Effect of PKCK2 mediated Snail phsphorylation on Wnt signaling

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Effect of PKCK2 mediated Snail phsphorylation on Wnt signaling

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The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

Youngkyung Kim

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This certifies that the Master's Thesis of Hyangtae Choi is approved.

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The Graduate School

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YoungKyung Kim

Table of Contents

LIST OF FIGURES

LIST OF TABLE

ABSTRACT

Effect of PKCK2 mediated Snail phosphorylation on Wnt signaling

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Protein kinase casein kinase 2 (PKCK2) is a serine/threonine kinase that has been known to play important roles in cell cycle control, cellular differentiation, and proliferation. Recently, PKCK2 is overexpressed in many types of human cancer. It is a positive regulator in Wnt signaling pathway, since the Wnt signaling pathway intermediate Dishevelled, β-catenin, and LZTS2 are a PKCK2 phosphorylation substrates and activating the transcription of Wnt target genes.

The zinc finger transcription factor, Snail, functions as a potent repressor of E-cadherin expression. Snail is phosphorylated by GSK3-β, resulting in β-TrCP-mediated ubiquitination and proteasomal degradation. Axin2 levels increase

in response to Wnt signaling, GSK3-β is exported from the nuclear compartment leaving Snail in its non-phosphorylated, transcriptionally active form. Thus, stabilization of Snail repressed E-cadherin expression is required for cell migration, invasion, and metastasis. However, little is known about PKCK2 mediated regulation of Snail.

This study presented the identification of Snail as a novel binding protein to PKCK2, the role of PKCK2-mediated phosphorylation on Snail, and its effect on Wnt signaling. The interaction between Snail and PKCK2 was initially identified by a yeast two-hybrid screening and was further confirmed by GST pull down and co-immunoprecipitation. Mutational analysis and *in vitro* kinase assay indicate that Snail contains multiple PKCK2 phosphorylation sites. In addition, it is demonstrated that PKCK2 stabilizes Snail by phosphrylation. PKCK2 induced an accumulation of Snail in the nucleus which further bloked phosphorylation by GSK3-β and snail active form repressed the E-cadherin level. Further, PKCK2 activity is required for the regulation of Snail-mediated Wnt signaling pathway.

Taken together, PKCK2 regulates the Snail stability through its phosphorylation, thereby modulates E-cadherin mediated transcriptional activity.

Key words: PKCK2, Wnt signaling, Snail, E-cadherin, GSK3-β, phosphorylation.

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Ⅰ. INTRODUCTION

Wnt signaling has central roles in a number of developmental processes and constitutively active Wnt signaling can lead to cancer formation[1, 2]. In the canonical Wnt signaling pathway, Wnt ligands bind to a co-receptor complex consisting of a Frizzled receptor and one of the LDL receptor-related proteins (LRPs), LRP5 or LRP6[3]. This is followed by the recruitment of Axin to the plasma membrane, resulting in the stabilization of β-catenin[4]. Stabilized β-catenin accumulates in the nucleus and complex with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of DNA-binding transcription factors to enhance expression of genes that regulate cell fate, polarity, and proliferation[5, 6].

Protein kinase casein kinase 2 (PKCK2) is a constitutively active, growth factor-independent serine/threonine protein kinase with key roles in cell cycle control, cellular differentiation, proliferation, and regulation of apoptosis[7-9]. These multiple roles stem from a complex structure, with two catalytic α and two regulatory β subunits existing as $\alpha_2\beta_2$, $\alpha\alpha`\beta_2$, or $\alpha`_2\beta_2$ configurations[10]. A variety of human cancers show changes in PKCK2 expression or activity[9, 11-13]. Recently, it was reported that PKCK2 confers TRAIL resistance to cancer cells through procaspase-2 phosphorylation[9], and that PKCK2 inhibition may enhance the cytotoxicity of natural killer (NK) cells[14]. PKCK2 positively modulates Wnt/β-catenin by β-catenin phosphorylation at Thr 393, which inhibits proteasomal degradation and increases the translocation and co-transcriptioal activity of β-catenin[15,16].

Epithelial to mesenchymal transition (EMT) is a process whereby cells lose cell - cell interactions and other epithelial properties whilst acquiring a more migratory and mesenchymal phenotype. EMT occurs at several stages of early development and an EMT like process is thought to occur during tumor progression[17-20]. A hallmark of EMTs is the functional loss of E-cadherin (CDH1) which mediates cell-cell interactions. Snail also regulates the expression of different target genes involved in EMT and other functions, like cell survival[21]. Importantly, Snail has been shown to be upregulated in various tumor types[22] and has more recently been observed expressed at the tumor stromal interface[23].

To date, several studies have examined the post-translational modifications that control Snail cell function. Snail consists of four C-terminal DNA binding zinc fingers and a regulatory region spanning amino acid 1-150[24] comprising an N-terminal SNAG (SNAIL and Gfi conserved) domain (1-9 amino acids) important for co-repressor interaction[25], and the Serine Rich Domain (SRD) which appears to control the function of a short leucine rich nuclear export sequence (NES) recognized by the CRM1 transporter[26-27]. The SRD was important for subcellular localization and that mutation of all serines to alanine residues in that region resulted in cytoplasmic localization of Snail, and consequent repressive dysfunction[26]. Additionally, separate two investigations[27-28] identified a short sequence in the SRD important for Snail post-translational regulation. This short sequence is conserved in several proteins, including β-catenin and IκB, and is termed destruction box (DB). The DB in the case of β-catenin contains two serines, the phosphorylation of which is critical for recognition and ubiquitination by the β-TrCP ubiquitin ligase [5]. This leads to proteasomal degradation and thereby constitutes an important shared control mechanism for the government of several transcriptional regulators simultaneously.

In the present study, it was examined whether Snail is a subatrate of PKCK2 and investigated the role of PKCK2-mediated phosphorylation on Snail, and its effect on Wnt signaling. This study demonstrates that PKCK2 binds to Snail directly *in vitro* and *in vivo*, and phosphorylates sites on Snail. Importantly, PKCK2-mediated

phosphrylation regulates stability of Snail in a proteasome dependent manner. Also, the Snail was phosphorylated by PKCK2 could block phosphorylation of GSK3-β and ubiqutin degradation via β-TrCP. As well as the snail phosphorylation by PKCK2 was repressed the E-cadherin level, thereby PKCK2-mediated Snail regulates modulating Wnt signaling pathway.

Ⅱ. MATERIALS AND METHODS

1. Cell culture and reagents

MCF7, human breast cancer cell line and HEK293, human embryonic kidney cell line, human esophageal cancer cell line , HCE4, were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100μg/ml streptomycin (Gibco-BRL) and 100units/ml penicillin (Gibco-BRL). MG-132 was purchased from Calbiochem (Darmstadt, Germany), prepared in 10mM stock in dimethyl sulfoxide (DMSO) (Sigma- Aldrich, St, Louis, MO, USA). The PKCK2 inhibitors, tetrabromobenzotriazole (TBB) was prepared in 50mM stock using DMSO (Sigma-Aldrich). Control cultures received the same amounts of DMSO, and final DMSO concentrations did not exceed 0.1%

2. Western blot analysis

Cells were lysed in RIPA buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, and 0.25% sodium deoxycholate]. Lysates were incubated for 15 min at 4℃ and cleared by centrifugation at 12,000 rpm for 15 min at 4℃. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amount of proteins was subjected to electrophoresis on SDS-polyacrylamide rels and then transferred to polyvinylidene fluoride (PVDF)

membrane (Millipore, Billerca, MA, USA). The blotted membranes were blocked in Tris-buffer (pH 7.4) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich) and 5% (w/v) nonfat Difco™ skim milk (BD Biosciences, Sparks, MD, USA) and probed with primary antibodies. The following antibodies were used : HDAC1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), α-tubulin (Oncogene Science, Cambridge, MA, USA), PKCK2 (Upstate, Charlottesville, VA, USA), Flag tag (Sigma-Aldrich), β-actin (Sigma-Aldrich). Secondary antibodies were anti-rabbit IgG peroxidase (Themo scientific, Rockford, IL, USA) and anti-mouse IgG horseradish peroxidase (Thermo scientific). The signals were developed by a standard enhanced chemiluminescence (ECL) method according to the manufacture's protocol (Roche, Indianapolis, IN, USA)

3. Immunoprecipitation (IP)

Cells were lysed in a buffer composed of 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM sodium orthovanadate, 1mM PMSF, and protease inhibitors. After centrifugation, 500 μg of the clarified cell lysate was pre-cleared with G plus/protein A-agarose (Santa Cruz Biotechnology Inc.), by incubating 1 h. The supernatant was collected and 2 μg of anti-Flag, anti-PKCK2 antibody were added. After overnight incubation, 50 μl of 50% slurry of G plus/protein A-agarose was added and the mixture was incubated for 2 h. The agarose bead was centrifuged, washed three times with ice-cold lysis buffer and two times with ice-cold TBS,

suspended in electrophoresis sample buffer, and boiled for 5 min. The immunoprecipitated protein was further analyzed by Western blotting. Non-immune mouse IgG or non-immune rabbit IgG (Santa Cruz Biotechnology Inc.) served as a negative control.

4. GST fusion proteins purification

After transforming *E. coli* (BL21-DE3) with GST-PKCK2α expression constructs, fusion protein expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma-Aldrich) for 6 h at 30℃. The cells were lysad by sonication in lysis buffer containing 1% triton X-100 and 1mM PMSF in phosphate-buffered saline (PBS) (Gibco-BRL). The GST-tagged recombinant proteins were purified using Glutathione Sepharose^{TM}4B (GE Healthcare Bio Science AB, Temecula, CA, USA) column chromatography. The fusion protein was eluted using elution buffer containing 10 mM glutathione, and was dialysed against PBS containing 10% glycerol (BIO BAGICS INC., Markham, Canada).

5. GST pull-down assay

Tree micrograms of GST or GST-PKCK2α was immobilized on 20 μl of Glutathione Sepharose^{TM}4B resin, and then incubated with cell lysates of the Flag-Snail-transfected HEK293 overnight at 4℃. After washing with PBS buffer,

the bound proteins were eluted by heating at 100℃ for 5 min with 2X Laemmli sample buffer and resolved by 12% SDS-PAGE, then detected by Western blotting. As a control, GST and GST-PKCK2α were shown by Coomassie Blue staining.

6. Histidine (His)-tagged Snail proteins purification in *E.coli*

The constructed pET-28a-His-Snail expression plasmids were transformed into *E. coli* BL21/pG-Tf2 (TaKaRa, Shiga, Japan). Protein expression was induced by the addition of 5 ng/ml tetracycline (tet) (Sigma-Aldrich) before the target protein induction by 0.5 mM IPTG (Sigma-Aldrich) for 24 h at 25°C in LB media containing 20 g/ml chloramphenicol and 25 g/ml kanamycin. Harvested cells were resuspended in 40 ml of lysis buffer [(50 mM NaH_2PO_4 (pH 7.5), 500 mM NaCl, 5 mM imidazole)], and sonicatded. The cell lysate was spun at 12,000 rpm for 30 min. The supernatant was applied by gravity Ni-NTA agarose column (Qiagen, Valencia, CA, USA). The column was washed with wash buffer [(50 mM NaH_2PO_4 (pH 7.5), 500 mM NaCl, 50 mM imidazole)], and then eluted with elution buffer [(50mM NaH_2PO_4 (pH 7.5), 500 mM NaCl, 300 mM imidazole)]. The elution was dialyzed against PBS containing 10% glycerol at 4 °C.

7. ***In vitro* kinase assay**

PKCK2 *in vitro* kinase activity assay was carried out as described previously[18]. Briefly, 3 μg of bacterially expressed GST-CS (CK2 substrate) proteins were

incubated with glutathione sepharose 4B beads for 1 hr, then they were washed twice using 1x kinase buffer [4 mM MOPS, pH 7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 μM sodium orthovanadate, and 200 μM DTT]. Then the beads were incubated either with 100 ng recombinant active PKCK2 (ATGen) or 100 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 20 min at 30°C. Reactions were stopped by washing three times using 1x kinase buffer. Samples were resuspended with 30 μl of 2x SDS sample loading buffer and subjected to SDS-PAGE followed by staining with coomassie brilliant blue and autoradiography.

8. Site-directed mutagenesis of Snail

To identify the site of PKCK2 phosphorylation in Snail, mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA). All mutant constructs were confirmed by DNA sequencing. For generation of the mutants the following mutagenetic primer pairs were used.

Table1. Primer pairs for mutagenesis

Mutants Name		Oligonucleotide sequence
T89A	Sense	5' GCAGAGCTGGCCTCCCTGTCAGATGAG 3'
	Antisense	5' CTCATCTGACAGGGAGGCCAGCTCTGC 3'
S90A	Sense	5' GCAGAGCTGACCGCCCTGTCAGATGAG 3'
	Antisense	5' CTCATCTGACAGGGCGGTCAGCTCTGC 3'
S92A	Sense	5' GCAGAGCTGACCTCCCTGGCCGATGAG 3'
	Antisense	5' CTCATCGGCCAGGGAGGTCAGCTCTGC 3'
S96A	Sense	5' TCAGATGAGGACGCCGGGAAAGGCTCC 3'
	Antisense	5' GGATCCTTTCCCGGCGTCCTCATCTGA 3'
T89AS90A	Sense	5' GCAGAGCTGGCCGCCCTGTCAGATGAG 3'
	Antisense	5' CTCATCTGACAGGGCGGCCAGCTCTGC 3'
S92E	Sense	5' GCAGAGCTGACCTCCCTGGAAGATGAG 3'
	Antisense	5' CTCATCTTCCAGGGAGGTCAGCTCTGC 3'
T89AS92E	Sense	5' GCAGAGCTGGCCTCCCTGGAAGATGAG 3'
	Antisense	5' CTCATCTTCCAGGGAGGCCAGCTCTGC 3'
S90AS92E	Sense	5' GCAGAGCTGACCGCCCTGGAAGATGAG 3'
	Antisense	5' CTCATCTTCCAGGGCGGTCAGCTCTGC 3'
T89AS90AS92E	Sense	5' GCAGAGCTGGCCGCCCTGGAAGATGAG 3'
	Antisense	5' CTCATCTTCCAGGGCGGCCAGCTCTGC 3'

9. Isolation of total RNA and reverse transcription polymerases chain reaction (RT-PCR/Real time PCR)

Total RNA was extracted using TRIZOL reagent® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First strand cDNA was synthesized from 2 μg of total RNA in a 20 μl volume using random hexamers and

SuperScript® Ⅱ Reverse Transcriptase (Invitrogen), followed by PCR using primers as follows. E-cadherin mRNA level as assessed by SYBR Green Real time-PCR in MCF7 cells. GAPDH was co-amplified for an internal control.

Ⅲ. RESULTS

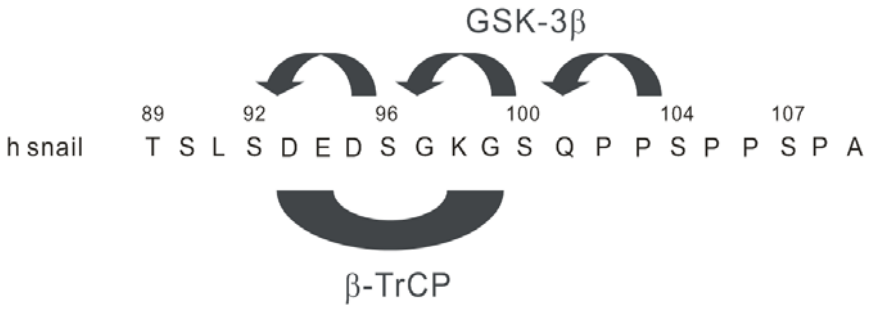
1. Phosphorylation of Snail

The human Snail amino acid sequence contains in excess of 20 serine and/or threonine residues. Of note, inspection of this region of the Snail protein revealed PKCK2α consensus motifs (S/T-X–X–D/E). Further, this region of the Snail protein also contained the DSGXXS destruction motif recognized by β-TrCP Consistent with the possibility that Snail is one of PKCK2α substrate, *in vitro* kinase assay was performed. HEK293 cells were transfected with Snail-Flag and lysates were prepared and were immunoprecipitated using ant-Flag antibody, and then immunoprecipitated A/G agarose beads were incubated with recombinant active PKCK2α and ^{32}P-labeled ATP. It was found that PKCK2α phosphorylates Snail-Flag. To examine whether the PKCK2α phosphorylates Snail specifically, the HEK293 cells transfected with Snail-Flag were treated with emodin, the well-known cell-permeable, selective, and fairly potent PKCK2 inhibitor and found that phosphorylation of Snail was strongly inhibited in the presence of emodin (Fig. 1A). To determine phosphorylation site(s) in Snail by PKCK2, amino acid 1-155 region of Snail was expressed in bacteria and purified. Snail contained serine or threonine residue within consensus motifs was mutated to uncharged, non-phosphorylatable alanine residue (T89A, S90A, S92A, S96A, T89AS90A) and each mutated constructs were expressed and purified. PKCK2 *in vitro* kinase assay

showed that serine-92 residue was directly phosphorylated by PKCK2 (Fig. 1A). Though studies Snail S92 was phosphorylated by PKCK2 (Fig. 1A). To test whether phosphorylation of Snail at S92 could result in Snail phosphorylation, a mutant Snail with a mimetic of a phosphate group and a mutant that cannot be phosphorylated were used. To mimic the effect of an acidic phosphate group at S92, the serine was substituted with an aspartic acid (92E). As a results PKCK2 *in vitro* kinase assay showed that S92E was phosphorylated by PKCK2 (Fig. 1B). Snail has phosphorylation of adjacent serine or threonine residue in the consensus sequence would impact on the phosphorylation of PKCK2. To confirm additional phosphorylation sites of Snail immediately adjacent to the S92 was mutated (T89A, S90A), preserved in S92E, and performed PKCK2 *in vitro* kinase assay. As a result, the mutant constructs (T89AS92E, S90AS92E, T89AS90AS92E) were not phosphorylated by PKCK2. These results indicate that, while S92 was phosphorylated additional phosphorylation of Snail was simultaneously phosphorylated by PKCK2 (Fig. 1B). To confirm the interaction between PKCK2α and Snail, *in vitro* binding assays were performed using recombinant proteins. The Snail-Flag was transfected into HEK293 cell and the lysate was assessed for its ability to form a complex with GST-PKCK2α or GST alone. When GST-PKCK2α or GST protein was pull-downed by glutathione-Sepharose beads, Snail was not observed with GST but with GST-PKCK2α (Fig. 1C). To examine whether PKCK2α interacts with Snail *in vivo*, HEK293 cells were co-transfected with both

HA-PKCK2α and Snail-Flag. After immunoprecipitation using anti-CK2α antibody, Snail was detected in the immunoprecipitates (Fig. 1C). Taken together, these results indicate that PKCK2α directly and specifically binds to Snail *in vitro* and *in vivo*.

A.


GSK-3β
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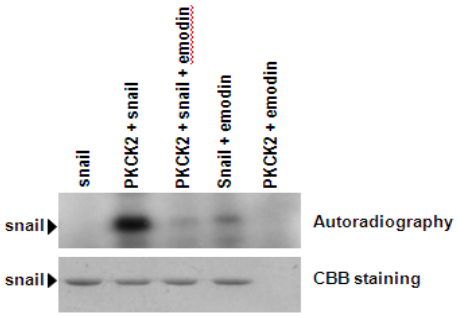
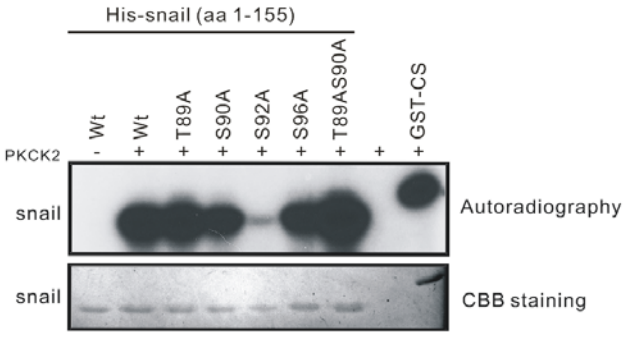
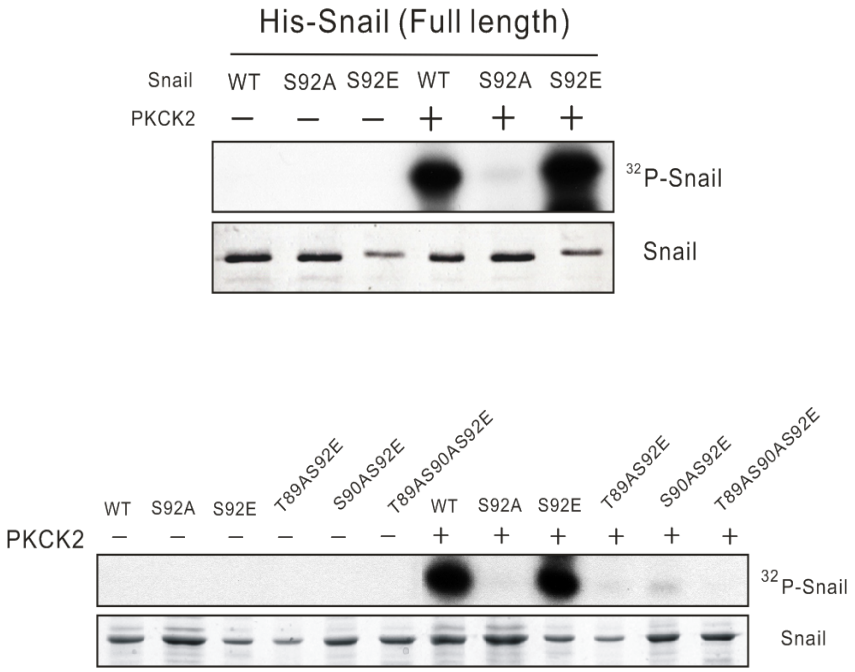
92
96
100
104
107
h snail
T S L S D E D S G K G S Q P P S P P S P A
β-TrCP


snail
PKCK2 + snail
PKCK2 + snail + emodin
Snail + emodin
PKCK2 + emodin
snail
Autoradiography
snail
CBB staining


His-snail (aa 1-155)
Wt
Wt
T89A
S90A
S92A
S96A
T89AS90A
GST-CS
PKCK2
-
+
+
+
+
+
+
+
snail
Autoradiography
snail
CBB staining

B.



C.

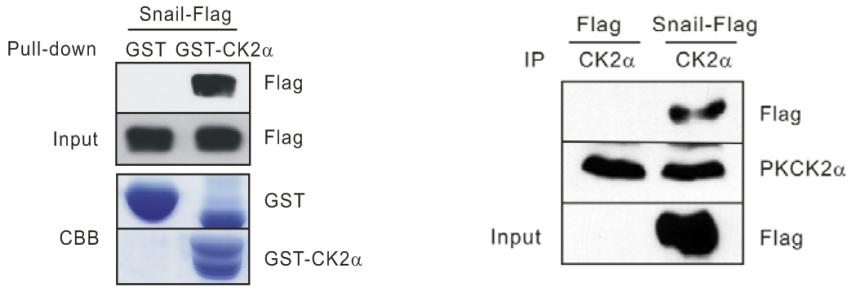


Figure 1. Snail phosphorylation by PKCK2. A. Sequence alignment of human Snail. Highlights are shown for conserved PKCK2 consensus motif. HEK293 cells were transfected with Snail-Flag and then, treated or untreated with emodin.

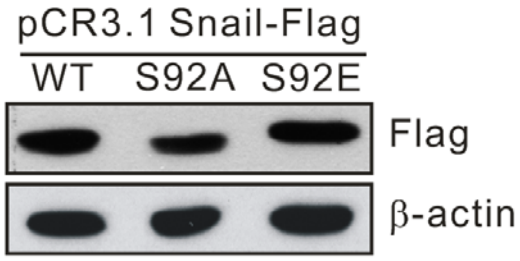
Immunoprecipitation was performed using anti-Flag antibody followed by *in vitro* kinase assay. Autoradiographic result showed that Snail was phosphorylated by PKCK2 in the absence of emodin treatment. The expression control was given Snail-Flag. His-tagged recombinant Snail (a,a1-155) wild type and mutants were expressed in *E.coli* and were performed PKCK2 *in vitro* kinase assay. Snail serine-92 is specifically phosphorylated by PKCK2α. The expression and loading controls were given His-Snail. B. The expression control was given Snail-Flag. His-tagged recombinant Snail (full-length) wild type and mutants were expressed in *E.coli* and were performed PKCK2 *in vitro* kinase assay. The expression and loading controls were given His-Snail. C. Bacterially expressed GST-PKCK2 was incubated with 1 mg of lysates prepared from HEK293 that was transfected with Snail-Flag. After pulling-down, the presence of Snail-Flag was documented by western analysis using anti-Flag antibody (*Upper*). Western results obtained from total cell lysates were given in '*Input*' to provide expression control (Flag). CBB represents expressed GST and GST-PKCK2 stained with Coomassie blue. HEK293 cells were co-transfected with Snail-Flag and HA-PKCK2α, and the cell lysates were subjected to immunoprecipitation with anti CK2α-antibody. Co-immunoprecipitated Snail (detected with anti-Flag antibody) is shown. The expression control (Flag) is given in the Input. The anti-rabbit IgG was used for a negative control.

2. Snail stabilization by PKCK2-mediated phosphorylation

To investigate the physiological role(s) of PKCK2-mediated phosphorylation on Snail, it was examinated whether stability of Snail protein is dependent on PKCK2 activity. Western analysis using lysates prepared from the MCF7 cell that had been transfected with Snail-Flag. To test whether phosphorylation of Snail at S92 could result in increased Snail stability, a mutant Snail with a mimetic of a phosphate group and a mutant that cannot be phosphorylated were used. To mimic the effect of an acidic phosphate group at S92, the serine was substituted with an aspartic acid (92E). Snail generated the catalytically non-phosphorylation by PKCK2 mutants S92A. Snail S92A mutant was expressed at reduced level in comparison with the wild-type Snail but S92E was increased stability of Snail (Fig. 2A). To confirm the physiological role(s) of PKCK2-mediated additional phosphorylation on Snail, it was examinated whether stability of Snail protein is dependent on PKCK2 activity. Western analysis using lysates prepared from the MCF7 cell that had been transfected with Snail-Flag (wild-type, mutants). Snail mutants (T89AS92E, S90AS92E, T89AS90AS92E) were significantly reduced the stability of Snail in comparison with the Snail S92E (Fig.2B). As well as additional phosphorylation sites of Snail was mutated to phopho-mimic status (T89ES92E, S90ES92E, T89ES90ES92E) were higher stability than T89AS90AS92E. In other words, the addition of phosphate groups on T89 and S90 would have enhanced the Snail stability (Fig 2B). To determine which phosphorylation site(s) is important for the

stability of Snail, MCF7 cells were transiently transfected with Snail-Flag and HA-PKCK2. Western analysis revealed that Snail stability of wild-type was increased when PKCK2 was over-expressed. However, the expression levels of Snail S92 mutants (S92A, S92E) were maintained without major changes. To determine which phosphorylation site(s) is important for the stability of Snail, MCF7 cells were transiently transfected with Snail-Flag and HA-PKCK2. Western analysis revealed that Snail stability of wild-type was increased when PKCK2 was over-expressed. However, the expression level of Snail S92 mutants (S92A, S92E) were maintained without major changes (Fig. 2C). To further confirm that PKCK2 phosphorylation may stabilize Snail, western analysis using lysates prepared from the MCF7 cells that had been transfected with Snail-Flag in the presence or absence of PKCK2 inhibitor, TBB showed that when PKCK2 activity was inhibited, the stability of exogenous Snail wild-type was markedly decreased. However, mutants level of Snail remained unchanged in the same condition (Fig. 2D). These results suggest that the decrease of Snail stability by PKCK2 inhibitor might be determined at post-translational level.

A,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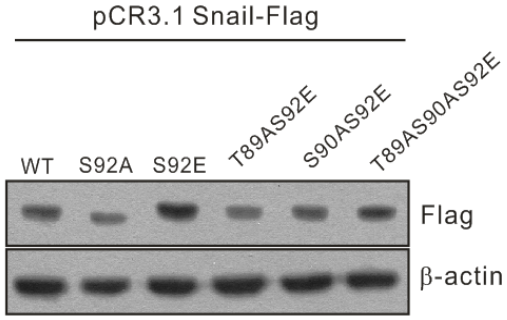
WT
S92A
S92E
Flag
β-actin

B.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S92E
T89AS92E
S90AS92E
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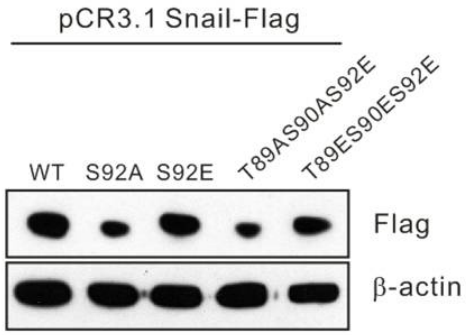
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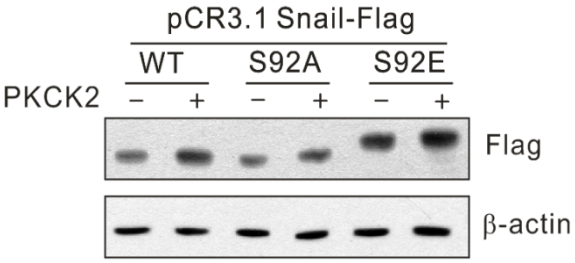
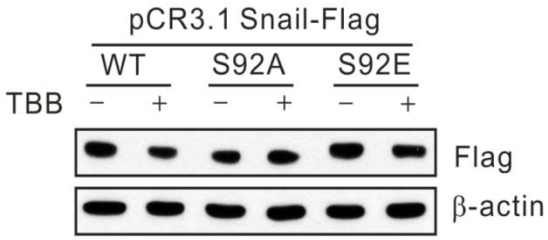
S92A
S92E
T89AS90AS92E
T89ES90ES92E
Flag
β-actin

C.



D.



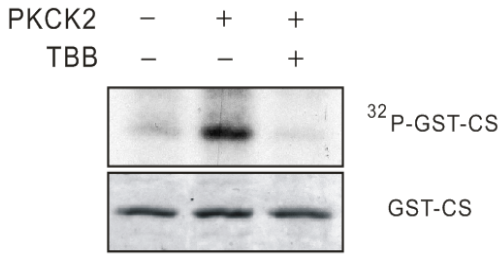


Figure 2. Snail stabilization by PKCK2-mediated phosphorylation. A. B. Western blot analysis results. MCF7 cells were transfected with Flag tagged

wild-type (WT) or indicated mutant Snail constructs. Equal amounts of cell lysates were blotted for anti-Flag and β-actin. C. MCF7 cells transfected with Snail- Flag wild-type (WT) and mutants (S92A, S92E) were transfected or not transfected with HA-PKCK2α. D. MCF7 cells transfected with Snail-Flag wild-type (WT) and mutants (S92A, S92E) were treated or untreated with TBB(100μM) for 6 hours after which cells were collected for lysate preparation. Equal amounts of cell lysates were blotted for anti-Flag and β-actin.

3. PKCK2-mediated phosphorylation of Snail Ubiquitin dependent degradation of Snail

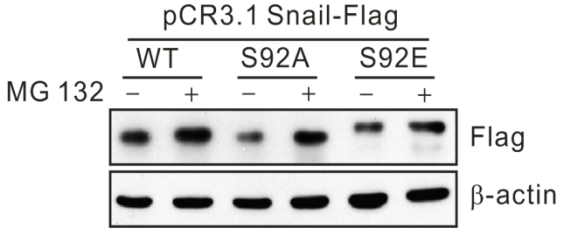
The Snail transcript encodes a series of β-catenin-like canonical motifs that support its GSK3-β dependent phosphorylation, β-TrCP-directed ubiquitination, and proteasomal degradation via a PKCK2-mediated phosphorylation of Snail. Since PKCK2-dependent phosphorylation has been shown to modulate the stability of target protein, the stability of Snail was examined with TBB treatment to elucidate the effect of PKCK2-mediated phosphorylation on the function of Snail. The result demonstrated that TBB treatment decreased the expression of Snail (Fig. 2D). To further study that PKCK2-mediated phosphorylation of Snail on ubiquitin dependent degradation of Snail, Snail wild-type and mutants (S92A, S92E) treated with MG132. MG132 treatment significantly enhanced Snail mutants level in MCF7 cell. Therefore, these studies strongly support the notion that PKCK2-mediated phosphorylation regulates Snail stability (Fig. 3A). To determine whether the phosphorylation reduced of Snail S92A, S92E by GSK3-β, to perform GSK3-β *in vitro* kinase assay with purified in *E.coli* His-Snail (full length). When expressed at similar levels, Snail S92E was phosphorylated by GSK3-β to a lesser extent than Snail S92A. Furthermore, PKCK2 induced an accumulation of Snail in the nucleus which further bloked phosphorylation by GSK3-β (Fig. 3B). Of note, inspection of this region of the Snail protein revealed a ^{92}S-X-X-DSG-X-X-S-X-X-X-S-X-X-S^{107} motif with considerable similarity to the

GSK3-β sensitive N-terminal phosphorylation motif found in β-catenin[5-6].

Further, this region of the Snail protein also contained the DSG-X-X-S destruction motif recognized by β-TrCP in β-catenin as well as the IκB protein[5-6]. This leads to proteasomal degradation and thereby constitutes an important shared control mechanism for the government of several transcriptional regulators simultaneously.

In Snail these serines lie at positions 96 and 100 ($D^{96}SGKG^{100}SQPP$), they are evolutionary conserved and are the target for the protein kinase GSK3-β. Mutation of these serines to alanine leads to increased Snail stability[27-28] also identified two additional conserved serines ($QPP^{104}SPP^{107}SPAP$) as targets for the serine/threonine protein kinase GSK3-β. Therefore, it leads to ubiquitination and degradation of Snail[28].

A.



B.

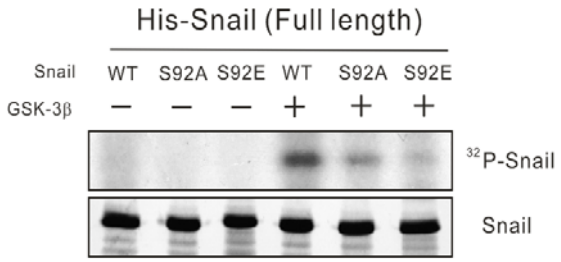


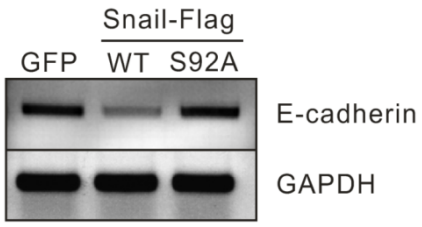
Figure 3. Effect of PKCK2-mediated phosphorylation on ubiquitin dependent degradation of Snail. A. MCF7 cells transfected with Snail-Flag (wild-type and mutants) were treated or untreated with MG132 (10μM) for 6 hours. B. The expression control was given Snail-Flag. His-tagged recombinant Snail (full-length) wild type and mutants were expressed in *E.coli* and were performed GSK-3β *in vitro* kinase assay. The expression and loading controls were given His-Snail.

4. Phosphorylation status dependent E-cadherin repressor function of Snail

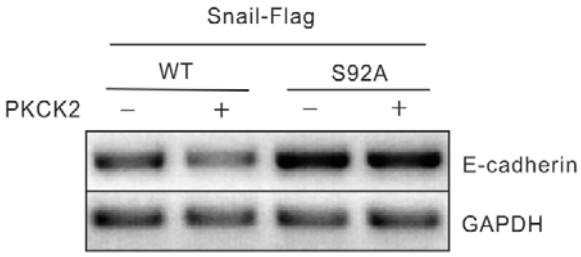
To examine the effect of PKCK2 dependent Snail phosphorylation on E-cadherin mediated transcription, MCF7 cells were transfected with Snail-Flag, empty vector (pcDNA3.1). E-cadherin mRNA level was measured in the presence of Snail wild-type or mutants using RT-PCR and Real-time PCR.

Snail has been proposed to play a key role during both development and cancer by virtue of its ability to repress E-cadherin expression and drive EMT[30-32]. Snail has been implicated in repression of E-cadherin transcription through the three E-box domains present in the proximal E-cadherin promoter region. Snail exerted only modest inhibitory effects on the wild-type E-cadherin mRNA level in MCF7 cells, equivalent amounts of the expression vectors Snail mutants(S92A) exerted lower repression effects on E-cadherin mRNA level (Fig. 4A). As well as Snail wild-type displayed an enhanced level to suppress E-cadherin mRNA level when PKCK2 over-expressed in MCF7 cells. However, the level of E-cadherin was maintained without major changes to Snail mutant (S92A) (Fig. 4B). To test whether phosphorylation of Snail at S92 could result in repressed E-cadherin mRNA level, a mutant Snail with a mimetic of a phosphate group and a mutant that cannot be phosphorylated were used. E-cadherin mRNA level was increased to Snail S92E mutant in comparison with the Snail S92A mutant. Similar results were also obtained when Real-time PCR was performed (Fig. 4C).

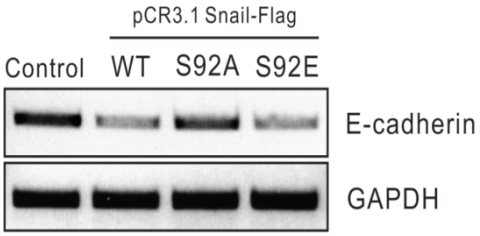
A.


Snail-Flag
GFP
WT
S92A
E-cadherin
GAPDH

B.


Snail-Flag
WT
S92A
PKCK2
–
+
–
+
E-cadherin
GAPDH

C.


pCR3.1 Snail-Flag
Control
WT
S92A
S92E
E-cadherin
GAPDH

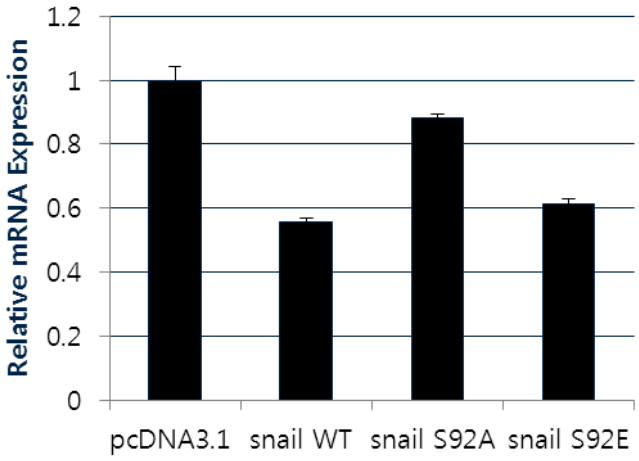


Figure 4. Phosphorylation status dependent E-cadherin repressor function of Snail. A~C. RT-PCR. GAPDH was used as an internal control. A. Snail-Flag and empty vector (pcDNA 3.1) transfected 4μg in MCF7 cells. B. Snail-Flag and empty vector (pcDNA 3.1) transfected 4μg or HA-PKCK2α 2μg in MCF7 cells. C. Snail-Flag and empty vector (pcDNA 3.1) transfected 4μg in MCF7 cells. D. E-cadherin mRNA level as assessed by SYBR Green qRT-PCR in MCF7 cells.

Ⅳ. DISCUSSION

PKCK2α is a ubiquitously expressed and constitutively active serine/threonine kinase. It forms a tetramer with two β regulatory subunits and two α catalytic subunits, although the α subunit also exists as a monomer. PKCK2 is involved in several cellular functions, including proliferation, cell cycle progression, and development. More than 300 potential substrates have been suggested for PKCK2, but the precise role and regulation of this protein kinase remains unclean as ever.

Here, this study demonstrated Snail binding to PKCK2α by a yeast two-hybrid, and confirmed this interaction *in vitro* and in mammalian cells by GST pull down and co-immunoprecipitation of co-expressed PKCK2α and Snail. The investigation of the functional relationship between Snail and PKCK2α showed a strong and selective phosphorylation of Snail at serine-92 by PKCK2. The phospho-mimic the effect of an acidic phosphate group at S92 was phosphorylated by PKCK2. Snail has phosphorylation of adjacent serine or threonine residue in the consensus sequence would impact on the phosphorylation of PKCK2. To confirm additional phosphorylation sites of Snail immediately adjacent to the S92 was mutated, preserved in S92E, and performed PKCK2 *in vitro* kinase assay. As a result, the mutant constructs were not phosphorylated by PKCK2. These results indicate that, while S92 was phosphorylated additional phosphorylation of Snail was simultaneously phosphorylated by PKCK2.

Because phosphorylation has variable effects on target proteins, it was investigated initially whether the stability of Snail is mediated on phosphorylation by PKCK2. In the presence of PKCK2 inhibitor, TBB, the stability of Snail was decreased dramatically and the expression level of Snail mutants that were un-phosphorylable Snail is also decreased than that of wild type. It was further found that PKCK2 stabilizes Snail by phosphorylation in a proteasome dependent manner. Thus, PKCK2 activity is required for stabilization of Snail.

The Snail transcript encodes a series of β-catenin-like canonical motifs that support its GSK3-β dependent phosphorylation, β-TrCP-directed ubiquitination, and proteasomal degradation via a PKCK2-mediated phosphorylation of Snail. PKCK2-mediated phosphorylation regulates Snail stability. To determine whether the phosphorylation reduced of Snail S92A, S92E by GSK3-β performed GSK3-β *in vitro* kinase assay. The Snail S92E was phosphorylated by GSK3-β to a lesser extent than Snail S92A. Furthermore, PKCK2 induced an accumulation of Snail in the nucleus which further bloked phosphorylation by GSK3-β.

The Snail protein revealed a ^{92}S-X-X-DSG-X-X-S-X-X-X-S-X-X-S^{107} motif with considerable similarity to the GSK3-β sensitive N-terminal phosphorylation motif found in β-catenin[5-6].

Further, this region of the Snail protein also contained the DSG-X-X-S destruction motif recognized by β-TrCP in β-catenin[5-6]. This leads to proteasomal degradation and thereby constitutes an important shared control mechanism for the government

of several transcriptional regulators simultaneously.

In Snail these serines lie at positions 96 and 100 ($D^{96}SGKG^{100}SQPP$), they are conserved and are the target for the GSK3-β. Mutation of these serines to alanine leads to increased Snail stability[27-28] also identified two additional conserved serines ($QPP^{104}SPP^{107}SPAP$) as targets for the serine/threonine protein kinase GSK3-β. Therefore, it leads to ubiquitination and degradation of Snail[28]. Following the GSK3-β dependent phosphorylation of β-catenin, a ^{32}DpSGXXpS motif was recognized by the E3 ubiquitin ligases β-TrCP, which mediated the ubiquitination of phosphorylated β-catenin and lead to proteasomal degradation[5-6.29].

The effect of PKCK2 dependent Snail phosphorylation on E-cadherin mediated transcription, E-cadherin mRNA level was measured in the presence of Snail. Snail was specifically phosphorylated mutants (S92A) exerted lower repression than Snail wild-type effects on E-cadherin mRNA level. As a result, Snail was regulated by PKCK2 effects on repression of E-cadherin mRNA level. Taken together, Snail was regulated by PKCK2 via its phosphorylation modulates the Wnt signaling and required for cell migration, invasion, and metastasis.

PKCK2 expression level or activity is up-regulated in a variety of human cancers [9, 11-13] and PKCK2 acts as a positive modulator in Wnt signaling[8,16], thereby PKCK2 could induce the tumor migration, invasion, and metastasis. In consideration of cancer condition, however, it is not very efficient for cancer that PKCK2 increases the protein stability of Snail in cancer. Snail has been proposed to playing a key role

during development and cancer by virtue of its ability to repress E-cadherin expression and drive EMT.

These facts implicate the benign tumor might activate the PKCK2 activity and delete the Snail expression at the same time. Therefore, benign tumor could be complete to be a malignant tumor. Taken together, it is likely to be considered the loss of Snail expression in cancer cells could be proportional to the malignant degree in cancer patients.

Ⅴ. CONCLUSION

1. Snail directly phosphorylation at serine 92 by PKCK2.

2. PKCK2 regulates stability of Snail.

3. PKCK2 regulates stability of Snail by inhibiting GSK3-β mediated ubiquitin dependent degradation pathway.

4. PKCK2 mediated Snail stabilization augment E-cadherin repressor function of Snail.

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ABSTRACT (in Korean)

PKCK2에 의한 Snail 인산화가 Wnt 신호전달에 미치는 영향

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김 영 경

프로테인 카이네이즈 케이신 카이네이즈 2 (PKCK2)는 serine/threonine 카이네이즈로써 세포 성장과 사멸, 그리고 증식에 중요한 효소이다. 최근 연구들에 의하면 PKCK2 는 세포사멸 억제 역할을 하며, 암세포에서 PKCK2 의 활성이 높다는 것이 밝혀졌다. 암 발생 과정에서 중요한 신호전달계의 하나인 Wnt 신호전달과정에 관여하는 Dishevelled, β-catenin, LZTS2 등의 여러 물질들이 PKCK2 에 의해 인산화되며, 인산화를 통해 Wnt 신호전달이 더욱 활성화됨이 보고되었다. E-cadherin 전사 억제자인 Snail 은 핵 내 GSK3-β 에 의해 인산화되면 ubiquitination 후 proteasome 으로 이동하여 degradation 된다. Wnt 신호전달체계가 활성화되어 Axin2

의 발현이 증가하면, Axin2 는 핵 내 GSK3-β를 세포질로 이동시킴으로써 핵 내 GSK3-β 에 의한 Snail 의 인산화가 이뤄지지 않게 됨으로써 안정화되고 결과적으로 E-cadherin 의 발현을 억제함으로써 암세포의 이동, 침윤 및 전이가 가능하도록 만들 수 있다. 그러나 여전히 Snail의 생물학적 기능에 대해서는 거의 알려져 있는 것이 없다.

본 연구에서는, Snail이 PKCK2와 상호작용하는 단백질임을 밝혔고, PKCK2에 의한 Snail의 인산화가 Wnt 신호전달체계에 어떠한 영향을 미치는지를 조사하였다.

먼저, Yeast two hybrid 실험을 통해, PKCK2와 Snail이 상호작용하는 것을 알아냈으며, 더 나아가 면역침전법을 이용해 *in vitro*와 *in vivo* 상에서도 서로 상호작용하는 것을 알 수 있었다. *In vitro* kinase assay와 돌연변이 분석을 통해서 Snail에는 PKCK2 인산화 부위가 존재하는 것을 알 수 있었으며, 이 인산화 부위를 PKCK2가 인산화시킴으로써, GSK3-β 에 의한 Snail의 인산화가 억제되고 Snail의 단백질을 안정화시키며 E-cadherin의 발현을 억제하는 것을 알 수 있었다. 즉, PKCK2의 활성은 Snail의 단백질 안정화를 조절하고, Wnt 신호 전달 체계를 조정한다.

종합하여 PKCK2의 인산화에 의해 Snail의 단백질 안정화가 조절되고, E-cadherin의 전사 활성에 관여한다.

핵심되는 말 : PKCK2, Wnt signaling, Snail, E-cadherin, GSK3-β, phosphorylation