A THESIS

FOR THE DEGREE OF MASTER

Functional Analysis of a Human Dual Specificity Phosphatase, DUSP28 in Cell Signaling

Thai Doan Luong

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Jun 2014

A THESIS

FOR THE DEGREE OF MASTER

Functional Analysis of a Human Dual Specificity Phosphatase, DUSP28 in Cell Signaling

Thai Doan Luong

(Supervised by Professor Jae-Hoon Kim)

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Jun 2014

Functional Analysis of a Human Dual Specificity Phosphatase, DUSP28 in Cell Signaling

Thai Doan Luong

(Supervised by professor Jae Hoon Kim)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Biotechnology

Jun, 2014

This thesis has been examined and approved

_____

Chairperson of the supervising committee

Professor Se Pill Park, Ph.D., College of Applied Life Science, Jeju National University, Korea

_____

Professor Somi Kim. Cho, Ph.D., College of Applied Life Science, Jeju National University, Korea

_____

Professor Jae Hoon Kim, Ph.D., College of Applied Life Science, Jeju National University, Korea

Department of Biotechnology

JEJU NATIONAL UNIVERSITY

Content

LIST OF ABBREVIATIONS

DUSP	Dual specificity phosphatase
MAPK	Mitogen-activated protein kinases
JNK	c-Jun N-terminal kinases
ERK	Extracellular signal regulated kinases
P38	p38 mitogen activated protein kinases
MKK4	Dual specificity mitogen activated protein kinase kinase 4
MKB	MAPK binding domain
DSP	Dual specificity phosphatase domain
NES	Nuclear export signal
NLS	Nuclear localization signal sequence
MKP	MAPK phosphatase
LDP	Low molecular mass dual specificity phosphatase

LIST OF FIGURES

1. ABSTRACT

Dual specificity phosphatases (DUSPs) consist of protein phosphatases that catalyze removal of the phosphate moiety from both tyrosine and serine/threonine residues in phosphorylated protein. Due to its structurally and functionally distinct features, the knowledge about DUSPs is quite restrictive with many controversies in their regulation and function.

In this study, we expressed human DUSP28 and subsequently determined its characteristics in Hela cells. DUSP28 was found to be localized in both cytoplasm and nucleus.

We determined its signaling regulation on the activation of JNK and p38 pathways by anisomycin treatment, together with ERK pathway activation by Epidermal Growth Factor (EGF) induction.

The results showed that DUSP28 overexpression reduced ERK and JNK phosphorylation but not p38 phosphorylation. Due to negative regulate the phosphorylation of JNK, DUSP28 also reduced the phosphorylation of c-Jun, a downstream factor of JNK. In addition, DUSP28 down-regulated MKK4 phosphprylation, an upstream factor of JNK, upon anisomycin treatment. As far as we know, this is a first report for DUSP 28 signaling in cells.

Key words: dual specificity phosphatase, c-jun N-terminal kinase (JNK), anisomycin

요약

이중 특이성 인산 가수분해효소 (DUSPs)는 인산화된 단백질에서 티로신과 세린/트레오닌 잔기로부터 인산기 제거를 촉진하는 인산 가수분해효소 단백질 그룹이다. DUSP들은 구조적 기능적으로 독특한 모습을 가지고 있으나, 기능에 있어서 밝혀지지 않은 부분이 많고, 여러 논쟁거리가 되고 있다. 본 연구에서 인간 유래의 DUSP28을 Hela 세포에 발현하여, 그 특성을 밝혀내고자 했다.. DUSP28은 세포질과 핵에서 국부적으로 발현하는 것으로 나타났다. 우리는 표피생장인자(EGF)에 의한 ERK 경로의 활성화와 함께 아니소마이신 처리에 의한 JNK와 p38 경로의 활성화 신호전달 조절 메커니즘을 연구하였다.

그 결과, DUSP28 과발현이 ERK와 JNK의 인산화를 감소시켰지만 p38 인산화에는 영향을 주지 않았다. JNK의 인산화 억제는 JNK의 하류인자인 c-Jun의 인산화 감소를 유도하였다. 또한, DUSP28은 JNK의 상류인자인 MKK4의 인산화를 억제하였다. 세포내에서 DUSP28의 신호전달에 관한 연구로서는 처음으로 보고되는 것이다.

키워드: dual specificity phosphatase, c-jun N-terminal kinase (JNK), anisomycin

2. INTRODUCTION

Cell signaling is a complex communicative systerm that governs cells to perceive and respond to enviromental stimuli. Complex multi-component signal transduction pathways provide opportunities for feedback, signal amplification, and interactions between multiple signals and signaling pathways in cells. Errors in signal transduction result in many diseases such as cancer, diabetes, autoimmunity, etc.

A signal transduction pathway is involved interaction of many proteins induced by internal or external signals. Upon stimulation, many factors will bind to their receptors at the cell surface. Several of these receptors are kinases that start to phosphorylate themselves and other proteins. This phosphorylation can increase its kinase activity and generate a binding site for a different protein and thus induce protein-protein interaction.

For example, epidermal growth factor (EGF) binds to its receptor (EGFR) and activates the receptor to phosphorylate itself. The phosphorylated receptor binds to an adaptor protein (GRB2), which couples the signal to further downstream signaling processes such as mitogen activated protein kinase/ERK (MAPK/ERK) pathway.

The MAPK protein is a kinase protein that can attach phosphate to target proteins such as Myc and Jun transcription factors, thus alter gene transcription and cell cycle progression ultimately. Some signaling transduction pathways response differently, depending on the amount and kind of signaling received by the cell.

Signal transduction pathways play essential roles in cellular processes and involve both activated or inactivated states of target proteins. A cellular signaling process is governed by the phosphorylation or dephosphorylation due to phosphatases and kinases (Philipp Selenko, 2003). Such tight regulation helps the physiological status of biological systerm in a homeotic balance (Hornber, 2005; Owens, 2007). Along with kinase proteins, phosphatases

act on signaling proteins in many transduction events. Unfortunately even keep the essential role as kinases, people used to recognize phosphatases merely as house keeping enzyme.

A short quote in a review article of professor Nicholas: *"There are many misconceptions surrounding the roles of protein phosphatases in the regulation of signal transduction, perhaps the most damaging of which is the erroneous view that these enzymes exert their effects merely as constitutively active housekeeping enzymes. On the contrary, the phosphatases are critical, specific regulators of signalling in their own right and serve an essential function, in a coordinated manner with the kinases, to determine the response to a physiological stimulus" (FEBS Journal280 (2013), 346–378).*

Around these years, the role of phosphatases has been gradually evaluated more deeply and correctly. We presently recognize phosphatases as critical and specific regulators in a coordination with kinases to decide appropriate and swift responses to physiological stimuli (Tonks et al., 2013; Lim et al., 2010). Therefore, characterization of phosphatase protein is very important and necessary in order to make clear full the signaling map.

There is a family of phosphatases for structurally and functionally distinct signaling proteins. Among them, dual specificity phosphatase proteins (DUSPs) have been proved as playing essential roles in signaling transduction, modulating the amplitude and duration of cellular signaling (Bermudez et al., 2009; Patterson et al., 2009; Lydia Tabernero et al., 2008).

Many researchers presently show the interaction between DUSPs and mitogen-activated protein kinases/MAP kinases proteins in order to keep the homeotic balance in cell signaling. Mitogen activated protein kinases (MAPKs) govern variety of physiological processes, including cell proliferation, differentiation, stress responses and apoptosis (Chang and Karin, 2001; Davis et al., 2000).

Mammalian MAPKs involve three major groups, which are classified on the basis of sequence similarity, differential activation by agonists and substrate specificity. They are (i)

the p42/p44 MAPKs or extracellular signal-regulated kinases (ERKs), (ii) the Jun N-terminal kinases (JNK1, JNK2, JNK3), and (iii) the p38 MAPKs (a, b, d and g) (Cohen, 1997).

MAPKs are activated by phosphorylation on threonine and tyrosine residues within a conserved signature sequence TxY by a MAPK kinase (MKK or MEK), which is in turn phosphorylated and activated by a MAPK kinase kinase (MKKK or MEKK) (Marshall et al., 1994; Qi and Elion, 2005). The component kinases of the MAPK module may interact sequentially, but can also be organised into signalling complexes via interactions with specific scaffold proteins (Garrington and Johnson, 1999; Morrison and Davis, 2003).

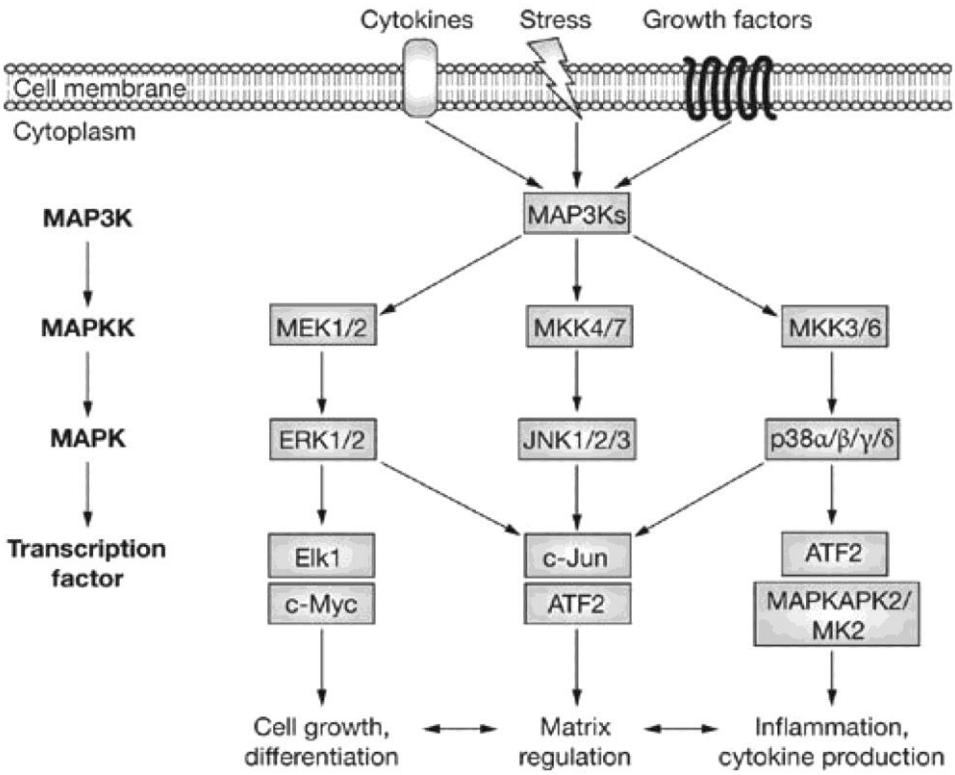

Figure 2.1 The simplified mitogen-activated protein kinase (MAPK) pathway

(Source: Nat Clin Pract Rheumatol (2007), 651-660)

DUSPs/MKP controls a broad signaling network of cellular processes. Briefly, the catalytic cleft contains an active cysteine, in which functionally forms a transient "DUSP-phosphate" intermediate and the release of dephosphorylated substrate. The conserved aspartate accepts a proton from a water molecule as a general acid, thereby neutralizing negative charge on the leaving group. The glutamine positions the nucleophilic water for increasing phosphate hydrolysis. The other residues in signature sequence HCX_2GX_2R also keep important duties in the dephosphorylation reaction (Farooq et al., 2004; Laiping Xie et al., 2002)

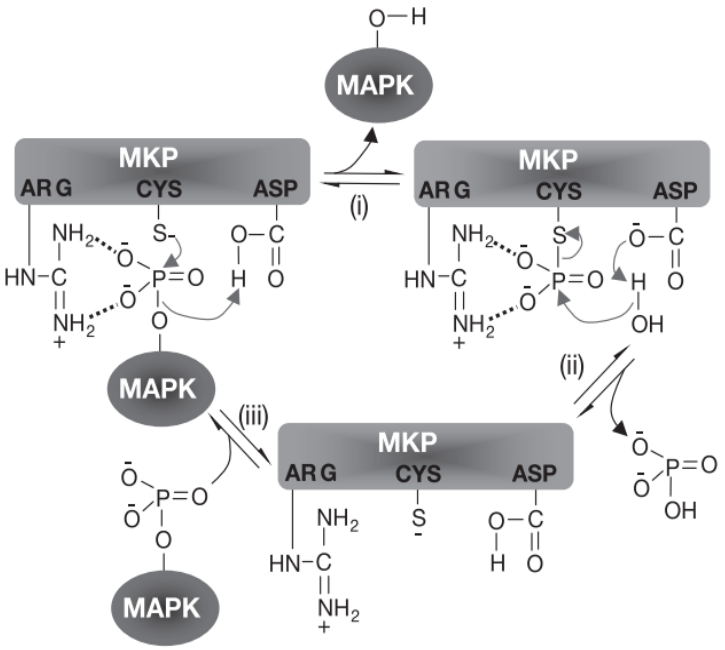

Figure 2.2 Catalytic mechanism of dephosphorylation of MAPKs by MKPs.

(i) Nucleophilic attack of the thiolate anion of the active site Cys of an MKP on the phosphate of pY of an MAPK. (ii) The active site Asp, acting as a general base, accepts a proton from a water molecule. (iii) The regenerated thiolate anion of the MKP binds phosphorylated MAPK and the catalytic cycle is repeated

(Source: Cellular Signalling (2004) 769–779, A. Farooq and M.-M. Zhou)

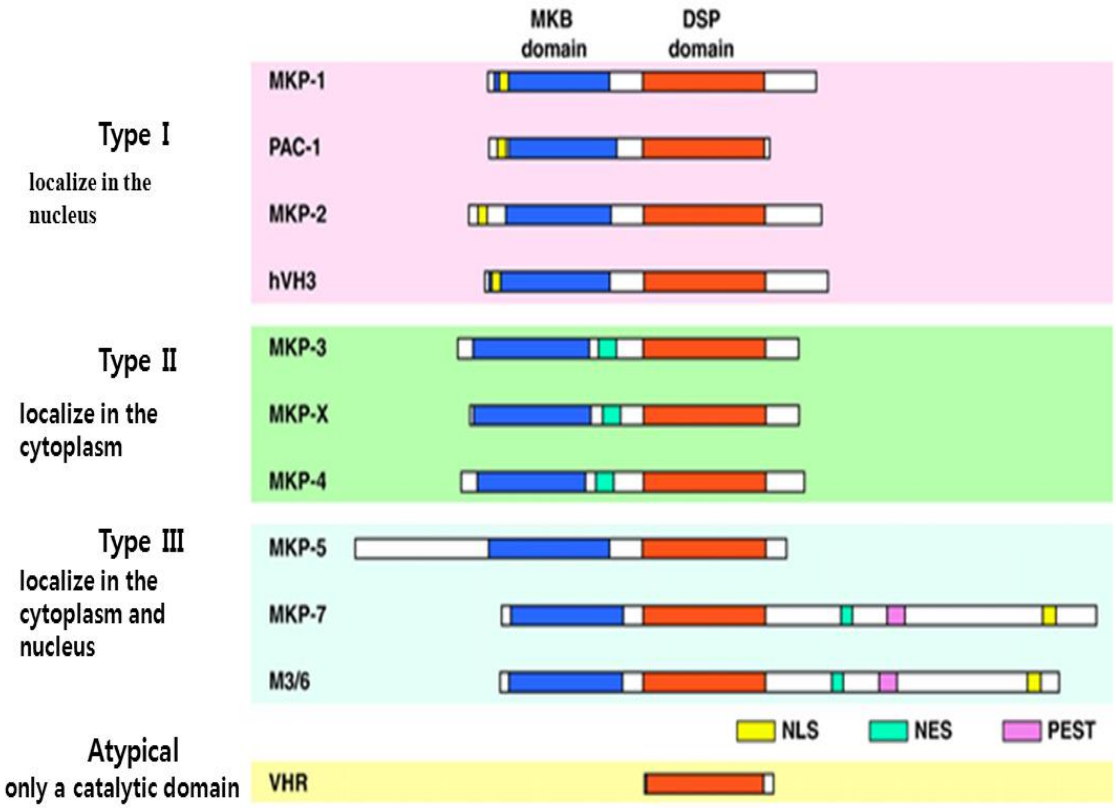

Figure 2.3 Classification and domain structure of the MKP family.

In addition to the MAPK binding (MKB) domain and dual-specificity phosphatase (DUSP) domain, nuclear localization signal (NLS), nuclear export signal (NES), and PEST sequences are indicated.

(Source: Biochimica et Biophysica Acta (2007) 1227–1237, K. Kondoh & E. Nishida)

DUSP28 contains virtually signature sequence HCX_2GX_2R with only conserved histidine is replaced to tyrosine. Studies of DUSP3/VH3 showed that histidine orients the cysteine residue in the catalytic pocket. The residue replacement may confers the catalytic specificities to DUSP28 lacking in other DUSPs.

Herein, we report the expression and characterization of DUSP28 in Hela cells. Upon induced with many stimulators, we studied the cell signalling regulation using DUSP28 wild type and phosphatase inactive C103S mutant.

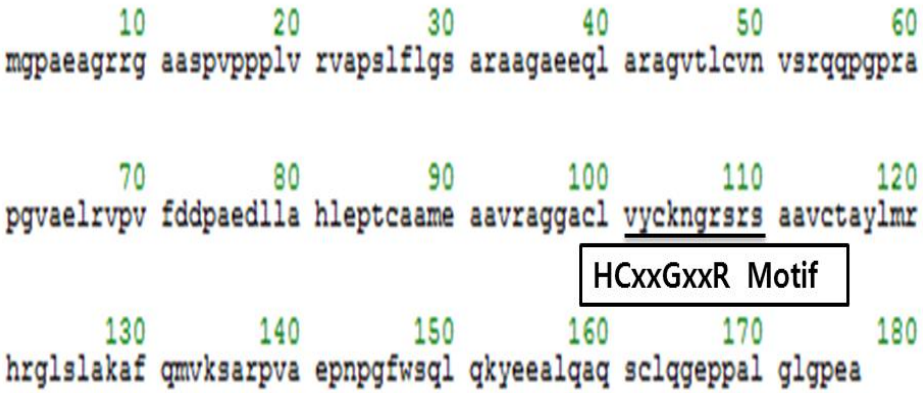

Figure 2.4 The signature sequence HCX_2GX_2R of DUSP28

3. MATERIALS AND METHODS

3.1 Materials

All constructs were transiently transfected into Hela cells, using WelFect-Q^{TM} Plus Transfection Reagent (WelGene).

We collected total protein from transfected cells using Lysis Buffer consisting of 1mM EDTA, 30mM Tris-HCl pH7.4, 150 mM NaCl, 1% Nonidet P-40, 10μg/ml Leupeptin, 5 μg/mlAprotinin, 2 mM Sodium Vanadate, 30 mM Sodium Pyrophosphate, 100 mM NaF, and 1mM PMSF.

Specific antibodies in immunobloting assays included antibody flag (Sigma Aldrich, Madrid, Spain), antibody p-ERK, peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), antibody p-JNK/p38/c-jun/MKK4/MKK6/MKK7 (Cell Signalling Technology, Izasa S.A, Barcelona, Spain), and antibody HA (Roche, USA).

3.2 Vector construction

3.2.1 pcDNA-DUSP28-flag expression constructs

In this study, we futher subcloned DUSP28 full lenght into the *NheI –XhoI* site of the mammalian expression vector pcDNA 3.1 with flag tagged C-terminus. Using 2 specific primers :

DUSP28_NheI_F; 5’-GCTAGCTAGCCACCATGGGACCGGCAGAAGCTGG-3’.

DUSP28_XhoI_R; 5’-GCCGCTCGA GAGCCTCAGGGCCCAACCCTA- 3’.

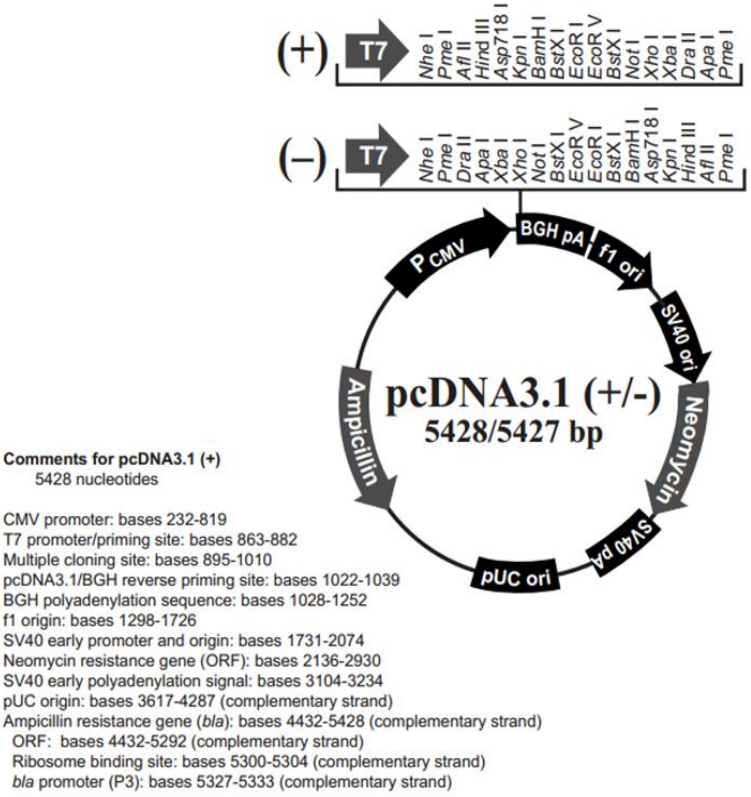

Figure 3.1 The information of mammalian expression plamid pcDNA 3.1

3.2.2 pEGFPN1-DUSP28 and pEGFPN1-CAXX constructs

In order to determine the subcellular localization of DUSP28. We tagged GFP into the C-terminus of DUSP28. In addition, a plasma membrane targeting CAAX motif, was attached at c-terminus of GFP. Using 2 specific primer:

CMV_F; 5’- CGCAAATGG GCGGTAGGCGTG-3’.

CAAX_R; 5'-GGCGTCTAGATCAGGAGAGCACACACTTGCAGCTCATGCAGCCGGG GCCCTTGTACAGCTCGTCCATGCC-3’.

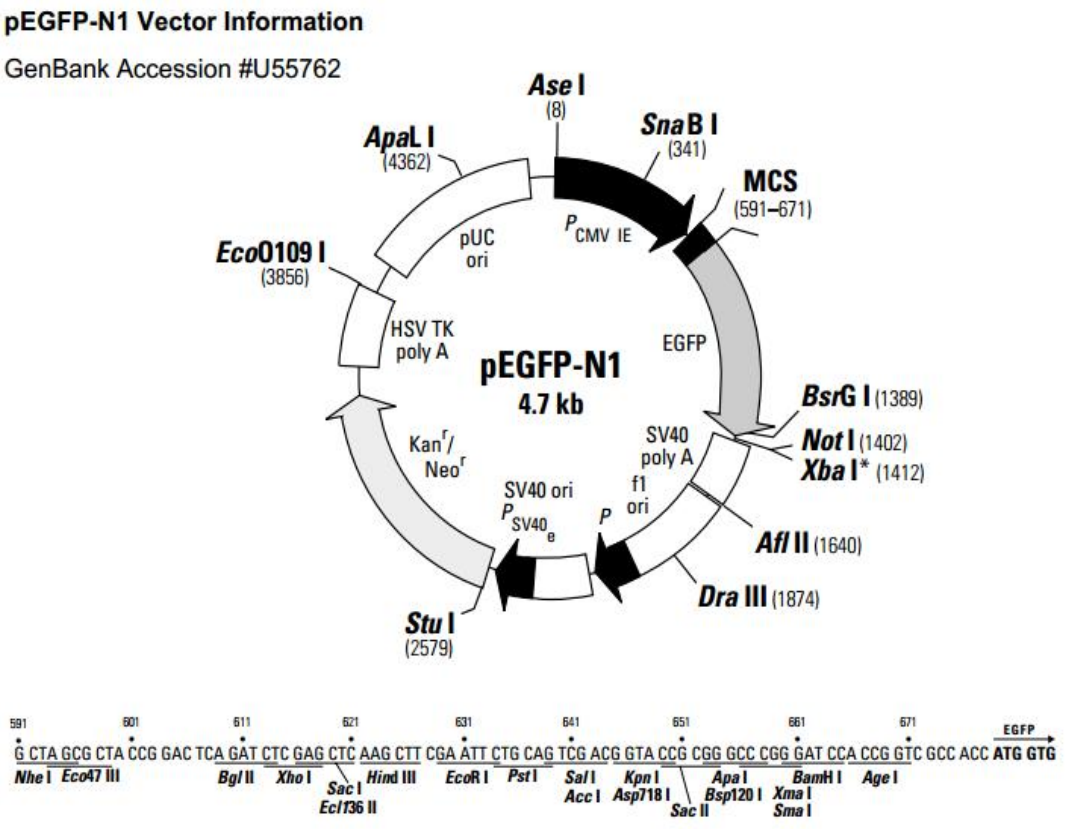

Figure 3.2 The information of mammalian expression plamid pEGFPN1

3.2.3 pcDNA-MKK4/MKK6/MKK7-HA constructs

In order to determine the signaling regulation of DUSP28 in MKK4/MKK6/MKK7, upstream kinases of JNK and p38 kinase, we futher subcloned MKK4/MKK6/MKK7 into eukaryotic expression vector pcDNA 3.1.

To pcDNA-MKK6-HA, we first used 2 specific primers to amplify the full lenght of MKK6 by PCR. We then inserted the templates into NheI –XhoI site of eukaryotic expression vector pcDNA 3.1 with HA tagged C-terminus.

MKK6_NheI_F; 5’-GACGATGCTAGCATGTCTCAGTCG-3’

MKK6_XhoI_R; 5’-GACGATGATGCTAGCTCTCAGTCG-3’

To pcDNA-MKK4-HA and pc-DNA-MKK7-HA. We first used 2 specific primers that matched to CMV promoter and BGH polyadenylation sequence in order to amplify MKK4/MKK7 full lenght (MKK4/MKK7 expression plasmids were kindly gifts). We then inserted them into NheI –XhoI site of the eukaryotic expression vector pcDNA 3.1 with HA tagged C-terminus.

3.3 Cell culture

Hela cells were cultured at 37˚C and 5% CO_2 in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Invitrogen).

3.4 Transient transfection

We used WelFect-Q^{TM} Plus Transfection Reagent (WelGene) according to the manufacturer's protocol. Under transfection condition, Hela cells were in serum starving condition for 6 hours before replaced by 10% FBS medium. 14-16 hours later, we replaced the old medium with BSA medium (1mg/ml) and kept culturing transfected cells overnight for assay.

3.5 Subcellular localization assay

We transient transfected pEGFPN1-DUSP28 or pEGFPN1 empty vector to Hela cells. Twenty four hours post-transfection, cells were then fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 buffered saline. We next stained cells with 1μg.mL^{-1} DAPI (Sigma) and viewed with fluorescence microscopy (Olymus IX70, Japan)

3.6 Subcellular fractionation assay

We havested transfected cells in Phosphate Buffer Saline (PBS) using Cell Scraper. Thereafter, we centrifuged 3000 rpm/min in 5 min to collect cells. Transfected cells were lysised by Buffer B and grinder. Lysis Buffer B involved 25 mM Tris-HCl pH 7.5, 250 mM Sucrose, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 1 mM PMSF, 10μg/ml Leupeptin ,and 10 μg/ml Aprotinin.

Briefly, we extracted cells inside a grinder (Wheaton, USA) using Lysis Buffer B

with 70 strokes. We next centrifuged the extracted solution (15.000 rpm/min, 4℃, 10 min) and collected the supernatant containing cytosolic and membrane proteins.

In order to separate membrane and cytosolic proteins, the supernatant were continuous centriguged in 100.000 g/min, 4℃ for 1 hr. The pellet containing membrane proteins and supernatant containing cytosolic proteins were then resuspended in Buffer B and heated at 95℃ before immunobloting assay.

3.7 Immunobloting assay

We lysised transfected cells with Lysis Buffer Total cellular protein concentration was determined by bradford protein assay (Bio-rad, USA). After electrophoresis through sodium dodecyl sulphate polyacrylamide gel, we transferred into nitrocellulose blotting membranes (Amersham, Germany).

Protein containing membranes were probed with specific primary and secondary antibodies thereafter blocked with 5% skim milk or 1% BSA. We used an enhanced chemiluminescent substrate to detect the cellular signaling bands (WEST -ZOL, iNtRON Biotechnology Inc., Seoul, Korea).

3.7 The regulation of DUSP28 on MAPK pathways

We cotransfected JNK/ERK/p38 together with DUSP28/DUSP28CS/ pcDNA into Hela cells. Transient transfected cells were next induced with 10 μg/ml anisomycin to JNK/p38 overexpressed cells or 0.02 μg/ml Epidermal Growth Factor (5 min) to ERK overexpressed cells.

The signaling effects of DUSP28 were determined by immunobloting assay with antibodies specific to activation form of JNK/ERK/p38.

4. RESULTS

4.1 DUSP28 subcellular localization

In order to determine the subcellular localization of DUSP28, we expressed GFP tagged DUSP28 into Hela cells.

We observed that GFP expressed in both cytoplasm and nucleus of Hela cells (Figure 4.2). The four amino acids CAAX motif was known to be sufficient for plasma membrane targeting, thus GFP-CAAX expression was shown around nucleus (Figure 4.3).

To GFP tagged DUSP28, we observed the strong expression in both cytoplasm and nucles, similar to GFP expression (Figure 4.1)

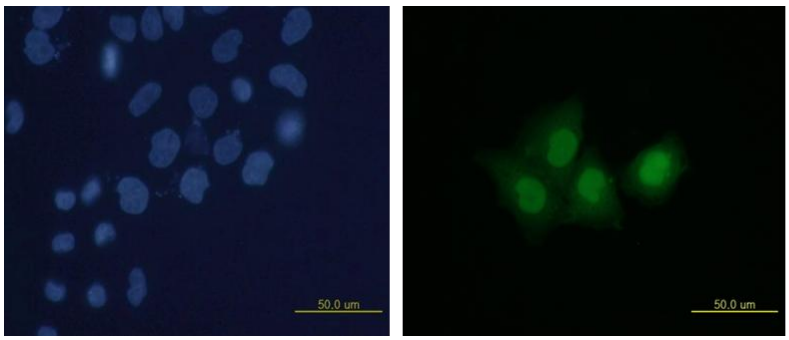

Figure 4.1 Subcellular localization of DUSP28

Hela cells were transient transfected with 3 μg of pEGFP-DUSP28. Cells were then fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 buffered saline. We next stained cells with 1 $\mu g.mL^{-1}$ DAPI (Sigma) before viewing with fluorescence microscopy (Olymus IX70, Japan)

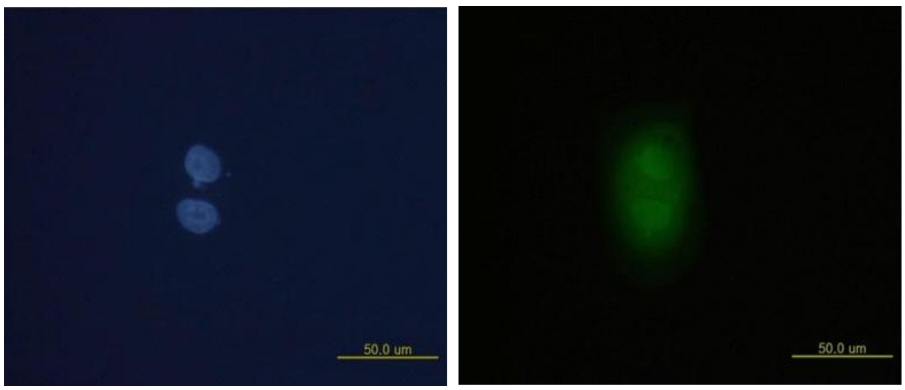

Figure 4.2 Subcellular localization of GFP

Hela cells were transient transfected with 3 μg of pEGFP vector. Cells were then fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 buffered saline. We next stained cells with 1μg.mL^{-1} DAPI (Sigma) before viewing with fluorescence microscopy (Olymus IX70, Japan)

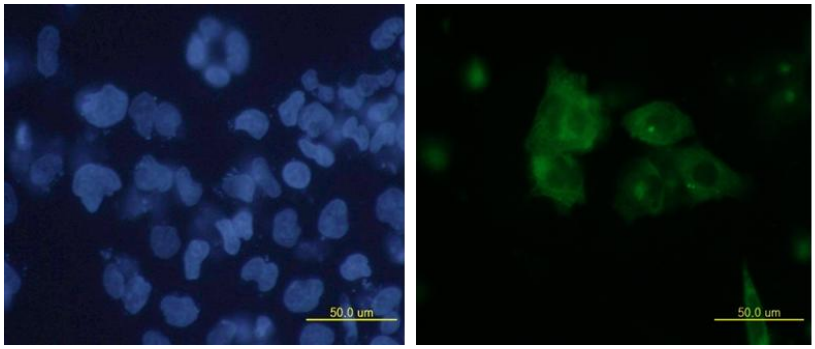

Figure 4.3 Subcellular localization of pEGFP-CAAX

Hela cells were transient transfected with 3 μg of pEGFP-CAAX. Cells were then fixed with 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 buffered saline. We next stained cells with 1μg.mL^{-1} DAPI (Sigma) before viewing with fluorescence microscopy (Olymus IX70, Japan)

4.2 DUSP28, a cytosolic/membrane phosphatase protein

The subcellular localization result showed DUSP28 expressed in both cytoplasm and nucleus in Hela cells. It raised a possibility that DUSP28 could bind to nucleus membrane in order to pervade inside nucleus and regulate its specific cell signaling.

To obtain this point, thereafter induction with anisomycin, DUSP28/DUSP CS together with GFP/GFP-CAAX were transfected into Hela cells.

The subcellular fractionation result show that in contrast to GFP/cytosolic protein, GFP-CAAX was shown binding to nucleus membrane corresponding to subcellular localization assay (Figure 4.2 lane 1-4).

As we expected, the subcellular fractionation assay showed that both DUSP28/DUSP CS expressed as cytosolic/plasma membrane protein (Figure 4.2 lane 5-8).

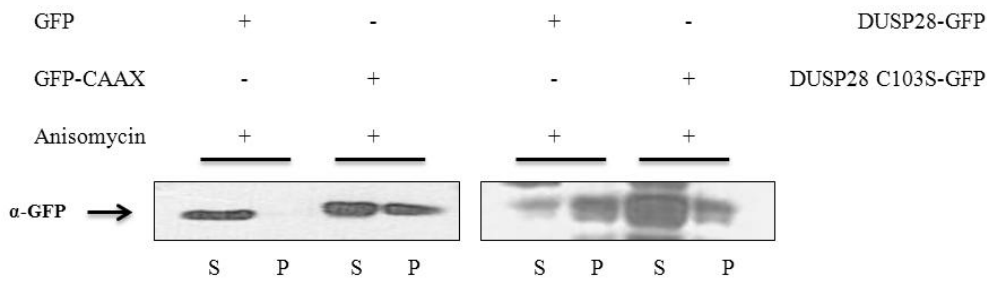

Figure 4.4 DUSP28 was targeted into nucleus membrane in Hela cells

Hela cells were transient transfected with 3 μg of pEGFP; pEGFP-CAAX; pEGFP-DUSP28 and pEGFP-DUSP28 CS. We then induced cells with 10 μg/ml anisomycin before cellular fractionation assay. The expression of cytosolic/membrane proteins were determined by immunobloting using GFP antibody.

4.3 Effects of DUSP28 overexpression on MAPK signaling pathways in Hela cells.

In mammals, three major MAPK pathways have been identified, including MAPK/ERK, SAPK/JNK, and p38 MAPK. Variety DUSPs are shown as important regulators in MAPK pathways (Ching-Yu Huang et al., 2012; Jeffrey 2007; Ulla Schwertassek et al., 2010).

We thus questioned “whether DUSP28 participates in signalling regulation involving MAPK pathways”.

Upon anisomycin or EGF treatment in Hela cells, the p-JNK/ERK/p38 level in DUSP28 CS and pcDNA overexpression were similar, meanwhile DUSP28 overexpression dramatically reduced p-JNK and p-ERK level (Figure 4.5; 4.6 and 4.7).

In contrast, we did not observed any alteration in p38 phosphorylation between DUSP28/DUSP28 CS/pcDNA under same condition (Figure 4.6; lane 1 and 3).

The Immunobloting result suggested that “DUSP28 is a negative regulator in JNK/ERK but not p38 pathways”.

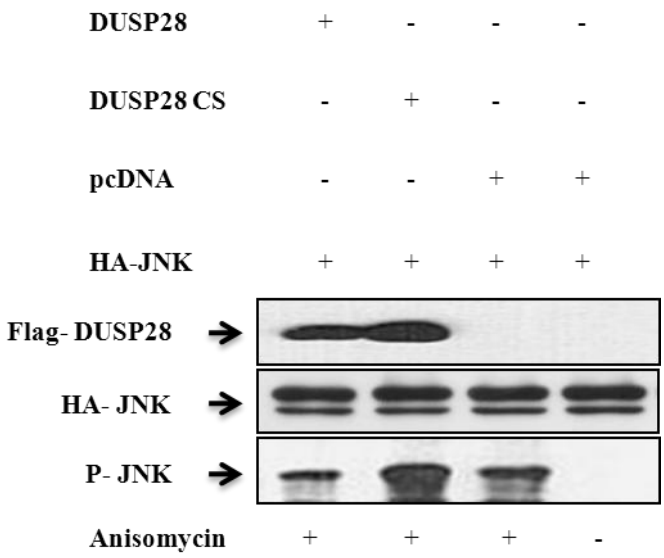

Figure 4.5 Effects of DUSP28 overexpression on JNK signaling pathway

We co-transfected 3 μg of DUSP28/DUSP28 CS/pcDNA together with 3 μg of JNK Cell were then induced with 10 μg/ml anisomycin.

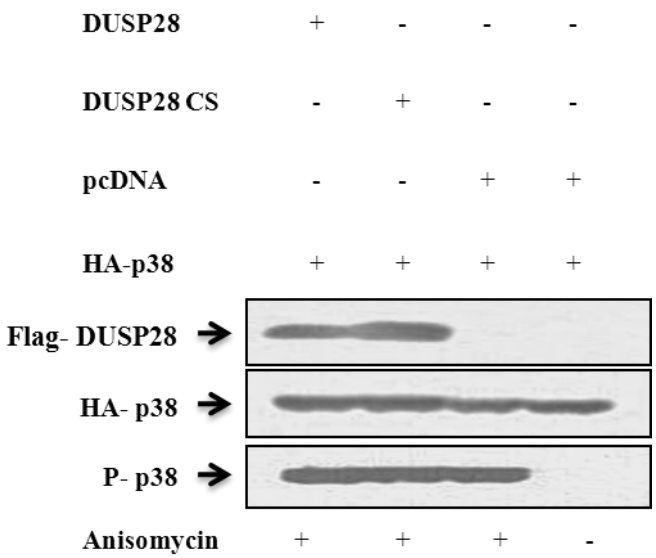

Figure 4.6 Effects of DUSP28 overexpression on p38 signaling pathway

We co-transfected 3 μg of DUSP28/DUSP28 CS/pcDNA together with 3 μg of p38. Cell were then induced with 10 μg/ml anisomycin.

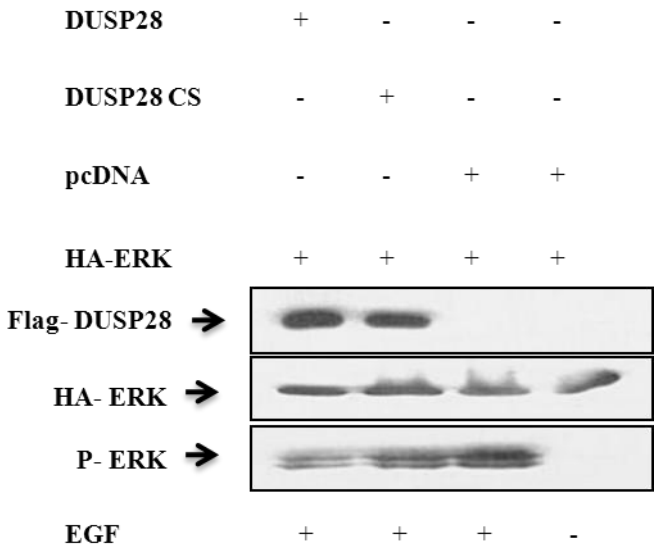

Figure 4.7 Effects of DUSP28 overexpression on ERK signaling pathway

We co-transfected 3 μg of DUSP28/DUSP28 CS/pcDNA together with 3 μg of ERK. Cell were then induced with 0.02 μg/ml EGF.

4.4 DUSP28 is a negative regulator of ERK and JNK pathways upon EGF and anisomycin treatment

In order to confirm the negative regulation of DUSP28, we studied time course activity of ERK and JNK pathway in DUSP28/pcDNA overexpression upon anisomycin/EGF treatment .

All of pcDNA/DUSP28 transfected cells showed the peak of ERK/JNK phosphorylation at 5 min/30 min.

In the case of pcDNA transfected cell, the ERK phosphorylation kept the high level until 20 min and 60 min to JNK phosphorylation (Figure 4.8 and 4.9).

Meanwhile, DUSP28 overexpressed cells briefly reduced the phosphorylation of ERK and JNK after approaching the peak at 5 min and 30 min induction respectively.

Upon these results above, we concluded that DUSP28 is a negative regulator of ERK and JNK pathways in EGF/anisomycin responsed cell signaling in Hela cells.

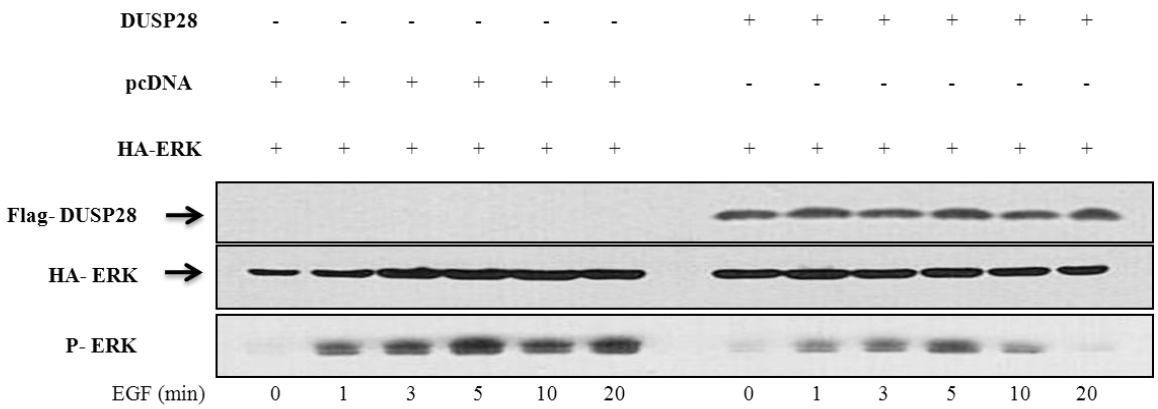

Figure 4.8 The time course assay of ERK phosphorylation upon DUSP28 overexpression in Hela cells

We co-transfected 2 μg of JNK together with 3 μg of DUSP28/pcDNA plasmids to Hela cells. We next induced cells with 0.02μg/ml Epidermal Growth Factor (EGF) in different time points.

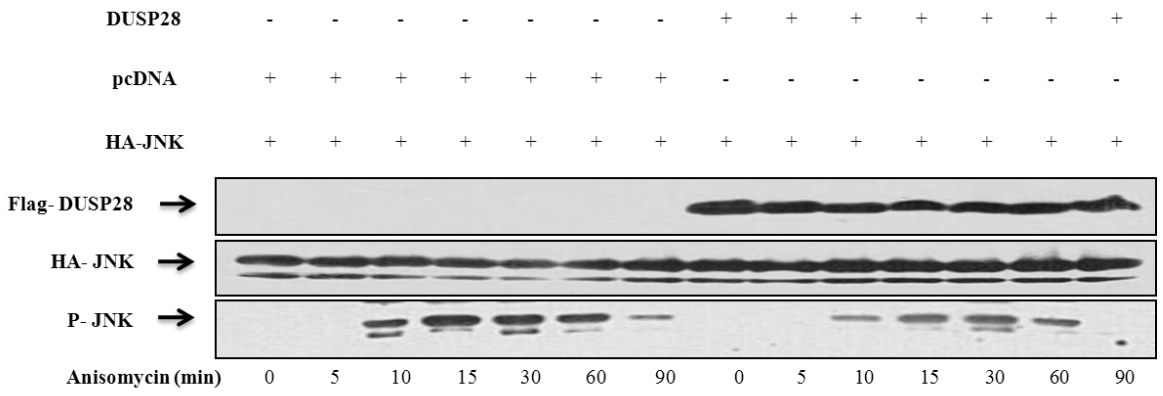

Figure 4.9 The time course assay of JNK phosphorylation upon DUSP28 overexpression in Hela cells

We co-transfected 2 μg of JNK together with 3 μg of DUSP28/pcDNA plasmids to Hela cells. Cells were next induced with 10 μg/ml anisomycin in different time points.

4.5 DUSP28 overexpression reduced the phosphorylation of c-jun

C-jun is activated through double phosphorylation by JNK in response to various extracellular stimuli, including UV irradiation, oxidative, anisomycine treatment ..etc. Phosphorylation of c-Jun increases the c-jun target genes.

In addition, the c-jun activity is also regulated by the ERK pathway. The activation of ERK increases c-jun transcription and stability through CREB and GSK3.

We showed that DUSP28 reduced JNK/ERK phosphorylation. We thus determined whether DUSP28 also regulates downstream factor c-jun or not. DUSP28/DUSP28 CS/pcDNA transfected cells were induced with anisomycin for 60 min.

The c-Jun phosphorylation in DUSP28 CS and pcDNA transfected cells was shown in similar level, meanwhile DUSP28 overexpression reduced c-jun phosphorylation (Figure 4.10).

We thus concluded that upon negative regulation of JNK and ERK phosphorylation, DUSP28 reduced downstream factor c-jun phosphorylation.

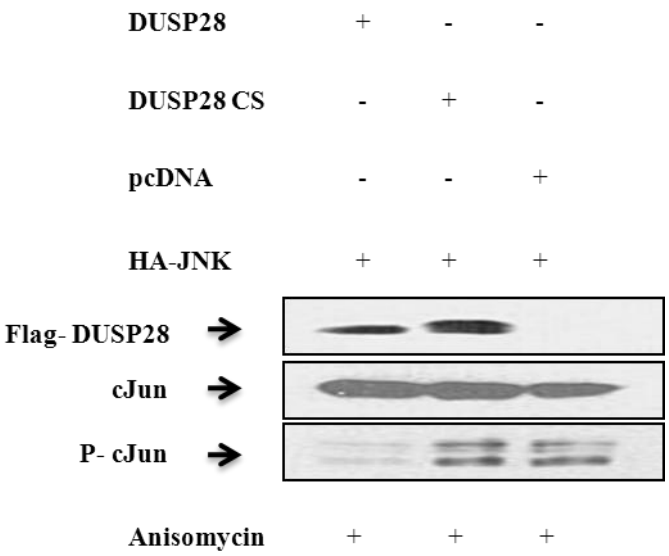

Figure 4.10 Effects of DUSP28 overexpression in JNK downstream factor (c-Jun) upon anisomycin treatment

We co-transfected 3 μg of DUSP28/DUSP28 CS/pcDNA together with 3 μg of JNK . Cells were then induced with 10 μg/ml anisomycin for 60 min.

4.6 DUSP28 overexpression reduced the phosphorylation of JNK upstream factor, MKK4

MKK4 and MKK7 are direct activators and uptream kinases of JNK in response to various enviromental stresses. Meanwhile MKK6 is a uptream kinase and directly phosphorylate p38 MAP kinase in response to cellular stress, cytokine or inflammatory.

We previously showed DUSP28 supressed JNK phosphorylation in response to cellular stress induced anisomycin. We next determined whether DUSP28 regulates JNK upstream kinases MKK4/MKK7 phosphorylation or not.

The immunobloting result showed that p-MKK4 level was similar in DUSP28 CS and pcDNA transfected cells while phosphorylation of MKK4 was decreased dramatically in DUSP28 overexpression cells (Figure 4.11) .

In addition, we did not observed any alteration in MKK6 phosphorylation between DUSP28/DUSP28 CS/pcDNA transfected cells. It was corresponding to our previous result that DUSP28 did not dephosphorylate p38 MAP kinase, downstream of MKK6 (Figure 4.13).

Meanwhile MKK7 kinase was not phosphorylated upon anisomycin treatment (Figure 4.12).

We thus concluded that upon anisomycin treatment in Hela cells, DUSP28 negative regulate MKK4 and thus reduced JNK and downstream factor c-jun phosphorylation.

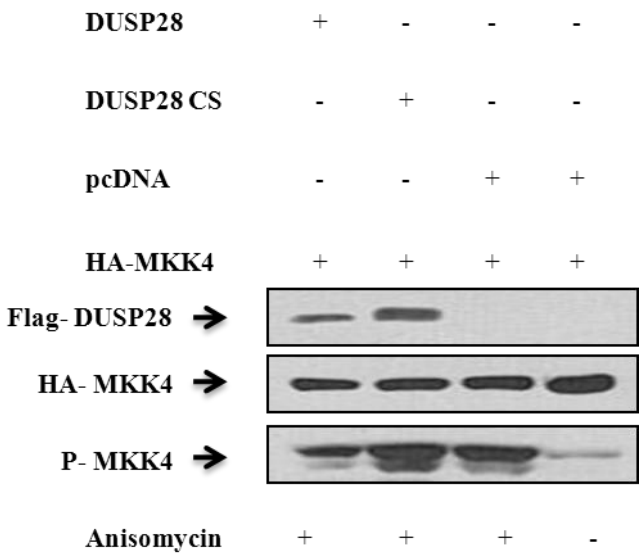

Figure 4.11 Effects of DUSP28 overexpression in MKK4, upstream factor of JNK

We co-transfected 3 μg of DUSP28/DUSP28CS/pcDNA together with 3 μg of MKK4 into Hela cells . Cells were then induced with 10 μg/ml anisomycin for 30 min.

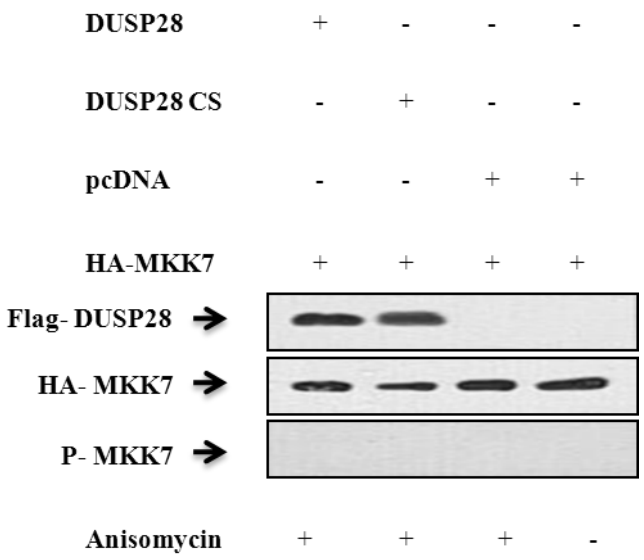

Figure 4.12 Effects of DUSP28 overexpression in MKK7, upstream factor of JNK

We co-transfected 3 μg of DUSP28/DUSP28CS/pcDNA together with 3 μg of MKK7 into Hela cells . Cells were then induced with 10 μg/ml anisomycin for 30 min.

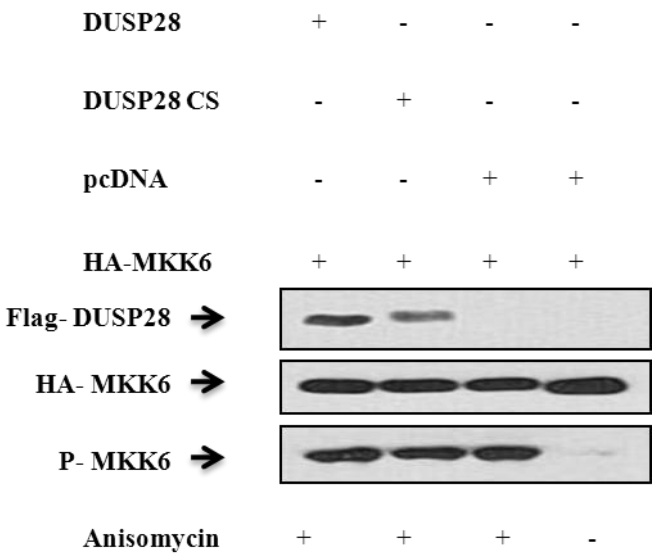

Figure 4.13 Effects of DUSP28 overexpression in MKK6, upstream factor of p38

We co-transfected 3 μg of DUSP28/DUSP28CS/pcDNA together with 3 μg of MKK6 into Hela cells . Cells were then induced with 10 μg/ml anisomycin for 30 min.

4. DISCUSSION

We can classify DUSPs into 3 major groups and atypical group upon the sequence similarity, protein structure and subcellular localization. In addition of functional MAPK binding domain (MKB) and dual specificity phosphatase domain (DSP), all major groups contain the private domains in order to specific function in their substrates (Kondoh, 2007).

Group I, such as MKP-1and MKP-2 (Wu, 2005), localizes in nuclear compartment due to containing a nuclear localization signal sequence (NLS). They play important role in the cellular signaling feedback in the nucleus. Group II, such as MKP-3 and MKP-4, contain a nuclear export signal (NES) and thus localize in cytoplasm (Maria Karlsson, 2004; Marco Muda, 1997). Group III, such as MKP-5 and M3/6, localizes in both cytoplasm and nucleus due to consist of both NLS and NES (Aspasia Theodosiou, 1999). Group III also contains PES sequence with unknown function. Atypical group, such as VHR, consist of low molecular proteisn since it consist only DSP domain (Ishibashi, 1992).

Due to simple struture and low molecular weight, atypical DUSPs's characteristics are quite flexible. Different members of atypical group show distinct specific substrate, signaling regulation and subcellular localization. Such as VHR is a negative regulator of ERK and JNK pathways in T cells (A. Alonso, 2001; J.L. Todd, 1999; J.L. Todd, 2002). Meanwhile, LDP-3 was shown as possitive regulator of JNK and p38 pathways in Cos-7 cells [32].

We previously showed that DUSP28 belong to atypical group with low molecular mas (composed of 176 amino acid residues). Upon using tyr1 (END(pY)INASL), tyr2 (DADW(pY)LIPQQG), and thr (RRA(pT)VA) as substrates, DUSP28 strongly dephosphorylated tyr and lower specific activity to thr/ser residues (Dae Gwin Jeong, 2011).

We first determined DUSP28's subcellular localization in Hela cells. We used Hela cell line because of the low endogenous level of DUSP28 containing (Data now shown). When overexpressed in Hela cells, DUSP28 was observed in both cytoplasm and nucleus.

Upon anisomycin treatment, the expression of DUSP28/DUSP28 CS in cytosolic and plasma membrane was different. DUSP28 was shown strong binding to plasma membrane while DUSP28 C103S was shown strong expression in cytosolic. Because of inactive phosphatase DUSP28 CS, we thus speculated that the cell singlaing regulation of DUSP28 was depended on its subcellular localization.

We next studied DUSP28 regulation on cell signaling. Due to the close relationship between DUSPs and MAPKs, we decided to check DUSP28 regulation on 3 main MAPK pathways including ERK, JNK and p38 MAPK pathways.

In the case of JNK pathway, we showed that DUSP28 overexpression suppressed the phosphorylation of JNK upon anisomycin treatment. Our findings thus raised the following possibilities. 1) DUSP28 dephosphorylates the critical residues of JNK required for its activation and thereby suppresses the JNK phosphorylation. 2) DUSP28 competes for ATP on the ATP-binding site of JNK to inhibit the its phosphorylation. 3) DUSP28 disturbs the physical interaction between JNK and JNK upstream kinases MKK4/MKK7. 4) DUSP28 suppresses the JNK upstream kinases MKK4/MKK7 phosphorylation or futher MKK4/7 activating MAPKKKs. Therefore, we examined the validity of each.

The immunobloting results showed that DUSP28 down-regulated MKK4, an upstream factor of JNK, but not directly JNK phosphorylation. DUSP28 overexpression dramatically reduced MKK4 phosphorylation but not MKK7 upon anisomycin treatment.

Therefore we ruled out third possibility that related to direct interaction between DUSP28 and JNK. Because of anisomycin preferentiallly phosphorlated MKK4, we will character DUSP28 regulation in stress induced MKK7 phosphorylation in futher studies.

Furthermore we are going to determine whether DUSP28 regulates MKK4 upstream kinases, MAPKKKs or not.

To ERK pathway, we showed that DUSP28 negative regulated ERK phosphorylation. However, we did not check the regulation of DUSP28 on the ERK upstream kinase, MEK1 phosphorylation. Upon the regulation on JNK upstream kinase, we speculated that DUSP28 might supressed MEK1 phosphoryaltion in order to control ERK pathway. Our futher work will focus on upstream kinases of ERK.

These results were consolidated with the time course and magnitude of JNK/ERK activation based on DUSP28 overexpresion. The phosphorylation of JNK/ERK in DUSP28 overexpressed cells has not only lower magnitude but also earlier extinguished than empty vector control.

In conclusion, we show that DUSP28 is a specific regulator of JNK/ERK pathway in environmental induced cellular signaling responses. Although the DUSP28 substrate is still unknown, the DUSP28 subcellular localization elicit that DUSP28 is a master physical converter that regulate the signaling from cytoplasm to nucleus.

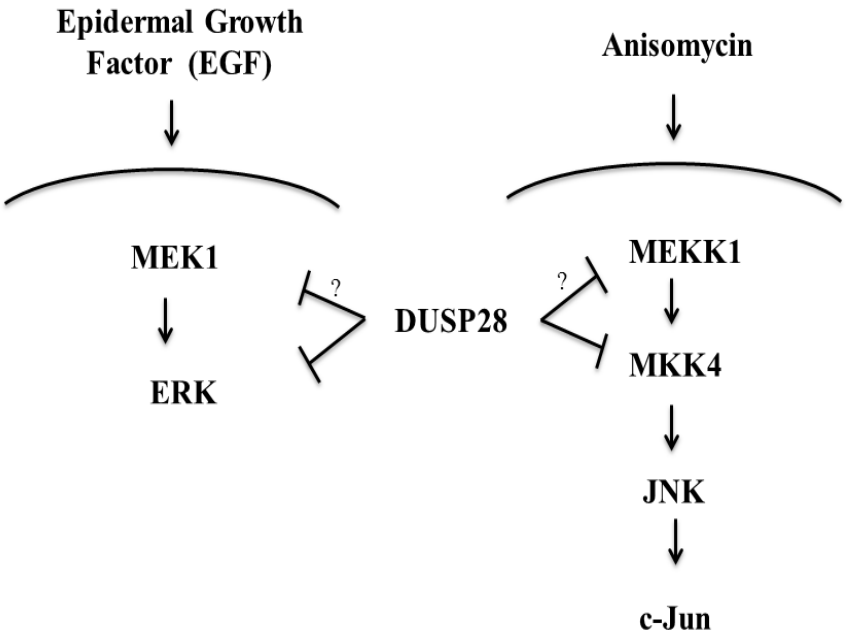

Figure 5.1 The proposed model for DUSP28 negative regulation in ERK and JNK pathways

REFERENCES

[1] Philipp Selenko (2003), Switching Off the Swich. *Structure*, Vol.11.

[2] Hornber (2005), Principles behind the multifarious control of signal transduction. ERK phosphorylation and kinase/phosphatase control. *FEBS Journal* 272, 244-258.

[3] Owens (2007), Differential regulation of MAP kinase signaling by dual specificity protein phosphatses. *Oncogene* 26, 3203-3213.

[4] K. Tonks (2013), Protein tyrosine phosphatases – from housekeeping enzymes to master regulators of signal transduction. *FEBS Journal* 280, 346-378.

[5] A. Lim (2010), Phosphotyrosine signaling: envoling a new cellular communication system. *Cell* Vol 142, 661-667.

[6] Bermudez (2009), The dual specificity MAP kinase phosphatases: critical roles in development and cancer. *Am J Physiol Cell Physiol* 299: C189-CS202

[7] Patterson (2009), Dual specificity phosphatases: critical regulators with diverse cellular targets. *Biochemical Journal* 418, 447-489.

[8] Lydia Tabernero (2008), Protein tyrosine phosphatases: structure–function relationships. *FEBS Journal* 275, 867–882.

[9] Farooq (2004), Structure and regulation of MAPK phosphatases. *Cellular Signalling* 16, 769-779.

[10] Laiping Xie (2002), Design and characterization of an improve protein tyrosine phosphatase substrate trapping mutant. *Biochemistry* 41,4032-4039.

[11] Dae Gwin Jeong (2011), Characterization of a Dual Specificity Protein Phosphatase, Human DUSP28. *Journal of Life Science* Vol.21. No.1, 31-35.

[12] Jaco L Todd (2002), Dual specificity protein tyrosine phosphatase VHR down regulates c-Jun N-terminal kinase (JNK). *Oncogene* 21, 2573 -2583.

[13] Ching-Yu Huang (2012), DUSPs, to MAP kinases and beyond. *Cell & Bioscience*, 2:24.

[14] L, Jeffrey (2007), Targeting dual specificity phosphatases: manipulating MAP kinase signaling and immune responses. *Nature reviews drug discovery* volume 6, 391-402.

[15] Ulla Schwertassek (2010), Myristoylation of the dual-specificity phosphatase c-JUN N-terminal kinase (JNK) stimulatory phosphatase 1 is necessary for its activation of JNK signaling and apoptosis. *FEBS Journal* 277, 2463–2473.

[16] Yu (2007), A novel amplification target, DUSP26, promotes anaplastic thyroid cancer cell growth by inhibiting p38 MAPK activity. *Oncogene* 26, 1178–1187.

[17] J. Staples (2010), Cross-talk between the p38 and JNK MAPK pathways mediated by MAP Kinase Phosphatase-1 determines cellular sensitivity to UV radiation. *The Journal of Biological Chemistry* volume 285, 34, 25928–25940.

[18] Tony (1998), Signal transduction by the c-Jun N-terminal kinase (JNK) from inflammation to development. *Current Opinion in Cell Biology* 10, 205-219.

[19] Davis (2000), Signal Transduction by the JNK Group of MAP Kinases. *Cell* Vol. 103, 239-252.

[20] Aspasia Theodosiou (2002), Differential effects of stress stimuli on a JNK inactivating phosphatase. *Oncogene* 21,2387 ± 2397.

[21] Hamdi (2005), DNA damage in transcribed genes induces apoptosis via the JNK pathway and the JNK-phosphatase MKP-1. *Oncogene* 24,7135 ± 7144.

[22] Sharon Biton (2011), NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-α feedforward signaling. *Cell* 145, 92-103.

[23] Kondoh (2007), Regulation of MAP kinases by MAP kinase phosphatases. *Biachimica et Biophysica Acta* 1773, 1227-1237.

[24] J.J. Wu, The noncatalytic amino terminus of mitogen-activated protein kinase phosphatase 1 directs nuclear targeting and serum response element transcriptional regulation. *Molecular and Cellular Biology* 25 (2005) 4792–4803

[25] Maria Karlsson (2004), Both nuclear-cytoplasmic shuttling of the dual specificity phosphatase MKP-3 and its ability to anchor MAP Kinase in the cytoplasm are mediated by a conserved nuclear export signal. *The Journal of Biological Chemistry* volume 279, 41882–41891.

[26] Marco Muda (1997), Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. *The Journal of Biological Chemistry* volume 272, 5141-5151.

[27] Aspasia Theodosiou (1999), MKP5, a new member of the MAP kinase phosphatase family, which selectively dephosphorylates stress-activated kinases. *Oncogene* 18, 6981 ± 6988.

[28] T. Ishibashi (1992), Expression cloning of a human dual-specificity phosphatase, *Proc. Natl. Acad. Sci. U. S. A.* 89, 12170–12174.

[29] A. Alonso (2001), Inhibitory role for dual specificity phosphatase VHR in T cell antigen receptor and CD28-induced Erk and Jnk activation, *The Journal of Biological Chemistry* Volume 276, 4766–4771.

[30] J.L. Todd (1999), Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. A novel role in down-regulating the ERK pathway, *The Journal of Biological Chemistry* Volume 274, 13271–13280.

[31] J.L. Todd (2002), Dual-specificity protein tyrosine phosphatase VHR down-regulates c-Jun N-terminal kinase (JNK), *Oncogene* 21, 2573–2583.

[32] Takagaki (2004), Characterization of a novel low-molecular-mass dual-specificity phosphatase-3 (LDP-3) that enhances activation of JNK and p38. *Biochemical Journal* 383, 447–455.

ACKNOWLEDGEMENT

First of all, I give my gratitude, honor and all my love to dad, mom, siblings and Ai Thao who give me a life, raise me up in difficulties. Family is my only word that never replaced. I also give my appreciation to Mrs Dieu, Mrs and Mr Ly who granted me in university studying time. Thanks for that, I could continue to write my ambitions.

This time to express my profound gratitude to my supervisor, my professor, my teacher: Prof. Kim Jae Hoon for all of things. His exemplary guidance, continual encouragement and belief given time to time take me into another step of scientific life. Thanks for his all things in order to help me throughout 2 years of MS course.

In addition, I would like to send my gratitude to Prof. Kim Somi for giving me an opportunity to study in Korea which is a promotive step in my life.

With all my respect and honor to say about Dr. Yun Jeong Hun, my second teacher in Korea, my dear brother and also exemplar in my scientific life. I have no word to show my deep sense of gratitude for his support, guidance in both of working companies and daily manners.

Alternatively, my appreciation to Dr. Han Song-E, thanks for all things she has given me in knowledge, lessons and experiences in studies which help me in various difficult stages. I also take this time to thank all of my labmates who supporting me throughout 2 years.

I give my deep thanks to staff members of Biology Department of Jeju National University for the valuable information given by them.

Last but not least, I thank all of my Vietnamese friends, especially Mr. Tin, Miss Thao and Mr Quyen who supporting not only in my work but also in my life.