Protein Kinase Casein Kinase 2 Regulates Anoikis

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Protein Kinase Casein Kinase 2 Regulates Anoikis

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Hyeonseok Ko

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This certifies that the Doctoral Dissertation of Hyeonseok Ko is approved.

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ABSTRACT

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The disruption of integrin-extracellular matrix interactions in normal epithelial cells induces anoikis. The acquisition of anoikis-resistance in cancer cells is regarded as a critical step for metastasis. The E- to N- cadherin switching enables tumor cells to invade, migrate, and metastasize and N-cadherin confers anoikis resistance to cancer cells. Little

was know about the mechanism of E- to N-cadherin switching, the regulatory mechanism of N-cadherin gene expression, and role (s) of N-cadherin in anoikis resistance. In this study, PKCK2 activity in cancer cell was involved in all those processes. β-catenin, a Wnt-signaling mediator was stabilized by PKCK2-mediated phosphorylation, thereby increasing its nuclear translocation. Nuclear β-catenin increased Axin2 transcription resulting in increased cytoplasmic shuttling of nuclear GSK3β, a kinase responsible for controlling Snail turnover, thereby stabilizing E-cadherin repressor, Snail and then, E-cadherin was down-regulated. In addition, it has been observed that an essential N-cadherin promoter region (-296 to -158) for N-cadherin gene expression and a transcription factor, myeloid zinc finger1 (MZF1) could bind to the region, and when MZF-1 was overexpressed, the N-cadherin promoter activity was increased. Interestingly, MZF1 protein stability was also regulated by PKCK2 activity. It has been known that when N-cadherin was expressed, PKB/Akt activity was increased. Consistent with this, PKB/Akt activity was high in N-cadherin expressing cancer cells and when N-cadherin was knocked-down by siRNA, PKB/Akt activity was decreased and anoikis-resistant esophageal cancer cells became susceptible to anoikis. In addition, PTEN phosphatase activity was inhibited by PKCK2 and when N-cadherin expressing cancer cells were

treated with PKCK2 inhibitor, emodin, PKB/Akt activity was decreased and anoikis-resistant cancer cells underwent anoikis.

Taken together, intracellular PKCK2 activity in cancer cells regulated E- to N-cadherin switching, N-cadherin up-regulation resulting in increasing PKB/Akt activity, thereby conferring cancer cells anoikis resistance. Therefore, downregulation of PKCK2 activity in cancer cells could reduce cancer metastasis.

Key words : PKCK2, cadherin switching, β-catenin/Axin2/GSK3β/snail signaling, anoikis, MZF1

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Ⅰ. INTRODUCTION

The cadherins are members of large family of trans-membrane glycoproteins that mediate calcium-dependent, homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture [1-4]. The linkage between cadherin and the actin cytoskeleton through the junctional complex, including α-catenin, β-catenin, γ-catenin,

vinculin, and p120^{cat}, contributes to stable cell-cell adhesion [3, 5-11]. The subtypes of classical cadherins, E-, N-, and P-cadherins, are expressed in a cell-, tissue-, and development-specific manner. E-cadherin is the major cadherin in polarized epithelial cells, whereas N-cadherin is expressed mainly in mesenchymal cells [12]. The function of E-cadherin during tumor progression is frequently replaced or even overruled by the expression of N-cadherin [13-8]. The switching of cadherin subtypes during tumor cell development might enable tumor cells to interact directly with other N-cadherin-expressing cells, such as fibroblasts and vascular endothelial cells, thus affecting tumor-host cell adhesion, tumor cell invasion, and migration [18-9].

Cell adhesion is a complex process, in which several adhesion molecules are involved. These adhesion molecules interact with an array of different structural components referred to as the extracellular matrix (cell-matrix adhesion) and neighboring homotypic or heterotypic cells (cell-cell adhesion) [20].

The adhesion of normal epithelial cells to extra-cellular matrix (ECM) is crucial for their survival and proliferation [21]. When adhesion is disrupted, normal epithelial cells rapidly undergo anoikis [22], a type of detachment-induced apoptosis [22-5]. Anoikis plays an important role in the maintenance of tissue homeostasis in organisms and also appears to

block the growth of detached cells at inappropriate locations, thus acting as an important safeguard against oncogenesis. Therefore, the acquisition of anoikis-resistance is regarded as a critical step during the metastatic transformation of a tumor [20, 26]. However, little was known about the role (s) of N-cadherin in anoikis.

PKCK2 is a constitutively active, growth factor-independent serine/threonine protein kinase composed of two catalytic α subunits and two regulatory β subunits. It plays a key role in cell cycle control, cellular differentiation, and proliferation and also participates in the regulation of apoptosis by phosphorylating some apoptosis-related factors [27-9]. Furthermore, PKCK2 plays a positive role in the Wnt/β-catenin signaling through phosphorylates β-catenin at Thr 393, leading to proteasome resistance and increased nuclear translocation and co-transcriptional activity [30-1]. The Wnț/β-catenin signaling play roles in human cancers, through mutation of genes encoding β-catenin itself or its regulators, chiefly APC, the adenomatous polyposis coli gene product, or Axin [31]. In addition, recent data have shown that Wnt/β-catenin signaling regulated stability of snail, transcriptional repressor of E-cadherin, through Axin2-mediated GSK3β shutling to cytoplasm [32-3].

In this study, it has been investigated that the mechanism of E- to N-cadherin

switching, regulatory mechanism of N-cadherin gene expression, and role (s) of N-cadherin in anoikis resistance. In the study presented here that PKCK2 activity in cancer cell was involved in all those processes. β-catenin, a Wnt-signaling mediator was stabilized by PKCK2-mediated phosphorylation, thereby increasing its nuclear translocation. Nuclear β-catenin increased Axin2 transcription resulting in increased cytoplasmic shuttling of nuclear GSK3β, a kinase responsible for controlling Snail turnover, thereby stabilizing E-cadherin repressor, Snail and then, E-cadherin was down-regulated. In addition, it has been showed that an essential N-cadherin promoter region (-296 to -158) for N-cadherin gene expression and a transcription factor, myeloid zinc finger1 (MZF1) could bind to the region, and when MZF-1 was overexpressed, the N-cadherin promoter activity was increased. Interestingly, MZF1 protein stability was also regulated by PKCK2 activity. It has been known that when N-cadherin was expressed, PKB/Akt activity was increased. Consistent with this, PKB/Akt activity was high in N-cadherin expressing cancer cells and when N-cadherin was knocked-down by siRNA, PKB/Akt activity was decreased and anoikis-resistant esophageal cancer cells became susceptible to anoikis. In addition, PTEN phosphatase activity was inhibited by PKCK2 and when N-cadherin expressing cancer cells were treated with PKCK2 inhibitor, emodin, PKB/Akt activity was decreased and

anoikis-resistant cancer cells underwent anoikis.

Taken together, intracellular PKCK2 activity in cancer cells regulated E- to N-cadherin switching, N-cadherin up-regulation resulting in increasing PKB/Akt activity, thereby conferring cancer cells anoikis resistance. Therefore, downregulation of PKCK2 activity in cancer cells could reduce cancer metastasis.

Ⅱ. MATERIALS AND METHODS

1. Cell culture and reagents

TE2, TE3, HCE4 and HC7, human esophageal cancer cell lines were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, MD) supplemented with 10 % fetal bovine serum (FBS) (Gibco-BRL, MD). A549 cells, human lung cancer cell line, and MDA MB-435, human breast cancer cell line, were cultured in RPMI medium supplemented with 10 % FBS. MDA MB-231, human breast cancer cell line was cultured in MEM medium supplemented with 10 % FBS. For the induction of anoikis, cells were plated on poly-HEMA [poly (2-hydroxy ethylmethacrylate); Sigma-Aldrich, St. Louis, MO] coated tissue culture plate as previously described [34]. TGF-β1 was purchased from BD Biosciences (Bedford, MA), prepared in 250 ng/ml stocks in 36 % acetonitrile/0.1 % trifluroacetic acid. MG-132 was purchased from Calbiochem (Darmstadt, Germany), prepared in 10 mM stock in dimethyl sulfoxide (DMSO). The PKB/Akt inhibitor LY294002 (Cell Signaling Technology, Inc., Beverly, MA) and PKCK2 inhibitor, emodin (Sigma-Aldrich) were kept as 10 mM and 5 mg/ml in DMSO, respectively.

2. Western blot analysis

The Western blot analysis was carried out as previously described [35]. Cells were lysed in RIPA buffer composed of 1 M Tris (pH 8.0), 5 M NaCl, 100 mM EGTA, 10 % NP-40, and 0.25 % sodium deoxycholic acid. Lysates were briefly vortexed and cleared by centrifugation at 12,000 rpm for 10 min at 4 ℃. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amount proteins were subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Co., Bedford, MA). The blotted membranes were immunostained with antibodies specific for the following antigens: PARP, phospho-AKT (Ser-473), AKT, phospho-PTEN (Ser-380/Thr-382) and PTEN were purchased from Cell Signaling Technology, Inc. (Beverly, MA). α-tubulin, procaspase-3 and vimentin were purchased from Santa Cruz Biotechnology Inc. (Dalaware Avenue, CA). N-cadherin and E-cadherin were purchased from Zymed Laboratories Inc. (South San Francisco, CA). cytokeratin-10 (BabCO, Richmond, CA), β-tubulin (Oncogene Science, Cambridge, MA), β-catenin (Transduction Laboratories, Lexington, KY), involucrin (NeoMarker, Fremont, CA), PKCK2 (Upstate, Charlottesville, VA), MZF1 (Atlas antibodies, Stockholm, Sweden) or HA (Sigma-Aldrich). The signals were

developed by a standard enhanced chemiluminescence (ECL) method according to the manufacturer's protocol (Amersham Pharmacia Biotech Inc., Uppsala, Sweden).

3. Cell proliferation assay

The cell proliferation assay was performed using sulforhodamine B solution (SRB) assay. Cells were plated on poly-HEMA coated tissue culture plates. The cells were collected and aspirated off the culture media. Then, the cells were fixed by adding 50 μl ice cold 10 % trichloroacetic acid (TCA) and incubated at 4 ℃ for 30-60 min. The cells were washed with tap water five times and allowed to air dry for 5 min. The cells were added 50 μl 0.4 % sulforhodamine B solution in 1 % acetic acid and incubated for 30 min at room temperature for staining. Following staining, the cells were washed four times with 1 % acetic acid to remove any unbound dye, and then allowed to air dry for 5 min. The cells were solubilized with 100 μl 10 mM Tris (pH 10.5) and placed on an orbital rotator for 5 min. Supernatants were collected and absorbance was read at 570 nm using UV-spectrophotometer.

4. cDNA microarray

The cDNA microarray was performed using GEArray^{TM}Q series kit (Superarray Bioscience Co. Frederick, MD) according to the manufacturer's protocol.

5. Immunoprecipitation (IP)

Immunoprecipitation was performed as previously described [36]. Cells were collected and lysed in 1 ml IP lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 % NP-40] with 1x complete protease inhibitor cocktail (Roche Diagnostics). The cell lysates were pre-cleared and then incubated with the indicated antibodies for 1 h at 4°C. The complexes were precipitated with Protein A/G-Sepharose beads (Santa Cruz Biotechnology Inc.), washed, and re-suspended in 40 μl 1x SDS-loading buffer. Non-immune mouse IgG (Santa Cruz Biotechnology Inc.) served as a negative control.

6. Small interfering RNA (siRNA)

The siRNAs were purchased from Dharmacon, Inc. (Option A4; Lafayette, CO). The target sequences for N-cadherin was CAUAUGUGAUGACCGUAAC (nucleotides 851-869). The siRNA against PKCK2 consists of 4 sets of duplex. Duplex 1, GAUCCACGUUUCAAUGAUAUU; Duplex 2, GAUGUACGAUUAUAGUUUGUU; Duplex 3, ACACAGACCUCGAAAUCUU; Duplex 4, GAGAGGAGGUCCCAACAU CUU. A scrambled siRNA was used as a control. Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen Life Technology, Carlsbad, CA).

7. Cell fractionation

Cells were incubated in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 1x complete protease inhibitor cocktail] for 30 min on ice. NP 40 (final 0.6 %) was added to the cells and vortexed vigorously for 20 sec, followed by centrifugation at 12,000 rpm for 30 sec. The supernatant was used as the cytoplasmic fraction. The pellet was washed with 1x phosphate buffered saline (PBS) for 3 times, and then buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1x complete protease inhibitor cocktail] was added and the pellet was resuspended. After 15 min incubation on ice, centrifuge was performed at 12,000 rpm for 5 min. The supernatant was used as the nuclear fraction.

8. Reporter assay using N-cadherin gene promoter

The N-cadherin promoter luciferase construct, pNcad-1310 contained the -1310/ +190 region of the human N-cadherin gene. Serial deletion constructs pNcad-1247, pNcad-667, pNcad-579, pNcad-296, pNcad-158, pNcad-139, and pNcad-89 were subcloned into pGL3 basic vectors. Each construct and pCMV-β-gal were transiently co-transfected into HCE4 cells using Lipofectamine Plus reagent (Invitrogen Life Technology). The cells were harvested and analyzed for luciferase activity after 24 h of transfection. Reporter activity

was normalized with β-galactosidase activity.

9. Chromatin immunoprecipitation (ChIP) assay

The *in vivo* molecular interaction between MZF1 and the N-cadherin promoter was analyzed using ChIP assay. HCE4 cells were transfected with pSG5-HA or pSG5-HA-MZF1 using Lipofectamine Plus. After 24 h of transfection, the cells were treated or untreated with emodin (20 μg/ml) for 24 h. For ChIP assay, chromatin was first isolated as follows. In brief, approximately 2×10^9 cells were first treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and then incubated with 100 mM Tris (pH 9.4) and 10 mM DTT at 30 ℃ for 15 min. The cells were then washed twice with PBS and resuspended in 600 μl of Sol A buffer [10 mM HEPES (pH 7.9), 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT] by pipetting. After a short spin (5 min at 3,000 rpm), the pellets were resuspended in Sol B buffer [20 mM HEPES (pH 7.9), 25 % glycerol, 0.5 % NP-40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA] containing protease inhibitors followed by vigorous pipetting in order to extract the nuclear proteins. After centrifugation at 13,000 rpm for 30 min, the nuclear pellets were resuspended in IP buffer [(1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl and protease inhibitors)] and sonicated to break the chromatin into fragments with an average length of 0.5-1 kb.

The ChIP assay was then performed with antibody against HA. The primers used for ChIP analysis were as follows. ChIP primers forward, 5'-GCTGCATCCCTCGCGGCTTC-3', reverse, 5'-GACAAATAGCGGGCCGCGGA-3'.

10. DNase I footprinting assay

The 234 bp (−296/−63) fragment of N-cadherin promoter region was labeled, amplified in one strand and purified as follows. The primer was labeled with polynucleotide kinase (PNK) and [γ-^{32}P]-ATP. The fragment was amplified with labeled primers, and then purified using PAGE. DNA-protein binding reactions were performed with 50,000 cpm of probe per reaction in a solution containing 10 mM HEPES (pH 7.9), 60 mM KCl, 7% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly (dI-dC) and the indicated amount of nuclear extract. After 30 min of incubation on ice, 5 μl of DNase I, freshly diluted in a solution containing 10 mM HEPES (pH 7.9), 60 mM KCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$ and 7 %(v/v) glycerol, was added to the reaction, which was then kept at room temperature for 2 min. Dilutions of DNase I ranged from 1:200 to 1:2000 of stock (10 units/μl), depending on the amount of protein in the reaction. Digestion reactions were stopped by the addition of 80 μl of a stop solution containing 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of yeast tRNA and 10 μg of

proteinase K. The samples were incubated for 30 min at 45 °C, extracted with phenol-chloroform, precipitated with ethanol, and resuspended in formamide dye. The samples were resolved in 6 % (w/v) polyacrylamide/7 M urea sequencing gel. The protected regions were mapped with reference to the migration of Maxam-Gilbert A-G sequencing products. The radioactivity in the dried gel was detected using X-ray film with a Kodak intensifying screen.

11. *In vitro* Transcription and translation (TNT)

HA tagged recombinant MZF1 transcription factor was *in vitro* transcribed and translated using TNT® quick transcription/translation systems (Promega) according to the manufacturer's instructions.

12. Electro mobility shift assay (EMSA)

To confirm the MZF1 binding on N-cadherin promoter, the [γ-^{32}P]-ATP labeled oligonucleotides were first prepared. The oligonucleotides used in this assay were as follows: 5'-TCCCCCGCCCCCTCCCAGCT-3'. The oligonucleotides were labeled with polynucleotide kinase (PNK) and [γ-^{32}P]-ATP, then purified by Sephadex™ G-50 column chromatography (Amersham Biosciences, NJ). The formation of protein-DNA complexes was induced by the same procedure as for the DNase I footprinting assay. After 30 min on

ice, the samples were resolved in 4 % (w/v) non-denaturing polyacrylamide gel polymerized in 0.5 x TBE [89 mM TBE (pH 8.3)] (where TBE is Tris/ borate/EDTA). For competition assays, a 100 fold molar excess of unlabelled competitor oligonucleotides was added to the reaction mixture before the addition of TNT product. The radioactivity in the dried gel was detected using X-ray film with a Kodak intensifying screen.

13. PKCK2 kinase assay *in vitro*

PKCK2 activity was measured as follows. Briefly, 3 μg of bacterially expressed GST-CS (CK2 Substrate) proteins were incubated with glutathione Sepharose 4B beads for 30 min, then they were washed twice using 1x kinase buffer (4 mM MOPS, pH7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 μM sodium orthovanadate, and 200 μM DTT). Then, the beads were incubated either with 50 ng recombinant active PKCK2 (Millipore) or 40 μg of cell lysates in final 50 μl of kinase reaction buffer [10 μl of 5x kinase buffer, 10 μl of magnesium/ATP cocktail solution (90 μl of 75 mM $MgCl_2$/500 mM ATP plus 10 μl (100 μCi) of [γ-^{32}P]-ATP] for 30 min at 30°C. Reactions were stopped by washing twice with 1x kinase buffer. Samples were resuspended with 30 μl of 1x SDS sample loading buffer and subjected to SDS-PAGE followed by staining with coomassie brilliant blue and autoradiography.

14. Cell invasion assay

The cell invasion assay was performed using the Cell Invasion Assay Kit purchased from CHEMICON International, Inc. (Temecula, CA).

Ⅲ. RESULTS

1. Anoikis in esophageal cancer cells.

To determine whether esophageal cancer cell lines are anoikis-sensitive or resistant, the cells were plated on the poly-HEMA coated tissue culture plates for 3, 24, and 48 h. Microscopic observations revealed that all the cell lines formed compact multi-cellular aggregates (MCA) by 16 h post-plating (Figure 1A). Lysates were prepared at each time points, and western blot analysis was performed using antibodies against PARP and procaspase-3. The processing of procaspase-3 and the cleavage of PARP were evident in TE2 and TE3 at 24 h after plating but not in the HCE4 and HCE7 cells until 48 h (Figure 1B, top). To confirm these results, cell viability assay was performed using SRB. The number of HCE4 or HCE7 cells was increased to 7.5 and 5 fold at 48 h after plating, respectively. In contrast, the number of TE2 or TE3 cells was decreased to 0.5 fold at same condition (Figure 1B, bottom). Taken together, the cell lines were grouped into anoikis-sensitive (TE2 and TE3) and anoikis-resistant (HCE4 and HCE7) lines. In the following experiments, TE2 cells were used as an anoikis-sensitive cell line and HCE4 cells were

used as an anoikis-resistant cell line.

2. E-cadherin is expressed only in anoikis-sensitive cell lines.

It has been hypothesized that there should be difference(s) in the expression level of cell adhesion molecules between anoikis-sensitive and resistant cell lines because anoikis was induced by detachment from the matrix. To determine which adhesion molecule(s) is differentially expressed between two cell lines, a cDNA microarray was performed using GEArray^{TM} Q series, Human Extracellular Matrix & Adhesion Molecules Gene Array which contains 96 genes encoding proteins important for the attachment of cells to their surroundings. It has been found that E-cadherin was expressed only in anoikis-sensitive TE2 cells (Figure 1C). The observation that both TE2 and HCE4 cells formed MCA (Figure 1A) suggested that other types of cell-cell adhesion molecule(s) might be involved in the cell-cell adhesion of HCE4 cells and, it has been found that N-cadherin instead of E-cadherin was expressed in HCE4 cells (Figure 1D). To rule out the possibility that the inability to detect E-cadherin in HCE4 might be due to the rapid degradation of E-cadherin, HCE4 cells were pre-treated with MG-132, a proteasome

inhibitor. Even after the MG-132 pre-treatment, E-cadherin was not detected in the HCE4 cells, indicating that epithelial E-cadherin was switched into mesenchymal N-cadherin in HCE4 cells (Figure 1D). In addition, the expression levels of keratin 10, involucrin, and vimentin were compared between TE2 and HCE4 cells. Keratin 10 and involucrin, both markers for terminal differentiation of epithelial cells, were expressed both in TE2 and HCE4 cells. Involucrin, however, was expressed at lower levels in HCE4 cells than in TE2 cells. Vimentin, a marker for mesenchymal cells, was expressed only in HCE4 cells (Figure 1D). These data indicated that HCE4 cells were dedifferentiated into mesenchymal cells. When the integrity of junctional complexes was examined, the N-cadherin expressed in HCE4 was linked to β-catenin (Figure 1E, HCE4; lane 6 and 8), as E-cadherin in TE2 cells (Figure 1E, TE2; lane 2 and 3). Taken together, these data indicated that when E-cadherin was switched into N-cadherin, the cells became resistant to anoikis.

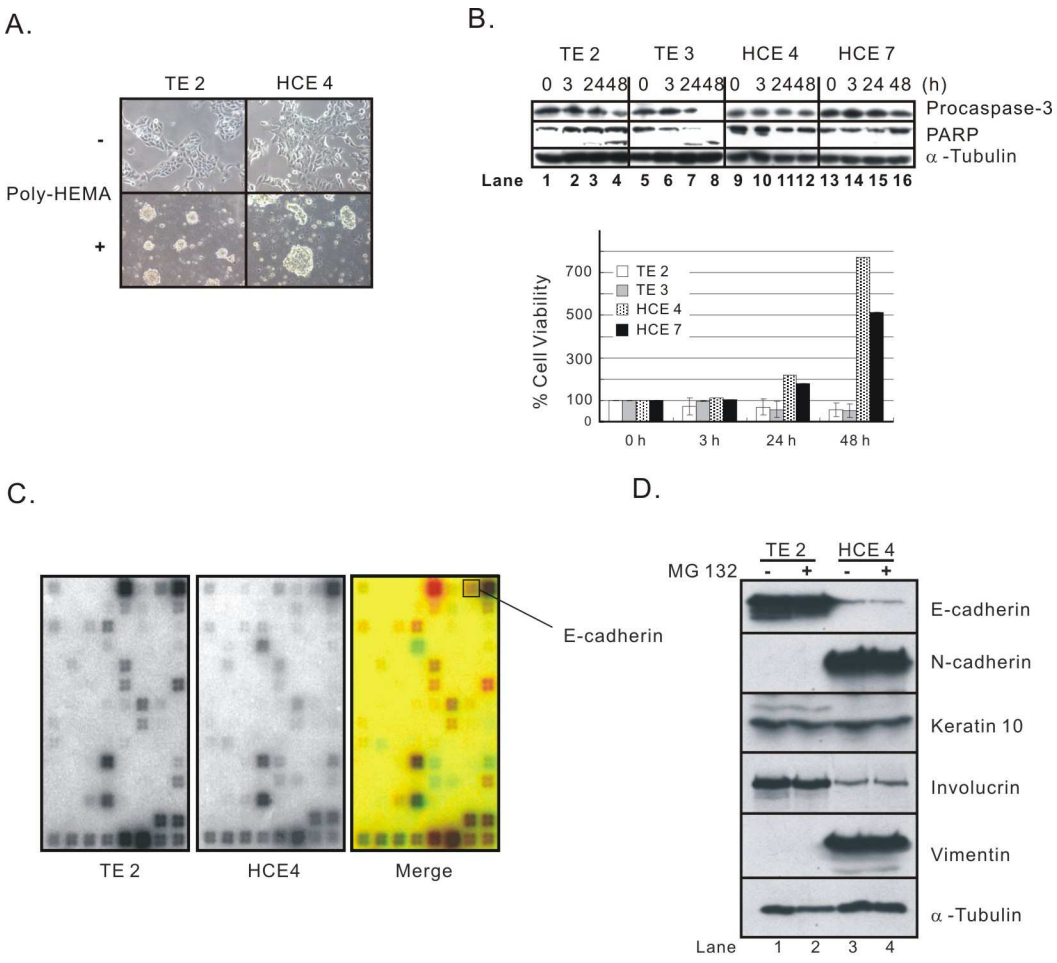

A.
TE 2
HCE 4
-
Poly-HEMA
+
B.
TE 2
TE 3
HCE 4
HCE 7
0 3 24 48 0 3 24 48 0 3 24 48 0 3 24 48 (h)
Procaspase-3
PARP
α -Tubulin
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
% Cell Viability
700
500
300
100
0
TE 2
TE 3
HCE 4
HCE 7
0 h
3 h
24 h
48 h
C.
E-cadherin
TE 2
HCE4
Merge
D.
MG 132
TE 2
HCE 4
- + - +
E-cadherin
N-cadherin
Keratin 10
Involucrin
Vimentin
α -Tubulin
Lane 1 2 3 4
E.
TE 2
HCE 4
G β E N G β E N
E-cadherin
N-cadherin
β-catenin
Lane 1 2 3 4 5 6 7 8

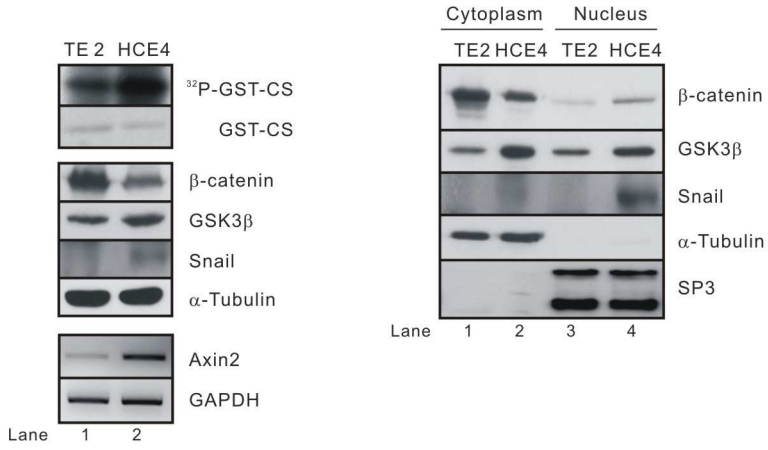
Figure 1. Anoikis in esophageal cancer cells and epithelial-mesenchymal transition in HCE4 cells. A, TE2 and HCE4 cells were plated on poly-HEMA-coated tissue culture plate. Twenty four hours after plating, the cells were observed using phase contrast microscope. TE2 and HCE4 cells formed compact MCAs. **B,** TE2, TE3, HCE4, and HCE7 cells ($1x10^6$ cells/well) were cultured on the poly-HEMA coated tissue culture plates for 3, 24, and 48 h. Cell lysates were prepared by directly adding 1xSDS loading buffer, and subjected to 12 % SDS-PAGE, and western blot analysis was performed using indicated antibodies (top). SRB cell viability was performed. The data was expressed as mean±SD for triplicate, and similar results were obtained from two independent experiments (bottom). **C,** cDNA microarray result using GEArray™ Q series, Human Extracellular Matrix & Adhesion Molecules Gene Array between TE2 and HCE4 cells. **D,** Western blot analysis was performed using indicated antibodies. The cells were treated or untreated with MG132 (10 μM), a proteasome inhibitor, for 6 h. **E,** N-cadherin formed complexes with β-catenin. The lysates from TE2 or HCE4 cells were immunoprecipitated with normal mouse IgG (G), anti-β-catenin (β), anti-E-cadherin (E), or anti-N-cadherin (N) antibody followed by western blot analysis using the antibodies indicated.

3. PKCK2 mediated up-regulation of Axin2 and stabilization of snail

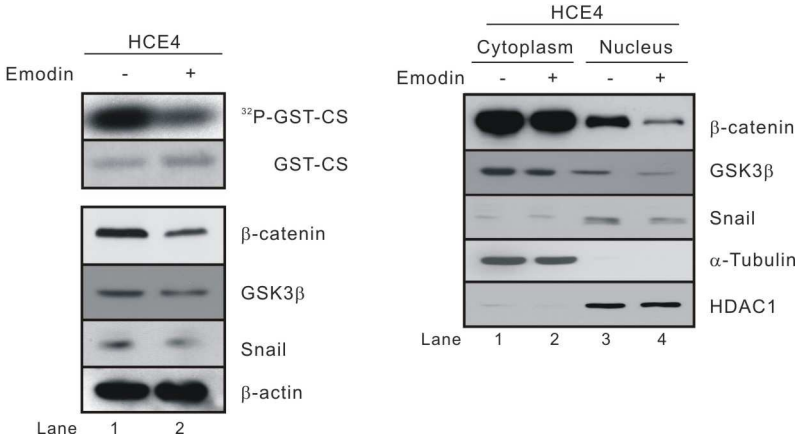
Recently it was reported that β-catenin-TCF-dependent signaling activates an EMT program in human breast cancer cells by inducing the expression of Axin2, thereby regulating nuclear GSK3β activity, the dominant kinase responsible for modulating the stability of the zinc finger transcription factor, Snail, a key regulator of E- to N-cadherin switching [32] and that inhibition of PKCK2 activity causes a reduction in LEF-1 transcriptional activity due to accelerated proteasome-dependent degradation of β-catenin [30]. Collectively, intracellular PKCK2 activity in cancer cells could stabilize snail and then, induce E-cadherin down-regulation. To substantiate this, it has been examined PKCK2 activity of N-cadherin expressing HCE4 cells and E-cadherin expressing TE2 cells. The result showed that PKCK2 activity was a lot higher in HCE4 than in TE2 cells (Figure 2A, left upper panel). Then, it has been checked the expression level of the molecules involved in Wnt signaling. As shown in Figure 2A, although total expression level of β-catenin was lower in HCE4 cells than that of TE2 cells, its nuclear expression level was higher than TE2 cells (left middle panel). Consistent with this, mRNA expression level of Axin2 was higher in HCE4 cells than that of TE2 cells (Figure 2A, left lower panel) and snail expression was only observed in HCE4 cells (Figure 2A). To confirm whether PKCK2 is

involved in the stabilization of snail and β-catenin, and the induction of Axin2 gene expression in HCE4 cells, HCE4 cells were treated with emodin, a PKCK2 inhibitor. When PKCK2 activity was inhibited (Figure 2B, left upper panel), the expression levels of β-catenin and snail were decreased (both in total and in nuclear fraction, Figure 2B). In addition, when the cells were transfected with either scrambled siRNA or siRNA against PKCK2 in the presence or absence of co-treatment of emodin, Axin2 mRNA level was markedly decreased (Figure 2C). The same results were reproduced when we compared anoikis sensitive MDA MB-231 that has low PKCK2 activity and anoikis resistant MDA MB-435 that has high PKCK2 activity (Figure 2D and E). Taken together, these results indicated that intra-cellular PKCK2 activity played important roles in E-cadherin down-regulation.

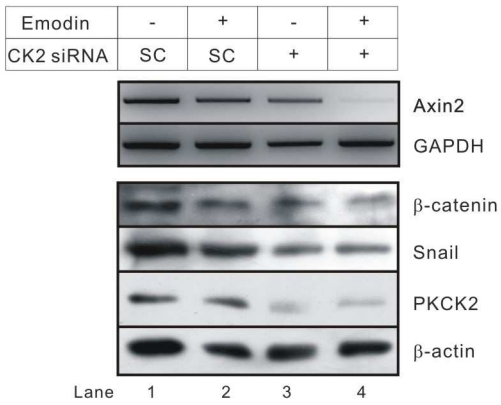
A.


TE 2 HCE4
32P-GST-CS
GST-CS
β-catenin
GSK3β
Snail
α-Tubulin
Axin2
GAPDH
Lane 1 2
Cytoplasm Nucleus
TE2 HCE4 TE2 HCE4
β-catenin
GSK3β
Snail
α-Tubulin
SP3
Lane 1 2 3 4

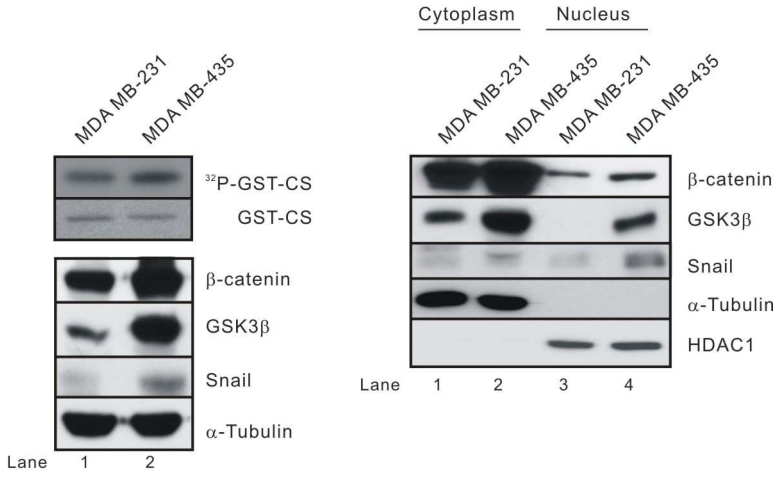
B.


HCE4
Emodin - +
32P-GST-CS
GST-CS
β-catenin
GSK3β
Snail
β-actin
Lane 1 2
HCE4
Cytoplasm Nucleus
Emodin - + - +
β-catenin
GSK3β
Snail
α-Tubulin
HDAC1
Lane 1 2 3 4

C.


Emodin - + - +
CK2 siRNA SC SC + +
Axin2
GAPDH
β-catenin
Snail
PKCK2
β-actin
Lane 1 2 3 4

D.


MDA MB-231
MDA MB-435
32P-GST-CS
GST-CS
β-catenin
GSK3β
Snail
α-Tubulin
Lane 1 2
Cytoplasm
Nucleus
MDA MB-231
MDA MB-435
MDA MB-231
MDA MB-435
β-catenin
GSK3β
Snail
α-Tubulin
HDAC1
Lane 1 2 3 4

E.

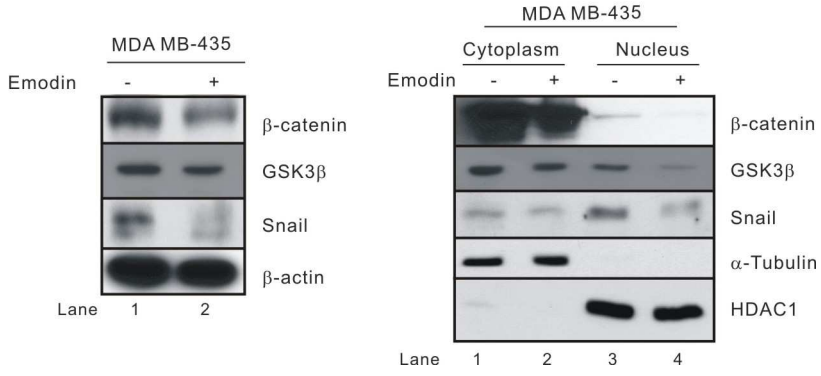

MDA MB-435
Emodin - +
β-catenin
GSK3β
Snail
β-actin
Lane 1 2
MDA MB-435
Cytoplasm
Nucleus
Emodin - + - +
β-catenin
GSK3β
Snail
α-Tubulin
HDAC1
Lane 1 2 3 4

Figure 2. PKCK2 involvement in E-cadherin down-regulation. Expression level of molecules involved in Wnt signaling. *In vitro* kinase assay was performed using lysates prepared from TE2 or HCE4 cells (**A,** left upper panel), or from MDA MB-231 or MDA MB-435 (**D,** left upper panel). GST-CS represents a GST-tagged recombinant CK2 substrate, and ^{32}P-GST-CS represents phosphorylated GST-CS (top). Coomassie blue staining of input GST-CS is shown (bottom). Western blot analysis was performed using total cell lysates (**A & D**; left, middle panel) or lysates from cytoplasmic or nuclear fraction (**A & D**; right panel). The indicated Abs were used. mRNA expression level of Axin2 in TE2 and HCE4 cells. RT-PCR was performed and GAPDH was used as an internal control. Effect of PKCK2 inhibition on the expression level of the molecules involved in Wnt signaling. HCE4 cells **(B)** or MDA MB-435 cells **(E)** were treated or untreated with emodin (20 μg/ml) for 24 h. *In vitro* kinase assay and western blot analysis were performed as described above. **C,** Effect of PKCK2 inhibition on the expression of Axin 2 mRNA. HCE4 cells were transfected with either scrambled siRNA or siRNA against PKCK2. After 48 h of transfection, the cells were treated or untreated with emodin for 24 h. RT-PCR was performed using cDNA generated form total RNA of each condition. Western blot analysis was also performed using lysates obtained from the cells treated with

the same condition as above.

4. Identification of a promoter region essential for N-cadherin gene expression in HCE4 cells.

Although a PKCK2 dependent down-regulation of E-cadherin was documented, the mechanism of N-cadherin up-regulation was still unknown. Accordingly, to examine whether there is an essential region in N-cadherin promoter for up-regulation of N-cadherin, N-cadherin promoter region (-1310 to +190) was cloned into pGL3 luciferase reporter plasmid (Figure 3). In order to localize a region (s) that was responsible for N-cadherin gene expression, 5' serial deletion of N-cadherin promoter was performed (Figure 4). The N-cadherin promoter activity was high until the 5' end of the promoter was deleted down to position -158 in N-cadherin expressing HCE4 cells (Figure 4B).

Figure 3. Nucleotide sequence of the human N-cadherin gene promoter region. The transcription start site was marked by +1 and binding sites for known transcription factors was shown in grey.

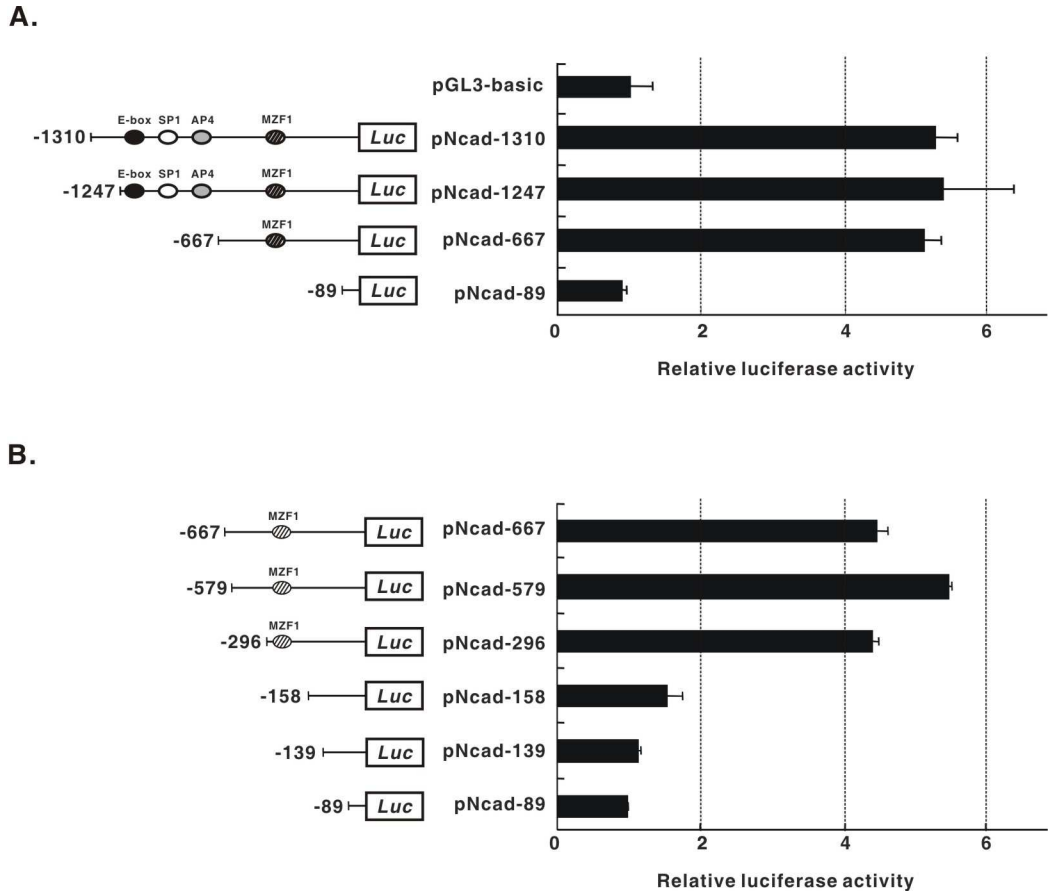
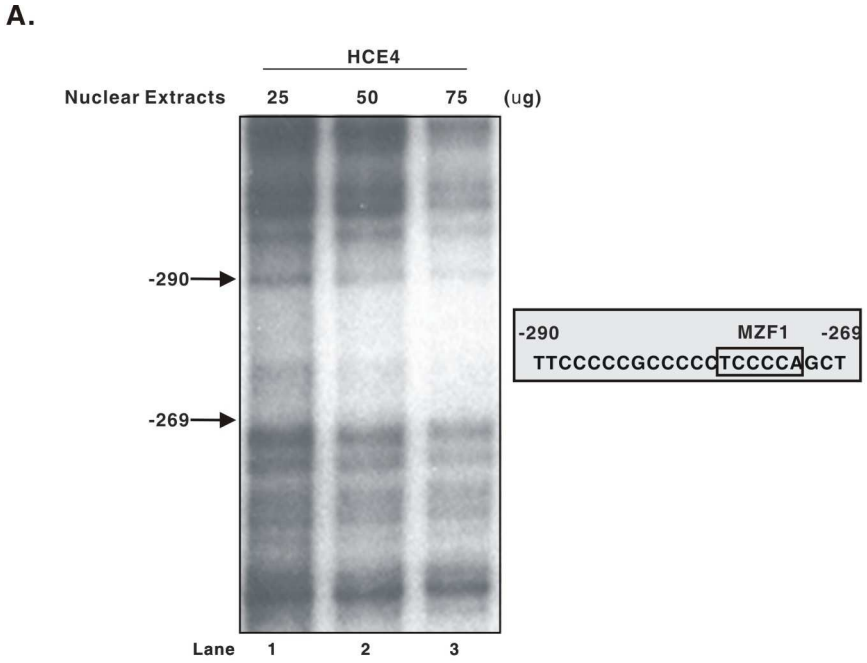


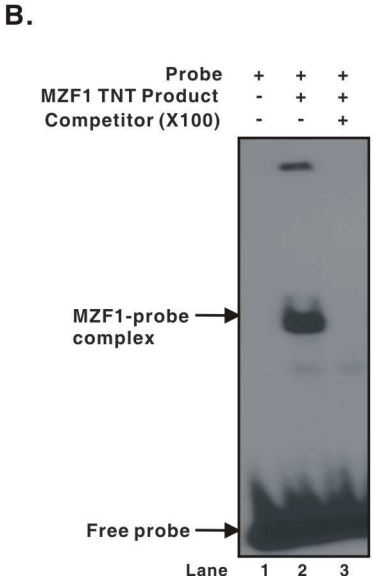
Figure 4. Identification of an essential promoter region required for N-cadherin gene expression. Luciferase reporter under the control of human N-cadherin promoter pNcad-1310 and its 5' serial deletion constructs were transfected into HCE4 cells. Normalized luciferase activities was shown as mean±SD of three independent experiments in a triplicate and was expressed as fold increase relative to the basal activity.

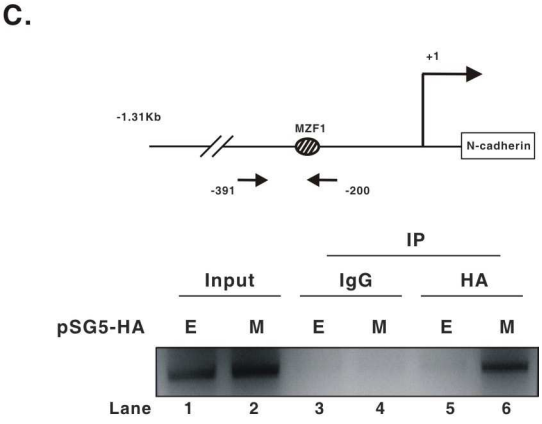
5. Identification of a putative transcription factor binding site in the human N-cadherin gene promoter

To locate a putative transcription factor binding site in the N-cadherin promoter, DNase I footprinting experiment was performed within the region -296 to -158. The experiment with nuclear extract prepared from HCE4 cells showed one protected region between -290 and -269 (Figure 5A). Computer analysis revealed that the site was compatible with the binding site for the Myeloid Zinc Finger-1 (MZF-1), that was characterized by a GA-rich binding core with the sequence TCCCCA (-277 to -272). To confirm the direct binding of MZF-1 to these sequences, EMSA was performed using the -290/-269 region of the N-cadherin gene as a probe. It has been prepared recombinant MZF-1 by *in vitro* transcription and translation. HA tag was fused to the N-terminal end of MZF-1. The results showed that MZF1 specifically bound to the probe (Figure 5B, lane 2). The addition of excess self-competitor to the reaction mixture competed with the binding of MZF-1 (Figure 5B, lane 3). To confirm the binding of MZF-1 to the N-cadherin promoter in HCE4 cells, the cells were transfected with pSG5-HA-MZF1 and then, ChIP assay was performed. Chromosomal DNA from HCE4 cells was cross-linked using formaldehyde, and an antibody specific to HA was used to immunoprecipitate the

chromatin fragment. A specific region of the N-cadherin promoter (-391/-200) was amplified by PCR (Figure 5C). To examine the effect of MZF1 on transcriptional activation of N-cadherin gene promoter, HEC4 cells were co-transfected with pSG5-HA-MZF1 and pNcad-667 or pNcad-139 reporter construct. When MZF1 was over-expressed, luciferase activity was 2.5 fold increased in the cells transfected with pNcad-667 but not in the cells with pNcad-139. Taken together, MZF1 bounds to TCCCCA core sequence (-277 to -272) of N-cadherin promoter and increased N-cadherin promoter activity.


A.
HCE4
Nuclear Extracts
25
50
75
(ug)
-290
-269
-290
MZF1
-269
TTCCCCCGCCCCCTCCCCAGCT
Lane
1
2
3


B.
Probe
MZF1 TNT Product
Competitor (X100)
MZF1-probe complex
Free probe
Lane
1
2
3


C.
+1
-1.31Kb
MZF1
N-cadherin
-391
-200
IP
Input
IgG
HA
pSG5-HA
E
M
E
M
E
M
Lane
1
2
3
4
5
6

D.

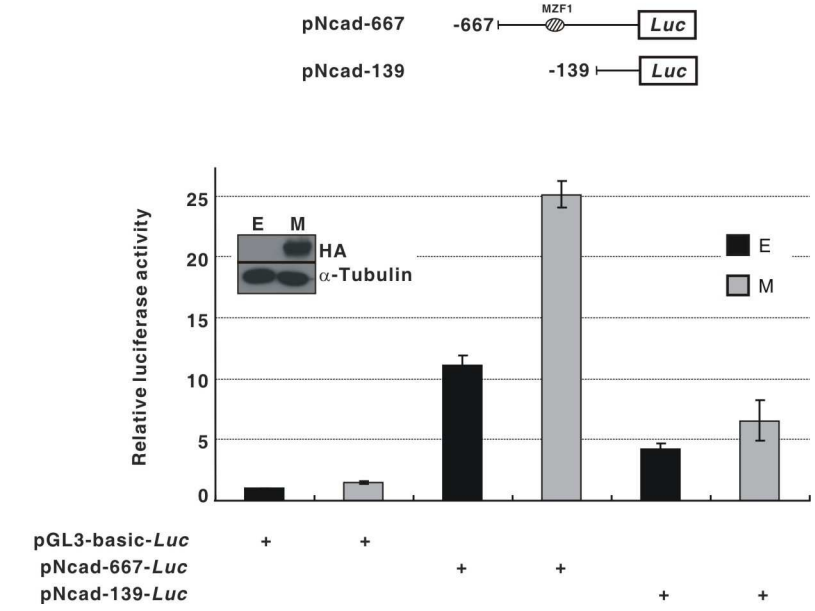


Figure 5. Regulation of N-cadherin promoter activity by MZF1 in HCE4 cells. A, DNase I footprinting assay with indicated amounts of nuclear extracts from HCE4 cells. The probes used were ^{32}P-end-labelled fragments of −296/−63. The protected region was indicated by position (-290/-269). The region contained MZF1 transcription factor binding site. **B**, Electrophoretic mobility shift assay. The assay was performed using HA-tagged *in vitro* transcription/translation product of MZF1 protein in 4% (wt/vol) nondenaturing polyacrylamide gel. Fifty thousand cpm (0.1 pmol) of ^{32}P-labeled N-cadherin promoter fragment (-290/-269) containing putative MZF1 binding site was used as a probe. **C,**

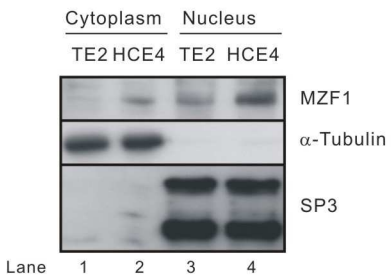
Schematic diagram of the N-cadherin promoter and the position of primers (denoted by arrows facing opposite directions above the promoter) used in the ChIP assay (top). HCE4 cells were transfected with an empty vector alone (pSG5-HA) (E) or a vector containing MZF1 cDNA (pSG5-HA-MZF1) (M). Cross-linked chromatin was prepared from HCE4 cells and then, immunoprecipitation was performed using indicated antibodies followed by PCR using primers described in the top (bottom). **D,** MZF1-mediated activation of N-cadherin promoter. HEC4 cells were co-transfected with pSG5-HA or pSG5-HA-MZF1, and pNcad-667 or pNcad-139 reporter construct. Normalized luciferase activities was shown as mean±SD of three independent experiments in a triplicate and was expressed as fold increase relative to the basal activity. The expression of HA-tagged MZF1 was shown in the *inset*. E and M represented pSG5-HA and pSG5-HA-MZF1, respectively.

6. Regulation of MZF1 expression by PKCK2.

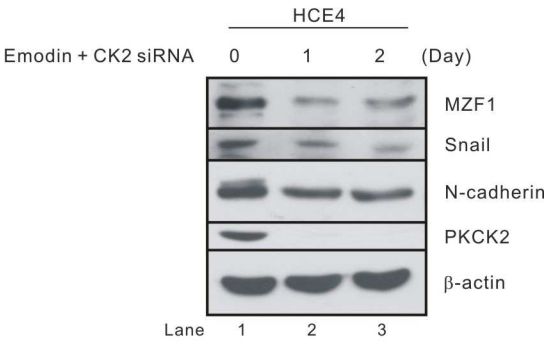
To examine whether the expression level of endogenous MZF1 is correlated with intracellular PKCK2 activity, cell fractionation was performed followed by western blot analysis using antibody against MZF1. The result showed that expression level of nuclear MZF1 in HCE4 cells was higher than that in TE2 cells (Figure 6A, lane 4). In addition, when PKCK2 activity was inhibited by the combination of emodin treatment and down-regulation of PKCK2 expression using siRNA (Figure 6B, PKCK2; lanes 2 and 3) , the expression of MZF1 was downregulated in HCE4 cells (Figure 6B, MZF1; lanes 2 and 3). Consistent with this, the expression level of N-cadherin was also down-regulated (Figure 6B, N-cadherin; lanes 2 and 3). In addition, when PKCK2 activity was inhibited by emodin treatment, the MZF1 binding to the N-cadherin promoter was decreased (Figure 6C, lane 5 vs. lane 6). Accordingly, then it has been examined whether PKCK2 phosphorylates MZF1. To test whether PKCK2 and MZF1 interact directly, HCE4 cells were transfected with HA-tagged MZF1. Immunoprecipitation followed by western blot analysis revealed that MZF1 interacts with PKCK2 (Figure 6D). Taken together, these results suggested that PKCK2 might phosphorylate MZF1 by direct interaction, thereby stabilizing MZF1 resulting in up-

regulation of N-cadherin gene expression.

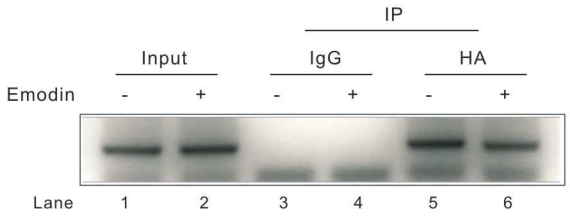
A.


Cytoplasm
Nucleus
TE2 HCE4 TE2 HCE4
MZF1
α-Tubulin
SP3
Lane 1 2 3 4

B.


HCE4
Emodin + CK2 siRNA 0 1 2 (Day)
MZF1
Snail
N-cadherin
PKCK2
β-actin
Lane 1 2 3

C.


IP
Input
IgG
HA
Emodin - + - + - +
Lane 1 2 3 4 5 6

D.

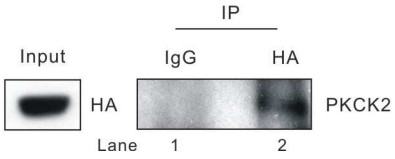

IP
Input
IgG
HA
HA
PKCK2
Lane 1 2

Figure 6. PKCK2-mediated stabilization of MZF1. A, Expression level of MZF1. Western blot analysis was performed using cytoplasmic and nuclear fraction of TE2 and HCE4 cells **B,** PKCK2 dependent stabilization of MZF1. HCE4 cells were transfected with PKCK2 siRNA for 1 or 2 days. Twelve hours before harvesting the siRNA transfected cells, emodin (20 μg/ml) was added. Lysates were prepared and western blot analysis was performed using antibodies indicated. **C,** Effect of PKCK2 activity on MZF1 binding to N-cadherin promoter. HCE4 cells were treated with emodin for 24 h. Cross-linked chromatin was prepared from HCE4 cells, immunoprecipitated using Abs indicated and then, PCR was performed. **D,** Direct interaction between MZF1 and PKCK2. 293A cells were transfected with pSG5-HA-MZF1, and then immunoprecipitation was performed using normal mouse IgG or anti-HA followed by western blot.

7. N-cadherin suppresses anoikis.

Although both TE2 and HCE4 cells formed MCA, only TE2 cells underwent apoptosis upon detachment. Therefore, it has been speculated that N-cadherin-generated survival signals might protect HCE4 cells from undergoing anoikis. To substantiate this, N-cadherin siRNA was used to knock-down endogenous N-cadherin. When N-cadherin was knocked-down (Figure 7A, left lower panel, N-cadherin; lane 2), detached HCE4 cells formed small clumps instead of compacted MCAs (Figure 7A, left upper panel; N), the cells underwent apoptosis (Figure 7A, left lower panel, PARP and Procaspase-3; lane 2). To investigate whether the cell death is caused by the disruption of N-cadherin mediated cell-cell adhesions or by the loss of survival signal(s) through N-cadherin knock-down, detached HCE4 cells were treated with EGTA to disrupt Ca^{2+} dependent N-cadherin mediated cell-cell adhesion. Although HCE4 cells could not form MCA in the presence of the EGTA treatment (Figure 7A, left middle panel), N-cadherin was still expressed (Figure 7A, left lower panel, N-cadherin; lane 3) and the cells did not undergo apoptosis (Figure 7A, left lower panel, PARP and Procaspase-3; lane 3). Consistent with these results, cell viability was decreased in the absence of N-cadherin (Figure 7A, right panel). Collectively,

these results indicated that anoikis resistance might result not from N-cadherin-mediated cell-cell adhesion but from N-cadherin generated survival signal(s). To examine whether the expression of N-cadherin suppresses anoikis in other cancer cell lines, it has been tested human breast cancer cell lines, MDA MB-231 and MDA MB-435. The results showed that N-cadherin was expressed only in MDA MB-435 cells and the cells were resistant to anoikis (Figure 7B). Taken together, N-cadherin protected cancer cells from undergoing anoikis.

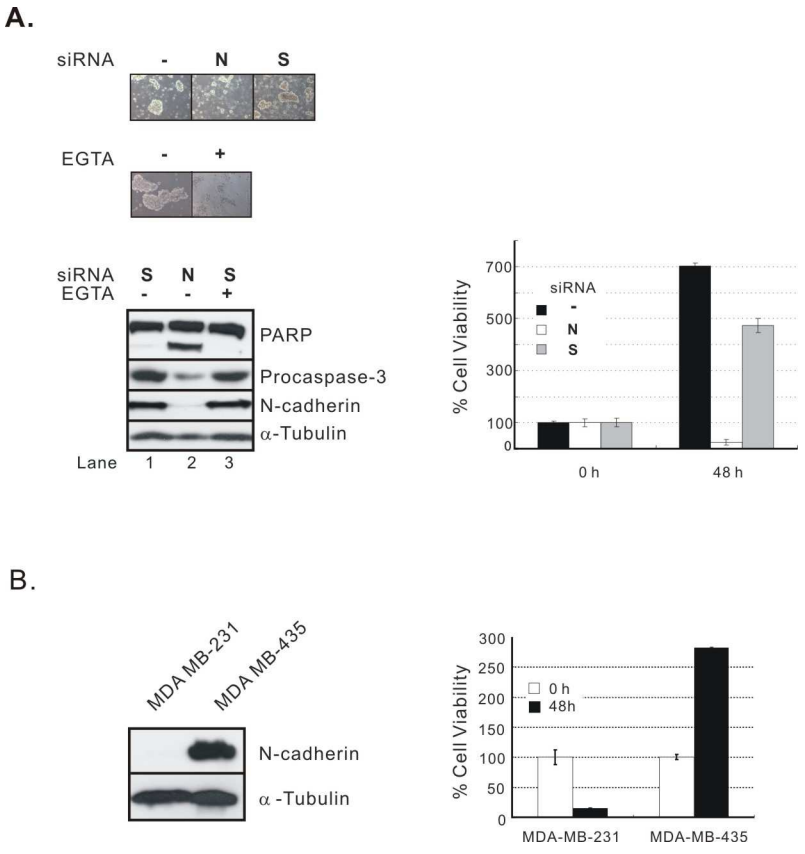


Figure 7. N-cadherin-mediated suppression of anoikis. A. Effect of N-cadherin expression on cell survival. HCE4 cells were transfected with either scrambled siRNA (S) or N-cadherin siRNA (N) for 48 h. The cells were then cultured on poly-HEMA-coated tissue culture plates for 36 h. Scrambled siRNA transfected HCE4 cells were plated on poly-HEMA-coated tissue culture plates and then subsequently treated or untreated with 2 mM EGTA for 12 h. The cells were observed using phase contrast microscope (left upper

and middle panels). Western blot analysis was performed using the antibodies indicated (left lower panel). SRB cell viability assay was performed. The data were expressed as mean±SD for triplicate, and similar results were obtained from two independent experiments (right panel). **B.** N-cadherin and anoikis in breast cancer cell lines. Western blot analysis was performed using lysates prepared form MDA MB-231 or MDA MB-435 cells (left). The cells ($1x10^6$ cells/well) were cultured on the poly-HEMA coated tissue culture plates for 48 h and then, SRB cell viability assay was performed. The data were expressed as mean±SD for triplicate, and similar results were obtained from two independent experiments (right).

8. PKCK2 regulates N-cadherin-mediated PKB/Akt signaling pathway through PTEN.

As PKB/Akt is a critical signal for cell survival, it has been checked the activity of PKB/Akt by documenting ser-473 phosphorylation of PKB/Akt [37-8]. Anoikis-resistant HCE4 cells showed higher PKB/Akt activity than anoikis-sensitive TE2 cells (Figure 8A). To examine whether PKB/Akt activity is critical for anoikis resistance of HCE4 cells, the cells were treated with a phosphatidylinositol 3-kinase (PI3-K) inhibitor, LY294002. When PKB/Akt was inhibited (Figure 8B, Akt-ⓟ; lanes 6-8), detached HCE4 cells became susceptible to anoikis (Figure 8B, Procaspase-3 and PARP; lanes 7 and 8). In addition, when N-cadherin was down-regulated by siRNA against N-cadherin (Figure 8C, N-cadherin; lane 2), PKB/Akt activity was also down-regulated (Figure 8C, Akt-ⓟ; lane 2). These results showed that N-cadherin activated PKB/Akt signaling, thereby protecting cancer cells from undergoing anoikis.

Previously, it was reported that PKCK2 mediated PTEN phosphorylation might inhibit its phosphatase activity [39-40]. Consistent with this report, when HCE4 cells were treated with emodin, PTEN phosphorylation was decreased (Figure 8D, PTEN-ⓟ; lane 2),

PKB/Akt activity was down-regulated (Figure 8D, Akt-ⓟ; lane 2) and then, the cells became to undergo apoptosis at 48 h after plating on poly-HEMA coated tissue culture plate (Figure 8D, Procaspase-3 and PARP, lane 2). Taken together, these results showed that PKB/Akt activation was a major survival signal for protecting cancer cells from undergoing anoikis and PKCK2 further activated PKB/Akt signal by phosphorylation mediated PTEN inhibition.

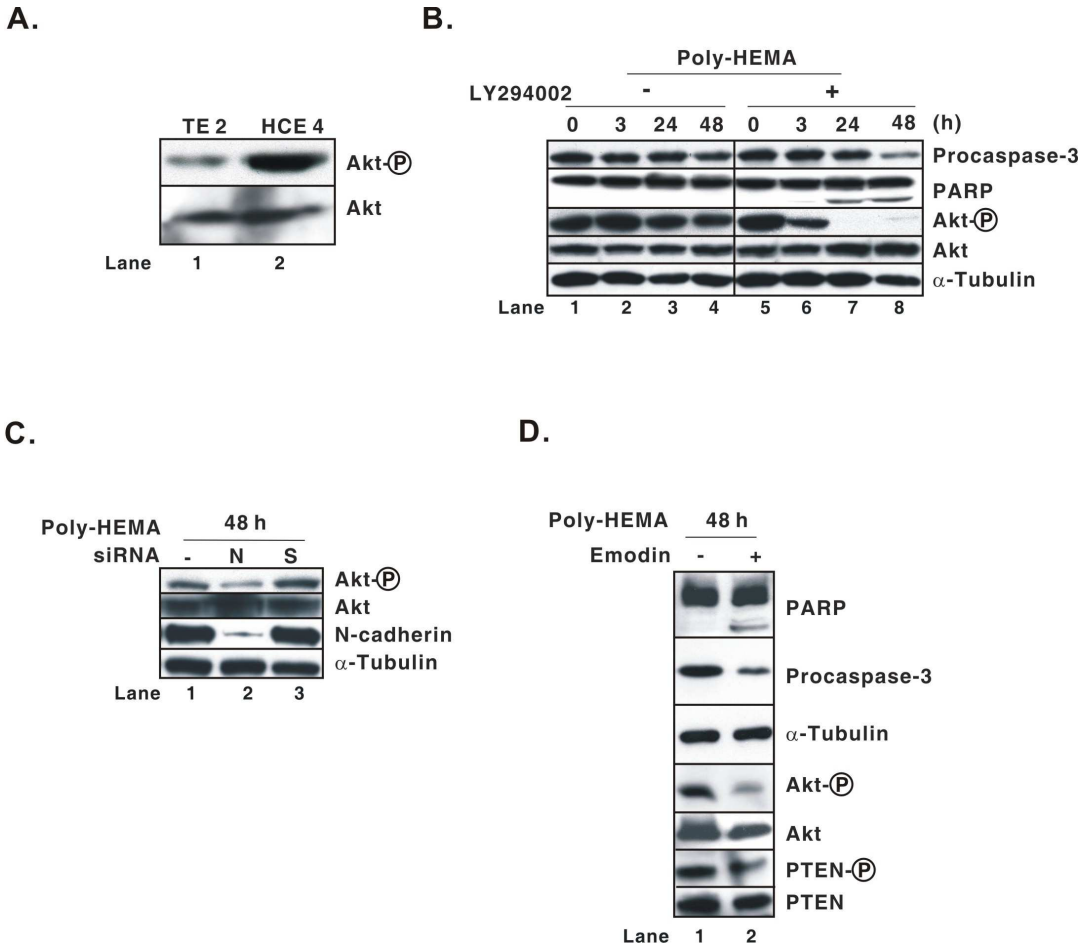


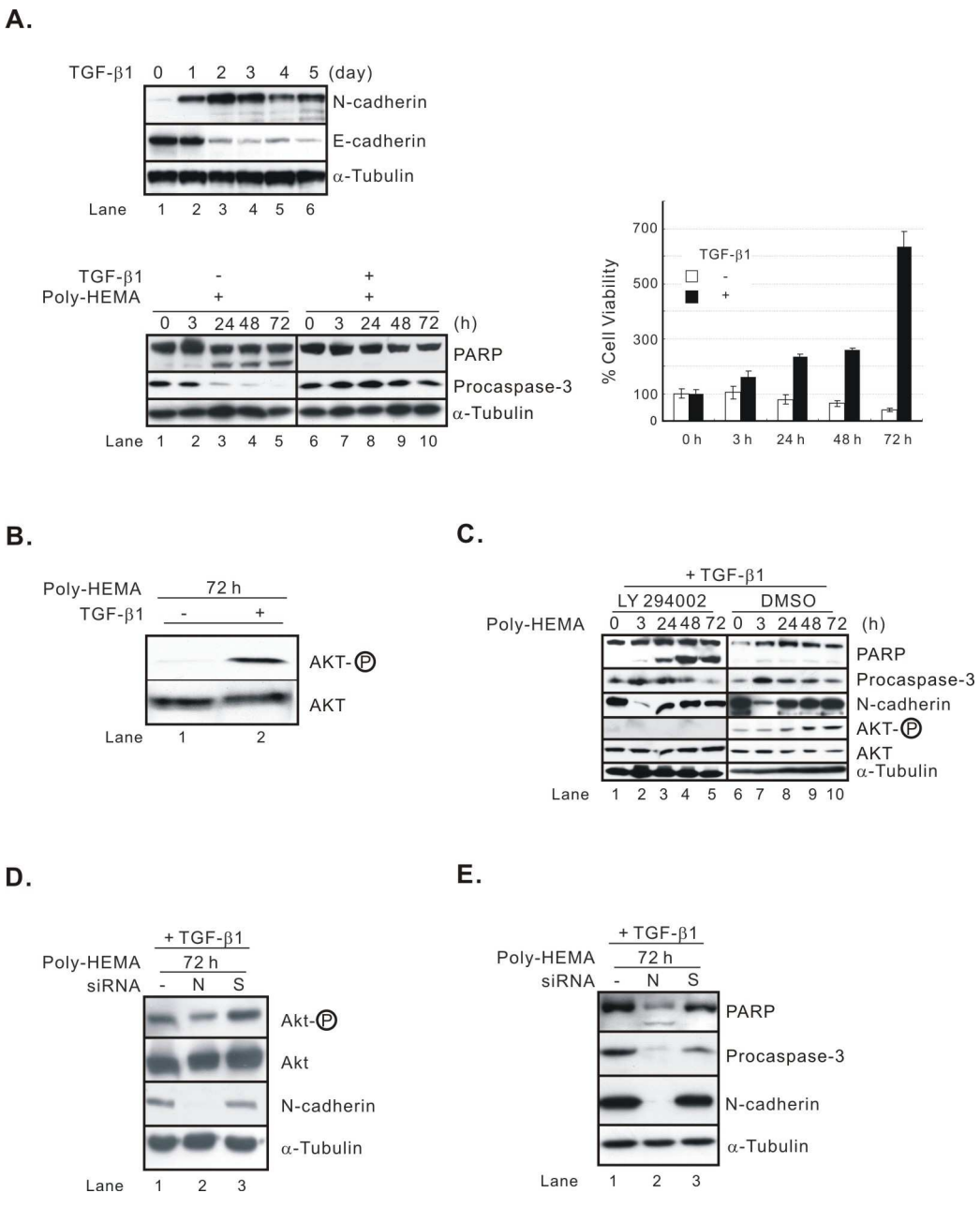
Figure 8. PKB/Akt activation by N-cadherin. A, N-cadherin dependent PKB/Akt activation. Cell lysates were prepared from TE2 and HCE4 cells and then western blot analysis was performed using antibody against phospho-Akt (ser 473, Akt-Ⓟ). The same blot was re-probed with anti-Akt antibody (Akt). **B,** PKB/Akt dependent protection from

anoikis. HCE4 cells were pre-treated or un-treated with LY294002 (25 μM) for 2 h and then subsequently cultured on poly-HEMA coated tissue culture plate for the indicated time points. Western blot analysis was performed using antibodies indicated. **C,** N-cadherin dependent activation of PKB/Akt. HCE4 cells were transfected with scrambled siRNA (S) or N-cadherin siRNA (N) for 48 h. The cells were then transferred to and cultured on poly-HEMA-coated tissue culture plates for 48 h. Western blot analysis was performed using the antibodies indicated. **D,** PTEN inactivation by PKCK2. HCE4 cells were pre-treated or un-treated with emodin for 2 h and then subsequently cultured on poly-HEMA coated tissue culture plate for 48 h. Western blot analysis was performed using antibodies indicated.

9. PKCK2 dependent E-to N-cadherin switching

To confirm the roles of the PKCK2 in E- to N-cadherin switching, thereby conferring anoikis resistance to cancer cells, it has been used A549, human lung cancer cell line because E- to N-cadherin switching by TGF-β1 has been well documented [41-3]. When A549 cells were treated with TGF-β1, N-cadherin replaced E-cadherin from 24 h after treatment (Figure 9A, left top). When N-cadherin replaced E-cadherin, the cells became resistant to anoikis (Figure 9A, left bottom; lanes 3-5 vs. lanes 8-10). SRB cell viability assay result also showed that the number of N-cadherin expressing A549 cells was increased to 6.2 fold at 72 h after plating on poly-HEMA coated plates but the number of E-cadherin expressing A549 cells was decreased to 0.3 fold (Figure 9A, right). Consistent with these results, PKB/Akt was activated by TGF-β1 treatment (Figure 9B). When PKB/Akt activity was inhibited by LY294002 treatment, the cells became susceptible to anoikis even in the presence of N-cadherin expression (Figure 9C, lanes 3-5 vs. lanes 8-10). There was no change in the expression level of N-cadherin after LY294002 treatment (Figure 9C). When N-cadherin was silenced by siRNA, PKB/Akt activation was decreased (Figure 9D, lane 2) and the cells underwent anoikis (Figure 9E, lane 2). To examine whether PKCK2 is involved in TGF-β1 mediated E-to N-cadherin switching, A549 cells

were pre-treated with emodin and then treated with TGF-β1. When the cells were treated with TGF-β1 alone, the cells became spindle shaped, but in the presence of emodin pre-treatment, the shape of the cells was not changed (Figure 9F, top). Consistent with this result, when emodin was pre-treated, TGF-β1 mediated E- to N-cadherin switching did not occur (Figure 9F, bottom). Taken together, these results indicated that PCKC2 was involved in E- to N-cadherin switching, thereby protecting cancer cells from undergoing anoikis.


A.
TGF-β1 0 1 2 3 4 5 (day)
N-cadherin
E-cadherin
α-Tubulin
Lane 1 2 3 4 5 6
TGF-β1 - +
Poly-HEMA + +
0 3 24 48 72 0 3 24 48 72 (h)
PARP
Procaspase-3
α-Tubulin
Lane 1 2 3 4 5 6 7 8 9 10
% Cell Viability
TGF-β1
-
+
0 h 3 h 24 h 48 h 72 h
B.
Poly-HEMA 72 h
TGF-β1 - +
AKT-Ⓟ
AKT
Lane 1 2
C.
+ TGF-β1
LY 294002 DMSO
Poly-HEMA 0 3 24 48 72 0 3 24 48 72 (h)
PARP
Procaspase-3
N-cadherin
AKT-Ⓟ
AKT
α-Tubulin
Lane 1 2 3 4 5 6 7 8 9 10
D.
+ TGF-β1
Poly-HEMA 72 h
siRNA - N S
Akt-Ⓟ
Akt
N-cadherin
α-Tubulin
Lane 1 2 3
E.
+ TGF-β1
Poly-HEMA 72 h
siRNA - N S
PARP
Procaspase-3
N-cadherin
α-Tubulin
Lane 1 2 3

F.

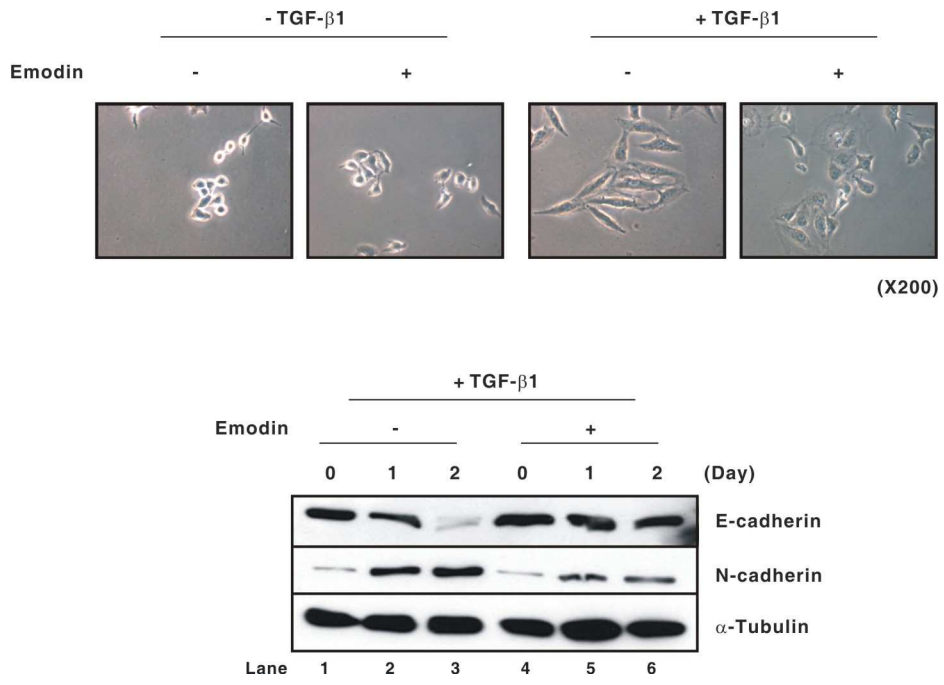


Figure 9. PKCK2 involvement in TGF-β1 mediated E- to N-cadherin switching in A549 lung cancer cell line. A. TGF-β1 mediated E- to N-cadherin switching and anoikis. A549 cells were treated with TGF-β1 (5 ng/ml) for the indicated time points and then western blot analysis was performed using antibodies indicated (left top). A549 cells were treated or un-treated with TGF-β1 for 2 days and then transferred to poly-HEMA coated tissue culture plates and cultured further for 72 h in the presence of TGF-β1. Western blot analysis was performed using antibodies indicated (left bottom). SRB cell viability of the

cells that were treated or untreated with TGF-β1 was performed. The data were expressed as mean±SD for triplicate, and similar results were obtained from two independent experiments (bottom). **B,** PKB/Akt activation and anoikis resistance. A549 cells were pre-treated or un-treated with TGF-β1 for 2 days and then subsequently cultured on poly-HEMA coated tissue culture plate for 72 h. Cell lysates were prepared and then western blot analysis was performed using antibody against phospho-Akt (ser 473, Akt-Ⓟ). The same blot was re-probed with anti-Akt antibody (Akt). **C,** A549 cells were pre-treated with TGF-β1 for 2 days and then treated or un-treated with LY294002 for 2 h. The cells were then transferred to and cultured on poly-HEMA coated tissue culture plate for the indicated time points in the presence of TGF-β1 and LY294002. Western blot analysis was performed using antibodies indicated. **D, E,** N-cadherin mediated PKB/Akt activation and anoikis resistance. A549 cells were pre-treated with TGF-β1 for 2 days and then transfected with either scrambled siRNA (S) or N-cadherin siRNA (N) for 48 h. The cells were then transferred to and cultured on ploy-HEMA-coated tissue culture plates for 72 h. Western blot analysis was performed using antibodies indicated. **F,** PKCK2 involvement in E- to N-cadherin switching. A549 cells were pretreated or untreated TGF-β1 for 1 h and then treated or untreated with emodin for 1 and 2 days. The cells were observed using

phase contrast microscope (top). Cell lysates were prepared from the same condition as described in **A.** Western blot analysis was performed using antibodies indicated (bottom).

10. PKCK2, N-cadherin, and Invasion

Previously it was reported that N-cadherin becomes up-regulated in invasive tumor cell lines and in tissues from the breast [44], prostate [19], and melanomas [45]. It has also been suggested that PKB/Akt enhances the invasiveness of pancreatic carcinoma cells via up-regulation of IGF-I [46] or via the increased secretion of matrix metalloproteinases-2 and -9 from immortalized mammary epithelial cells and ovarian carcinomas [47]. Consistent with these findings, N-cadherin expressing HCE4 cells in this study showed higher invasive ability than TE2 cells (Figure 10A) and the knock-down of N-cadherin by siRNA decreased invasiveness of HCE4 cells (Figure 10B).

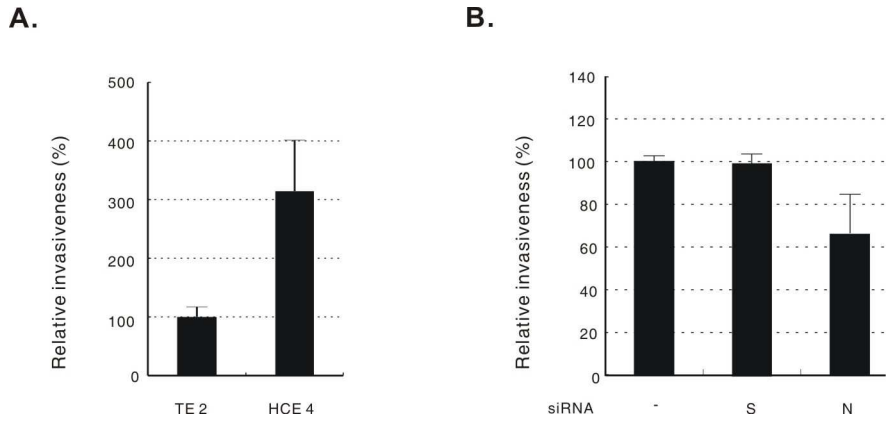


Figure 10. N-cadherin-mediated promotion of invasion. A, Invasiveness of TE2 and HCE4 cells was measured by Cell Invasion Assay Kit (CHEMICON).B. HCE4 cells were transfected with scrambled siRNA (S) or N-cadherin siRNA (N) for 72 h. Cell invasion assay was performed. Experiments were done in triplicate (mean value ± SD), and similar results were obtained from two independent experiments.

Ⅳ. DISCUSSION

The disruption of integrin-extracellular matrix interactions in normal epithelial cells induces anoikis. The acquisition of anoikis-resistance in cancer cells is regarded as a critical step for metastasis. The E- to N- cadherin switching enables tumor cells to invade, migrate, and metastasize and N-cadherin confers anoikis resistance to cancer cells. Little was know about the mechanism of E- to N-cadherin switching, the regulatory mechanism of N-cadherin gene expression, and role (s) of N-cadherin in anoikis resistance. In the study presented here that intracellular PKCK2 activity in cancer cells regulated E- to N-cadherin switching, N-cadherin up-regulation resulting in increasing PKB/Akt activity, thereby conferring cancer cells anoikis resistance.

The activation of the canonical Wnt signaling pathway can stabilize snail, an E-cadherin repressor through β-catenin/Axin2/GSK3β cascade [32] and the signaling can be potentiated by PKCK2 mediated β-catenin phosphorylation at Thr 393, leading to proteasome resistance and increased protein and co-transcriptional activity [30-1]. PKCK2 is frequently activated in human cancers and can induce mammary tumors and lymphomas when expressed in transgenic mice [48-50]. Consistent with these reports, the expression levels

of nuclear β-catenin, Axin2, and snail in cancer cells that have high PKCK2 activity were higher than in cancer cells that have low PKCK2 activity (Figure 2) and E-cadherin was down-regulated (Figure 1 C & D). However, when PKCK2 was inhibited, those increased expressions were reversed (Figure 2B, C, & E). Accordingly, high PKCK2 activity in cancer cells could mimic the constitutive activation of canonical Wnt signaling. Previous report showed that N-cadherin-mediated stabilization of fibroblast growth factor-1 (FGFR-1) constitutively activates ERK resulting in phosphorylation of E-cadherin. Phosphorylated E-cadherin may then recruit Hakai, an E3 ligase, that targets E-cadherin for degradation to the proteasome pathway by ubiquitination [51]. However, it has been showed that even in the presence of MG132, a proteasome inhibitor, E-cadherin was not observed in N-cadherin expressing HCE4 cells (Figure 1D, lane 4).

While E-cadherin is down-regulated, why N-cadherin is up-regulated? Thus, in the present study, it has been searched and found a MZF1 binding site in N-cadherin gene promoter that was responsible for the gene expression. Previously, it was reported that MZF1 is an important transcription factor regulating N-cadherin promoter activity and expression in osteoblasts [52]. MZF-1 DNA-binding consensus sequences are 5'-TGGGGA-3' [53] and the sequence is also found in N-cadherin promoter (-150/-145) and was reported

to be important for N-cadherin gene expression in osteoblasts [52]. However, in the present study, the MZF1 binding site found in N-cadherin promoter was located at (-277/-272) and it was inverted oriented (5'-TCCCCA-3'). Based on reporter assay (Figure 5A), the MZF1 binding site found in this study was a main MZF1 binding site in cancer cells. Since it has been documented PKCK2 involvement in E-cadherin down-regulation in cancer cells that have high PKCK2 activity, the next procedure was to question whether PKCK2 is also involved in MZF1 regulation. It has been found that there was a direct interaction between PKCK2 and MZF1 (Figure 6D), that MZF1 was highly expressed in cancer cells that have higher PKCK2 activity (Figure 6A), and that MZF1 expression was down-regulated by PKCK2 inhibition suggesting that PKCK2 might stabilize MZF1 by phosphorylation (Figure 6B).

When E-cadherin was switched into N-cadherin, the cancer cells became resistant anoikis (Figure 1, 7, and 8). Consistent with previous reports [18, 54], N-cadherin could activate PKB/Akt, thereby conferring anoikis resistance to cancer cells (Figure 8 and 9). N-cadherin expression itself could activate PKB/Akt and EGTA-mediated disruption of N-cadherin-mediated cell-cell adhesion did not alter anoikis resistance of cancer cells (Figure 7A). PTEN regulates cancer cell survival by inhibiting PI3-kinase-PKB/Akt dependent

signaling via its phosphatidylinositol (3,4,5)P_3 phosphatase activity [39]. PKCK2 negatively regulates PTEN activity by phosphorylation at Ser 370 and 385 [40, 55-6]. Consistent with these, PKCK2 inhibition could restore PTEN activity by de-phosphorylation, and thus, PKB/Akt became inactivated, and then, the cells became anoikis sensitive (Figure 8D).

So far, in the study demonstrated here that PKCK2 involvement in E-cadherin down-regulation, N-cadherin up-regulation, and activation of PKB/Akt via PTEN using different cancer cell lines. Therefore, A549, human lung cancer cell line was used because TGF-β1-mediated E- to N-cadherin switching has been well documented [41-2, 57]. As expected, E- to N-cadherin switching was observed by TGF-β1 treatment (Figure 9A). With this cadherin switching, PKB/Akt was activated (Figure 9B) and the cells became resistant to anoikis (Figure 9A). When PKCK2 was inhibited, this cadherin switching was inhibited confirming that PKCK2 activity was required for E-to N-cadherin switching (Figure 9F).

N-cadherin switching could give cancer cells favor(s) for tumor-host cell adhesion, tumor cell invasion, and migration [18-9]. Consistent with these, N-cadherin expressing cancer cells were highly invasive than E-cadherin expressing ones (Figure 10).

Taken together, it has been showed that PKCK2 played critical roles in E-cadherin

down-regulation, N-cadherin up-regulation, and PKB/Akt activation, thereby acquiring anoikis resistance. Therefore, down-regulation of PKCK2 activity in cancer cells could reduce cancer invasion and metastasis.

Ⅴ.CONCLUSION

The E- to N-switching of cadherin subtypes may affect tumor-host cell adhesion, tumor cell invasion, and migration. Normally, the disruption of integrin-extracellular matrix interactions in epithelial cells induces anoikis and thus, the acquisition of anoikis-resistance is regarded as a critical step for cancer metastasis. However, little was known about the mechanism of E- to N- cadherin switching, regulatory mechanism of N-cadherin gene expression, role (s) of N-cadherin in anoikis resistance. In the present study, it has been reported that intracellular PKCK2 activity was involved in E-to N-cadherin switching by modulating PKCK2 mediated β-catenin stabilization. Stabilized nuclear β-catenin in turn could stabilize E-cadherin repressor, Snail, thereby repression of E-cadherin in cancer cell lines. PKCK2 also stabilized myeloid zinc finger transcription factor, MZF-1, thereby stimulating N-cadherin gene expression. Expressed N-cadherin could activate PKB/Akt and PKCK2 could inhibit PTEN by phosphorylation, thereby, augmenting PKB/Akt activation. In summary, intracellular high PKCK2 activity could confer anoikis resistance to cancer cells.

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ABSTRACT (in Korean)

Protein Kinase Casein Kinase 2에 의한 Anoikis 조절 기작

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고 현 석

암세포의 발달 과정 중에 일어나는 E-cadherin 에서 N-cadherin 으로의 대체 발현은 암세포의 이동과 침윤을 유도해서 결국에는 전이가 되게 한다. 정상 상피 세포에서 integrin-세포기질 사이의 결합 억제는 anoikis 를 유도한다. Anoikis 에 대한 저항성 획득은 암세포의 전이 과정에서 중요한 단계로 알려져 있다. 높은 protein kinase casein kinase 2 (PKCK2) 활성도는 인간의 암세포에서 자주 발견되며, 세포의 성장과 자가 사멸과 같은 다양한 세포 활성을 조절한다. 따라서, 본 연구에서는 E-cadherin 에서 N-

cadherin 으로의 발현 변화 기작과 N-cadherin 유전자 발현의 조절 기작과 anoikis 저항성 획득에서 N-cadherin 의 역할에 대해 연구했다. 먼저 anoikis 에 민감한 식도암 세포주인 TE2 와 TE3 세포주는 E-cadherin 을 발현하지만 anoikis 에 저항성을 획득한 식도암 세포주인 HCE4 와 HCE7 세포주는 N-cadherin 을 발현하는 것을 알 수가 있었다. HCE4 세포주에서 E-cadherin 의 발현 감소는 높은 PKCK2 활성도에 의한 β-catenin/Axin2/GSK3β 신호 전달 기작을 통해서 E-cadherin 의 전사 억제 인자인 snail 의 안정성을 증가시킴으로써 유도될 것이라고 추측할 수 있었다. 또한 N-cadherin 유전자 발현 조절 기작을 밝혀내기 위해 N-cadherin 프로모터를 연구한 결과, 프로모터 활성을 담당하는 부위를 찾았고 그 부위에 결합하는 전사 활성인자인 myeloid zinc finger 1 (MZF1)이 N-cadherin 의 프로모터 활성도를 높인다는 것을 알 수 있었다. 뿐만 아니라 MZF1 는 PKCK2 에 의해 인산화 되며 발현이 조절됐다. 다음으로, anoikis 저항성 획득에서 N-cadherin 의 역할을 살펴보았다. N-cadherin 에 의한 anoikis 저항성 획득은 PKB/Akt 활성화에 의해 매개되었고, anoikis 에 대한 저항성을 획득한 암세포에 N-cadherin siRNA 를 통해 N-cadherin 발현을 억제한 결과 PKB/Akt 활성도는 감소하였고 동시에 anoikis 에 민감하게 변화되는 것을 알

수 있었다. 또한 높은 PKCK2 활성도에 의한 PTEN 단백질의 비활성화는 PKB/Akt 활성도를 유지시켜서 anoikis 에 대한 저항성 획득을 가능하게 하였다. 이런 결과들은 transforming growth factor-β 1 (TGF-β1)에 의해 E-cadherin 에서 N-cadherin 으로 발현이 대체되는 폐암세포주인 A549 세포주에서 다시 확인할 수 있었다. TGF-β1 가 전 처치 되지 않았을 경우 A549 세포주는 E-cadherin 을 발현하며 anoikis 에 대해 민감했지만, TGFβ1 에 의해 N-cadherin 이 발현되는 A549 세포주는 PKB/Akt 활성도가 증가 되었고 동시에 anoikis 에 대한 저항성을 획득했다. A549 세포주의 anoikis 저항성 획득에서 N-cadherin 의 역할은 N-cadherin siRNA 를 통해 다시 한번 확인하였다. 뿐만 아니라 TGF-β1 에 의한 E-cadherin 에서 N-cadherin 으로의 대체 발현이 PKCK2 활성도에 의해 조절된다는 것을 알 수 있었다.

이런 결과들로 미루어 볼 때 PKCK2 는 암세포에서 일어나는 E-cadherin 에서 N-cadherin 으로의 대체 발현뿐 아니라 전이 과정에서 중요한 단계인 anoikis 에 대한 저항성 획득을 조절한다는 것을 알 수 있었다.

핵심되는 말 : PKCK2, cadherin 대체 발현, β-catenin/Axin2/GSK3β 신호 전달 기작, anoikis, MZF1