17β-estradiol induces *MUC5B* gene expression through Ras/Raf-ERK MAP kinase-RSK1-CREB activation in human airway epithelial cells

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Directed by Professor Jeong-Gweon Lee

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This certifies that the Doctoral Dissertation of Yoo-Sam Chung is approved.

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박사과정 동안 관심을 주신 이정권 교수님과 실험에 많은 배려와 관심을 기울여 주신 윤주헌 교수님께 감사드립니다. 또한 바쁘신 중에도 세심하게 논문을 검토하여 주신 박전한 교수님, 이재면 교수님, 윤호근 교수님께도 감사의 말씀을 드립니다.
실험실에서 많은 가르침과 도움을 주신 최혜정 선생님, 문욱열 선생님, 박영덕 선생님, 안영은 선생님, 박성하 선생님께도 감사드리며 하시는 연구에 많은 진전이 있으시기를 바랍니다.
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ABSTRACT

17β-estradiol induces *MUC5B* gene expression through Ras/Raf-ERK MAP kinase-RSK1-CREB activation in human airway epithelial cells

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(Directed by Professor Jeong-Gweon Lee)

MUC5B is a major mucin of the human respiratory tract, and it is not clear how *MUC5B* expression is regulated in various airway diseases. The goal of this study was to determine the mechanisms by which E2 induces *MUC5B* gene expression in airway epithelial cells. It was found that 17β-estradiol (E2), a sex hormone, stimulate *MUC5B* gene overexpression by interaction with estrogen receptor α (ER α) and by acting through ERK-MAP kinase (MAPK). Co-treatment with E2 and ER antagonist ICI 182,780 blocked both E2-induced ERK-MAPK activation and *MUC5B* gene

expression. It was also found that the activation of p90 ribosomal S 6 protein kinase 1 (RSK1), cAMP-response element-binding protein (CREB) and cAMP-response element (CRE) (-956 region of the *MUC5B* promoter) responsive signaling cascades via ERK-MAPK are crucial at the mediation *MUC5B* gene expression. This study further showed that CREB was recruited to CRE together with CREB- binding protein (CBP) and steroid receptor coactivator-1 (SRC-1). This coincided with increased histone H3 acetylation (AcH3) and up-regulation of *MUC5B* gene expression. Taken together, these studies give additional insights into the molecular mechanism of hormone-induced *MUC5B* gene expression and enhance our understanding of abnormal mucin secretion in response to hormonal imbalances.

Key words : MUC5B, estradiol, signal pathway, MAP kinase

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I. INTRODUCTION

Mechanicochemical proteins produced by airway epithelial cells are essential components of airway mucus, which plays an important role in protecting the airway from bacterial and viral attacks. Mucin hypersecretion is commonly observed in many respiratory diseases, such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis, and cystic fibrosis.[1-5] To date, twenty distinct mucin genes have been identified: *MUC1*[6], *MUC2*[7], *MUC3*[8], *MUC4*[9], *MUC5AC*[10], *MUC5B*[11], *MUC6*[12], *MUC7*[13], *MUC8*[14], *MUC9*[15], *MUC10*[16], *MUC11*[17], *MUC12*[17], *MUC13*[18], *MUC15*[19],

MUC16[20], *MUC17*[21], *MUC18*[22], *MUC19*[23], and *MUC20*[24]. Of these, *MUC5AC* and *MUC5B* have been shown to be major components of respiratory secretions.[25-26] Expression of such mucin genes is increased by inflammatory mediators, such as LPS[27], TNF-α[28], IL-1β[28], and neutrophil elastase (NE)[29]. In addition to such inflammatory mediators, it is known that the sex hormone estrogen also induces glandular hyperplasia and increases mucus in the nasal mucosa.[30-31]

MUC5B is one of the major mucins secreted from the human airway submucosal glands. In chronic airway diseases, increases in mucin secretion could be achieved through marked enlargement of the submucosal glands, accompanied by increases in the number of cells involved in MUC5B synthesis.[32]. Currently, there are a few reports suggesting that estrogen may be involved in *MUC5B* gene expression in the endocervix.[33]. However, the precise mechanism has not been determined.

Many estrogenic actions are mediated by intracellular estrogen receptors (ERs) that function as ligand-activated transcription factors to regulate the expression of estrogen-responsive genes.[34] E2 rapidly activates adenylate cyclase, increasing intracellular levels of active phospholipase C to generate inositol 1,4,5-triphosphate and diacylglycerol, stimulating nitric oxide synthase to generate nitric oxide and activating the extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated

protein kinase (MAPK) pathway.[35-37] The molecular mechanisms of receptor medicated signaling pathway are not fully understood.

The goal of this study was to elucidate details of the mechanism by which E2 induces *MUC5B* gene expression in airway epithelial cells. This study revealed that ERK MAPK was essential for E2-induced *MUC5B* gene expression in human airway epithelial cells, and that p90 ribosomal S 6 protein kinase 1 (RSK1) mediated the E2-induced phosphorylation of the cAMP response element binding (CREB) protein. In addition, the transcription activities of *MUC5B* promoter regions showed that the CRE in the *MUC5B* promoter is important for E2-induced *MUC5B* gene expression.

II. MATERIALS AND METHODS

1. *Reagents*

ICI 182,780 was obtained from Tocris (Ellisville, MO, USA). PD98059 and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho p44/42 MAP kinase (Thr-202/Tyr-204, p-ERK1/2), anti-p44/42 MAP kinase (ERK1/2), anti-phospho-p38 MAP kinase (Thr-180/Tyr-182), anti-phospho-SAPK/c-Jun NH_2-terminal kinase MAP kinase (Thr-183/Tyr-185), anti-phospho-RSK1 (Ser-380), anti-RSK1/2/3, anti-phospho-CREB (Ser-133), anti-CREB, anti-phospho-ER-α (Ser-118), and anti-ER-α (62A3) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-CBP, anti-SRC-1, anti-H3, and anti-AcH3 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The cDNA construct expressing a dominant-negative version of Ras (Ras-N17) and Raf1 was kindly provided by Dr. H. Kim (University of Korea, Seoul, Korea).

2. *Cell culture*

Epithelia were isolated from scrapings of the inferior turbinate of healthy adult volunteers. None of the volunteers had any history of allergic symptoms,

nasal polyps, or asthma. They did not have a history of smoking and did not take any medicines for the past 6 months. The volunteers' permission and the approval from the Institutional Review Board at Yonsei University College of Medicine were obtained for the use of the specimens. The epithelial cells from the turbinates were treated with 1% pronase (Type XIV protease, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 18 to 20 h at 4 °C. To remove fibroblasts, endothelial cells, and myoepithelial cells, isolated cells were placed in a plastic dish and cultured for 1 h at 37 °C. Isolated epithelial clusters were divided into single cells by incubating them with 0.25% trypsin/EDTA for 5 min. Passage-2, normal human nasal epithelial (NHNE) cells were seeded in 0.5 ml of culture medium onto 24.5-mm, 0.45-μm pore size Transwell clear culture inserts (Corning Inc., Corning, NY, USA). Cells were cultured in a 1:1 mixture of bronchial epithelial cell growth medium (Clonetics, San Diego, CA, USA) and Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA, USA) containing supplements described previously.[38] Cultures were grown submerged, and culture medium was changed on the first day and every other day thereafter. The human lung mucoepidermoid carcinoma cell line, NCI-H292, was purchased from the American Type Culture Collection (ATCC,

CRL-1848, Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) in the presence of penicillin-streptomycin at 37 ℃ in a 5% CO_2 humidified chamber. DLD-1 cells were also obtained from ATCC (CCL-221). For all experiments involving cell culture, phenol-red free RPMI-1640 (Invitrogen) supplemented with 5% dextran/charcoal–treated FBS (Hyclone, Logan, UT, USA) was used.

3. *RT-PCR*

Total RNA was isolated using TRIzol (Invitrogen) from NHNE cells treated with E2 (10^{-9} M). cDNA was synthesized with random hexamers (PerkinElmer Life Sciences, Boston, MA, USA and Roche Applied science, Indianapolis, IN, USA) using Moloney murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences). Oligonucleotide primers for PCR were designed based on the GenBank™ sequence of *MUC5B* (GenBank™ accession no. AJ012453, 5′ primer CTGCGAGACCGAGGTCAACATC; 3′ primer TGGGCAGCAGGAGCACGGAG). The following PCR conditions used involved 35 cycles: denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, and polymerization at 72 ℃ for 30 s. The oligonucleotide primers for β2-microglobulin (used as a control gene for the RT-PCR) were designed based on

the GenBank™ human sequence (GenBank™ accession no. XM007650, 5′ primer CTCGCGCTACTCTCTTTCTGG; 3′ primer GCTTACATGTCT CGATCCCACTTAA). PCR conditions used involved 23 cycles as follows: denaturation at 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and polymerization at 72 ℃ for 30 s. The PCR products were run in a 1.5% agarose gel and visualized with ethidium bromide under a transilluminator.

4. Real-time quantitative PCR

Primers and probes were designed using the PerkinElmer Life Science Primer Express® software purchased from PE Biosystems. Commercial reagents (TaqMan PCR Universal PCR master mix, PE biosystems) and conditions were applied according to the manufacturer's protocol. Three microgram of cDNA (reverse transcription mixture), oligonucleotides at a final concentration of 800 nM for each primer, and 200 nM TaqMan hybridization probe were used in a 25-μl volume. The probe for real-time PCR was labeled with carboxylfluorescein (FAM) at the 5′-end and with the quencher carboxytetramethylrhodamine (TAMRA) at the 3′-end. The following primers and TaqMan probes were used: *MUC5B*, forward (5′-CTACCTGGACAACCACTACTGC-3′), reverse (5′-

TGGTGACAGTGAGGACGATATCC-3′) and TaqMan probe (FAM-CTGCCACTGCCGCTGCCGCC-TAMRA), β2-microglobulin (B2M), forward (5′-CGCTCCGTGGCCTTAGC-3′), reverse (5′-GAGTACGCTGGATAGCCTCCA-3′) and TaqMan probe (FAM-TGCTCGCGCTACTCTCTCTTTCTGGC-TAMRA). Real-time PCR was performed on a PE Biosystems ABI PRISM® 7300 sequence detection system (Foster City, CA, USA). The thermocycler parameters were 50 ℃ for 2 min and 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. All reactions were performed in triplicate. The relative quantity of *MUC5B* mRNA was determined using a comparative threshold method, and the results normalized against β2-microglobulin as an internal control. Data were analyzed using the Student's *t* test for paired and unpaired values.

5. *Western blot analysis*

NCI-H292 or NHNE cells were grown to confluence in 6-well plates. After treatment with 10^{-9} M E2, the cells were lysed in 1× lysis buffer (125 mM Tris, pH 7.8, 10 mM EDTA, 10 mM DTT, 50% glycerol, and Triton® X-100). Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA,

USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5 and 150 mM NaCl) for 2 h at room temperature, followed by overnight incubation with primary antibodies in TBS-T (0.5% Tween 20 in TBS). After washing with TBS-T, the blots were incubated with anti-rabbit or anti-mouse antibody (Cell Signaling Technology) in TBS-T for 1 h at room temperature and visualized with ECL reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

6. ***Immunodetection and quantitation of*** **MUC5B** ***mucin secretion***

The immuno-dot blotting assay for detection of intracellular mucin produced by cultured cells has been described previously in detail.[39] In brief, after treatment with E2, cultured cells were lysed in 1× lysis buffer. Equal amounts of whole cell lysates were applied to a nitrocellulose membrane (Bio-RAD Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5 and 150 mM NaCl) for 2 h at room temperature, followed by overnight incubation with anti-human MUC5B antibody (1:200, Santa Cruz Biotechnology, Inc.) in TBS-T (0.5% Tween 20 in TBS). After washing with TBS-T, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG in TBS-T for 1 h

at room temperature and the signal detected by means of chemiluminescence (ECL kit, Santa Cruz Biotechnology, Inc.). The data were represented as mean ± standard deviation (SD) of triplicate from the same experiment.

7. *Immunofluorescence microscopy*

For the histological study, cells were washed three times with PBS and fixed in 3% paraformaldehyde solution [3% (wt/vol) paraformaldehyde, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.4, in PBS] for 10 min. The cells were washed three times with PBS, permeabilized in 0.2% Triton® X-100/PBS for 5 min, and washed three times with PBS. The cells were then blocked with 10% normal goat serum (Jackson Immuno Research labs Inc., West Grove, PA, USA) for 1 h, and washed with PBS. ER-α was detected using the ER-α, phospho-ER-α, polyclonal antibody (1:75, Cell Signaling Technology), and incubated for 24 h at 4℃, followed by washes in PBS. The aforementioned procedure was repeated with an appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100, Jackson Immuno Research labs Inc). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), and the coverslips were mounted on slides with Vectashield Mounting Medium (Vector Larboratories, Inc., Burlingame, CA, USA) and examined using a Zeiss LSM

510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA).

8. ***Transient transfection and luciferase analysis***

NCI-H292 cells were transiently transfected with pGL3-basic (Promega, Madison, WI, USA), pGL3-MUC5B (-1329/+92), pGL3-MUC5B (-956/+92), and pGL3-MUC5B (-649/+92) constructs using the FuGENE6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions, incubated for 24 h, treated with 10^{-9} M E2 for 24 h, harvested, and assayed for luciferase activity using the luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. β-galactosidase activity was also assayed to standardize sample transfection efficiencies. To confirm that the luciferase activity of each construct was caused by E2, the activity of each construct was assayed in the absence of E2.

9. ***Small interfering RNA (siRNA) treatment***

The role of ERK1/2 in mediating the estrogen effects was examined using ERK1-siRNA (siERK1) or ERK2-siRNA (siERK2) to silence the ERK1 or ERK2 gene. The siERK1 (Santa Cruz Biotechnology, Inc. sc-29307), siERK2 (Santa Cruz Biotechnology, Inc. sc-35335), and the siRNA negative control (Santa Cruz Biotechnology, Inc. sc-37007) were used. The role of RSK1 in

mediating the estrogen effects was examined using RSK1-siRNA (siRSK1) to silence the RSK1 gene. The siRSK1 gene sequence used was AAUUGUCUCCUUUAC CACGUAGCCG, and siRSK1 (Stealth™, human CREB gene Acc no. NM001006665) was chemically synthesized by Invitrogen Research. The role of CREB in mediating the estrogen effects was examined using CREB-siRNA (siCREB) to silence the CREB gene. The siCREB gene sequence used was UUACAGCUGCAUCUCCACUCUGCUG, and siCREB (Stealth™, human CREB gene Acc no. NM134442.2) was chemically synthesized by Invitrogen Research. The siRNA negative control (Stealth™, 12935-300, Invitrogen) was used. NCI-H292 cells were seeded the night before transfection at a density of 30-50% confluence by the time of transfection. Forty nanomol of siERK1/2, siRSK1, siCREB and siRNA negative control were used for transfection using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were maintained in culture for 3 days before harvesting and further analyses. The efficiency of the siRNA knockdown was determined by Western blot analysis with the appropriate antibodies.

10. ***ChIP Assays***

For ChIP assays, chromatin was isolated as described elsewhere.[40] In brief, approximately 2×10^9 NCI-H292 cells in 150-mm dishes were treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and fixed with 125 mM glycine at room temperature for 5 min. The cells were rinsed twice with PBS and resuspended in 1 ml of solution A (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA) by pipetting. After a short spin (5 min at 3000 rpm), the pellets were resuspended in solution B (10 mM HEPES, pH 6.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) containing protease inhibitors, by vigorous pipetting to extract nuclear proteins. After centrifuging at 4,000 rpm for 5 min, the nuclear pellets were resuspended in immunoprecipitation buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1% SDS, 0.5% Empigen BB) containing protease inhibitors, and sonicated to break the chromatin into fragments with an average length of 0.5-1 kb. The following antibodies were used in the assay: 2 μg of anti-phospho- CREB (Ser-133), anti-CBP, anti-H3, and anti-AcH3 antibodies and 2 μg of rabbit IgG as a negative control. The CRE (-956 to -753) primers used for ChIP analysis are as follows: forward (5′-ACGCGTGAGGTATTGCAGCGCGGACG-3′) and reverse (5′-GGTTGAGGAAGCAGCTGCTC-3′), with the PCR product being 203 bp.

The primers for the negative controls (-3960 to -3761) were designed to amplify the DNA fragment upstream of the CRE element: forward (5′-CCAGGCACTGGCTCTGAGA-3′) and reverse (5′-GAGGGTCCCATCGTGTGAC-3′), yielding a PCR product of 199 bp.

III. RESULTS

1. *E2 induces MUC5B gene expression and* MUC5B *protein secretion*

To determine whether E2 can induce *MUC5B* gene expression within NHNE cells, RT-PCR was carried out after treating the cells with various concentrations of E2. As the dose of E2 was increased from 10^{-10} to 10^{-8} M, the level of *MUC5B* gene expression and intracellular MUC5B protein expression were gradually increased. As shown in Fig. 1A and B, E2 at a concentration of 10^{-9} M significantly elevated *MUC5B* gene expression and MUC5B protein (3.0-fold increase). Accordingly, 10^{-9} M E2 was used for all subsequent experiments. To determine whether E2 induced *MUC5B* gene expression in a time-dependent manner, *MUC5B* gene expression was examined after exposure to 10^{-9} M E2 for various periods. The level of *MUC5B* gene expression and MUC5B protein expression were increased in a time-dependent manner (Fig. 1C and D) and reached a peak after a 24 hr exposure to E2 (3.1-fold increase). Accordingly, all subsequent experiments were treated for 24 h.

E2 is known to rapidly induce cytoplasmic signal transduction pathways, resulting in the phosphorylation of target proteins.[34-37]

2. *E2 increases ER*α *Ser- 118 phosphorylation*

Activation of Ser118 in the N-terminal activation function 1 domain of ERα is related to the phosphorylation of MAPK.[41] E2 rapidly induced phosphorylation of ERα Ser-118 in NHNE cells, with a peak in detectable p-ERα between 5 to 20 min after the introduction of E2 (Fig. 2A). To determine whether ICI 182,780, a specific ERα and ERβ antagonist, inhibited ERα Ser118 phosphorylation, NHNE cells were treated with 5 μM ICI 182,780. ICI 182,780 inhibited ERα Ser-118 phosphorylation induced by E2 (Fig. 2B) and suppressed E2-induced *MUC5B* gene expression and p-ERK1/2 activation (Fig. 2C), indicating that E2-induced *MUC5B* gene expression was ERα-mediated. As shown by immunofluorescence staining in NHNE cells (Fig. 2D), phospho-ERα Ser118 was distributed equally in the cytoplasm and the nucleus in the resting state, while phospho-ERα Ser118 re-localized to the cytoplasm and cell membrane upon treatment with E2. These results suggest that E2 induces *MUC5B* gene expression by ERα and pERK1/2, implicating their involvement in E2-induced *MUC5B* gene expression.

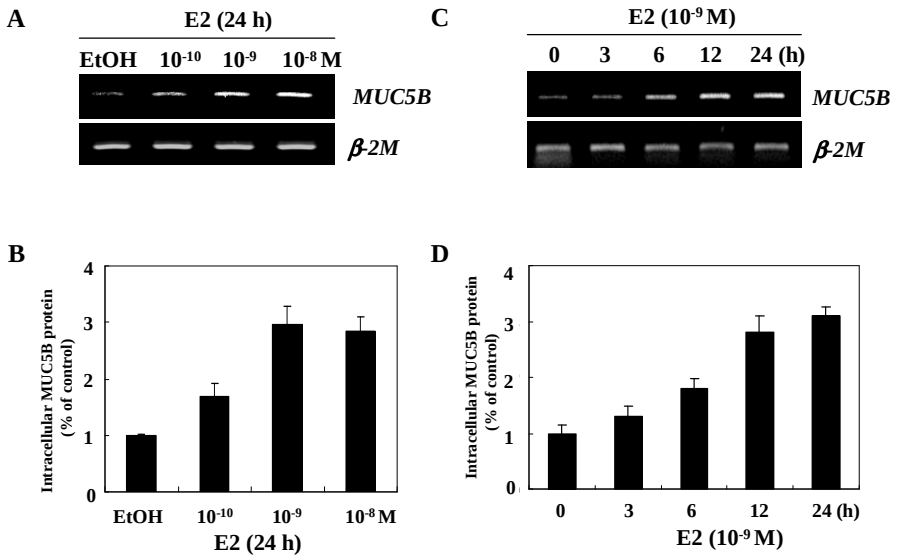

Fig. 1. Effect of E2 on *MUC5B* gene expression and intracellular MUC5B mucoprotein in NHNE cells. Cells were treated with E2 (10^{-10}, 10^{-9}, and 10^{-8} M) for 24 h (A, B) or treated with E2 (10^{-9} M) for 0, 3, 6, 12, and 24 h (C, D). Cell lysates were harvested for RT-PCR or immuno-dot blot analysis. Total RNA was isolated, and RT-PCR was performed using the specific primers described in (A, C) "Experimental Procedures." And equal amounts of whole cell lysates were processed for immuno-dot blot analysis as described in (B, D) "Experimental Procedures." Expression of β2-microglobulin (β2M) was analyzed as a control.

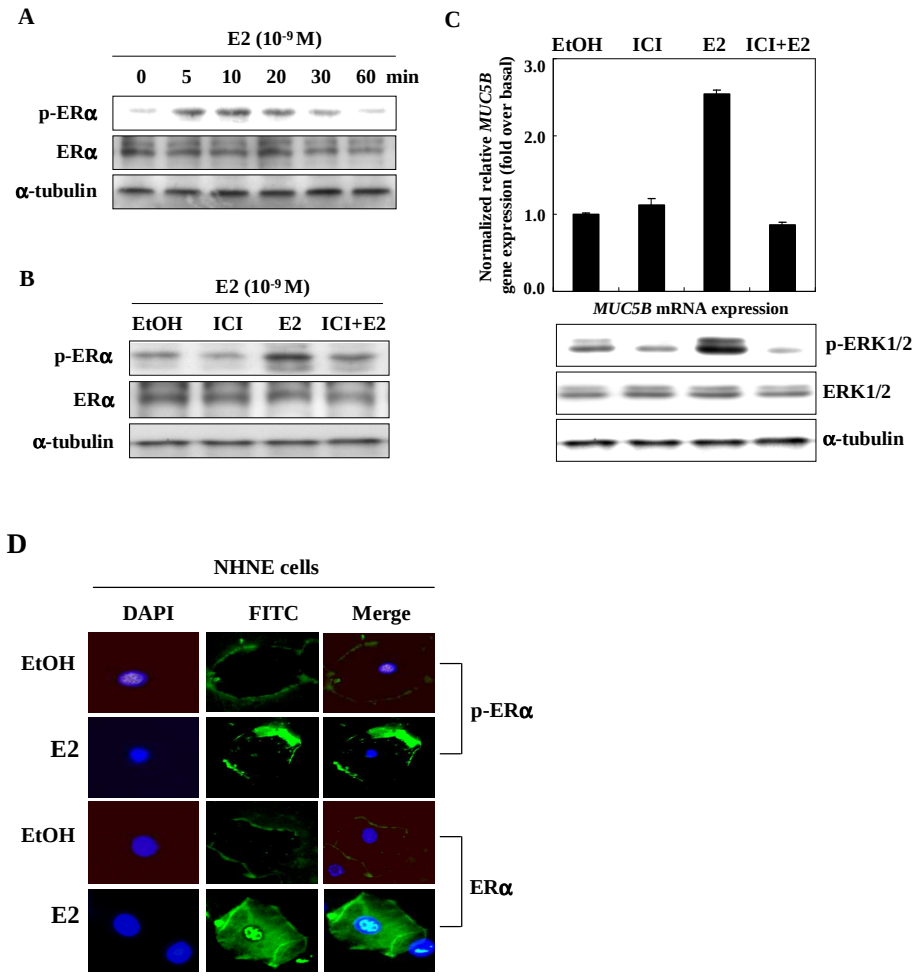

Fig. 2. Effect of ICI182,780 on *MUC5B* gene expression in NHNE cells. Cells were treated with E2 (10^{-9} M) for 5, 10, 20, 30, and 60 min, and total cell lysates were processed for Western blot analysis as described in "Experimental Procedures." α-tubulin was used as an internal control (A). Cells were pretreated for 1 h with 5 μM ICI182,780 and stimulated for 10 min with E2

prior to the collection of cell lysates for Western blot analysis. α-tubulin was used as an internal control (B). Pretreated cells were stimulated for 24 h with E2 prior to collection for real-time quantitative PCR (C, upper panel) or for 10 min prior to Western blot analysis (C, lower panel). The cellular localization of the ERα in NHNE was analyzed by immunofluorescence (D).The data shown are a representative experiment repeated at least three times with less than 10% variation.

3. ***E2 induces MUC5B gene expression via ERK1/2 MAPK signaling***

To investigate which MAPK signal pathway was activated by E2 in NHNE cells and NCI-H292 cells, Western blot analysis was performed using phospho antibodies. As shown in Fig. 3A, E2 activated ERK1/2 MAPK in both NHNE cells and NCI-H292 cells (data not shown) with a peak activation at 10 min. However, activations of phospho-p38 or -c-Jun NH_2-terminal kinase were not detected.

To investigate the possible involvement of the ERK1/2 MAPK pathway in E2-induced *MUC5B* gene expression, the real-time PCR was performed with pretreatment with 30 μM PD98059 for 1 h. PD98059 inhibited *MUC5B* gene expression and p-ERK1/2 activation (Fig. 3B). These results indicate that the activation of ERK1/2 MAPK is closely related to E2-induced *MUC5B* gene expression. For further confirmation, the NCI-H292 cells were transfected with siRNA ERK1, siRNA ERK2 or siRNA negative control. The siRNA ERK1 or siRNA ERK2 transfection was found to suppress E2-induced ERK1 or ERK2 MAPK activity and E2-induced *MUC5B* gene expression (Fig. 3C). These results indicate that the activation of ERK1/2 MAPK is essential for E2-induced *MUC5B* gene expression in NCI-H292 cells.

4. *Effect of Ras DN and Raf DN on E2-induced MUC5B gene expression*

Ras and Raf are upstream targets of activated MAPK.[42] To examine whether the Ras/Raf/MAPK pathway plays a role in E2-induced *MUC5B* gene expression, cells were transiently transfected with constructs encoding Ras DN (Ras N17) or Raf1 DN (craf1, a kinase-defective form of Raf1). The transient overexpression of Ras or Raf1 DN suppressed the E2-induced ERK1/2 MAPK phosphorylation, and E2-induced *MUC5B* gene expression (Fig. 4A and B). These results suggest that the activation of ERK1/2 MAPK by E2 occurs via a Ras/Raf-dependent pathway to induce *MUC5B* gene expression in NCI-H292 cells.

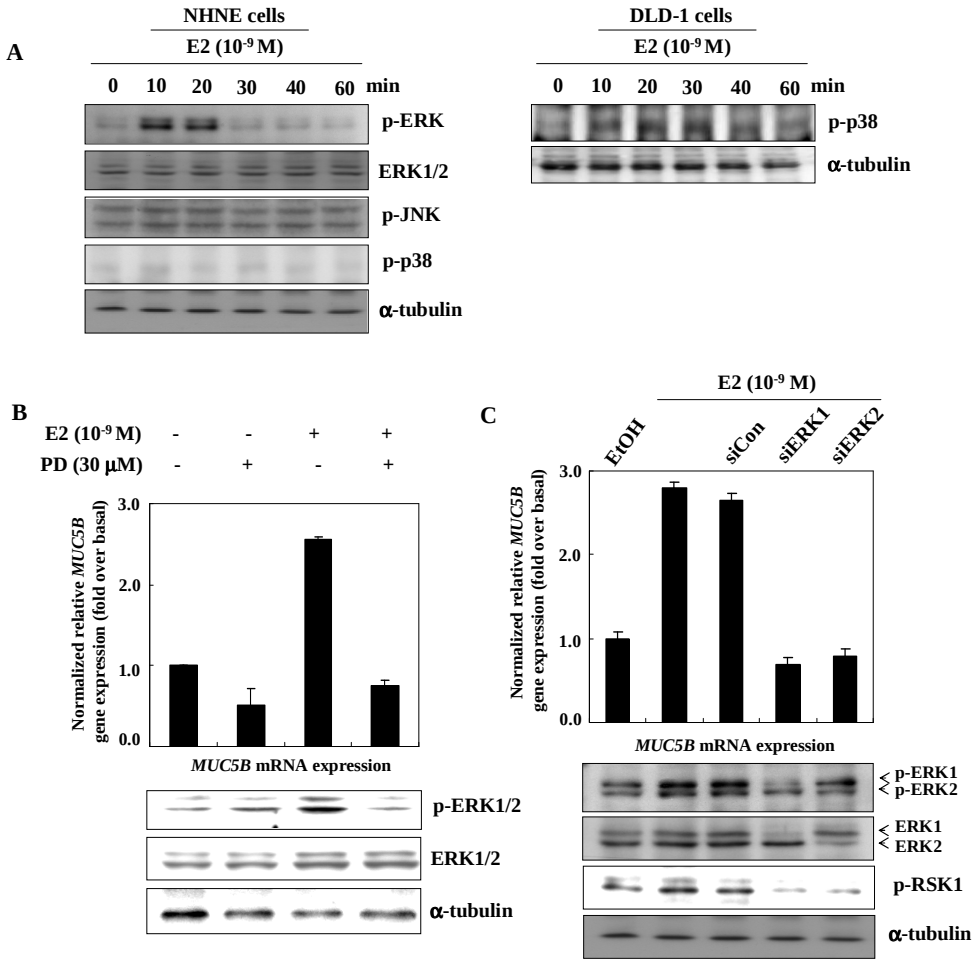

Fig. 3. E2 induces *MUC5B* gene expression by ERK MAP kinase signaling in NHNE cells. The cells were treated with E2 (10^{-9} M) for 10, 20, 30 40, and 60 min, and total cell lysates were processed for Western blot analysis as described in "Experimental Procedures." α-tubulin was used as an internal control (A). NHNE cells were pretreated for 1 h with 30 μM PD98059 and stimulated for 24 h with E2 prior to collection of total RNA for real-time

quantitative PCR (B, upper panel). Pretreated cells were stimulated for 10 min with E2 prior to the collection of cell lysates for Western blot analysis (B, lower panel). NCI-H292 cells were transfected with siERK1 (40 nM), and siERK2 (40 nM) or siRNA negative control (40 nM) and stimulated with E2 for 24 h prior to real-time quantitative PCR (C, upper panel) or for 10 min prior to Western blot analysis (C, lower panel). The data shown are a representative experiment repeated at least three times with less than 10% variation.

5*. RSK1 is required for E2-induced MUC5B gene expression*

To identify the molecules involved in the down-stream signaling of ERK1/2 MAPK in E2-induced *MUC5B* gene expression, RSK1 and MSK1 which are substrates of CREB[43-45] were examined. The phosphorylation of RSK1 by E2 peaked at 10 min and decreased by 60 min after E2 stimulation (Fig. 5A). E2 did not affect phosphorylation of MSK1 (data not shown). These results suggest that RSK1 acts as a downstream signaling mediator of ERK1/2 MAPK. To determine whether RSK1 plays an important role in E2-induced *MUC5B* gene expression, an RSK1 silencing study was performed, as RSK1 is a mediator of cytokine-induced CREB phosphorylation at Ser 133. Three days after transfection, cells were collected, and the effects of siRNA were analyzed by Western blot analysis. siRNA specific for RSK1 suppressed the levels of p-RSK1 (Fig. 5B), while the siRNA negative control exerted no effect. Knocking down RSK1 led to a decreased level of the *MUC5B* gene expression as assessed by quantitative real-time PCR, while the negative control showed no effect (Fig. 5B). These results indicate that RSK1 is an essential downstream mediator of ERK MAPK signaling for E2-induced *MUC5B* gene expression.

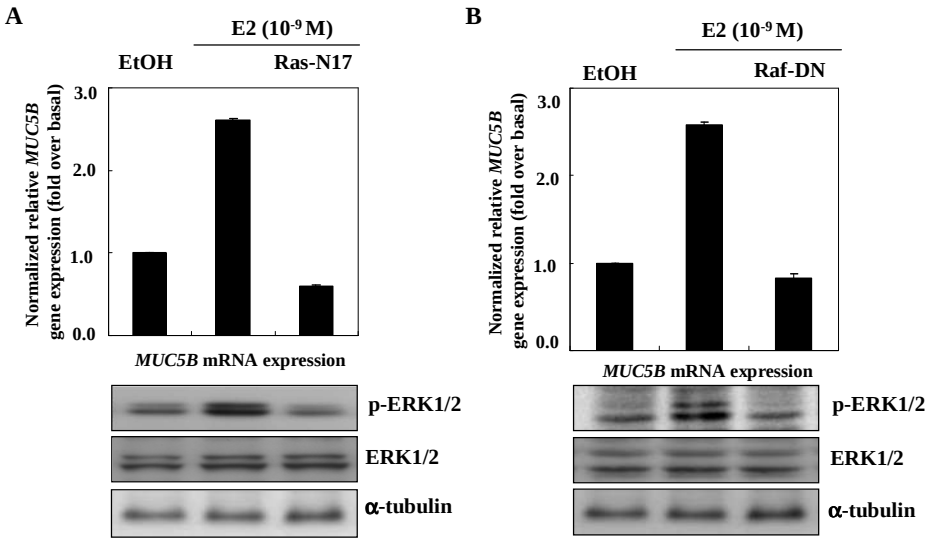

Fig. 4. Effect of transient transfection with Ras DN and Raf DN on E2-induced *MUC5B* gene expression in NCI-H292 cells. The cells were transiently transfected with dominant-negative Ras or Raf (Ras-N17, Raf-DN) and stimulated with E2 for 10 min prior to Western blot analysis (A and B, lower panel). Transfected cells were stimulated with E2 for 24 h prior to real-time quantitative PCR (A and B, upper panel). The data shown are from a representative experiment repeated at least three times with less than 10% variation.

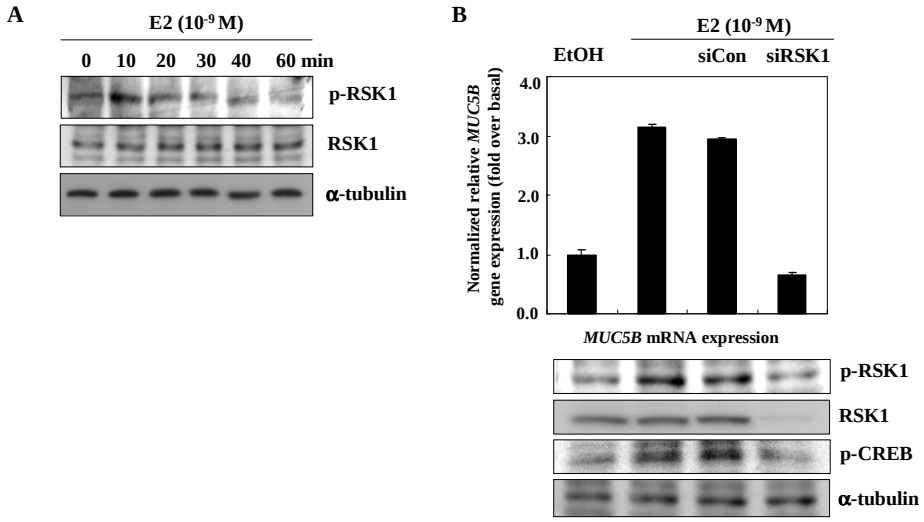

Fig. 5. Effect of RSK1 on E2-induced *MUC5B* gene expression. The cells were stimulated for the indicated times with E2, and total cell lysates were processed for Western blot analysis as described in "Experimental Procedures." α-tubulin was used as an internal control (A). The cells were transfected with siRSK1 and stimulated with E2 for 24 h prior to real-time quantitative PCR (B, upper panel). Transfected cells were stimulated with E2 for 10 min prior to Western blot analysis (B, lower panel). The data shown are a representative experiment repeated at least three times with less than 10% variation.

6. ***Effect of CREB on E2-induced MUC5B gene expression***

To determine if CREB plays a role in E2-induced *MUC5B* gene expression, Western blot analysis was performed using an anti phospho-CREB antibody. The phosphorylation of CREB by E2 peaked at 20 min and decreased at 40 min (Fig. 6A). To test the role of CREB in transcriptional regulation by E2, siRNA was used to knock down CREB in NCI-H292 cells. Three days after transfection, cells were collected, and the effects of siRNA were analyzed by Western blot analysis. siRNA specific for CREB suppressed the levels of p-CREB (Fig. 6B), while the siRNA negative control exerted no effect. Knocking down CREB led to a decreased level of the *MUC5B* gene expression as assessed by quantitative real-time PCR, while the negative control showed no effect (Fig. 6B). These results indicate that activation of CREB is essential for E2-induced *MUC5B* gene expression by ERK MAPK and RSK1.

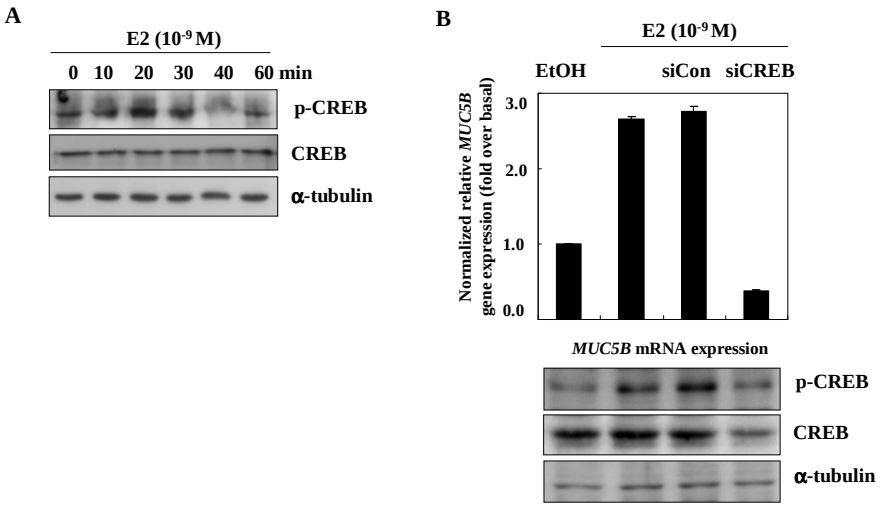

Fig. 6. Effect of CREB on E2-induced *MUC5B* gene expression. The cells were treated with E2 (10^{-9} M) for 10, 20, 30, 40, and 60 min, and total cell lysates were processed for Western blot analysis as described in "Experimental Procedures." α-tubulin was used as an internal control (A). The cells were transfected with siCREB (40 nM) or siRNA negative control (40 nM) and stimulated with E2 for 24 h prior to real-time quantitative PCR (B, upper panel). Transfected cells were stimulated with E2 for 20 min prior to Western blot analysis (B, lower panel). The data shown are a representative experiment repeated at least three times with less than 10% variation.

7. *CRE is required for E2-induced MUC5B transcription*

Various promoter deletion clones, such as -956 to +92, and -649 to +92, were constructed based on the -1329 to +92 clone. NCI-H292 cells were transiently transfected with these constructs and treated with E2 (10^{-9} M) for 24 h. As shown in Fig. 7A, E2 selectively increased the luciferase activity of the -956 to -649 region of the *MUC5B* promoter. However, its effect was reduced on fragments covering the -649 to +92 region, suggesting that the -956 to -649 region of the *MUC5B* promoter is required for responding to E2.

Although it has been clearly established that CREB binds to the *MUC5B* promoter *in vitro*,[46,47] to investigate whether the CRE is required for targeting CREB to the *MUC5B* promoter by E2, ChIP assays were carried out using a siRNA against CREB. Three days later, the cells were treated with or without E2 for 20 min and processed for ChIP using an anti-phospho-CREB antibody, and the purified genomic DNA was amplified with primers specific to the *MUC5B* promoter (-956/-649). CREB led to an increase in the binding of CRE to the *MUC5B* promoter. However, the binding of CREB was abolished by treatment with CREB siRNA, confirming the effectiveness and specificity of the siRNA (Fig. 7B). These data suggests that CREB binds to the CRE on the

MUC5B promoter, and that CRE is critical for the up-regulation of *MUC5B* transcriptional activity by E2.

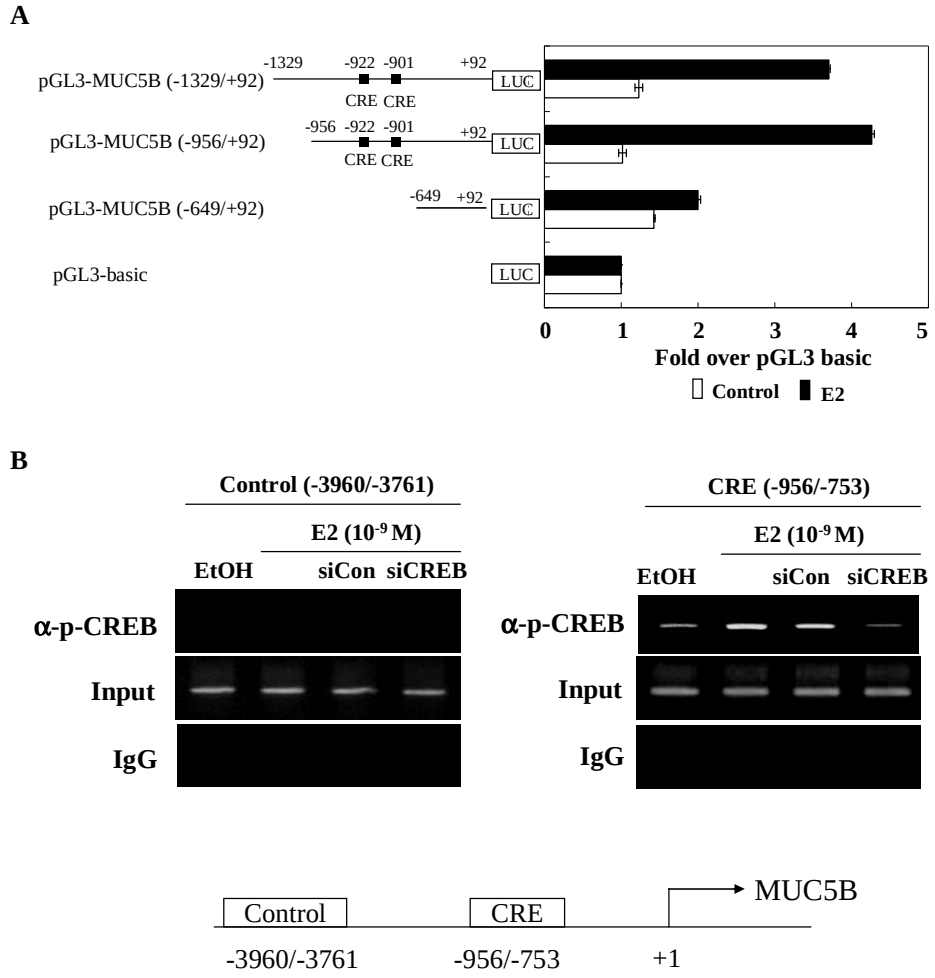

Fig. 7. CRE is required for E2-induced *MUC5B* transcription. NCI-H292 cells were transiently transfected with various *MUC5B* promoter luciferase reporter constructs, and stimulated with E2 for 24 h. Luciferase activity was then measured in E2-treated or -untreated cells. The luciferase activities were corrected for transfection efficiency using the β-galactosidase activity of the cell lysates to standardize the values. The values shown are mean ± SD of

experiments performed in triplicate (A). The ChIP assays were performed with cells transfected with siRNA CREB or siRNA negative control, then treated with E2 (10^{-9}M) for 20 min. The cells were immunoprecipitated using CREB antibody or control IgG that either amplified the CREB binding flanking region in *MUC5B* promoter (CRE site) or a region further upstream that does not contain a CREB binding site (non-CRE site) (B). IgG, goat antirabbit IgG (negative control for ChIP). The data shown are a representative experiment repeated at least three times with less than 10% variation.

8. ***E2 induces the recruitment of coactivators to MUC5B promoter***

Activation of gene expression involves recruitment of coactivator proteins that function as bridging factors connecting sequence-specific transcription factors to the basal transcription machinery.[48,49] Thus, to determine whether E2 treatment led to recruitment of coactivators to the CREB, cells were treated with or without E2 for 20 min and processed for ChIP using antibodies against CBP, SRC-1, H3, and AcH3. As shown in Fig. 8, E2 treatment increased the recruitment of CBP and SRC-1, and increased H3 acetylation to the *MUC5B* promoter. These data showed a good correlation between the increase in CBP and SRC-1 recruitment and increase of CREB induced by E2. Taken together, these results indicate that E2 activates CREB transcriptional activity by increasing the recruitment of CBP and SRC-1 to the *MUC5B* promoter.

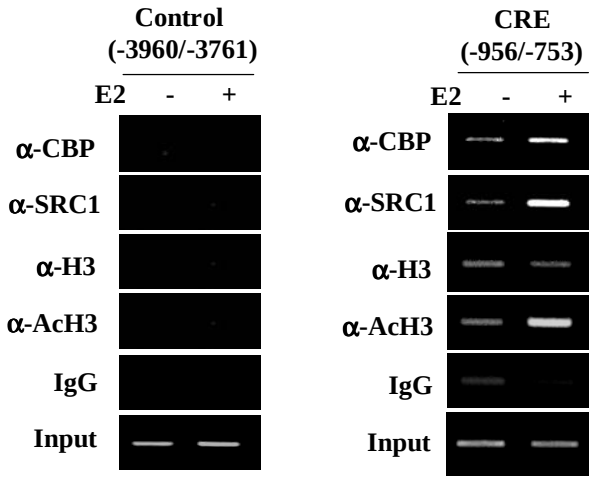

Fig. 8. E2 increases the recruitment of coactivators CBP and SRC-1 to the CREB. ChIP assays were carried out using 2 μg of CBP, SRC-1, H3, and AcH3 antibodies and specific primers to amplify the CRE site in the *MUC5B* promoter. DNA fragments containing the CRE site in *MUC5B* promoter were amplified by PCR. This figure is a representative of more than three separate experiments.

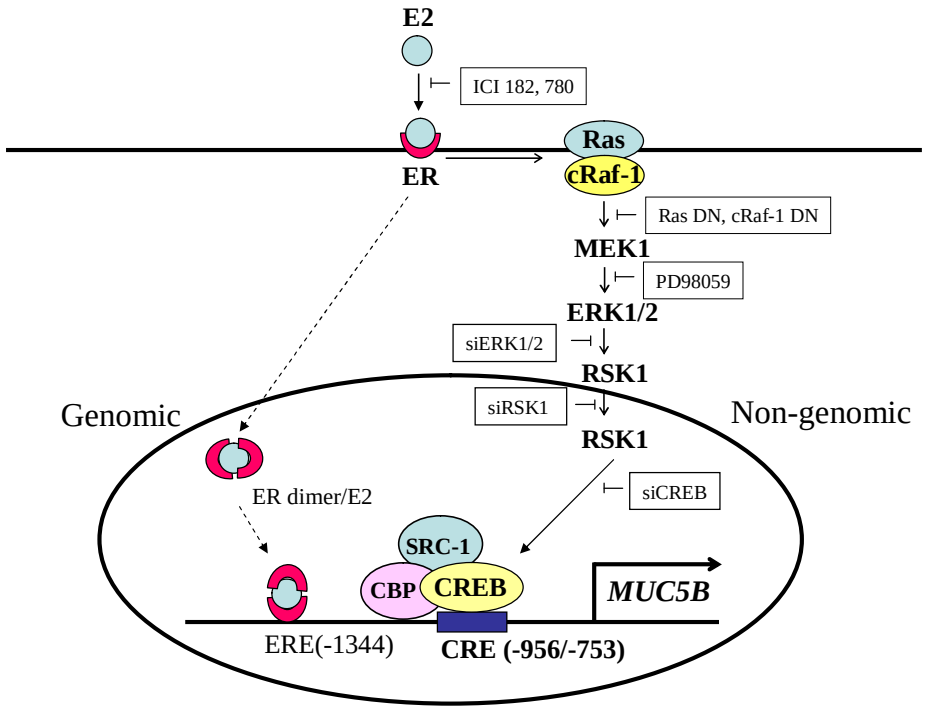

Fig. 9. The signaling pathway regulating E2 induction of *MUC5B* gene expression in human airway epithelial cells. E2 activates cytoplasmic ER. Then, E2 activates Ras-Raf which led to activation of MEK leading to ERK activation. Finally, the culminates in activation of the CREB transcription factor and binding of the specific sites with the *MUC5B* gene promoter.

IV. DISCUSSION

Airway inflammation and mucus hypersecretion are common features of various airway diseases, such as rhinosinusitis, asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis.[1,3-5] *MUC5AC* and *MUC5B* are the major genes expressed in the human airway, and their glycoprotein products are the most abundant in mucus secretions.[25-26] In the normal distribution pattern of the airway epithelium, MUC5AC is produced by goblet cells, while MUC5B is usually produced in the submucosal glands.[50] Interestingly, Chen *et al.*[51] reported that, in addition to the expression by submucosal glands, MUC5B could be expressed by surface airway epithelial cells in airway tissue sections obtained from patients with COPD and asthma. In various chronic airway diseases, MUC5B is present in the most tenacious portions of the mucus.[52-54] As such, MUC5B may be the most important mucin associated with mucus viscosity. Therefore, the molecular mechanism of MUC5B secretion regulated by inflammatory cytokines or hormones in the airway is very important, and the understanding of this mechanism may offer new therapeutic strategies for inhibiting airway mucus hypersecretion.

However, the molecular mechanism by which *MUC5B* is up-regulated by

17β-estradiol (E2) remains poorly understood. In this study, the involvement of E2 in the up-regulation of *MUC5B* gene expression in human airway epithelial cells was investigated.

Although previous work on *MUC5AC* gene expression showed that cytokines such as IL-β and TNF-α activate at least two MAP kinases, ERK and p38 MAP kinase[44], E2 was shown in this study to only activate ERK MAP kinase in *MUC5B* gene expression. Recently, Chen *et al.*[55] also reported that IL-17 induced *MUC5B* gene expression through Janus kinases (JAK) 2 and ERK MAP kinase in human, monkey, and mouse airway tissues. *MUC5B* gene expression was increased by defensin or uridine 5′-triphosphate (UTP) through ERK1/2 MAP kinase pathway.[56,57] These finding support the notion that ERK MAP kinase is an important signaling molecule in *MUC5B* gene expression.

In the present study, the downstream signaling molecules of ERK MAP kinase were investigated. RSK1 and CREB were identified as essential downstream molecules of ERK MAP kinase activation in E2-induced *MUC5B* gene expression. RSK1 is known to be regulated by MAPK/ERK and is currently the best candidate for mediation of cytokine-induced CREB phosphorylation at Ser^{133}.[43,58,59] Recently, Song *et al.*[44] reported that IL-1β and

TNF-α induced *MUC5AC* gene expression through CREB phosphorylation in primary human nasal epithelial cells and NCI-H292 cells. In addition, Cho *et al.*[45] reported that CREB activation is required for PGE2-induced *MUC8* gene expression. These findings support this study that CREB activation is involved in the downstream signaling of ERK MAPK and RSK1 for E2-induced *MUC5B* gene expression. Gerritsen *et al.*[60] reported that p300 and CBP act as coactivators of p65 transactivation as well as SRC-1 of p160 and may play important roles in the cytokine-induced expression of various immune and inflammatiory genes. These findings suggest that CREB may interact directly or indirectly with other transcription factor(s), and that non-DNA binding transcriptional coactivators, such as p300, CBP, and SRC-1 which are thought to function as bridging proteins between DNA-binding factors and the basal transcription factors, play a role as integrators of diverse signaling pathways affecting *MUC5B* gene expression.

Interestingly, Van Seuningen *et al.*[46] reported that co-transfection of Sp1 with fragment 1896 (-956/-1) led to an increase of *MUC5B* gene expression in mucus-secreting LS173T and Caco-2 cells, suggesting that Sp1 may also be an important transcription factor.

The results indicate that the -956/-753 region of the *MUC5B* promoter is critical for E2-induced *MUC5B* gene expression. In this region of the *MUC5B* promoter, there are two possible CREB binding sites in the -922 and -901 region, and since the sequence of these two sites are identical, it can be inferred that both of these sites are important for E2-induced *MUC5B* gene expression.

In summary, this study demonstrated that the Ras-Raf-ERK MAPK-RSK1-CREB cascade is essential for E2-induced *MUC5B* gene expression. Furthermore, E2 may facilitate the recruitment of CBP and SRC-1 to CREB, leading to the up-regulation of *MUC5B* gene expression in human airway epithelial cells (Fig. 9). This is the first study to report E2-induced *MUC5B* gene expression occurring by non-genomic action.

V. CONCLUSION

This study demonstrated that ERK MAPK is essential for E2-induced *MUC5B* gene expression in human airway epithelial cells, and that p90 ribosomal S 6 protein kinase 1 (RSK1) mediate the E2-induced phosphorylation of the cAMP response element binding (CREB) protein. In addition, the CRE in the *MUC5B* promoter is important for E2-induced *MUC5B* gene expression.

The abbreviations used are: E2, 17-Estradiol; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; RSK1, p90 ribosomal S 6 protein kinase; CREB, CRE binding protein; DN, dominant negative; siRNA, small interfering RNA.

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Abstract in Korean

기도상피세포에서 17β-estradiol 에 의한 MUC5B 유전자 발현의 신호전달 경로

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MUC5B 는 사람의 호흡기에서 주요 점액 성분의 하나이나 여러 기도질환에서 그 발현의 조절기전은 잘 알려져 있지 않다. 본 연구에서는 estradiol 이 어떤 기전을 통하여 사람 코점막상피세포와 NCI-H292 세포에서 *MUC5B* 유전자 발현을 유도하는지 알아보고자 하였다. 17β-estradiol (E2)은 성호르몬으로 estrogen receptor α (ERα)와 결합하여 ERK-MAP kinase (MAPK)를 통하여 *MUC5B* 유전자 발현을 자극하였다. E2 와 함께 ER 길항제인 ICI 182, 780 을 투여하면 E2 에 의해 유도되는 ERK-MAPK 활성과 *MUC5B* 유전자의 발현이 모두

억제되었다. E2 는 주요 MAP kinase 중 p38 과 JNK 는 활성화시키지 않았으나 ERK1/2 는 활성화시켰고 MEK1 길항제인 PD98059 를 투여하였을 때 E2 에 의한 ERK1/2 활성과 *MUC5B* mRNA 발현이 모두 억제되었다. 또한 siRNA 로 ERK1 이나 ERK2 를 knockdown 시켰을 때 E2 에 의한 ERK1, ERK2 의 활성과 *MUC5B* mRNA 발현이 모두 억제되었다. Ras 와 Raf 의 dominant-negative construct 로 transient transfection 시킨 세포에서도 E2 에 의한 ERK1/2 의 활성과 *MUC5B* mRNA 발현이 모두 억제되었다. siRNA 로 RSK1 을 knockdown 시켰을 때 E2 에 의한 *MUC5B* mRNA 발현이 억제되었다. siRNA 로 CREB 을 knockdown 시켰을 때 E2 에 의한 *MUC5B* mRNA 발현이 억제되었다.

일련의 *MUC5B* promoter construct 를 제작하여 luciferase assay 를 시행한 결과, -956bp 에서 -649bp 사이에 존재하는 cAMP-response element (CRE) site 가 ERK-MAPK 를 통한 *MUC5B* 유전자 발현에 중요하다는 것을 알 수 있었다. E2 를 처리하였을 때 CREB 이 -956bp 에서 -753bp 사이의 *MUC5B* promotor 에 결합하는 것을 ChIP assay 를 통하여 확인할 수 있었다. CREB 은 또한 CRE site 에 CREB-binding protein (CBP) 과 steroid receptor coactivator-1 (SRC-1)과 함께 결합하여 histone acetrylation 과 *MUC5B* 유전자 발현을 증가시켰다.

따라서 17β-estradiol 은 기도상피세포에서 estrogen receptor, Ras, Raf, MEK1, ERK1/2 MAPK, RSK1 로 신호가 전달되며 CREB 이 CBP, SRC-1 과 함께 *MUC5B* promoter 에 결합하여 *MUC5B* 유전자 발현을 유도한다.

핵심되는 말: MUC5B, estradiol, MAP kinase, 신호전달

PUBLICATION LIST

: None