The Effect of MZF-1 phosphorylation by PKCK2 on cancer cells

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Directed by Professor **Kun-Hong Kim**

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

Seong-Rak Kim

December 2010

This certifies that the Master's Thesis of Seong-Rak Kim is approved.

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December 2010

ACKNOWLEDGEMENTS

I would like to express gratitude to my graduate advisor Professor Dr. Kun-Hong Kim. His guidance and suggestion were critical in successful outcome of my master degree thesis project. Also I would like to thank the other members of my dissertation committee, Professor Dr. Jong-In Yook and Professor Dr. Tae-Il Kim for their criticism and thoughtful suggestions.

I would like to thank Professor Dr. Kyung-Sup Kim, Dr. Yong-Ho Ahn, Professor Dr. Sang-Wook Park, Professor Dr. Jae-Woo Kim and Professor Dr. Ho-Geun Yoon. They provided wonderful research environment and useful knowledge. I would like to thank all the graduate students and technical support staff of the Department of Biochemistry and Molecular Biology at Yonsei University College of Medicine.

Finally, I would like to express special thanks to my family for their love and support. This work may not have been completed without their support and encouragement.

Seong-Rak Kim

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ABSTRACT

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on cancer cells

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The epithelial mesenchymal transition (EMT) is an important phenomenon during metastasis of cancer cells. When EMT occurs, E- to N- cadherin switching is a

prerequisite. Protein kinase casein kinase 2 (PKCK2) is messenger-independent serine/threonine protein kinase and involved in apoptosis, cell survival, Wnt signaling pathway, DNA repair, and cell cycle. Recently, it is reported that PKCK2 controls the expression of E-cadherin by phosphorylating β-catenin, the key molecule of Wnt signaling pathway and controls the stability of the snail which is the transcriptional repressor of E-cadherin. Myeloid Zinc Finger 1 (MZF1) is a transcription factor which plays an important role in blood cell development. Recently, it is reported that MZF1 is transcription factor for N-cadherin gene expression in osteoblast along with Sp1. Also, PKCK2 phosphorylation consensus motif was found in MZF-1 and thus, it was examined whether MZF-1 could be one of a substrates of PKCK2 and what could be the impact of MZF-1 phosphorylation by PKCK2 on N-cadherin expression.

First, to find which serine / threonine residues within MZF-1 protein can be phosphorylated by PKCK2, KinasePhos2.0 program was used. And then, to confirm that searched residues came through PKCK2 in fact with phosphorylation or not, immunoprecipitation, *In vitro* kinase assay, and site-directed mutagenesis were performed. Consequently, PKCK2 phosphorylates 27th serine residue of MZF-1. In addition, PKCK2-mediated MZF-1 phosphorylation occurred through a direct

interaction with PKCK2. And it was proved through the glutathione S-transferase (GST) pull down assay and immunoprecipitation *in vitro* and *in vivo*. Next, to confirm whether PKCK2-mediated MZF-1 phosphorylation affects the MZF-1 protein was examined, western blot analysis and dual luciferase reporter assay were used. When TBB, the specific inhibitor of PKCK2, was treated, expression of the MZF-1 protein was reduced and N-cadherin promoter activity decreased. Also, it was shown that the phenomenon appears equally in the experiment using the S27A, the non-phosphorylated mutant of MZF-1.

Taken together, PKCK2-mediated MZF1 phosphorylation directly stabilizes MZF-1 protein. Due to this, the promoter activity of N-cadherin is increased and it controls the E- to N-cadherin switching. Thus, these findings demonstrate that PKCK2 has an effect on the metastasis of cancer cell, ultimately.

Key words: Cancer, EMT, PKCK2, MZF-1, N-cadherin

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Ⅰ. INTRODUCTION

In order for a cancer cell to be metastasized, many physiological changes occur in cancer cell. Among the changes, one of the representing phenomena is

epithelial mesenchymal transition (EMT). And there is the E- to N- cadherin switching as one of the important phenomenon prerequisite for EMT.

The cadherins are members of large family of transmembrane glycoproteins that mediate calcium-dependent, homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture.[1-2] The cadherin has subtypes of largely E-, N-, and P-cadherin. Among them, the E-cadherin is the subtype which is expressed importantly in the polarized epithelial cell while the N-cadherin is the subtype which is expressed importantly in the mesenchymal cell.[3] Usually, when the tumor progresses, the E-cadherin is often substituted by the N-cadherin.[4-9] During the tumor cell development, switching of cadherin subtypes allows tumor cells to interact directly with other N-cadherin expressing cells such as fibroblast and vascular endothelial cell to ultimately let the tumor-host cell adhesion, tumor cell invasion and migration to occur effectively to be equipped with conditions for the tumor metastasis. [9-10] Recently, it is reported that E-cadherin expression which is important in the E- to N- cadherin switching is regulated by Wnt/β-catenin signaling.[11-12] However, the regulation mechanism of N-cadherin expression has not been known clearly yet.

PKCK2 is a constitutively active, growth factor-independent serine/threonine protein kinase composed of two catalytic α subunits and two regulatory β subunits. It plays a key role in cell cycle control, cellular differentiation, and proliferation and also participates in the regulation of apoptosis by phosphorylating some apoptosis-related factors.[13-15] Also, when PKCK2 phosphorylates 393rd threonine of β-catenin, it positively regulates Wnt signaling pathway by blocking proteasome dependent degradation of β-catenin and, so it helps translocation β-catenin into nucleus. [16-17] In addition, recent data have shown that Wnt/β-catenin signaling regulated stability of snail, transcriptional repressor of E-cadherin, through Axin2-mediated GSK3β shuttling into cytoplasm. [11-12]

Myeloid Zinc Finger 1 (MZF-1) is a transcription factor which plays an important role in blood cell development. It consists of 1 SCAN domain and 13 zinc finger domains. According to the previous report, the abnormal expression of MZF-1 is related to cancer development.[18-19] And, recent data have shown that the MZF-1 controls the promoter activity and expression of N-cadherin along with Sp1 in the osteoblast.[20] Also, PKCK2 phosphorylation consensus motif was found in MZF-1 from previous studies.

Therefore, this study is intended to reveal that MZF1 is phosphorylated by the PKCK2, find out the phosphorylation site(s) of MZF1 by PKCK2, investigate how PKCK2-mediated MZF1 phosphorylation affects MZF1 protein and N-cadherin promoter activity. Thus, it was shown that MZF-1 phosphorylation by PKCK2 has an effect on the metastasis of cancer cell, ultimately

Ⅱ. MATERIALS AND METHODS

1. Cell lines and reagent

HCE4, human esophageal cancer cell line and HEK293, human embryonic kidney cell line and MCF7, human breast cancer 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). All the cell lines were grown at 37°C in 5% CO_2. The PKCK2 inhibitors, tetrabromobenzotriazole ,TBB (Sigma-Aldrich, St. Louis, MO, USA) were prepared in 20 mM stock using DMSO (Sigma-Aldrich). Control cultures received the same amounts of DMSO, and final DMSO concentrations did not exceed 0.1%.

2. 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 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 for 1 h. The supernatant was collected and 2 μg of anti-FLAG, anti-HA antibody or anti-myc antibody was 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.

3. PKCK2 *in vitro* kinase activity assay

PKCK2 activity was measured as follows. Briefly, 3 μg of bacterially expressed GST-CS (CK2 Substrate) proteins were incubated with glutathione Sepharose 4B beads for 60 min, and they were washed twice using 1x kinase buffer (4 mM MOPS, pH7.2, 5 mM β-glycerolphosphate, 1 mM EGTA, 200 μM sodium orthovanadate, and 200 μM DTT). Then, the beads were incubated 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 twice with 1x kinase buffer. Samples were resuspended with 30 μl of 2x SDS sample loading buffer and subjected to 12% SDS-PAGE followed by staining with coomassie brilliant blue and dried on whatman papers. The ^{32}P incorporation was detected by autoradiography

4. Site-directed mutagenesis of MZF1

To identify the site of PKCK2 phosphorylation in MZF1, 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 :

Table.1 Primer pairs for mutagenesis

Mutant name		Oligonucleotide sequence
S27A	Sense	5' TAGAGGACGCTGAGGAGGAG 3'
	Antisense	5' CTCCTCCTCAGCGTCCTCTA 3'
S27E	Sense	5' TAGAGGACGAGGAGGAGGAG 3'
	Antisense	5' CTCCTCCTCCTCGTCCTCTA 3'

5. GST pull down assay

Three micrograms of GST or GST-PKCK2α was immobilized on 20μl of Glutathione Sepharose™ 4B resin, and then incubated with cell lysates of the HA-MZF1-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.

6. 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, and0.25% sodium deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF) (USB, Cleveland, OH, USA) protease inhibitor cocktail (R&D Systems, Minneapolis, MN, USA), and 1 mM sodium orthovanadate (Sigma-Aldrich). Lysates were briefly vortexed and cleared by centrifugation at 12,000 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 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 : HA tag (Sigma-Aldrich and Covance, New York city, NY, USA), Myc tag (Cell Signaling, Danvers, MA, USA), β-actin (Sigma-Aldrich). 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..

7. Dual-luciferase reporter assay

HCE4 cells and MCF7 cells were seeded in 6-well plate and co-transfected with HA-MZF1, myc-PKCK2α, or empty vector along with several pGL3-Ncad promoter vectors that serial truncated N-cadherin promter regions and renilla luciferase reporter plasmid (pRL-TK) using PolyExpress™ (Boa biotech, Seoul, South Korea). After 48 h of transfection, the cells were treated with the PKCK2

inhibitor, TBB and then washed with PBS and harvested. Cell lysates were prepared using 200 μl of Passive Lysis buffer (Promega, Madison, WI, USA). Aliquots of 20 μl of cleared lysate were assayed for luciferase activity by using a Dual-luciferase® reporter assay system (Promega). Luciferase activity of N-cadherin promoter plasmid was normalized to the luciferase activity of the pRL-TK. All the experiments were performed independently in triplicate.

Ⅲ. RESULTS

1. The PKCK2 phosphorylates the 27th serine residue of MZF-1.

In order to find out phosphorylation site(s) within MZF-1 by PKCK2, the KinasePhos 2.0 which is a program that finds the serine/threonine residue which has a possibility of phosphorylation based on PKCK2α phosphorylation consensus motifs (S/T-X-X-D/E) was used. Consequently, 11 residues (6 serine and 5 threonine) that can be phosphorylated by PKCK2 within MZF-1 were found (Fig. 1A). Also, these existed between the N-terminal of the MZF-1 protein and 360th amino acid. Based on this, to determine phosphorylation site(s) in MZF-1 by PKCK2, protein expression plasmids containing MZF-1 fragments [S1 (fragment 1): aa 1-240, S2: aa 120-360] were constructed (Fig. 1 B). To find out which side of the fragments phosphorylated by the PKCK2, each MZF-1 fragment expression vector was transfected on the HEK293 cell ,human embryonic kidney cell line, and then the *In vitro* Kinase assay after immunoprecition by using the HA antibody. As a result, the phosphorylation occurred strongly on the MZF-1 S1 side (Fig. 1 C). This result shows that the

phosphorylation site(s) by PKCK2 exists in between 1 and 120aa. Since phosphorylation site(s) between 1 and 120aa found by using the KinasePhos 2.0 previously were 27th serine and 111th serine, these residues substituted to non-phosphorylated alanine by using site-directed mutagenesis. First, 27th serine was substituted to non-phosphorylated alanine and *in* vitro kinase assay was performed as in the above method. Fortunately, it was shown that disappearing of phosphorylation strongly (Fig. 1 D). Also, to confirm this result, protein of 1 – 120aa of MZF-1 was custom-manufactured by ATGen (Seongnam-si, South Korea), and then *in vitro* kinase assay was performed. As a result, strong phosphorylation was found (Fig. 1 E).

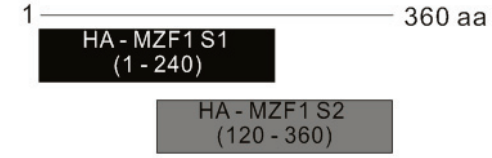
Taken together, these results indicate that PKCK2 phosphorylates MZF-1 and phosphorylation site is 27th serine residue.

A.

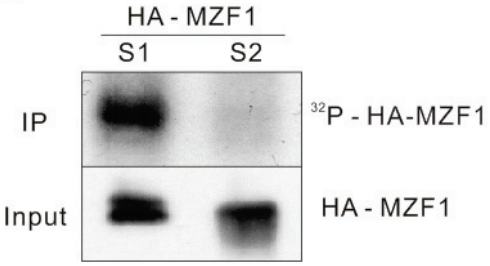
Summary Result				
Protein Name	Predicted Phosphorylated Sites			
	Serine(S)	Threonine(T)	Tyrosine(Y)	Histidine(H)
MZF1_HUMAN	6	5	0	0

```
MRPAVLGSPD RAPPEDEGPV MVKLEDSEEE GEAALWDPGP EAARLRFRCF RYEEATGPQE 60
---------- ---------- ------S--- ---------- ---------- ---------- CK2

ALAQLRELCR QWLRPEVRSK EQMLELLVLE QFLGALPPEI QARVQGQRPG SPEEAAALVD 120
---------- ---------- ---------- ---------- ---------- S--------- CK2

GLRREPGGPR RWVTVQVQGQ EVLSEKMEPS SFQPLPETEP PTPEPGPKTP PRTMQESPLG 180
---------- ---------- ---------- -------T-- ---------- ---------- CK2
---------- ---------- ---------- ---------- ---------- --T------- CK2

LQVKEESEVT EDSDFLESGP LAATQESVPT LLPEEAQRCG TVLDQIFPHS KTGPEGPSWR 240
------S--- ---------- ---------- ---------- ---------- ---------- CK2
---------- --S------- ---------- ---------- ---------- ---------- CK2
---------- ---------- ---T------ ---------- ---------- ---------- CK2
---------- ---------- ---------T ---------- ---------- ---------- CK2
---------- ---------- ---------- ---------- ---------- -------S-- CK2

EHPRALWHEE AGGIFSPGFA LQLGSISAGP GSVSPHLHVP WDLGMAGLSG QIQSPSREGG 300

FAHALLLPSD LRSEQDPTDE DPCRGVGPAL ITTRWRSPRG RSRGRPSTGG GVVRGGRCDV 360
---------- --S------- ---------- ---------- ---------- ---------- CK2
---------- ---------- ---------- ---------- -------T-- ---------- CK2
```

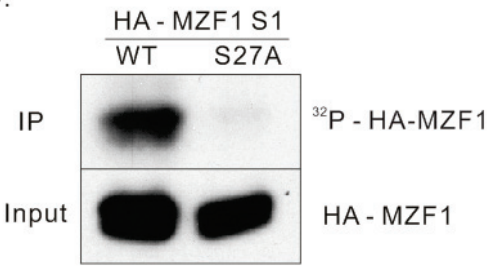
B.



C.



D.



E.

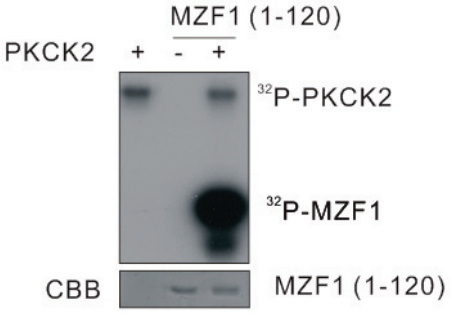


Figure 1. MZF1 is phosphorylated by PKCK2. **A,** The KinasePhos 2 is used to find the residue with the possibility of phosphorylation within MZF1 by PKCK2. **B,** Schematic diagram of expressed MZF1 fragments. **C and D,** HEK293 cells were transfected with HA-MZF1 fragments. Immunoprecipitation was performed using anti-HA antibody followed by *in vitro* kinase assay. Autoradiographic result showed that MZF1 was phosphorylated by PKCK2 (^{32}P-HA-MZF1). The expression and loading controls were given (HA-MZF1). D are shown as the wild type and S27A mutants of HA-MZF1 S1 fragment. **E,** *In vitro* kinase assay was performed using bacterially expressed MZF1protein (1-120aa). ^{32}P-MZF1 represents phosphorylated MZF1 protein. ^{32}P-PKCK2 represents auto-phosphorylated PKCK2. Coomassie blue staining of input MZF1 protein is shown (CBB).

2. PKCK2 and MZF-1 interacts directly.

To find out whether or not PKCK2 phosphorylates MZF-1 by a direct interaction, following studies were performed. First, *in vitro* binding assays were performed using recombinant proteins. HA-MZF-1 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, MZF-1 was observed not with GST alone but with GST-PKCK2α (Fig. 2 A). And then, to examine whether PKCK2α interacts with MZF-1 *in vivo*, HEK293 cells were co-transfected with both HA-PKCK2α and Flag- MZF-1. After immunoprecipitation using anti-HA antibody, MZF-1 was detected in the immunoprecipitates (Fig. 2 B).

Taken together, these results indicate that PKCK2α directly and specifically binds to MZF-1 *in vitro* and *in vivo*.

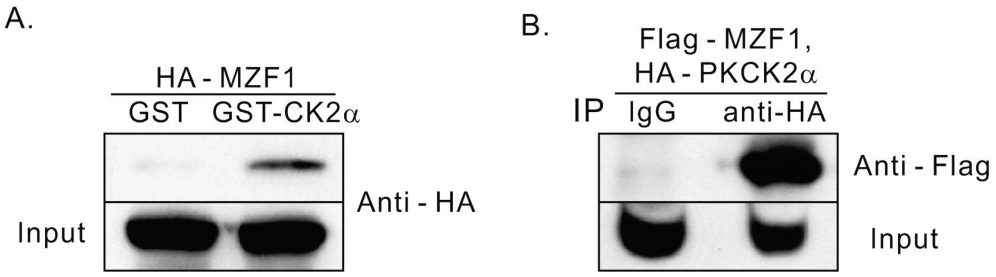


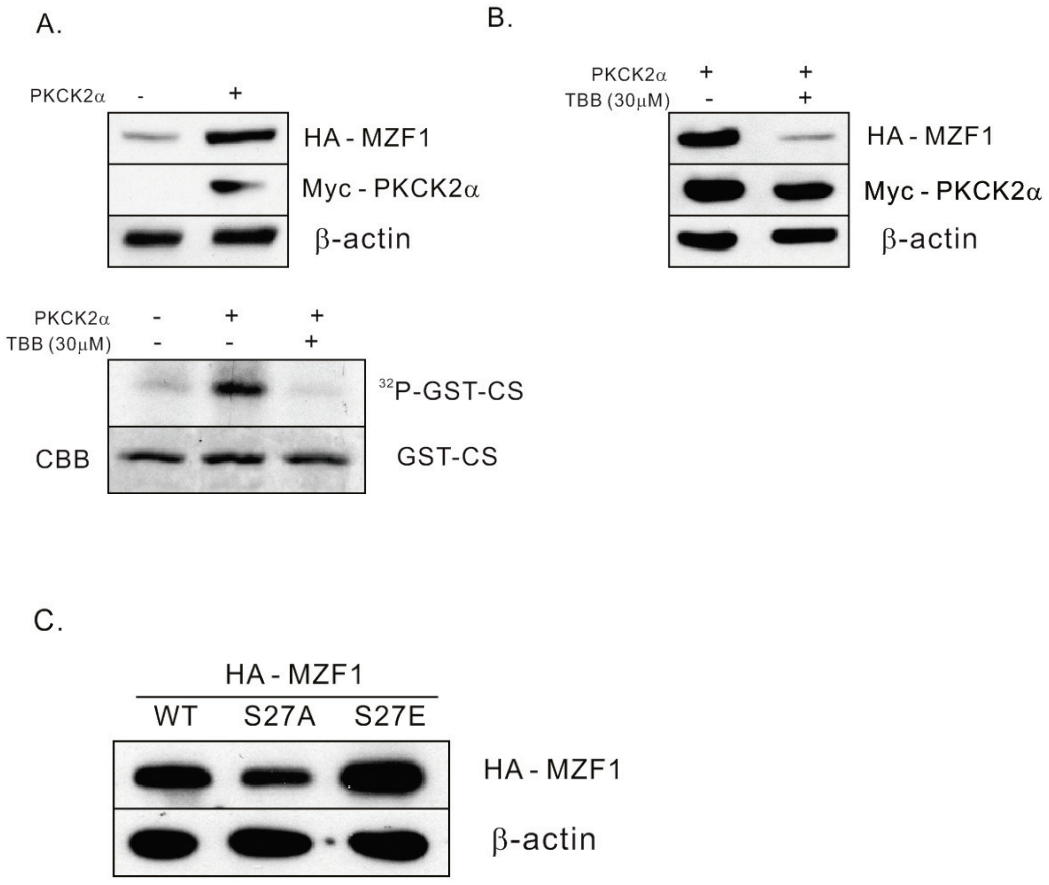
Figure 2. PKCK2 interacts with MZF-1 *In Vitro* and *In Vivo.* **A,** Bacterially expressed GST-PKCK2α was incubated with 1 mg of lysates prepared from HEK293 that was transfected with HA-MZF-1. After pulling-down, the presence of HA-MZF-1 was documented by western analysis using anti-HA antibody. Western results obtained from total cell lysates were given in '*Input'* to provide expression control (HA). **B,** HEK293 cells were co-transfected with Flag-MZF-1 and HA-PKCK2α, and the cell lysates were subjected to immunoprecipitation with anti HA-antibody. Co-immunoprecipitated MZF-1 (detected with anti-Flag antibody) is shown. The expression control (Flag) was given in the Input. The anti-rabbit IgG was used for a negative control.

3. MZF-1 phosphorylation by PKCK2 stabilizes MZF-1 protein.

To determine how PKCK2-mediated MZF-1 phosphoryaltion affects the MZF-1 protein, changes of expression level of MZF-1 protein according to PKCK2 activity were observed. MCF7 cell, known with low PKCK2 activity, was co-transfected with either MZF-1 and empty vector or MZF-1 and PKCK2. And then, after 48 hours, expression level of MZF-1 protein was observed by using western blot analysis. As a result, when MZF-1 expressed along with PKCK2, expression level of MZF-1 protein dramatically increased. (Fig. 3 A ; upper). Then, to confirm that it was due to the PKCK2 activity, expression level of MZF-1 protein was observed after tetrabromobenzotriazole(TBB), specific PKCK2 inhibitor, treatment. As a result, increased expression of MZF-1 decreased again despite inserting PKCK2 (Fig. 3 B). Also, to confirm whether or not PKCK2 activity was reduced by TBB treatment, *In Vitro* Kinase assay was performed. As a result, increased PKCK2 activity by inserting PKCK2 dramatically decreased again despite inserting PKCK2 (Fig. 3 A; lower). Then, to confirm whether or not such phenomenon is directly correlated with PKCK2-mediated MZF-1 phosphorylation, mutant construct which has substituted the 27th serine, phosphorylation site of MZF-1, with non-phosphorylated alanine (S27A) and phospho-mimicked glutamic

acid (S27E) was manufactured. Then, HCE4, known with high PKCK2 activity, was transfected with either wild type(WT) or S27A or S27E, and after 48 hours, the expression level was observed respectively. As a result, it was shown that the expression level of S27A decreased and S27E increased as compared WT (Fig.3 C). This result shown that expression level of MZF-1 protein is regulated PKCK2 mediated MZF-1 phosphorylation directly.

Comprehensively, such results show that MZF-1 protein is stabilized by PKCK2 activity and such phenomenon is correlated with PKCK2 mediated MZF-1 phosphorylation directly.


A.
PKCK2α - +
HA - MZF1
Myc - PKCK2α
β-actin
PKCK2α - + +
TBB (30μM) - - +
32P-GST-CS
CBB
GST-CS
B.
PKCK2α + +
TBB (30μM) - +
HA - MZF1
Myc - PKCK2α
β-actin

C.
HA - MZF1
WT S27A S27E
HA - MZF1
β-actin

Figure 3. PKCK2-mediated MZF1 phosphorylation stabilizes MZF1 protein.

A, Western blot analysis results. MCF7 cells were co-transfected either with HA-MZF1, myc-empty or HA-MZF1, myc-PKCK2α (upper). *In vitro* kinase assay was performed using same cell lysates. ^{32}P-GST-CS represents phosphorylated GST-CS. Coomassie blue staining of input GST-CS is shown (CBB) (lower). **B**, MCF7 cells were co-transfected with HA-MZF1, myc-PKCK2α, and then treated or untreated with TBB. Equal amounts of cell lysates were blotted for anti-HA, anti-myc and β-actin. **C,** HCE4 cells transfected either with HA-MZF1 wt or S27A or S27E. Equal amounts of cell lysates were blotted for anti-HA and β-actin.

4. The phosphorylation of MZF-1 by PKCk2 increases the N-cadherin promoter activity.

Recently, it is reported that MZF-1 is transcription factor for N-cadherin gene expression in osteoblast along with Sp1.[20] Thus, in order to find the MZF-1 biding region which could affect the N-cadherin promoter activity, a N-cadherin promoter 5' serial deletion constructs was manufactured and then, promoter activity assay was performed. Consequently, it was shown that the promoter activity which was maintained at a high level in the deletion construct including parts (-150, -277) assumed to be the MZF-1 binding region became low in the deletion construct in which that region is disappeared (Fig. 4 A). Then, to confirm whether or not the MZF-1 binds on that part to control the N-cadherin promoter activity, MCF7 cell were co-transfected with N-cadherin promoter and MZF-1 and then, promoter activity assay was performed. As a result, it was shown that increase of N-cadherin promoter activity when MZF-1 existed (Fig. 4 B). Then, to confirm whether or not such a phenomenon is controlled by the PKCK2 activity, MCF7 cell were co-transfected with MZF-1 and PKCK2 then, transfected cells were treated TBB and then, promoter activity assay was performed. As a result when TBB was treated, N-cadherin promoter activity dramatically decreased despite inserting of PKCK2 (Fig. 4 C).

Finally, to confirm whether or not such a control of N-cadherin promoter activity by PKCK2 activity is directly related to the phosphorylation of MZF-1, HCE4 cell were transfected with either MZF-1 WT or S27A, non-phosphorylated mutant, or S27E, phospho-mimicked mutant and then, promoter activity assay was performed. As a result, N-cadherin promoter activity decreased in S27A and slightly increased in S27E compared to WT (Fig. 4 D).

Comprehensively, such results indicate that MZF-1 is important transcription factor to regulate N-cadherin promoter activity. And phosphorylation of MZF-1 by the PKCK2 directly controls the N-cadherin promoter activity.

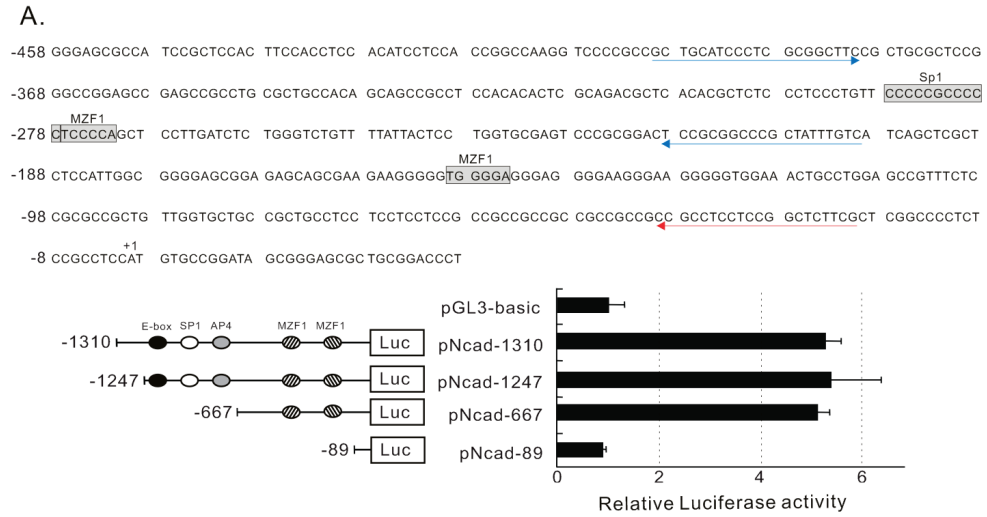

A.
-458 GGGAGCGCCA TCCGCTCCAC TTCCACCTCC ACATCCTCCA CCGGCCAAGG TCCCCGCCGC TGCATCCCTC GCGGCTTCCG CTGCGCTCCG
-368 GGCCGGAGCC GAGCCGCCTG CGCTGCCACA GCAGCCGCCT CCACACACTC GCAGACGCTC ACACGCTCTC CCTCCCTGTT CCCCCGCCCC
-278 CTCCCCAGCT CCTTGATCTC TGGGTCTGTT TTATTACTCC TGGTGCGAGT CCCGCGGACT CCGCGGCCCG CTATTTGTCA TCAGCTCGCT
-188 CTCCATTGGC GGGGAGCGGA GAGCAGCGAA GAAGGGGGTG GGGAGGGAG GGGAAGGGAA GGGGGTGGAA ACTGCCTGGA GCCGTTTCTC
-98 CGCGCCGCTG TTGGTGCTGC CGCTGCCTCC TCCTCCTCCG CCGCCGCCGC CGCCGCCGCC GCCTCCTCCG GCTCTTCGCT CGGCCCCTCT
-8 CCGCCTCCAT GTGCCGGATA GCGGGAGCGC TGCGGACCCT
MZF1
Sp1
MZF1
+1
E-box SP1 AP4
MZF1 MZF1
-1310
-1247
-667
-89
Luc
pGL3-basic
pNcad-1310
pNcad-1247
pNcad-667
pNcad-89
0
2
4
6
Relative Luciferase activity
B.
Relative Luciferase activity
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Empty
MZF1
pGL3 - basic
pNcad -667
C.
Relative Luciferase activity
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MZF1 + Empty + DMSO
MZF1 + CK2a + DMSO
MZF1 + CK2a + TBB 30μM
pGL3 - basic
pNcad -667
D.
Relative Luciferase activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MZF1 WT
MZF1 S27A
MZF1 S27E
pNcad -667

Figure 4. PKCK2-mediated MZF1 phosphorylation up-regulates N-cadherin promoter activity. **A,** The transcription start site was marked by +1 and binding sites for known transcription factors was shown in grey. Luciferase reporter under the control of human N-cadherin promoter pNcad-1310 and its 5' serial deletion constructs were transfected into HCE4 cells. Normalized luciferase activities were shown as mean±SD of three independent experiments in a triplicate and were expressed as fold increased relative to the basal activity. **B,** MCF7 cells were transfected with HA-MZF1, or empty vector along with pGL3-Ncad -667 plasmid in triplicate. After 2 days, relative luciferase activities were measured. pRL-TK plasmid was used for co-transfection in all the experiments to normalize luciferase activity. **C,** MCF7 cells were co-transfected with HA-MZF1, myc-empty vector, or HA-MZF1, myc-PKCK2α along with pGL3-Ncad -667 plasmid in triplicate. After 2 days, transfected cells were treated or untreated with TBB for 12hr, and then relative luciferase activities were measured. **D,** HCE4 cells were transfected with HA-MZF1 [WT or S27A or S27E] along with pGL3-Ncad -667 plasmid in triplicate. After 2 days, relative luciferase activities were measured.

Ⅳ. DISCUSSION

In the metastasis of cancer cells, the epitherial Mesenchymal transition (EMT) is an important phenomenon. And one of the crucial prerequisite for EMT to occur is the E- to N- cadherin switching. It triggers weakness in an interaction among tumor cells and increases the mobility by the decrease of E-cadherin which induces a strong cell-cell adhesion by manifestation in the polarized epithelial cell mainly and by the increase of N-cadherin which is usually manifested in the mesenchymal cell[3-9]. Also, it allows a direct interaction with other N-cadherin expressing cells such as fibroblast and vascular endothelial cell to make an easy migration and invasion of cancer cells by making a direct interaction possible and ultimately induces the metastasis of cancer cells[9-10]. Although many studies have been done relatively on the mechanism of decrease of E-cadherin, not many have been known about the increase of N-cadherin.

PKCK2 is a ubiquitously expressed and constitutively active serine/threonine kinase. It forms a tetramer with two α regulatory subunits and two β 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[21], but the precise role and regulation of this protein kinase remains unclean as ever. Recently, it is reported that PKCK2 controls the expression of E-cadherin by phosphorylating β-catenin, the key molecule of Wnt signaling pathway and controls the stability of the snail which is the transcriptional repressor of E-cadherin.[11-12].

As an important transcription factor to the blood cell development, there have been reports that the abnormal manifestation of MZF-1 is related to cancer. [18-19] Also, recently, it is reported that the MZF-1 controls the promoter activity and expression of N-cadherin along with Sp1 in osteoblast. [20] Thus, based on the possibility that the PKCK2 which controls the expression of E-cadherin through stabilizing the snail can control the expression of N-cadherin through MZF-1 as its medium, this study has been initiated.

First, phosphorylation site(s) by PKCK2 within MZF-1 were searched using KinasePhos 2.0, 11 residues that can be phosphorylated by PKCK2 within MZF-1 were found. And among them, a research was performed to which residue was actually phosphorylated by the PKCK2. And it was found to be the 27th serine

residue. Then, to confirm whether or not the phosphorylation by the PKCK2 can actually control the expression of N-cadherin, the physiological effect of PKCK2-mediated MZF-1 phosphorylation was studied. It was found that the phosphorylation of MZF-1 by PKCK2 stabilizes the MZF-1 protein directly and up-regulates the N-cadherin promoter activity by binding in the N-cadherin promoter region.

Comprehensively, the PKCK2 not only down-regulates the E-cadherin by the snail as its medium, but also up-regulates the N-cadherin through MZF-1 as seen in the study. Ultimately, PKCK2 has an important role in the metastasis as a key molecule which controls the E- to N- cadherin switching

V. CONCLUSION

The PKCK2 interacts with MZF1 directly to phosphorylate the 27th serine residue of MZF-1. And phosphorylation of MZF-1 by PKCK2 directly stabilizes the MZF1 protein and increases activity of N-cadherin promoter.

Comprehensively, the PKCK2 up-regulates the N-cadherin promoter activity by phosphorylating the MZF-1 to control the N-cadherin. Thus, PKCK2 controls the metastasis of cancer cells.

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ABSTRACT (in Korean)

암 세포에서 PKCK2에 의한
MZF-1 인산화의 효과

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암세포가 전이를 일으키는데 있어 epithelial mesenchymal transition(EMT) 는 중요한 현상이다. 이러한 EMT가 일어나는데 E- to N-

cadherin switching은 그 전제조건이 된다. 프로테인 카이네이즈 카제인 카이네이즈2 (PKCK2)는 신호 전달과 무관하게 활성을 갖는 serine/threonine 프로테인 카이네이즈로써 세포 사멸, 세포 생존, DNA 복구 및 세포 주기 조절 등에 관여한다. 최근에 PKCK2가 Wnt signaling pathway의 핵심 인자인 β-catenin을 인산화 시켜 E-cadherin 의 전사 억제자인 snail 의 안정성을 유지시켜 줌으로써 E-cadherin 의 발현을 억제시킨다는 내용이 보고되었다. Myeloid Zinc Finger-1 (MZF-1) 은 혈액 세포 발달에 중요한 역할을 하는 전사 인자인데 최근 보고에 의하면 MZF-1이 osteoblast에서 Sp1과 함께 N-cadherin의 promoter 활성과 단백질 발현을 높인다는 보고가 있었다. 또한 선행 조사 결과 MZF-1에도 PKCK2에 의해 인산화가 일어날 수 있는 보존된 자리가 존재함을 알 수 있었다. 이를 토대로 본 연구에서는 MZF-1과 PKCK2의 관계를 규명하여 보고자 하였다.

먼저 KinasePhos 2.0 프로그램을 통하여 PKCK2에 의하여 인산화가 일어날 수 있는 MZF-1 단백질 내의 serine/threonine 잔기들을 찾아 그들 중 어느 잔기가 실제로 PKCK2에 의해 인산화가 되는지를 면역침전법 및 *In Vitro* 카이네이즈 분석법, 그리고 돌연변이 분석을 통하여 알아보았다. 그 결과 PKCK2가 MZF1의 27번째 serine 잔기를 인산화 시킨다는 것을 알 수 있었다. 또한 PKCK2에 의한 MZF-1의 인산화가 PKCK2와 MZF-1의 직접적인 상호작용에 의한 것임을 알아내었으며 이는 *In Vitro*와 *In Vivo* 상에서 면역침전법을 통해 밝혀냈다. 다음으로 PKCK2에 의한 MZF-1의

인산화가 MZF-1 단백질에 어떠한 영향을 미치는지 살펴보았다. PKCK2의 특이적인 억제제인 TBB를 처리하였을 때 MZF-1 단백질의 발현이 감소하였으며 또한 N-cadherin promoter의 활성이 줄어드는 것을 알 수 있었고 이는 MZF-1의 비인산화 돌연변이인 S27A를 이용한 실험에서도 동일하게 나타나는 현상 임을 알 수 있었다.

종합하여 볼 때, PKCK2에 의한 MZF-1의 인산화는 직접적으로 MZF-1 단백질을 안정화 시키고 이로 인해 N-caherin의 promoter 활성을 증가시킴으로 N-cadherin의 발현을 조절하여 E- to N-cadherin switching을 유발하여 궁극적으로는 암세포의 전이에 영향을 준다고 할 수 있겠다.

핵심되는 말: 암, EMT, PKCK2, MZF1, N-cadherin