

# Interleukin-20 Promotes Migration of Bladder Cancer Cells through Extracellular Signal-regulated Kinase (ERK)-mediated MMP-9 Protein Expression Leading to Nuclear Factor (NF- $\kappa$ B) Activation by Inducing the Up-regulation of p21<sup>WAF1</sup> Protein Expression\*<sup>§</sup>

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**Background:** The role of interleukin-20 (IL-20) in tumor migration remains to be elucidated.

**Results:** IL-20 induces cell migration via ERK1/2-mediated NF- $\kappa$ B/MMP-9 regulation by inducing p21<sup>WAF1</sup> expression.

**Conclusion:** p21<sup>WAF1</sup> is essential for the cell migration induced by IL-20.

**Significance:** This work provides novel insights into the molecular events of IL-20-mediated cancer cell migration indicating it is dependent on p21<sup>WAF1</sup> expression.

The role of inflammatory cytokine interleukin-20 (IL-20) has not yet been studied in cancer biology. Here, we demonstrated up-regulation of both IL-20 and IL-20R1 in muscle-invasive bladder cancer patients. The expressions of IL-20 and IL-20R1 were observed in bladder cancer 5637 and T-24 cells. We found that IL-20 significantly increased the expression of matrix metalloproteinase (MMP)-9 via binding activity of NF- $\kappa$ B and AP-1 in bladder cancer cells and stimulated the activation of ERK1/2, JNK, p38 MAPK, and JAK-STAT signaling. Among the pathways examined, only ERK1/2 inhibitor U0126 significantly inhibited IL-20-induced migration and invasion. Moreover, siRNA knockdown of IL-20R1 suppressed migration, invasion, ERK1/2 activation, and NF- $\kappa$ B-mediated MMP-9 expression induced by IL-20. Unexpectedly, the cell cycle inhibitor p21<sup>WAF1</sup> was induced by IL-20 treatment without altering cell cycle progression. Blockade of p21<sup>WAF1</sup> function by siRNA reversed migration, invasion, activation of ERK signaling, MMP-9 expression, and activation of NF- $\kappa$ B in IL-20-treated cells. In addition, IL-20 induced the activation of I $\kappa$ B kinase, the degradation and phosphorylation of I $\kappa$ B $\alpha$ , and NF- $\kappa$ B p65 nuclear translocation, which was regulated by ERK1/2. IL-20 stimulated the recruitment of p65 to the MMP-9 promoter region. Finally, the IL-20-induced migration and invasion of

cells was confirmed by IL-20 gene transfection and by addition of anti-IL-20 antibody. This is the first report that p21<sup>WAF1</sup> is involved in ERK1/2-mediated MMP-9 expression via increased binding activity of NF- $\kappa$ B, which resulted in the induction of migration in IL-20/IL-20R1 dyad-induced bladder cancer cells. These unexpected results might provide a critical new target for the treatment of bladder cancer.

Bladder cancer is one of the most common tumors of the urinary tract that can cause cancer deaths in men (1). More than 90% of urinary bladder tumors are composed of transitional cell carcinoma that arises from the transitional epithelium (2). Urinary bladder tumors have been classified into two distinct categories as follows: nonmuscle-invasive bladder cancer and muscle-invasive bladder cancer (MIBC)<sup>3</sup> (3, 4). More than 75% of patients present with nonmuscle-invasive bladder cancer, consisting of low grade and superficial bladder tumors. Nearly 15% of tumors are MIBC with incident high grade bladder tumors that infiltrate the muscular layers of the bladder, which can take the form of metastatic disease and subsequently lead to death (4). Etiological factors involved in bladder carcinogenesis remain poorly identified, and effective molecular markers are still limited.

Tumor invasion and migration have been associated with the ability of tumor cells to break down the surrounding tissues and with proteolytic degradation of the extracellular matrix (ECM)

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<sup>3</sup> The abbreviations used are: MIBC, muscle-invasive bladder cancer; IKK, I $\kappa$ B kinase; EDC, *N*-ethyl-*N'*-dimethylaminopropyl carbodiimide; ECM, extracellular matrix; MMP, matrix metalloproteinase; CDK, cyclin-dependent kinase; HUVEC, human umbilical vein endothelial cell.

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components (5, 6). The migration of tumor cells requires alteration of the ECM and the basement membrane by proteases such as matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, in the process of tumor cell metastasis (5, 6). Over the last decade, it has become apparent that increased expression of MMPs is correlated with metastatic potential (4–6). Several studies have demonstrated that the gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa), which degrade type IV collagen, have a direct impact on the biological behavior of tumor cells during pathological processes such as tumor invasion and migration (5, 6). Previous studies have shown that MMP-9 supports crucial roles in the progression of bladder tumor *in vitro* and *in vivo* (4). Many studies have shown that growth factors and cytokines can stimulate MMP-9 expression in several types of cells (7–10). Further studies have demonstrated that the transcription factors NF- $\kappa$ B, Sp-1, and AP-1 are key transcriptional regulators responsible for the induction of MMP-9 in cancer cells (7–11).

In mammalian cells, the G<sub>1</sub>-S cell cycle stage represents a critical check point for cells to induce growth arrest or proliferation (12). The G<sub>1</sub>-S cell cycle progression is regulated by complexes of cyclin-dependent kinases (CDKs) and cyclins (12). A CDK inhibitor, p21<sup>WAF1</sup>, binds to CDK or CDK-cyclin complexes, thereby preventing the kinase activity, which leads to the inhibition of cell cycle progression (12, 13). In addition to modulating the cell cycle, p21<sup>WAF1</sup> proteins play significant roles in apoptosis, proliferation, and cell migration (13). Recent efforts to identify the malignant potential of tumor cells have explored the role of cell cycle regulators in tumor progression (14, 15). However, the molecular mechanism of cell cycle inhibitors in tumor progression remains to be investigated.

Interleukin-20 (IL-20) was a member of the IL-10 family of cytokines (16, 17). IL-20 is highly associated with potent inflammatory diseases such as psoriasis, contact hypersensitivity, rheumatoid arthritis, and atherosclerosis (18). IL-20 receptor complexes are divided into two alternative types. Type I is composed of IL-20R1/IL-20R2 chains, and type II consists of an IL-22R1/IL-20R2 heterodimer (16, 17). IL-20 can stimulate STAT3 activation in keratinocytes (16). IL-20 treatment has activated MAPK, such as ERK1/2, p38 MAPK, and JNK, in human umbilical vein endothelial cells (HUVEC) (19). Experiments with IL-20-stimulated GBM8901 glioblastoma cells cultures induced the activation of JAK2/STAT3 and ERK1/2 pathways (20). Although IL-20 was described as a potent pro-inflammatory cytokine in several inflammatory diseases, little is known about its role and mechanism in the migration involved in tumor progression.

In this study, we used 5637 and T-24 bladder carcinoma cell lines to investigate the roles of IL-20 and IL-20 receptor in the regulation of tumor cell migration. In addition, we report the novel finding that p21<sup>WAF1</sup> is a key regulator of IL-20-induced migration, which is mediated by the MMP-9, transcription factors, and signaling pathways in bladder cancer cells.

### EXPERIMENTAL PROCEDURES

**Ethics Statement**—The Ethics Committee of Chungbuk National University approved the protocol used for this study. Written informed consent was obtained from all patients

involved in this study. The Institutional Review Board of Chungbuk National University approved the collection and analysis of all samples.

**Clinical Samples**—The clinical samples were obtained from 62 primary bladder cancer samples (62 MIBCs), 58 samples of histologically normal-looking surrounding tissues, and 10 samples of normal bladder mucosae from patients with benign diseases.

**Tissue Samples**—All tumors were macro-dissected, typically within 15 min of surgical resection. Each bladder cancer specimen was confirmed by pathological analysis of a part of the tissue sample in fresh-frozen sections from cystectomy and transurethral resection specimens, then frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until use.

**RNA Extraction**—Total RNA was isolated from tissue using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol. The quality and integrity of the RNA were confirmed by agarose gel electrophoresis and ethidium bromide staining, followed by visual examination under ultraviolet light.

**Real Time-PCR**—Real time-PCR assays were performed in the original and independent cohorts, using a Rotor Gene 3000 PCR system (Corbett Research, Mortlake, Australia). GAPDH was analyzed in parallel as an internal control. Real time PCRs containing primers and SYBR Premix EX Taq (Takara Bio Inc., Otsu, Japan) were carried out in micro-reaction tubes (Corbett Research). Spectral data were captured and analyzed using Rotor-Gene Real Time Analysis Software 6.0 Build 14 (Corbett Research). For amplification, IL-20 sense (5'-TTGCCTTCAG-CCTTCTCTCT-3') and IL-20 antisense (5'-CCTTCCTCAG-GTATCCTCTA-3'), IL-20R1 sense (5'-TACAATGGACTCC-ACCAGAG-3') and IL-20R1 antisense (5'-ACCGTCCA-CTTTCAGCCCAT-3') primers were used. GAPDH was analyzed in parallel as an endogenous RNA reference gene, and data were normalized to the expression of GAPDH.

**Cell Cultures**—Human bladder carcinoma cell lines (5637 and T-24) were obtained from the American Type Culture Collection. The cells were maintained in DMEM (4.5 g of glucose/liter) supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (Biological Industries, Beit Haemek, Israel) at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub>-humidified incubator.

**Materials**—IL-20 and polyclonal antibodies to IL-20 were purchased from R&D Systems (Minneapolis, MN). Polyclonal antibodies to ERK, phospho-ERK, p38 MAPK, phospho-p38 MAPK, JNK, phospho-JNK, phospho-I $\kappa$ B $\alpha$  (Ser-32/36), and phospho-p65 (Ser-536) were obtained from Cell Signaling (Danvers, MA). U0126, SP600125, and SB203580 were obtained from Calbiochem. Polyclonal antibodies to JAK1, JAK2, JAK3, STAT1, STAT2, STAT3, STAT5, phospho-JAK1, phospho-JAK2, phospho-JAK3, phospho-STAT1, phospho-STAT2, phospho-STAT3, phospho-STAT5, IL-20R1, IL-22R1, IL-20R2, p65, I $\kappa$ B $\alpha$ , lamin B, and tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IKK- $\alpha$  and anti-IKK- $\beta$  antibodies were obtained from Imgenex (San Diego). The polyclonal MMP-9 antibody was obtained from Chemicon. Small interfering RNA (siRNA) oligonucleotides targeting IL-20R1 (5'-CUUACACUGUGCAGUAUUUUU-3'), IL-20R2 (5'-CUUUCACAAUGGUUCUAGAUU-3'), IL-22R1 (5'-CACUUUCUAGUCCUAAACAUU-3'), p21<sup>WAF1</sup>

(5'-CUGUCAGUCAGUCGUAGUAUU-3'), p27 KIP1 (5'-CAA-ACGUGCGAGUGUCUAAUU-3'), p53 (5'-CCACUUGAUG-GAGAGUAUU-3'), and Scramble (5'-CUGUCAGUCAGUC-GUAGUAUU-3') were designed and synthesized from Genolution (Seoul, Korea).

**Immunoblot**—Growth-arrested cells were treated with IL-20 in the absence of 10% FBS for various durations at 37 °C. The cells were then washed twice with cold PBS and freeze-thawed in 250  $\mu$ l of lysis buffer (containing, in mmol/liter, HEPES (pH 7.5) 50, NaCl 150, EDTA 1, EGTA 2.5, DTT 1,  $\beta$ -glycerophosphate 10, NaF 1, Na<sub>3</sub>VO<sub>4</sub> 0.1, phenylmethylsulfonyl fluoride 0.1, 10% glycerol, 0.1% Tween 20, 10 g/ml leupeptin, and 2  $\mu$ g/ml aprotinin) and then scraped into 1.5-ml tubes. The lysates were placed on ice for 15 min and then centrifuged at 12,000 rpm for 20 min at 4 °C. The protein concentration of the supernatant was determined using a Bradford reagent method (Bio-Rad). Equal amounts of cellular proteins were resolved by electrophoresis on a 0.1% SDS, 10% polyacrylamide gel under denaturing conditions. The proteins were transferred electrophoretically to nitrocellulose membranes (Hybond, Amersham Biosciences). After blocking in 10 mmol/liter Tris-HCl (pH 8.0), 150 mmol/liter NaCl, and 5% (w/v) nonfat dry milk, the membranes were treated with primary antibodies for 90 min, followed by incubation with peroxidase-conjugated secondary antibodies for 45 min. The immunocomplexes were detected using a chemiluminescence reagent kit (Amersham Biosciences). The experiments were repeated at least three times for the immunoblotting studies (10).

**IKK Assay**—We performed an immunocomplex kinase assay using GST-I $\kappa$ B $\alpha$  as the substrate. Briefly, whole cell lysates (500  $\mu$ g) were collected in ice-cold lysis buffer (containing, in mmol/liter, HEPES (pH 7.5) 50, NaCl 150, EDTA 1, EGTA 2.5, DTT 1,  $\beta$ -glycerophosphate 10, NaF 1, Na<sub>3</sub>VO<sub>4</sub> 0.1, phenylmethylsulfonyl fluoride 0.1, and 10% glycerol, 0.1% Tween 20, 10  $\mu$ g/ml leupeptin, and 2  $\mu$ g/ml aprotinin). Lysates were precleared by incubation with 0.25  $\mu$ g of the appropriate control IgG together with 20  $\mu$ l of protein A/G plus (25%, v/v) agarose conjugate for 30 min at 4 °C, all of which was followed by centrifugation. Supernatants were then incubated with 1  $\mu$ g of anti-IKK- $\alpha$  and - $\beta$  for 2 h at 4 °C and then 20  $\mu$ l of protein A/G plus agarose was added and incubated at 4 °C on a rocker platform overnight. After several washes with IP buffer and PBS, beads containing IKK- $\alpha$ / $\beta$  were incubated with 0.5  $\mu$ g of GST-I $\kappa$ B $\alpha$  substrate and 200  $\mu$ M ATP in 20  $\mu$ l of kinase buffer (50 mM Tris-Cl (pH 7.4), 20 mM MgCl<sub>2</sub>, 20 mM  $\beta$ -glycerophosphate, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 0.5 mM DTT, 1 mM benzamidine, 10  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml leupeptin) at 30 °C for 30 min. The kinase reactions were stopped by the addition of 5  $\mu$ l of 5 $\times$  Laemmli's loading buffer and were heated at 100 °C for 5 min; the gel was dried, and the radioactive bands were visualized. To determine the total amounts of IKK- $\alpha$  and IKK- $\beta$  in each sample, 30  $\mu$ g of whole cell proteins were resolved on 10% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK- $\alpha$  or anti-IKK- $\beta$  antibody.

**Preparation of IL-20-conjugated QD565**—The carboxyl QD565 nanoparticles were covalently conjugated with IL-20 (concentration: 10 ng/ml) by incubation for 1 h at room temperature with the addition of *N*-ethyl-*N'*-dimethylaminopropyl

carbodiimide (EDC) to enhance the coupling efficiency between the amine and the carboxyl groups (21, 22). The reaction ratio of the QD565 particles to the IL-5 and EDC was 1:2:1000. The QD565-IL-20 was centrifuged at 15,000 rpm for 15 min to remove the unconjugated free IL-20 and EDC; this was followed by several washing steps using Tris buffer solution (10 mM Tris-HCl (pH 7.4)). After a brief sonication, the final conjugated products were mixed using a Tris borate buffer solution (10 mM Tris-HCl, 10 mM sodium borate (pH 7.4); Sigma).

**Confocal Microscopy of IL-20-QD565 Nanoparticles from the Cells**—The cells were seeded into precoated gelatin 6-well plates, and sterile coverslips were put in place. The cells were then washed twice with phosphate-buffered saline (PBS). The antibody-conjugated QD565 nanoparticles described above were introduced with docking cells and incubated for 4 h at 37 °C. The cells were then fixed using a 3.7% formaldehyde solution (Sigma) and were rinsed three times with PBS for 10 min. The coverslips from the 6-well plates were placed onto glass slides after mounting medium containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Vector Laboratories, Inc., CA) was added. The fluorescence signal was detected using confocal laser scanning microscopy (Carl Zeiss LSM 510, Carl Zeiss, Jena, Germany).

**Wound-healing Migration Assay**—Cells were plated on 6-well dishes and grown to 90% confluence in 2 ml of growth medium. The cells were damaged using a 2-mm-wide tip and were then treated with IL-20. They were allowed to migrate, and photographs were taken through an inverted microscope ( $\times$ 40 magnification).

**Invasion Assay**—The ability of cells to invade through Matrigel-coated filters was determined using a modified 24-well Boyden chamber (Corning Costar, Cambridge, MA; 8- $\mu$ m pore size). Cultured cells were trypsinized and suspended in DMEM, which was layered on the coated insert filters. Cells were stimulated with IL-20. The lower chamber contained IL-20 in the same concentration. Plates were incubated at 37 °C for 24 h. Membranes were washed with phosphate-buffered saline, and noninvading cells on the upper surface were removed using cotton swabs. Cells invading the lower surface of the membrane were fixed and stained with 0.01% crystal violet in 20% ethanol. Photographs were taken of 10 fields at  $\times$ 20 magnification per sample.

**Zymography**—Conditioned medium was electrophoresed in a polyacrylamide gel containing 1 mg/ml gelatin. The gel was then washed at room temperature for 2 h with 2.5% Triton X-100 and subsequently at 37 °C overnight in a buffer containing 10 mM CaCl<sub>2</sub>, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.5). The gel was stained with 0.2% Coomassie Blue and photographed on a light box. Proteolysis was detected as a white zone in a dark blue field (10).

**Plasmid Construction**—To obtain the full-length cDNA of IL-20, first-strand cDNA synthesis was performed using a HelixCript<sup>TM</sup> First-strand cDNA synthesis kit (NanoHelix Co., Ltd., Korea) according to the manufacturer's instructions, with 5  $\mu$ g of total RNA from EJ human bladder cancer cells. The full-length cDNA was obtained by PCR with first-strand cDNA as a template using a 5' primer containing a HindIII site, 5'-AGCT AAG CTT C ATG AAA GCC TCT AGT CTT-3' and a

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3' primer containing a XbaI site, 5'-CTAG TCT AGA CTA TTC TGT CTC CTC CAT CCA TT-3'. The PCR was performed as follows: 95 °C for 60 s, 30 cycles of 95 °C for 20 s, 56 °C for 40 s, 72 °C for 120 s, and 72 °C for 5 min. The PCR product (0.53 kb) was cloned into pHelix-TA-vector (NanoHelix Co., Ltd., Korea) and determined by DNA sequencing. The inserted fragment (0.53 kb) was cut out by digestion with HindIII and XbaI and then inserted into the corresponding sites of pcDNA3 (Invitrogen), which was designated pcDNA3-IL-20. Cells were transfected with either pcDNA3-IL-20 or pcDNA3 (empty vector, no insert) in 100-mm dishes using Superfect reagent (Qiagen, Valencia, CA), according to the manufacturer's protocol.

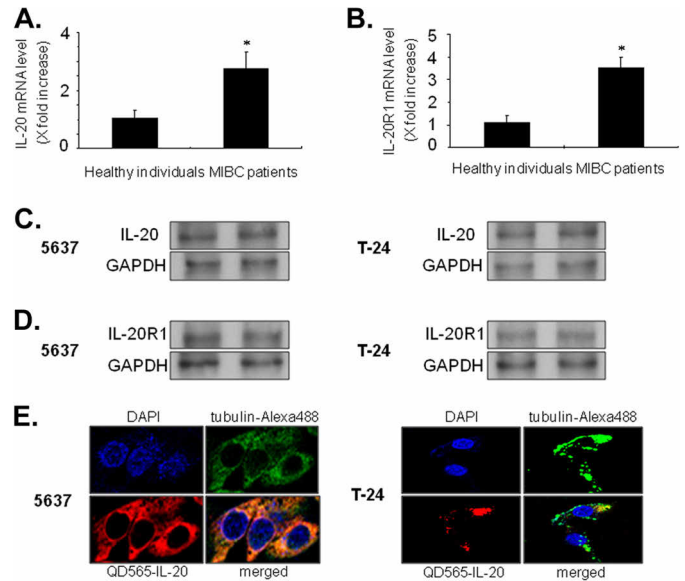
**Transfection**—Cells were transfected with siRNA using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocols. After the indicated incubation with IL-20, the cells were studied via immunoblot, zymography, electrophoretic mobility shift assay (EMSA), wound-healing migration, and invasion.

**Nuclear Extracts and EMSA**—Cultured cells were collected by centrifugation, washed, and suspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF. After 15 min on ice, the cells were vortexed in the presence of 0.5% Nonidet P-40. The nuclear pellet was then collected by centrifugation and extracted in a buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF for 15 min at 4 °C.

The nuclear extract (10–20 µg) was preincubated at 4 °C for 30 min with a 100-fold excess of an unlabeled oligonucleotide spanning the –79 MMP-9 *cis* element of interest. The sequences were as follows: AP-1, CTGACCCCTGAGTCAGC-ACCTT; NF-κB, CAGTGGAAATCCCCAGCC; mNF-κB (MT), CAGTGGAAATGGCCAGCC; and Sp-1, GCCCATTCCTTCCGCCCCAGATGAAGCAG. The reaction mixture was then incubated at 4 °C for 20 min in a buffer (25 mM HEPES buffer (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05 M NaCl, and 2.5% glycerol) with 2 µg of poly(dI/dC) and 5 fmol (2 × 10<sup>4</sup> cpm) of a Klenow end-labeled ([<sup>32</sup>P]ATP) 30-mer oligonucleotide, which spanned the DNA-binding site in the MMP-9 promoter. The reaction mixture was electrophoresed at 4 °C in a 6% polyacrylamide gel using a TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) running buffer. The gel was rinsed with water, dried, and exposed to x-ray film overnight (10). For supershift assays, nuclear extracts were incubated with antibodies against p65 of NF-κB for 30 min at 37 °C. Then the complex was analyzed by EMSA. Preimmune serum was used as the negative control.

**Cell Cycle Analysis via Fluorescence-activated Cell Sorter (FACS)**—Cells were harvested, fixed in 70% ethanol, and stored at –20 °C. The cells then were washed twice with ice-cold PBS and incubated with RNase and the DNA intercalating dye propidium iodide. Cell cycle phase analysis was performed using a FAC StAR flow cytometer equipped with Cell FIT software (BD Biosciences).

**Chromatin Immunoprecipitation (ChIP) Assay**—Cells were incubated with IL-20. DNA that had been immunoprecipitated by anti-p65 antibody was purified. The DNA pellet was resuspended in H<sub>2</sub>O, and PCR amplification was carried out with the



**FIGURE 1. mRNA levels of IL-20 and its receptor IL-20R1 are overexpressed in patients with MIBC.** A and B, IL-20 and IL-20R1 mRNA expression was analyzed by real time PCR in 62 MIBC patients tissues versus 68 healthy individual tissues. Results are represented as IL-20 and IL-20R1 mRNA expression relative to GAPDH mRNA expression. \*,  $p < 0.01$  compared with healthy individuals. C–E, IL-20 and its receptor IL-20R1 are expressed in bladder cancer 5637 and T-24 cells. C and D, 5637 and T-24 cells were cultured with medium containing 10% FBS for 24 h. IL-20 and IL-20R1 proteins in the cell lysates were detected by immunoblot. E, detection of IL-20 in 5637 and T-24 bladder cancer cells using confocal microscopy analysis. Both cell lines were stained with antibodies against QD565-conjugated IL-20 (red). Both nuclei (anti-DAPI) and cytoplasm (anti-tubulin-Alexa488) were counterstained with DAPI (blue) and tubulin-Alexa488 (green).

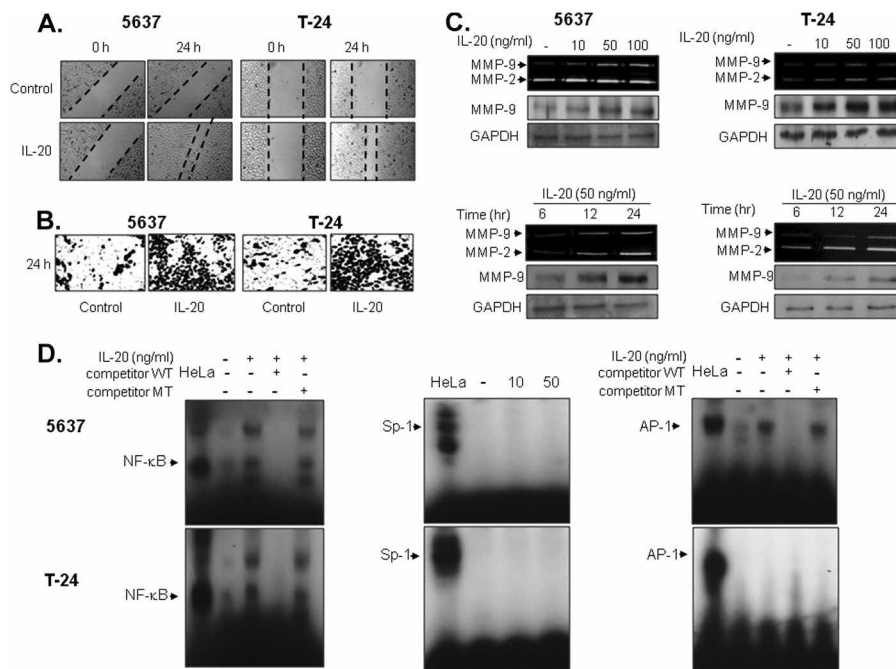
forward primer 5'-TGTCCTTTACTGCCCTGA-3' and the reverse primer 5'-ACTCCAGGCTCTGTCTCTCTT-3', which were specifically derived from the MMP-9 promoter region (–657 to –484). PCR products were analyzed on ethidium bromide-stained agarose gels (2%). The input represents the PCR products from chromatin pellets prior to immunoprecipitation.

**Statistical Analysis**—Where appropriate, data were expressed as the mean ± S.E. Data were analyzed using factorial analysis of variance and Fisher's least significant difference test. Statistical significance was set at  $p < 0.05$ .

## RESULTS

**Expression of IL-20 and IL-20R1 Was Elevated in Patients with MIBC**—A previous study using RNA microarray analysis showed elevated expression of IL-20 and its receptor IL-20R1 in patients with MIBC (23). In this study, to verify the previous clinical analysis, the mRNA levels of IL-20 and IL-20R1, type I receptor of IL-20, were determined in 62 MIBC samples and 68 samples from healthy individuals using real time PCR. The results from this study demonstrated that both IL-20 and IL-20R1 mRNA levels were significantly higher in patients with MIBC than in healthy individuals (Fig. 1, A and B). These results suggest that IL-20 and IL-20R1 expression is correlated with progression of bladder cancer.

**Detection and Subcellular Localization of IL-20 and IL-20R1 in Human Muscle-invasive Bladder Cancer, 5637 and T-24 Cells**—Immunoblot analysis was performed to confirm whether IL-20 and IL-20R1 are expressed in bladder cancer



**FIGURE 2. IL-20-stimulated migration and expression of MMP-9 via inducing the activation of NF- $\kappa$ B and AP-1 in bladder cancer cells.** *A*, wound-healing assays revealed a significant increase in the wound closure rates of 5637 and T-24 cells after IL-20 (50 ng/ml) stimulation for 24 h ( $\times 40$  magnification). *B*, cells were plated in Boyden chambers in the absence or presence of IL-20. After 24 h, photos were taken of invaded cells. *C*, cells were treated with increasing concentrations of IL-20 for the indicated times, and culture supernatants from IL-20-treated cells were then analyzed for the production of MMP-9 using gelatin zymography. Cell lysates of IL-20-treated cells were subjected to immunoblot analysis for MMP-9 and GAPDH. *D*, after serum starvation for 24 h, cells were incubated with various concentrations of IL-20 for 24 h, and the activation of NF- $\kappa$ B, AP-1, and Sp-1 was measured by EMSA. To stimulate activity of NF- $\kappa$ B and AP-1, the IL-20-treated cells were incubated with an unlabeled NF- $\kappa$ B oligonucleotide probe (WT) or a mutant (MT) oligonucleotide probe.

5637 and T-24 cells. The protein expression of IL-20 and IL-20R1 was endogenously observed in both cell lines with medium containing 10% FBS (Fig. 1, *C* and *D*). Next, to study the subcellular localization of IL-20 in 5637 and T-24 cells, we examined the expression pattern of IL-20 using immunofluorescence confocal microscopy analyses. IL-20 fluorescent staining was easily detected in both the cytoplasm and peri-nuclear areas in 5637 and T-24 cells (Fig. 1*E*). These results demonstrated that IL-20 and IL-20R1 protein were expressed in human bladder cancer 5637 and T-24 cells.

**IL-20 Promoted Migration and MMP-9 Expression in Bladder Cancer Cells**—Because pro-inflammatory cytokines have been implicated in the migration of tumor cells (24–26), an *in vitro* wound-healing assay and an invasion assay were examined to determine the migration of cancer cells induced by IL-20. As a first step, we examined the effect of IL-20 on the wound-healing migration of bladder cancer cells. Treatment of these cells with IL-20 (50 ng/ml) migrated into the wounded area and revealed an evident increase in the wound closure rates of both 5637 and T-24 cells within 24 h (Fig. 2*A*). However, in the vehicle-treated cells (control), the wound remained open after 24 h (Fig. 2*A*). In a similar manner, exposure to IL-20 showed a significant increase, in comparison with control cells (Fig. 2*B*), in invasiveness through the Boyden chambers. The role and significance of MMPs in cell migration have already been demonstrated in several tumor cell types (4–6). To establish a link between cell migration and MMPs with our system, we investigated the effect of IL-20 on the expression of MMPs in bladder cancer cells. Treatment with IL-20 resulted in significant induction of MMP-9 expression in a concentration- and time-

dependent pattern, as determined by analysis of culture supernatants by gelatin zymography (Fig. 2*C*). Immunoblot analysis of bladder cancer cells showed that there was a strong induction in the levels of MMP-9 protein upon treatment with IL-20 (Fig. 2*C*). In addition, IL-20 treatment increased the levels of MMP-2 in both types of bladder cancer cells (Fig. 2*C*).

**IL-20 Activated NF- $\kappa$ B and AP-1 Transcription Factors in Bladder Cancer Cells**—Previous studies have shown that the MMP-9 expression was correlated directly with tumor grade, invasion, and metastasis in bladder cancer (4). In this study, we focused on the regulatory roles of MMP-9 in IL-20-treated bladder cancer cells. The MMP-9 gene promoter contains several transcription factors, including NF- $\kappa$ B, Sp-1, and AP-1 that are verified as binding sites in humans and mice (7–11, 27). We first examined whether IL-20 can induce the activation of NF- $\kappa$ B, Sp-1, and AP-1 in bladder cancer cells. Both bladder cancer cell lines were treated with IL-20 at different concentrations, and the activation of transcription factors was studied by EMSA using nuclear extracts containing several DNA-binding motifs of bladder cancer cells. The results revealed that treatment with IL-20 significantly increased the DNA binding activity of NF- $\kappa$ B and AP-1 in 5637 cells (Fig. 2*D*). In T-24 cells, the DNA binding activity of NF- $\kappa$ B was only observed in response to IL-20 treatment (Fig. 2*D*). The addition of excess unlabeled NF- $\kappa$ B and AP-1 completely removed the binding to the oligonucleotides, whereas the addition of mutated oligonucleotides did not alter the DNA binding (Fig. 2*D*). However, no specific binding complexes to Sp-1 were detected in either line of cells treated with IL-20 (Fig. 2*D*). These results demonstrate that the

## Role of p21<sup>WAF1</sup> in IL-20-induced Migration

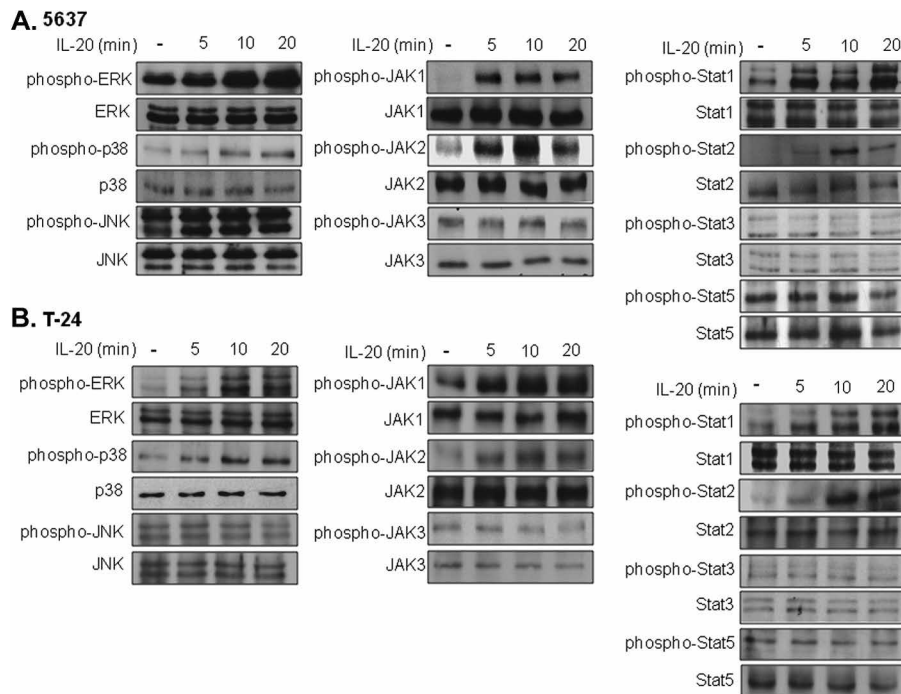


FIGURE 3. IL-20-induced activation of MAPK and JAK-STAT signaling pathways in bladder cancer cells. *A* and *B*, cells were treated with IL-20 (50 ng/ml) for the indicated time intervals, and the activation of MAPK and JAK-STAT was studied by immunoblotting with ERK1/2, JNK, p38, JAK1, JAK2, JAK3, STAT1, STAT2, STAT3, and STAT5 using specific antibodies.

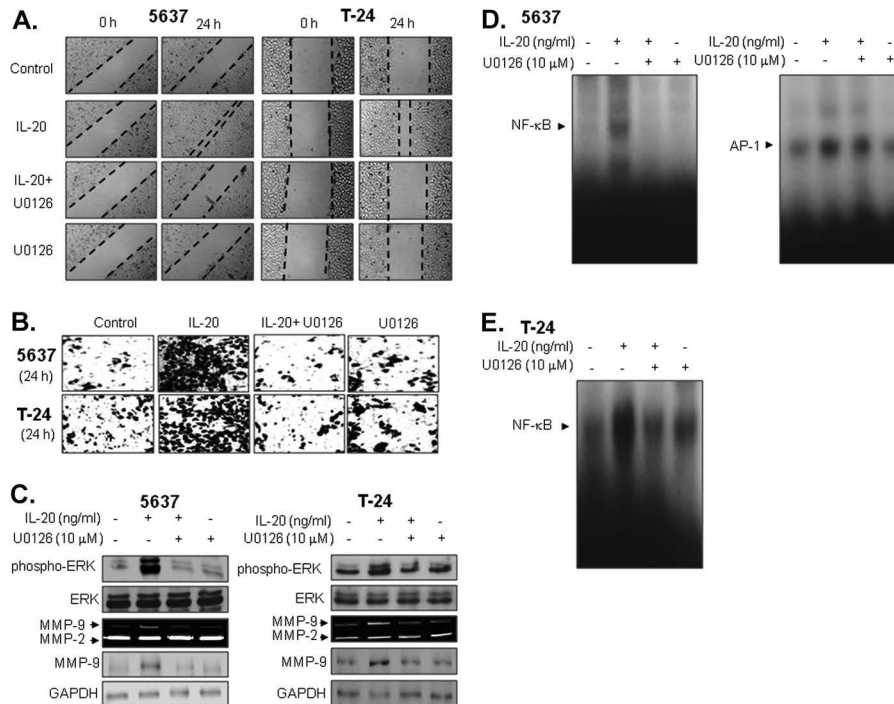
activation of NF- $\kappa$ B and AP-1 binding is involved in IL-20-induced MMP-9 expression in bladder cancer cells.

**IL-20-stimulated Activation of MAPK and JAK-STAT Signaling in Bladder Cancer Cells**—To determine the signaling pathways that lead to various molecular responses in bladder cancer cells in response to IL-20, we investigated the roles of upstream signaling molecules MAPK and JAK-STAT signaling. Bladder cancer cells were treated with IL-20 for different time intervals ranging from 0 to 20 min. Treatment of 5637 cells with IL-20 led to a significant increase in the activation of ERK1/2, JNK, and p38 MAPK (Fig. 3*A*). Stimulation of T-24 cells with IL-20 resulted in the activation of ERK1/2 and p38 MAPK (Fig. 3*B*). In addition, the results indicated that the levels of activation of JAK1, JAK2, STAT-1, and STAT-2 were up-regulated in IL-20-treated 5637 cells (Fig. 3*A*). Moreover, treatment with IL-20 stimulated the activation of JAK1, JAK2, STAT1, and STAT2 in T-24 cells (Fig. 3*B*). These data indicated that MAPK and JAK-STAT signaling was involved in IL-20-induced bladder cancer cell responses.

**ERK1/2 Signaling Pathway Is Involved in the Migration of Bladder Cancer Cells Induced by IL-20**—To understand whether the activation of MAPK and JAK signaling in response to IL-20 was responsible for IL-20-induced migration, bladder cancer cells were preincubated with U0126 (ERK1/2-specific inhibitor), SP600125 (JNK1 inhibitor), SB203580 (p38 kinase inhibitor), piceatannol (JAK1-specific inhibitor), and AG490 (JAK2-specific inhibitor) followed by IL-20 treatment. As shown in Fig. 4, *A* and *B*, pretreatment with U0126 blocked the IL-20-induced wound-healing migration and invasion of both bladder cancer cell lines. However, other inhibitors (SP600125, SB203580, piceatannol, and AG490) had no significant effect on wound-healing migration in IL-20-stimulated bladder cancer

cells (supplemental Figs. S1, *A* and *B*, and S2, *A* and *B*). Similar results were observed in an invasion assay (supplemental Figs. S1, *A* and *B*, and S2, *A* and *B*). Thus, we hypothesized that IL-20 promotes the migration of bladder cancer cells through the activation of ERK1/2.

**ERK1/2 Is Associated with NF- $\kappa$ B-mediated Control of MMP-9 Expression in IL-20-induced Migration of Bladder Cancer Cells**—The present results demonstrate that IL-20 stimulated MMP-9 expression through activation of NF- $\kappa$ B and AP-1 binding in bladder cancer cells. Moreover, our results showed that inhibition of ERK1/2 blocked the IL-20-induced migration of bladder cancer cells. We then investigated whether ERK1/2 signaling includes the molecular pathways that lead to the binding activation of NF- $\kappa$ B or AP-1, and hence the expression of MMP-9, in IL-20-treated bladder cancer cells. To this end, bladder cancer cells were treated with U0126 in the presence or absence of IL-20, and immunoblot and zymographic analyses were performed. The immunoblot results indicate that pretreatment of both bladder cancer cell lines with U0126 blocked the IL-20-induced activation of ERK1/2 (Fig. 4*C*). Zymographic and immunoblot analysis showed that IL-20-induced MMP-9 expression was also inhibited in the presence of U0126 (Fig. 4*C*). To further determine the role of ERK1/2 in the transcriptional regulation connected with the induction of MMP-9 expression in IL-20-stimulated cells, we performed an EMSA using the NF- $\kappa$ B and AP-1 motifs. IL-20-induced activation of NF- $\kappa$ B binding was significantly suppressed by the addition of U0126 in both cell lines (Fig. 4, *D* and *E*), although AP-1 binding activity was not affected in the presence of U0126 in IL-20-treated 5637 cells (Fig. 4*D*). These results suggest that the ERK1/2 signaling is involved in the



**FIGURE 4. Role of U0126 in the induction of migration, ERK1/2 activation, and transcription factor-mediated MMP-9 expression.** Cells were preincubated in serum-free medium with U0126 (10  $\mu$ M) for 40 min followed by addition of IL-20 (50 ng/ml) and incubation for additional 24 h. **A** and **B**, wound-healing migration and invasion assay was performed, as described under "Experimental Procedures." **C**, production of MMP-9 in culture supernatants was measured by gelatin zymography. MMP-9 expressions from cell lysates were analyzed by immunoblot. In addition, the cells were pretreated with U0126 (10  $\mu$ M) for 40 min for the detection of phospho-ERK1/2. The cells were further incubated with IL-20 (50 ng/ml) for 10 min. Immunoblot analysis was then performed using cell lysates. **D** and **E**, cells were cultured with IL-20 (50 ng/ml) in the absence or presence of inhibitor and nuclear proteins extracted. Activation of NF- $\kappa$ B and AP-1 DNA binding was analyzed by EMSA using radiolabeled oligonucleotide probes.

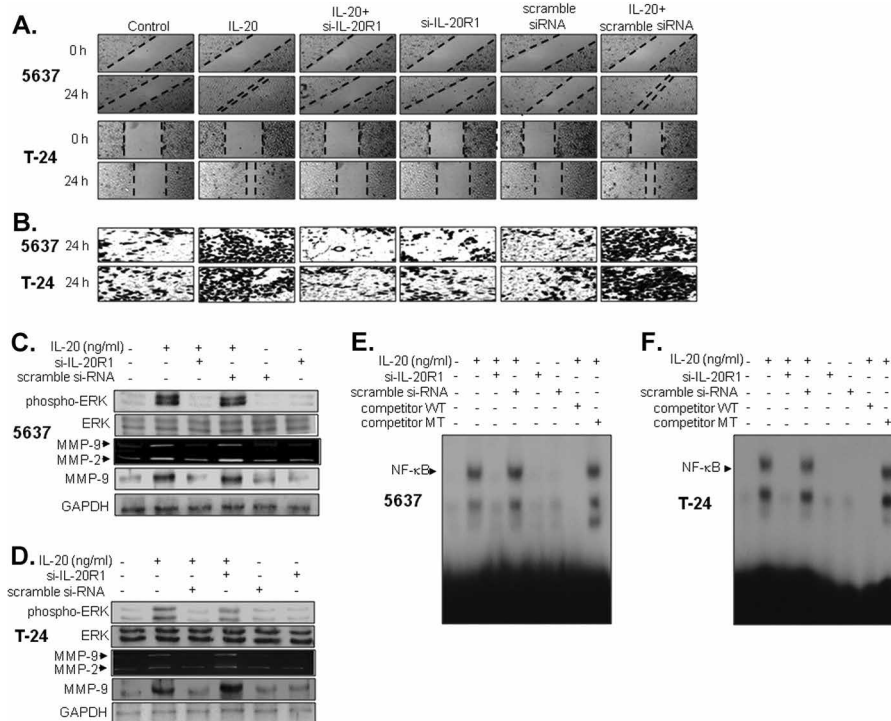
IL-20-mediated control of MMP-9 expression via the activation of NF- $\kappa$ B binding in bladder cancer cells.

**Involvement of IL-20R1 in Migration, MMP-9 Expression, ERK1/2 Activation, and Binding Activities of NF- $\kappa$ B in IL-20-treated Bladder Cancer Cells**—IL-20 transduces its signal through both the IL-20 receptors (IL-20R1/IL-20R2 and IL-22R1/IL-20R2 heterodimeric receptor) (16, 17). To functionally determine whether IL-20 can signal through these putative receptors, the potential role of IL-20 in bladder cancer cells was further confirmed by a receptor-specific siRNA knockdown system, including si-IL-20R1, si-IL-20R2, and si-IL-22R1. To examine the effectiveness and specificity of receptor-specific siRNA, bladder cancer cells were transfected with si-IL-20R1, si-IL-20R2, si-IL-22R1, and scrambled siRNA, followed by IL-20 treatment. Bladder cancer cells transfected with si-IL-20R1, si-IL-20R2, and si-IL-22R1 had reduced protein levels of IL-20R1, IL-20R2, and IL-22R1 (supplemental Fig. S1, C and D). Moreover, the protein levels of IL-20R1, IL-20R2, and IL-22R1 were not affected in the transfection of scrambled siRNA into both cell lines (supplemental Fig. S1, C and D), suggesting that all the siRNA molecules were effective and specific. Following transfection with si-IL-20R1, we performed the wound-healing migration and invasion assay. As shown in Fig. 5, A and B, we observed that the knockdown of IL-20R1 decreased the IL-20-induced migration and invasion of both bladder cancer cell lines, compared with control cells or cells transfected with scrambled siRNA. However, unexpectedly, the knockdown of IL-20R2 and IL-22R1 did not affect the migration and invasion of bladder cancer cells induced by IL-20

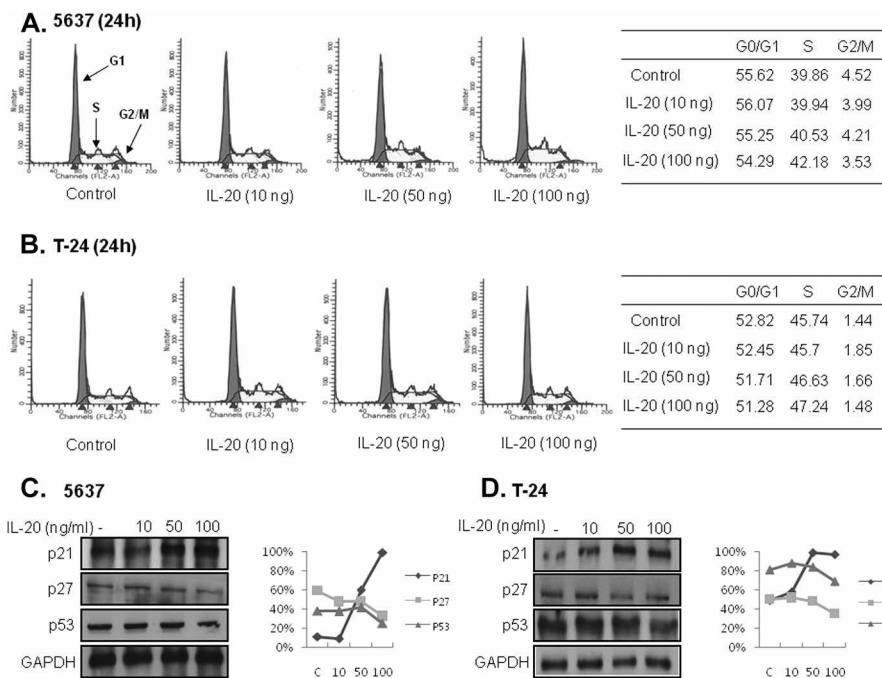
(supplemental Fig. S3, A and B). Thus, we carefully suggest that IL-20 may mediate the migration of bladder cancer cells via its IL-20R1 receptor. Therefore, we next examined ERK1/2 activation and MMP-9 expression in IL-20-treated bladder cancer cells following transfection of si-IL-20R1. Knockdown of IL-20R1 significantly inhibited the increased activation of ERK1/2 and expression of MMP-9 in 5637 and T-24 cells in response to IL-20 (Fig. 5, C and D). Furthermore, si-IL-20R1 effectively suppressed the IL-20-induced binding activity of NF- $\kappa$ B in all the cells (Fig. 5, E and F). These findings demonstrate that IL-20 may mediate migration, ERK1/2 activation, MMP-9 expression, and NF- $\kappa$ B DNA binding activity in human bladder cancer cells through the binding of IL-20R1.

**Induction of Cell Cycle Inhibitor p21<sup>WAF1</sup> in IL-20-treated Bladder Cancer Cells**—Cell cycle regulators have been implicated in disease progression in patients with bladder, colorectal, and breast cancer (14, 28–30). To determine the patterns of cell cycle progression in IL-20-mediated cellular effects, we investigated the cell cycle profile using the FACS approach in IL-20-treated bladder cancer cells. FACS analysis showed that cell populations in the G<sub>1</sub>-, S-, and G<sub>2</sub>/M-phases of the cell cycle were not affected in any of the bladder cancer cells following treatment with IL-20 for 12 or 24 h (Fig. 6, A and B, and supplemental Fig. S4, A and B). Previous studies have shown that the alterations of the tumor suppressor gene p53 and cell cycle inhibitors p21<sup>WAF1</sup> are crucial components in tumor development, recurrence, and survival in patients with bladder tumors (31, 32). Therefore, we examined the expression levels of cell cycle inhibitory molecules such as p21<sup>WAF1</sup>, p27 KIP1, and p53.

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**FIGURE 5. Involvement of IL-20R1 in migration, ERK1/2 activation, and transcription factor-mediated MMP-9 expression in IL-20-treated bladder cancer cells.** *A* and *B*, cells were transfected with either si-IL-20R1 or scrambled siRNA, then treated with IL-20 (50 ng/ml) for 24 h, and the wound-healing migration and invasion assay was measured. *C* and *D*, expression of MMP-9 was studied by gelatin zymography and immunoblot using culture supernatants and cell lysates. For the analysis of ERK1/2 activation, cells were transfected with si-IL-20R1 or scrambled siRNA, and then treated with IL-20 for 10 min. The cell lysates were subjected to immunoblot analysis. *E* and *F*, after transfection of si-IL-20R1 or scrambled siRNA, cells were treated with IL-20 (50 ng/ml) in the absence of serum for 24 h. NF-κB activity was assessed with EMSA. An unlabeled NF-κB oligonucleotide probe (*WT*) or a mutant (*MT*) oligo probe was used as a competitor control.

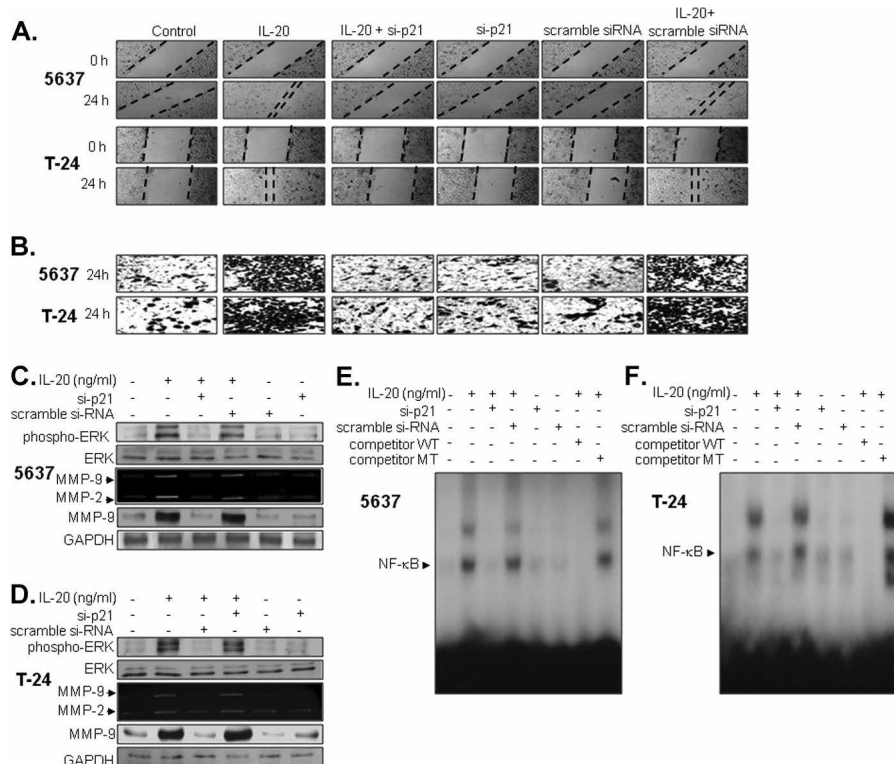


**FIGURE 6. IL-20-induced up-regulation of p21<sup>WAF1</sup> in bladder cancer cells.** *A* and *B*, after serum starvation for 24 h, cells were treated with the indicated concentrations of IL-20 for 24 h. Cell cycle distribution was then performed by flow cytometric analysis. The percentage of cells in each population is shown in the *table*. *C* and *D*, cells were incubated with the indicated concentrations of IL-20 for 24 h. The cell lysates were subjected to immunoblot analysis using antibodies specific for p21<sup>WAF1</sup>, p27 KIP1, and p53. The results from representative experiments were normalized to GAPDH expression. The quantification of each protein was normalized to GAPDH.

Bladder cancer cells were treated with IL-20 for 24 h, and p21<sup>WAF1</sup> protein levels were determined. The results of immunoblot analysis indicated that the level of p21<sup>WAF1</sup> was

increased by IL-20 in a dose-dependent manner (Fig. 6, *C* and *D*). However, the expression levels of p53 and p27 KIP1 proteins were degraded by IL-20 (Fig. 6, *C* and *D*). These unex-





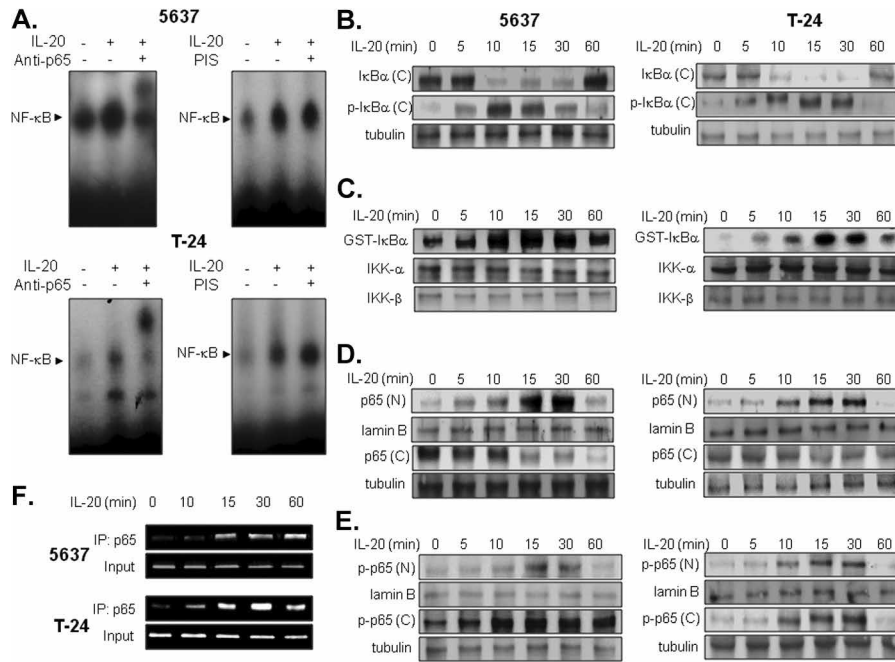
**FIGURE 7. Role of p21<sup>WAF1</sup> in the IL-20-mediated stimulation of migration, ERK1/2 activation, and transcription factor-mediated MMP-9 expression in bladder cancer cells.** *A*, cells were transfected with si-p21 or scrambled siRNA for 24 h, and cells were then scratched with pipette tips and stimulated with IL-20 (50 ng/ml). After 24 h, the healing areas of migrated cells were visualized under a phase contrast inverted microscope. *B*, cells were transfected with the si-p21 or scrambled siRNA and stimulated with IL-20 (50 ng/ml). After 24 h, an invasion assay was performed. *C* and *D*, cells were transiently transfected with si-p21 or scrambled siRNA and treated with IL-20 (50 ng/ml) for 24 h. Zymographic and immunoblot analyses were performed from cultured medium and cell lysates for MMP-9 expression. For the activation of ERK1/2, cells were transfected with si-p21 or scrambled siRNA. The cells were then incubated with IL-20 for 10 min. The cell lysates were subjected to immunoblot. The activation of ERK1/2 was measured. *E* and *F*, after transfection of either si-p21 or scrambled siRNA, the cells were stimulated with IL-20 for 24 h. The NF- $\kappa$ B DNA binding activity was examined by EMSA using nuclear extracts from the cells. An unlabeled NF- $\kappa$ B oligo probe (WT) or a mutant (MT) oligonucleotide probe was used as a competitor control.

pected results suggest that p21<sup>WAF1</sup> might be an important factor in the regulation of IL-20-stimulated bladder cancer cell responses.

*siRNA-mediated Knockdown of p21<sup>WAF1</sup> Restored Migration, MMP-9 Expression, ERK1/2 Activation, and Binding Activity of NF- $\kappa$ B in IL-20-stimulated Bladder Cancer Cells*—The results reported above prompted us to examine whether p21<sup>WAF1</sup> is involved in IL-20-mediated cellular responses. To this end, bladder cancer cells were transfected with a p21<sup>WAF1</sup>-specific siRNA (si-p21) or a scrambled siRNA and then treated with IL-20. As shown in Fig. 7, *A* and *B*, IL-20 treatment of bladder cancer cells or of cells transfected with the scrambled siRNA induced wound-healing migration and invasive ability. These stimulatory effects were nearly completely suppressed by transfection with si-p21 in 5637 and T-24 cells (Fig. 7, *A* and *B*). The knockdown of p21<sup>WAF1</sup> was confirmed by immunoblot analysis (supplemental Fig. S1, *C* and *D*). IL-20-induced activation of ERK1/2 was also reversed in the presence of si-p21 transfection in 5637 and T-24 cells (Fig. 7, *C* and *D*). In addition, the increased MMP-9 expression induced by IL-20 was suppressed in bladder cancer cells transfected with si-p21, as shown by gelatin zymography and immunoblot analysis (Fig. 7, *C* and *D*). Finally, to investigate the possible implication of transcription factor NF- $\kappa$ B in the regulation of MMP-9 in response to IL-20 by p21<sup>WAF1</sup>, we performed an EMSA. As shown in Fig. 7, *E* and

*F*, NF- $\kappa$ B DNA binding activity was almost abolished by transfection of si-p21 in both cell lines. However, the cells transfected with si-p27 and si-p53 did not affect the IL-20-induced cellular response, including the ability of wound-healing migration and invasion, ERK1/2 activation, MMP-9 expression, and NF- $\kappa$ B DNA binding activity in both bladder cancer cell lines (supplemental Figs. S5, *A–F*, and S6, *A–F*). These results demonstrate that p21<sup>WAF1</sup> must be involved in cell migration via ERK1/2-mediated MMP-9 expression by inducing the activation of NF- $\kappa$ B binding in IL-20-treated bladder cancer cells.

*I $\kappa$ B $\alpha$  Degradation and NF- $\kappa$ B p65 Translocation Induced by IL-20 in Bladder Cancer Cells*—Cell activation by cytokines has led to the phosphorylation and degradation of I $\kappa$ B $\alpha$ , followed by NF- $\kappa$ B translocation into the nucleus (33). To elucidate the effect of IL-20 on the I $\kappa$ B $\alpha$  proteolytic pathway, we examined the phosphorylated and protein levels of I $\kappa$ B $\alpha$  by immunoblot analysis. As shown in Fig. 8*B*, IL-20 caused a rapid degradation of I $\kappa$ B $\alpha$  at 10, 15, and 30 min compared with the control but was restored to control levels at 60 min in both cell lines (Fig. 8*B*). We next examined the level of I $\kappa$ B $\alpha$  phosphorylation because phosphorylation of I $\kappa$ B $\alpha$  results in its degradation. A substantial increase in I $\kappa$ B $\alpha$  phosphorylation was highly recorded at 10 and 15 min in IL-20-treated 5637 cells (Fig. 8*B*). In the case of T-24 cells, IL-20 induced the phosphorylation of I $\kappa$ B $\alpha$  at 10, 15, and 30 min (Fig. 8*B*). To determine whether IL-20-induced deg-



**FIGURE 8. IL-20-induced activation of IKK, phosphorylation and degradation of IκBα, and translocation of p65 subunits in bladder cancer cells.** *A*, cells were treated with IL-20 (50 ng/ml) for 24 h and then incubated with the indicated antibody for 30 min. They were assayed by EMSA. Preimmune serum (PIS) was included as the negative control. *B*, IL-20-induced phosphorylation and degradation of IκBα. The cells were incubated with IL-20 (50 ng/ml) for the indicated times. Cytoplasmic extracts were isolated and subjected to immunoblot using antibodies against IκBα or phospho-IκBα. Tubulin was used as a loading control protein. *C*, activation of IKK by IL-20. The cells were incubated with IL-20 (50 ng/ml) for differing time periods. Whole cell extracts were immunoprecipitated with antibody against IKK-α/β and subjected to immune complex kinase assay. To examine the effect of IL-20 on the level of expression of IKK proteins, whole cell extracts were examined by immunoblot using anti-IKK-α and anti-IKK-β antibodies. *D* and *E*, IL-20 induced the nuclear translocation of p65. The cells were either untreated or pretreated with IL-20 (50 ng/ml) for the indicated times. Cytoplasmic (C) and nuclear extracts (N) were prepared and analyzed by immunoblot using antibodies against p65 or phospho-p65 (p-p65). For loading control of nuclear and cytoplasmic protein, anti-tubulin and lamin B antibodies were used, respectively. *F*, ChIP analysis of the MMP-9 NF-κB-binding site. The cells were treated with IL-20 for the indicated time intervals. DNA immunoprecipitated by an anti-NF-κB p65 antibody was purified. Immunoprecipitated (IP) DNA was amplified by PCR using primers. The input represents PCR products from chromatin pellets prior to immunoprecipitation.

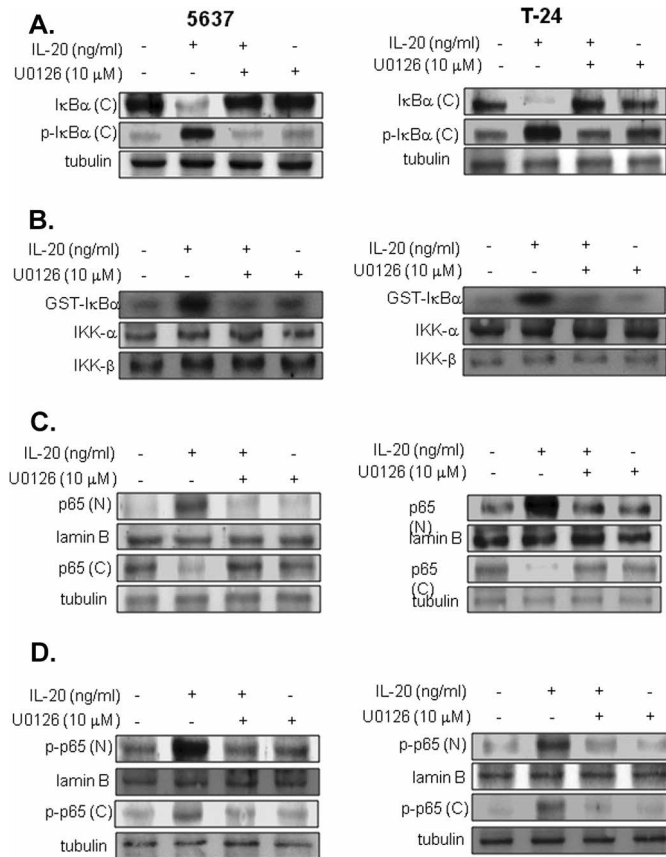
radiation and phosphorylation of IκBα was due to the activation of IKK, we analyzed the IKK activity in IL-20-treated cells. IL-20 treatment induced kinase activity of IKK in both cell lines (Fig. 8C), but it did not affect the expression of immunoprecipitated IKK-α or IKK-β proteins (Fig. 8C).

The classic form of NF-κB is a heterodimer that consists of the p50 and p65 subunits (34, 35). The p65 subunit contains a supershift assay using p65 antibody to characterize the p65 subunit responsible for DNA binding. Incubation of the nuclear extract from cultured bladder cancer cells with antibodies directed against the p65 subunit showed a supershift of the NF-κB band in the presence of IL-20 (Fig. 8A). Preimmune serum did not affect the DNA binding (Fig. 8A). These results suggest that IL-20 may target the p65 subunit of NF-κB.

Because the p65 subunit is indispensable for the activation of NF-κB, we investigated the nuclear translocation of p65 subunits in IL-20-treated cells. The nuclear translocation of p65 was increased transiently in both cells after IL-20 treatment (Fig. 8D). For cytoplasmic extracts, the levels of the p65 subunit were largely decreased by IL-20 at 15 and 30 min in 5637 and T-24 cells (Fig. 8D). We also examined the effect of IL-20 on the phosphorylation of p65 subunits, which is required for the transcriptional activity of NF-κB (36). The treatment of IL-20 with both types of bladder cancer cells stimulated p65 phosphorylation in the nucleus and cytoplasm (Fig. 8E).

To further investigate whether the p65 subunit induced by IL-20 interacts with the *MMP-9* promoter region, a ChIP-PCR assay was performed. The results demonstrated that IL-20 stimulated the binding of p65 subunits to the *MMP-9* promoter region (Fig. 8F). Together, these results demonstrate that IL-20 stimulated IKK activation, IκBα degradation and phosphorylation, NF-κB p65 nuclear translocation, and recruitment of p65 to the *MMP-9* promoter region in bladder cancer cells.

*U0126, an ERK1/2-specific Inhibitor, Suppressed Phosphorylation and Degradation of IκBα, Activation of IKK, and NF-κB p65 Nuclear Translocation in IL-20-treated Bladder Cancer Cells*—U0126 inhibited IL-20-induced activation of NF-κB in bladder cancer cells (Fig. 4, D and E). Therefore, we investigated whether U0126 affects the molecular mechanism of NF-κB induced by IL-20. U0126 treatment almost inhibited degradation of IκBα by IL-20 in both cells (Fig. 9A). In addition, treatment with U0126 blocked the IL-20-induced phosphorylation of IκBα (Fig. 9A). Moreover, results from the immune complex kinase assay showed that U0126 suppressed IL-20-induced IKK activity in both cells (Fig. 9B). We next investigated whether U0126 regulates IL-20-induced nuclear translocation of p65 subunits. Treatment of cells with U0126 impeded the translocation of p65 to the nucleus from the cytoplasm in bladder cancer cells (Fig. 9C). Furthermore, IL-20 failed to induce the phosphorylation of p65 subunits in the presence of U0126 in the cytoplasm and nuclear exposure (Fig. 9D).



**FIGURE 9. ERK1/2 inhibitor U0126 suppressed the IL-20-induced activation of IKK, phosphorylation and degradation of IκBα, and translocation of p65 subunits in bladder cancer cells.** *A*, *C*, and *D*, U0126 inhibited the phosphorylation and degradation of IκBα and translocation of p65 subunit in cells. The cells were untreated or treated with U0126 (10 μM) for 4 h and then stimulated with IL-20 (50 ng/ml) for 15 min. Cytoplasmic (C) and nuclear (N) extracts were prepared and analyzed by immunoblot using antibodies against IκBα, phospho-IκBα, p65, or phospho-p65 (p-p65). For loading control of nuclear and cytoplasmic protein, antibodies specific for tubulin and lamin B were used, respectively. *B*, U0126 inhibits IL-20-induced IKK activity. The cells were untreated or treated with U0126 (10 μM) for 4 h, followed by treatment with IL-20 for 15 min. The IKK proteins were then immunoprecipitated with anti-IKK-α/β antibody. The levels of activation of GST-IκBα were detected by IKK assay. The immunoprecipitated IKK protein levels were examined from whole cell extracts by immunoblot using anti-IKK-α and anti-IKK-β antibodies.

*Effect of the IL-20 Gene and the Anti-IL-20 Antibody on Wound-healing Migration and Invasion in Bladder Cancer Cells*—To investigate direct effect of IL-20 on wound-healing migration and invasion in bladder cancer cells, both types of bladder cancer cells were transfected either with IL-20 cDNA or an empty vector. The wound-healing and invasion ability of 5637 and T-24 cells transfected with the *IL-20* gene was increased in 24 h, compared with control or cells transfected with empty vector (supplemental Fig. S7, *A* and *B*). Furthermore, the effects of anti-IL-20 antibody on wound-healing migration and invasion were then examined by adding antibodies to the culture medium. The results showed that the increased ability of wound-healing migration and invasion induced by IL-20 was strongly reversed in the presence of an anti-IL-20 antibody in both cells (supplemental Fig. S7, *C* and *D*). These results suggest that IL-20 must be involved in the wound-healing migration and invasion of bladder cancer cells.

## DISCUSSION

IL-20 is a pro-inflammatory cytokine associated with psoriasis, rheumatoid arthritis, and cardiovascular diseases (16–18). Several lines of study have demonstrated that IL-20 stimulated activation of JAK-STAT and MAPK signaling in several types of cell lines, including keratinocytes, HUVEC, and GBM8901 glioblastoma cells (16, 19, 20). The recent results of immunotherapy using anti-IL-20 antibody showed the suppression of breast cancer-induced osteolysis (37). However, the role and molecular mechanism of IL-20 in tumor migration have not previously been characterized. In this study, we report the molecular events involved in the migration of bladder cancer cells in response to IL-20.

Cumulative studies have demonstrated that cytokines produced by various kinds of stimuli, including inflammation, promote invasion and migration of tumor cells (24–26). The enhanced invasive and proliferative capacity of tumor cells has been associated with the presence of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and IL-17 (24, 25, 38–40). In this study obtained from real time PCR, the expression levels of IL-20 and IL-20R1 mRNA were up-regulated in patients with MIBC. In addition, our results showed that the expression of both IL-20 and IL-20R1 was found in 5637 and T-24 bladder cancer cells. Based on these findings, we postulated that IL-20 might play a pivotal role in the development and progression of bladder cancer. We then initiated this study of how IL-20 might contribute to the regulation of tumor progression in bladder cancer.

The main events in the metastatic process are generally recognized to be the migration and invasion of tumor cells, which require degradation of ECM components by proteolytic enzymes, such as MMP-2 and MMP-9 (5, 6). A number of factors, including growth factor and cytokine, have been shown to induce cell migration: EGF, IL-1β, IL-6, IL-8, IL-18, ephrin B1, TGF-β, PDGF, FGF, VEGF, and insulin (41, 42). Here, we found that IL-20 induced the migration of bladder cancer cells, independent of cell cycle progression. The effects of IL-20 on cell migration were confirmed by overexpression of the *IL-20* gene or by the addition of anti-IL-20 antibody. This result is consistent with a recent report regarding the role of IL-20 in promoting the growth and migration of breast cancer cells (37). In addition, transfection of IL-20R1-specific siRNA (si-IL-20R1) into bladder cancer cells significantly inhibited IL-20-induced migration and invasion. However, unexpectedly, the blockage of other receptors, IL-20R2 and IL-22R1, had no effect on the migration and invasion of bladder cancer cells induced by IL-20. These effects of IL-20 may work through binding of the IL-20R1 receptor, which results in cell migration and invasion.

The expression of MMP is known to play a role in bladder cancer progression and migration in clinical and animal studies (4). Enhanced levels of MMP-2 and MMP-9 expression have been found in bladder cancer progression (43–48). MMP-9 levels were increased in high grade and advanced stage bladder tumors, which are correlated with MIBC (4, 44, 46). Based on previous results, it was concluded that MMP-9 expression might contribute to enhanced invasive bladder cancer progression (4). Despite the obviously important role of cytokines in

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the progression of bladder cancer (4, 38–40), the processes by which IL-20 are implicated in MMP-9 regulation were not completely understood. We thus hypothesized that IL-20 could affect the regulatory mechanism of MMP-9 in bladder cancer cells. In support of this hypothesis, we found that exogenous IL-20 promoted MMP-9 expression via activation of NF- $\kappa$ B and AP-1 binding in 5637 bladder cancer cells. In the case of T-24 cells, our results indicated that the NF- $\kappa$ B site is a main factor in the IL-20-mediated transcriptional activation of MMP-9. In addition, transfection with si-IL-20R1 significantly reduced MMP-9 expression and the binding activity of NF- $\kappa$ B induced by IL-20 in both bladder cancer cell lines. These results suggest that the IL-20 receptor, IL-20R1, is required for the up-regulation of IL-20-induced MMP-9 via activation of NF- $\kappa$ B in bladder cancer cells, which leads to the destruction of ECM and influences cell migration and invasion in bladder cancer progression.

Many cytokines mediate signaling through distinct pathways in several cell lines, such as MAPK and the JAK-STAT pathway (16, 18–20). In this study, we found that IL-20 significantly induced ERK, JNK, and p38 MAPK in 5637 cells. Activation of ERK and JNK was stimulated by IL-20 in T-24 cells. In addition, our results showed the induction of JAK1, JAK2, STAT1, and STAT2 activation in both 5637 and T-24 cells. Using inhibitors of each signaling molecule, this study showed that ERK1/2 signaling is involved in the migration and invasion of bladder cancer cells in response to IL-20. Furthermore, we demonstrated that ERK1/2 is indispensable for NF- $\kappa$ B-mediated MMP-9 expression in IL-20-treated bladder cancer cells. However, other signaling pathways, including JNK, p38 MAPK, JAK1, and JAK2, had no effect on IL-20-induced cell migration and invasion. Previously, several lines of study have demonstrated that IL-20 induced the signaling of MAPK and JAK2/STAT3 in IL-20-treated cells, such as keratinocytes, HUVEC, and GBM8901 glioblastoma cells (16, 19, 20). However, the identification and role of the signaling pathway in IL-20-mediated cell migration and invasion have remained largely unknown to date. The present results represent the first report that ERK1/2 signaling is a crucial factor responsible for the IL-20-mediated migration and invasion of bladder cancer cells. We also identified the involvement of transcription factor NF- $\kappa$ B in the ERK-mediated control of the MMP-9 expression in IL-20-treated bladder cancer cells.

Although the importance of cell cycle regulation has been demonstrated in tumor development and progression (14, 31, 32), little is currently known regarding the effect of IL-20 on bladder cancer cell response. Many studies have demonstrated that the mutation of cell cycle inhibitory genes, such as p53, is associated with bladder cancer stage, progression, and prognosis (32, 49). Recently, the importance of the expression of cyclin D1 and the shuttling of cyclin E during the development of bladder cancer has been demonstrated (50, 51). Although previous reports have shown the role of the loss of p21<sup>WAF1</sup> expression in predicting poor outcomes in MIBC patients (31), these findings contradict others suggesting that p21 expression can provide a poor prognosis in patients with bladder cancer (52). Several lines of evidence have shown that the expression of p21<sup>WAF1</sup> was initially considered an anti-growth factor, based

on its ability to inhibit cell proliferation (12, 13). However, other studies have demonstrated that p21 expression is also involved in the regulation of cellular processes, including proliferation and migration, which results in a tumor-promoting oncogenic factor under certain circumstances (12, 13). The involvement of cell cycle regulation in the progression of bladder cancer has recently been investigated (13). However, the molecular mechanism of cell cycle regulators coordinated with the migration and invasion of cancer cells remains to be clarified. In this study, we examined the immunoblot analysis and then observed the elevated expression levels of p21<sup>WAF1</sup> induced by IL-20 in both bladder cancer cell lines. In an attempt to understand how p21<sup>WAF1</sup> mediates cell migration and invasion, we introduced the specific siRNA knockdown system of p21<sup>WAF1</sup> (si-p21). Our results support the novel notion that p21<sup>WAF1</sup> is an essential factor in the migration and invasion of bladder cancer cells in response to IL-20. Our data further extended these findings by indicating that p21<sup>WAF1</sup> regulated ERK1/2-mediated MMP-9 expression through the activation of NF- $\kappa$ B binding in the IL-20-stimulated migration and invasion of bladder cancer cells. These results suggest that p21<sup>WAF1</sup> may mediate the role of tumor migration and invasion, which results in the progression of bladder cancer cells. Future studies must examine the exact mechanism of p21<sup>WAF1</sup> in IL-20-mediated ERK1/2, MMP-9, and NF- $\kappa$ B in bladder cancer cells.

The classical NF- $\kappa$ B proteins are dimeric transcription factors (p50/p65) and regulate many genes involved in mammalian immune systems, proliferation, cell survival, and adhesion (35). In this study, we found that IL-20 induced NF- $\kappa$ B activation through IKK activity, which led to phosphorylation and degradation of I $\kappa$ B $\alpha$  and the induction of nuclear translocation and phosphorylation of the p65 subunit of NF- $\kappa$ B. IL-20 stimulated the recruitment of the p65 subunit to the MMP-9 promoter region. In addition, our chemical inhibitor U0126 study demonstrated that an IL-20-induced classical NF- $\kappa$ B pathway is regulated by ERK1/2 signaling. Further studies are required to investigate the issue of whether the alternative NF- $\kappa$ B pathway is involved in the IL-20-induced migration and invasion of bladder cancer cells.

The accumulated studies have suggested that inflammatory mediators, including cytokines, are crucial constituents of the local environment of tumors and are involved in the migration, invasion, and metastasis of malignant cells (24–26). Inflammatory cytokines released by cancer cells or inflammatory cells have been identified as a tumor-promoting factor (24–26). Conversely, several studies have demonstrated that inflammatory cytokines are a tumor inhibitory factor (24–26). In this study, we demonstrated that MMP-9 is produced by bladder cancer cells in response to IL-20, which in turn results in the breakdown of ECM, subsequently leading to the migration and invasion of cancer cells. In addition, using a siRNA approach, we carefully suggested the role of IL-20R1, not IL-20R2 and IL-22R1, in the regulation of the IL-20-induced migration and invasion of bladder cancer cells as a binding partner. We now propose that IL-20 can signal via the IL-20R1 receptor, which may stimulate the various actions of bladder cancer cell responses, such as activation of signaling molecules, enhanced migration and invasion, and MMP-9 expression.

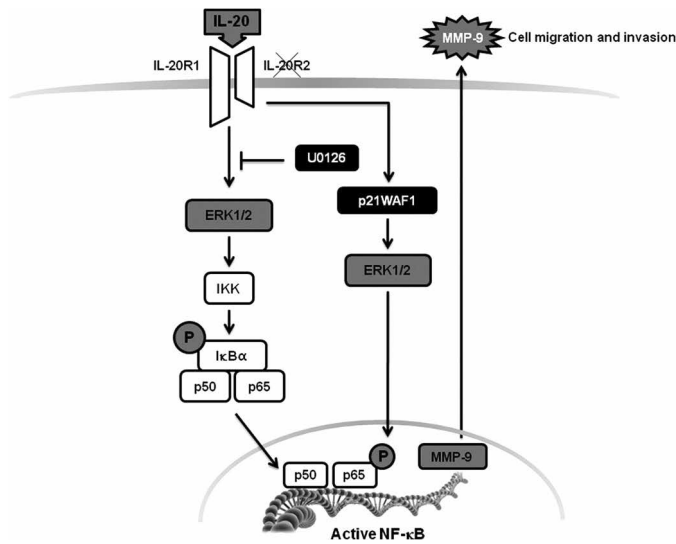


FIGURE 10. Molecular events of the IL-20/IL-20R1 dyad-mediated bladder cancer cell migration.

In conclusion, based on the present results, we showed elevated expression levels of IL-20 and IL-20R1 in MIBC patients, compared with healthy individuals. We then focused on the role of IL-20 in regulating the migration and invasion of cancer cells. We proposed the novel notion that IL-20 signaling, by binding IL-20R1, stimulates the migration and invasion of bladder cancer cells. In addition, the results of this study demonstrated that cell cycle inhibitor p21<sup>WAF1</sup> is required for cell migration and invasion via ERK1/2-dependent MMP-9 expression, by inducing the activation of NF-κB in IL-20-treated bladder cancer cells. Moreover, IL-20 stimulated the classical NF-κB pathway, which is regulated by ERK1/2 signaling. The identified signaling pathways regulating IL-20-induced migration and invasion in bladder cancer cells are summarized in Fig. 10.

Taken together, the results indicate that IL-20 produced by tumor cells is an inducer having a role in migration and invasion that leads to the progression of bladder cancer cells, which could enable the development of a range of potential therapies targeting malignant cells. The efficacy of the *IL-20* gene *in vivo* will be the focus of our future studies to elucidate the progression and invasion of bladder tumors.

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