Regulation of PKCK2 activity in TGFβ-mediated epithelial-mesenchymal transition

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Regulation of PKCK2 activity in TGFβ-mediated epithelial-mesenchymal transition

Directed by Professor Kunhong Kim

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ABSTRACT

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E- to N-cadherin switching is a molecular hallmark of epithelial-mesenchymal transition (EMT) that might be a critical process in invasion and metastasis of cancer cells.

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Recently, it was reported that overexpression of PKCK2α catalytic subunit could induce E-to N- cadherin switching. As it has been well known that TGF-β could induce the cadherin switching in A549, human non-small cell lung cancer cell line, this study was designed to examine whether PKCK2 is required for TGF-β mediated EMT. Results show that in the absence of PKCK2 activation, TGF-β could not induce the cadherin switching in A549 cells. PKCK2 activity began to increase after 24 hr of TGF-β treatment and achieved at its maximum at approximately 48 hr after treatment. TGF-β receptor I (TβRI) inhibitor abolished TGF-β mediated EMT and PKCK2 activation. As PKCK2 activity was increased, the expression of PKCK2 catalytic or regulatory subunit was examined. In the presence of TGF-β signaling, there was no change in the level of α catalytic subunit but the level of β regulatory subunit was down-regulated. β subunit knockout increases PKCK2 activity and induces E-to N-cadherin switching and imbalance between the expression level of catalytic subunit and regulator subunit could regulate PKCK2 activity. TGF-β signaling dependent down-regulation of CK2β was a proteasome dependent and TβRI kinase activity dependent process. C terminus of HSC70-Interacting Protein (CHIP) was found to be an E3 ubiquitin ligase of CK2β down regulation. As CK2β is phosphorylated by CK2α and phosphorylated CK2β was not down-regulated by TGF-β signaling. It was found that when CK2β is phosphorylated by CK2α, as yet unknown protein phosphatase could be involved for the de-phosphorylation of CK2β and de-phosphorylated CK2β could undergo CHIP mediated

ubiquitination dependent degradation. The involvement of phosphatase was evidenced by the observation that protein phosphatase inhibitor, okadaic acid treatment could block CK2β down regulation induced by TGF-β signaling.

Taken together, PKCK2 activation is required during TGF-β mediated EMT process. Imbalance between the expression level of CK2α and CK2β could regulate the PKCK2 activity. Therapeutic perturbation of PKCK2 activation in cancer cells could inhibit EMT and prevent metastasis.

Key words: Protein Kinase Casein Kinase (PKCK2), Transforming Growth Factor-β (TGF-β), Epithelial Mesenchymal Transition (EMT), C-terminus of HSC70 - Interacting Protein (CHIP), Phosphatase

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I. INTRODUCTION

Epithelial mesenchymal transition (EMT) is a critical phenotypic change in the metastasis of cancer cells. During EMT, cells lose the characteristics of an epithelial cell,

such as strong cell-to-cell interaction. Rather they acquire the characteristics of mesenchymal cells such as loose cell-to-cell interaction, secretion of strong proteases, and increased motility. The ability of migration and invasion to surroundings increases, finally leading to metastasis.[1-3] Cadherin is a member of glycoprotein family that involves in homotypic cell to cell adhesion.[4,5] During EMT, E- to N- cadherin switching occurs, and this is one of the most important molecular characteristics of EMT.[6-12]

Protein kinase Casein Kinase 2 (PKCK2) is a pleiotropic, ubiquitous, highly conserved and constitutively active protein serine/threonine kinase that forms a heterotetramer composed of 2 catalytic α subunit (α, α') and 2 regulatory β subunit. PKCK2 is involved in many cellular processes such as cell cycle control, cellular differentiation, proliferation, and regulation of apoptosis.[13,14] Previously, it has been reported that the expression or activation of PKCK2 changes in many cancer cells and that the overexpression of the catalytic α subunit of PKCK2 induces tumor formation, suggesting that PKCK2 can potentially be a therapeutic target for cancer treatment.[15-21] Recently, it was reported that artificial overexpression of PKCK2 catalytic α subunit alone could induce E- to N- cadherin switching in TE2, human esophageal cancer cells.[22]

TGF-β superfamily is a ubiquitous cytokine involved in various cellular processes such as cell growth, cell-cell adhesion, migration, cell-fate determination, differentiation, and apoptosis. TGF-β transmits signal to the inside of the cells through activation of

serine/threonine kinase receptor termed TGF-β receptor 1 (TβR1). Both Smad pathway which transmits signal by activating Smad, an intracellular signal transducer, and non-Smad pathway which transmits signal without Smad exist. It is currently well known that TGF-β plays a crucial role in cancer progression and induces E- to N- cadherin switching in several types of cancer cells.[23] In particular, it has been reported that TGF-β serves a crucial role in triggering EMT in epithelial cells and acts as an inducer for cancer metastasis.[24,25]

Therefore, this study was designed to confirm whether PKCK2 is generally required for signaling induced EMT such as TGF-β signaling.

II. MATERIALS AND METHODS

1. Cell lines and reagent

TE2, human esophageal cancer cell line and HEK293, human embryonic kidney cell line were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100 μg/ml streptomycin (Gibco-BRL) and 100 units/ml penicillin (Gibco-BRL). A549, non small cell lung cancer (NSCLC) cell line and HT29 and SW620, colorectal cancer cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 100 μg/ml streptomycin (Gibco-BRL) and 100 units/ml penicillin (Gibco-BRL). All the cell lines were grown at 37°C in 5% CO_2. The PKCK2 inhibitors, tetrabromobenzotriazole (TBB; Sigma-Aldrich, St. Louis, MO, USA) and the reversible cell-permeable proteasome inhibitor, MG132 (Sigma-Aldrich) were prepared in 20 mM stock using DMSO (Sigma-Aldrich). The TβRI specific inhibitor, SB431542 (Sigma-Aldrich) were prepared in 10 mM stock using DMSO. The inhibitor of protein biosynthesis in eukaryotic organisms, cycloheximide (CHX; Sigma-Aldrich) were prepared in 30 mM stock using DMSO. A protein phosphatase inhibitor, okadaic acid (OA; Sigma-Aldrich) were prepared in 10 μM stock using DMSO. Control cultures received the same amounts of DMSO, and final DMSO concentrations did

not exceed 0.1%. TGF-β (R&D Systems, Minneapolis, MN, USA) were prepared in 10 ng/μl stock using 2 nM HCl containing distilled water. Control cultures received the same amounts of distilled water.

2. RNA interference, lentiviral expression, and generation of stable cell lines

siRNA mediated inhibition of C terminus of HSC70-Interacting Protein (CHIP) expression was carried out according to the manufacturer's protocol (Santa Cruz Biotechnology Inc. Dalaware Avenue, CA, USA). Cells were transfected with siRNA using Lipofectamine® RNAiMAX (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer's protocols. For overexpression of PKCK2α, the lentivirus vector Lentipgk-GFP mCMV-HA-PKCK2α IRESpuro was purchased from Macrogen (Seoul, Korea) and co-transfected into 293T cells with packaging plasmids. The resulting supernatant containing PKCK2α-expressing lentivirus was used to transduce cells according to the manufacturer's protocol (Macrogen). Transduced cells were selected using puromycin (1 μg/mL). A human shRNA kit for silencing PKCK2α or Smad4 expression was purchased from Origene Technologies Inc. (Rockville, MD, USA). To obtain stable transfectants, cells were transfected with the plasmid and then treated with puromycin (2 μg/mL) for selection.

3. Western blot analysis

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, 1 mM phenyl methylsulfonyl fluoride (PMSF) (USB, Cleveland, OH, USA) protease inhibitor cocktail (R&D Systems) and 1 mM sodium orthovanadate (Sigma-Aldrich). Lysates were briefly vortexed and cleared by centrifugation at 13,200 rpm for 20 min at 4℃. Supernatants were collected and transferred to a fresh tube. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amount of protein extracts were subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co., Bedford, MA, USA). The blotted membranes were blocked in 100mM Tris-buffer (pH 7.4) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich) and 5% (w/v) non-fat Difco^{TM} skim milk (BD Biosciences, Sparks, MD, USA) and probed with primary antibodies. The following antibodies were used: HA tag (Covance, New York city, NY, USA), Myc tag, GFP, Smad2/3, phosphor Smad2/3, CHIP (Cell Signaling, Danvers, MA, USA), Flag tag, β-actin (Sigma-Aldrich), 6X His tag, PKCK2β (R&D Systems), E-cadherin, N-cadherin (Invitrogen Life Technology), PKCK2α (Millipore Co.), Hdac I, Smad4, and Vimentin (Santa Cruz Biotechnology, Inc.). Secondary antibodies were anti-rabbit IgG peroxidase (Thermo scientific, Rockford, IL, USA) and anti-mouse IgG horseradish peroxidase (Thermo scientific). The signals were developed by Lumi-Light

Western Blotting Substrate (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol.

4. *In vitro* kinase activity assay

Intracellular PKCK2 activity was measured using an in vitro kinase assay as follows. Briefly, 3 μg of bacterially expressed GST-CS (<u>G</u>ST-tagged recombinant <u>C</u>K2 <u>S</u>ubstrate) protein was incubated with glutathione Sepharose 4B beads for 60 minutes and washed twice with 1x kinase buffer [4 mM MOPS, pH 7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 mM sodium orthovanadate, and 200 mM Dithiothreitol (DTT)). The beads were then incubated with 100 μg cell lysate in a final volume of 50 μL kinase reaction buffer (10 μL of 5x kinase buffer), 10 μL magnesium/ATP cocktail solution [90 μL of 75 mM $MgCl_2$/500 mM ATP plus 10 μL (100 μCi) [γ-^{32}P]-ATP] for 20 minutes at 30℃. Reactions were stopped by washing twice with 1x kinase buffer. Samples were resuspended with 30 μL of 2x SDS sample loading buffer, subjected to 12% SDS-PAGE followed by staining with Coomassie brilliant blue, and dried on Whatman papers. Incorporation of ^{32}P was detected by autoradiography.

5. Dual-luciferase reporter assay

A549 vector control (VC) and CK2α knockdown (CKD) cells were seeded in 6-well

plate and co-transfected with Smad binding element (SBE) promoter and renilla luciferase reporter plasmid (pRL-TK) using ViaFect™ (Promega, Madison, WI, USA). After 24 hr of transfection, the cells were treated with TGF-β for 24 hr and then washed with PBS and harvested. Cell lysates were prepared using 200 µl of Passive Lysis buffer (Promega). Aliquots of 20 µl of cleared lysate were assayed for luciferase activity by using a Dual-luciferase® reporter assay system (Promega). Luciferase activity of SBE promoter plasmid was normalized to the luciferase activity of the pRL-TK. All the experiments were performed independently in triplicate.

6. Cell fractionation

Cells were swelled in buffer A composed of 10 mM HEPES, pH 7.9 , 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1x protease inhibitor cocktail and 1 mM sodium orthovanadate. Samples were adjusted to 0.6% Nonidet P-40 (NP-40) and vigorously vortexed for 10 sec. Nuclei were pelleted by centrifugation at 13,200 rpm for 30 sec at 4℃. Supernatants were collected and used as the cytoplasmic fraction. Washing the pellet with PBS, Pellet were lysed in buffer C composed of 20 mM HEPES, pH 7.9 , 0.4 M NaCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 x protease inhibitor cocktail and 1 mM sodium orthovanadate by sonication. Lysates were cleared by centrifugation at 13,200 rpm for 20 min at 4℃. Supernatants were collected and used as the nucleus fraction.

7. Semi-quantitative RT PCR

Total RNA was isolated using easy-spin^{TM} total RNA extraction kit (iNtRON biotechnology, Gyeonggi, Korea), according to the manufacturer's instructions. Total RNA concentration was determined using a NanoDrop and 2 μg RNA was reverse transcribed to cDNA by SuperScriptII First-Strand cDNA Synthesis kit (Invitrogen Life Technology). cDNA was used to perform subsequent PCR amplification using TaKaRa ExTaq polymerase (TaKaRa Bioscience, Kyoto, Japan). Sequence information for each primer is as follows: PKCK2 F, 5'- TGGATATGTGGAGTTTGGGTTGT-3'; PKCK2 R, 5'-AAAAATGGCTCC TTCCGAAAG-3'; PKCK2 F, 5'-TCTGCTGAGTTGGGAGTGAG-3'; PKCK2 R, 5'-CA GACAACAAACCAGACCCA-3'; GAPDH F, 5'-GTGAAGGTCGGAGTCAACGGATTT - 3'; GAPDH R 5'-GCTGATGATCTTGAG GCTGTTGTCA-3'

8. Immunoprecipitation (IP)

Cells were lysed in a buffer composed of 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and 1x protease inhibitor cocktail. Pre-clearing of lysates were performed by 1h of incubation in the presence of G plus/protein A-agarose (Santa Cruz Biotechnology Inc.). The supernatant was collected and 2 μg of anti-FLAG, anti-HA antibody or anti-myc antibody was added. After overnight

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incubation, 50 μl of 50 % slurry of G plus/protein A-agarose was added and the mixture was incubated for 2 hr. 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 blot immunostaining. Non-immune mouse IgG or non-immune rabbit IgG (Santa Cruz Biotechnology Inc.) served as a negative control.

9. CRISPR/Cas9 gene knock out system

Recombinant Cas9 protein expression construct and 3 candidates of sgRNA constructs were purchased from ToolGen, Inc. (Seoul, Korea). All experiments were performed according to the manufacturer's manual.

10. Cell migration assay

The cell migration assay was conducted using a specific wound assay chambers purchased from Ibidi GmbH (Martinsried, Munich, Germany). All experiments were performed according to the manufacturer's manual.

11. Site-directed mutagenesis

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 primer pairs were used (Table 1).

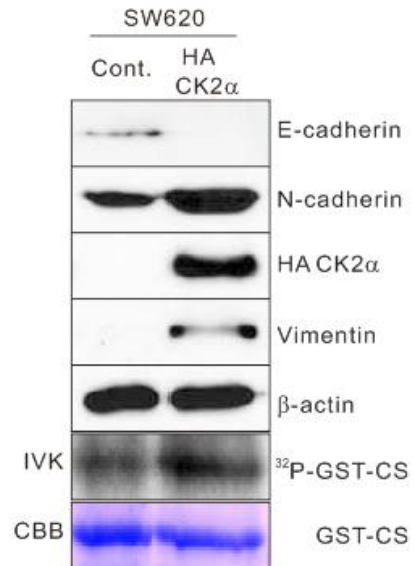Table 1. Primer sequences for mutagenesis

Name of mutant		Primer Sequences
S2AS3AS4A	Forward	5' GACGTGAAGATG**GCCGCCGCA**GAGGAGGTGTCC 3'
	Reverse	5' GGACACCTCCTC**TGCGGCGGC**GATCTTCACGTC 3'
S2ES3ES4E	Forward	5' GACGTGAAGATG**GAAGAAGAA**GAGGAGGTGTCC 3'
	Reverse	5' GGACACCTCCTC**TTCTTCTTC**GATCTTCACGTC 3'
S209A	Forward	5' AGCAACTTCAAG**GCC**CCAGTCAAGACG 3'
	Reverse	5' CGTCTTGACTGG**GGC**CTTGAAGTTGCT 3'
S209E	Forward	5' AGCAACTTCAAG**GAG**CCAGTCAAGACG 3'
	Reverse	5' CGTCTTGACTGG**CTC**CTTGAAGTTGCT 3'

III. RESULTS

1. PKCK2α over-expression could induce EMT in cancer cells

Recently, it was reported that overexpression of the α catalytic subunit of PKCK2 induces E- to N- cadherin switching in TE2, human esophageal cancer cell line (Fig. 1A). To generalize this result, CK2α–overexpressing stable cell lines were generated using A549, HT29, and SW620 as previously described.[22] E- to N- cadherin switching was induced in these cell lines (Fig. 1B and C), indicating that increase in PKCK2 activity could induce E- to N-cadherin switching in cancer cells without tissue specificity.

A. TE2

GFP | HA CK2α

E-cadherin
N-cadherin
HA CK2α
GFP
β-actin

IVK — ³²P-GST-CS

CBB — GST-CS

B. A549

Cont. | HA CK2α

E-cadherin
N-cadherin
HA CK2α
β-actin

IVK — ³²P-GST-CS

CBB — GST-CS

C. HT29

Cont. | HA CK2α

E-cadherin
N-cadherin
HA CK2α
Vimentin
β-actin

IVK — ³²P-GST-CS

CBB — GST-CS

SW620

Cont. | HA CK2α

E-cadherin
N-cadherin
HA CK2α
Vimentin
β-actin

IVK — ³²P-GST-CS

CBB — GST-CS
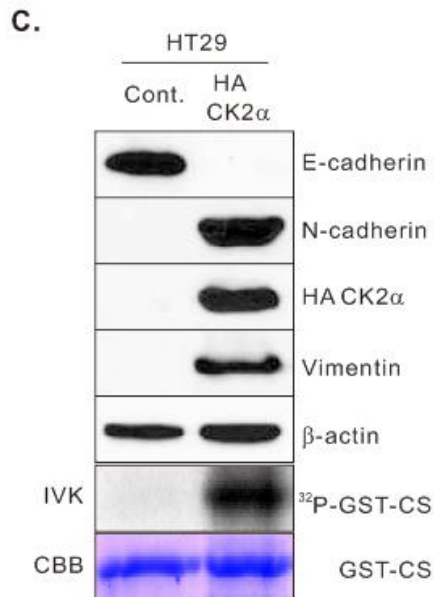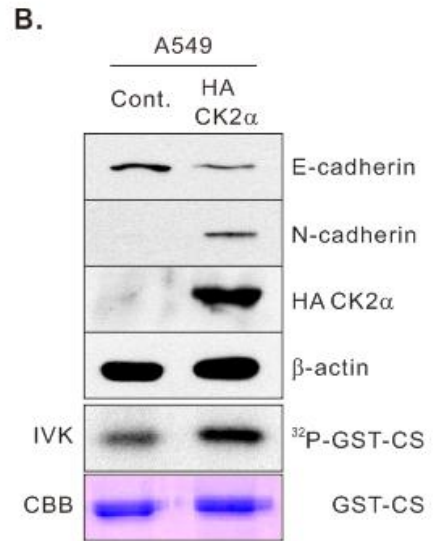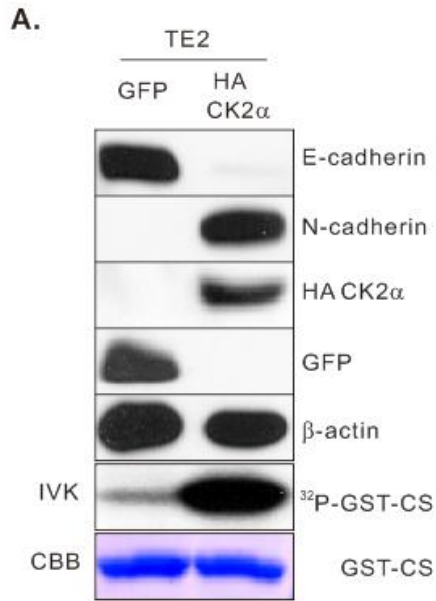
Figure 1. EMT is induced by PKCK2α over-expression. (A) TE2 cells were transduced with lentivirus-expressing HA-tagged PKCK2 α catalytic subunit. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-HA, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity was performed. ^{32}P-GST-CS represents phosphorylated GST-CS and GST-CS represents Coomassie blue stained input GST-CS. The same procedure was conducted on **(B)** A549 cells and **(C)** HT29 and SW620 cells, respectively.
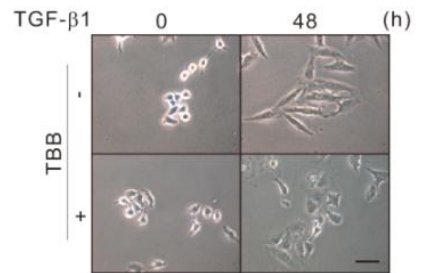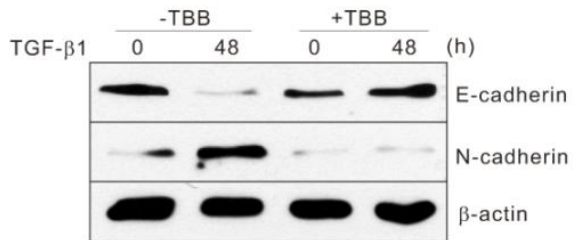
2. PKCK2 is required for TGF-β mediated EMT

A549 has been reported as a good cell line model to see TGF-β mediated EMT[26] and in addition, increase in PKCK2 activity could induce EMT in A549 cells (Fig. 1B). Accordingly, to examine the involvement of PKCK2 in TGF-β mediated EMT in A549, the cells were pre-treated with tetrabromobenzotriazole (TBB), PKCK2 specific inhibitor before TGF-β treatment. In the presence of TBB, there was no characteristic morphological change of mesenchymal cells were observed and E- to N-cadherin switching was not induced (Fig. 2A). To confirm these results, CK2α knockdown stable cell line was generated in A549 using shRNA. E- to N-cadherin switching was not induced and migration ability was not increased even in the presence of TGF-β treatment (Fig. 2B,C). These results indicate that PKCK2 activity is required for TGF-β mediated EMT.
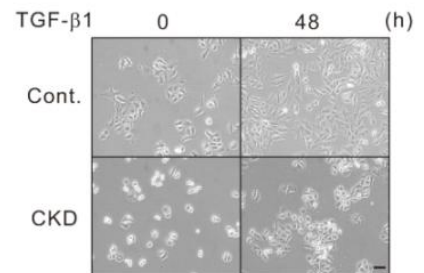
Then, to examine whether PKCK2 activity is changed during TGF-β mediated EMT, A549 cells were treated with TGF-β at different time points. Results showed that PKCK2 is activated during TGF-β mediated E- to N- cadherin switching and maximum activation was achieved approximately 48 hr after TGF-β treatment (Fig. 2D). To examine whether TGF-β receptor I (TβRI) kinase activity is required for PKCK2 activation, A549 cells were pre-treated with SB431542, TβRI specific inhibitor before TGF-β treatment. The results showed that EMT was not induced and PKCK2 activity did not increase even upon TGF-β treatment (Fig. 2E). This indicates that TβRI kinase activity is essential for E- to N- cadherin switching

and PKCK2 activation.

A.

B.

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C.

	Cont.		CKD	
TGF-β1	−	+	−	+

0 day

1 day

200 μm

D.

TGF-β1   0   24   48   72   (h)

E-cadherin
N-cadherin
CK2α
β-actin

IVK   ³²P-GST-CS
CBB   GST-CS

E.

TGF-β1   −   +   +   +
SB431542   −   −   5   10   (μM)

E-cadherin
N-cadherin
β-actin

IVK   ³²P-GST-CS
CBB   GST-CS

``

20
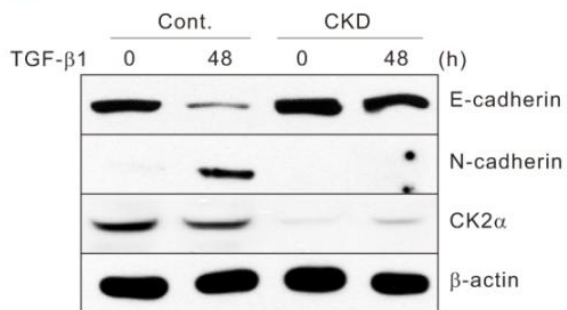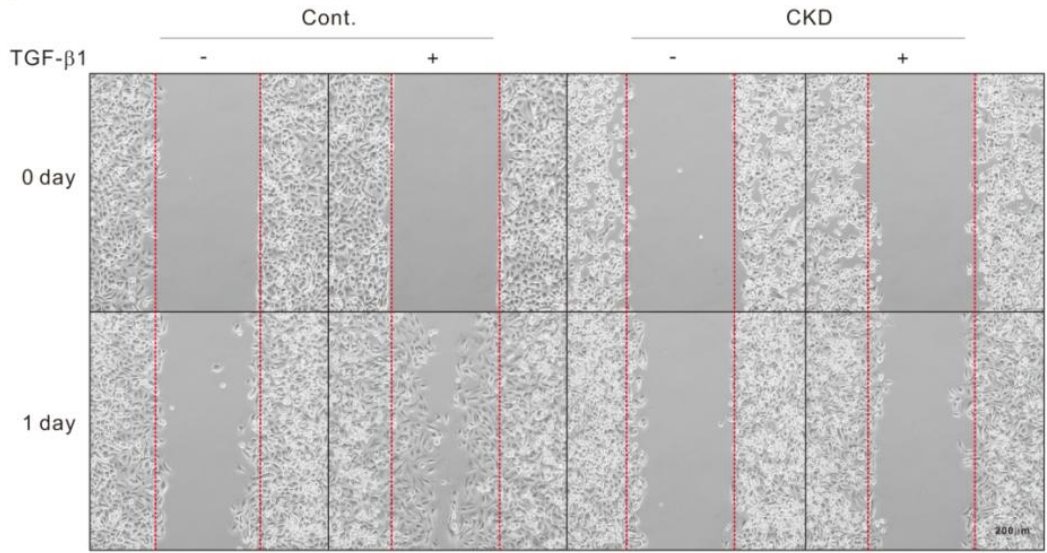
Figure 2. PKCK2 is activated during TGF-β mediated EMT. (A) A549 cells were pre-treated or untreated with TBB (10 μM), and then treated with TGF-β for 48 hr. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin and β-actin Ab. Photograph was taken using phase contrast microscopy. **(B)** A549 cells were stably transfected with shRNA against PKCK2 catalytic α subunit and treated with puromycin for selection. A549 vector control (con.) and CK2α knockdown (CKD)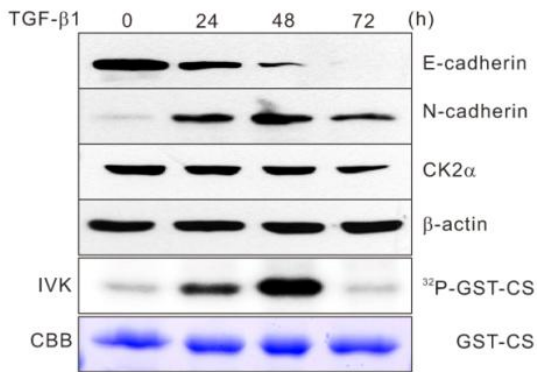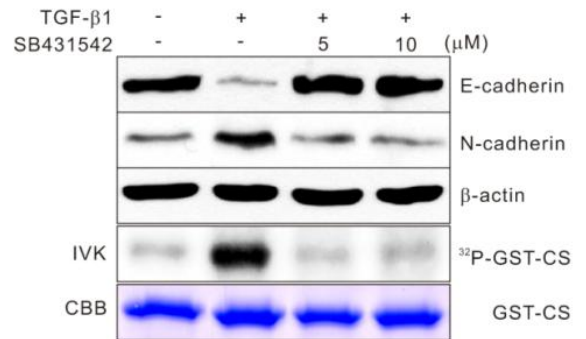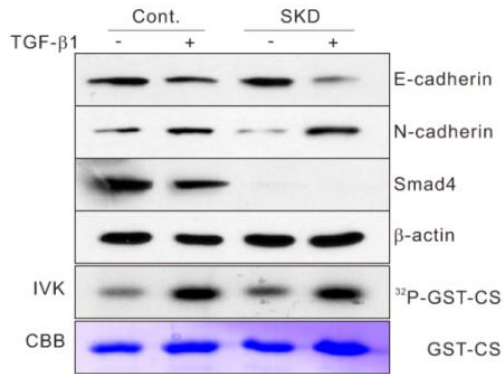 cells were treated with TGF-β for 48 hr. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CK2α, and anti-β-actin Ab. **(C)** Migration of A549 cont. and CKD was analysed with wound assay culture inserts (Ibidi) in the presence of TGF-β for 24 hr. Representative pictures of the beginning (0 day) and the end (1 day) of the experiments are shown. **(D)** A549 cells were treated with TGF-β for the indicated times. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CK2α, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed. **(E)** A549 cells were pre-treated or untreated with SB431542, and then treated with TGF-β for 48 hr. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed.

3. Canonical Smad pathway is not required for EMT

Then, to examine the molecular mechanism of PKCK2 activation during TGF-β signaling, following experiments were performed. It has been known that TGF-β transmits signal through Smad pathway and/or non-Smad pathway.[27] Upon TGF-β activation, Smad4 along with phosphorylated Smad2/3 moves into the nucleus.[28,29] To investigate whether Smad pathway is required for TGF-β mediated EMT, Smad4 knockdown stable cell was generated in A549 cells. Even in the absence of Smad4 expression, E- to N-cadherin switching was induced and PKCK2 activity was regulated by TGF-β treatment (Fig. 3A). To examine the effect of PKCK2 on canonical Smad signaling pathway, CK2α knockdown A549 cells were treated with TGF-β. Results showed that no changes were observed in Smad signaling, suggesting that Smad signaling is required for E-to N-cadherin switching and PKCK2 is not required for canonical Smad signaling.

A.

Cont.　　　SKD

TGF-β1　−　+　　−　+

E-cadherin

N-cadherin

Smad4

β-actin

IVK　　　　　　　　　　　32P-GST-CS

CBB　　　　　　　　　　　GST-CS

B.

Cont.　　　CKD

TGF-β1　−　+　　−　+

P-Smad2

Smad2

IVK　　　　　　　　　　　32P-GST-CS

CBB　　　　　　　　　　　GST-CS

C.

Relative luciferase activity (% of control)

■ TGFβ1 −
□ TGFβ1 +

3,000

2,000

1,000

0

VC　　CKD

D.

Nucleus

Cont.　　　CKD

TGF-β1　−　+　　−　+

Smad4

HDAC1

23
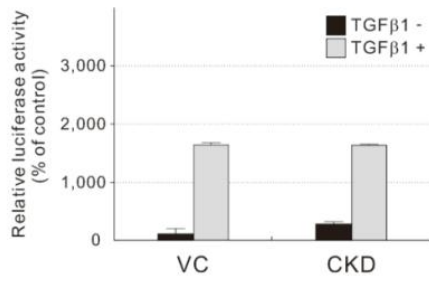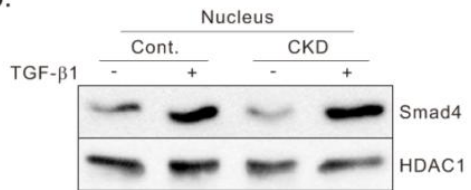
Figure 3. Canonical Smad pathway is not required for EMT. (A) A549 cells were stably transfected with shRNA against Smad4 and treated with puromycin for selection. A549 vector control (con.) and Smad4 knockdown (SCD) cells were treated with TGF-β for 48 hr. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-Smad4, and anti-β-actin Ab. *In vitro* kinase assay 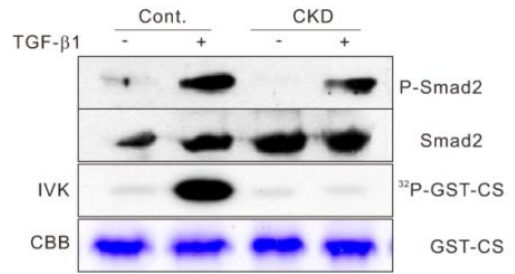for intracellular PKCK2 activity were performed. **(B)** A549 cont. and CKD cells were treated with TGF-β for 48 hr. Equal amounts of cell lysates were blotted for anti-phospho-Smad2/3, anti-Smad2/3, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed. **(C)** A549 cont. and CKD cells were transfected with pGL3-Smad binding elements (SBE) plasmid in triplicate. After 2 days, relative luciferase activities were measured. pRL-TK p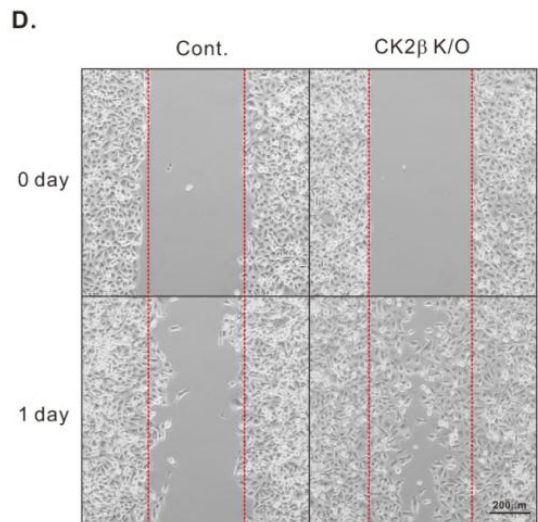lasmid was used for co-transfection in all the experiments to normalize luciferase activity. **(D)** A549 con. and CKD cells were treated with TGF-β for 48 hr. Cells were fractionated and equal amounts of nuclear lysates were blotted for anti-Smad4 and anti-HDAC 1 Ab.

4. PKCK2β is down-regulated by TGF-β signaling

Previously, it was documented that PKCK2 is activated during TGF-β mediated EMT. It was assumed that the imbalance in the expression level between PKCK2 catalytic and regulatory subunits could be the reason for increase in PKCK2 activity. Consistent with the assumption, it was reported that the imbalance between PKCK2 subunits caused by the reduction of β regulatory subunit is linked to the increase in molecular targets associated with EMT in tissue samples of breast cancer patients.[30,31] Based on this, the expression of PKCK2 subunits during TGF-β mediated EMT was examined. The results showed that the expression of endogenous catalytic α subunit did not change during the experiment but the expression of endogenous regulatory β subunit gradually decreased. With these changes, PKCK2 activity gradually increased (Fig.4 A). Hereafter, to mimic TGF-β signaling, TβRI constitutively active (CA) plasmid construct was generated through site-directed mutagenesis and used instead of TGF-β whenever needed. To confirm the above results, CK2α or CK2β was co-expressed with TβRI CA form. Results showed that no change in exogenous CK2α expression but exogenous CK2β expression was decreased by TGF-β signaling. While mRNA expression level was not changed by the signaling (Fig. 4B), the reduction in expression of CK2β might occur at protein level.

To mimic the decrease in CK2β expression level, CK2β knockout cell line was generated in A549 cells using CRISPR/Cas9 gene knockout system and the cells were

treated with TGF-β. Result showed that PKCK2 activity was increased and EMT was induced and migration ability was increased in CK2β knockout cells even in the absence of TGF-β treatment (Fig. 4C, D). To support this result, cells were co-transfected with different amount of CK2α and CK2β expression constructs. The results showed that PKCK2 activity became increased when the amount of CK2α expression construct increased (Fig. 4E).

A.

A549

TGF-β1 0 1 3 6 12 24 (h)

E-cadherin
N-cadherin
CK2α
CK2β
β-actin

IVK ^{32}P-GST-CS

CBB GST-CS

B.

His-TβRI CA - + - +
HA-CK2α + + - -
myc-CK2β - - + +

His
HA
Myc
β-actin

His-TβRI CA - + - +
HA-CK2α + + - -
myc-CK2β - - + +

TβRI
CK2α
CK2β
GAPDH

C.

A549

Cont. CK2β K/O

E-cadherin
N-cadherin
CK2α
CK2β
β-actin

IVK ^{32}P-GST-CS
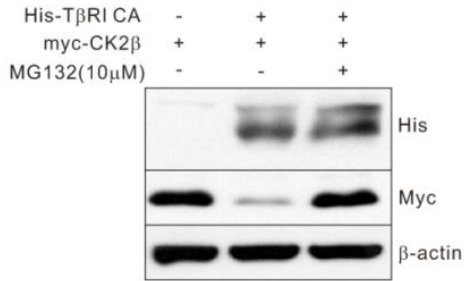
CBB GST-CS

D.

Cont. CK2β K/O

0 day

1 day

200μm

27

E.
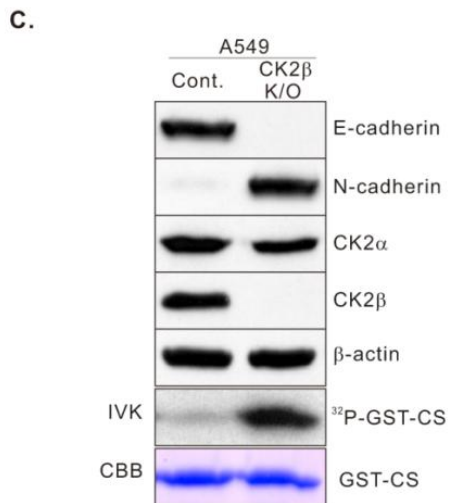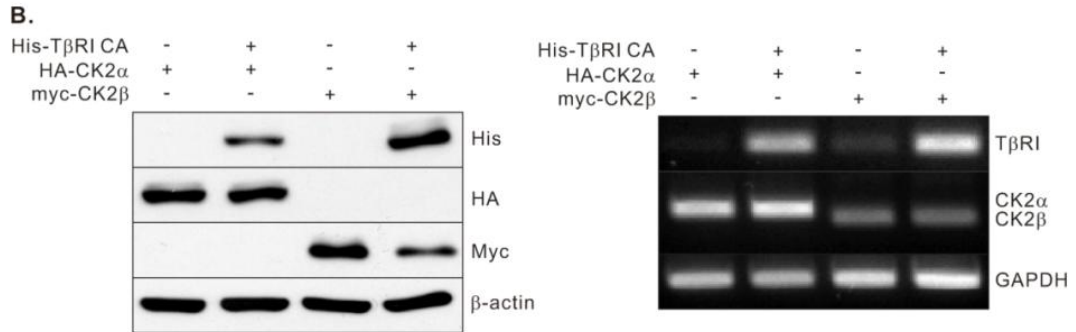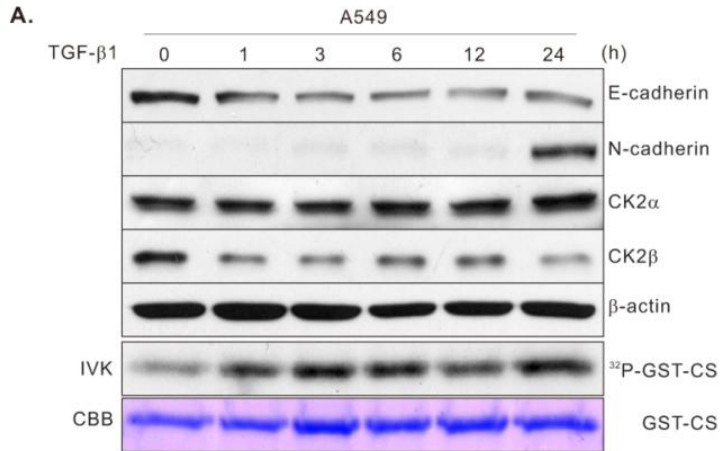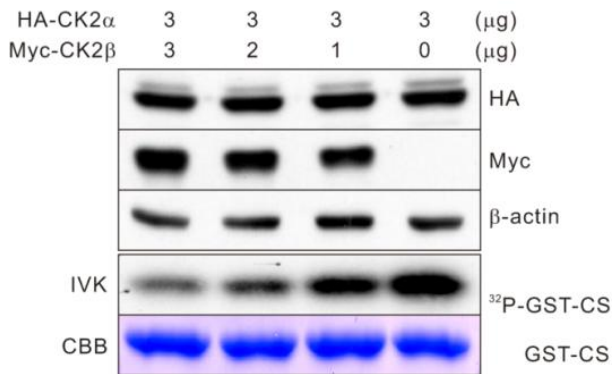
Figure 4. PKCK2β is down-regulated by TGF β signaling. (A) A549 cells were treated with TGF-β for the indicated times. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CK2α, anti-CK2β, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed. **(B)** HEK293 cells were co-transfected with HA-CK2α or myc-CK2β along with His-TβRI CA. Equal amounts of cell lysates were blotted for anti-His, anti-HA, anti-myc, and anti-β-actin Ab (left panel). Semiquantitative RT PCR was performed (right panel). **(C)** CK2β knockout A549 cells were generated using CRISPR/Cas9 systems. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CK2α, anti-CK2β, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed. **(D)** Migration of A549 cont. and CK2β K/O was analysed with wound assay culture inserts (Ibidi) for 24 hr. Representative pictures of the beginning (0 day) and the end (1 day) of the experiments are shown. **(E)** HEK293 cells were co-transfected with HA-CK2α and myc-CK2β. Equal amounts of cell lysates were blotted for anti-HA, anti-myc, and anti-β-actin Ab. *In vitro* kinase assay for intracellular PKCK2 activity were performed.

5. Proteasome dependent degradation of PKCK2β

To examine whether degradation of CK2β is proteasome dependent, cells were treated with MG132. The results showed that CK2β was degraded by TGF-β signaling, but the degradation was not observed in the presence of MG132 (Fig.5A). To examine whether CK2β is ubiquitinated by TGF-β signaling, an ubiquitination assay was performed. The results showed that CK2β is poly-ubiquitinated when TβRI CA was expressed (Fig. 5B). To investigate whether these poly-ubiquitination occurs in a TβRI kinase activity dependent manner, either TβRI CA or TβRI kinase dead (KD) were co-expressed with CK2β. In the presence of TβRI CA, CK2β expression was decreased but with TβRI KD, no decrease was observed (Fig. 5C). To confirm these results, A549 cells were pre-treated with SB431542 or MG132 before TGF-β treatment. Similarly, decrease in CK2β expression was not observed in the presence of MG132 and EMT was also not induced. In addition, decrease in CK2β expression was delayed and EMT was inhibited by the presence of SB431542 (Fig. 5D).

A.

His-TβRI CA	−	+	+
myc-CK2β	+	+	+
MG132(10μM)	−	−	+

His

Myc

β-actin

B.

IP : anti - myc

HA-Ub	+	+	+	+
His-TβRI CA	−	+	−	+
myc-CK2β	−	−	+	+

HA-Ub

input

Myc

His

C.

| His-TβRI | − | CA | KD |
| myc-CK2β | + | + | + |

His

Myc

β-actin

D.

	DMSO			SB431542			MG132			
TGF-β1	0	12	24	0	12	24	0	12	24	(hr)
myc-CK2β	+	+	+	+	+	+	+	+	+	

E-cadherin

N-cadherin

myc

β-actin

30
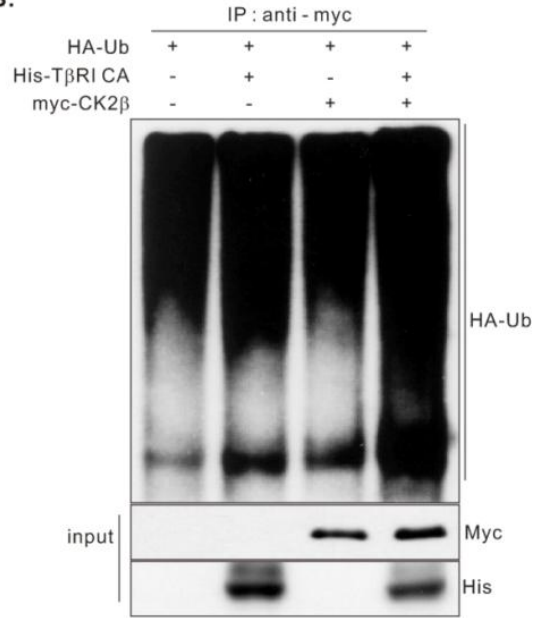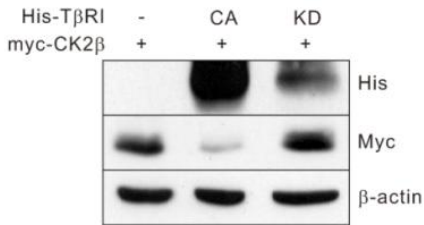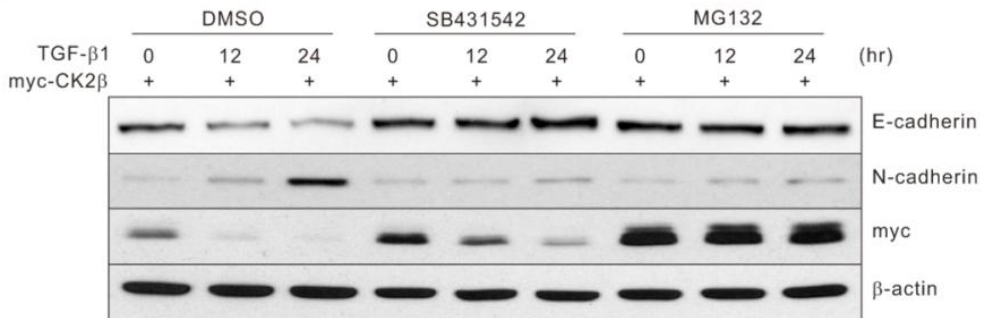
Figure 5. Proteasome dependent degradation of PKCK2β. (A) HEK293 cells were co-transfected with His-empty or His-TβRI CA along with myc-CK2β, and then treated or untreated with MG132 (10 μM). Equal amounts of cell lysates were blotted for anti-His, anti-myc, and anti-β-actin Ab. **(B)** HEK293 cells were co-transfected with indicated plasmids, and then treated with MG132 for 12 hr. Immunoprecipitation was performed using anti-myc Ab. Co-immunoprecipitated ubiquitins (detected with anti-HA Ab) is shown. The expression controls (myc, His) were given in the Input. **(C)** HEK293 cells were co-transfected with His-empty, His-TβRI CA, or His-TβRI KD along with myc-CK2β. Equal amounts of cell lysates were blotted for anti-His, anti-myc, and anti-β-actin Ab. **(D)** A549 cells were transfected with myc-CK2β, and then, pre-treated with DMSO, SB431542 (10 μM), and MG132 for 12 hr, respectively, and then, treated with TGF-β for the indicated times. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CK2α, anti-CK2β, and anti-β-actin Ab.

6. CHIP is an E3 ubiquitin ligase for PKCK2β degradation

To investigate which E3 ligase might be involved in the ubiquitinaiton of CK2β, some of E3 ligases involved in TGF-β signaling were co-expressed with CK2β. Results showed that among tested E3 ligases, CHIP most effectively down-regulated CK2β expression (Fig. 6A). Similarly, in the presence of MG132, CHIP could not degrade CK2β (Fig. 6B), and ubiquitination of CK2β was increased in the presence of CHIP (Fig. 6C). Moreover, CKβ was more degraded in the presence of CHIP and TGF-β signaling than either CHIP alone or TGF-β signaling alone (Fig. 6D). To examine the interaction between CHIP and CK2β, immunoprecipitation (IP) was performed and showed an interaction between CHIP and CK2β (Fig. 6E). To examine the effect of the absence of CHIP on CK2β expression, siRNA against CHIP was used for knockdown. The expression of CK2β in CHIP knockdown A549 cells were not changed even in the presence of TGF-β, suggesting that CHIP is the E3 ubiquitin ligase for CK2β degradation (Fig. 6F).

A.

B.

C.

D.

33
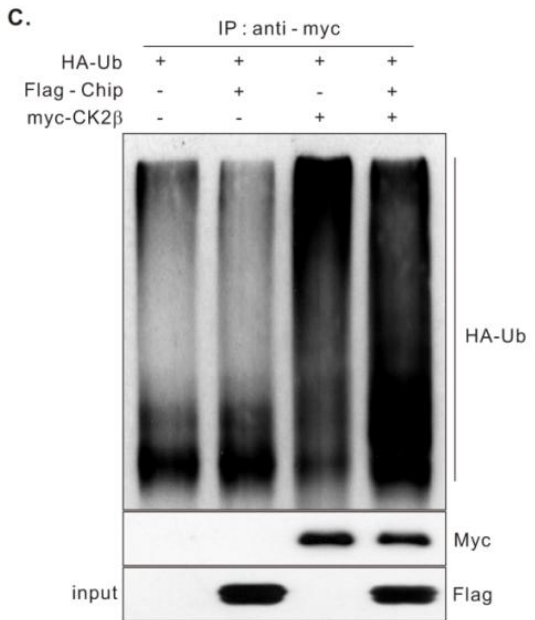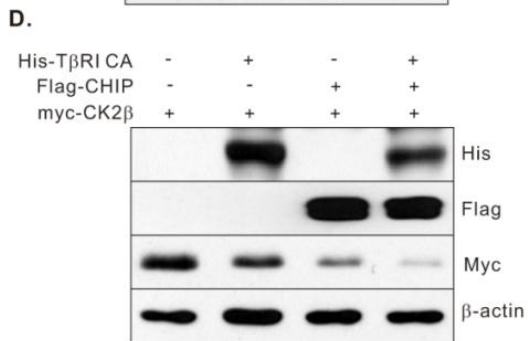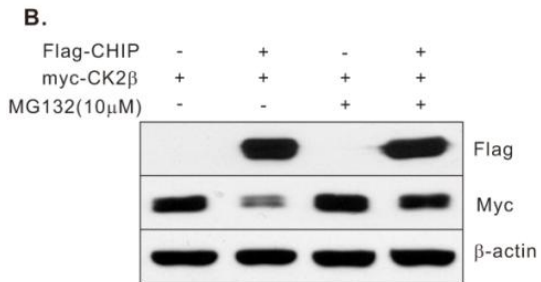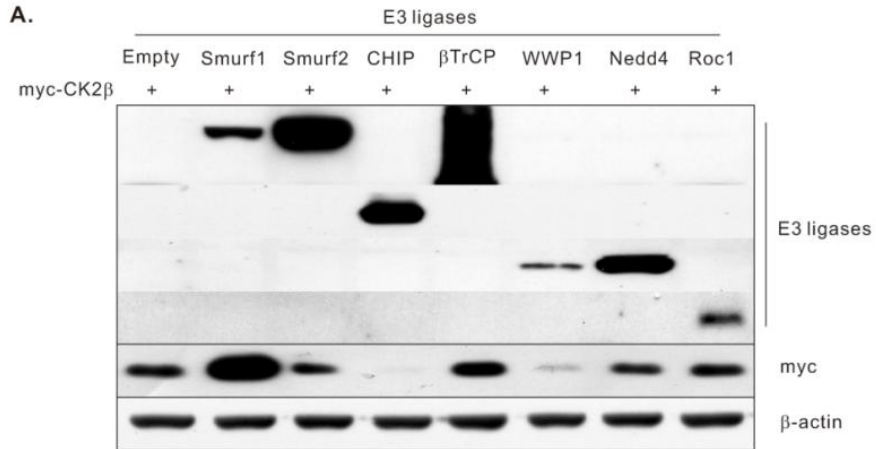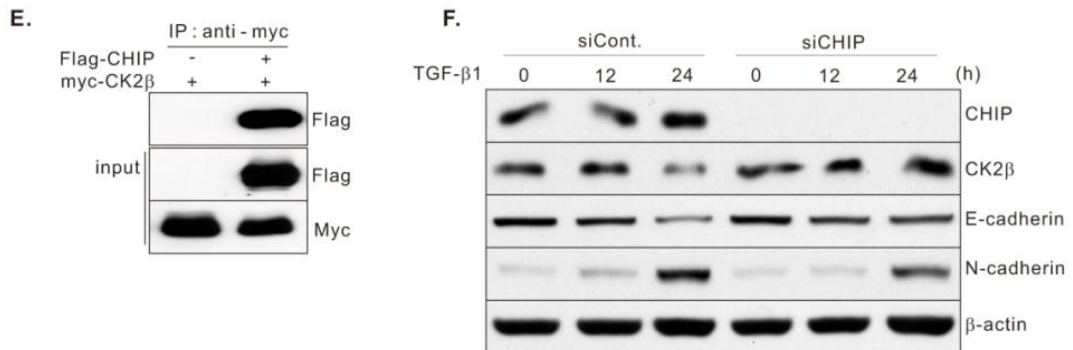
Figure 6. CHIP as an E3 ubiquitin ligase for PKCK2β degradation. (A) HEK293 cells were co-transfected with indicated E3 ligase plasmids along with myc-CK2β. Equal amounts of cell lysates were blotted for anti-Flag, anti-myc, anti-HA and anti-β-actin Ab. **(B)** HEK293 cells were co-transfected with Flag-empty or Flag-CHIP along with myc-CK2β, and then treated or untreated with MG132 (10 μM). Equal amounts of cell lysates were blotted for anti-Flag, anti-myc, and anti-β-actin Ab. **(C)** HEK293 cells were co-transfected with indicated plasmids, and then treated with MG132 for 12 hr. Immunoprecipitation was performed using anti-myc Ab. Co-immunoprecipitated ubiquitins (detected with anti-HA Ab) is shown. The expression controls (myc, Flag) were given in the Input. **(D)** HEK293 cells were co-transfected with indicated plasmids. Equal amounts of cell lysates were blotted for anti-His, anti-Flag, anti-myc, and anti-β-actin Ab. **(E)** HEK293 cells were co-transfected with Flag-CHIP and myc-CK2β, and the cell lysates were subjected to immunoprecipitation using anti-myc Ab. Co-immunoprecipitated CHIP (detected with anti-Flag Ab) is shown. The expression control (Flag, myc) was given in the Input. **(F)** A549 cells were transfected with siRNA against CHIP, and then, treated with TGF-β for the indicated times. Equal amounts of cell lysates were blotted for anti-E-cadherin, anti-N-cadherin, anti-CHIP, anti-CK2β, and anti-β-actin Ab.

34

7. Possible involvement of protein phosphatase in PKCK2β degradation

To examine the effect of CK2α on TGF-β signaling mediated CK2β degradation, the level of CK2β expression was examined during TGF-β signaling in the presence or absence of CK2α expression. Results showed that in the presence of CK2α, CK2β mobility was shifted and the expression was not down-regulated (Fig.7A) indicating that CK2β is phosphorylated by CK2α and the phosphorylated CK2β might not be degraded by TGF-β signaling. To examine whether CK2α mediated phosphorylation of CK2β could stabilize CK2β, cells were treated with CHX for the indicated times and found that CK2β phosphorylation resulted in the stabilization of CK2β (Fig.7B).

To examine whether de-phosphorylation of CK2β is prerequisite for degradation by TGF-β signaling, transfected cells were treated with OA. In the absence of OA, exogenous CK2β was down-regulated by TβRI CA expression, but in the presence of OA, CK2β was not down-regulated (Fig.7C). To confirm this result, A549 cells were pre-treated with OA and then treated with TGF-β for the indicated times. In the absence of OA, CK2β is down-regulated during TGF-β signaling, but in the presence of OA, no change was observed (Fig. 7D).

A.

His-TβRI CA	-	+	+	-	+	+
HA-CK2α	-	-	-	+	+	+
myc-CK2β	+	+	+	+	+	+
MG132(10μM)	-	-	+	-	-	+

His

HA

Myc

β-actin

B.

HA-CK2α	-	-	-	+	+	+
myc-CK2β	+	+	+	+	+	+
CHX(30μM)	0	2	4	0	2	4	(h)

Myc

HA

β-actin

C.

His-TβRI CA	-	-	+	+
myc-CK2β	+	+	+	+
OA (2nM)	-	+	-	+

His

Myc

β-actin

D.

	- OA (2nM)							+ OA (2nM)					
TGF-β1	0	1	3	6	12	24	(h)	0	1	3	6	12	24

CK2α

CK2β

β-actin

36
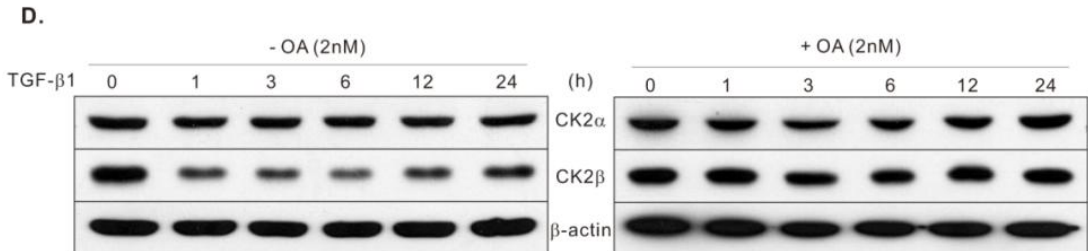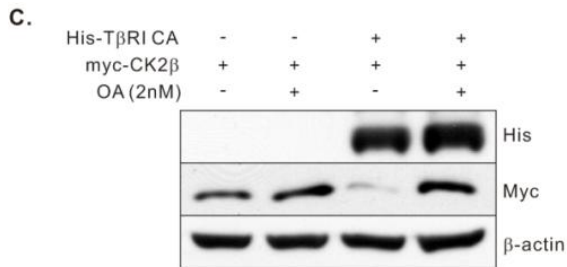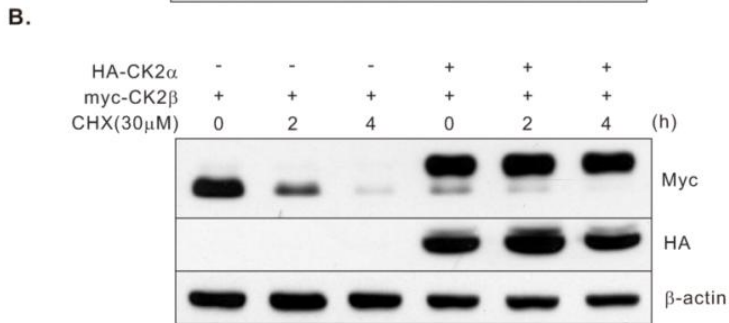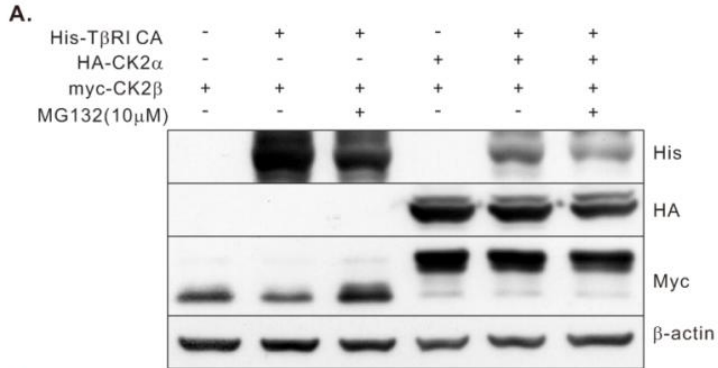
Figure 7. Possible involvement of protein phosphatase in PKCK2β degradation. (A) HEK293 cells were co-transfected with indicated plasmids and then treated with MG132 (10 μM) for 12 hr. Equal amounts of cell lysates were blotted for anti-His, anti-HA, anti-myc, and anti-β-actin Ab. **(B)** HEK293 cells were co-transfected with indicated plasmids and then treated with CHX (30 μM) for indicated times. Equal amounts of cell lysates were blotted for anti-HA, anti-myc, and anti-β-actin Ab. **(C)** HEK293 cells were co-transfected with indicated plasmids and then treated with OA for 12 hr. Equal amounts of cell lysates were blotted for anti-His, anti-myc, and anti-β-actin Ab. **(D)** A549 cells were pre-treated with OA (2 nM) for 12 hr and then treated with TGF-β for the indicated times. Equal amounts of cell lysates were blotted for anti-CK2α, anti-CK2β, and anti-β-actin Ab.

8. De-phosphorylation mediated degradation of PKCK2β

CK2α multiply phosphorylates CK2β on serine 2, serine 3, serine 4, and serine 209.[32,33] To examine the effect of CK2β phosphorylation on its degradation, non-phosphorylatable CK2β mutant, in which 4 serine residues are replaced with alanine (4A mutant) or phosphomimic CK2β mutant, in which 4 serine residues are replaced with glutamic acid (4E mutant) was generated. Ubiquitination assay showed that 4A mutant was more strongly ubiquitinated than wild type (WT) or 4E mutant (Fig. 8A). To examine the effect of TGF-β signaling on CK2 β phosphomutants, each mutant was co-expressed with TβRI CA. Results showed that the expression of 4A mutant was decreased by TGF-β signaling similarly to WT but the expression of 4E mutant was not (Fig. 8B). To examine the effect of CK2β phosphorylation on the interaction with CHIP, each phosphomutant was co-expressed with CHIP. Results showed that the interaction between CK2β and CHIP became weaker when CK2β is phosphorylated by CK2α or when phosphomimic CK2β was co-expressed with CHIP than when CK2β is not phosphorylated (Fig. 8C).

A.

IP : anti - myc

| HA-Ub | + | + | + | + |
| myc-CK2β | - | WT | A | E |

HA-Ub

input — myc

B.

myc-CK2β

| | WT | | A | | E | |
| His-TβRI CA | - | + | - | + | - | + |

His

Myc

β-actin

C.

IP : anti - myc

Flag-CHIP	-	+	-	+	+	+
HA-CK2α	-	-	-	-	+	-
myc-CK2β	-	-	+	+	+	E

Flag

Myc

input — Flag

HA

Myc
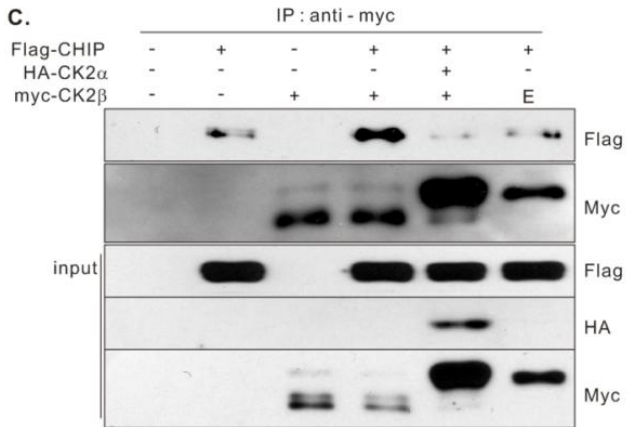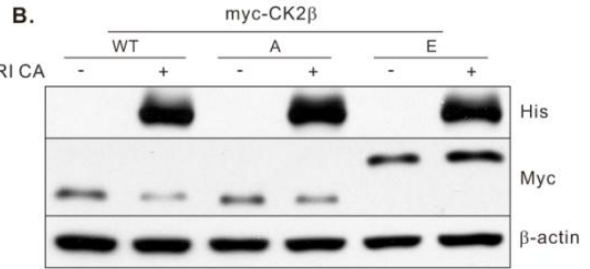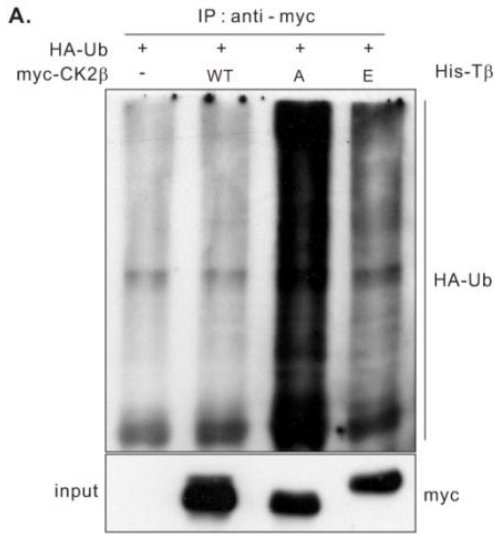
Figure 8. De-phosphorylation mediated degradation of PKCK2β. (A) HEK293 cells were co-transfected with indicated plasmids and then treated with MG132 for 12 hr. Immunoprecipitation was performed using anti-myc Ab. Co-immunoprecipitated ubiquitins (detected with anti-HA Ab) is shown. The expression control (myc) was given in the Input. **(B)** HEK293 cells were co-transfected with indicated plasmids. Equal amounts of cell lysates were blotted for anti-His, anti-myc, and anti-β-actin Ab. **(C)** HEK293 cells were co-transfected with indicated plasmids. Immunoprecipitation was performed using anti-myc Ab. Co-immunoprecipitated CHIP (detected with anti-Flag Ab) and CK2β (detected with anti-myc Ab) are shown. The expression controls (Flag, HA, and myc) were given in the Input.

IV. DISCUSSION

EMT is important for cancer progression and metastasis. TGF-β is one of the cytokines that are strongly associated with EMT. Recently, it was reported that the over-expression of PKCK2 catalytic α subunit alone could induce E- to N-cadherin switching. This study shows that PKCK2 activation is required during TGF-β mediated EMT process and that PKCK2 activity is regulated by the imbalance between the expression level of CK2α and CK2β.

Molecular mechanism of PKCK2 activity regulation is still largely unknown. While PKCK2 has been known as a growth factor-independent and constitutively active protein kinase,[34] EGF could activate PKCK2.[35] The correlation between PKCK2 activity and expression of the PKCK2α catalytic subunit remains controversial. Recent studies examining cell lines or primary tumors showed that intracellular PKCK2 activity correlates with the expression level of the PKCK2α catalytic subunit.[25,36] However, results in cancer cell lines showed that expression levels of the PKCK2α catalytic subunit did not correlate with intracellular PKCK2 activity.[37] Although CK2β subunit regulates catalytic activity, it plays an important roles in forming tetrameric PKCK2 honoenzyme[38] and in regulating catalytic site accessibility of substrates.[39] EMT was induced either with overexpression of CK2α catalytic subunit alone[22] or CK2β silencing (Fig. 4C),[30] suggesting that down-

regulation of regulatory CK2β subunit could result in increase in PKCK2 activity. As documented in these experiments, an increase in the expression of subunit ratio (CK2α/CK2β) could induce increase in PKCK2 activity, thereby inducing EMT.[31]

MG132 could protect TGF-β signaling mediated CK2β down-regulation (Fig. 5), suggesting the involvement of proteasome in CK2β degradation. Many E3 ubiquitin ligases such as Smad ubiquitin regulatory factor 1 (Smurf1),[40,41] Smurf2,[42-45] neural precursor cells-expressed developmentally down-regulated 4 (Nedd4-2),[37,46] WWP1 /TGIF-interacting ubiquitin ligase 1 (Tiul1),[46-48] Itch/atrophin-1 interacting protein 4 (AIP4),[49] or CHIP[50] was reported to be involved in TGF-β signaling. Some of those E3 ubiquitin ligases were screened and only CHIP down-regulated the expression level of CK2β (data not shown). Over-expression and siRNA mediated knockdown of CHIP confirmed that CHIP is the E3 ubiquitin ligase for CK2β degradation (Fig. 6).

As PKCK2 activation (Fig. 2C & D) and CK2β down-regulation (Fig. 5C) require TβRI kinase activity. Phosphorylation is often required for substrate recognition by E3 ubiquitin ligase but CK2β is not phosphorylated by TβRI (data not shown). Instead, CK2β was not degraded in the presence of OA (Fig. 7D), indicating that de-phosphorylation is required for the degradation. CK2β is phosphorylated by CK2α and these phosphorylation protected CK2β from TGF- β signaling mediated degradation. Consistent with this, CHIP did not interact with phosphomimic form of CK2β (Fig. 8C). Accordingly, as yet unknown

phosphatase might be activated during TGF-β signaling for CK2β de-phosphorylation. As PKCK2 activation and CK2β down-regulation require TβRI kinase activity, protein phosphatase 2A (PP2A) could be a possible phosphatase involved in CK2β de-phosphorylation. Activated TβRI interact directly with the regulatory subunit Bα of PP2A. [51,52]

In advanced cancers, TGF-β could enhance invasiveness and metastasis through inducing EMT. In this study, PKCK2 activation and requirement in TGF-β signaling induced EMT. Accordingly, perturbations of PKCK2 activation could have potential therapeutic benefit against a subset of patient population with tumor showing high PKCK2 activity.

V. CONCLUSION

Upon the activation of TGF-β signaling, as yet unknown protein phosphatase is activated by TGF-β receptor I then, activated phosphatase dephosphorylates PKCK2 regulatory β subunit. CHIP binds to de-phosphorylated CK2β undergoes proteasome dependent degradation through ubiquitination. CK2β degradation results in imbalance between the CK2α catalytic subunit and CK2β regulatory subunit, thereby resulting in PKCK2 activity increase (Fig. 9). The activated PKCK2 directly and indirectly affects the molecules involved in EMT.
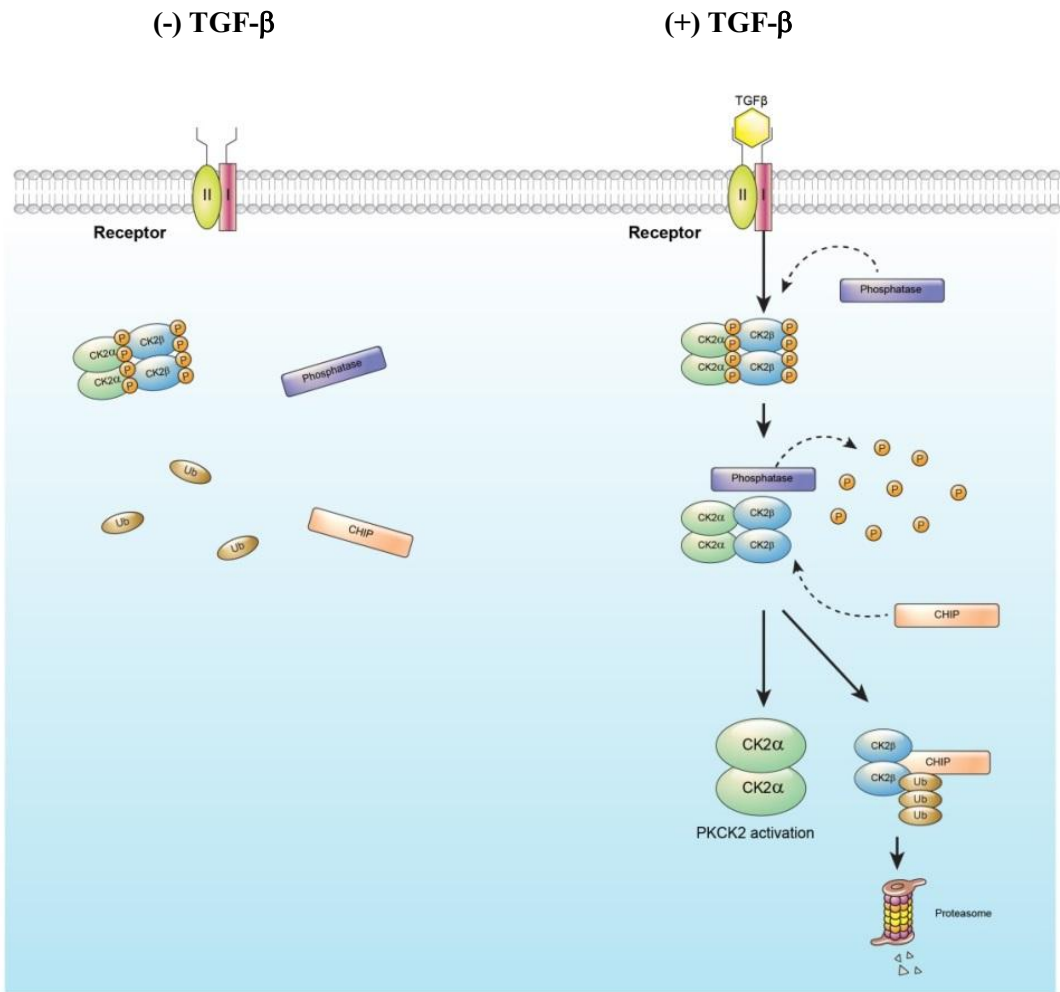
Figure 9. A schematic cartoon for explaining molecular mechanism of TGF-β mediated PKCK2 activation.

REFERENCES

1.   Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009;139:871-90.

2.   Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-8.

3.   Nieto MA. The ins and outs of the epithelial to mesenchymal transition in health and disease. Annu Rev Cell Dev Biol 2011;27:347-76.

4.   Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol 1999;147:631-44.

5.   Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol 2003;19:207-35.

6.   Cavallaro U, Schaffhauser B, Christofori G. Cadherins and the tumour progression: is it all in a switch? Cancer Lett 2002;176:123-8.

7.   Christofori G. Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. Embo j 2003;22:2318-23.

8.   Tang A, Eller MS, Hara M, Yaar M, Hirohashi S, Gilchrest BA. E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci 1994;107 (Pt 4):983-92.

9.   Hsu MY, Wheelock MJ, Johnson KR, Herlyn M. Shifts in cadherin profiles between human normal melanocytes and melanomas. J Investig Dermatol Symp Proc 1996;1:188-94.

10.   Scott GA, Cassidy L. Rac1 mediates dendrite formation in response to melanocyte stimulating hormone and ultraviolet light in a murine melanoma model. J Invest Dermatol 1998;111:243-50.

11.   Li G, Satyamoorthy K, Herlyn M. N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. Cancer Res 2001;61:3819-25.

12.   Tomita K, van Bokhoven A, van Leenders GJ, Ruijter ET, Jansen CF, Bussemakers MJ, et al. Cadherin switching in human prostate cancer progression. Cancer Res 2000;60:3650-4.

13.   Ahmad KA, Wang G, Slaton J, Unger G, Ahmed K. Targeting CK2 for cancer therapy. Anticancer Drugs 2005;16:1037-43.

14.   Song DH, Sussman DJ, Seldin DC. Endogenous protein kinase CK2 participates in

Wnt signaling in mammary epithelial cells. J Biol Chem 2000;275:23790-7.

15. Unger GM, Davis AT, Slaton JW, Ahmed K. Protein kinase CK2 as regulator of cell survival: implications for cancer therapy. Curr Cancer Drug Targets 2004;4:77-84.

16. Trembley JH, Chen Z, Unger G, Slaton J, Kren BT, Van Waes C, et al. Emergence of protein kinase CK2 as a key target in cancer therapy. Biofactors 2010;36:187-95.

17. Shin S, Lee Y, Kim W, Ko H, Choi H, Kim K. Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. Embo j 2005;24:3532-42.

18. Song K, Chen Y, Goke R, Wilmen A, Seidel C, Goke A, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. J Exp Med 2000;191:1095-104.

19. Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene 2001;20:3247-57.

20. Kim JS, Eom JI, Cheong JW, Choi AJ, Lee JK, Yang WI, et al. Protein kinase CK2alpha as an unfavorable prognostic marker and novel therapeutic target in acute myeloid leukemia. Clin Cancer Res 2007;13:1019-28.

21. Scaglioni PP, Yung TM, Cai LF, Erdjument-Bromage H, Kaufman AJ, Singh B, et al. A CK2-dependent mechanism for degradation of the PML tumor suppressor. Cell 2006;126:269-83.

22. Ko H, Kim S, Jin CH, Lee E, Ham S, Yook JI, et al. Protein kinase casein kinase 2-mediated upregulation of N-cadherin confers anoikis resistance on esophageal carcinoma cells. Mol Cancer Res 2012;10:1032-8.

23. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442-54.

24. Massague J. TGFbeta in Cancer. Cell 2008;134:215-30.

25. Moustakas A, Heldin CH. Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci 2007;98:1512-20.

26. Kasai H, Allen JT, Mason RM, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). Respir Res 2005;6:56.

27. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003;425:577-84.

28. Masuyama N, Hanafusa H, Kusakabe M, Shibuya H, Nishida E. Identification of two Smad4 proteins in Xenopus. Their common and distinct properties. J Biol Chem 1999;274:12163-70.

29. Sirard C, Kim S, Mirtsos C, Tadich P, Hoodless PA, Itie A, et al. Targeted disruption in murine cells reveals variable requirement for Smad4 in transforming growth factor beta-related signaling. J Biol Chem 2000;275:2063-70.

30. Deshiere A, Duchemin-Pelletier E, Spreux E, Ciais D, Forcet C, Cochet C, et al. Regulation of epithelial to mesenchymal transition: CK2beta on stage. Mol Cell Biochem 2011;356:11-20.

31. Deshiere A, Duchemin-Pelletier E, Spreux E, Ciais D, Combes F, Vandenbrouck Y, et al. Unbalanced expression of CK2 kinase subunits is sufficient to drive epithelial-to-mesenchymal transition by Snail1 induction. Oncogene 2013;32:1373-83.

32. Zhang C, Vilk G, Canton DA, Litchfield DW. Phosphorylation regulates the stability of the regulatory CK2beta subunit. Oncogene 2002;21:3754-64.

33. Gotz C, Kartarius S, Schetting S, Montenarh M. Immunologically defined subclasses of the protein kinase CK2 beta-subunit in prostate carcinoma cell lines. Mol Cell Biochem 2005;274:181-7.

34.   Allende JE, Allende CC. Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB J 1995;9:313-23.

35.   Ji H, Wang J, Nika H, Hawke D, Keezer S, Ge Q, et al. EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin. Mol Cell 2009;36:547-59.

36.   Giusiano S, Cochet C, Filhol O, Duchemin-Pelletier E, Secq V, Bonnier P, et al. Protein kinase CK2alpha subunit over-expression correlates with metastatic risk in breast carcinomas: quantitative immunohistochemistry in tissue microarrays. Eur J Cancer 2011;47:792-801.

37.   Kuratomi G, Komuro A, Goto K, Shinozaki M, Miyazawa K, Miyazono K, et al. NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem J 2005;386:461-70.

38.   Graham KC, Litchfield DW. The regulatory beta subunit of protein kinase CK2 mediates formation of tetrameric CK2 complexes. J Biol Chem 2000;275:5003-10.

39.   Gyenis L, Litchfield DW. The emerging CK2 interactome: insights into the

regulation and functions of CK2. Mol Cell Biochem 2008;316:5-14.

40.   Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurf1

interacts with transforming growth factor-beta type I receptor through Smad7 and

induces receptor degradation. J Biol Chem 2001;276:12477-80.

41.   Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH. A SMAD ubiquitin ligase

targets the BMP pathway and affects embryonic pattern formation. Nature

1999;400:687-93.

42.   Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, et al. Smad7

binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for

degradation. Mol Cell 2000;6:1365-75.

43.   Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin E3 ligase mediating proteasome-

dependent degradation of Smad2 in transforming growth factor-beta signaling. J

Biol Chem 2000;275:36818-22.

44.   Zhang Y, Chang C, Gehling DJ, Hemmati-Brivanlou A, Derynck R. Regulation of

Smad degradation and activity by Smurf2, an E3 ubiquitin ligase. Proc Natl Acad

Sci U S A 2001;98:974-9.

45.   Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, et al. TGF-beta

induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. Nat Cell Biol 2001;3:587-95.

46. Moren A, Imamura T, Miyazono K, Heldin CH, Moustakas A. Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J Biol Chem 2005;280:22115-23.

47. Komuro A, Imamura T, Saitoh M, Yoshida Y, Yamori T, Miyazono K, et al. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene 2004;23:6914-23.

48. Seo SR, Lallemand F, Ferrand N, Pessah M, L'Hoste S, Camonis J, et al. The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. EMBO J 2004;23:3780-92.

49. Bai Y, Yang C, Hu K, Elly C, Liu YC. Itch E3 ligase-mediated regulation of TGF-beta signaling by modulating smad2 phosphorylation. Mol Cell 2004;15:825-31.

50. Li L, Xin H, Xu X, Huang M, Zhang X, Chen Y, et al. CHIP mediates degradation of Smad proteins and potentially regulates Smad-induced transcription. Mol Cell Biol 2004;24:856-64.

51. Griswold-Prenner I, Kamibayashi C, Maruoka EM, Mumby MC, Derynck R.

Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. Mol Cell Biol 1998;18:6595-604.

52.   Petritsch C, Beug H, Balmain A, Oft M. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. Genes Dev 2000;14:3093-101.

ABSTRACT (in Korean)

TGFβ에 의한 상피간엽전이에서 PKCK2의 활성조절

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E- to N- cadherin switching은 암세포의 전이와 침윤 과정에서 중요한 과정인 상피간엽전이 (EMT)의 분자적 특징이다. 최근에 PKCK2α catalytic subunit의 과 발현이 E- to N- cadherin switching을 유도함이 보고되었다. 비소세포형 폐암세포주인

A549에서 TGF-β가 cadherin switching을 유도한다는 것이 잘 알려져 있으므로, 본 연구는 TGF-β에 의한 상피간엽전이에서 PKCK2를 필요로 하는지에 대해 확인하고자 하였다. 먼저, A549 세포주에서 PKCK2 활성화가 없는 상황에서, TGF-β는 cadherin switching을 유발하지 못하였다. PKCK2 활성은 TGF-β 처리 후 24시간부터 증가하기 시작하여 처리 후 48시간에 최대로 증가하였다. 하지만 TGF-β receptor I (TβRI) 억제제는 TGF-β에 의한 상피간엽전이와 PKCK2 활성화를 억제하였다. 다음으로 PKCK2 활성이 증가될 때, PKCK2의 subunit의 발현을 확인하였다. TGF-β 신호전달이 있을 때, catalytic α sub unit의 발현수준에는 변화가 없지만 regulatory β subunit의 발현수준은 감소하였다. 또한 β subunit knockout은 PKCK2 활성을 증가시키고 E- to N- cadherin switching을 유도하였다. 이는 catalytic subunit과 regulatory subunit의 발현수준 사이의 불균형이 PKCK2 활성을 조절함을 보여주었다. 다음으로, TGF-β 신호전달에 의한 CK2β의 발현감소는 proteasome 의존적이고 TβRI kinase 활성에 의존적인 과정이라는 것을 확인하였다. 우선, C-terminus of HSC70-Interacting Protein (CHIP)이 CK2β의 발현감소에 작용하는 E3 ubiquitin ligase 라는 것을 발견하였다. CK2β는 CK2α에 의해 인산화 되는데, 인산화 된 CK2β는 TGF-β 신호전달에 의해 발현이 조절되지 않았다. 우리는 CK2β가 CK2α에 의해 인산화 될 때, 아직 밝혀지지 않은 protein phosphatase가 CK2β의 탈 인산화에 개입하며, 탈 인산화 된 CK2 β는 CHIP에 의해 유도되는 proteasome 의존적인 분해과정을 겪는다는 것을 확인하였다. 이러한 protein phosphatase의 개입은 protein phosphatase

억제제인 okadaic acid를 처리하였을 때 TGF-β 신호전달에 의해 유도되는 CK2β의 발현 감소가 억제된다는 것을 확인함으로 증명하였다.

종합하여 볼 때, PKCK2의 활성화는 TGF-β에 의한 상피간엽전이 과정 동안 필요하다. CK2α와 CK2β의 발현수준 사이의 불균형은 PKCK2 활성을 조절한다. 암세포에서 PKCK2 활성화의 치료적인 변화는 상피간엽전이를 억제하고 전이를 막는 것이다.