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The Regulatory Mechanism of the *LY6K* Gene Expression in Human Breast Cancer Cells*[S]

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Background: LY6K is a candidate cancer biomarker that promotes invasion and metastasis.
Results: AP-1 activation is required for the *LY6K* expression and interfered by SNP242 or methylation.
Conclusion: AP-1 promotes *LY6K* expression that regulates cell mobility, whereas SNP242 or methylation reduces the metastasis.
Significance: Understanding the regulatory mechanisms of the *LY6K* is important for investigating breast cancer risk.

LY6K is a cancer biomarker and a therapeutic target that induces invasion and metastasis. However, the molecular mechanisms that determine human *LY6K* transcription are completely unknown. To elucidate the mechanisms involved in human *LY6K* gene regulation and expression, multiple *cis*-elements were predicted using TRANSFAC software, and the *LY6K* regulatory region was identified using the luciferase assay in the human *LY6K* gene promoter. We performed ChIP, EMSA, and supershift assays to investigate the transcription factor activity on the *LY6K* promoter, and the effect of a SNP and CpG site methylation on AP-1 transcription factor binding affinity. AP-1 and the CREB transcription factor bound to *LY6K* promoter within −550/−1, which was essential for LY6K expression, but only the AP-1 heterodimer, JunD, and Fra-1, modulates *LY6K* gene transcriptional level. A decrease in LY6K was associated with the SNP242 C allele, a polymorphic G/C-SNP at the 242 nucleotide in the *LY6K* promoter region (rs2585175), or methylation of the CpG site, which was closely located with the AP-1 site by interfering with binding of the AP-1 transcription factor to the *LY6K* promoter. Our findings reveal an important role for AP-1 activation in promoting *LY6K* gene expression that regulates cell mobility of breast cancer cells, whereas the SNP242 C allele or methylation of the CpG site may reduce the risk of invasion or metastasis by interfering AP-1 activation.

The human LY6K (lymphocyte antigen 6 complex locus K) belongs to the Ly-6/urokinase-type plasminogen activator receptor (uPAR)[2] superfamily, which can be divided into two subfamilies based on the glycosylphosphatidylinositol-anchoring signal sequence: one is the glycosylphosphatidylinositol-anchored transmembrane proteins, which include the retinoic acid-induced gene E (human LY6E), the E48 antigen (human LY6D), LY6H, prostate stem cell antigen, CD59, or protectin, lynxl, and uPAR (1–3). The other is a secretory protein without a glycosylphosphatidylinositol-anchoring signal sequence, which includes SLURP-1 and SLURP-2 (4). The glycosylphosphatidylinositol-anchored uPAR modulates tumor invasion, growth, and metastasis via the integrin-related Ras/ERK signaling pathway (5, 6).

The LY6K was first identified as a molecular marker for head-and-neck squamous cell carcinoma (7). Previous studies have shown that cancer-testis antigen LY6K could be a diagnostic biomarker and a therapeutic target for breast cancer, non-small cell lung carcinoma, bladder cancer, and esophageal squamous cell carcinoma (8–11). In breast cancer, elevated LY6K induces cell invasion and metastasis by activating the Raf-1/MEK/ERK signaling pathway and up-regulating expression of matrix metalloproteinase proteins MMP-2 and MMP-9 (12). Likewise, LY6K regulates cell growth, migration, and invasion in bladder cancer cell lines (10). However, the molecular mechanisms that mediate regulation of *LY6K* gene expression in cancer are unknown.

The activating protein-1 (AP-1) transcription factors are ubiquitously expressed and are composed of the Jun family (c-Jun, JunB, JunD) homodimers, Jun heterodimers with Fos (c-Fos, FosB, Fra-1, Fra-2), or ATF (activating transcription factor) proteins. The AP-1 dimeric complex binds to the DNA regulatory sequence, called the TPA response element, which regulates gene expression in response to a variety of stimuli, including growth factors, cytokines, and ultraviolet radiation (13, 14). The dimeric combination of the AP-1 family determines the genes that are regulated by AP-1 (15, 16). Cellular growth, proliferation, survival, differentiation, and environmental stress signals induce formation of the AP-1 transcriptional complex and modulate AP-1 transcriptional activity (14, 17). Activating AP-1 may contribute to breast and other tumor cell growth, invasive ability, and metastasis (18, 19).

Single nucleotide polymorphisms (SNPs) are the most frequent type of variation in the human genome, occurring once every several hundred base pairs throughout the genome (20). SNPs are polymorphic markers that provide a comprehensive

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[S] This article contains supplemental Table 1 and Figs. 1–3.

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[2] The abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; CREB, cAMP-responsive element-binding protein; AP-1, activating protein-1; luc, luciferase; NE, nuclear extract; ADR, adriamycin resistance; mt, mutant.

tool for analyzing the human genome and identifying specific genes and genomic regions linked to cancer phenotypes (21). Functional SNPs located on the promoter modulate gene expression leading to hormone or drug sensitivity and disease susceptibility (22, 23).

DNA methylation occurs when a methyl group is added to a CpG site and is an epigenetic event that plays an important role in gene regulation. In particular, methylation of the gene promoter CpG islands is tightly linked to histone modifications and nucleosome remodeling mechanisms that cause gene silencing (24, 25). Aberrant DNA methylation in human cancers is one of the most consistent epigenetic mechanisms that activates or silences gene expression (25).

In the present study, we identified the regulatory region essential for *LY6K* promoter activity. We also provide the first evidence that *LY6K* gene expression is regulated by SNP or DNA methylation in the proximal promoter region. Our findings suggest that the AP-1 transcription factor affects invasion and metastasis by regulating *LY6K* expression in breast cancer cells and that SNP242 or DNA methylation of a specific CpG site within the *LY6K* promoter down-regulates *LY6K* by effectively interfering with AP-1 binding, suggesting a reduced risk of breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast carcinoma cells used in this study included the MCF7 and MCF7-ADR, sublines, which are adriamycin-resistant (obtained from Roswell Park Cancer Institute, Buffalo, NY). Cells were grown in Dulbecco's modified Eagle's medium (WelGENE, Inc., Daejeon, Korea) containing 10% fetal bovine serum (WelGENE) and maintained at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air.

LY6K Promoter Constructs—Promoter constructs encompassing the region from −2.0 kb to 0.2 kb relative to the transcription start site of the human *LY6K* gene were amplified from human genomic DNA using specifically designed forward and reverse primers containing the XhoI and HindIII restriction enzyme sites, respectively. Each fragment was digested with XhoI and HindIII purified using the HiYield Gel/PCR extraction kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's recommendations and cloned into the pGL3-basic vector (Promega, Madison, WI). Deleted constructs from −500 to −1 and from −1550 to −1050 were also amplified by PCR with primers, including specific restriction enzyme sites, which were purified using the HiYield gel/PCR extraction kit (RBC Bioscience), and cloned into the pGL3-basic vector.

Transient Transfection and Luciferase Reporter Assay—MCF7-ADR cells were seeded in a 12-well plate at 1×10^5 per well and temporarily transfected with 1 μg of the *LY6K* promoter reporter vector using the FuGENE HD transfection reagent (Roche Applied Science). Cells were co-transfected with 40 ng of the phRL-CMV vector to verify transfection efficiency. After 24–48 h of transfection, the cells were lysed in 1× passive lysis buffer (Promega), and luciferase activity was measured using the Dual-Luciferase reporter assay (Promega) and a luminometer (Tuner Designs, Sunnyvale, CA) according to the manufacturer's instructions.

Site-directed Mutagenesis—The DNA fragments for AP-1 and SNP242 point mutated sites were created by PCR using specifically designed primers (supplemental Table 1) by incorporating the required base pair substitutions and using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The template for this process was the pGL3-*LY6K*-0.5 vector. The PCR cycling parameters were as follows: 1 cycle of 95 °C for 30 s, 12 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 1 min for point mutation. The insert sequence was verified by DNA sequence analysis.

Chromatin Immunoprecipitation Assay—The imprint chromatin immunoprecipitation kit (CHP1, Sigma-Aldrich) was used for the ChIP assay. Briefly, cells were fixed with 1% formaldehyde and quenched with 1.25 M glycine, and the DNA was sheared by sonication on ice (total time, 15 min; on time, 30 s; off time, 2 min). The sheared DNA was incubated with IgG as a negative control, and c-Jun (sc-1694), JunB (sc-8051), JunD (sc-74), c-Fos (sc-52), FosB (sc-28213), Fra-1 (sc-183), Fra-2 (sc-604), and PAX3 (sc-81351) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in a strip well overnight at 4 °C. The strip well was washed, the cross-link was reversed, and the DNA was purified with the GenElute Binding Column G (C6863, Sigma-Aldrich). Prepared samples were amplified using specifically designed ChIP primers for the region of interest (supplemental Table 1). PCR conditions for the ChIP analysis were as follows: 1 cycle of 95 °C for 2 min, 50 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, 72 °C for 5 min, and storage at 4 °C.

Nuclear Extracts and Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared using a nuclear extraction kit (Panomics, Inc., Santa Clara, CA), according to the manufacturer's instructions. The sequences of the predicted transcription binding site probe within the 550-bp human *LY6K* promoter region are presented in Table 1. Double-stranded oligonucleotides containing the AP-1 and CREB consensus sites were purchased from Promega. The sequences of the wild type and mutant probes containing the transcription binding site are presented in supplemental Table 1. For binding assays, 10 μg of nuclear extract samples were incubated with purified γ-^{32}P-labeled probe using G-50 Sephadex columns (Roche Applied Science) at room temperature for 30 min in a buffer containing 100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM ethylenediaminetatraacetic acid (EDTA), 10% glycerol, 1 mM DTT, proteinase inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml luepeptin) and 0.2 μg poly(dI-dC) in a 20-μl reaction volume. DNA-protein complexes were analyzed on 4% non-denaturing polyacrylamide gels that had been prerun at 200 V for 30 min in 0.5× Tris borate-EDTA buffer. The gels ran at 150 V for 2 h, dried under vacuum at 80 °C for 1 h, and then exposed to a FLA-7000 cassette. For competition assays, 10 μg of nuclear extract was incubated with unlabeled probe for 15 min before adding the purified γ-^{32}P-labeled probe.

Supershift Assay—Antibodies against the AP-1 factor (Santa Cruz Biotechnology), CREB (9104, Cell Signaling Technology, Inc., Danvers, MA), CBP (sc-369, Santa Cruz Biotechnology) or PAX3 (sc-81351, Santa Cruz Biotechnology), and 10 μg of nuclear extracts were incubated for 90 min and then added to the purified γ-^{32}P-labeled probe for 15 min at room tempera-

ture. The gels were run at 180 V for 3 h, dried under vacuum at 80 °C for 1 h, and then exposed to a FLA-7000 cassette.

RNA Interference—MCF7-ADR cells were transfected with several small interfering RNA (siRNAs) at a concentration of 30 nM for 48 h using Lipofectamine RNAi Max (Invitrogen), according to the manufacturer's protocol. siRNAs specific to *LY6K* (sc-77440), *JunD* (sc-35728), c-*Fos* (sc-29221), *Fra-1* (sc-35405), and *Fra-2* (sc-35407) were used to silence target genes. A control siRNA (sc-37007), which had no considerable homology to any known human sequence, was transfected as the negative control. All siRNAs were purchased from Santa Cruz Biotechnology.

Real-time RT-PCR—Total cellular RNAs were extracted with a NucleoSpin RNA/Protein kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. A total of 5 μg of RNA was used for reverse transcription with the M-MLV Reverse Transcription kit (Promega). A 100–300-ng portion of template cDNA was mixed with 1 μl of primer mix of forward and reverse preimers (supplemental Table 1) and 5 μl of SYBR Green Mastermix (Applied Biosystems, Foster City, CA).

Co-immunoprecipitation—Reciprocal IP was performed using 100-mm dishes of MCF7-ADR cells. The cells were washed with 1× cold PBS and harvested in 1 ml of 1× cold PBS following centrifugation at 12,000 rpm for 1 min at 4 °C. The pellets were lysed by vortexing with 100 μl of IP150 buffer/Nonidet P-40 (150 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10% glycerol, 1% Nonidet P-40, and 1 mM EDTA) and incubated for 15 min in ice. Lysates were diluted with 400 μl of IP150 buffer without Nonidet P-40 (37.5 mM NaCl, 6.25 mM Tris-HCl (pH 8.0), 2.5% glycerol, and 0.25 mM EDTA) and centrifuged at 12,000 rpm for 5 min at 4 °C to remove debris. The supernatants were incubated at 4 °C with anti-JunD rabbit polyclonal antibody (sc-74), anti-Fra-1 rabbit polyclonal antibody (sc-183), and anti-IgG rabbit polyclonal antibody as a negative control for 90 min. Immunoglobulin G (IgG)-Sepharose beads were used to immunoprecipitate the proteins. Following the incubation, the beads were washed three times with IP wash buffer (225 mM NaCl, 5 mM Tris-HCl (pH 8.0), 0.125% Nonidet P-40 and 0.25 mM EDTA). The washed beads containing the bound proteins were suspended in 20 μl of 6× SDS gel loading buffer and heated at 100 °C for 5 min. The released polypeptides in 15 μl of heated sample were resolved on 10–12% polyacrylamide gels.

Western Blot Analysis—Proteins from breast cancer cells were extracted using a NucleoSpin RNA/protein kit, (Macherey-Nagel), according to the manufacturer's instructions. Equal amounts of extracted protein were loaded on polyacrylamide gels and transferred to polyvinyl difluoride membranes. The membranes were blocked with 5% skim milk and incubated with specific primary antibodies. The antibody bound membranes were washed and then incubated with secondary antibodies and an anti-biotin marker. The membranes with bound antibodies were analyzed using the Amersham Biosciences ECL Plus Western blotting detection system (GE Healthcare). The primary antibodies were LY6K (sc-87282), JunD (sc-74), c-Fos (sc-52), Fra-1 (sc-183), and Fra-2 (sc-604) from Santa Cruz Biotechnology and anti-biotin (7075) from Cell Signaling Technology. The secondary antibodies were HRP-conjugated goat anti-rabbit IgG polyclonal (SAB-300J), and goat anti-mouse IgG F(ab′)2 polyclonal (SAB-100J), from Assay Designs (Ann Arbor, MI).

Statistical Analysis—The data, normalized to base line or control activity in individual experiments, were presented as mean ± S.E. Statistical significance was assessed by a Student's paired *t* test using GraphPad InStat Software (GraphPad Software, Inc., San Diego, CA). For all analyses, p values < 0.05 were considered statistically significant. In all figures, an *asterisk* represents a p value < 0.01.

RESULTS

Identification of the Putative LY6K Promoter Region—We have previously reported that LY6K is a potential breast cancer biomarker that affects invasive ability and metastasis (8). To investigate the regulatory mechanism of the *LY6K* gene, a series of *LY6K*-luc promoter constructs −2050/+182 were transfected into MCF7-ADR cells, and the levels of promoter activity were measured. The pGL3-control vector and the pGL3-basic vector were transfected as positive and negative controls, respectively. Transcriptional activity of *LY6K*-luc was normalized with the pGL3-basic vector, and we confirmed that the activity significantly increased −550/−200 and −1550/−1050 bps (Fig. 1*A*). Then, truncated constructs were specifically designed with deletions from −500 to −1 and from −1550 to −1050 to identify a more important site for controlling gene expression. The deletion from −550 to −1 sharply and significantly reduced promoter activity. However, deletion of −1550/−1050 did not alter promoter activity (Fig. 1*B*). These findings suggest the presence of a putative transcription binding site within the −550/−1 that regulates basal *LY6K* gene expression.

The AP-1 Transcription Factor Regulates LY6K Gene Expression—Given that promoter activity was reduced in −550/−1 deleted constructs, we investigated the DNA binding motif using TRANSFAC gene tool software, which is schematically listed in Table 1 and presented in Fig. 1*C*. We examined which transcription factor bound the *LY6K* promoter using EMSA (supplemental Fig. S1). We observed the formation of a prominent DNA-protein complex for the double-stranded oligonucleotide probes containing AP-1, cAMP-responsive element (CRE), and TATA sequences of the *LY6K* promoter. Various sized *LY6K* promoter constructs ligated to the luciferase reporter gene were transiently transfected into MCF7-ADR cells (Fig. 1*C*). The shorter pGL3−73 construct, which encompassed nucleotides −73 to +182, contained a minimal TATA box. This construct represented basal *LY6K* promoter activity. The pGL3−133 construct showed basal-like activity in the MCF7-ADR cell line. The pGL3−312 construct directed a 5-fold increase above basal transcription activity, suggesting the presence of important *cis*-regulatory elements, AP-1 and NF-κB, between nucleotides −133 and −312 of the *LY6K* promoter (Fig. 1*C*). Based on these results, we focused our study on the region encompassing nucleotides −312 to −133 of the *LY6K* promoter. The *LY6K* gene had the rs2585175 SNP located at nucleotide 242 within −312 to −133 of the *LY6K* promoter (called SNP242). We designed mutant constructs for AP-1 and SNP242 using site-directed mutagenesis to deter-

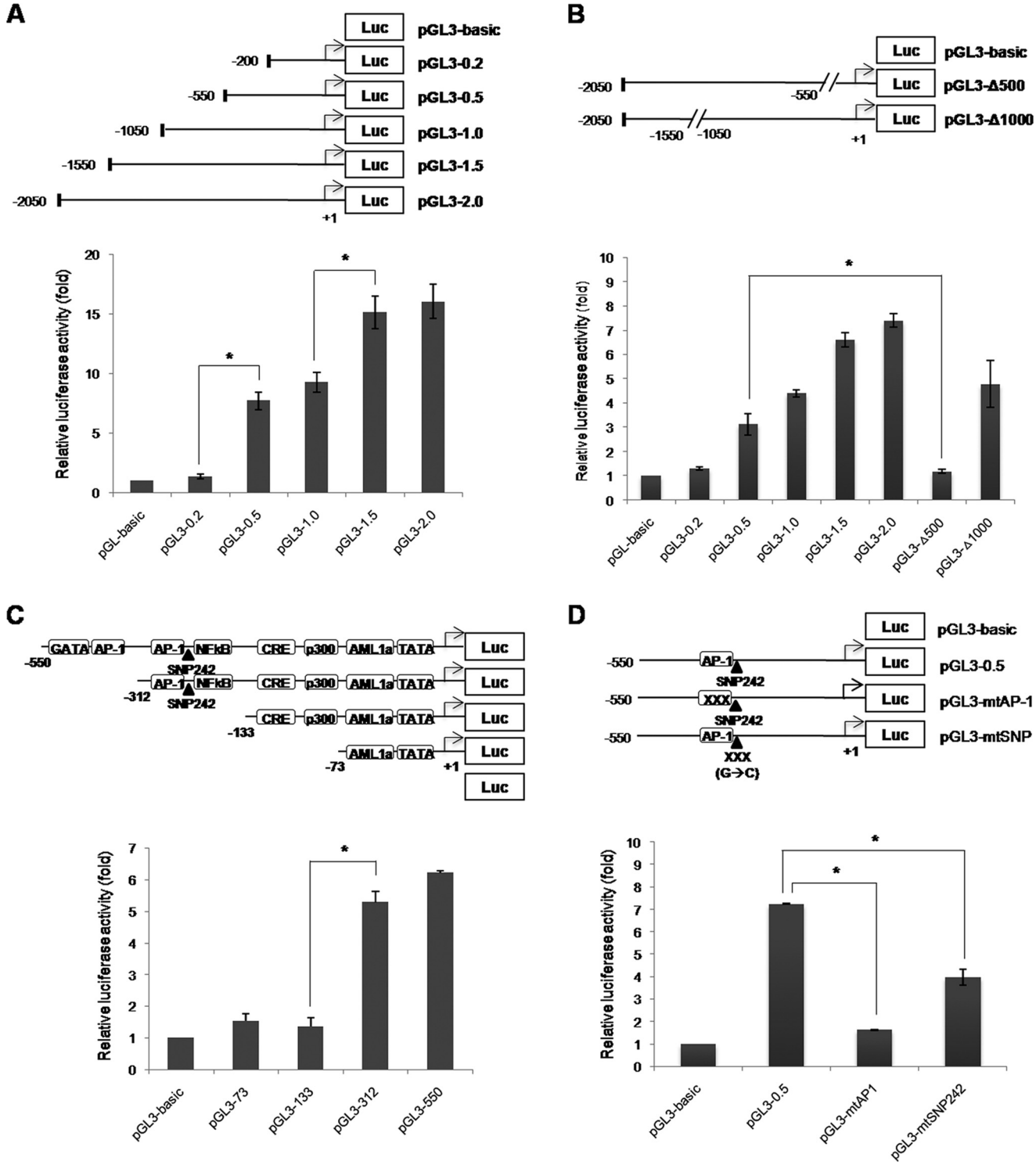

FIGURE 1. **Transcriptional activity of the *LY6K*-luc reporters in MCF7-ADR cells.** *A*, series constructs of the human *LY6K* gene promoter from −2050 to +182 cloned into the pGL3-luc vector. Transcriptional activity of the *LY6K* promoter was determined by transient transfection into the MCF7-ADR cell line and by quantifying the *LY6K* promoter linked to firefly luciferase activity. Promoter activity of *LY6K*-luc in MCF7-ADR cells was normalized with the pGL3-basic vector, which was transfected as a negative control. *B*, pGL3-Δ500 and Δ1000 are truncated constructs of the *LY6K* promoter which were missing bp −500 to −1 and −1550 to −1050, respectively. *C*, deletion analysis of the human *LY6K* promoter. Schematic presentation of the promoter elements in the *LY6K* promoter, based on the predicted *LY6K* promoter sequence. *D*, site-directed mutated *LY6K*-luc constructs. Substitution of two base pairs of the AP-1 binding site GA for CT, or a single base pair of SNP242 G for C. Site-directed mutated *LY6K*-luc reporter was co-transfected with phRL-CMV into MCF7-ADR cells. Cell lysates were measured using the luciferase assay.

mine the functional role of AP-1 and SNP242 in regulating the *LY6K* promoter. The pGL3-basic vector was transfected as the negative control, and transcriptional activity was measured. As shown in Fig. 1*D*, the normalized promoter activity of the AP-1 mutated *LY6K*-luc vector significantly decreased ~8-fold, suggesting that the transcription factor bound to the AP-1 consen-

TABLE 1
Consensus sequences found in the 550-bp human LY6K promoter region (−550/−1)

Binding sites	Sequence	Location (bp)
TATA box	5′-TATAA-3′	−30 to −25
AML1a	5′-TGTGGT-3′	−63 to −58
p300	5′-CGCTGGAGTGTGTC-3′	−105 to −92
CREB	5′-TGACCTCA-3′	−123 to −116
NF-κB	5′-GGGGAGACCC-3′	−230 to −221
AP-1	5′-TGAGTCA-3′	−252 to −246, −502 to −492
GATA3	5′-AGATAG-3′	−519 to −514

sus binding site within the −550 regulatory elements in the *LY6K* promoter. Interestingly, the single base pair mutated *LY6K*-luc reporter SNP242 decreased transcriptional activity 2-fold in MCF7-ADR cells. Taken together, we conclude that a transcription factor bound to the AP-1 binding site within the *LY6K* promoter and regulated expression of the *LY6K* gene and that the SNP242 mutation (G→C) reduced levels of the *LY6K* gene transcript.

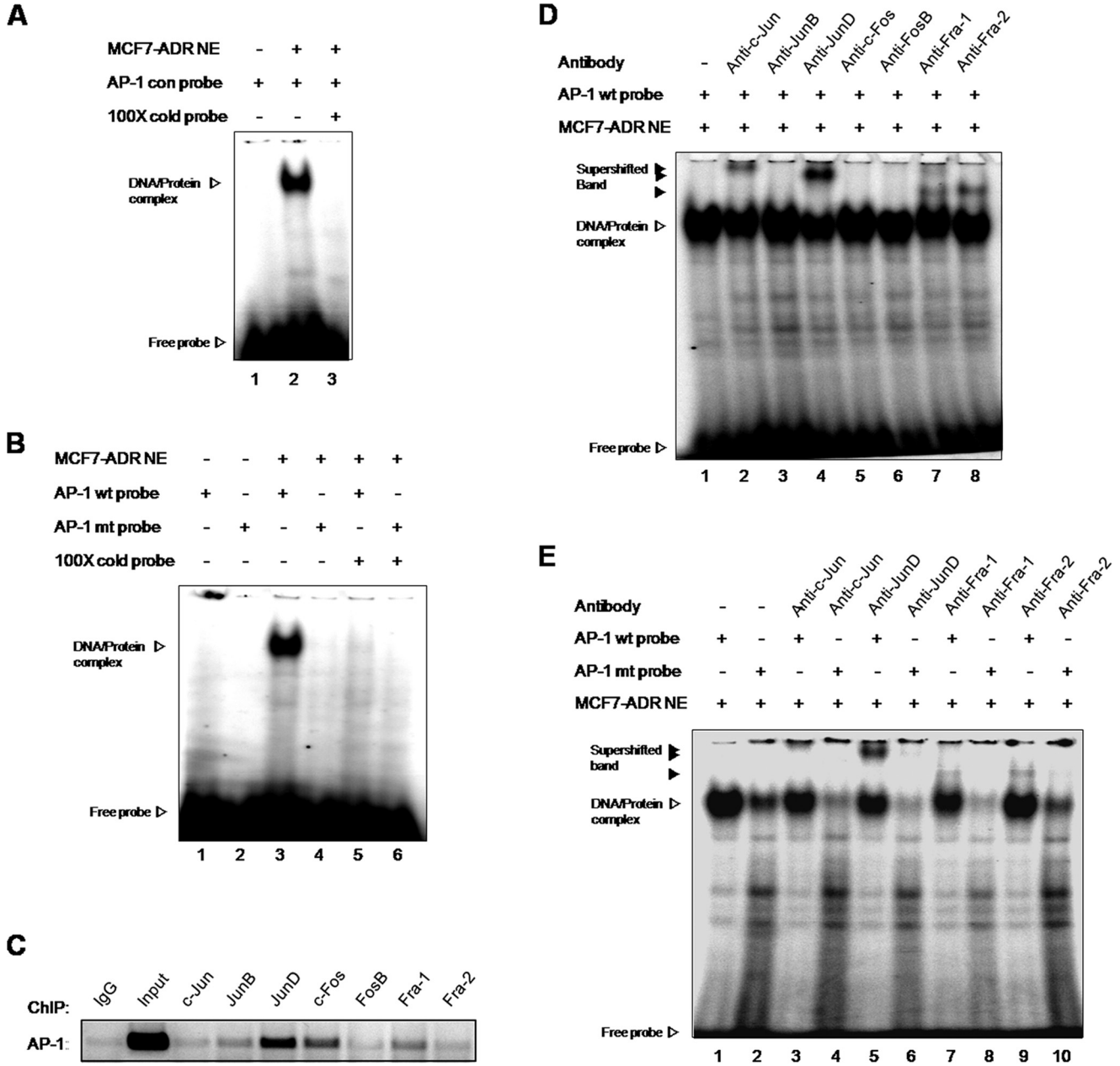

FIGURE 2. **Identification of AP-1 binding site in *LY6K* promoter using EMSA and supershift analysis with wild type (wt) and mutant (mt) 22-bp (−260/−239) probes.** *A*, EMSA with consensus AP-1 probe containing the consensus AP-1 binding site (TGAGTCA) was used to validate the presence of the AP-1 factor using the NE from MCF7-ADR cells in the presence or absence of 100× unlabeled wt AP-1 probe. *B*, representative radiographs of EMSA analysis using wt or mt AP-1 probes labeled with γ-^{32}P. *Lanes 1* and *2* were loaded only with the γ-^{32}P-labeled wt and mt probe. *Lanes 3* and *5* are the wt 22-bp probe containing the AP-1 binding site in the presence or absence of 100× unlabeled probe. *Lanes 4* and *6* show the mt AP-1 probe in the presence or absence of competitor. *C*, chromatin immunoprecipitation (ChIP) assay of the AP-1 site within the *LY6K* promoter region −253/−246 using AP-1 family antibodies. *D*, supershift analysis with the double-strand oligonucleotide probes containing the AP-1 binding site using NE from MCF7-ADR cells and the following AP-1 family antibodies: c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2. *E*, representative autoradiographs of supershift assays using a mixture of NE from MCF7-ADR cell with JunD or Fra-1 or Fra-2 antibodies wt 22-bp probes are shown. Equal amounts of protein were loaded in each lane. Specific shifted DNA complex is shown as an *unfilled arrowhead* to the *right* of each panel. Supershifted complexes are shown as a *filled arrowhead* to the *right* of the panel. *con*, consensus.

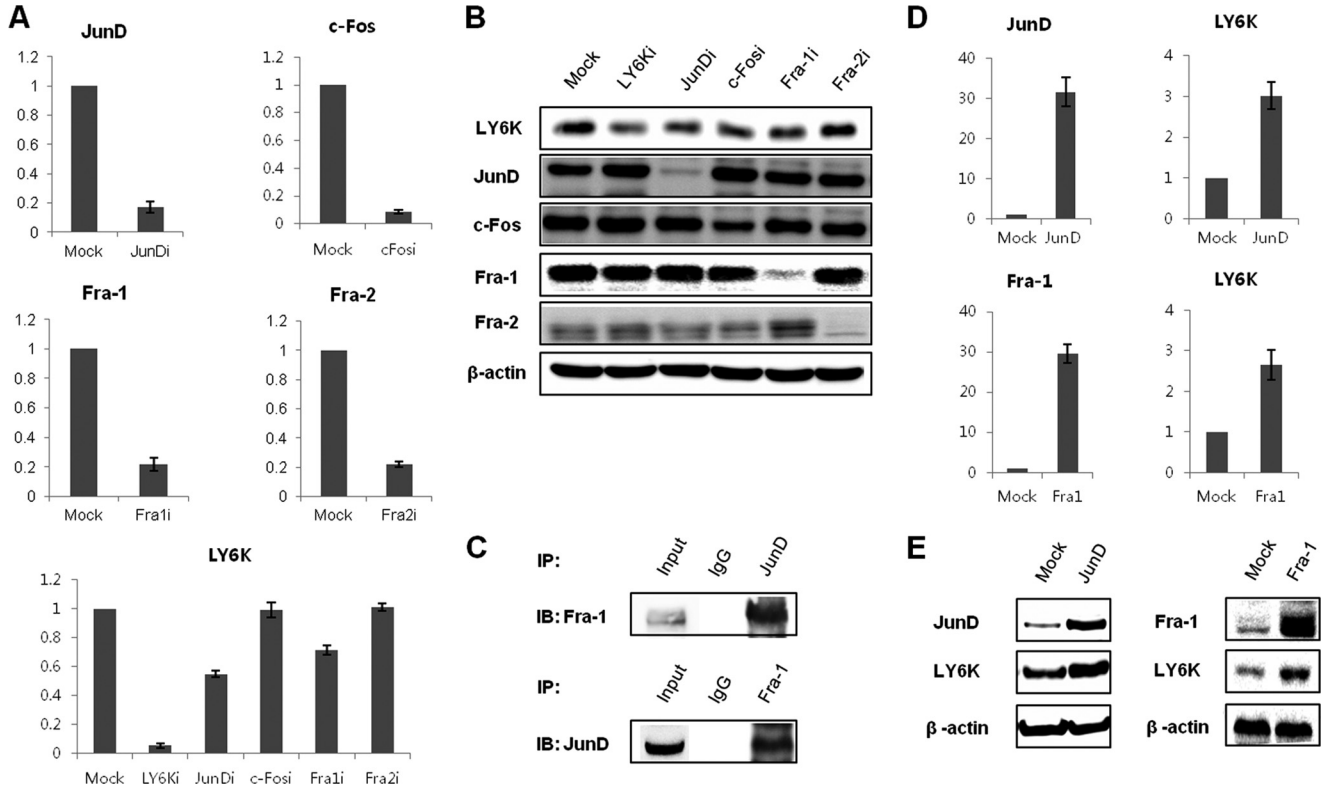

FIGURE 3. **JunD and Fra-1 regulate *LY6K* gene expression.** *A* and *B*, RNA and protein levels of the JunD, c-Fos, Fra-1, Fra-2, and LY6K genes were determined by real-time PCR and Western blot in siRNA or untransfected cells. *C*, immunoblots (*IB*) showing that JunD co-immunoprecipitates with Fra-1 from lysates of MCF7-ADR cells. Anti-IgG rabbit polyclonal antibody and input, 5% of total lysate, were used for negative and positive immunoprecipitation (*IP*) controls, respectively. *D* and *E*, RNA and protein levels of JunD, Fra-1, and LY6K genes were determined by real-time PCR and Western blot in transfected or untransfected cells. All values have been normalized to the 18 S rRNA level and are the averages of three independent readings. Equal amounts of protein were loaded in each lane.

Identified AP-1 Binding Site within 22 bp—The EMSA was conducted with the wt and mt AP-1 probe to confirm that the AP-1 binding site was within the *LY6K* promoter. First, it was determined whether the nuclear extract (NE) from MCF7-ADR cells had the AP-1 factor that bound to the consensus AP-1 probe in the presence or absence of unlabeled probe (Fig. 2*A*). Then, binding of NE from MCF7-ADR cells to the wt 22-bp probe within the *LY6K* promoter was investigated (Fig. 2*B*). A shifted band was observed in *lane 3*, which was a mixture of NE from MCF7-ADR cells with the wt 22-bp oligonucleotide probe (Fig. 2*B*). The shifted band disappeared in *lanes 4* and *5* in the presence of the unlabeled or AP-1 mutated probe. Therefore, it was concluded that the shifted band was a complex of the transcription factor and radiolabeled oligonucleotide. The EMSA was performed with the wild type and mutant CREB probes to confirm which transcription factors bound the CRE element of the LY6K promoter. The CRE binding factors present within the NE of MCF7-ADR cells demonstrated significant binding to a consensus CRE probe (supplemental Fig. 2*A*). We observed the formation of one prominent DNA-protein complex for the wild type CRE probe analyzed (supplemental Fig. 2*B*). In a supershift experiment, we confirmed that adding anti-CREB antibody produced a supershift complex but that the anti-CREB binding protein CBP antibody had no effect (supplemental Fig. 2, *C* and *D*). These results suggest that the AP-1 and CREB transcription factors bind to the *LY6K* promoter within −253/−246 and −130/−111 but that only AP-1 modulates LY6K gene transcription levels.

AP-1 Factor Binds to the AP-1 Binding Site within the LY6K Promoter—ChIP and supershift assays were used to demonstrate *in vitro* interactions between the AP-1 transcription factor and the AP-1 binding site within the *LY6K* promoter. First, a ChIP analysis using c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 antibodies was carried out and confirmed by PCR. Input and IgG were used as positive and negative controls, respectively. The results revealed that JunD directly bound to the DNA fragment containing the AP-1 binding site of *LY6K* (Fig. 2*C*). c-Fos and Fra-1 also bound to the *LY6K* promoter, but the signal was weak (Fig. 2*C*). Next, we examined which AP-1 transcription factors bound to the wt 22-bp probe contained at the AP-1 binding site. As shown in Fig. 2*D*, mixtures with JunD, Fra-1, and Fra-2 antibodies were supershifted. In the presence of the mutant AP-1 probe, supershifted bands were completely abolished compared with that of the wt AP-1 probe (Fig. 2*E*). Taken together, AP-1 factor binding to the proximal AP-1 binding site of the *LY6K* promoter suggested that these factors might be important in regulating *LY6K* promoter activity.

Functional Role of JunD and Fra-1 in LY6K Expression—To determine which AP-1 transcription factor forms a dimer with JunD, we used siRNAs specifically directed against *LY6K*, *JunD*, c-*Fos*, *Fra-1*, and *Fra-2* to silence their expression in MCF7-ADR cells. Transfection of several siRNAs abrogated LY6K, JunD, c-Fos, Fra-1, and Fra-2 mRNA and protein expres-

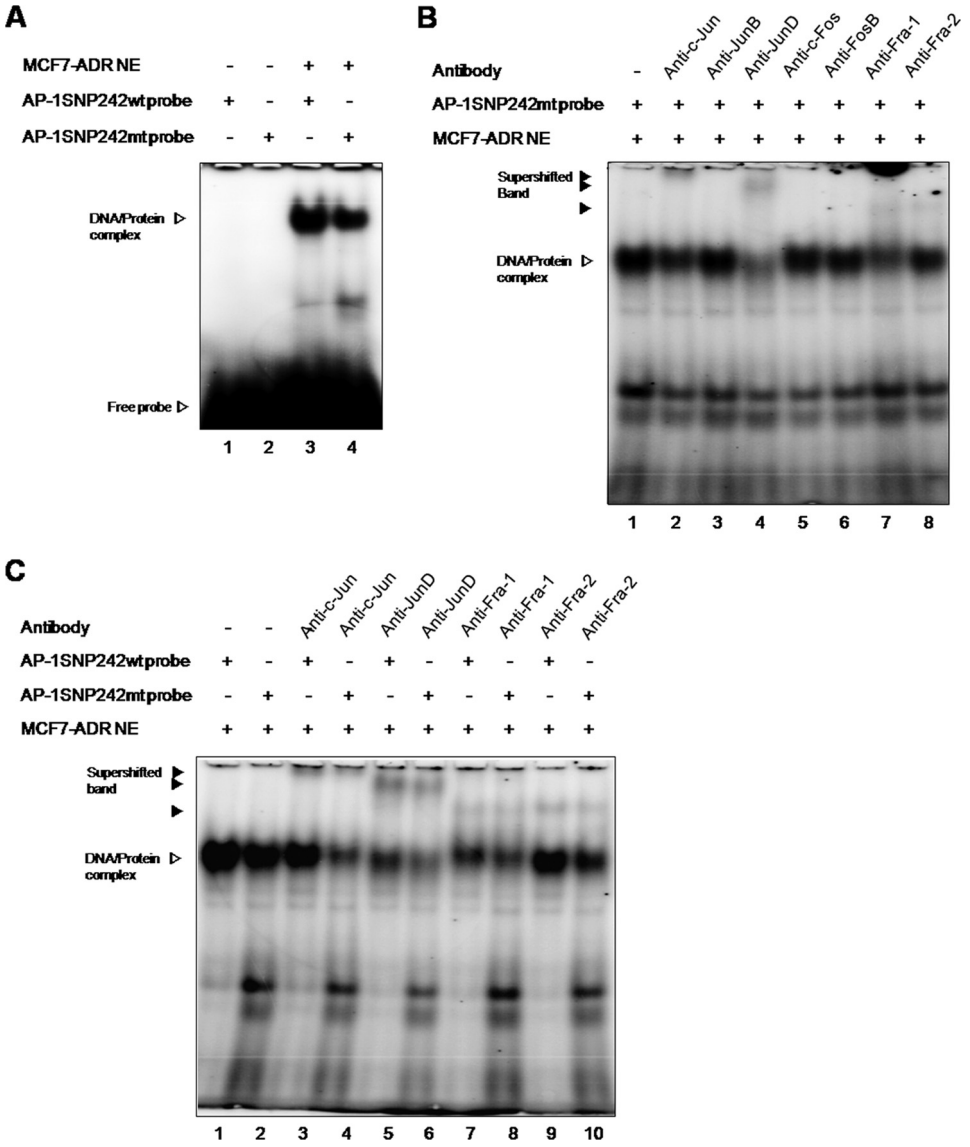

FIGURE 4. ***In vitro* interaction of SNP242 within *LY6K* promoter elements and AP-1.** *A*, EMSA was performed using the double-stranded oligonucleotide probe containing the AP-1 binding site with SNP242 wt or SNP242 mt in the presence or absence of NE from MCF7-ADR cells. *B* and *C*, the supershift assay was performed using double-stranded AP-1wtSNP242wt or AP-1wtSNP242mt oligonucleotide probe in the NE from MCF7-ADR cells and in the presence or absence of antibodies. Equal amounts of NE protein were loaded each lane. Specific shifted DNA and supershifted complexes are shown by *unfilled* and *filled arrowheads*, respectively, to the *right* of the panel.

sion, respectively, in MCF7-ADR cells assayed by real-time PCR and Western blotting, respectively (Fig. 3, *A* and *B*). Interestingly, we found that blocking JunD or Fra-1 expression induced a decrease in LY6K mRNA and protein level in MCF7-ADR cells, whereas transfection of the control siRNA did not alter this expression (Fig. 3, *A* and *B*). Endogenous proteins were immunoprecipitated to confirm the endogenous interaction between JunD and Fra-1. Western blot analysis of endogenous protein immunoprecipitates obtained with either anti-JunD or anti-Fra-1 antibodies revealed that JunD strongly co-immunoprecipitated with Fra-1, suggesting that these proteins may co-function in *LY6K* expression (Fig. 3*C*). The JunD and Fra-1 expression vectors were transfected in MCF7 cells to investigate whether increased levels of JunD and Fra-1 are able to modulate LY6K. We confirmed that LY6K expression level was up-regulated by elevated JunD or Fra-1 RNA and protein (Fig. 3, *D* and *E*). Therefore, it was concluded that the heterodimer of the AP-1 factors, JunD and Fra-1, regulates *LY6K* gene expression by directly binding to the *LY6K* promoter.

The Effect of SNP242 on AP-1 Binding Activity—Transcriptional activity of a single base pair of mutated constructs, pGL3-luc-mtSNP242, significantly decreased (Fig. 1*D*). SNP242 was closely located at a distance of three nucleotides from the AP-1 binding site; thus, we hypothesized that substituting G for C in SNP242 might interfere with AP-1 binding in the *LY6K* promoter. The newly designed EMSA probes, wt SNP242 and mt SNP242 containing the AP-1 binding site, were used to investigate whether they affected AP-1 binding activity. The density of the shifted band in *lane 4* was weaker than that in *lane 3*, indicating that the AP-1 factor bound less to the probes (Fig. 4*A*). Then, a supershift assay was performed with the AP-1wtSNP242wt probe using the AP-1 family factor (Fig. 4*B*)

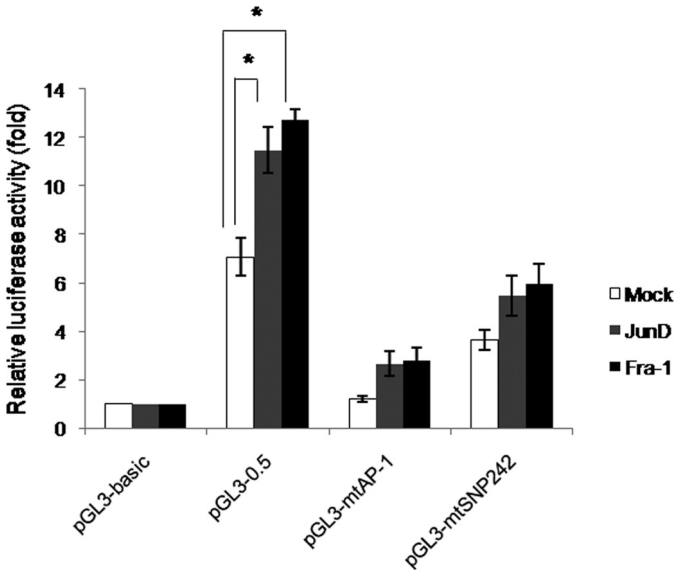

FIGURE 5. **Transcriptional activity of the *LY6K*-luc reporters in the absence or presence of AP-1 factors JunD and Fra-1.** The MCF7-ADR cells were transfected with the wild type AP-1, mutated AP-1, or mutated SNP242 *LY6K*-luc vectors in the absence or presence JunD or Fra-1 expression vector. Promoter activity of *LY6K*-luc was normalized with the pGL3-basic vector, which was transfected as a negative control. *, $p < 0.01$ (Student's paired *t* test *versus* mock).

and the AP-1wtSNP242mt probes using c-Jun, JunD, Fra-1, and Fra-2 antibodies, which bind to the *LY6K* gene promoter (Fig. 4*C*). We observed that the mixture of antibodies in *lanes 6*, *8*, and *10* produced weaker bands than those in *lanes 5*, *7*, and *9* (Fig. 4*C*). These results suggest that AP-1 bound to the *LY6K* promoter and activated transcription and that the presence of SNP242 (G→C) repressed AP-1 activity by reducing its DNA binding affinity to the *LY6K* promoter.

Functional Role of JunD and Fra-1 on the LY6K Promoter—To further examine the functional role of AP-1 factor, JunD and Fra-1, and the identified AP-1 binding site within the *LY6K* promoter, we compared the activity of the pGL3-0.5, the mutated AP-1 or the mutated SNP242 constructs in the absence or presence of the JunD or Fra-1 expression vector (Fig. 5). Overexpressed JunD or Fra-1 significantly up-regulates the transcriptional activity of the pGL3-0.5 vector, respectively, with slightly effects on the activity of the mutated AP-1 and mutated SNP242 *LY6K*-luc constructs (Fig. 5). Similarly, in other breast cancer cell lines, MCF7 and MDA-MB-231, overexpression of JunD or Fra-1 up-regulated the luciferase activity of the pGL3-0.5, without any significant effect on the activity of the mutated AP-1 and mutated SNP242 *LY6K*-luc constructs (supplemental Fig. 3). Therefore, these results support the conclusion that AP-1 factor, JunD and Fra-1, directly bind to identified AP-1 binding site within the *LY6K* promoter, and its binding affinity was affected by SNP242 in MCF7-ADR cells.

SNP242 Is Associated with LY6K Down-regulation—The EMSA was performed using SNP242 wt and SNP242 mt probes to confirm the hypothesis that substitution of G for C might generate transcription factor binding sites. In the presence of the SNP242-mutated probe, the shifted band appeared in *lane 4* (Fig. 6*A*). NE protein from MCF7-ADR cells bound to the SNP242 mt probe, and the band shift disappeared in the presence of competitor and the SNP242 wt probe (Fig. 6*A*). Therefore, it was concluded that the shifted band was a complex of transcription factors and radiolabeled oligonucleotides. We investigated the DNA binding motif using TRANSFAC software to predict the transcription factor that binds to *de novo* transcription binding sites. PAX3, a member of the paired-box transcription factor family, binding sites are created by SNP242 mutation. The PAX3 protein was used in the supershift assay to confirm PAX3 protein binding to the *de novo* PAX3 site (Fig. 6*B*). PAX3, which bound to the SNP242 mt probe, slightly supershifted in the presence of anti-PAX3 antibody (Fig. 6*B*). The *LY6K* mRNA level was compared in four cell lines that were wt for SNP242 (G/G) to the levels found in five cell lines for SNP242 (C/C) and measured by real-time PCR. The presence of SNP242 correlated with low *LY6K* transcript expression when compared with the levels seen in cells that were wt for SNP242 (G/G) (Fig. 6*C*). A ChIP analysis using PAX3 antibodies was carried out and confirmed by PCR using the MCF7-ADR (SNP242 G/G) and MCF7 (SNP242 C/C) cell lines. Input and IgG were used as positive and negative controls, respectively. The results revealed that PAX3 directly bound to the SNP242 (C/C) DNA fragment of *LY6K* (Fig. 6*D*). Taken together, PAX3 transcription factor bound to the *de novo* PAX3 binding site generated by mutated SNP242 (C/C), which decreased AP-1 activity on the *LY6K* promoter by interfering with AP-1 factor binding.

The Effect of CpG Site Methylation on AP-1 Binding Activity—The pGL3-*LY6K* promoter plasmids were *in vitro*-methylated by SssI methylase to further investigate the role of epigenetic modification in the regulation of gene expression. After MCF7-ADR cells were transfected with the methylated plasmids, the luciferase activities in these transfectants significantly decreased compared with those found in the cells transfected with unmethylated pGL3−312 and pGL3−550 (Fig. 7*A*). The probe containing the AP-1 binding site was methylated at two CpG sites by SssI methylase to investigate the effect of methylation on AP-1 binding activity. The two CpG sites were located 2 and 10 bp downstream of the AP-1 binding site, respectively. The methylated probe was incubated with the nuclear proteins from MCF7-ADR cells (Fig. 7*B*). Binding of the nuclear proteins to the methylated oligonucleotide probe significantly reduced compared with binding to the unmethylated probe (Fig. 7*B*). This result suggests that methylation at the CpG sites close to the AP-1 binding site interfered with AP-1 transcription factor binding to the AP-1 site.

DISCUSSION

LY6K has been reported as a testicular cancer antigen that is not only a target antigen for head-and-neck squamous cell carcinoma but also a diagnostic marker for breast cancer and a serologic biomarker and therapeutic target for lung and esophageal carcinomas (7–9). Activating LY6K results in an increase in cell invasive motility and metastasis, whereas a low level of LY6K reduces cell mobility in breast cancer cells (12).

The interaction of vascular endothelial growth factor (VEGF) with its type-2 receptor activates the MAPK pathway to stimulate uPAR expression, which induces angiogenesis (26). uPAR, a member of the Ly-6 superfamily, has AP-1 and NF-κB putative binding sites at the 5′-flanking region within the GC-rich

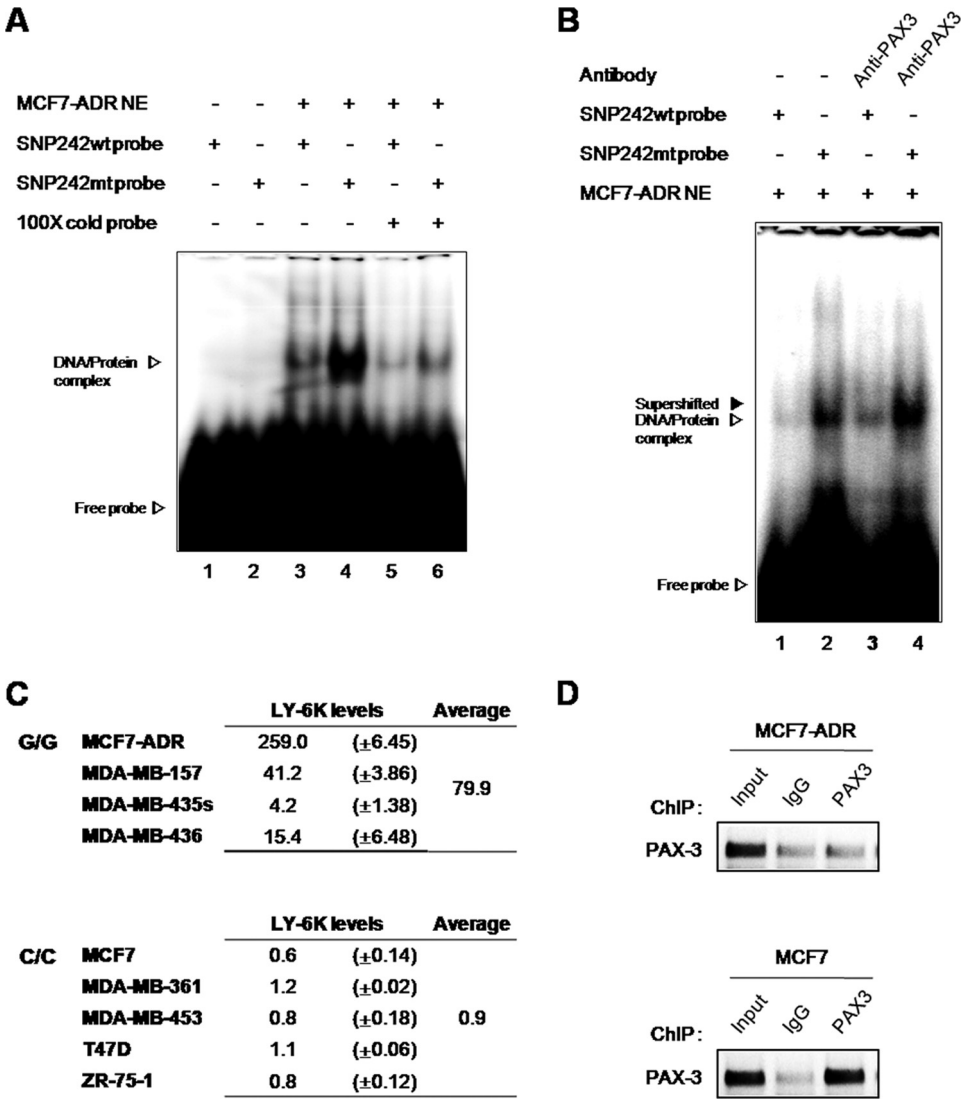

FIGURE 6. **A PAX3 transcription factor binding site is created by a G/C SNP at position −242 in the *LY6K* promoter.** *A*, the differential binding of transcription factor to the *LY6K* promoter SNP242 G allele and C allele was analyzed by EMSA. *B*, rs2585175 was predicted to create a PAX3 transcription binding site. The supershift assay was performed using a double-strand oligonucleotide probe containing the SNP242 C allele in the presence of the NE from MCF7-ADR cells and the PAX3 antibody. Equal amounts of NE protein were loaded in each lane. The shifted DNA-protein complex (*unfilled arrowhead*) and supershifts (*filled arrowhead*) are indicated to the *right* of the panel. *C*, the level of *LY6K* mRNA from the human breast cancer cell lines was measured by real-time RT-PCR. All values were normalized to 18 S rRNA level. *D*, ChIP assay of the PAX3 site within the *LY6K* promoter with PAX3 antibodies using MCF7-ADR (SNP242 G/G) and MCF7 (SNP242 C/C) cell lysates.

sequence and fewer TATA and CAAT boxes (27, 28). Similarly, a computer analysis of transcription factor binding sites screening the *LY6K* promoter showed fewer CAAT boxes and TATA boxes and putative AP-1 binding sites within the GC-rich region. Therefore, LY6K expression related to angiogenesis may be regulated by the VEGF signaling pathway similar to uPAR synthesis.

Promoter activity assays using a series of reporter gene constructs containing progressive 5′ deletions or specific mutations allowed us to determine regions in the *LY6K* promoter necessary for efficient activity. EMSA assays were used to detect one prominent complex corresponding to a specific protein-DNA interaction with the AP-1 and CRE sites (supplemental Fig. S1). Our results suggest that the AP-1 transcriptional activator directly binds the AP-1 binding site within the *cis*-element of the *LY6K* promoter. In contrast, CREB-1 did not affect *LY6K* expression but bound to the CRE motif. Therefore, the activity of the AP-1 transcription factor, an upstream transducer of LY6K and a member of the Ly-6/uPAR family, is critical for activating LY6K.

The AP-1 binding site identified in this study appears in the regulatory regions of many genes involved in proliferation, cell growth, cellular mobility, and the stress response. More recent studies have been carried out to identify the relationship between AP-1 transcription factors and breast cancer cells. Studies reported to date suggest that AP-1 plays a critical role in breast cancer, including breast cell transformation and breast cancer cell growth, proliferation, survival, invasion, and metastasis (29, 30). Based on our results, we suggest that the AP-1 family members JunD and Fra-1 play an important role in cell mobility of breast cancer cells by promoting *LY6K* gene expression.

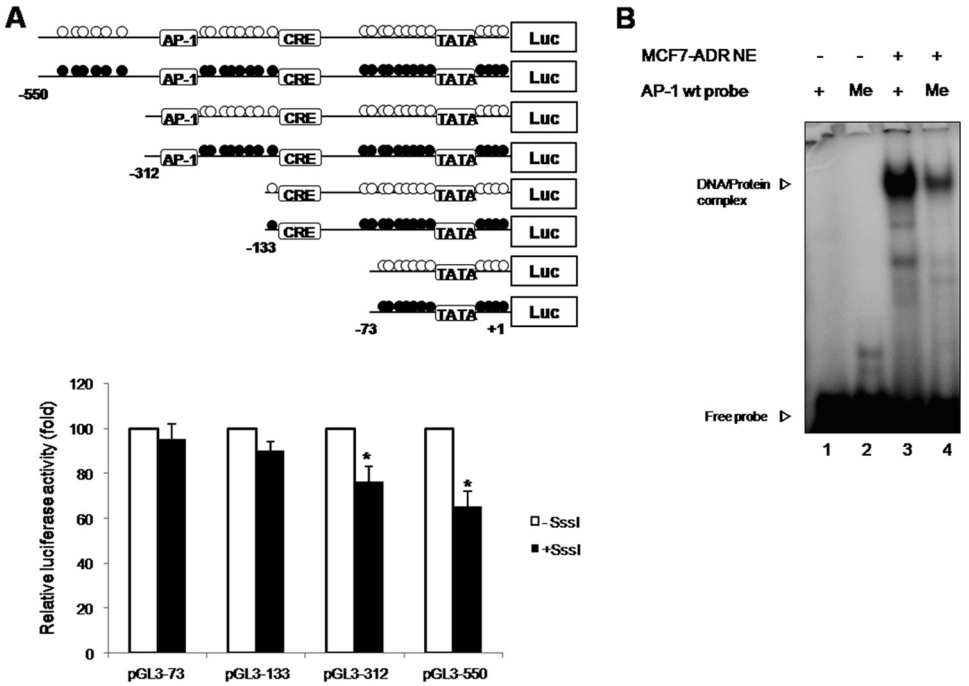

FIGURE 7. **CpG site methylation affect to AP-1 binding activity.** *A*, reduction of luciferase activities in cells transfected with methylated plasmids. MCF7-ADR cells were transfected with the SssI methylase-treated plasmid. The *open* and *filled bars* represent the unmethylated and methylated transfectants respectively. *B*, comparison of the *in vitro* binding capacity of the methylated and unmethylated probes by EMSA. The ^{32}P-labeled probe was methylated by SssI methylase. Shifted bands are indicated by the *unfilled arrowheads* to the *right* of the panel.

The Jun/Fos combination of the AP-1 complex induces strong proliferation, malignant transformation, and a powerful aggressiveness phenotype (31, 32). This is because the Jun/Fos heterodimer is more stable with a longer half-life and stronger DNA binding affinity within the promoter than those of the Jun/Jun homodimer (33). The Jun and Fos protein dimer binding affinity to the asymmetric heptanucleotide (TGA(C/G)TCA) is higher than that to the symmetric octanucleotide (TGACGTCA) (34). We conclude that the JunD/Fra-1 heterodimer complex is an AP-1 transcription factor that binds with the highest affinity within the *LY6K* promoter, indicating that *LY6K* gene expression is unregulated by the combination of JunD/Fra-1, which could promote the aggressiveness phenotype.

A functional SNP is a useful polymorphic marker for diagnosis and personalized cancer treatment. In particular, a localized promoter of specific genes contributes to the cancer phenotype and regulates gene expression by disrupting or creating transcription factor sites or interfering with transcription factor binding (21). Our results demonstrate that *LY6K* expression is dependent not on the AP-1 transcription factor but on SNP242 by creating a *de novo* PAX-3 binding site that leads to a decrease in AP-1 binding affinity. This observation suggests that the *LY6K* SNP242 C allele may reduce the carcinogenesis risk of breast cancer and metastasis (Fig. 8).

DNA methylation, which leads to gene silencing, is one of the epigenetic modifications, and at least three hypotheses explain this silencing (25). First, the suppression is mediated by binding between methylated DNA and a family of methyl-CpG binding proteins, leading to a change in chromatin structure. Second, gene silencing is caused by inhibiting the binding of sequence-specific transcription factors to their binding sites containing

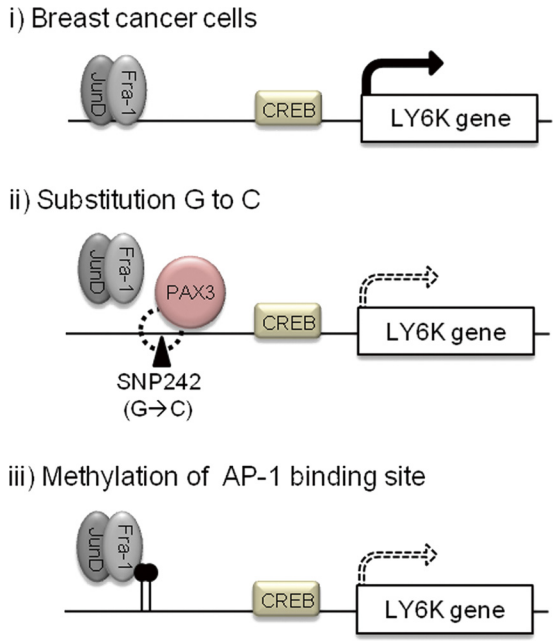

FIGURE 8. **Schematic diagram of the mechanisms by which AP-1 transcription factor regulates *LY6K* expression, which activates the ERK signaling pathway to mediate cell invasive motility and metastasis in human breast cancer cells.** Both substituting SNP242 G for C (G→C), which creates the PAX3 binding sites and methylation at the CpG site are closely localized on AP-1 binding site and reduce AP-1 binding affinity by interfering with AP-1 binding, which decreases *LY6K* gene expression (*dotted arrow*).

CpG (35). Third, CpG site modifications affect transcription factor binding affinity but are not part of the binding site (36). We also demonstrated that methylation at a CpG site closely located to the AP-1 binding site may reduce the risk

of breast cancer by effectively interfering with AP-1 binding (Fig. 8).

In summary, we investigated the molecular mechanisms that govern *LY6K* gene transcription by demonstrating that the binding of the JunD and Fra-1 transcription factors to the *LY6K* promoter regulated gene activation *in vitro*. Activated JunD and Fra-1 promoted LY6K expression at the RNA and protein levels. CREB also bound to the CRE within the *LY6K* promoter but did not affect LY6K expression. We also demonstrated that the SNP242 C allele and methylation of a CpG site close to the AP-1 binding site reduced AP-1 binding affinity by binding the PAX3 transcription factor and by interfering with AP-1 binding, respectively, which lead to *LY6K* down-regulation. Therefore, activation of JunD and Fra-1 induced invasion and metastasis of breast cancer cells by regulating the *LY6K* gene transcription levels via the ERK signaling pathway and by SNP242 or methylation, which reduced AP-1 binding affinity (Fig. 8). The regulatory mechanisms involved in LY6K silencing are very important for investigating breast cancer risk and for application to early diagnosis and prevention of breast cancer.

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