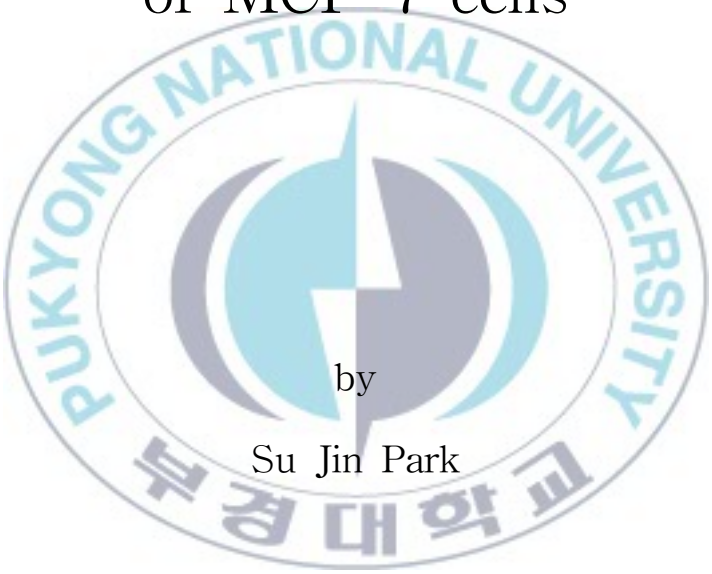
Thesis for the Degree of Doctor of Philosophy

Porphyra yezoensis peptide inhibits the metastasis and proliferation of MCF-7 cells



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February 2014

Porphyra yezoensis peptide inhibits the metastasis and proliferation of MCF-7 cells

김 펩타이드의 MCF-7 세포 증식 및 전이 억제 기전

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by

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A thesis submitted in partial fulfillment of requirements
for the degree of Doctor of Philosophy

Department of Food and Life Science, Graduate School
Pukyong National University

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Abbreviation

PPY: Peptide of *Porphyra yezoensis*

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxmethoxy-phenyl) 2H-tetrazolium

DAPI: 4,6-diamido-2-phenylindole

IGF-IR: Insulin-like growth factor I receptor

mTOR: Mammalian target of rapamycin

p70S6K: 70-kDa ribosomal protein S6 kinase

PDK1: Phosphoinositide-dependent kinase-1

PTEN: Phosphatase and tensin homolog

CDK: cyclin-depenent kinase

FAK: focal adhesion kinase

TG2: Tissue transglutaminase 2

LC3: Light chain 3

ATG: Autophagy-related gene

TGF-β1: Transforming growth factor β1

RT-PCR: Reverse transcription polymerase chain reaction

mRNA: Messenger RNA

EMT: Epithelial-to-Mesenchymal Transition

ECM: extracellular matrix

DEPC: diethylpyrocarbonate

E-cadherin: epithelial-cadherin

MMP: Matrix Metalloproteinase

APC: Adenomatous polyposis coli

GSK3β: glycogen synthase kinase 3 beta

TCF: T-cell factor

LEF: Lymphoid enhancer factor

ICAM: Intracellular adhesion molecule-1

DVL: Disheveled

RANi: RNA interference

siRNA: small interfering RNA

dsRNA: doble-stranded RNA

김 (*Porphyra yezoensis*) 펩타이드의 MCF-7 세포 증식 및 전이 억제 작용 메카니즘

박 수 진

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요 약

방사무늬돌김 (*Porphyra yezoensis*)은 그 영양가치 뿐만 아니라 콜레스테롤 저하작용, 향균효과 및 항암효과 등 다양한 생리효과를 가진다는 사실이 밝혀지면서 많은 관심의 대상이 되고 있다. 최근 김에 함유된 생리기능성분을 활용한 고부가가치 기능성식품 소재와 기술개발에 관심을 가지게 되었고 이에 대한 연구가 활발히 진행되고 있지만, 포피란과 같은 단당류와 당단백질 수준에서의 연구만 진행되고 있는 실정이다. 하지만, 김은 약 36%의 많은 단백질을 함유하고 있으나 단백질에 관한 기능성을 연구하는 보고는 거의 없는 실정이다. 뿐만 아니라, 방사무늬돌김으로 부터 분리된 당단백질은 현재 부분적으로 항암의 효능이 밝혀져 있기는 하지만, 펩타이드 수준에서의 연구는 전무한 상태이다.

본 연구에서는 방사무늬돌김으로 부터 분리한 펩타이드를 이용하여 인간 유방암 세포주인 MCF-7 세포의 성장 및 전이억제 메카니즘 기전에 관하여 밝히고자 하였다.

MTS assay결과 *Porphyra yezoensis*의 peptide (PPY)는 인간 유방암 세포 MCF-7의 증식억제 효과를 나타내었으며, DAPI staining assay에서도 동일하게 농도 의존적으로 세포 사멸을 확인할 수 있다. 이러한 결과는 인간 유방암 세포주인 MCF-7에서 PPY가 세포증식 억제 효능에 영향을 주고 있을 뿐만 아니라, 형태학적인 변화에서도 PPY가 인간 유방암 세포인 MCF-7에서 apoptosis 유도와 관련되어 있다고 여겨진다.

PPY가 세포사멸유도에 관련된 기전을 확인하기 위하여, 먼저, Insulin-like growth factor (IGF-I)와 관련된 세포사멸신호인 AKT 단백질이 농도 의존적으로 감소하며 AKT의 인산화에도 영향을 미치는 것으로 나타났다. 뿐만 아니라, IGF-IR과 ERK의 인산화가 감소함으로써 PPY가 인간유방암세포의 증식억제 효능을 나타내었다. 그리고 mTOR기전 관련 인자를 확인한 결과에서도 mTOR, p70S6K 등의 관련 단백질이 감소함으로써, PPY로 인하여 암세포의 증식억제 효능과, mTOR 관련인자인 p70S6K 와 Tissue transglutaminase2 (TG2) protein의 감소로 인하여 인간 유방암 세포 MCF-7의 전이억제 효과도 확인하였다.

이어서 프로그램화된 세포죽음의 또 다른 형태로써 type Ⅱ라고 불리는 autophagy에 관련인자를 확인함으로써 PPY가 세포 사멸 기전과 관련있음을 보고자 했다. Autophagy 유도시 발현이 증가하는 단백질로써 LC3와 Beclin-1이 있는데, Beclin-1은 autophagosome을 형성하는 중요한 역할을 하며, LC3는 autophagy가 일어나면 LC3의 두 가지 형태 중에 LC3 Ⅰ form이 LC3Ⅱ로 전환되면서

autophagosome의 막에 결합하기 때문에 직접적인 autophagosome의 직접적인 지표가 된다. 본 연구는 PPY 처리에 의하여 apoptosis 뿐만 아니라, autophagy가 동시에 유발됨으로써 확실한 세포 사멸 기전을 보여주고 있는 것으로 확인하였다.

Transforming growth factor-β (TGF-β)는 세포들에서 강력한 성장억제제로 작용하고 있으며 실제 apoptosis의 조절에 중요한 역할을 하는 인자로 알려져 있다. 동시에, epithelial-to-mesenchymal transition (EMT)의 발현에 관여함으로써 세포내 migration과 invasion에 영향을 주어 세포의 부착 능력을 억제한다. 본 연구에서는 PPY가 EMT 관련 단백질의 발현을 조절함으로써 세포와 세포 사이 전이를 억제해주는 결과로 보여진다. 본 연구에서는 PPY가 TGF-β1 signal의 억제 확인과 EMT관련 인자의 변화를 확인함으로써 PPY가 인간 유방암 세포내의 성장과 전이 억제에 중요한 key로 작용함을 확인하였다. 동시에 Wnt signal을 확인함으로써, TGF-β1의 발현과 세포와 세포사이의 부착 관련 단백질을 조절하는 결과로 보여진다. 이로써, PPY가 Wnt와 TGF-β1 signal의 발현에 관여함으로써, 인간 유방암 세포 MCF-7의 전이 및 부착 능력을 억제하는 것으로 나타났다.

Small interfering RNA (siRNA)는 특정 유전자의 mRNA와 상보적으로 결합하여 그 유전자의 단백질 발현을 억제, 전사단계에서 유전자의 발현을 억제하는 RNA간섭 (RNAi)으로 알려져 있다. 또한 유전자의 이중가닥 RNA (double-stranded RAN, dsRNA)를 세포 내로 주입하는 RNAi는 RNA-induced silencing complex (RISC)나 dicer RNase Ⅲ 같은 세포내 효소들에 의해 그 유전자의 mRNA를 특이적으로 분해하여 유전자의 발현을 억제하는 방법이다. 이들은 ribozyme이나 antisense ODN을 이용하는 유전자 억제방법보다 훨씬 더 효율적으로 목적하는 유전자의 발현을 억제시킬 수 있어 최근 들어 다양한 실험 연구와 치료 방법에 응용되고 있다. 본 실험에서는 target gene을 mTOR과 TGF-β1으로 선택하여 실험하고자 했다. mTOR siRNA와 TGF-β1 siRNA와 Lipofectamin을 사용하여 관련 gene을 knock-down 시킨 후, PPY를 농도별로 처리하여 그 결과를 보고자 하였다. non-target mTOR과 non-target TGF-β1과 비교하였을 시, mTOR siRNA와 TGF-β1 siRNA 처리한 군에서 발현량이 현저히 감소함을 확인하였다. 이는 Target gene의 발현억제를 확인함으로써, mTOR과 TGF-β1 signal에 PPY가 관련 있음을 보여주는 결과이다.

이로써, PPY가 성장 관련 기전인 IGF-IR과 mTOR 관련 단백질을 억제함으로써 세포사멸 경로와 apoptosis가 유도됨을 확인하였고, 동시에 autophagy target인 mTOR과 Atg 단백질을 확인함으로써 autophagy 또한 유도됨을 확인하였다. 이는 PPY가 인간 유방암 세포 MCF-7의 증식 억제에 관여함을 보여주는 결과로 사료되어진다. 그리고 TGF-β1과 Wnt 경로의 억제와 EMT 관련 인자를 확인함으로써, PPY가 인간 유방암 세포 MCF-7의 전이억제에 영향을 줌을 확인하였다. 지금까지의 결과를 통하여 PPY가 MCF-7인간 유방암 세포의 성장 및 전이를 억제하는 것으로 나타났다.

Ⅰ. Introduction

1. Inhibitory effect of PPY on MCF-7 cells

Seaweeds have received a great deal of attention from researchers in recent years. Seaweeds contain high amounts of proteins, vitamins, minerals and several polysaccharides in seaweeds have diverse biological activities, including effects on the immune system and cancer. A number of studies from seaweeds have various bioactivities including antitumor and immuno-modulatory activities (Zedong *et al*., 2011). For example, Porphyran, a polysaccharide produced by the red alga that is consumed in Asia, has been shown to decrease cholesterol levels and to have antibiotic and anticancer effects (Koo *et al.*, 1995). In addition, fucoidan is known for its anticoagulant and antioxidative effects (Nishinso *et al.,* 1991).

As increase in seaweeds production, it is produced 992,000 tons in 2012. Among them, laver 320,000 tons were produced (MAFRA, 2012). The laver is most farming of seaweeds production in Korea and appeared frequently in the diet. The kind of laver is *Porphyra tenera, Porphyra yezoensis* and *Porphyra suborbiculata* and so on. The majority of farming laver in Korea is *Porphyra yezoensis. Porphyra yezoensis*, classified as Bangiophyceae (Saga *et al*., 2002), more specifically, it is a foliose red algal genus of Porphyra, comprising approximately 70 species (Brodie 2003). *Porphyra yezoensis* are found mostly in littoral zones such as rocky intertidal and subtidal zones (Toshiki *et al*., 2012). It is an intertidal marine red algae that has

received increasing attention as a model organism owing to its important role in biological research (Liu *et al*., 2012).

Although several studies have examined the polysaccharides found in the extracts of *Porphyra yezoensis,* the effect of particular proteins has not been reported. Peptide from the marine alga *Porphyra yezoensis* (PPY) play roles in antitumor cell signaling mechanism but the mechanism behind these activity are not well understand. Therefore, this study determined the structure of the *Porphyra yezoensis* peptide to examine it activity.

Insulin-Like growth factor I (IGF-I) and its cognate receptor Insulin-Like growth factor I receptor (IGF-IR) contribute to normal cell function and to tumorigenesis. Insulin-like growth factor-I (IGF-IR) is significant in cell growth, differentiation, and survival (Butler *et al*., 1998). Overexpression of IGF-IR and related proteins results in cancer cell proliferation and survival (Rubini 1997; Reiss *et al*., 1998; Butler *et al*., 1998). The role of IGF-I signaling in tumor growth has been demonstrated in vivo using nucleic-acid based strategies. The choice of treatment with PPY was based on the knowledge that the insulin-like growth factor I (IGF-1R) drove important cell survival pathways. In this study aimed to determine whether *Porphyra yezoensis* derived peptide induced apoptosis by mechanism involving IGF-IR and cell death pathway. This study extracted PPY that has an apoptotic effect on MCF-7 cells. This study determined that PPY induces cell cycle arrest and activates the IGF-1R signaling pathway. Apoptosis is important in the normal

development and differentiation of a wide variety of tissue. Apoptosis is characterized by several unique feature, including cell shrinkage, chromatin condensation, DNA fragmentation, the cell surface expression of phosphatidylserine, and membrane blebbing (Khan *et al.*, 2008; Burz *et al*). Apoptosis plays an important role by which cells undergo death to control cell proliferation. Generally, regulation in this form of death comprises the participation of p53 and Bcl-2 family. Bcl-2 family proteins are known as important regulators of apoptosis, (Hague *et al.*, 1998; Antonsson *et al.*, 2000) and involve anti or pro-apoptotic members, such as Bcl-2 protein can be regulated by post-translational modifications, such as Bcl-2, Bcl-xL, Mcl-1 and Bax (Yin *et al.*, 1994; Reed *et al.*, 1997). The activation of Bcl-2 protein can be regulated by post-translational modification, such as phosphorylation to Akt, mTOR, and p70S6K (Ricky *et al.*, 2002; Malaguarnera *et al.*, 2004). The Akt cell survival via various molecular mechanisms that include phosphorylation and the inactivation of pro-apoptotic proteins, such as Bad, glycogen synthase kinase-3 (GSK-3), Forkhead, and Caspaase 9 (Khwaja 1999; McCormick 2004; Westfall *et al.*, 2005). Interestingly, this down-stream effector for PI3K/mTOR is constitutively activated in many type of human tumors, including breast cancer.

Mammalian target of rapamycin (mTOR) is a highly conserved 289-kDa Ser/Thr kinase that has been identified in yeast and all eukaryotes. It belongs to the phosphoinositide kinase family of protein kinases. In mammals, mTOR mainly regulates two important down-stream substrates, p70S6 kinase (p70S6k) and eukarvotic initation

factor 4B binding protein 1(4EBP1). Abnormal activation of the mTOR signaling pathway has been implicated in a range of human diseases, especially human cancer such as colorectal cancer and esophageal squamous cell carcinoma (Hay *et al.*, 2004). Thus, high concentrations of PPY considerably decreased mTOR, p70S6k, PDK1 protein level. This results showed effect PPY in MCF-7 cells growth inhibition.

Autophagy begins with the formation of double-membrane vesicles, known as autophagosome, that engulf cytoplasmic constituents. The autophagosomes then fuse with lysosomes, where the sequestered contents undergo degradation and recycling. Monoallelic loss of the essential autophagy gene, Beclin1, has been found in 40 to 75% of human breast, prostate, and ovarian cancer, suggesting that autophagy may play a role in preventing these tumor (Qu *et al.*, 2003). The production of PtIns3P by the Beclin1 is essential for the recruitment other autophagy-related gene (Atg) products that are critical for autophagosome formation. During the initiation phase, formation of the Atg5-Atg12 complex promotes the recruitment and conversion of cytosolic-associated protein light chain 3 (LC3-I) to the membrane-bound, lipidated form, LC3-Ⅱ (Minako *et al.*, 2007). Therefore, a trend of increased expression of autophagy-associated protein such as LC3, Beclin1, Atg5 and Atg7. These results supported the idea that induced autophagy in MCF-7 cells.

It was also investigated the activation of the IGF-IR and mTOR pathway in breast cancer cells may contribute to tumor cell proliferation and survival.

2. Inhibitory effect of MCF-7 cells metastasis by PPY

Transforming growth factor-β1 (TGF-β1) is a potent regulator of epithelial-to-mesenchymal transition (EMT) in which cells may undergo physiological and morphological changes including loss of cell contact and polarity with increased migratory and invasive abilities. In several types of advanced cancers, TGF-β1 has been shown to promote cancer cell invasion and metastasis; whilst inhibition of TGF-β1 receptor function by expressing dominant negative suppress cell invasion (Arteaga *et al.*, 1998; Arteaga 1997). This study have clearly shown that either activation of TGF-β1 signaling suppression could enhance cell invasion, a detailed analysis is warranted to determine enhanced TGF-β1 signaling is associated with downregulation of Smad expression pertinent to cell invasion.

Wnt signaling, which is important in promoting and maintaining the undifferentiated state of stem cells (Nusse 2008), has been shown to prepare cells for TGF-β1 induced EMT during development (Herve *et al.*, 2009). In cancer, overactive Wnt signaling is frequently detected and cooperates with TGF-β1 induce EMT (Scheel *et al.,* 2011). Similarly, oncogenic Ras signaling cooperates with TGF-β1 in inducing EMT in mammary epithelial cells (Oft *et al.,* 1996). In fact, also other pathways promoting cellular sternness like Notch and Hedghog signaling cooperate with TGF-β1 to induce EMT (Fuxe *et al.*, 2009; Maitah *et al.*, 2011; Zavadil *et al.,* 2004). Thus, These data suggested that PPY can inhibit Wnt/β-Catenin signaling. The specific aim of this study is to assess expression of

TGF-β1 and Wnt as well as cell-cell adhesion. In this study also examined the effect of cell-cell adhesion mechanism especially β-Catenin through activation of related pathway.

Epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial cells lose their polarized organization and cell-cell junctions, undergo changes cell shape and in cytoskeletal organization and acquire mesenchymal characteristics, such as fibroblast-like cell morphology and inhibited cell migration and invasion (Hay 1968). Thus, this study indicated that PPY treatment may have played a role in promoting the activation of TGF-β1 leading to EMT.

The processes of cell migration and invasion are integral to embryonic development and the functioning of adult organism. Deregulation of these processes contributes to numerous diseases. Ras GTPases and in particular members of Rho subfamily of GTPases play critical roles in cell migration and invasion (Rossmon *et al.*, 2005). Members of the Ras superfamily of GTPases, most notably the Rho proteins, play a prominent role in cell migration (Burridage *et al.*, 2004). Thus, the Rac, Cdc42 and Rho GTPases are essential for growth factor-and cytokine-stimulated chemotaxis in fibroblasts macrophages and neutrophils (Anand-Apte *et al.*, 1997; Antonsson *et al.*, 2000; Allen *et al.*, 1998; Roberts *et al.*, 1999). In addition, Rac, Cdc42, and Rho are necessary for the invasive behavior of cancer cells and fibroblast. The signaling pathways that are activated by Rho proteins to regulate cell migration and invasion

are under intense scrutiny (Etienne *et al.*, 1992; Sahai *et al.*, 2002; Ridley *et al.*, 1998). Accordingly, PPY regulates migration and invasion through activation of the Ras signal pathway. Moreover, PPY treatment regulated the cell migration by Rho family members. In this study examined the effect of the TGF-β1 and Wnt signal activation by PPY on MCF-7 cells, more, these results showed that PPY treatment inhibited epithelial-mesenchymal transition (EMT) involved in progression of cancers to invasive state.

3. Specific mRNA knock-down in MCF-7 cells

RANi promises to revolutionize key areas of medical research, as demonstrated by the preliminary finding obtained in the fields of cancer, infectious diseases and neurodegenerative disorders. RNAi technology is rapidly spreading in research laboratories worldwide, as it is associated with a number of practical and theoretic advantages over preexisting methods of suppressing gene expression (Fire 1998). Over the past decade RNA interference (RNAi) has emerged as a nature mechanism for silencing gene expression. This ancient cellular antiviral response can be exploited to allow specific inhibition of the function of any chosen target gene. RNAi is proving to be an invaluable research tool, allowing much more rapid characterization of the function of known genes. More important, RNAi technology considerably bolsters functional genomic to aid in the identification of novel genes involved disease processes (Simone 2004).

Mammalian target of rapamycin (mTOR) plays a critical role in

cell cycle regulation. Rapamycin, a known inhibitor for mTOR, can inactivate mTOR specifically (Francis 1996). Because mTOR regulates cell proliferation, it has been extensively investigated as a potent target anti-cancer therapies (Law 2005). In this study, mTOR was knocked down using small interfering RNA (siRNA) conjugated with lipofectamin in MCF-7 cells. Compared with non-targeting siRNA transfection, p70S6k and PDK1 protein levels were significantly suppressed by mTOR siRNA treatment in MCF-7 cells.

Transforming growth factor (TGF)-β1 is the dominant stimulus for extracellular matrix (ECM) production by MCF-7 cells. This study was designed to investigate the anti-cancer effects of using short interference RNA (siRNA) to target TGF-β1 in MCF-7 cells and its mechanism. Therefore, TGF-β1 siRNA can partially inhibit or block the expression of TGF-β1 and reduced the impact of PPY on the proliferation and activation of MCF-7 cells.

Ⅱ. Material and Methods

1. Materials

PPY (Peptide of P*orphyra yezoensis*) was synthesized by the PEPTRON (Daejeon, Korea). The MCF-7 cells were obtained from the KCLB (Korean Cell Line Bank). SuperSignal® West Pico Chemiluminescent Substrate, and BCA protein assay kit purchased from Pierce Co., Ltd. (Rockford, IL). Feal bovine serum (FBS) was purchase from Hyclone Laboratory Inc. (Logan, UT). RPMI 1640 and Penicillin-streptomycin mixture were purchased from Gibco BRL (Life Technologies, Gibco BRL., Gaitherberg, MD). Bovine serum albumin (BSA), gelatin (electrophoresis type A) were purchased from Sigma (St. Louis, Mo). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate solution (MTS/PMS) (CellTiter 96® aqueous non-radioactive cell proliferation assay kit) was purchased from Promega (Madison, WI). Precision Plus Protein Standards Dual Color was purchased from Bio-Rad Laboratories company (Hercules, CA). Low-range rainbow molecular weight markers (RPN755) was purchased from Amersharm Pharmacia Bioscience (Pscataway NJ). Culture dish, 6-/12-/96-well plate, corning tube, scrapper were purchased from Corning (Glendale, Arizona) and Falcon (Becton Dickinson Labware). 4,6-diamidio-2-phenylindole (DAPI) solution was purchased from Sigma Aldrich. TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). cDNA was synthesized using the

Oligo (dT) primer and RedSafe nucleic acid staining solution by iNtRON Biotechnology Inc., (Seongnam, Korea). cDNA was added to 2X TOPsimple^{TM} DyeMIX-nTaq was purchased from Enzynomics Inc., (Daejeon, Korea). Nucleotide sequence for mRNA (Cosmogenetech, Korea), and siRNA targeting gene were designed and synthesized by GenePharma (Shanghai GenePharma Co., Ltd). Cell transfection with Lipofectamin 2000 (Invitrogen, Carlsbad, CA). TOPflash/FOPfalsh reporter system was purchased from Millipore (Billerica, MA, USA). Antibodies against PI3K, Akt, phospho-Akt, IGF-IR, IRS, Shc, ERK, Cyclin D, CDK6, CDK2, p21, p27, β-catenin, E-cdherin, FAK, phosph-FAK, NF-KB, p53, Bcl-2, Bad, Bid, Bax, Beclin1. ATG5, ATG7, TGF-β1, Samd2, Smad4, Occludin, Vimentin, Wnt, Frizzed, ICAM, AXIN, TCF, LCF1, Raf, MEK, ELK, RhoA, RhoB, Rac1, CDC42, MMP2, GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against LC3, Smad3, Ras were purchaesd from Cell Signaling Technology (Beverly, MA). Antibodies against mTOR, p70S6K, PDK, pTEN, RPS6, eF4B, Snail, GSK3β were purchaesd from Bioworld technology. Antibody against TG2 was purchaesd from ABcam. Peroxidase-conjugated gote anti-mouse and anti-rabbit IgG were purchased from GE Healthcare Bioscience (Piscataway, NJ). Horseradish peroxidase (HRP) conjugated ExactaCruz^{TM} A was purchased from Santa Cruz Biotechnokogy.

2. Peptide preparation from *Porphyra yezoensis*

PPY (Peptide of *Porphyra yezoensis*) was synthesized by the PEPTRON (Daejeon, Korea). Purification of the PPY was performed on

Shimadzu Prominence HPLC apparatus and controlled using the software package Class-VP, 6.14 (Kyoto, Japan), using a c18 column (Shiseido capcell pak) in 0.1% TFA/water and a gradient of 10-70% acetonitrile in 0.1% TFA, with a flow rate of 1 ml/min and using UV detection at 220 nm.

3. Cell culture

The MCF-7 cells were obtained from the (Korean Cell Line Bank) KCLB were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 ug/ml penicillin and 100 ng/ml streptomycin at 37 ℃ in a humidified atmosphere with 5% CO_2.

4. Cell proliferation assay

Cell proliferation was estimated using a CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega, Madison, WI), which is based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyrmethoxy-phenyl)-2H-tetrazolium (MTS) into a formazan product soluble in tissue medium. The cells were seeded onto 96-well plates at 2×10^4 cells per well in 100 ul medium and allowed to attach for 24 h. The cell monolayer was washed with phosphate-buffered saline (PBS) to remove unattached cells. The attached cells were maintained in serum-free medium (SFM) for 12 h and then washed with PBS. The cells were incubated with fresh SFM containing various concentration (0-500 ng/ml) of peptide for 24 h. Subsequently, the cells were incubated with 10 ug/ml MTS solution for 30 min, and

the absorbance of each well was measured at 490 nm using a SpectraMAX 340-pc multiplate reader (Molecular Devices, Sunnyvale, CA).

5. DAPI staining assay

Cell were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5 ug/ml 4,6-diamidio-2-phenylindole (DAPI) solution for 10 min at room temperature. The cells were washed two times with PBS and analyzed by a fluorescent microscope.

6. Western blot analysis

6.1. Preparation of total cell lysates

The cells were cultured to 80% confluence at 37 ℃. Cells were then maintained in SFM for 6 h. After 6 h, cells were incubated with SFM or SFM containing 500 ng/ml PPY for the indicated times and then harvested. To prepare total cell lysates, the cells were washed twice with PBS and suspended in lysis buffer (50 mM Tris (pH 7.4), 1 mM ethylene glycol tetraacetic acid (EGTA), 150 mM Nacl, 1% Triton (X-100, 0.25% sodium deoxycholate) containing Protease Arrest 100X. After incubation for 30 min at 4 ℃, the extracts were centrifuged at 12,000 rpm for 15 min. The supernatant was used as the total cell lysates.

6.2. Preparation of nuclear extract

To prepare nuclear extracts, the cells were suspended in 500 ul buffer A (10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.05% TritonX-100) containing protease inhibitor. After incubation at 4 ℃ for 15 min, the mixtures were centrifuged at 12,000 g for 15 min. Released nuclei were resuspended in buffer B (10 mM Tris (pH 7.4), 5 mM $MgCl_2$) and buffer C (10 mM Tris (pH 7.4), 4 mM $MgCl_2$, 1 M NaCl) containing protease inhibitors for 30 min at 4 ℃. Then 80% glycerol was added to the supernatant (15 min, 12,000 g), to a final concentration of 20%.

6.3. Western blot analysis

Protein (100 ug/ml) were separated using 7.5~15% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane were blocked with 1% bovine serum albumin (BSA) in TBS-T (10 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% Tween-20) and then incubated overnight with the indicated primary antibodies (diluted 1:1000) in TBS-T containing 1% BSA with gentle shaking at 4 ℃. The secondary antibody was a peroxidase-conjugated goat anti-mouse or rabbit antibody (diluted 1:10000). Signals were detected using an ECL Western blotting kit (Amersham, Piscataway, NJ, USA).

7. Gelatin zymography

MMP-2 and MMP-9 activities were analyzed using

SDS-substrate gels by adding gelatin (1 mg/ml) to the 10% acrylamide separating gel. Cells in 6-well plates were treated with SFM containing various concentration (0-500 ng/ml) of PPY for 24 h. Tumor-conditioned medium (TCM) was mixed with substrate gel sample buffer (13.3% SDS, 40% glycerol, 42 mM Tris-HCl (pH 6.5), 0.013% bromophenol blue) and loaded onto the gel without prior boiling. Following electrophoresis, the gels were washed three times in 2.5% Triton X-100 for 10 min at room temperature to remove SDS. The gels were then incubated at 37 ℃ overnight in incubation buffer (50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$). The gels were stained with 0.5% CBB G-250 in 40% methanol, and 7% acetic acid for 1 h at room temperature, and de-stained in the same solution without Coomassie blue. Gelatin-degrading enzyme were identified as clear bands against the blue background of stained gel.

8. Apoptosis assay

Annexin V & Dead Cell Assay was performed utilizing Muse™ Cell Analyzer from Millipore (Billerica, MA) following manufacturer's instruction. Briefly, after the indicated treatments, the cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) and the event for dead late apoptotic, early apoptotic, and live cells were counted.

9. Wound healing assay

MCF-7 cells in 100-mm dishes were cultured to 80% confluence.

The medium was replaced with SFM, and the cells were cultured for 24 h, after which the cells were wounded by scraping with a pipette tip. The medium was replaced with SFM containing PPY, and the cells were cultured for 24 h. Wound closure was determined from photographs taken using a microscope at ×200 magnification.

10. mRNA expression

The mRNA expression levels of specific genes were evaluated by reverse-transcription polymerase chain reaction (RT-PCR). MCF-7 cells were seeded onto 6-well plates and were cultured for 24 h, after which the medium was replaced with SFM containing PPY for 24 h. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA), and total RNA was converted to cDNA using oligo (dT) primers (iNtRON Bioerchnology Inc., Seongnam, Korea). For PCR amplification, the cDNA and specific primers were added to 2 X TOPsimple^{TM} DyeMIX-nTaq (Enzynomics, Inc., Daejoen, Korea) and 0.1% diethylpyrocarbonate (DEPC) water. The amplified products were analyzed on 1% agarose gels stained with RedSafe^{TM} nucleic acid staining solution (iNtRON Biotechnology, INC).

11. siRNA-mediated silencing of mTOR, TGF-β1

Using the nucleotide sequence for mTOR, TGF-β1 mRNA from Cosmogenetech. The control and mTOR siRNA sequence are as follow: mTOR sense siRNA 5′ugaacccugccuuugucaugc3′, mTOR antisense siRNA 5′gcaugacaaaggcaggguuca3′ and TGF-β1 sense siRNA

5′uucuccgaacgugucacgutt3′, TGF-β1 antisense siRNA 5′acgugacacguucggagaatt3′. Briefly, MCF-7 cells were transfected with control siRNA (non-targeted) or mTOR siRNA, TGF-β1 siRNA (target) using a lipofectamin (Invitrogen, Carlsbad, CA) as per the manufacturer′s instruction. The cells were cultured in the presence of transfection mixture for 72 h and the following day, the transfection mixture was replaced by fresh RPMI medium. After the transfection, cells were collected for apoptosis.

12. β-Catenin/T-cell factor-lymphoid enhancer factor-1 transcription reporter assay

The nuclear activity of endogenous β-Catenin in MCF-7 cells was analysed by using the TOPflash/FOPflash reporter system (Millipore, Billerica, MA, USA). Cell grown in 24-well dish were transfected with either the TOPflash or FOPflash plasmid using lipofectamine regent. Six hours after transfection, the culture medium was replaced by RPMI supplemented with 10% FBS in the presence of absence. 72 hours after transfection, cells were washed with PBS and then lysed with the reporter lysis buffer (Promega). Luciferase activities in cell lysates were measured with the Promega Dul-Luciferase Reporter System and normalized to control β-gal luciferase signal.

Table 1. Primers sequence For RT-PCR

Target gene		Primers Sequence
Ras	Sense	5′-CCC GTC CTC ATG TAC TGG TC-3′
	antisense	5′-ATC TTG GAT ACG GCA GGT CA-3′
MEK	Sense	5′-CGA TGG ATC CCC CAA GAA GAA GCC GAC G-3′
	antisense	5′-CGA TCT CGA GTT AGA CGC CAG CAG CAT G-3′
Raf	Sense	5′-GAT GAT GGC AAA CTC ACG GAT TC-3′
	antisense	5′-AAG GCA GTC GTG CAA GCT CA-3′
ELK	Sense	5′-CAC TTC TGG AGC ACC CTG AGT C-3′
	antisense	5′-AGA GGC CAT CCA CGC TGA TA-3′
Smad2	Sense	5′-TAG GTG GGG AAG TTT TTG CTG A-3′
	antisense	5′-CGT CTG CCT TCG GTA TTC TG-3′
Smad3	Sense	5′-GGA GGG CAG GCT TGG GGA AAA TG-3′
	antisense	5′-GGG GAG GGT GCC GGT GGT GTA ATA-3′
Smad4	Sense	5′-AAG GTG AAG GTG ATG TTT G-3′
	antisense	5′-GAG CTA TTC CAC CTA CTG AT-3′
Snail	Sense	5′-TATGCTGCCTTCCCAGGCTTG-3′
	antisense	5′-ATGTGCATCTTGAGGGCACCC-3′
Axin	Sense	5′-ACC GAA AGT ACA TTC TTG ATA AC-3′
	antisense	5′-TCC ATA CCT GAA CTC TCT GC-3′
ICAM	Sense	5′-CACCTCCTGTGACCAGCCCA-3′
	antisense	5′-AACAGGACGGTCGCTGAGGG-3′
Frizzled	Sense	5′-CAG AGC GGG GCA GCA GTA CAA-3′
	antisense	5′-GCG CGG GCA GGA GAA CTT-3′
Rho A	Sense	5′-CTC ATA GTC TTC AGC AAG GAC CAG TT-3′
	antisense	5′-ATC ATT CCG AAG ATC CTT CTT ATT-3′
Rho B	Sense	5′-ATG GCG GCC ATC CGC AAG AAG C-3′
	antisense	5′-TCA TAG CAC CTT GCA GCA GTT G-3′
Cdc42	Sense	5′-CGA CCG CTA AGT TAT CCA CAG-3′
	antisense	5′-GCA GCT AGG ATA GCC TCA TCA-3′
Rac1	Sense	5′-GGA CAC AGC TGG ACA AGA AGA-3′
	antisense	5′-GGA CAG AGA ACC GCT CGG ATA-3′
TGF-β1	Sense	5′-GCA GAA CCC AAA AGC CAG AGT G-3′
	antisense	5′-CCA TAA CTA CCG TGG AGG TTG A-3′
Wnt 1	Sense	5′-TGC ACG CAC ACG CGC GTA CTG CAC-3′
	antisense	5′-CAG GAT GGC AAG AGG GTT CAT G-3′
Occluddin	Sense	5′-TCA GGG AAT ATC CAC CTA TCA CTT CAG-3′
	antisense	5′-CAT CAG CAG CAG CCA TGT ACT CTT CAC-3′
β-Catenin	Sense	5′-GAA ACG GCT TTC AGT TGA GC-3′

	antisense	5′-CTG GCC ATA TCC ACC AGA GT-3′
GSK-3β	Sense	5′-CAG CAA GGT GAC AAC AGT GG-3′
	antisense	5′-GGA ACA TAG TCC AGC ACC AGA-3′
C-myc	Sense	5′-CCA GGA CTG TAT GTG GAG CG-3′
	antisense	5′-CCT GAG GAC CAG TGG GCT GT-3′
GAPDH	Sense	5′-CAG CCG AGC CAC ATC G-3′
	antisense	5′-TGA GGC TGT TGT CAT ACT TCT C-3′

Ⅲ. Results and Discussion

Part 1. Preparation of *Porphyra yezoensis* peptide

1. Peptide of *Porphyra yezoensis* (PPY)

A peptide having potent were purified. The amino acid sequenced of the purified peptides were analysed as: PPY (K-K-A-A-E), molecular mass was determined to be 546 Da (Fig.1).

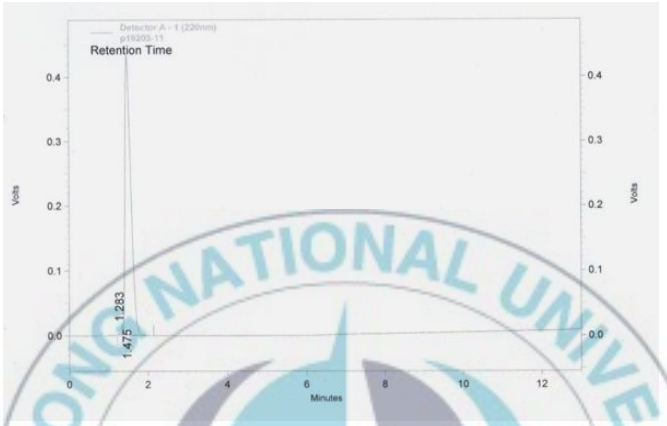


Fig. 1. Separation of peptide *Porphyra yezoensis* (PPY)

Separation of peptide *Porphyra yezoensis* (PPY) by HPLC (capcell pak C18 column).

Part 2. Inhibitory effect of PPY on MCF-7 cells

1. Inhibitory effect of PPY on proliferation of MCF-7 cells

Proliferation and death of cells are carefully and continuously balanced in multi-cellular organism. Imbalances in the cooperation of these processes can result in diseases such as cancer due to over proliferation and reduced cell death (Overholtzer *et al.*, 2007; Coates *et al.*, 2010). Cancer begins when a cell breaks free from the normal restrictions on cell division and begins to follow its own agenda for proliferation (Brita *et al.*, 2005; Yilmaz *et al.*, 2009; Shinichi 2010).

Using MCF-7 cell the effect of peptide from *Porphyra yezoensis* (PPY) on the proliferation of these cells were evaluated by MTS assay. The conversion level of MTS to colored formazan by succinate dehydrogenase is proportional to the number of living cells.

PPY inhibition proliferation of MCF-7 cells, as determined by the MTS assay. After exposure to 0~500 ng/ml PPY for 24 h exposure to 500 ng/ml PPY inhibited growth by 60%. In MTS assay, PPY a dose-dependent effect on the growth of MCF-7 cells (Fig.2). After exposure to 0-500 ng/ml PPY for 24 h, relative cell number decreased in a concentration-dependent manner. Compared with untreated cells, that MCF-7 cells exhibit inhibition of cell growth after exposure to PPY for 24 h. Moreover, In accordance with the results of MTS assay, PPY induced apoptotic cell death in a dose-dependent manner in MCF-7 cells by DAPI assay (Fig.3). To confirm that PPY decreased cell proliferation, this result observed cell morphology. The

cells treated with PPY appeared to decrease in number compared to untreated cells. The DAPI staining showed that PPY inhibited the proliferation of MCF-7 cells in a time-dependent manner. DAPI assay confirmed that PPY treatment induced cell death. The result showed effect PPY in MCF-7 cell growth inhibition.

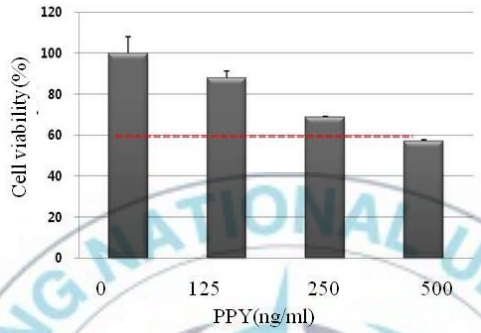


Fig. 2. Effect of PPY on the MCF-7 cells proliferation.

MCF-7 cells were treated with various concentrations of PPY for 24 hours, and cytotoxicity was determined by the MTS assays.

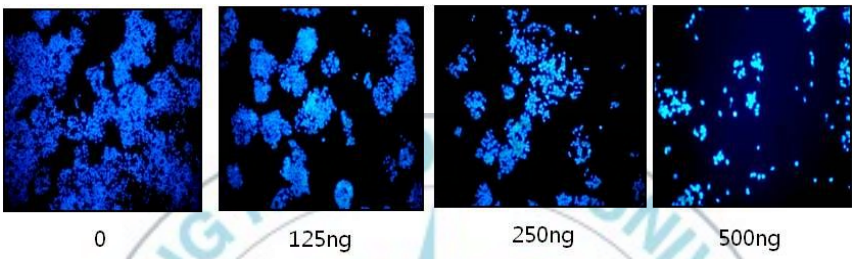


Fig. 3. Effect of PPY on cell morphology of MCF-7 cells

PPY caused morphological changes in MCF-7 cancer cells with DAPI staining assay. After 24 h of PPY (0-500 ng/ml), cells were observed under an optical microscope. Photographs were taken at a magnification of ×200.

2. Activation of PI3K-AKT pathway

PI3K/Akt pathway is mainly associated with growth. PI3K/Akt signaling pathways are known to be important for cell differentiation and proliferation. The PI-3K signal transduction pathway regulates cancer survival, apoptosis, and a number of diverse cellular processes such as growth, proliferation, cell transformation, metastasis, membrane trafficking, and cell survival (Carlos *et al.*, 2004). Moreover, activated PI-3K induces colon cancer proliferation and can convert differentiated human gastric and colon carcinoma cells to a less differentiated and more malignant phenotype (Kobayashi *et al.*, 1999). In the PI3K/AKT pathway, growth factors activate PI3K via tryosine kinase receptors, and activated PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), converting PI(4,5)P2 to PI(3,4,5)P4, a lipid second messenger (Czech 2003).

Akt binds to PI(3,4,5)P3 and changes conformation following phosphorylation by 3-phosphoinositide-dependent PDK1. Activated Akt phosphorylates numerous substrates that are involved in various cellular formations like cell growth, cell cycle, apoptosis and glucose metabolism (Liang *et al.*, 2003; Fresno *et al.*, 2004) AKT, as a major down-stream modulator of PI3K, enhances cell proliferation via a variety of molecular mechanisms, including phosphorylation and inactivation of pro-apoptotic genes (Malaguarnera *et al,.* 2004; Carlos *et al,.* 2004; Croci *et al,.* 2004).

In the present study, the involvement of this pathway in PPY-induced down regulation on MCF-7 cancer cell proliferation. To

examined whether PPY stimulates the activation p85, a subunit of PI3K. PPY caused decrease activation of Akt and Akt phosphortlation in MCF-7 cells treated with PPY. Moreover, PPY caused decreased activation of p85. These results indicate that the activation of the PI-3K/Akt pathway is also involved in PPY nduced inhibition of MCF-7 cells. These results indicated that the characterization of PPY-induced decrease of MCF-7 cells involved in the regulation of the PI3K signaling pathway (Fig.4).

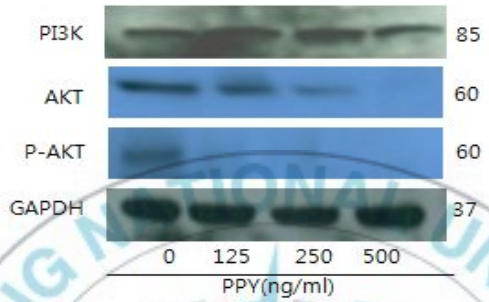


Fig. 4. Effect of PPY on the expression of PI3K/AKT pathway

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown PI3K, AKT and p-AKT proteins.

3. The expression of IGF-IR

Insulin-like growth factor-I receptor (IGF-IR) is significant in cell growth, differentiation, and survival (Butler *et al.*, 1998). Overexpression of IGF-IR and related proteins results in cancer cell proliferation and survival. Thus, IGF-IR is involved in malignant transformation (Rubini *et al.*, 1997; Reiss *et al.*, 1998). IGF-I signaling plays a role in cancer development and progression (Baserga *et al.*, 2003; Werner *et al.*, 2000). IGF-I protected cancer cells against apoptosis. The mechanisms by which IGF-I and IGF-I receptor interact with cell death pathways remain unclear. Therefore, it is important to elucidate the relationships between IGF-I and IGF-I receptors and apoptotic pathways. (Remacle-Bonnet *et al.*, 2000).

To further investigate the mechanism of PPY induced inhibition, this study used IGF-IR protein to examine whether peptide activates the signaling pathway in MCF-7 cells. The signaling activity of IGF-IR is crucial to the control of apoptosis and proliferation processes, and IGF-IR activation results in autophosphorylation (Rubini *et al.*, 1997). Therefore, these results suggest the effect of PPY on IGF-IR expression. MCF-7 cells were treated with different concentration of PPY for 24 h, Fig.5, shows western blot analysis of apoptosis-associated protein in MCF-7 cells. Expression level of IGF-IR were decrease in MCF-7 cell in a PPY dose dependent manner. The protein level of ERK was decrease in 125 ng/ml PPY treated MCF-7 cells, whereas phosphorylation of ERK was inhibited by PPY. These results also found that treatment with PPY resulted in

activation of the intrinsic apoptosis pathway by decrease the ERK and phosphor ERK in MCF-7 cells (Fig.5).

Induction of apoptosis has become a predominant mechanism by which chemotherapeutic agent exerted cytotoxicity (Wang *et al.*, 2008). The data presented here provided evidence of the apoptotic effect of PPY in MCF-7 cells.

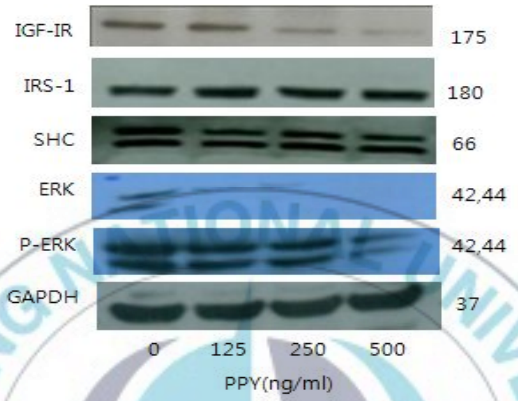


Fig. 5. Effect of PPY on the expression of IGF-IR pathway

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown IGF-IR, IRS-1, SHC, ERK and p-ERK proteins.

4. Expression of cell cycle-related protein

Cell cycle progression is a highly ordered and tightly regulated process that involves multiple checkpoint at extracellular growth signal, cell size, and DNA integrity are monitored. Deregulation of cell cycle has been recognized as the hallmark of cancer progression in most malignant tumor. Furthermore, it has been shown to induce an aberrant form of mitosis celled mitotic catastrophe and also may be involved in triggering apoptosis (Liu *et al.*, 2009).

Apoptosis is regulated by proteins that are involved in cell cycle progression. There is accumulation evidence that manipulation of the cell cycle may prevent or induce an apoptotic response depending upon the cellular context. Because cellular context is crucial, these proteins may serve to tie cell death with proliferative signals even though they may not be part of the cell's apoptotic machinery (Pucci *et al.*, 2000).

These results showed that PPY treatment causes a dose dependent decrease in the level CyclinE and CDK6. In contrast, p21, p27 increase in a dose dependent manner after PPY treatment (Fig.6). Reduces expression of p27 has been observed in several human cancers, and p27 is associated with the induction of apoptosis in cancer cells (Naumann *et al.*, 1999). As shown in Fig.6, PPY treatment decreased protein expression of Cdk6 and CyclinE, whereas levels of Cdk2 were largely unchanged. The Cdk inhibitor p21 and p27 suppresses the activity of the Cyclin E/Cdk complex. Reduced expression of p27 has been observed in several human cancers, and p27 is associated with the induction of apoptosis in cancer cells

(Naumann *et al.*, 1999; Murillo *et al.*, 2002).

In order to determined that PPY also affects the cell cycle regulators of sub-G1 phase, expression levels of these regulator were examined. The progression of the cell cycle is largely controled by coordinated successive activation of Cdk, which is in turn modulated by an association with regulatory subunits, Cyclins, and group of Cdk-inhibitory proteins (Fig.7). Cyclin E is highly expressed during the G1 to S phase transition, and Cdk, including Cdk2, Cdk4, Cdk6 are critical regulators during the phase transition (Murillo *et al.*, 2002). Therefore, these results suggest that PPY may be modulating the sub-G1 arrest via upregulation of p21 and downregulation of Cdk6 and Cyclin E.

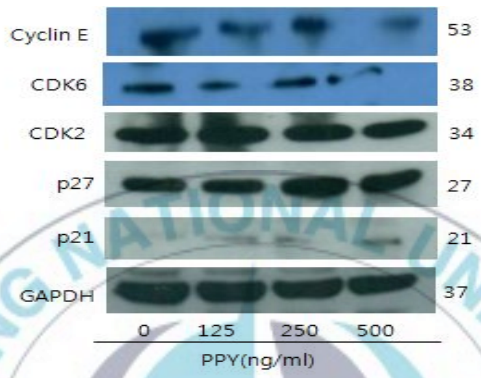


Fig. 6. Effect of PPY on the expression of cell-cycle related proteins

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown CyclinE, CDK6, CDK2, p27 and p21 proteins.

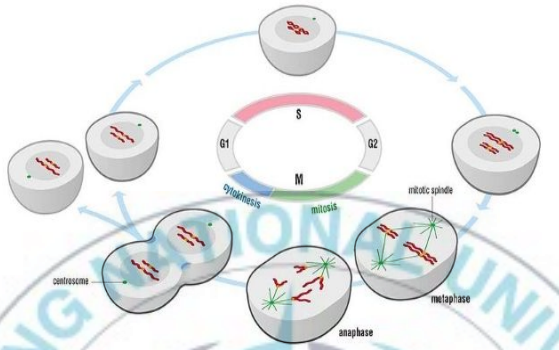


Fig. 7. Schematic model for mammalian cell cycle progression

The cell cycle is divided into 4 broadly defined pahses; the G1(growth and preparation of the chromosome for repilactaion), S(synthesis of DNA), G2(preparation for mitosis), M(mitosis) phase. Beata G (2000).

5. Induced apoptosis by PPY

This study was the mode of cell death and cell cycle arrest induced in breast cancer cells by PPY. The induction of cell death through apoptosis by the PPY on breast cancer cells was confirmed by Annexin-V. This study performed Annexin V staining assay for cell apoptosis rate by Muse Annexin V and Dead Cell kit.

MCF-7 cells were treated with PPY and apoptotic and necrotic cells were detected by Annexin V and 7-aminoactinomycin D (AAD) staining, respectively. In this assay, cells in the early stage of apoptosis are Annexin V-positive and 7-AAD-negative, and those in late apoptosis are Annexin V-positive and 7-AAD-positive. In the present study, PPY treatment increased the percentage of apoptotic MCF-7 cells, in a dose-dependent manner. Control cells comprised 8.15% apoptotic cells, 0.45% necrotic cells and 91.40% living cells (Fig.8). Treatment with 125, 250 and 500 ng/ml PPY for 24 h resulted in 15.75, 20.70 and 25.80% apoptotic cells, respectively. In this study, the cells treated with the highest concentration (0-500 ng/ml) of PPY were observed to undergo apoptosis. The apoptosis rate increased 25.80% in final PPY concentration (500 ng/ml) compared with the control group (Fig.8). This indicates that even at the highest concentration, the mode of cell death is still the preferable one, apoptosis. During early apoptosis, the externalization of the phospholipid phosphatidylserine occurs at the cell membrane and could be detected by Annexin V. Therefore, Annexin could be used as probe for detection of apoptosis (Vangestel *et al.*, 2010).

As apoptosis is believed to play a major role in the survival and proliferation of the neoplastic cells, failure to activate apoptosis represents as one of the major obstacles to the success of a particular cancer treatment (Cummings *et al.,* 2004).

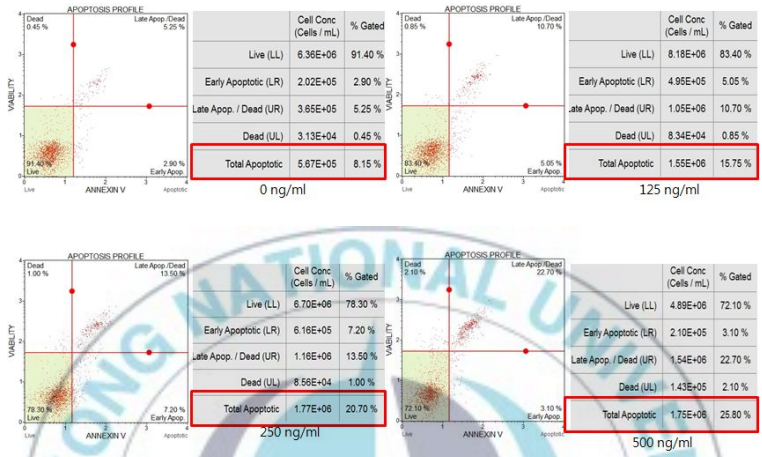


Fig. 8. PPY induced apoptosis of the MCF-7 cells.

Cells cultured in 6-well plates were treated with PPY (0-500 ng/ml) and then collected in medium containing 1% FBS. The percentages of apoptotic and necrotic cells were determined using a Muse Annexin V and Dead Cell kit as described in Materials and method. Cells in the early stage of apoptosis are Annexin V-positive and 7-AD-negative, and those in late apoptosis are Annexin V-positive and 7-AD-positive.

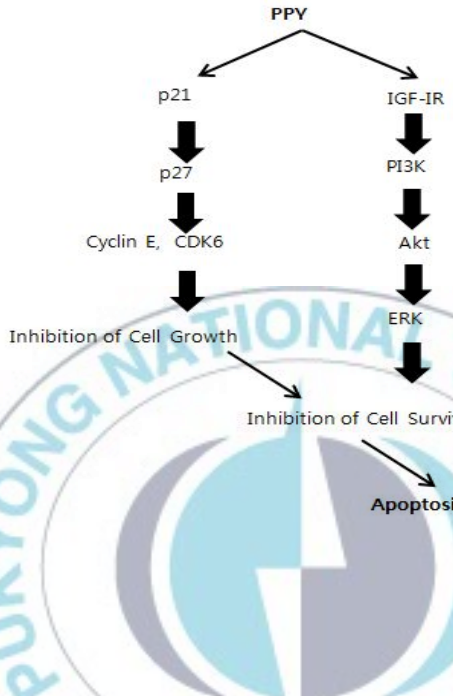


Fig. 9. The proposed model of PPY induced apoptosis in MCF-7 cells

6. Expression of mTOR pathway

Mammalian target of rapamycin (mTOR) is a serine-threonine kinase that plays an important role in the regulation of cell proliferation and protein synthesis through the activation of its downstream target ribosomal p70S6 kinase (Varma *et al.*, 2007). mTOR is activated by several stimuli such as growth factor, nutrients through receptor tyrosine kinase (RTK), phosphatidyl inositol 3 kinase (PI3K) and Akt/PKB signaling cascde (Xu *et al.*, 2004). The mTOR pathway is involved in the development of many human cancers (Faivre *et al.*, 2006), and it examined the activation of mTOR pathway in MCF-7 cells (Fig.10). The mammalian target of rapamycin (mTOR) is also a serine/threonine protein kinase that regulates cell growth by integrating nutrient-and growth factor-derived signal (Fingar *et al.*, 2004; Dann *et al.*, 2006). As shown in Fig.10, mTOR and p70S6K were decrease dose-dependent on MCF-7 cells by PPY. High concentrations of PPY considerably decrease PDK1 protein level. In this study shown that PPY can increase PTEN protein level in a dose-dependent manner in MCF-7 cells, and this was accompanied by decrease of RPS6. Therefore, these results showed effect PPY in MCF-7 cell growth inhibition.

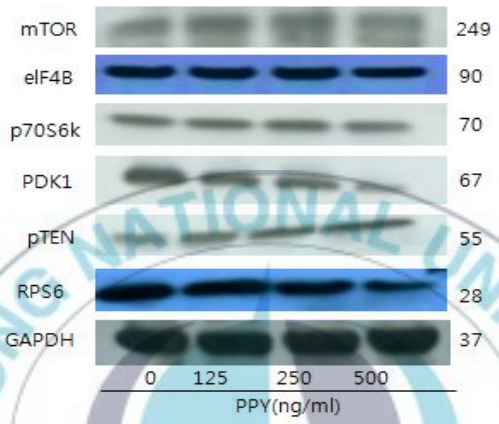


Fig. 10. Effect of PPY on the expression of mTOR pathway

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown mTOR, eIF4B, p70S6K, PDK1, pTEN and RPS6 proteins.

7. p70S6K plays an important role in metastasis

mTOR and p70S6K are an important part of signaling pathway in etiology of several types of cancers and other metabolic disorders like obesity and diabetes. Understanding the behavior of upstream and downstream targets of mTOR and p70S6K is an important process in providing insights into various processed in diseased situation as compared to normal (Sarbassov *et al.,* 2005). The 70-kDa ribosomal protein S6 kinase (p70S6K) is a Serine/threonine kinase that regulates protein translation by phosphorylating ribosome protein S6 (Jefferies *et al.*, 1997; Manteuffel *et al.*, 1997). p70S6K has been associated with breast cancer, poor prognosis, and metastasis, but the mechanisms associated with the role of p70S6K in metastasis are not well understood. To determine the downstream targets and mechanisms that may play a role in metastasis, this study used western blot analysis to detect proteins that may be critical in cell attachment, motility, invasion, and metastasis (Akar *et al.,* 2010). The aim of this study was to investigate if p70S6K is involved in breast cancer metastasis.

PPY cause decrease activation of p70S6K in MCF-7 cells. This study also observed the downregulation of TG2, B-Catenin, p-FAK protein (Fig.11). Downregulation of p70S6K also inhibited tissue transglutaminase 2 and β-Catenin expression in the MCF-7 cells. The results of this study show that p70S6k is involved in metastasis in MCF-7 cells.

Tissue transglutaminase (TG2), a member of the

transglutaminase family, is a calcium-dependent enzyme that catalyzes the covalent cross-linking of proteins. This multifunctional protein is expressed ubiquitously and abundantly, and has been implicated in a variety of cellular processes, such as cell differentiation, death, inflammation, migration, and wound healing (Fesus *et al.*, 2005; Fesus *et al.*, 2002). Expression level of TG2 were decrease in MCF-7 cells in a PPY dose-dependent manner. The expression proteins TG2 was decrease, when cells were activated by PPY.

P70S6K plays an important role metastasis by regulating key proteins such as Cyclin D1, PDCD4, focal adhesion kinase. E-Cadherin, β-Catenin, and tissue transglutaminase 2 that are essential for cell attachment, survival, and invasion, as well as metastasis in breast cancer (Fig.12).

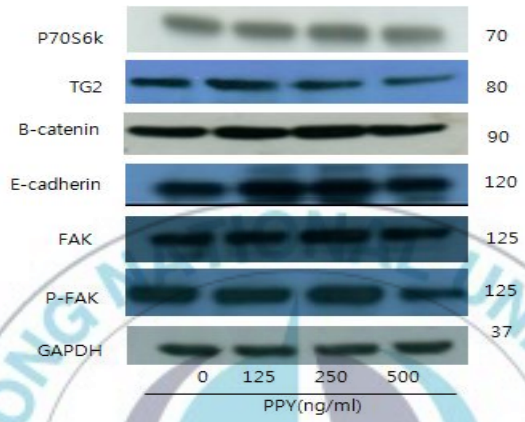


Fig. 11. Targeting p70S6K inhibition of cells migration and metastasis by PPY

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown p70S6K, TG2, β-Catenin, E-Cadherin, FAK and p-FAK proteins.

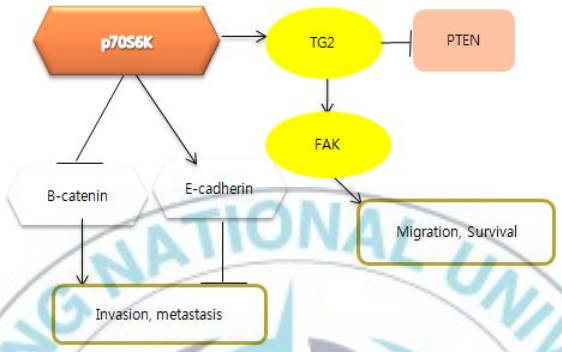


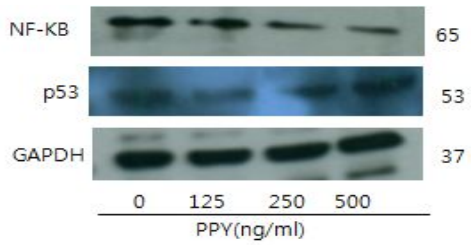
Fig. 12. p70S6K plays an important role in metastasis by regulating key proteins, such as focal adhesion kinase, β -Catenin, E-Cadherin and tissue transglutaminase 2 that essential cell attachment, survival, invasion and metastasis in MCF-7 cells

8. Activation of NF-kB and Bcl-2 family

The Akt/mTOR signaling is crucial to many widely divergent physiological processes which include transcription, differentiation, apoptosis, and metabolism (Yuan *et al.*, 2008). Akt plays a crucial role in determining cell fate by regulating fundamental transcriptional factors, such as nuclear factor-kappa B (NF-κB)(Pearson *et al.*, 2008). NF-kB induce upregulation of Bcl-2 family members, and the balance between anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax plays a major role in regulating apoptosis. NF-KB which plays a pivotal role in cell survival regulates vast number of genes related to apoptosis such as Bcl-2, Bax and Fas (Robort *et al.*, 2007). Bcl-2 family members such as Bax and Bad promote apoptosis, whereas, other members including Bcl-2 and Bcl-Xl exert anti-apoptosis effects (Juliane *et al.*, 1998).

As shown in Fig.13 activity NF-κB and Bcl-2 were decreased whereas p53 and Bad, Bax activity were increased by PPY. This result indicated that PPY controls apoptosis regulator gene expression by down-regulation of p53 and up-regulation of NF-κB activity to PPY induced apoptosis in MCF-7 cells. Importantly, this study also observed that the p53/NF-κB and PI3K/Akt/mTOR pathway are affected by PPY and aided explaining the cellular functional relationship between NF-κB, Bcl-2 family genes, and mTOR under PPY induced apoptotic conditions in MCF-7 cells. This results showed that PPY might modulate anti-cancer and Akt/mTOR signaling.

(A)



(B)

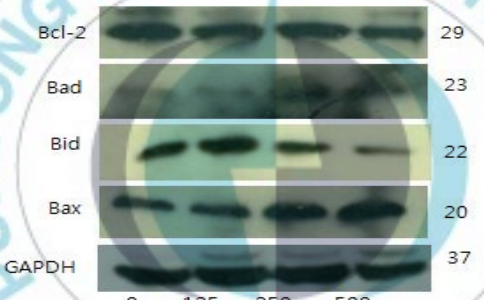


Fig. 13. Effect of PPY on the expression of NF-κB, p53(A) and Bcl-2 family(B)

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown NF-kB, p53, Bcl-2, Bad, Bid and Bax proteins.

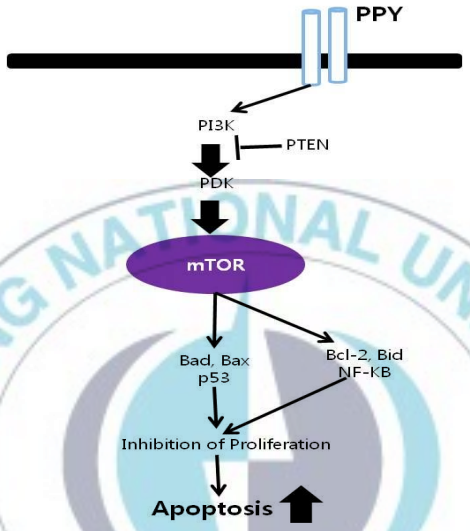


Fig. 14. The propose model of NF-κB and Bcl-2 family in MCF-7 cells

9. The role of autophagy

Autophagy is signaling pathway which leads to degradation of cellular components by lysosomal activity. Due to an enhanced supply of monomers originating from the cells own resources, this process is essential for survival during stress. In addition, autophagy has been shown to be important in a variety of other cellular processes including the recycling of aged or damaged organelles, remodeling of cellular structures during development, cell death, and protection against bacterial infection (Levine *et al.,* 2004). The relationship between autophagy and cancer has been a hot topic for years, and currently there are numerous studies shedding light onto different aspects of this relationship. Recent studies suggest that autophagy may cooperate with apoptosis to promote cell death (Alfred *et al.,* 2009; Corcelle *et al.,* 2009; Levine *et al.*, 2009).

As shown in Fig.15, a trend of increased expression of autophagy-associated proteins such as LC3, Beclin1, Atg5 and Atg7. Here we detected MCF-7 cells treated with 500 ng PPY 24 h displayed an increase LC3 protein compared with untreated cells. As a specific marker for autophagy, LC3 was widely used to monitor autophagy. Lipidation of microtubule-associated protein LC3, as an autophagy marker, coats autophagosomes during autophagy and is converted to LC3-Ⅱ resulting in the appearance of the delayed electrophoretic mobility in gel (Chan *et al.*, 2000). Beclin1 is an essential autophagic gene that contributes to the initial vesicle nucleation and formation of autophagosome, whereas Atg5 participates

in autophagic vesicle elongation and completion (Zhang *et al* 2013).

As shown in Fig.16, this section to briefly summarize what we know about the anatomy of autophagy, and the role of Atg and other proteins involved in the formation and maturation of autophagosomes. For more detailed discussions, readers should consult recent reviews dedicated to the morphology of autophagy (Esklinen *et al.,* 2005; Fingar *et al.,* 2004), the origin of the membrane source for autophagy (Juhasz 2006; Mijaljica *et al* 2006; Reggiori *et al*., 2005), the role Atg proteins during the formation of autophagosomes (Klionsky 2005; Suzuki *et al.,* 2007) and the molecular events that govern the maturation of autophagosomes (Esklinen *et al*., 2005)

These results supported the idea that PPY induce autophagy in MCF-7 cells. These in vitro data suggested that PPY inhibits tumor growth and induces apoptosis in MCF-7 cells. In addition, this study demonstrated that PPY induced autophagy via the Akt/mTOR pathway.

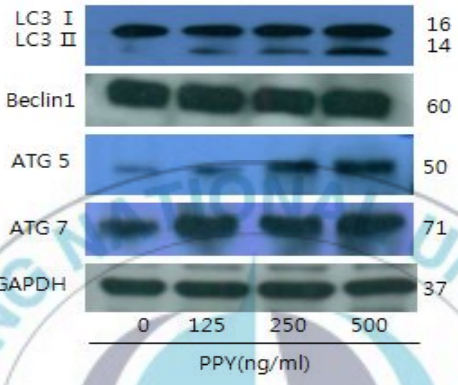


Fig. 15. Effect of PPY on the expression of Autophagy related proteins

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown mTOR, Beclin1, ATG5 and ATG7 proteins.

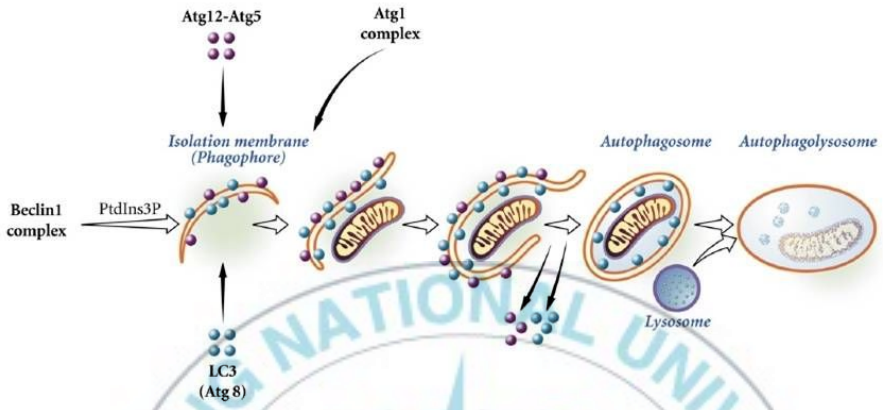


Fig. 16. The proposed model of the role of autophagy

The process of autophagy is regulated by Autophagy-related gene (Atg) and their homologes in different eukaryotic cells. The diagram shows the main stages of autophagosome development: phagophore formation, elongation, autophagosome formation and its fusion with lysosome (Parkhitko *et al*., 2013).

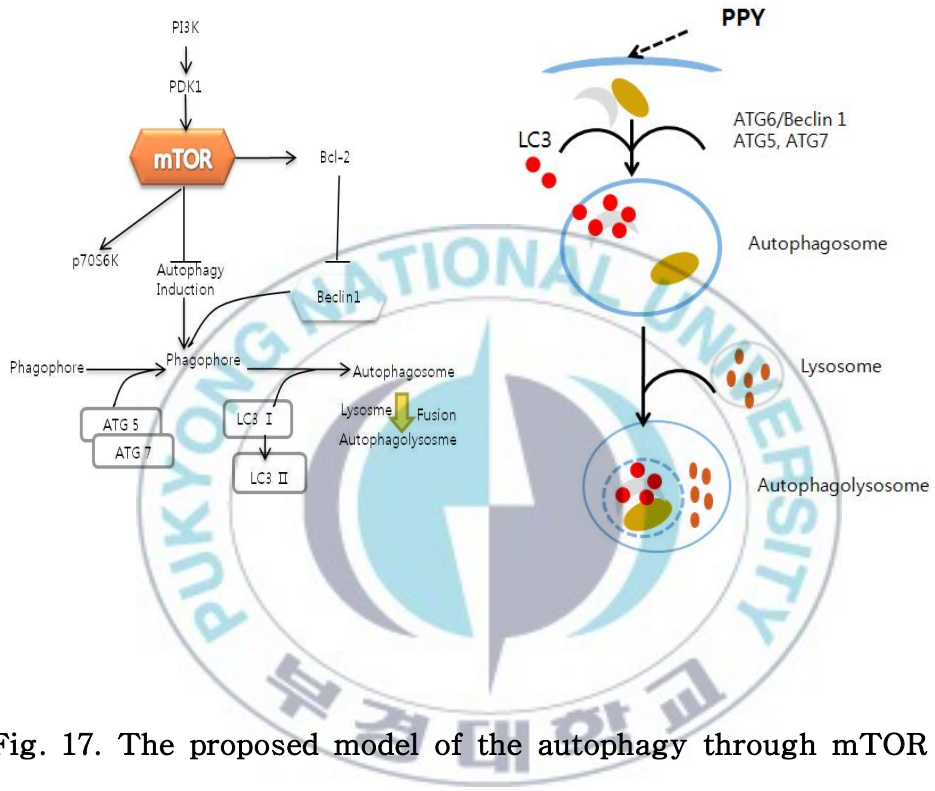


Fig. 17. The proposed model of the autophagy through mTOR

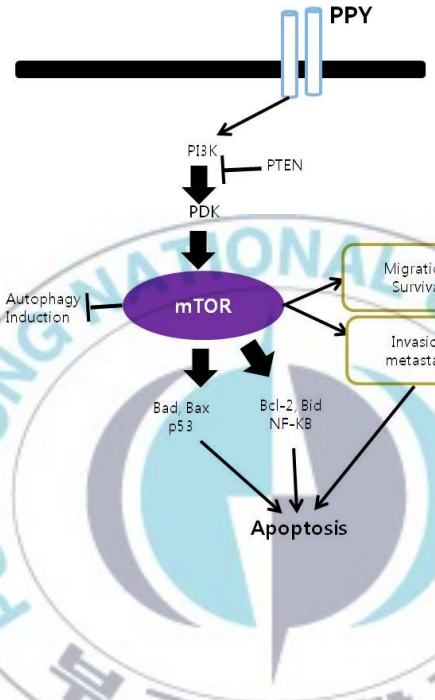


Fig. 18. The proposed model of the autophagy and metastasis through mTOR signaling pathway

Part 3. Inhibitory effect of metastasis by PPY

1. Effect of cell death in MCF-7 cells by co-treatment TGF-β1 with PPY

Transforming growth factor-β1 (TGF-β1) is a potent regulator in promoting the invasion and proliferation of breast cancer cells (Yang *et al.*, 2010). In several types of advanced cancers, TGF-β1 has been shown to promote cancer cell invasion and metastasis; whilst inhibition of TGF-β1 receptor function by expressing dominant negative suppress cell invasion (Artega *et al.*, 1998; Arteage 1997).

In order to evaluate whether TGF-β1 affects change of cells in time-dependent manner, the cells were incubated with 0-2 ng/ml TGF-β1 for 0-48 h. To identify the effect of PPY on cell death, cells were treated with or without TGF-β1 (0-2 ng/ml). The results showed that microscopic analysis confirmed that PPY treatment induced cell death. In this study, TGF-β1 was used to induced cell death of MCF-7 cells, with 48 hours and 1 ng/ml of TGF-β1 treatment, MCF-7 cells undergo a dramatic morphological change, from compact, cobblestone-like epithelial structures (Fig.19). Change of cell morphology and decreased of cell numbers became pronounced dose-dependent and time-dependent manner. Microscopic analysis showed that TGF-β1 treatment was that apoptotic cells and apoptosis than control group.

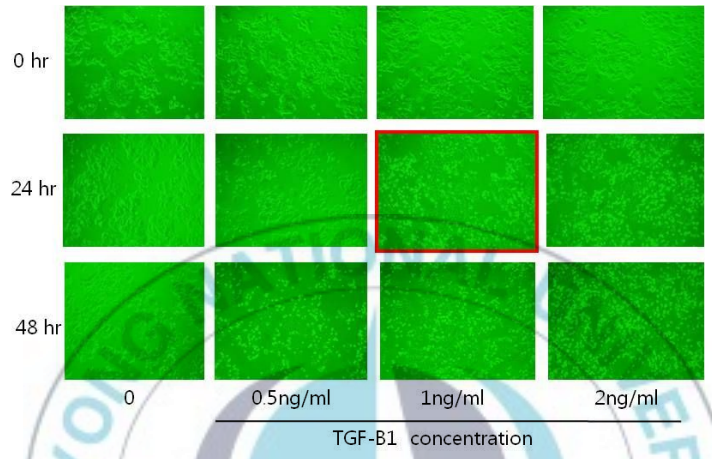


Fig. 19. Morphological changes in MCF-7 cells by TGF-β1

The cells were treated with SFM (control) or TGF-β1 (0.5-2 ng/ml) for indicated times. After the indicated times, the morphologies were photographed using phase contrast optics.

2. Activation of TGF-β1 pathway

Transforming growth factor-β1 (TGF-β1) signaling transduction pathway participates in many human disorders, including atherosclerosis, fibrosis and cancer (Blobe *et al.*, 2000). Smad-dependent pathway is a specific TGF-β1 signaling pathway participate in cancer development (Bierie *et al.*, 2006; Derynck *et al.*, 2003). Smads family is a group of TGF-β1 signal-specific intracellualr signal transducers, and Smad4 is a key protein that mediates all the Smad signaling pathway-specific ligands, and play a critical role in TGF-β1 signaling pathway (Hahn *et al.,* 1996; Sam *et al.,* 1996).

Many studies have focused on Smad-dependent TGF-β1 signaling pathway with tumor. The role of TGF-β1/Smad on tumor cell growth and metastasis in MCF-7 cells is not fully understood (Bierit *et al.*, 2006). This study used a series of cell function assays to examine the role of TGF-β1/Smad signaling in MCF-7 cells. This results observed the effects inhibition of growth on TGF-β1 and expression level of Smad is downregulation in dose-dependent manner (Fig.20, 21). This indicates that there is a Smad-dependent PPY induced growth inhibition. In this study, TGF-β1 signaling pathway induced cell apoptosis when PPY treatment, the obvious apoptosis phenomenon may be related to a certain apoptosis pathway mediated by decreased Smad expression. These results suggest that restoration of Smad expression in MCF-7 cells is crucial for TGF-β1 playing its role in inhibiting cell growth. In different cell types, TGF-β1 can both inhibit cell proliferation and induce apoptosis (Hague *et al.,* 1998;

Wimmell *et al.,* 2003).

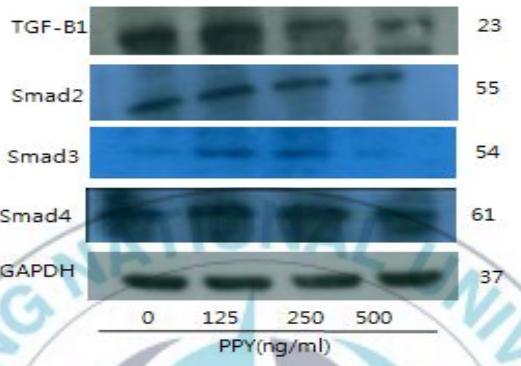


Fig. 20. Effect of PPY on the expression of TGF-β1 signaling by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown TGF-β1, Smad2, Smad3 and Smad4 proteins by Western blot.

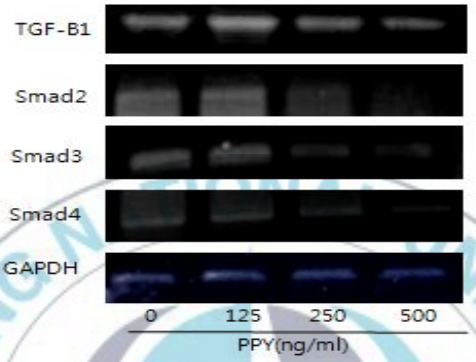


Fig. 21. Effect of PPY on the expression of TGF-β1 signaling by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown TGF-β1, Smad2, Smad3 and Smad4 proteins by RT-PCR.

3. Smad expression by stimulated TGF-β1

The transforming growth factor beta (TGF-β) family of signaling molecules are key regulators of both developmental, and malignant processes (Walsh *et al.*, 2011). Many signaling pathway are implicated the biological effects of TGF-β stimulation, and many molecules are involved in TGF-β signaling, such as the extracellular signal-regulated kinase (ERK), c-jun NH_2-terminaet kinase (JNK), p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), etc. while among the net the Smad-dependent pathway is a specific signaling pathway participate in cancer development (Bierie *et al.*, 2006; Derynck *et al.,* 2003)

TGF-β1 stimulates inhibit cell proliferation through Smad-mediated pathway. In the western blot analysis, it was clear that PPY with TGF-β1 1ng/ml treatment sufficiently decreased TGF-β1, Smad2 and Smad4 protein expression compared to the only TGF-β 1 treatment group (Fig.22). Collective analysis of this data showed that the PPY have inhibitor activity against gene expression of these proteins.

In this study, have shown that the death of MCF-7 cells under stimulation of TGF-β1, suggesting the possibility of using PPY to prevent and reduce MCF-7 cells through directly binding to TGF-β1 and blocking its signal transductions. This study gives further insight into the specific pathway that are required for TGF-β1 activation and highlights the importance of Smad levels and TGF-β1 activity in cells, particularly breast cancer cells.

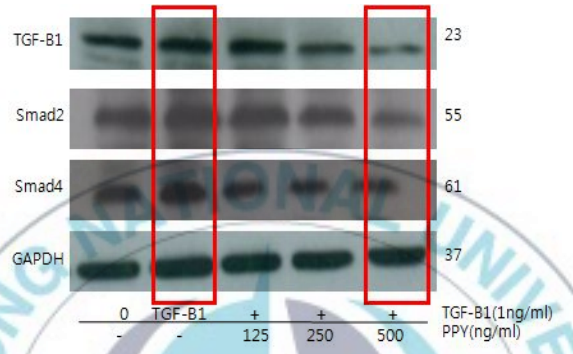


Fig. 22. Activation of Smad signaling pathway by PPY in MCF-7 cells

Cells were treated with several concentrations of PPY for 24 h and stimulated with TGF-β1 for 48 h. Cell membranes were prepared and analyzed by Western blot analysis using TGF-β1, Smad2, Smad3 and Smad4 antibodies.

4. Activation of Epithelial-to-mesenchymal transition markers

Transforming Growth Factor β (TGF-β) is a major driving force of the Epithelial-to-Mesenchymal (EMT) genetic program, which becomes overactive in the pathophysiology of many related human diseases (Cufi *et al.,* 2010). Tissues in our bodies are mainly composed of two different types of cells-mesenchymal and epithelial cells. Mesenchymal cells are solitary, capable of migrating and interacting with matrix proteins in interstitial tissues. On the contrary, epithelial cells interact with each other to for cell layers, which act as barriers that protect our bodies from the environment (Amanda *et al.*, 2010). Epithelial-to-mesenchymal transition (EMT) is a cellular process during which epithelial cells lose their polarized organization and cell-cell junctions, undergo changes in cell shape and in cytoskeletal organization and acquire mesenchymal characteristics, such as fibroblase-like cell morphology and increased cell migration and invasion (Hay 1968). EMT involves the loss of epithelial markers, such as the tight junction proteins claudins and occludins the adherens junction proteins E-cadherin, α and β-catenin, and cytokeratins. Concomitantly, a number of mesenchymal markers are increased in their expression, including N-cadherin, vimentin, fibronectin, matrix metalloproteinase, integrin α_V and β_1 and smooth muscle actin (Fig.23). (Thiery *et al.,* 2006; Yang *et al.,* 2008; Kang *et al.*, 2004; Christofori 2006). EMT-promoting transcription factors, such as snail and zeb proteins bind inactivate promoters of genes encoding junction proteins like E-cadherin, claudins and occludin (Peinado *et al.*, 2007). During

EMT, cells lose their epithelial polarity and dissolve their adherent and tight junctions, favoring a high plasticity in cell-cell adhesion and communication with the extracellular matrix through focal adhesions (Christofori 2006).

In this study, it was determined whether PPY affects the expression level of EMT of occludins, snail and vimentin in MCF-7 cells. Western blot analyses and RT-PCR confirmed that the amount of occludins, snail proteins decreased in dose-dependent manner after treatment with PPY (Fig.24, 25). By contrast, As shown in Fig.24, 25, PPY treatment significantly increased the expression level of vimentin. This expression pattern is consistent with EMT seen in other cell types (Yang *et al.*, 2008). These findings indicated that PPY treatment may have played a role in promoting the activation of TGF-β1 leading to EMT.

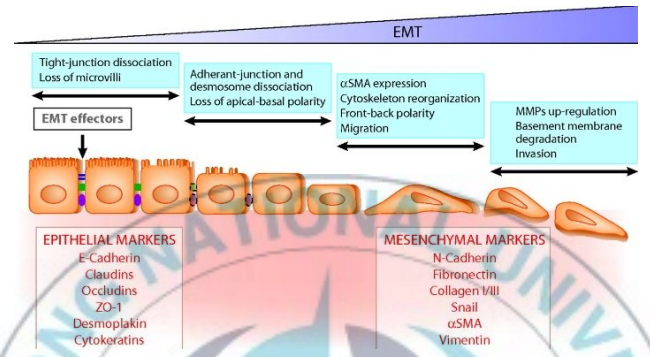


Fig. 23. Key events during EMT.

The diagram shows four key steps that are essential for the completion of the entire EMT course and the most commonly used epithelial and mesenchymal markers (Aroeira *et al.,* 2007).

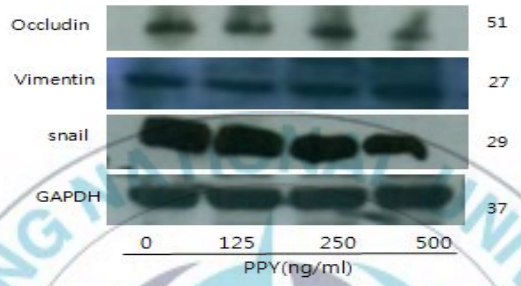


Fig. 24. Effect of PPY on the expression of EMT marker by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Occludin, Vimentin and Snail proteins.

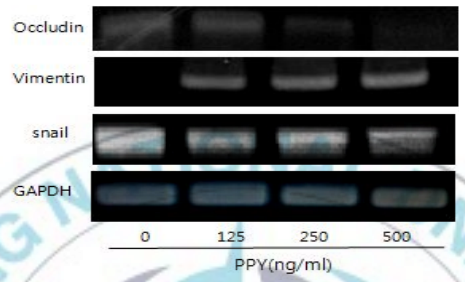


Fig. 25. Effect of PPY on the expression of EMT marker by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Occludin, Vimentin and Snail protein.

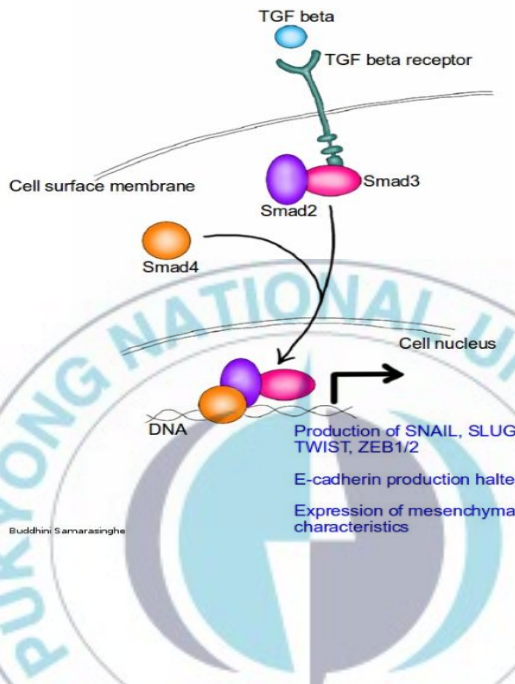


Fig. 26. TGF-β signaling pathway for the induction of EMT.

TGF-β binds to TGF-β receptor on cell surface. This activates Smad2 and Smad3 proteins, which go on to combine with Smad4. The resulting Smad group of proteins then moves into the cell nucleus and begins the production of SNAIL, SLUG, TWIST and ZEB1/2 proteins. These proteins now halt the production of E-cadherin and enable the expression of mesenchymal characteristics. (Samarasinghe B 2013).

5. Activation of Wnt signal

The canonical Wnt signalling pathway plays a critical role in development and disease. The key player of the pathway is β-Catenin. Its activity is mainly regulated by the destruction complex consisting of APC, Axin and GSK3. In the nucleus, the complex formation of β-Catenin and TCF initiates target gene expression (He *et al.*, 2004). Wnt signaling, which is important in promoting and maintaining the undifferentiated state of stem cells, has been shown to prepare cells for TGF-β1-induced EMT during development (Nusse *et al.*, 2008; Herve *et al.,* 2009). In cancer, overactive Wnt signaling is frequently detected and cooperates with TGF-β1 induced EMT (Scheel *et al.*, 2011). The principal regulatory mechanism that controls the nuclear accumulation of β-Catenin is the activity of the so-called destruction complex, consisting of the scaffolding proteins Axin and Adenomatous polyposis (APC) and the kinase Glycogen synthase kinase 3 (GSK3) (Kishide *et al.,* 1998; Yost *et al.*, 1996; Hart *et al.,* 1998).

Axin has a molecular weight of ~110 kDa (Chia *et al.,* 2005). It can interact directly with the CRM1 receptor, although via non-classical NES sequences. Although cytoplasmic at steady state, Axin shuttles in fact into and out of the nucleus. Axin may also have yet unkoknown functions in the nucleus (Wiechens *et al.*, 2004).

The kinase GSK3 is a ~50 kDa protein (Kim *et al.*, 2009). It exhibits a bipartite NLS, which was found to be both necessary and sufficient for nuclear localization. GSK3 is highly active in the nucleus It was proven that GSK3 has a nuclear function in downregulating the

activity of β-Catenin (Bijur *et al.*, 2003).

APC is a large ~310 kDa protein. It exhibits at lease two NES interacting with the CRM1 nuclear export factor and has been proven to shuttle between cytoplasm and nucleus (Neufeld *et al.,* 2000), but the functional relevance of this is still controversial (Rosin-Arbesfeld *et al.*, 2000).

Expression levels of the sub-factors of these genes, Axin, GSK-3β and β-Catenin were measured in same manner. This study observed a increased in protein levels of these Axin, GSK-3β. Axin induced inhibition of Wnt. In addition, this study observed a decrease in protein levels of Wnt, Frizzled and ICAM in a dose-dependent manner upon PPY treatment (Fig.27, 28). The Wnt/Frizzled protein receptor demonstrates that cell proliferation is associated with cancer cells (He *et al.*, 2004).

These results suggested that this inhibition is a general effect for Wnt-stimulated cells. The Wnt/Frizzled protein receptor demonstrates that cell proliferation is associated with cancer cells (He *et al.,* 2004). Therefore, inhibition of cancer cell proliferation and attachment is required to suppress Wnt/Frizzled. This study observed the expression levels of these genes by Western blot analysis and RT-PCR. Cells were treated with various concentration of PPY for 24 h. As a results observed a decrease in protein levels of Wnt, Frizzled in a dose-dependent manner upon PPY treatment (Fig.27). Additionally, mRNA expression levels revealed the same results (Fig.28). Expression of a sub-factor of Wnt signaling was due to reduced expression of a

receptor that was also observed in these experiments. These results confirmed inhibition of Wnt and Frizzled receptors. Therefore, expression levels of sub-factors of these genes (Axin, GSK-3β and β-Catenin) were measured in the same manner. The β-Catenin was degraded by Axin, GSK-3β complex in normal cells. In addition, Axin induced inhibition of Wnt (Doble *et al.,* 2009). As a results, this study confirmed increased expression of Axin. GSK-3β increased to decompose the role of β-Catenin is involved in cell adhesion (Fig.29, 30). Target gene expression occurs following accumulation of β-Catenin in the nucleus and entering of the TCF/LEF binding factor. This promotes transcription of C-myc, which promotes cell division by the β-Catenin/TCF/LEF complex (Brown *et al.,* 2001). Therefore, this study examined the expression levels of ICAM, C-myc by Western blot and RT-PCR. Cells were treated with PPY for 24 h. These results observed the downregulation of protein and mRNA levels of these genes in a dose-dependent manner with PPY (Fig.31, 32).

To address whether PPY inhibits the expression of endogenous Wnt target genes at the mRNA and protein levels, this study treated cells with PPY for RT-PCR analyses, and the result showed that PPY treatment reduced the expression of Wnt target genes such and Axin and C-Myc. These data suggested that PPY can inhibit Wnt/β-Catenin signaling. In this study focused on cell inhibition, cell motility and proliferation through decreased β-Catenin by downregulation of Wnt.

A model depicting the novel role of PPY in Wnt signaling (Fig.

34). In the presence of Wnt, Wnt binds to receptor Frizzled and co-receptor LRP5/6, and Disheveled (DVL) binds to Frizzled. The Axin protein complex including β-Catenin and GSK-3β proceeds to the membrane and Axin binds to phosphorylated LRP5/6. β-Catenin is released and accumulates in the cytoplasm, then enters the nucleus to associate with TCF and activate the transcription of Wnt target genes (Brown *et al.,* 2001). PPY acts to disrupt the interaction of β-Catenin with TCF4, contributing to the decrease in Wnt signaling (Fig.34) (Chen *et al.*, 2012).

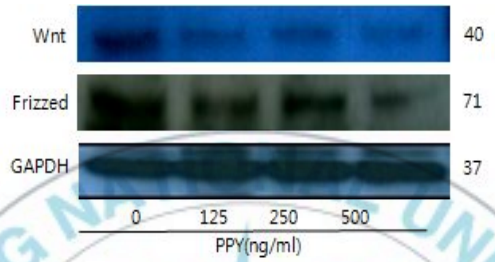


Fig. 27. Effect of PPY on the expression of Wnt and Frizzled by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Wnt and Frizzled proteins.

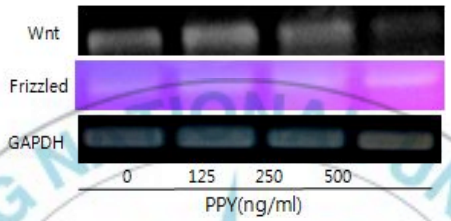


Fig. 28. Effect of PPY on the expression of Wnt and Frizzled by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Wnt and Frizzled proteins.

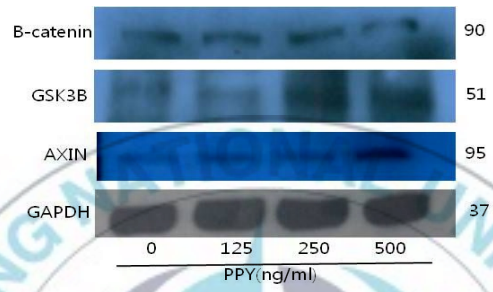


Fig. 29. Effect of PPY on the expression of β-Catenin, GSK-3β, and AXIN by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown β-Catenin, GSK-3β and AXIN proteins.

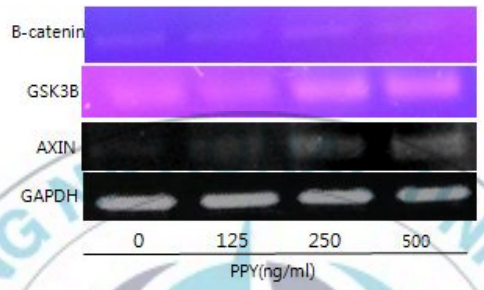


Fig. 30. Effect of PPY on the expression of β-Catenin, GSK-3β, and AXIN by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown β-Catenin, GSK-3β and AXIN proteins.

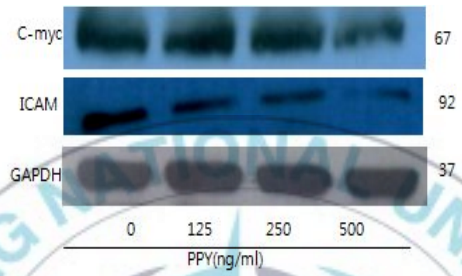


Fig. 31. Effect of PPY on the expression of C-myc and ICAM by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown C-myc and ICAM proteins.

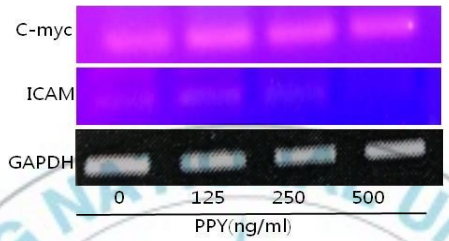


Fig. 32. Effect of PPY on the expression of C-myc and ICAM by RT-PCR

Cells were treated with varying concentrations of PPY (0-500ng/ml) for 24 h. The data shown C-myc and ICAM proteins.

6. Activation of TCF/LEF

In the nucleus, it forms a complex with transcription factor from the LEF/TCF family, which initiates target gene expression. In the nucleus, β-Catenin interacts with T-Cell Factor (TCF)/Lef transcription factors and activates cell transcription of transcription factors and activaties cell transcription of target genes. Target gene expression occurs following accumulation of β-Catenin in the nucleus and entering of the TCF/LEF binding factor (Jeong Y *et al.* 2000). In the absence of β-Catenin degradation, its accumulation in the nucleus induced activation of transcription (TCF/LEF), which can have a significant effect on cell proliferation (Clevers 2006; MacDonal *et al.,* 2009).

Many studies have focused on a reduction by expression of the TCF/LEF transcription factor activated in cancer cells, as well as an increase in the expression of a variety of factors including C-myc, ICAM-1, which are involved in the promotion of cancer cell cycle and proliferation (Kunnumakkara *et al.,* 2009; Tharakan *et al.,* 2010).

Therefore, this study examined the expression levels of LEF, TCF by Western blot analysis. Cells were with PPY for 24 h. These results observed the downregulation of protein levels of these genes in dose-dependent manner with PPY (Fig.33). In addition, this study suggested that this inhibition is a general effect for Wnt-stimulated cells. In conclusion, PPY can inhibit the growth of MCF-7 cells by blocking the intrinsic Wnt signaling through TCF/LEF proteins. In the presence of PPY, the formation of LEF/TCF is disrupted, contributing

to the reduction in Wnt signaling and inhibition of cell growth.

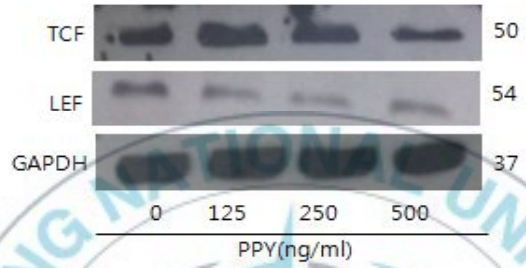


Fig. 33. Effect of PPY on the expression of transcription factor from the LEF/TCF by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown TCF and LEF proteins.

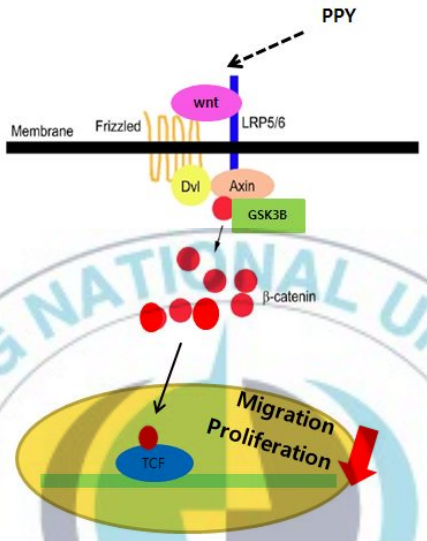


Fig. 34. The proposed model of the Wnt/β-Catenin by PPY

7. PPY inhibits the TCF promoter activity

β-Catenin acts to regulate the transcription of genes through the binding of a complex of β-Catenin and T cell Factor (TCF) family of transcription factors to specific promoter elements (Chiara *et al*., 2012). In the nucleus, β-Catenin interacts with T-cell Factor (TCF)/Lef transcription factors and activates cell transcription of target genes that promote cell proliferation, differentiation and tissue development (Colosimo *et al.,* 2010). The decrease of nuclear β-Catenin by PPY treatment suggested that β-Catenin nuclear signaling might have been attenuated. This study next evaluated the effect of PPY on the transcriptional activities of β-Catenin in MCF-7 cells by using the TOPflash/FOPflash reporter system. As shown in Fig.35, PPY-treatment for 24 h reduced luciferase activity (TOPFlash) in MCF-7 cells. This data showed, in MCF-7 cells observe changes in the transcriptional activity of TOPflash luciferase reporter plasmid (Fig. 35).

β-Catenin is known to relieve the inhibition of TCF/lymphoid enhancer factor by repressors leading to transcriptional activation of target genes, such as C-myc, metalloproteinase-2 (MMP2) and Cyclin D1. The TOPflash luciferase reporter plasmid contains three copies of the consensus T-cell factor (TCF) binding sites upstream of the luciferase gene, whereas its negative control version (FOPflash) carries mutation at these binding sites (Chen *et al*., 2012). In the nucleus, β -Catenin interacts with T-cell Factor (TCF)/Lef transcrption factors and activates cell transcription of target genes, including Cyclin D1,

C-myc and metalloproteases that promote cell proliferation, differentiation and tissue development (Chiara *et al.*, 2012). Furthermore, increased β-Catenin activity was found to be significantly correlated with the poor prognosis of breast cancer patients. Consistent with these clinical data, numerous animal studies have shown that aberrant activation of Wnt/β-Catenin signalling, either by overexpression of canonical Wnt proteins or by direct stabilisation of β-Catenin, can lead to mammary tumourigenesis (Clever 2006; Karim *et al.*, 2004; Huber *et al.*, 2001; Brown 2001).

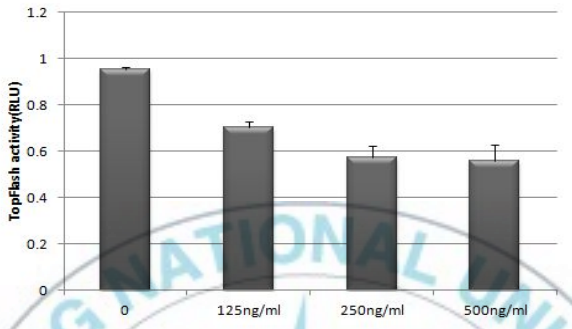


Fig. 35. Effect of PPY on the expression on the transcriptional activity of β-Catenin/TCF

The cells were co-transfected with reporter genes harbouring Tcf-4 binding sites (TOPflash) or a mutant Tcf-binding site (FOPflash), respectively, and β-galactosidase gene. After transfection cells were treated with PPY at 0-500 ng/ml at 0.1 uM as indicated Luciferase activity was determined 24 h post-transfection, normalized against values for the corresponding β-galactosidase activity.

8. Activation of Ras signal

Signaling through the WNT/β-Catenin and the Ras (rat sarcoma)/MAPK (mitogen-activated protein kinase) pathways play a key role in the regulation of various physiological cellular processes including proliferation, differentiation, and cell death (Eva *et al.,* 2013). Normally, signaling through the RAS/RAF/MAPK cascade is initiated by the binding of growth factor to receptor tyrosine kinases, thus recruiting the GRB2/SOS (growth factor receptor-bound protein 2/son of evenness) protein complex to the inner membrane. The monomeric G-protein Ras is activated whereby a signaling cascade via the kinases RAF, MEK (MAPK kinase), and ERK (extracellular signal-regulated kinase) is launched. Phosphorylated ERK translocates to the nucleus, where it interacts with transcription factors to activate transcription of known target genes such as ELK, C-Fos and c-JUN, which regulate proliferation, differentiation, and apoptosis (Seger *et al.*, 1995; Pearson *et al.*, 2001). Physiologically, signaling through Wnt/β-Catenin or the RAS/MAPK cascade is initiated by the binding of WNTs or growth factor to the extracellular domains of their respective receptor. Different mechanisms have been unraveled by which the two pathways affect each other at the level of receptor activation, as will be detailed in the following (Torre *et al.*, 2001).

To determine whether PPY can activate the Ras-Mek-Elk pathway in MCF-7 cells. Incubation with 0-500 ng/ml PPY induced activation Ras, Raf and Mek that measurable by Western blot and RT-PCR (Fig.36, 37). As shown in Fig.36, 37 active Ras dramatically

decreased, while either Raf and Mek significantly decreased by PPY on MCF-7 cells. Ras expression has been suggested as a marker for tumor aggressiveness of breast cancer including the digress of invasion and tumor recurrence (Mendelsohn *et al*., 2000).

These results indicated that PPY regulates cell death through activation of the Ras-Mek-Elk signal pathway. These signaling pathways are known to be important for cell differentiation and proliferation. The Ras/Raf/Elk pathway is specifically related to cell proliferation and survival (Go *et al*., 2011).

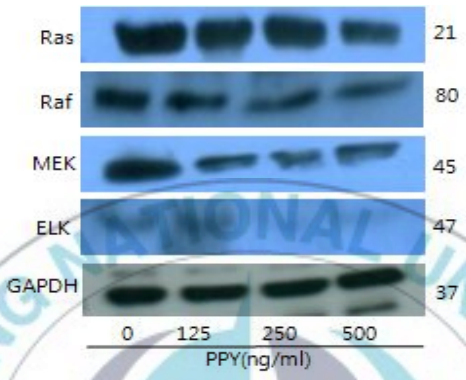


Fig. 36. Effect of PPY on the expression of Ras signaling pathway by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Ras, Raf, MEK and ELK proteins.

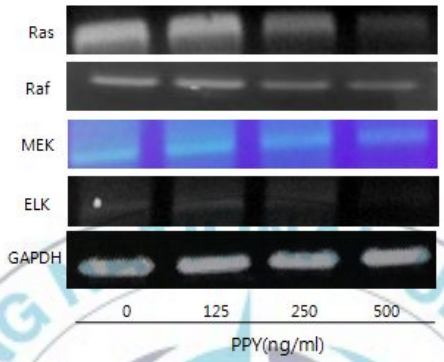


Fig. 37. Effect of PPY on the expression of Ras signaling pathway by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Ras, Raf, MEK and ELK proteins.

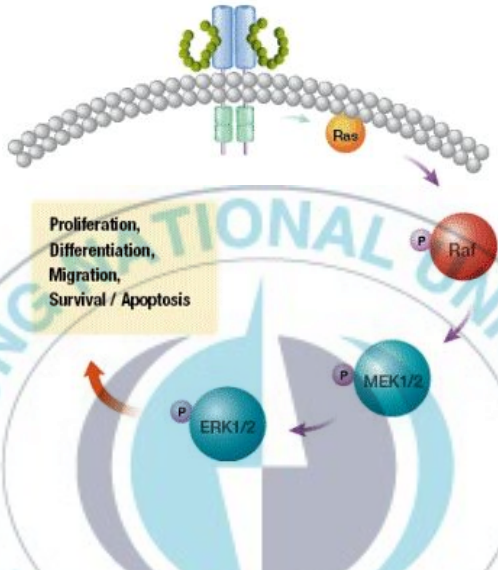


Fig. 38. The proposed model of the Ras signaling. 2012 *National Cancer Institute.*

9. Activation of Rho family

The Rho family of GTPases belongs to the Ras superfamily; they are monomeric low molecular weight proteins. There are 20 known mammalian Rho protein members, divided in part into subfamilies including Rho, Rac, Cdc42, Rnd, RnoD, RhoF, RhoH, and RhoBTB. Rho GTPases exist in either an inactive GDP-bound state, or an active GTP-bound state during which the GTPase can interact with downstream effector (Zhai *et al.*, 2006; Boureux *et al.*, 2007; Rossman *et al.,* 2005; Etienne *et al.*, 2002; Vega *et al.*, 2007; Riou *et al.,* 2010; Chardin 2006). The Rho family of small guanosine triphosphatases (GTPases), which include RhoA, RhoB, C-Raf, Rac-1, and Cdc42, are critical in regulating actin reorganization associated with cell growth, migration, transformation, and gene expression (Wennerberg *et al.*, 2004). RhoA, Rac and Cdc42 are well-characterized members of the Rho family of GTPases and have been decribed as key regulators of cell migration (Nobes *et al.*, 1995). RhoA was first described to promote the assembly of contractile actomyosin filaments, or stress fibers, and Rac promoted the assembly of a peripheral actin meshwork, including lamellipodia (Ridley *et al.*, 1992; Ridley *et al.,* 1992).

In the present study, the protein and mRNA expression levels of the small GTPases Rho A, Rho B, Rac-1 and Cdc42 were determined by Western blot analysis and RT-PCR analysis of MCF-7 cells treat with PPY for 24 h (Fig.39, 40). Treatment with PPY dose-dependently downregulated the protein and mRNA expression levels Rho A, Rac-1

and Cdc42 (Fig.40). In contrast, PPY treatment upregulated the protein and mRNA expression levels of Rho B, small GTPase that is involved in the inhibition of cancer cell growth (Fig.39, 40).

During the process of cell migration, actin-driven protrusions at the leading edge of cell motility are driven by Rac activation, whereas actomyosin contractility at the cell body and rear are coordinated by active Rho, with Cdc42 regulating cell polarity through integrating extracellular directional cues (Etiennee *et al.*, 2001). Rac and Cdc42 share an overlapping set of downstream effector to enact cytoskeletal changes. Signaling via Pak and MAPK activation lead to actin reorganization downstream of either Rac or Cdc42 (Srinivasan *et al.*, 2003; Cotteret *et al.*, 2002).

While these signaling pathways are well-characterized, the understanding of Rho GTPase regulation of cell movement has become more complex. Tumor cell motility has been characterized to occur not only in a mesenchymal pattern with a spindle like shape and an obvious leading cell edge, but also in an amoeboid fashion with cycles of expansion and contraction of the cell body, potentially dependent upon the environment through which the cells move (Pankova *et al.*, 2010; Victoria *et al.*, 2010). Moreover, biosensor capable of visualizing active Rho family members have implicated that both Rho and Rac can be active at leading edge protrusions (Machacek *et al.*, 2009). Thus the environmental regulation and spatiotemporal control of Rho GTPases are key factors in the regulation of cytoskeletal dynamics toward cell locomotion.

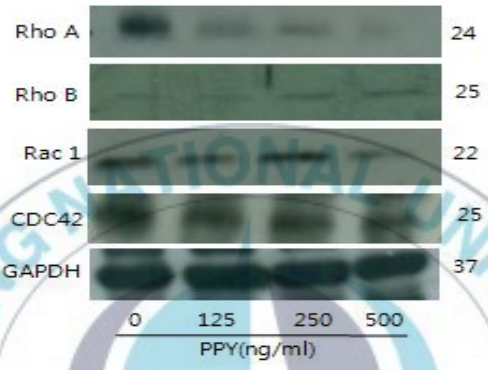


Fig. 39. Effect of PPY on the expression of Rho family by Western blot

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Rho A, Rho B, Rac1 and CDC42 proteins.

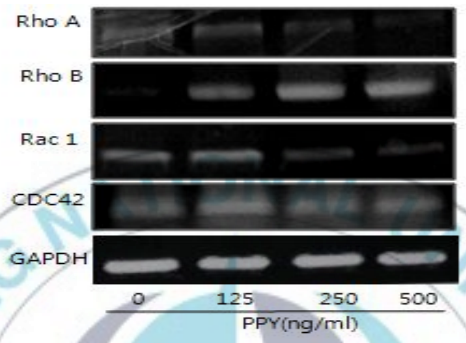


Fig. 40. Effect of PPY on the expression of Rho family by RT-PCR

Cells were treated with varying concentrations of PPY (0-500 ng/ml) for 24 h. The data shown Rho A, Rho B, Rac1 and CDC42 proteins.

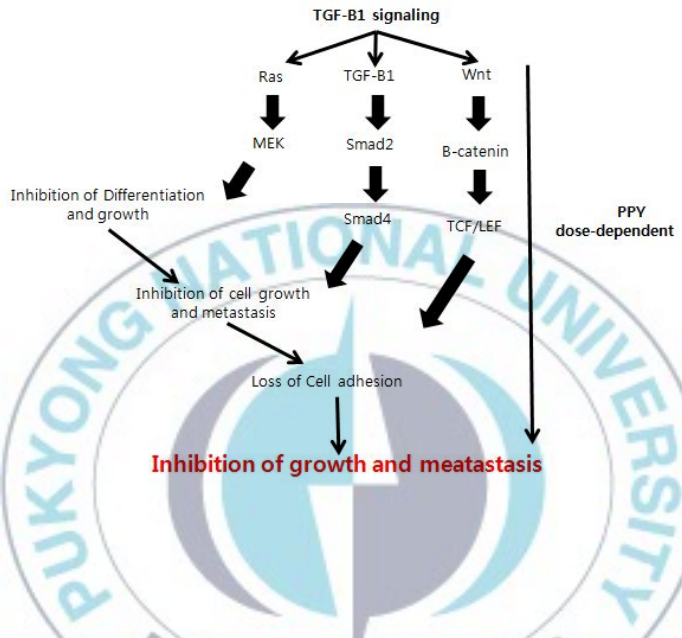


Fig. 41. The proposed model for signaling associated with MCF-7 cells by TGF-β1 signaling pathway

10. Activation of MMP-2 and MMP-9

Metastasis invariably indicates a poor prognosis even when the control of primary cancers is effectively achieved by surgery, chemotherapy or radiation therapy. Once tumor mass is found in a distant tissue from the primary site, it often signals involvement of multiple organ. The unresectable nature of the disseminated tumors and the levels of pain caused by the bone and nerve involvement shift the treatment plan from curative to palliative with an aggressive pain management plan. Due to the biological and technical difficulties such as multi-step nature of metastasis, complex cross communication between metastatic and host stromal cells, and difficulties in studying metastatic step using in vivo model systems, molecular mechanism underlying each metastatic step are still poorly understood (Stamenkovic *et al*., 2000).

The protease involved are matrix metalloproteinases (MMPs) a family of zinc ion dependent endopeptidases. They are capable of digesting different components of the ECM and the basement membrane (Robert *et al*., 2003). The ECM gives structural support to cells and plays a central role in cell adhesion, differentiation, proliferation and migration. For metastasis to occur, there must be alterations of the cell-cell and cell-matrix attachment followed by a proteolytic degradation of the ECM and migration of tumor cells. Such degradation of ECM is catalyse by MMPs (Hiroshi *et al.,* 1994). Both MMP2 and MMP9 expression and activity are important for experimental metastasis.

Matrix metalloproteinase-9 (MMP9), an enzyme synthesized and secreted by both metastatic and host cells, is one of the classic metastasis-promoting genes implicated with metastasis of many types of human cancers (Coussen *et al.,* 1996; Deryugina *et al.,* 2006; Stamenkovic 2000). MMP-9 has been initially suggested to contribute to tumor metastasis by cleaving various extracellular matrix molecules, which allows metastatic cells to be more motile and invasive (Chang *et al.,* 2001; Egeblad *et al.,* 2002).

This study focused on the role of MMPs in MCF-7 cells. This study have also detected MMP-9, MMP-2 in the conditioned medium of MCF-7 cells, as analyzed by Western blot and gelatin zymography. This data showed, it has detected MMP-2, MMP-9 on MCF-7 cells surface; using an MMP-specific substrate, this result observed activity in intact MCF-7 cells, and as expected for an MMP, this activity was inhibited by PPY. In this previous study, expression of MMPs was confirmed. Therefore, MMP-2 and MMP-9 levels were evaluated in MCF-7 cells following treatment with PPY. MMP-2 levels were decreased by PPY as determined by Western blot analysis. In addition, gelatin zymography assays indicated that MMP-2 and MMP-9 levels decreased with PPY. Moreover at the highest concentration (500 ng/ml), both MMP-2 and MMP-9 expression levels were decreased (Fig.42).

These results led us to conclude that MMP-9 is expressed by MCF-7 cells and is located on the cell surface as well as in the conditioned medium. Although both MMP-2 and MMP-9 gelatinases

have been found on the cell surface, this is to our knowledge, the first description of MMP-9 activity on the cell membrane. MMP-2 and MMP-9 are important mediators of basement membrane degradation of gelatin and factors involved in angiogenesis and cancer cell invasion are known to induced MMP-2 and MMP-9 (Brooks *et al.*, 1996; Strongin *et al.*, 1995; Olson *et al.,* 1998).

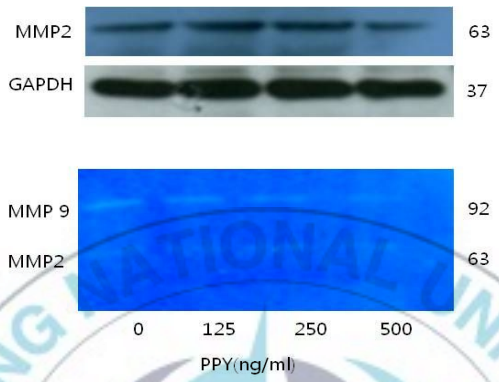


Fig. 42. Effect of PPY on the expression of MMP2 and MMP9 by Western blot and Zymography

Cells were treated with several concentrations of PPY for 24 h. Western blot and gelatin zymography analysis were performed as described. Using anti-MMP-2 antibodies.

11. Inhibition of wound-healing migration

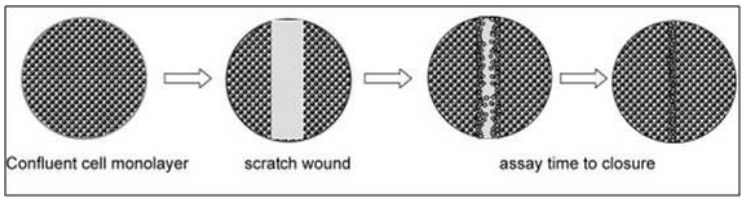
Tumor cell migration in one of the most important events contributing to tumor dissemination, and its prevention may arrest malignant evolution (Kohn *et al.*, 1009). Cell migration is a process that is critical at many stage of embryonic development, and is essential for tissue repair and immune function (Anne *et al.*, 2003). Importantly, deregulated motile behavior contributes to pathological processes including tumor angiogenesis metastasis, atherosclerosis, and arthritis (Hood *et al.*, 2002; Heldin *et al.*, 1999; DeVires *et al.*, 1999).

The effect of PPY on MCF-7 cells migration was examined using a wound-healing assay. As results, MCF-7 cells treated with PPY exhibited dose-dependent inhibition of migration into the cell-wounded zone on 100-mm dishes, indicating a PPY induced inhibition of cell mobility (Fig.43). This study used a wound-healing assay to confirm cell migration and observed the denuded zone through a microscope. As shown in Fig.43, the gap (denuded zone) between the cells was inhibited dose-dependent by PPY. The cell migration distance is determined by measuring the width of wound divided by two and by subtracting this value from the initial half-width of the wound.

Migration of this kind usually involves tissue invasion and shows similarity to the events leading to the initiation of metastasis is one of the reasons for studying such migration. The cells that migrate are initially immobile and are induced to become migratory (Pernille 2002). This transition is clearly an essential first step of the process,

but it is poorly understood at the molecular level. Initiating cell migration involves changes in cell adhesion. During the actual migration, the cells invade a complex tissue, guided by cues present in this environment. These can be permissive cues or instructive cues. Finally, cells reach a target and stay there to perform a differentiated function (Bronner 1993). Collectively, these finding suggest that PPY inhibits MCF-7 cells migration.

(A)



(B)

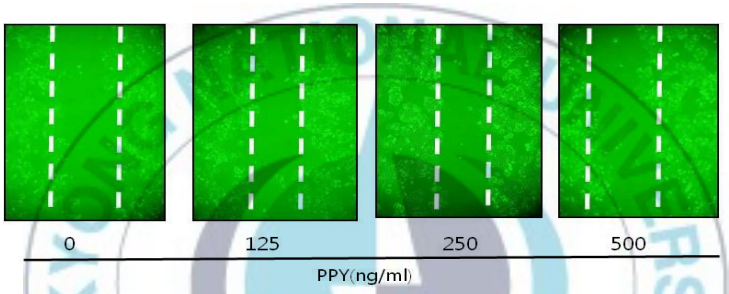


Fig. 43. PPY caused morphological changes in MCF-7 cells with cell migration

PPY inhibits wound-healing migration of MCF-7 cells. Cells were cultured in 100-mm dishes for 24 h, and then the cell layer was wounded by scraping. The medium was replaced with medium containing 1% FBS and PPY. The denuded zone of cells was photographed using a microscope at×200 magnification, and the degree of recovery was measured. Scratch wound assay (A), Phase micrographs of MCF-7 cells after monolayer wounding (B).

Part 4. Effect of PPY on specific mRNA knock-down

1. siRNA/lipofectamin induced mTOR knock-down

RNAi interference (RNAi) is a powerful tool to knock down specific mRNA expression levels by exploiting a natural intracellular regulatory phenomenon in mammalian species (Hannon *et al.*, 2004; Jones *et al.*, 2004; Aigner 2006). Gene silencing using short interfering RNAs (siRNA) has many potential therapeutic applications (Antonin *et al.*, 2007). RNAi is now commonly used in biological and biomedical research to study the effect of blocking expression of a given gene. As the effect is rarely complete, it is generally termed a "known-down" to distinguish it from the "knock-out" achieved by deletion of the gene (Simone *et al.*, 2004).

In this study, mTOR knocked down using small interfering RNA (siRNA) conjugated with PPY in MCF-7 cells. MCF-7 cells transfected with siRNA/lipofectamin complex of different PPY ratio (0, 125, 250, 500 ng/ml) were assayed for mTOR expression. Total protein was harvested three days after siRNA treatment. Western blot results showed significant reduction of mTOR expression by siRNA when the PPY ratio was 500 ng/ml (Fig.44). Furthermore, an PPY ratio of 500 sufficient to knock down mTOR expression in MCF-7 cells in vitro. Compared to controls, mTOR siRNA complexed with lipofectamin reduces mTOR protein expression dramatically in MCF-7 cells (Fig.44). As shown in Fig. 44, Compared with non-targeting siRNA transfection, p70S6k and PDK protein levels were significantly

suppressed by mTOR siRNA treatment in vitro.

Transfection of mTOR siRNA in MCF-7 cells produced mTOR gene silencing monitored by Western blotting three days post-transfection. Knock-down of mTOR occurs only when the PPY ratio was 500 ng/ml, indicating that siRNA specifically knocked down mTOR protein in MCF-7 cells. Compared to non-targeting siRNA complexes, mTOR siRNA complexes reduced mTOR protein expression in MCF-7 cells dramatically as shown in Fig.44. Thus, these data confirm that mTOR siRNA suppressed the targeted gene specifically through the RNAi mechanism.

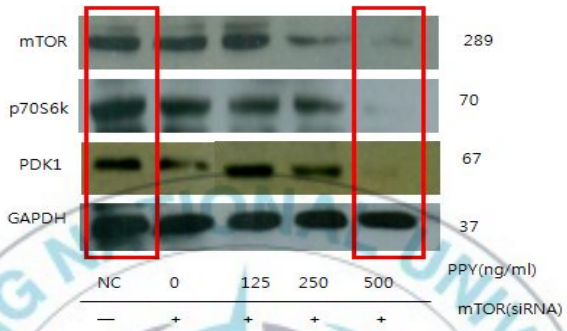


Fig. 44. Effect of PPY on the expression of mTOR siRNA/lipofectamin transfection

Western blot analysis for mTOR signaling expression after mTOR siRNA/lipofectamin transfection.

2. siRNA/lipofectamin induced TGF-β1 knock-down

RNA interference (RNAi) is the regulatory mechanism most eukaryotic cells and uses small double-stranded RNA (dsRNA) molecules as triggers to homology-dependent control of gene activity (Atsushi *et al.*, 2006). On a genomic scale, it is an effective tool for regulating gene function. RNAi is widely used to treat viral infection, cancer, immunodeficiency disease (Qing *et al.*, 2011). Therefore, with the deeper understanding of the molecular mechanism of cancer cells and the development of genetic therapy technique, gene therapy may be one of the effective treatments for MCF-7 cells.

In this study used siRNA that TGF-β1 in order to prevent MCF-7 cells, and we investigated the possible anticancer mechanism of siRNA TGF-β1. This study examined the transfection efficiency of TGF-β1 siRNA in the MCF-7 cells culture by measuring the expression of TGF-β1 gene 72 h after transfection. The TGF-β1 siRNA interfered with 60-80% of the negative vector (Fig.45). As shown in Fig. 45, PPY 500 ng/ml treatment TGF-β1 siRNA protein expression was significantly decreased compared to TGF-β1 negative control.

These results indicated that TGF-β1 siRNA can partially inhibit or block the expression of TGF-β1 and reduce the impact of PPY on the proliferation and activation of MCF-7 cells.

In summary, this study have successfully designed and constructed TGF-β1 siRNA. The plasmid effectively silence TGF-β1 gene expression in MCF-7 cells. TGF-β1 siRNA significantly down

regulated the expression of TGF-β1 which could inhibit activation and proliferation. These results suggested that gene silencing of TGF-β1 by siRNA can efficient and more specific approach of MCF-7 cells.

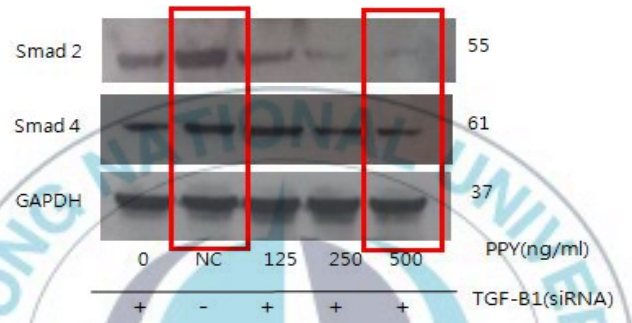


Fig. 45. Effect of PPY on the expression of TGF-β1 siRNA/lipofectamin transfection

Western blot analysis for TGF-β1 signaling expression after TGF-β1 siRNA/lipofectamin transfection.

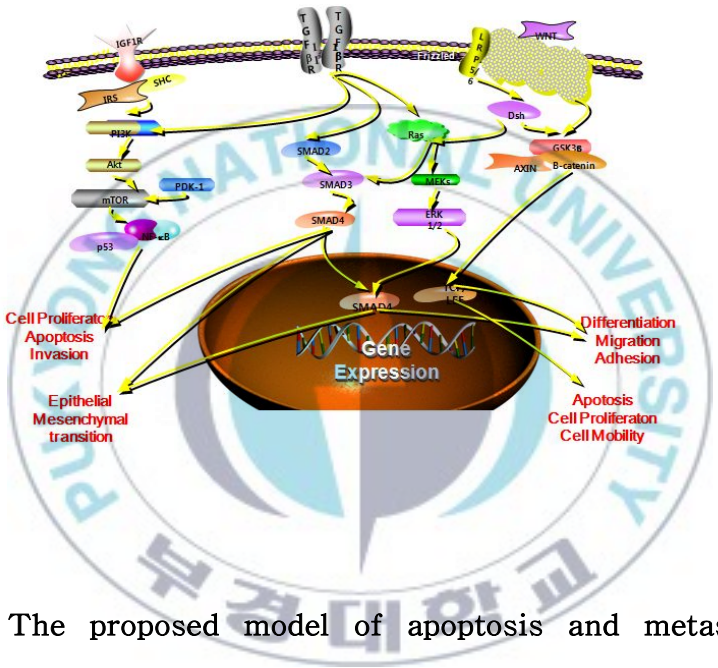


Fig. 46. The proposed model of apoptosis and metastasis on MCF-7 cells by PPY

Ⅳ. Discussion

1. Inhibitory effect of PPY on MCF-7 cells

The aim of present study was to determine whether peptide from *porphyra yezoensis* (PPY) could inhibited on proliferation of MCF-7 cells and to identify the signals related to this proliferation. MCF-7 cells were induced to undergo apoptosis by treatment with PPY. Apoptosis was morphologically confirmed by examining the nuclear morphology after DAPI staining assay. To confirm that PPY induced cells apoptosis, this study observed cell morphology. The cells treated with PPY appeared to decrease in number compared to untreated cells. Microscopic analysis confirmed that PPY treatment induced cell death.

These results indicate that activation also contributes to PPY decrease through the activation of the PI3K/Akt pathway. The PI3K/Akt pathway has been identified as key player in cell survival (Kandel *et al.*, 1999; Parrizas *et al.*, 1997). Akt also functions in normal growth as seen in Akt-knockout mice, which show retarded growth. Once the p85 subunit is positioned, the p110 subunit of PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates Akt (Carrie *et al.,* 2005).

PPY induced MCF-7 cells apoptosis by downregulation the expression of IGF-IR and IRS-I, which would initiate the extrinsic apoptosis pathway, and the active forms of SHC were detected. Signaling through IGF-IR stimulates proliferation, promotes angiogenesis and metastasis, and induced apoptosis. There is now

abundant evidence indicating that signaling through the IGF-IR pathway is important for survival of breast cancers, as well as in vitro cell lines such as MCF-7 cells (Hells 2004; Ibrahim *et al.*, 2005). These studies showed that PPY treatment was effective in growth inhibition and apoptosis induction. It was suggested that IGF-IR could be used as successful with PPY in the treatment of MCF-7 cells.

In next section investigated the expression of the mTOR pathway in MCF-7 cells. The expression of mTOR decreased with PPY. Theses results suggest that mTOR signaling proteins are activated in cells. Activation of the mTOR signaling pathway may inhibited to tumor cell proliferation and survival of MCF-7 cells.

In conclusion in these results, this study have investigated the effective PPY on the inhibition of MCF-7 cells proliferation, as well as the possible mechanisms of growth inhibition. This study previously showed PPY apoptosis cells and identified regulation of the IGF-IR and mTOR signaling pathway in MCF-7 cells.

2. Inhibitory effect of MCF-7 cells metastasis

Elevated levels of TGF-β1 are found in cancer patients and at invasive fronts in human cancer tissues, and are frequently associated with tumor metastasis and poor prognosis (Massague 2008; Chod *et al.*, 2008; Dalal *et al.*, 1993). In addition to its role in inducing EMT, TGF-β1 contributing immune cell recruitment, polarization and secretion of other EMT mediators (Mantovani *et al.*, 2002; Flavell *et al.*, 2010; Yang *et al.*, 2010). To investigate the effect of TGF-β

1/Smads signaling pathway on inhibition of MCF-7 cells development and metastasis by Western blot. This data indicate that Smad protein lose its expression in MCF-7 cells. This indicates that TGF-β1 signal at tumor metastasis by Smad-dependent pathway.

The antitumor and metastatic effect of PPY has been shown to be mediated by inhibiting the activation of Ras/Mek that is accompanied by a decreased expression level of MMP in MCF-7 cells (Chang *et al.*, 2001). In this study, the results showed decreased protein levels of Raf, Rac, Cdc42 and RhoA. All these findings indicated that PPY is involved in the inhibition of the downregulation of FAK/PI3K/AKT and small GTPase signals. Also, PPY reduced MCF-7 cells invasion and migration. Thus, these results suggested that FAK/PI3K/AKT pathway and small GTPase family proteins were involved in PPY induced suppression of invasion and migration.

The Wnt signaling pathway is not only involved in various biological processes including cell proliferation, differentiation, motility, survival and/or apoptosis, but also plays pivotal roles in embryonic development and maintenance of homeostasis in mature tissues (Juan *et al.*, 2002). In this results, this study found that PPY is able to inhibit Wnt signaling and cell survival based on its ability to inhibit the β-Catenin/TCF-mediated transcriptional activity and reduced the expression of Wnt target genes in MCF-7 cells. These results found that PPY target a component of the Wnt pathway by disrupting the formation of the β-Catenin/TCF complex, a result that provides a basis for the design of more effective, PPY derivatives that target the

β-Catenin/TCF complex.

3. Specific mRNA knock-down in MCF-7 cells

siRNA is of substantial current interest as a sequence-specific posttranscription gene silencing tool for the genetic analysis and for translational therapeutic applications in various mammalian cells (Aagaard *et al.*, 2007; Almeida *et al.*, 2005). To facilitate released siRNA internalization by MCF-7 cells, mTOR siRNA was complexed with lipofectamin at different PPY (0-500 ng/ml). Compared to non-targeting siRNA complexes, mTOR siRNA complexes, reduced mTOR mRNA expression in MCF-7 cells dramatically as show in Fig.34. Thus, these data confirm that mTOR siRNA suppressed the targeted gene specifically through the RNAi mechanism. Nearly 70% decrease in activation after mTOR siRNA treatment is observed compared to non-targeting siRNA transfection, supporting that mTOR function specifically.

A major advantage of using RNAi versus other antisense-based approaches for therapeutic applications is that utilizes cellular machinery that efficiently allows the targeting of complementary transcripts, which often results in the highly potent down-regulation of gene expression (Aagaard *et al.*, 2007).

Strategies aimed at disrupting TGF-β1 synthesis and/or signaling pathways markedly decrease Smad in experimental models (Shek *et al.*, 2004). Therefore, this study used RNAi to target TGF-β1 for this experiment. Western blot analysis showed that in TGF-β1

siRNA and PPY 500 ng/ml treatment, Smad expression decreased significantly compared to non-targeting TGF-β1. These results suggested that gene silencing of TGF-β1 by siRNA can be an efficient and more specific approach.

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