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Epiregulin expression by Ets-1 and ERK signaling pathway in Ki-*ras*-transformed cells

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ABSTRACT

Epiregulin belongs to the epidermal growth factor family, binds to the epidermal growth factor receptor, and its expression is upregulated in various cancer cells, but the regulatory mechanism is unclear. We investigated the regulatory mechanism of epiregulin expression in Ki-*ras*-transformed cancer cells. In 267B1/Ki-*ras* cells, the RAF/MEK/ERK pathway was constitutively activated, epiregulin was up-regulated, and the expression and phosphorylation of Ets-1 were augmented. The inhibition of ERK by PD98059 decreased epiregulin and Ets-1 expression and suppressed the growth of 267B1/Ki-*ras* cells. A chromatin immunoprecipitation assay demonstrated that Ets-1 was bound to human epiregulin promoter, and this binding was abolished by PD98059. Silencing of Ets-1 by RNA interference decreased cellular epiregulin transcript expression. We suggest that the Ki-*ras* mutation in 267B1 prostate cells constitutively activates the RAF/MEK/ERK pathway and induces the activation of the Ets-1 transcription factor, ultimately leading to the increased expression of epiregulin.

Epiregulin is a relatively newly determined member of the epidermal growth factor (EGF) family. The amino acid sequence of epiregulin is 24–50% identical to that of other EGF family members [1]. Epiregulin directly binds EGF receptor (EGFR) and ErbB-4 and activates EGFR, ErbB-2, ErbB-3, and ErbB-4 [2]. Epiregulin plays a role as an autocrine growth factor in epithelial cells [3]. Its expression is increased in human pancreatic cancer cell lines [4], human colon cancer cells [5], hTERT-immortalized fibroblasts [6], human metastatic bladder cancer cell lines [7], and metastatic human breast cancer cells [8]. Nevertheless, the regulatory mechanisms of epiregulin gene expression in various cancer cells are unclear.

Mutated variants of Ras are found in 30% of all human cancers. Mutated Ras proteins activate both the RAF/MEK/ERK and the PI3K/Akt pathways. Specifically, Ki-*ras* activates the RAF/MEK/ERK pathway, whereas Ha-*ras* preferentially activates the PI3K/Akt pathway [9]. Activated ERKs (phospho-ERKs) in the RAF/MEK/ERK pathway are translocated to the nucleus where they phosphorylate and regulate various transcription factors such as c-myc, Ets, CREB, and c-Jun, ultimately leading to changes in gene expression [10]. The E26 transformation-specific sequence (Ets) family of proto-oncogenes was first detected in the avian erythroblastosis retrovirus E26 [11] and share a highly conserved DNA-binding motif termed the Ets domain. Approximately 85 amino acid residues of the conserved Ets-domain mediate binding to purine-rich GGAA/T core consensus DNA sequences that contain additional flanking nucleotides [12]. Ets genes have altered expression patterns in human neoplasias such as thyroid, pancreas, liver, prostate, colon, lung, breast carcinomas and leukemias [13]. The overexpression of Ets-1 and Ets-2 is closely related with prostate cancer carcinogenesis and progression [14,15]. The phosphorylation of Ets-1 and Ets-2 is regulated by ERK via the RAF/MEK/ERK pathway on threonine 38 and threonine 72, respectively [16]. Ets-1 and Ets-2 activate the promoters of extracellular matrix-degrading proteases, MMP-9, and invasion-associated urokinase uPA in response to epidermal growth factors [17], and these genes are associated with clinical features such as lymph node status and prostate cancer prognosis.

The 267B1 human fetal prostate epithelial cell line immortalized by the SV40 T antigen is non-tumorigenic in nude mice and does not form colonies in soft agar. Conversely, 267B1/Ki-*ras* of the *v*-Ki-*ras*-transformed cell derivative is tumorigenic in nude mice and forms colonies in soft agar [18]. We performed microarray analyses to explore genes and regulatory pathways that control cell growth by ras activation (Ki-*ras*) in the 267B1 cell line. Interestingly, the epiregulin transcript was significantly upregulated in our Ki-*ras*-transformed human prostate cells. Using the human prostate cell

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line 267B1 and 267B1/Ki-*ras* cells, we investigated the regulatory mechanism of epiregulin expression in Ki-*ras*-transformed 267B1 prostate epithelial cells.

Materials and methods

Reagents and antibodies. PD98059 was obtained from EMD Biosciences, Inc. (Darmstadt, Germany). Primary antibodies against to Ets-1, Ets-2, ERK, P-p38, p38, JNK, β-tubulin, and all of secondary antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). P-ERK and P-JNK antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Ets-1 [Thr^{38}] antibody was purchased from Novus Biologicals, Inc. (Littleton, CO).

Reverse transcription-polymerase chain reaction (RT-PCR). 267B1 and 267B1/Ki-*ras* cells were maintained in RPMI 1640 medium (Hyclone, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone), and were cultured in a humidified CO_2 incubator at 37 °C. In order to conduct RT-PCR, total RNA was isolated using easy-BLUE™ total RNA extraction kit (iNtRon Biotechnology, Korea) as described by the manufacturer's protocol. Reverse transcription was performed using the ProSTAR™ (Stratagene, LaJolla, CA) and the cDNA was amplified by PCR. The forward and reverse primers used in the amplification were: 5′-CGG AAT TCT CCA GTG TCA GAG GGA CAC A-3′/5′-CCG CTC GAG ATC CCT GCC CAT AAG TTT-3′ for human epiregulin, 5′-TCA GAC GCT CCA TCC CAT CAG CTC G-3′/5′-TGC CAT CAC TCG TCG GCA TCT GGC T-3′ for human Ets-1, and 5′-ACC ACA GTC CAT GCC ATC AC-3′/5′-TCC ACC ACC CTG TTG CTG TA-3′ for GAPDH.

Cell proliferation assay (MTS assay). Approximately 0.5×10^4 cells per well were seeded into a 96-well microplate in 200 μl of complete medium. The cell number was measured using an MTS

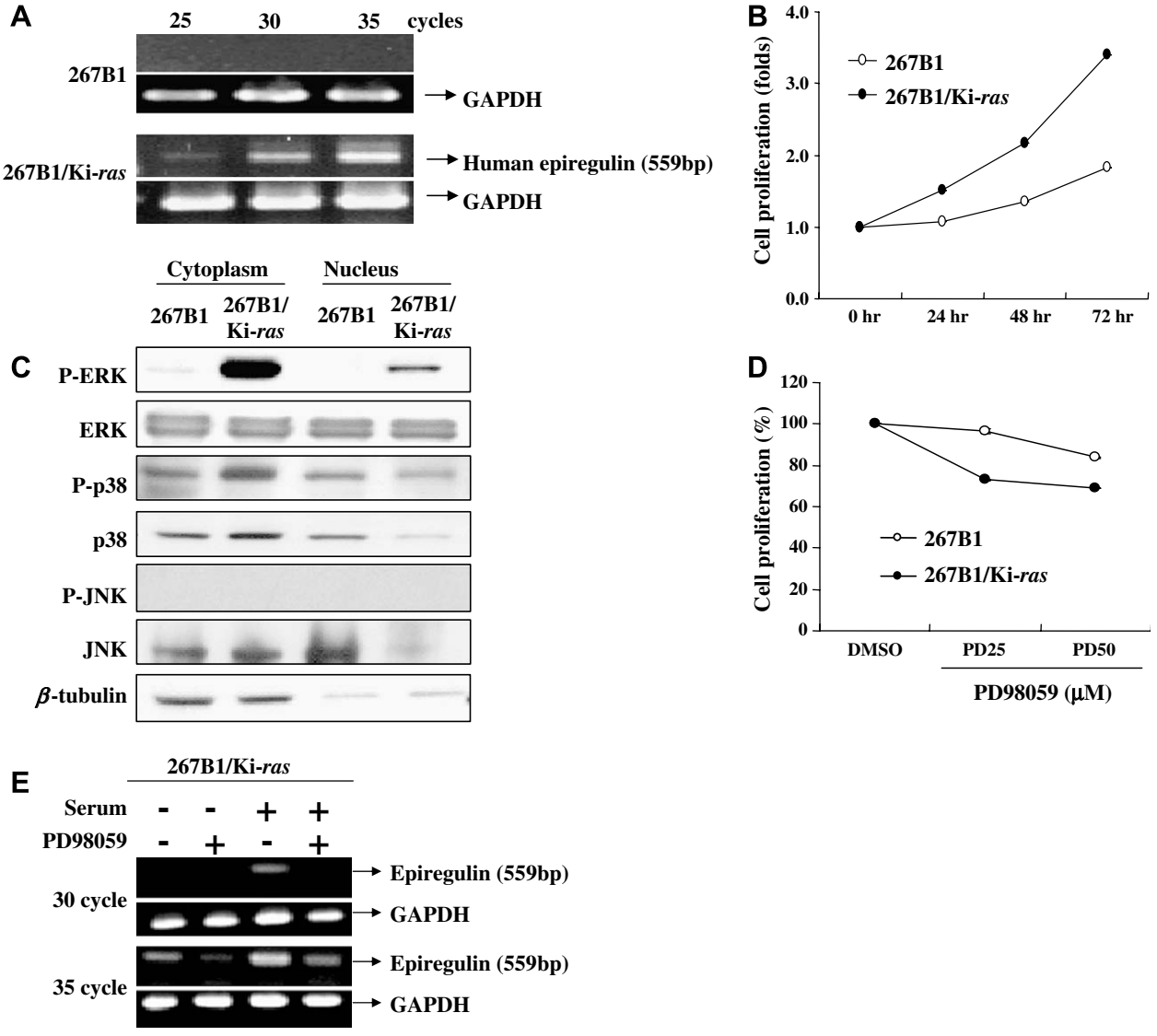

Fig. 1. Epiregulin expression and constitutive activation of the ERK MAPK pathway in Ki-*ras*-transformed cells (267B1/Ki-*ras*). (A) Epiregulin expression in the Ki-*ras*-transformed cell line. The mRNA expression levels of epiregulin in 267B1 prostate epithelial cells and 267B1/Ki-*ras*-transformed prostate epithelial cells were analyzed by RT-PCR. PCR products shown are from 25, 30, and 35 cycles. (B) Cell proliferation assay of 267B1 and 267B1/Ki-*ras* cells. Cells were grown in 96-well plates for up to 72 h, and the numbers of cells were measured by MTS assay. Two independent experiments were performed in triplicate; points represent the mean ± SD from a representative experiment of three experiments. (C) Constitutive activation of ERK/MAPK in 267B1/Ki-*ras* cells. Cytoplasmic and nuclear extracts were subjected to immunoblot analysis using specific antibodies against P-ERK, ERK, P-p38, p38, P-JNK, JNK, and β-tubulin as described in Materials and methods. (D) Inhibition of proliferation of Ki-*ras*-transformed cells by PD98059. After 267B1 and 267B1/Ki-*ras* cells were grown for 24 h, the cells were incubated further with PD98059 for 48 h, and the relative number of cells was estimated by MTS assay. (E) Influence of serum factors and ERK MAPK inhibition on epiregulin expression. After 267B1/Ki-*ras* cells were incubated in the absence or presence of PD98059 for 24 h in complete or serum-deficient medium, RT-PCR of epiregulin was performed. GAPDH was used as a control.