause of cancer deaths worldwide. Approximately 80% of the gastric cancers in Western countries are at an advanced stage at diagnosis (1). Chemotherapy does not significantly affect the length or quality of life of advanced gastric cancer patients due to local invasion and metastasis. An understanding of the detailed mechanisms of invasion and metastasis in gastric cancer would be helpful in improving the treatment outcome.

*H. pylori* is believed to be the major etiological factor in the development of gastric cancer. Large-scale epidemiological studies have confirmed the strong association between *H. pylori* infection and both cancer and its histological stages (2). Animal models have also demonstrated the importance of *H. pylori* in gastric carcinogenesis (3). Recently, studies have indicated that *H. pylori* plays an important role, not only in the development of gastric cancer, but also in the process of tissue remodeling, angiogenesis, tumor invasion and metastasis (4).

Cancer invasion and metastasis are multifactorial processes that require the coordinated action of cell-secreted proteolytic enzymes and their inhibitors (5). Urokinase-type plasminogen activator (uPA), its inhibitors, and uPA receptor (uPAR) form a complex proteolytic system that has been implicated in cancer invasion and metastasis. uPA, a serine protease, has the ability to convert plasminogen to active plasmin (6). Furthermore, the uPA-uPAR binding interaction can independently affect cell motility, integrin function, and gene expression (7). In many cancers, uPAR expression is required for the invasive and metastatic phenotype (8). uPAR overexpression increases the ability of cancer cells to penetrate a barrier of a reconstituted basement membrane. In contrast, the blockade of the uPAR by the expression of a catalytically

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**Key words:** *helicobacter pylori*, urokinase plasminogen activator receptor, reactive oxygen species, NF- $\kappa$ B, gastric cancer

inactive enzyme or an antisense uPAR cDNA significantly decreases the invasiveness of cancer cells. In gastric cancer, increased levels of uPA and uPAR have been clearly demonstrated to be essential for maintaining the invasive and metastatic phenotypes, and these increases are considered to be prognostically significant (9).

The level of uPAR synthesis is increased by a diverse set of agents including the epidermal growth factor, vascular endothelial growth factor, hepatocyte growth factor, fibroblast growth factor, transforming growth factor, okadaic acid and phorbol ester (8,10). An analysis of the genomic structure of uPAR reveals many potential targets for the regulation at both the transcriptional and posttranscriptional levels. Within its 3'-flanking region, the uPAR gene contains a single-copy AUUUA sequence that is responsible for the destabilization of various mRNAs (11). Within its 5'-flanking region, the uPAR gene contains the putative binding sites for an activator protein-1 (AP-1), AP-2, and Sp1 but no potential TATA or CAAT boxes (12).

An *H. pylori* infection has been reported to induce a number of genes in cells that are potential determinants of angiogenesis and metastasis including vascular endothelial growth factor and cyclooxygenase-2. However, it is unclear how *H. pylori* can activate these specific genes (13). A myriad of intracellular signals has been suggested to mediate the effects of *H. pylori*, including the activation of mitogen-activated protein kinase (MAPK), the release of arachidonic acid, and the regulation of the transcription factors, AP-1 and NF- $\kappa$ B (14). In addition, *H. pylori* stimulates the production of reactive oxygen species (ROS), which is a class of highly reactive, diffusible, and ubiquitous molecules in various cell types (15).

In this study, it was found that *H. pylori* induces the expression of uPAR via ROS and the NF- $\kappa$ B signaling pathway, and stimulates the invasiveness of human gastric cancer AGS cells.

## Materials and methods

**Bacterial strains.** The *H. pylori* strains, 26695 and J99, were obtained from the American Type Culture Collection (Manassas, VA, USA). The *H. pylori* strain 51 was isolated from a human biopsy obtained from a patient with a duodenal ulcer at Gyeongsang National University Hospital, Jinju, Korea (16). The frozen *H. pylori* strains were thawed and grown on brucella agar containing DMCD (1 mg/ml), vancomycin (10  $\mu$ g/ml), nalidixic acid (25  $\mu$ g/ml) and amphotericin B (1  $\mu$ g/ml) at 10% CO<sub>2</sub> and 37°C. One loop of bacteria grown overnight was inoculated on a brucella agar plate enriched with DMCD (1 mg/ml) and cultured overnight under the same conditions.

**Cell culture and culture conditions.** Human gastric carcinoma AGS cells were obtained from the American Type Culture Collection. The cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The effect of *H. pylori* on uPAR expression was determined by harvesting the cells cultivated with various numbers of *H. pylori* and examining the expression of uPAR by

Western and Northern blot analyses. The role of the specific signaling pathways in uPAR induction by *H. pylori* was examined by pretreating the AGS cells with 0-20 mM N-acetyl cysteine (NAC, an antioxidant, Sigma Chemical Co., St. Louis, MO, USA) and 0-20  $\mu$ M BAY11-7082 (a specific inhibitor of NF- $\kappa$ B, Calbiochem, San Diego, CA, USA) for 1 h prior to exposure to *H. pylori*.

**Western blot analysis.** The cells were washed in phosphate-buffered saline (PBS), detached using Trypsin-EDTA buffer, and stored at -70°C until needed. The protein was extracted with a RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and protease inhibitors (aprotinin, leupeptin, PMSF, benzamidine, trypsin inhibitor, sodium orthovanadate). Fifty micrograms of the proteins was then separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked in a PBS solution containing 5% nonfat dry milk, incubated with the primary antibodies in a blocking solution overnight at 4°C, and washed three times with TPBS [Tween-20 (0.1%), PBS] at 10-min intervals. Horseradish peroxidase-conjugated secondary antibodies (Amersham Corp., Arlington Heights, IL, USA) were used to detect the immunoreactive proteins by chemiluminescence. The following antibodies were used: anti-uPAR (American Diagnostica Inc, Greenwich, CT, USA) and anti-I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The total protein levels were assayed by washing the blotted membrane with a stripping solution [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7)] for 30 min at 50°C, and the membrane was then reprobed with the anti- $\beta$ -actin mouse monoclonal antibody (Sigma Chemical Co.).

**Matrigel invasion assay.** A cell invasion assay was performed using a BioCoat™ matrigel invasion chamber (Becton-Dickinson, Bedford, MA, USA) according to the manufacturer's protocol. Briefly, 10<sup>5</sup> cells in 300  $\mu$ l were added to each chamber and allowed to invade the matrigel for 24 h. The noninvading cells on the upper surface of the membrane were removed from the chamber, and the invading cells on the lower surface of the membrane were stained using a Quick-Diff stain kit (Becton-Dickinson). The chambers were washed twice with water and allowed to air dry. The number of invading cells was counted using phase-contrast microscopy. The effect of the anti-uPAR antibody on the *H. pylori*-induced cell invasion was determined by preincubating the *H. pylori*-treated cells with 1  $\mu$ g/ml of the neutralizing antibody to uPAR for 10 min and adding them to the invasion chamber.

**Measurement of intracellular H<sub>2</sub>O<sub>2</sub>.** The level of intracellular H<sub>2</sub>O<sub>2</sub> was measured using 5- and 6-carboxyl-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes, Eugene, OR, USA) according to the procedure reported by Hwang *et al.* (17). Briefly, the cells were grown in serum-starved RPMI-1640 medium supplemented with 0.5% FBS for an additional 2 days. The cells were then stabilized in serum-free RPMI-1640 medium without phenol red for at least 30 min before being exposed to *H. pylori* for 0-90 min. The effect of NAC was assessed by pretreating the cells with NAC for 30 min and incubating them with the H<sub>2</sub>O<sub>2</sub>-sensitive



SPANDIDOS PUBLICATIONS are DCFDA (5 ng/ml) for 10 min. The cells were immediately under a laser-scanning confocal microscope. The DCF fluorescence was excited at 488 nm using an argon laser, and the emission evoked was filtered with a 515-nm-long pass filter.

**Northern blot analysis.** The total RNA extraction and Northern blot hybridization were performed as previously described (18). Each cDNA probe was radiolabeled with [ $\alpha$ - $^{32}$ P]deoxyribonucleoside triphosphate using a random-priming technique with the Rediprime labeling system (Amersham Corp.). The probed nylon membranes were exposed to radiographic film (Life Technologies, Inc., Grand Island, NY, USA).

**Measurement of uPAR promoter activity.** The transcriptional regulation of uPAR was examined using the transient transfection with a uPAR promoter-luciferase reporter construct (pGL3-uPAR) (18). The plasmid pGL3/uPAR-promoter was kindly provided by Dr Y. Wang (Australian National University, Canberra, Australia). The AGS cells ( $5 \times 10^5$ ) were seeded and grown until they reached 60-70% confluence, and pRLTK (an internal control plasmid containing the herpes simplex thymidine kinase promoter linked to the constitutively active Renilla luciferase reporter gene) and pGL3-uPAR were then cotransfected into cells using FuGENE 6 (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's protocol. pRLTK and pGL3 were cotransfected as a negative control. The cells were incubated in the transfection medium for 20 h and treated with *H. pylori* for 4 h. The effects of the inhibitors on the uPAR promoter activity were determined by pretreating cells with the inhibitors for 1 h prior to the addition of *H. pylori*. The cotransfection studies were performed in the presence or absence of 2  $\mu$ g dominant-negative mutants of I- $\kappa$ B $\alpha$ , I- $\kappa$ B $\beta$  and NF- $\kappa$ B-inducing kinase (NIK). The dominant-negative mutants of I- $\kappa$ B $\alpha$  and I- $\kappa$ B $\beta$  (19) and NIK (20) were kindly provided by Dr D.W. Ballard (Vanderbilt University School of Medicine, Nashville, TN, USA) and Dr W.C. Greene (University of California, San Francisco, CA, USA), respectively. The cells were harvested using a passive lysis buffer (Dual-Luciferase Reporter assay system; Promega, Madison, WI, USA), and the luciferase activity was determined using a single sample luminometer according to the manufacturer's protocol.

**Extraction of nuclear proteins.** AGS cells at 80-90% confluence were incubated overnight in the medium containing 5% FBS and then treated with various numbers of *H. pylori*. The cells were then re-suspended in 500  $\mu$ l cold buffer A [50 mM Tris (pH 7.4), 150 mM NaCl, 0.2 mM EDTA, 3% (v/v) glycerol, and 1.5 mM MgCl<sub>2</sub>]. After the cells had been allowed to swell for 5 min on ice, they were lysed with 500  $\mu$ l of buffer B [identical to buffer A except containing 0.05% Nonidet P-40 (Sigma)]. The homogenate was gently layered onto an equal volume cushion of buffer C [10 mM Tris (pH 7.4), 25% (v/v) glycerol, and 1.5 mM MgCl<sub>2</sub>] and centrifuged for 5 min at 200  $\times$  g. The white nuclear pellet was re-suspended in 75  $\mu$ l of a cold high-salt lysis buffer [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM

EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride]. This suspension was agitated for 30 min at 4°C and then microcentrifuged for 15 min at 4°C. The resulting supernatant was stored in aliquots at -80°C.

**Electrophoretic mobility shift assay (EMSA).** EMSA was carried out using a Gel Shift assay system (Promega). Briefly, an oligonucleotide containing the consensus sequence of NF- $\kappa$ B (5'-AGT TGA GGG GAC TTT CCC AGG-3') was end-labeled with [ $\alpha$ - $^{32}$ P]adenosine triphosphate (3000  $\mu$ Ci/mmol; Amersham Pharmacia Biotech., Buckinghamshire, UK) using T4 polynucleotide kinase, purified in Microspin G-25 columns (Sigma) and used as the probe for EMSA. The nuclear extract proteins (6  $\mu$ g) were pre-incubated with the binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 4% (v/v) glycerol, and 0.05 mg/ml poly(deoxyinosine-deoxycytosine)] for 5 min and then incubated with the radiolabeled probe for 15 min at 37°C. Each sample was electrophoresed in a 5% nondenaturing polyacrylamide gel in 0.5X Tris borate-EDTA buffer at 150 V for 4 h. The gel was then dried and subjected to autoradiography.

**Transient transfection of NF- $\kappa$ B-reporter.** The NF- $\kappa$ B-reporter construct was purchased from Clontech (Palo Alto, CA). After the cells reached 80-90% confluence, they were washed with RPMI-1640 and incubated with RPMI-1640 in the absence of serum and antibiotics for 5 h. The cells were then transfected with 1  $\mu$ g NF- $\kappa$ B-reporter containing pGL3 vector using FuGENE 6 (Boehringer Mannheim) for 24 h. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation (ChIP) was performed using the ChIP assay kit (Upstate, Lake Placid, NY) according to the manufacturer's recommendations. The cells grown on 100-mm dishes were collected and cross-linked using 1% formalin for 10 min at room temperature. The extracts were sonicated until the DNA fragments were 500-800 bp in size. The cell extracts were then subsequently incubated with 2  $\mu$ g IgG or the antibody against NF- $\kappa$ B (Santa Cruz, sc-7151) overnight at 4°C. The extracts were incubated with salmon sperm DNA/protein A-sepharose beads (Upstate, Charlottesville, VA) for 1 h. After extensive washing of the beads, the proteins were eluted and reversed by heating them for 4 h at 65°C. After DNA purification, PCR was performed for 35-40 cycles. The following primers were used for the NF- $\kappa$ B site (284 bp): 5'-TTTTACAGCAGTGGAGAGCG-3' (sense) and 5'-TCAGGAAGGAGGCAGTTTTG-3' (antisense).

## Results

**Effect of *H. pylori* on the induction of uPAR and cell invasiveness.** AGS cells were cocultured with 3 different strains of *H. pylori* (26695, 51, J99), and the level of the uPAR protein in the cells was measured. As shown in Fig. 1A, all the examined *H. pylori* strains induced uPAR in the gastric AGS cells. It was also found that *H. pylori* induced uPAR in a dose-dependent manner at a bacterium/cell ratio



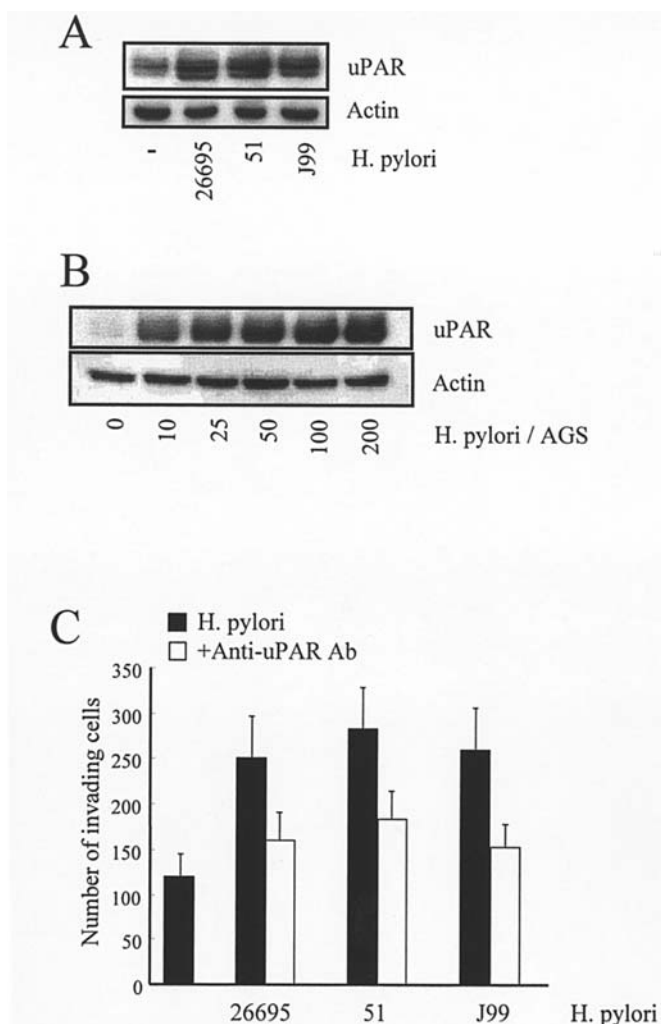


Figure 1. Induction of uPAR and the cell invasiveness by *H. pylori* in human gastric AGS cells. (A and B) Western blot analysis was performed to determine the level of the uPAR protein in the AGS cells. (A) The AGS cells were incubated with the 3 different *H. pylori* strains (26695, 51, J99) for 8 h at a bacterium/cell ratio of 100:1. (B) The cells were incubated with *H. pylori* 51 for 8 h at a bacterium/cell ratio of 0-200:1. (C) AGS cells ( $10^5$ ) were incubated with the 3 different strains of *H. pylori* ( $10^7$ ) in BioCoat marigel invasion chambers for 24 h in the presence or absence of 50  $\mu$ g/ml anti-PAR antibodies (anti-uPAR). After incubation, the invading cells on the undersurface of the chambers were counted using phase contrast light microscopy after staining with a Diff-Quick stain kit. The data represents the means  $\pm$  SD from triplicate measurements.

of 1:1-200:1 (Fig. 1B). It was suggested that uPAR is essential for the invasive phenotype of cancer cells (18). The role of *H. pylori*-induced uPAR in the AGS cell invasion was examined by incubating the cells with specific antibodies to uPAR in a modified Boyden invasion chamber. As shown in Fig. 1C, the cell invasiveness was increased remarkably by incubation with the *H. pylori* strains. However, the *H. pylori*-treated cells partially lost their matrigel invasiveness after being incubated with uPAR neutralizing antibodies. The nonspecific IgG did not affect the *H. pylori*-induced cell invasiveness (data not shown). This suggests that the uPAR induced by *H. pylori* plays an important role in the invasiveness of gastric cancer cells.

*Involvement of ROS in uPAR induction by H. pylori.* The underlying signaling in the *H. pylori*-induced uPAR

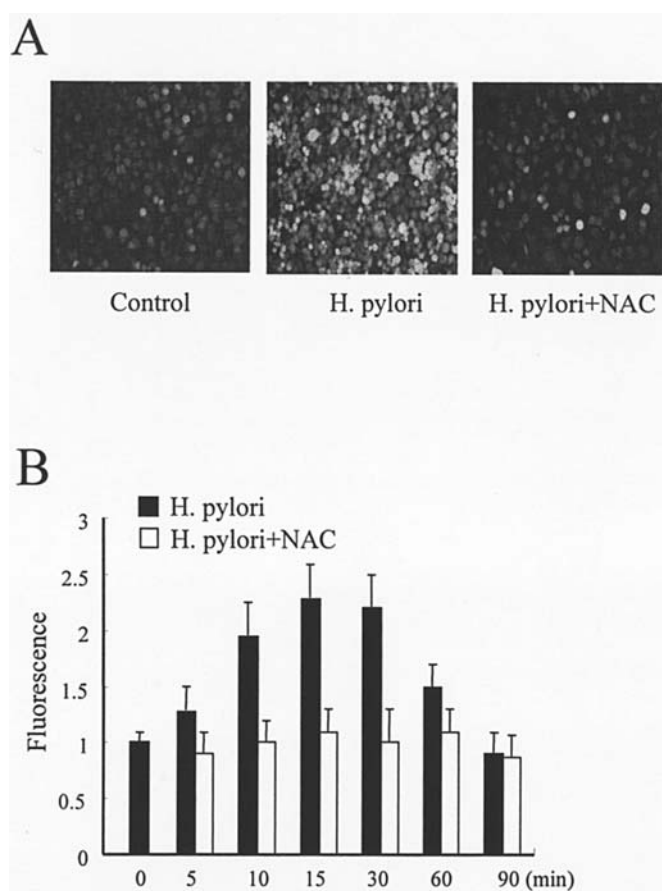


Figure 2. Production of ROS by *H. pylori*. Synchronized quiescent AGS cells, after being pretreated or not with 5 mM NAC for 1 h, were incubated with *H. pylori* 51 for 0-90 min at a bacterium/cell ratio of 100:1. The cells were then incubated in the dark for 10 min with 5 mg/ml DCFDA. The DCF fluorescence was imaged with a confocal laser scanning fluorescence microscope (A) and then quantified (B).

expression was investigated by determining the change in the level of ROS in AGS cells treated with *H. pylori* using the  $H_2O_2$ -sensitive fluorophore, DCFDA. The results showed that *H. pylori* induced the production of ROS in the cells (Fig. 2A and B). The level of intracellular ROS increased progressively after incubating the cells with *H. pylori* reaching a peak at ~15 min, with a subsequent slow decline thereafter. Pretreating the cells with 5 mM NAC, an ROS scavenger, almost completely inhibited the production of ROS (Fig. 2A and B). The effects of NAC on the expression of uPAR mRNA by *H. pylori* was next examined. As shown in Fig. 3A, the NAC pretreatment partially blocked the *H. pylori*-induced expression of uPAR mRNA. The effect of NAC on the transcriptional regulation of the uPAR gene was also examined. To this end, AGS cells were transiently transfected with the promoter-reporter construct (pGL3-uPAR) of the human uPAR gene and the luciferase gene. The AGS cells transfected with pGL3-uPAR showed a 4-fold increase in the promoter activity after the *H. pylori* treatment. When the transfected cells were pretreated with NAC before the *H. pylori* treatment, the induction of the uPAR promoter activity by *H. pylori* was inhibited in a dose-dependent manner (Fig. 3B). In addition, exogenous  $H_2O_2$  alone induced uPAR expression (Fig. 3C).

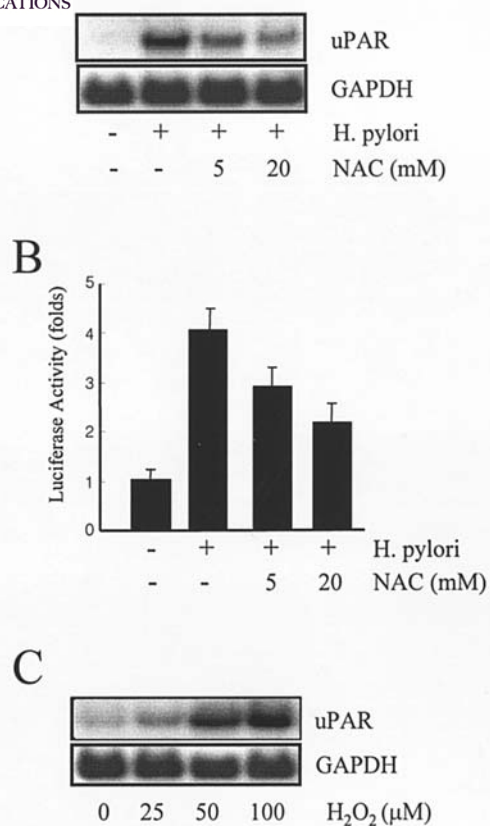


Figure 3. Involvement of ROS in *H. pylori*-induced uPAR expression. (A) AGS cells, after being pretreated with 0-20 mM NAC for 1 h, were incubated with *H. pylori* 51 for 4 h at the bacterium/cell ratio of 100:1. After incubation, the uPAR mRNA levels in the cell lysates were determined by Northern blot analysis. (B) AGS cells, after being pretreated with 0-20 mM NAC for 1 h, were transiently transfected with 1 μg pGL3-uPAR. The transfected cells were incubated with *H. pylori* 51 at a bacterium/cell ratio of 100:1 for 4 h. After incubation, the cells were lysed and the luciferase activity was measured using a luminometer. The data represent the means ± SD from triplicate measurements. (C) AGS cells were incubated with 0-100 μM H<sub>2</sub>O<sub>2</sub> for 4 h, and the uPAR mRNA in the cell lysates was determined by Northern blot analysis.

**Activation of NF-κB by *H. pylori*.** *H. pylori* was further examined to determine if it could activate the transcription factor NF-κB in gastric AGS cells. As shown in Fig. 4A, the *H. pylori* treatment caused a remarkable increase in the amount of NF-κB that could form complexes with the radiolabeled oligonucleotide probes in EMSA. The activation of NF-κB is usually associated with the induction of I-κB phosphorylation, which is followed by its degradation by proteasome and NF-κB nuclear translocation (21). Therefore, the change in the amount of I-κB in AGS cells was determined by Western blotting using an antibody to I-κB. The result showed that the AGS cells treated with *H. pylori* showed a loss of the I-κB protein in a dose-dependent manner (Fig. 4B). The extent to which *H. pylori* modulated NF-κB-dependent transcription activity was next evaluated using NF-κB-reporter (luciferase). As shown in Fig. 4C, the *H. pylori* treatment caused increases in the NF-κB-dependent transcription activity in a dose-dependent manner.

**Involvement of NF-κB in uPAR induction by *H. pylori*.** The role of the transcription factor NF-κB in the induction of uPAR

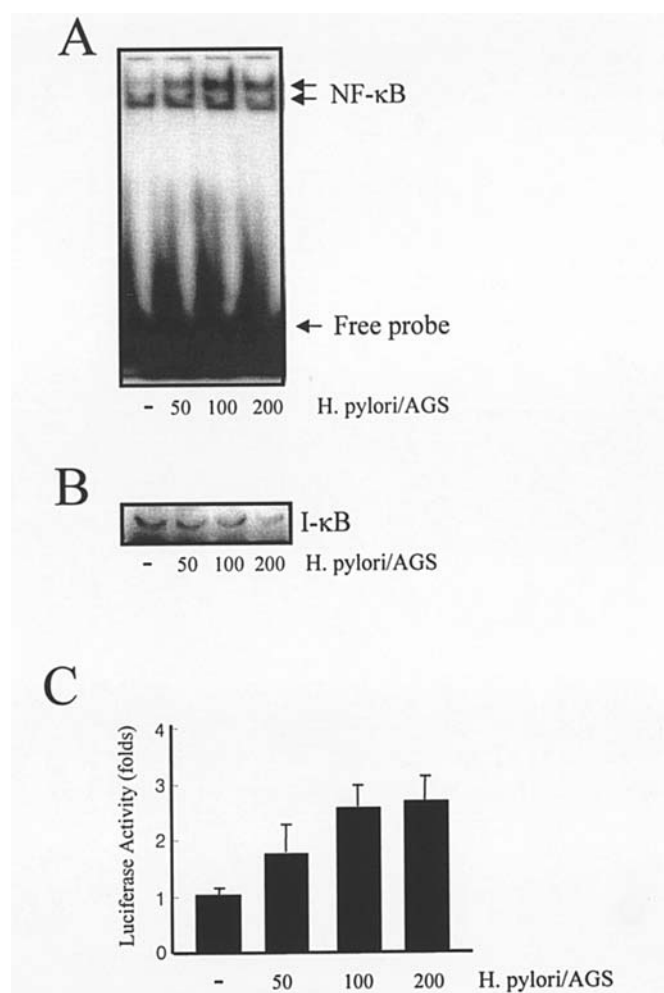


Figure 4. Activation of the transcription factor NF-κB by *H. pylori*. (A) AGS cells were treated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 0-200:1. The nuclear extracts from the cells were analyzed by EMSA for the activated NF-κB using a radiolabeled oligonucleotide probe. (B) AGS cells were treated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 0-200:1. The levels of the I-κB subunit of NF-κB in the cell lysates were determined by Western blot analysis. (C) AGS cells were transiently transfected with the pNF-κB-Luc-reporter construct. The transfected cells were incubated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 0-200:1. After incubation, the cells were lysed and the luciferase activity was determined using a luminometer. The data represents the means ± SD from triplicate measurements.

by *H. pylori* was examined by pretreating the AGS cells with 0-20 μM BAY11-7082 (a specific inhibitor of NF-κB) before the *H. pylori* treatment. BAY11-7082 blocked the *H. pylori*-induced uPAR mRNA expression and uPAR promoter activity in a dose-dependent manner (Fig. 5A and B). The importance of NF-κB in the induction of uPAR by *H. pylori* was confirmed by transfecting the AGS cells with the uPAR promoter-reporter in which the NF-κB site was mutated. As shown in Fig. 5B, a mutation of the NF-κB binding site significantly reduced the *H. pylori*-induced activation of the uPAR promoter. The effects of the dominant-negative mutant forms of I-κB and NIK on the uPAR promoter were investigated. The results showed that inhibition of the NF-κB signals significantly inhibited the *H. pylori*-induced uPAR promoter activity (Fig. 5C). In order to further confirm that transcription factor NF-κB is essential in *H. pylori*-induced uPAR, the binding of NF-κB on the uPAR promoter was

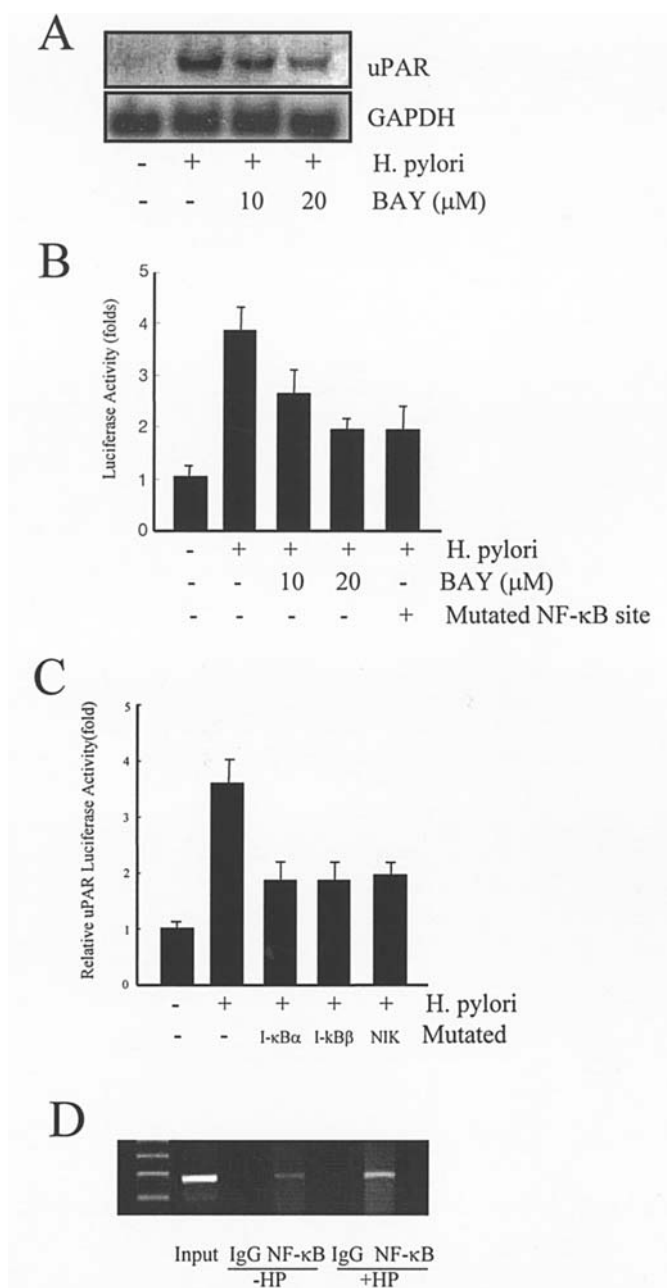


Figure 5. Involvement of NF- $\kappa$ B on *H. pylori*-induced uPAR expression. (A) AGS cells pretreated with 0-20  $\mu$ M BAY11-7082 (BAY) were incubated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 100:1. After incubation, the level of uPAR mRNA in the cell lysates was determined by Northern blot analysis. (B) AGS cells were transiently transfected with pGL3-uPAR. The transfected cells were pretreated with 0-20  $\mu$ M BAY11-7082 (BAY) for 1 h, and incubated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 100:1. Where indicated, mutated pGL3-uPAR (converting the NF- $\kappa$ B site GGGAGGAGT to GGATCCAGT) was transfected into the cells. (C) The dominant-negative mutants of I- $\kappa$ B $\alpha$  and  $\beta$ , and NIK were cotransfected with pGL3-uPAR into the AGS cells. After incubation with *H. pylori* 51 (HP) for 4 h at a bacterium/cell ratio of 100:1, the uPAR promoter activity in the cell lysates was determined. The data represents the means  $\pm$  SD from triplicate measurements. (D) AGS cells were incubated with *H. pylori* 51 for 4 h at the bacterium/cell ratio of 100:1. After incubation, the binding of NF- $\kappa$ B to the NF- $\kappa$ B binding sites on the human uPAR promoter sequences in the gastric AGS was determined by ChIP.

examined by chromatin immunoprecipitation. As shown in Fig. 5D, the level of binding of endogenous NF- $\kappa$ B to the NF- $\kappa$ B binding site on the uPAR promoter sequences was

increased after the *H. pylori* treatment. This suggests the involvement of NF- $\kappa$ B in the uPAR induction by *H. pylori*.

**Activation of NF- $\kappa$ B by ROS.** Considering that *H. pylori* can generate reactive oxygen species and that NF- $\kappa$ B is a redox-sensitive transcription factor, it is natural to speculate on the role of ROS with regard to NF- $\kappa$ B activation in *H. pylori*-mediated uPAR induction. The AGS cells were pretreated with NAC before the *H. pylori* treatment and the level of NF- $\kappa$ B-DNA binding was examined in EMSA. As shown in Fig. 6A, NAC inhibited the *H. pylori*-induced NF- $\kappa$ B-DNA binding. The effects of NAC on the NF- $\kappa$ B-dependent transcription activity were next examined. As shown in Fig. 6B, the NAC pretreatment blocked the *H. pylori*-induced NF- $\kappa$ B-dependent transcription activity in a dose-dependent manner. In addition, the exogenous  $H_2O_2$  alone induced the NF- $\kappa$ B-DNA binding (Fig. 6C). These results suggested that the intracellular  $H_2O_2$  produced by *H. pylori* contributed to the activation of NF- $\kappa$ B in gastric AGS cells.

**Effects of NAC and BAY11-7082 on the *H. pylori*-induced cell invasiveness.** This study showed that *H. pylori* induced uPAR through ROS and NF- $\kappa$ B, and the uPAR induced by *H. pylori* plays an important role in the invasiveness of gastric cancer cells (Fig. 1). The effects of NAC and BAY11-7082 on the cell invasiveness were investigated to determine if the ROS and NF- $\kappa$ B are required for this process. As shown in Fig. 7, both NAC and BAY11-7082 inhibited the *H. pylori*-induced cell invasiveness in a dose-dependent manner.

## Discussion

*H. pylori* is a bacterium that lives in the stomach and duodenum. Although the pathogenesis of gastroduodenal diseases caused by this bacterium is not well understood, some immunologic mechanisms are believed to be involved in gastric inflammation. Recently, considerable effort has been directed at defining the role of *H. pylori* in the development and progression of gastric cancer as stimulated by the following observations: i) epidemiologic studies have indicated that an infection with *H. pylori* is a risk factor for gastric carcinoma (22,23), ii) long-term studies suggest that the association between an *H. pylori* infection and gastric cancer ranks with that between smoking and lung cancer (24), iii) *H. pylori* induces various genes associated with gastric cancer angiogenesis and metastasis including vascular endothelial growth factor (25), IL-8 (26) and MMPs (26) and iv) Mongolian gerbils with *H. pylori* inoculated into the stomach develop chronic gastritis, intestinal metaplasia, and adenocarcinoma (27).

It was previously reported that *H. pylori* induced uPAR expression and stimulated the cell invasiveness in human gastric cancer AGS cells, suggesting that the overexpression of uPAR induced by *H. pylori* is involved in the increased cell invasiveness (18). However, the precise mechanisms for how *H. pylori* upregulates the uPAR are unknown. Recently, Choi *et al* (28) demonstrated that the overexpression of uPAR in human gastric carcinomas correlates with their invasiveness and tumorigenicity. The plasminogen activation system and MMPs play key roles in the degradation of the



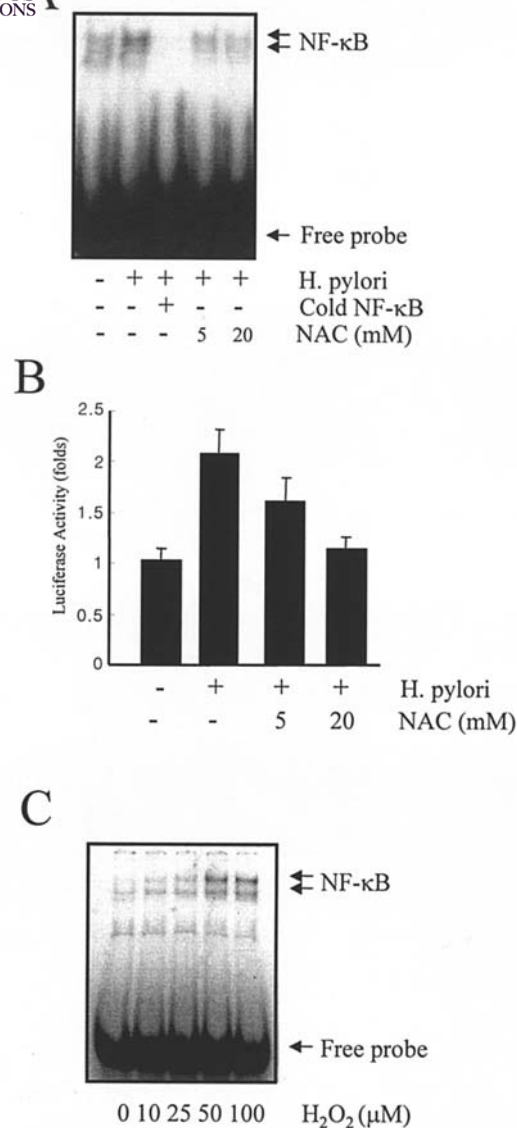


Figure 6. Effect of ROS produced by *H. pylori* on the activation of NF-κB. (A) The AGS cells, after being pretreated with 0-20 mM NAC, were incubated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 100:1. After incubation, the nuclear extracts from the cells were analyzed by EMSA for activated NF-κB using a radiolabeled oligonucleotide probe. (B) The AGS cells were transiently transfected with the pNF-κB-Luc-reporter construct. The transfected cells were pretreated with 0-20 mM NAC, and incubated with *H. pylori* 51 for 4 h at a bacterium/cell ratio of 100:1. After incubation, the cells were lysed and the luciferase activity was determined using a luminometer. The data represents the means  $\pm$  SD from triplicate measurements. (C) The AGS cells were incubated with 0-100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 4 h, and the level of activated NF-κB in the nuclear extracts from the cells were analyzed by EMSA.

basement membrane and extracellular matrix in tissue remodeling, cell invasion, and angiogenesis. uPA, a serine protease, has the ability to convert a plasminogen to active plasmin and activates the MMPs and growth factors (6). Gooz *et al* (26) reported that the levels of MMPs and their inhibitors secreted by AGS gastric carcinoma cells were increased by an *H. pylori* infection.

The results showed that *H. pylori* induced the production of ROS in AGS cells. The increase in the level of ROS was detectable within 10 min after exposing the cells to *H. pylori*. The production of ROS precedes the induction of uPAR

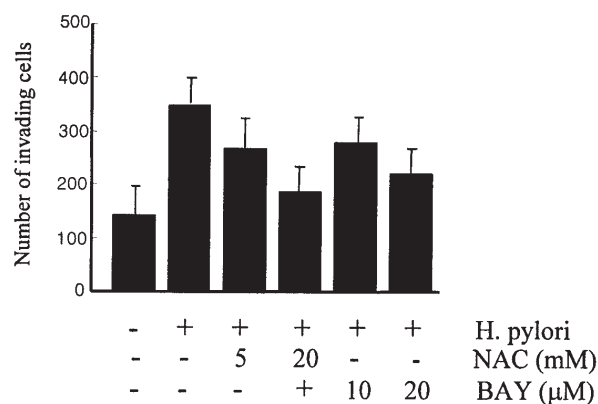


Figure 7. Effects of NAC and BAY11-7082 (BAY) on the *H. pylori*-induced AGS cell invasiveness. AGS cells ( $10^5$ ) were incubated with *H. pylori* 51 ( $10^7$ ) in BioCoat matrigel invasion chambers for 24 h in the presence of 0-20 mM NAC or 0-20  $\mu$ M BAY. After incubation, the invading cells on the undersurface of the chambers were counted using phase contrast light microscopy after staining with a Diff-Quick stain kit. The data represents the means  $\pm$  SD from triplicate measurements.

expression, and this upregulation is attenuated by NAC, a ROS scavenger. In addition, exogenous ROS alone induced the expression and promoter activity of uPAR. These results indicate a role of ROS in the activation of the uPAR gene by *H. pylori*. The molecular mechanism for ROS production by *H. pylori* remains unclear. Bagchi *et al* (29) reported that human gastric cells incubated with an 87-kDa cytotoxin produced from *H. pylori* enhanced the production of ROS, as determined by the reduction of cytochrome c and the level of hydroxyl radicals and DNA damage. Teshima *et al* (30) reported that gastric epithelial cells contain phagocyte NADPH oxidase components (gp91-, p22-, p67-, p47-, and p40-phoxes), and that these cells produce ROS through the activation of nonphagocytic NADPH oxidase in response to *H. pylori*. In addition to the gastric mucosa, it was suggested that the sources of ROS production are probably the host neutrophils, which are activated by the soluble factors of *H. pylori*. Davies *et al* (31) reported elevated levels of ROS in duodenal biopsies from patients with active duodenal ulcers. The chemiluminescence was higher in *H. pylori*-positive tissue than in negative tissue when samples were grouped according to the equivalent macroscopic or microscopic damage. This difference was in part accounted for by a higher level of neutrophil infiltration in the *H. pylori*-positive mucosa. However, when the biopsy specimens with equivalent neutrophil infiltration were compared directly, the *H. pylori*-positive specimens gave greater chemiluminescence than the negative tissue specimens.

The results suggest that the ROS produced by *H. pylori* can stimulate the cell invasiveness by upregulating uPAR expression in gastric AGS cells. Several laboratories have reported convincing evidence that antioxidants offer protection against tumor invasion and metastasis using both *in vivo* and *in vitro* studies. Asc-2-O-phosphate-6-O-palmitate, a lipophilic and autooxidation-resistant derivative of ascorbic acid was shown to inhibit an invasion by fibrosarcoma cells using its potent antioxidant activity (32). In addition, the administration of Cu-Zn superoxide dismutase, an enzyme that scavenges superoxide, successfully reduced the lung

metastasis of mouse MethA sarcoma and Lewis lung carcinoma cell lines (33). Zhang *et al* (34) reported that the exogenous ROS produced by the hypoxanthine-xanthine oxidase system increased the invasion of mesothelial cells.

The following observations suggest that NF- $\kappa$ B is also involved in the signaling pathway for the induction of uPAR by *H. pylori* in AGS cells: i) the *H. pylori* treatment increased the amount of NF- $\kappa$ B, which could form a complex with the radiolabeled oligonucleotide probe in EMSA; ii) AGS cells treated with *H. pylori* showed a loss of the I- $\kappa$ B $\alpha$  protein; iii) the *H. pylori* treatment also resulted in an increase in the NF- $\kappa$ B-dependent transcriptional activity, as revealed by the Luc reporter construct assay; iv) the induction of the uPAR promoter activity by *H. pylori* was inhibited by inhibiting the NF- $\kappa$ B signals; and v) the level of binding of endogenous NF- $\kappa$ B to the NF- $\kappa$ B binding site on the uPAR promoter sequences was increased after the *H. pylori* treatment, as determined by chromatin immunoprecipitation.

Overall, these results suggest that ROS are involved in the signaling pathway for NF- $\kappa$ B activation by *H. pylori* in gastric AGS cells. As shown in Fig. 6, NAC inhibited the NF- $\kappa$ B-DNA binding and NF- $\kappa$ B-dependent transcription activity induced by *H. pylori*. Furthermore, exogenous H<sub>2</sub>O<sub>2</sub> alone induced NF- $\kappa$ B-DNA binding. The mechanism by which ROS stimulates the NF- $\kappa$ B pathway is unclear. Li and Engelhardt (35) reported that the recruitment of NIK to TRAF6 is mechanistically important for the ROS induction of NF- $\kappa$ B activation. Storz and Toker (36) showed that protein kinase D (PKD) is essential for ROS-induced NF- $\kappa$ B activation by inducing the tyrosine phosphorylation of IKK. Src and Abl mediate PKD activation in response to H<sub>2</sub>O<sub>2</sub> stimulation, via the phosphorylation of Tyr463 in the PKD pleckstrin homology domain. Similarly, Fan *et al* (37) reported that ROS controlled NF- $\kappa$ B activation through the c-Src-dependent tyrosine phosphorylation of I- $\kappa$ B. However, ROS-induced NF- $\kappa$ B activation is strongly dependent on the cell type.

This study reports that the ROS produced by *H. pylori* induces the activity of NF- $\kappa$ B, which subsequently up-regulates uPAR expression resulting in increased cell invasiveness in gastric cancer cells. Understanding the mechanism by which uPAR expression is regulated by *H. pylori* may lead to the development of new strategies for treating gastric carcinomas. Therefore, further study is needed to elucidate the detailed regulatory mechanism.

## Acknowledgements

The authors are very grateful to Dr Y. Wang (Australian National University, Canberra, Australia) for the uPAR promoter-reporter construct, to Dr D.W. Ballard (Vanderbilt University School of Medicine, Nashville, TN) for the I- $\kappa$ B $\alpha$  and I- $\kappa$ B $\beta$  constructs and to W.C. Greene (University of California, San Francisco, CA) for the NIK construct. M.H. Kim was financially supported by the Brain Korea 21 Project, Center for Biomedical Human Resources at Chonnam National University in 2006. This research was supported by the Korean Science and Engineering Foundation through the Medical Research Center (R13-2002-013-00000-0) at Chonnam National University.

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