รายงานวิจัยฉบับสมบูรณ์

โครงการ ผลของการเกิดไอเอสจี15 คอนจูเกชันต่อการทำงานของโปรตีนยับยั้งมะเร็งแมสปิน

โดย ผศ. ดร. คงธวัช ชัยรัชวิทย์

เดือน ปี ที่เสร็จโครงการ

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รูปแบบ Abstract (บทคัดย่อ)
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(ชื่อโครงการ) ผลของการเกิดไอเอสจี15 คอนจูเกชันต่อการทำงานของโปรตีนยับยั้งมะเร็งแมสปิน

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บทคัดย่อ

แมสปิน หรือ เซอร์ปิน บี5 จัดอยู่ในตระกูลของโปรตีนยับยั้งเซอรีนโปรติเอส แมสปินเป็นเซอร์ปินที่มีคุณสมบัติต้านมะเร็งเนื่องจากสามารถยับยั้งการเจริญ การลุกลามและแพร่กระจายของมะเร็งได้ทั้งในหลอดทดลองและในสัตว์ทดลอง แม้ว่ากลไกพื้นฐานการทำงานของแมสปินยังคงไม่ชัดเจน ทว่ามีการศึกษามากมายได้นำเสนอวิธีการทำงานของแมสปินซึ่งรวมถึงการยับยั้งฤทธิ์ของ 26 เอส โปรติเอโซมภายในเซลล์มะเร็ง วัตถุประสงค์ของการศึกษานี้เพื่อพิสูจน์ว่าบริเวณทำงาน (อาร์ ซี แอล) ของแมสปินรับผิดชอบต่อการยับยั้งฤทธิ์ของโปรติเอโซมหรือไม่ และค้นหาผลกระทบของการเกิดคอนจูเกชั่นระหว่างแมสปินและไอเอสจี15 ต่อระบบการเกิดคอนจูเกชั่นของยูบิควิติน การใส่ยีนเข้าไปในเซลล์มะเร็งเต้านมเพื่อทำให้มีการแสดงออกของแมสปิน, โอวัลบูมิน หรือ มิวแตนท์ ซึ่งเป็นลูกผสมระหว่างแมสปินกับโอวัลบูมินแล้ววิเคราะห์การทำงานของโปรติเอโซมโดยการวัดด้วยวิธีฟลูออเรสเซนส์และทำการตรวจสอบคอนจูเกตของยูบิควิติน-โปรตีนด้วยวิธีWestern blotting ผลการทดลองพบว่าการแสดงออกของแมสปินและมิวแตนท์ลดฤทธิ์ของโปรติเอโซมแต่เพิ่มการสะสมคอนจูเกตของยูบิควิติน-โปรตีนในเซลล์ ซึ่งบ่งบอกได้ว่าอาร์ซีแอลของแมสปินเป็นบริเวณที่เพียงพอแต่ไม่จำเป็นต่อการทำงานนี้ของแมสปิน นอกจากนี้ การเกิดคอนจูเกชั่นของแมสปินกับโปรตีนที่คล้ายยูบิควิตินที่ชื่อว่า ไอเอสจี15 เกิดขึ้นได้โดยการแสดงออกรีคอมบิแนนท์แมสปิน, ไอเอสจี15, ยูบีอี1 แอล และ ยูบีซีเอช8 ในเซลล์มะเร็ง ซึ่งถูกวิเคราะห์โดยวิธี nickel pull-down assay และ immunoblotting ระบบไอเอสจี 15 คอนจูเกชั่นกระตุ้นการสะสมของยูบิควิติน-โปรตีนคอนจูเกตให้มีเพิ่มมากขึ้น ซึ่งเสนอแนะว่าการดัดแปลงแมสปินด้วยไอเอสจี15มีบทบาทสำคัญต่อหน้าที่ของแมสปินต่อระบบของยูบิควิติน-โปรติเอโซม ภายในเซลล์

คำหลัก: แมสปิน ไอเอสจี 15 ยูบิควิตินโปรติเอโซม คอนจูเกชั่น

ABSTRACT

Maspin or SERPIN B5 belongs to the serine protease inhibitor superfamily of proteins. Maspin is a tumor suppressor SERPIN because it can inhibit growth, invasion and metastasis of cancer *in vitro* and *in vivo*. Although the underlying mechanism of maspin activity is still mystifying, several reports have proposed models of its actions including the inhibition of 26S proteasome activity of carcinoma cells. The aims of this study are to investigate whether the reactive center loop (RCL) of maspin is responsible for inhibiting the proteasome activity and determine an effect of maspin-ISG15 conjugation on cellular ubiquitin conjugating system. Using mammalian transfection system, 6xHis-FLAG-maspin, -ovalbumin or two maspin/ovalbumin chimeric mutants were expressed in mammary carcinoma cell line MDA-MB-231. The 20S proteasome activity was measured using a fluorometric assay, and the ubiquitin-protein conjugates detected by Western blotting. Expression of wild-type maspin and the mutants decreased the proteasome activity, but increased the accumulation of ubiquitin-protein conjugates indicating that the RCL of maspin is sufficient, but not necessary for its activity. Furthermore, conjugation of maspin by a ubiquitin-like protein, ISG15 was shown by co-expressing recombinant maspin, ISG15, UBE1L, and UbcH8 in the carcinoma cells and analyzed by a nickel pull-down assay and immunoblotting. The accumulation of ubiquitin-protein conjugates was further enhanced by ISG15 conjugation system suggesting that modification by ISG15 play a pivotal role on the function of maspin toward the cellular ubiquitin-proteasome system.

Keywords: Maspin, ISG15, Ubiquitin, Proteasome, Conjugation

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Introduction

Cancer is one of the major public health problems worldwide, and now has a very high mortality rate in Thailand. Cancer cells are typically classified by uncontrolled cell growth and cell division leading to a mass of unwanted cells called a tumor. Besides, many cancer cells have an ability to relocalize to distant tissues and these distant settlements of cancer cells consequently account for the major cause of death in cancer.

Carcinogenesis is a complicated multi-step process in which initiation and progression of cancer in cells involve genetic/epigenetic aberrations of human genome. A typical cancer cell does not result from a single mutation, but rather the multiple mutations of numerous genes. Such alterations in the genome lead to either over-activation (*e.g*. proto-oncogenes) or inactivation (*e.g* tumor suppressor genes) of specific groups of genes. Altered regulation of these genes in cancer cells eventually causes uncontrolled-cell proliferation, invasion, and metastasis. Understanding gene regulation at the transcriptional and post-transcriptional levels as well as post-translational modifications of the protein is thus necessary for development of novel efficient cancer therapy.

Maspin (mammary serine protease inhibitor) is a 42 kDa protein and a member of the serine proteinase inhibitor (SERPIN) superfamily of proteins including α1-anti-trypsin, plasminogen activator inhibitor and ovalbumin (Irving JA, et al. 2000). Despite its similarity to other SERPIN members, maspin does not directly exert its biological functions as a proteinase inhibitor (Bass R, et al. 2002, Pemberton PA, et al. 1995). Several studies have revealed the tumor

suppressor role of maspin as an effective inhibitor of cancer cell invasion and metastasis (Bailey CM, et al. 2006, Seftor RE, et al. 1998). Down-regulation of maspin in carcinoma tissues is also correlated with progression of tumors (Hojo T, et al. 2001, Ito R, et al. 2004, Khalkhali-Ellis Z. 2006, Maass N, et al. 2001). Therefore, maspin becomes a promising target for both prognosis/diagnosis and therapeutic intervention against cancer (Sheng S. 2004).

Maspin has been characterized as a class II tumor suppressor since it effectively inhibits tumor invasion and metastasis (Sager R, et al. 1997, Zou Z, et al. 1994). Re-expression of maspin in carcinoma cell lines lead to inhibition of cell invasion and metastasis both *in vitro* and *in vivo* (Maass N, et al. 2000, Streuli CH. 2002). The underlying mechanisms of action by which maspin elicits its anti-tumor and anti-metastatic effects are not well established and still remain to be solved. Earlier studies have characterized the functional domain of maspin (Figure 1) and further revealed that the functional domain called reactive center loop (RCL) is required for the anti-invasive effect of maspin (Ngamkitidechakul C, et al. 2003, Sheng S, et al. 1996, Zhang M, et al. 1993). Deletion of maspin's RCL or substitution with the ovalbumin RCL completely abolishes maspin activities. The mode of maspin action appears to mediate RCL binding to a cell surface receptor since addition of anti-RCL maspin antibody can block the effect of exogenously added maspin (Sheng S, et al. 1996). However, other functions for intracellular maspin cannot be excluded because immunohistochemical analysis shows prominent nuclear and cytoplasmic staining of maspin (Abd El-Wahed MM, et al. 2005, Marioni G, et al. 2006, Pemberton PA, et al. 1997). In order to elucidate the biological

function(s) of maspin, several maspin-binding proteins have been identified using the yeast two-hybrid approach. Glutathione S-transferase (Yin S, et al. 2005) and interferon regulatory factor 6 (Bailey CM, et al. 2005) are among the maspin's binding partners and their interactions suggest the role of maspin in cellular responding to oxidative stress and in maintaining the normal cellular phenotype, respectively.

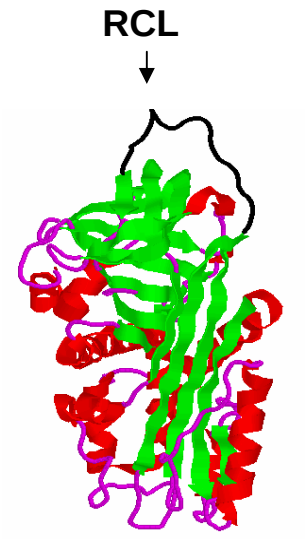

Figure 1 Structure of Maspin

The regulation of maspin expression has been extensively studied at the transcriptional level and shown to involve an epigenetic mechanism (Akiyama Y, et al. 2003, Futscher BW, et al. 2002, Maass N, et al. 2002, Zou Z, et al. 2000).The transcription of *maspin* gene is regulated at the 5' upstream of the *maspin* promoter by cytosine methylation (Maass N, et al. 2002). Hypermethylation of the promoter of the *maspin* gene is often found and plays a role on gene silencing in several cancers *e.g*. breast, thyroid, skin, and colon (Bettstetter M, et al. 2005, Boltze C, et al. 2003, Primeau M, et al. 2003, Wada K, et al. 2004).

Re-expression of maspin in cancer cell lines modifies several phenotypes including a reorganization of actin cytoskeleton, a reduction in invasive property, an induction of apoptosis, and an alteration in protein degradation through the ubiquitin (Ub) pathway, which may be fundamental to anti-invasive activity of maspin (Chen EI, et al. 2005). Shotgun proteomics analysis revealed that the re-expression of maspin has widespread effects on the tumor cell proteome. In most cases, protein expression was affected without changes in mRNA levels, indicating that maspin has a significant influence on posttranscriptional regulation of protein levels. Maspin has a major impact on the composition and function of the tumor cell proteasome. Maspin expression reduces the chymotrypsin-like activity of the 26S proteasome via the down-regulation of the β5 subunit of the proteasome leading to the accumulation of high molecular weight ubiquitin conjugates in all maspin-transfected cells. These observations indicate that maspin's mechanism of anti-invasion may be mediated through the ubiquitin-proteasome pathway.

Ubiquitin (Ub) is a highly conserved 76 amino acid protein (8,565 Da) that is present in all eukaryotic organisms (Goldstein G, et al. 1975). Ub has no enzymatic activity per se but exerts its biological function through a covalent ligation to a variety of cellular proteins by an ATP-dependent process (Pickart CM. 2001). Polyubiquitin chains formed on the target protein result in selective targeting to the 26S proteasome for protein degradation (Hershko A, et al. 1992). The covalent ligation of Ub occurs at the essential carboxyl terminal glycine of Ub, and is required to target proteins for degradation by the 26S

proteasome. This ligation of Ub on target proteins requires a cascade of enzymatic reactions as shown in Figure 2.

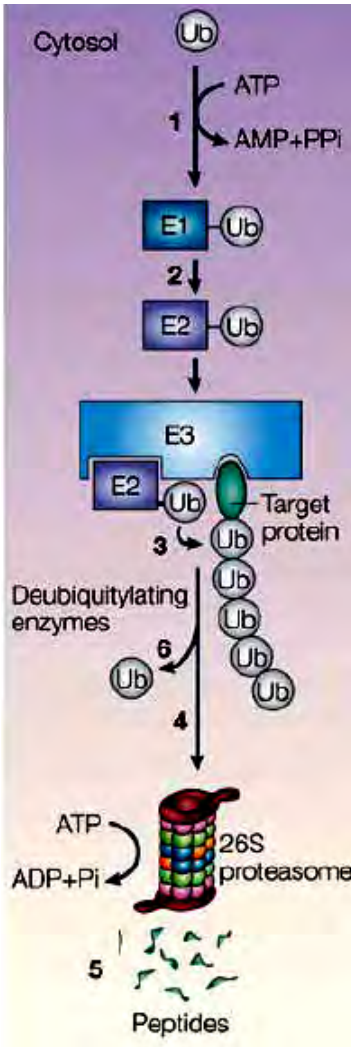

Figure 2 The Ubiquitin- Proteasome System

(Ciechanover A. 2005)

The initial step requires a **Ub**iquitin **a**ctivating enzyme or Uba1 (E1) that catalyzes formation of a ternary complex in which E1 contains both a non-covalently bound Ub adenylate and a Ub thiolester (Haas AL, et al. 1982). The Ub moiety of the E1-Ub thiolester is then transferred to an active site cysteine of a **Ub**iquitin **c**arrier protein (Ubc or E2) to form a high energy thiolester E2-Ub (Jentsch S, et al. 1990). While eukaryotes generally contain a single E1, the

E2s represent a superfamily of related paralogs (Haas AL, et al. 1997). All members of the E2 superfamily share a common folding and a conserved core domain comprising *ca*. 150 residues within which is the active site cysteine (Hemelaar J, et al. 2004). Finally, the Ub moiety of the E2-Ub thiolester is transferred to the target protein to form an isopeptide bond between the carboxyl-terminal glycine of Ub and an ε-amino group of a specific lysine residue(s) on the target protein. The reaction is catalyzed by a very large set of Ub ligases (E3) which confer target protein specificity. Once the polyubiquitin degradation signal is formed on the substrate protein, it is recognized and degraded by the 26S proteasome (Gregori L, et al. 1990).

The proteasome is a large multi-catalytic protease that degrades polyubiquitinated proteins to small peptides (Ciechanover A. 2005). This proteolytic machine regulates the turnover of the vast majority of proteins expressed in the cell, and therefore controls the complement of proteins expressed in a cell. It is composed of two sub complexes: a 20S core particle (CP) that carries the catalytic activity, and a regulatory 19S regulatory particle (RP) (Figure 3). One important function of the 19S RP is to recognize ubiquitinated proteins and unfold substrates to facilitate entry into the barrel-shaped 20S CP. The 20S proteasome possesses chymotrypsin-like, trypsin-like, and caspase-like protease subunits. Studies using cell permeable proteasome inhibitors revealed that the Ub-proteasome pathway is responsible for 80–90% of protein turnover in cells and is essential for the regulation of virtually all cellular processes (Lee DH, et al. 1998).

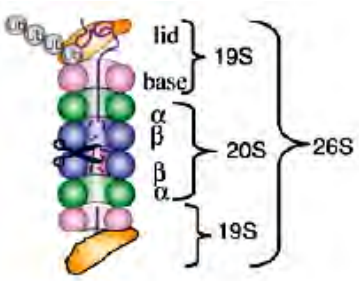

Figure 3 The Composition of 26S Proteasome

(Ciechanover A. 2005)

Maspin expression has a major impact on the complement of proteins in cells that are marked for 26S proteasome degradation by polyubiquitination. Such observations suggest that maspin's mechanism of anti-invasive action results from its ability to inhibit (and/or regulate) protein degradation by the proteasome. Therefore, it is of the particular interest to elucidate whether the functional domain of maspin (RCL) also involves in the ubiquitin-26S proteasome activities in order to connect the anti-metastatic effect of maspin and the ubiquitin-26S proteasome system.

In contrast to its transcriptional regulation, little is known about the post-translational modification of maspin protein. Phosphorylation on one or more tyrosine residues of maspin was detected; however, the biological effect has not been verified (Odero-Marah VA, et al. 2002). Conjugation of an ubiquitin-like protein, ISG15, to maspin has been previously shown in carcinoma cells (Zhao C, et al 2005). Like ubiquitin, ISG15 is conjugated to cellular proteins by a mechanism similar to that of ubiquitin (Loeb KR, et al. 1992). However, unlike ubiquitination, the ISG15 conjugation does not target the protein to proteasome degradation (Hamerman JA, et al. 2002, Malakhov MP, et al. 2003). Overexpression of ISG15 is also associated with decreased

polyubiquitination of target proteins and their turnover in tumor cells (Desai SD, et al. 2006). It has not yet been shown whether the ISG15 conjugation to maspin is crucial for modulation of maspin stability and/or activities.

Ubiquitin-like proteins are a family of proteins that function to post-translationally modify cellular targets in a pathway parallel to, but distinct from, that of Ub (Jentsch S, et al. 2000). Members of the Ub-like protein superfamily include ISG15, SUMO, NEDD8/RUB1, APG12, HUB, and FAT10 (Walters KJ, et al. 2004, Yuan Q, et al. 2004). Conjugation system of Ub-like proteins is well characterized, but does not share common conjugation machinery with that of Ub. For example, the activating enzyme or E1 and the carrier protein or E2 for the ISG15 conjugation pathway have been identified as UBE1L (Pitha-Rowe I, et al. 2004) and UbcH8 (Zhao C, et al. 2004), respectively. Like ISG15, both UBE1L and UbcH8 are induced by IFN (Nyman TA, et al. 2000, Zhao C, et al. 2004). Using bioinformatics approaches, IFN-inducible EFP and Herc5 were identified and then experimentally proven to be ISG15 E3 ligases (Dye BT, et al. 2007).

Unlike SUMO and NEDD8, a large number of protein targets are modified by ISG15 upon treatment of Type I interferon (IFN-α/β) (Loeb KR, et al. 1992). The serine protease inhibitor 2a (Serpin 2a) is the first identified target (Hamerman JA, et al. 2002). Using high-throughput immunoblotting and proteomic approaches, both IFN inducible and constitutively expressed proteins are identified as ISG15 targets including maspin as shown in Figure 4 (Giannakopoulos NV, et al. 2005, Malakhov MP, et al. 2003). Proteins conjugated with Ub-like proteins are not targeted for degradation by the 26S

proteasome (Narasimhan J, et al. 1996). Interestingly, modification of target proteins by ISG15 seems to inhibit their activities. Two ISG15-identified targets are Ub-E2 enzymes, Ubc13 and UbcH6, and their modification by ISG15 inhibits their ability to form a thiolester linkage with Ub (Takeuchi T, et al. 2005, Zou W, et al. 2005). The third characterized target is phosphatase 2Cβ and its enzymatic activity is also suppressed by ISG15 modification (Takeuchi T, et al. 2006). Moreover, the ISG15 pathway negatively regulates the Ub pathway. The elevated expression of ISG15 and its protein conjugates possibly modulates protein stability by antagonizing Ub-conjugation and reducing protein polyubiquitination. Interestingly, maspin is also conjugated by an ubiquitin-like protein, ISG15 through three sequential enzymatic reactions similar to the ubiquitin conjugation pathway (Zhao C, et al. 2005). Thus, the significance of ISG15 modification on maspin needs to be further elucidated.

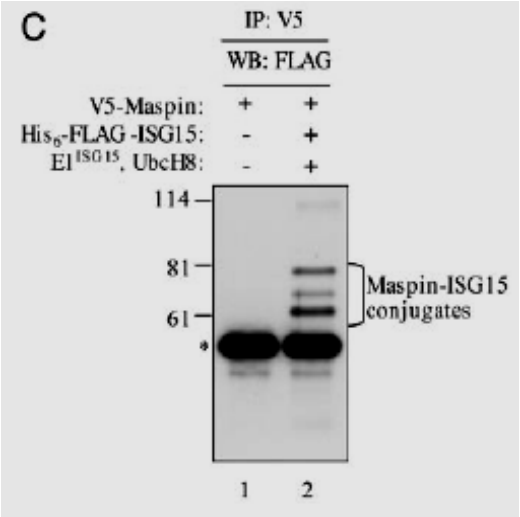

Figure 4 ISG15 Conjugation of Maspin

(Malakhov MP, et al. 2003)

In summary, characterization of the molecular mechanism by which maspin modulates the ubiquitin-26S proteasome system would directly lead to better understanding the molecular mechanism of maspin's anti-invasive function. Two specific aims are therefore proposed in the study to determine whether: 1) the reactive center loop (RCL) of maspin is required for inhibition of the chymotrypsin-like activity of the 26S proteasome, and 2) ISG15-conjugation by maspin reduce the accumulation of high molecular weight ubiquitin-protein conjugates. The basic knowledge from this finding will profoundly contribute to novel therapeutic approaches for treatment of invasive cancer.

Materials and Methods

Subcloning of cDNA into mammalian expression vectors and site directed mutagenesis

The full length cDNAs of 6xHis-FLAG tagged-maspin, ovalbumin, and maspin/ovalbumin chimeric mutants (MOM: maspin mutant containing the RCL of ovalbumin, OMO: ovalbumin mutant containing the RCL of maspin) in pYEP vectors and the 8xHis-TEV-maspin-pcDNA are kindly provided by Professor S.S. Twining, Medical College of Wisconsin, USA. All of cDNAs in the pYEP were later subcloned into the pcDNA mammalian expression vector (pcDNA 3.1) (Invitrogen). The full length cDNAs of human ISG15, human UBE1L and human UbcH8 are kindly provided by Professor A.L. Haas, Louisiana State University, USA. cDNAs of ISG15 and UBE1L were subcloned into pFLAG (Sigma Aldrich, USA), and UbcH8 cDNA was subcloned into a pcDNA3.1. All

cDNAs were subcloned into indicated plasmids by PCR-based method as described elsewhere (Jung V, et al. 1993).

The selected lysine residue was mutated to arginine using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). pCDNA 6xHis-FLAG maspin was used as a template. The correct coding sequence and a single nucleotide mutation were confirmed by DNA sequencing.

Cell culture and transfection

Human mammary adenocarcinoma MDA-MB-231, human cervical carcinoma HeLa, and human lung carcinoma A549 cell lines (ATCC) were regularly maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin at 37 °C in a humidified incubator of 5% CO_2. These cells were transfected with pcDNA-6xHis-FLAG-maspin wild type or pcDNA-6xHis-FLAG-chimeric mutant expression vectors using TurboFect reagent according to the manufacturer's protocol (Fermentus). Transfection of empty vector and pcDNA-6xHis-FLAG-ovalbumin was used for the controls.

To determine the effect of ISG15-conjugation, pcDNA-8xHis-TEV-maspin was co-transfected with pFLAG-ISG15, pFLAG-UBE1L, and pcDNA-UbcH8 as previously described by Zhao *et al* 2005. Briefly, IFN-β was added at 24 h post-transfection to the cultures at the final concentration of 1,000 units/ml and the cultures were further incubated for another 24 hours. Transfection of pcDNA-6xHis-FLAG-maspin alone or pFLAG-ISG15 alone was used for the controls. All transfectant cells were maintained in the same media with addition

of G418 neomycin. The expression of these cDNAs was confirmed by Western blot.

Western blot analysis

Total cell lysate of transfectants was collected using a mammalian lysis buffer (GE Health-Amersham Bioscience, Piscataway, NJ) including protease inhibitor mix (GE Health-Amersham Bioscience, Piscataway, NJ). Cell debris was removed by centrifugation at 14,000 x g for 10 min at 4^{o}C. The supernatant was subjected to 10 or 12% sodium dodecyl sulfate -polyarylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and blocked with 5% nonfat milk. The blot was incubated with primary antibody; monoclonal anti-human maspin antibody (BD Bioscience, USA), or monoclonal anti-FLAG® M2, or polyclonal anti-actin antibody (Sigma Aldrich, USA), or polyclonal anti-ISG15 antibody or polyclonal anti-ubiquitin antibody (kindly provided by Professor A.L. Haas, Louisiana State University, USA) for 2 h at room temperature. Then, the blot was washed three times, followed by a corresponding HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature. Finally, the specific band was visualized by chemiluminescence ECL detection system (GE Health-Amersham Bioscience, Piscataway, NJ).

Fluorometric 26S proteasome assay

Cells were harvested and homogenized in ice-cold lysis buffer without addition of protease inhibitors. Cellular chymotrypsin-like activity of 20S proteasome was assayed using the fluorogenic peptide substrate Suc-LLVY-AMC (Millipore Chemicon, Billerica, MA). Briefly, 10 ug of the protein lysates

was added to the reaction buffer diluted from the 20x reaction buffer (500 mM HEPES, 10 mM EDTA, pH 7.6) so that the final reaction volume was 90 µl. Triplicate reactions were performed for each cell lysate. The diluted lysates were incubated for 5 min for equilibration at 37°C. During incubation, a 20x substrate solution was made by diluting the fluorogenic peptide substrate stocks in reaction buffer. To each reaction, 10 µl of the appropriate 20x substrate stock solution was added; the final substrate concentration was 10 µM. Next, the fluorescence activity (Ex: 380 nm; Em: 460 nm) was measured in a fluorescence microplate reader.

Induction of ISG15 and ISG15 conjugates

IFN-β (kindly provided by Professor A.L. Haas, Louisiana State University, USA) was added into a confluent cell culture at final concentration of 1,000 units/ml. After 24 hours induction, total protein lysates were collected directly in SDS-sample loading buffer (62.5 mM Tris-HCl pH 6.8, 10 % Glycerol, 2% SDS, 0.01% bromphenol blue). They were separated in a 12% SDS-PAGE and followed by Western blotting as described above. The blot was then stripped and reprobed with a specific anti-actin antibody for normalization.

A nickel affinity pull-down assay

Ni-NTA agarose beads were equilibrated by pre-absorbing with 1 mg/ml bovine serum albumin at 4°C overnight and then washed with a washing buffer (50mM phosphate buffer pH 8.0, 150 mM NaCl, and 0.1% Tween-20). Total cell lysate was prepared using ice-cold mammalian lysis buffer containing protease inhibitor mix without EDTA. Then, the supernatant was mixed with the beads and incubated at room temperature for 2 hours with gentle rocking.

Next, the beads were washed five times with washing buffer containing 0.1% Tween-20, and further eluted by 50mM phosphate buffer pH 8.0 containing 250 mM Imidazole.

Statistical analysis

All experiments were tested at least three times in each experiment. The differences in the mean value among the groups are determined by one-way ANOVA with Student–Newman–Keuls test using the SPSS 11.5 software (SPSS Inc., Chicago, IL). Data were expressed as mean ± S.D. with P value < 0.05 indicating significance.

Results and Discussion

Inhibition of cancer invasion and metastasis is one of the major biological activities of tumor suppressor maspin (Seftor RE, et al. 1998). However, information on the molecular mode of maspin action is quite limited. Either addition of exogenous recombinant maspin (Sheng S, et al. 1996) or transfection of maspin cDNA into carcinoma cells (Shi HY, et al. 2001) effectively inhibits the invasion and metastasis both *in vitro* and *in vivo*. Importantly, mutagenesis studies revealed that the reactive center loop (RCL) of maspin is necessary and sufficient for the inhibition of cancer cell invasion (Ngamkitidechakul C, et al. 2003). In this study, the cDNAs of maspin and maspin/ovalbumin chimeric mutants (MOM and OMO) were subcloned into the pcDNA mammalian expression vector. Initially, condition of transfection was tested in A549 at two different ratios of transfection reagent (μL) to μg DNA (3:1 and 6:1). As shown in Figure 5, without maspin transfection, A549 cells produced undetectable level of endogenous maspin. However, recombinant

maspin was successfully produced within 24 hrs after transfection with pcDNA-8xHis-TEV-maspin by using the only ratio of 3 to 1. Subsequently, transfection of all constructs into MDA-MB-231 cells was carried out using the 3 to 1 ratio (Figure 6).

Transfection of pcDNA-6xHis-FLAG-maspin and the mutant vectors into MDA-MB-231 showed a time-dependent expression (24 vs 48 hr). As shown in Figure 6, more than two-fold increased expressions of maspin and MOM were detected at 48-h as compared to 24-h post transfection. Unlike maspin and MOM, transfection of pcDNA-6xHis-FLAG-ovalbumin (OV) and OMO mutant gave a lower level of expression at 48 h post-transfection.

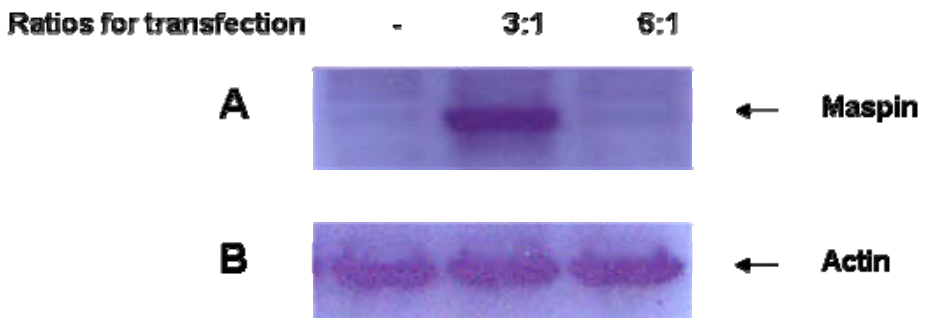

Figure 5 Ectopic Expression of Recombinant Maspin in A549 Cells. A pcDNA-8xHis-TEV-maspin vector was transfected into A549 using two different ratios of the transfection reagent (μl) to μg plasmid DNA (3:1 and 6:1). After 24 hours transfection, total cell lysates were subjected to Western blot analysis using a specific monoclonal anti-Maspin (A). The blot was stripped and reprobed with a specific polyclonal anti-actin (B).

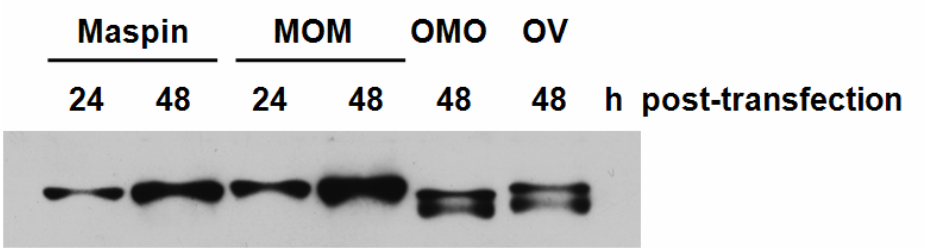

Figure 6 Expressions of Recombinant 6xHis-FLAG Maspin, Mutants (MOM, OMO) and Ovalbumin (OV) in MDA-MB-231 Cells. After 24 or 48 h post-transfection, total cell lysate was analyzed for recombinant protein expression using monoclonal anti-FLAG-M2 antibody.

Effect of maspin and mutants on cellular proteasome activity and ubiquitin conjugation

Not only does re-expression of maspin in breast carcinoma cell lines inhibit their invasiveness, but also alters the carcinoma's proteome by inhibiting the chymotrypsin-like activity of 20S proteasome (Chen El, et al. 2005). The functional domain responsible for maspin's proteomic regulation was herein proposed as the RCL domain if the biological function of maspin results from its ability to inhibit the proteasome activity. Therefore, the cell lysates of maspin and mutant transfectants were analyzed for 20S proteasome activity as shown in Figure 7. Similar to the previous report (Chen El, et al. 2005), maspin was able to reduce the proteasome activity of MDA-MB-231 cells in our assay. Significantly, the proteasome activity was also reduced in MDA-MB-231 transfected with either OMO mutant containing maspin's RCL or MOM mutant containing ovalbumin's RCL. However, ovalbumin significantly had no inhibitory effect on this activity. The result suggests that not only is the RCL, but other

region of the maspin molecule also sufficient for regulating the proteasome activity.

Since function of the proteasome is regularly associated with ubiquitin conjugating system, free ubiquitin and ubiquitin conjugates were also determined in the MDA-MB-231 transfectants. The level of free ubiquitin was somewhat similar among the transfectants and control, except in the OV transfectant that had a slightly higher free ubiquitin level than the other (Figure 8). Interestingly, MDA-MB-231 transfectants with maspin, MOM and OMO showed a significant higher level of ubiquitin-protein conjugates than that in the control. The result was consistent with the proteasome assay in which the activity was reduced in these transfectants, leading to the accumulation of high molecular weight of ubiquitin-protein conjugates. Therefore, the proteasome and ubiquitin conjugating system are regulated by maspin, and the reactive center loop (RCL) of maspin is sufficient, but unnecessary for the inhibition of proteasome activity.

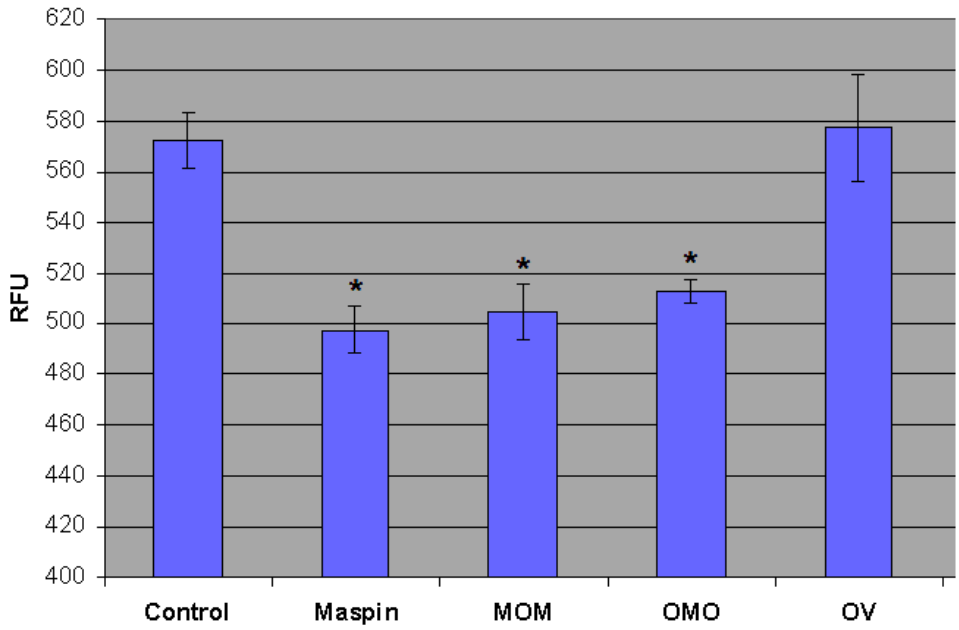

Figure 7 Effect Of Maspin and Other Mutants' Transfection on the Proteasome Activity of MDA-MB-231. Total cell lysate of the transfectants was analyzed for the cellular chymotrypsin-like activity of 20S proteasome using the fluorogenic peptide substrate Suc-LLVY-AMC by measuring at Ex: 380 nm; Em: 460 nm (* $p < 0.05$ relative to control)

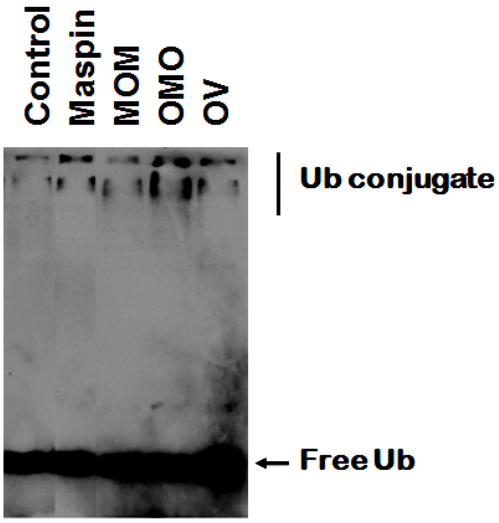

Figure 8 Effect of Maspin and Mutants on the Accumulation of High Molecular Weight Ubiquitin-protein Conjugates. After 48-h transfection of either maspin or mutant constructs, total protein lysate of MDA-MB-231 cells was collected and analyzed by immunobloting using anti-ubiquitin antibody.

Effect of maspin on ISG15-conjugation and the accumulation of high molecular weight ubiquitin-protein conjugates

It is noteworthy that specific activities of many proteins can be regulated by post-translational modifications. ISG15 conjugation to cellular target proteins seems to regulate the activity of target proteins (Takeuchi T, et al. 2005). ISG15 is highly elevated and extensively conjugated to cellular proteins in many tumors and tumor cell lines. In addition, the increased level of ISG15 has been shown to reduce levels of polyubiquitinated proteins. Importantly, ISG15-conjugation of maspin (Zhao C, et al. 2005) has been demonstrated in carcinoma cells; however, the consequence of this

modification remains mysterious. In this study, the effect of ISG15-conjugation on maspin's ability to accumulate the ubiquitinated proteins was investigated.

Interferon-β (IFN-β) is known to induce free ISG15 and ISG15 conjugates. Initially, A549 cells were tested for ISG15 induction by IFN-β since they have been extensively used to study ISG15 conjugation system (Narasimhan J, et al. 1996). As shown in Figure 9, both free ISG15 and ISG15 conjugates were clearly induced by IFN-β suggesting that A549 cells possess complete ISG15 conjugation machinery.

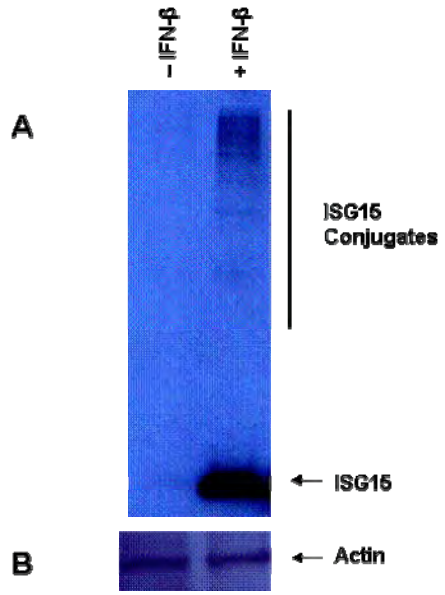

Figure 9 Induction of Free ISG15 and ISG15 Conjugates by IFN-β in A549 Cells. 1 x 10^3 units/ml IFN-β was added into A549 cells. After 24 hours incubation, total cell lysates were subjected to Western blot analysis using a specific polyclonal anti-ISG15 (A). The blot was stripped and reprobed with a specific polyclonal anti-actin (B).

To determine the effect of ISG15-conjugation on maspin expression, 1,000 units/ml of IFN-β was added to the cultures at 24 or 48 h post-transfection with pcDNA-8xHis-TEV-maspin, and the cultures were further incubated for another 24 hours. As shown in Figure 10, recombinant maspin was synthesized after 24 hours and continually to 48 hours after transfection. Comparable level of synthesized recombinant maspin was observed in the absence or presence of IFN-β (Figure 10A) suggesting that IFN-β has no effect on recombinant maspin synthesis.

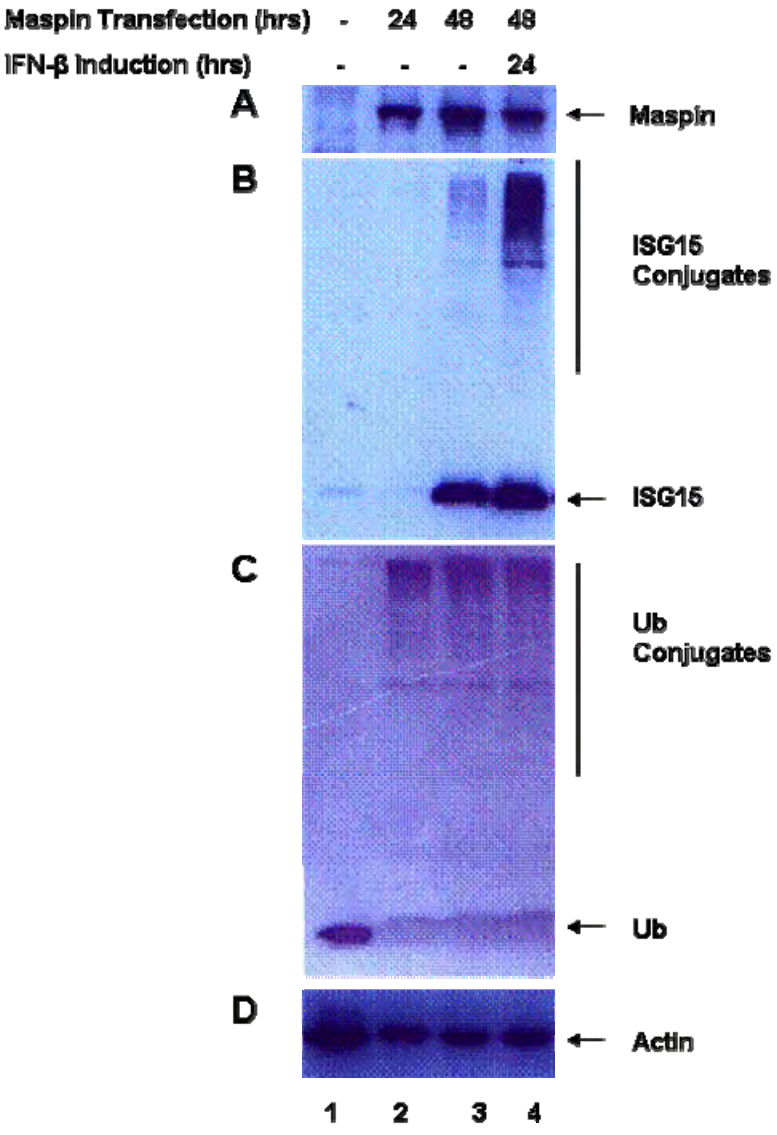

Figure 10 Induction of Free ISG15 and ISG15 Conjugates by Maspin Expression in A549 cells. At 24 h post-transfection with pcDNA-8xHis-TEV-maspin, A549 cells were later treated with IFN-β (1,000 units/ml). After another 24 or 48 h incubation, total cell lysates were collected and subjected to Western blot analysis using a specific monoclonal anti-Maspin (A), a specific polyclonal anti-ISG15 (B), a specific polyclonal anti-Ub (C), or a specific polyclonal anti-actin (D).

Interestingly, free ISG15 and ISG15 conjugates were clearly increased in maspin transfectant cells with or without addition of IFN-β. While the level of free ISG15 was similar, the increased level of ISG15 conjugates without IFN-β treatment was significantly lower than those in the transfectant cells treated with IFN-β (Figure 10B lane 3 and 4). Furthermore, the induction of ISG15 conjugation by either IFN-β induction or maspin transfection did not alter the level of ubiquitin (Ub) conjugation (Figure 10C). Thus, ISG15 conjugation by IFN-β induction did not affect maspin's ability to accumulate the ubiquitin conjugated proteins.

Although ISG15 conjugation was induced in A549 cells transfected with maspin cDNA, the maspin-ISG15 conjugate was undetectable (data not shown). To study the effect of maspin-ISG15 conjugation, overexpression of both E1 and E2 for ISG15 machinery was performed along with 8xHis-TEV-maspin and FLAG-ISG15 transfection in MDA-MB-231 cells. As shown in Figure 11, recombinant FLAG-ISG15 was conjugated to cellular proteins in the co-transfection experiment without addition of IFN-β detected by anti-FLAG M2

antibody. The observed conjugation of FLAG-ISG15 was detected only in co-transfection of FLAG-ISG15 and ISG15 conjugation machinery (both FLAG-UBE1L and UbcH8).

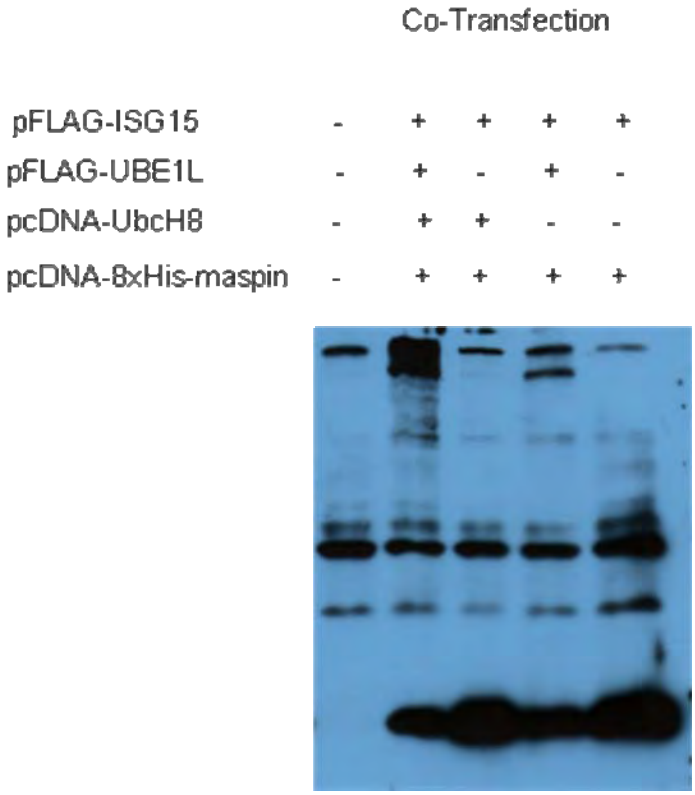

Figure 11 Induction of Recombinant ISG15 Conjugates by Co-transfection in MDA-MB-231 cells. pcDNA-8xHis-TEV-maspin and pFLAG-ISG15 with or without ISG15 conjugation machinery (pFlag-UBE1L and pcDNA-UbcH8) were co-transfected into MDA-MB-231 cells. After 48 h incubation, total cell lysates were collected and subjected to Western blot analysis using a monoclonal anti-FLAG M2 antibody.

In order to detect recombinant maspin-ISG15 conjugate, maspin-ISG15 conjugates were first pull-downed by a nickel column and then detected by antibodies against either the FLAG tag or maspin. As shown in Figure 12, 8xHis-maspin was effectively conjugated to FLAG-ISG15 in MDA-MB-231 transfectants. Next, analyzed by Western blotting using anti-ubiquitin antibody, the level of high molecular weight ubiquitin-protein conjugates was very much enhanced in the transfectants as compared with the control (Figure 13), suggesting that maspin-ISG15 conjugation likely affects the ubiquitin conjugation system. Since maspin was capable of inhibiting the proteasome activity (Figure 7) and ISG15 conjugation did not affect the ubiquitin conjugation (Figure10 C), ISGylation of maspin could directly enhance its activity leading to an increased accumulation of ubquitin-protein conjugates. It is however unknown whether ISG15 regulates the proteasome inhibitory activity of maspin. Additional experiments are necessary to investigate the effect of maspin-ISG15 conjugation on the proteasome activity.

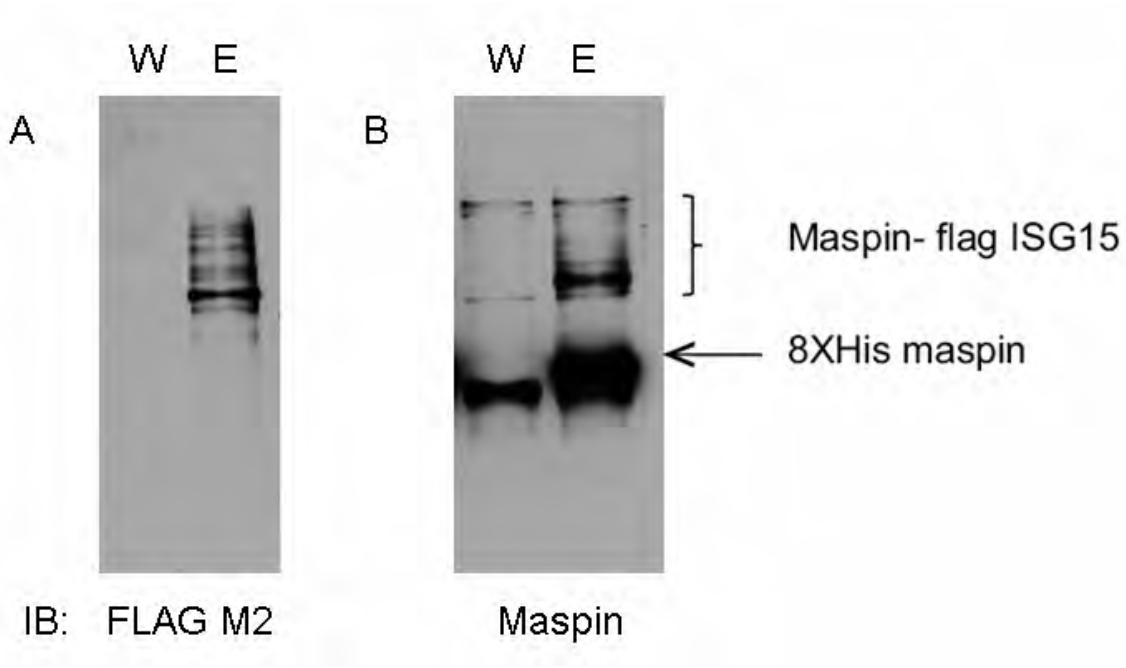

Figure 12 Conjugation of Maspin-flag ISG15. MDA-MB-231 cells were co-transfected with pcDNA-8xHis-TEV-maspin, pFLAG-ISG15, pFLAG-UBE1L, and pcDNA-UbcH8. Maspin-ISG15 conjugates were isolated by a nickel pull-down assay. Fractions (W: wash; E: elutent) were collected and subjected to Western blotting using anti-FLAG M2 (A) and anti-maspin antibodies (B).

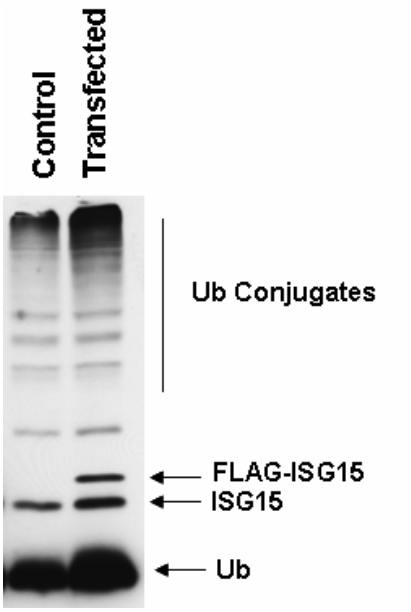

Figure 13 Effect of Maspin-ISG15 Conjugation on the Accumulation of High Molecular Weight Ubiquitin-protein Conjugates. After 48-h cotransfection of pcDNA-8xHis-TEV-maspin, pFLAG-ISG15, pFLAG-UBE1L, and pcDNA-UbcH8, total protein lysate of MDA-MB-231 cells was collected and analyzed by Western bloting using anti-ubiquitin antibody.

In order to determine the ISG15 conjugating site(s) on maspin, initially, ISG15 conjugation machinery (FLAG-ISG15, FLAG-UbE1L, and UbcH8) along with either 6xHis-FLAG-maspin or two maspin/ovalbumin chimeric mutants, 6xHis-FLAG-MOM or 6xHis-FLAG-OMO, were co-transfected into HeLa cells. After 48-h co-transfection, recombinant ISG15 conjugates were pulled down by nickel beads and then detected by Western blot analysis using anti-FLAG M2 (Figure 14A) or anti-maspin (Figure 14B) antibodies. As shown in Figure 14, both 6xHis-FLAG-maspin and 6xHis-FLAG-MOM formed a conjugate with ISG15 in a comparable amount whereas; conjugation of 6xHis-FLAG-OMO to

ISG15 was significantly less. Since anti-maspin antibody successfully detected only recombinant maspin and MOM, free OMO was not observed in Figure 14B. The results indicate that ISG15 conjugation site is not located in the reactive site loop of maspin.

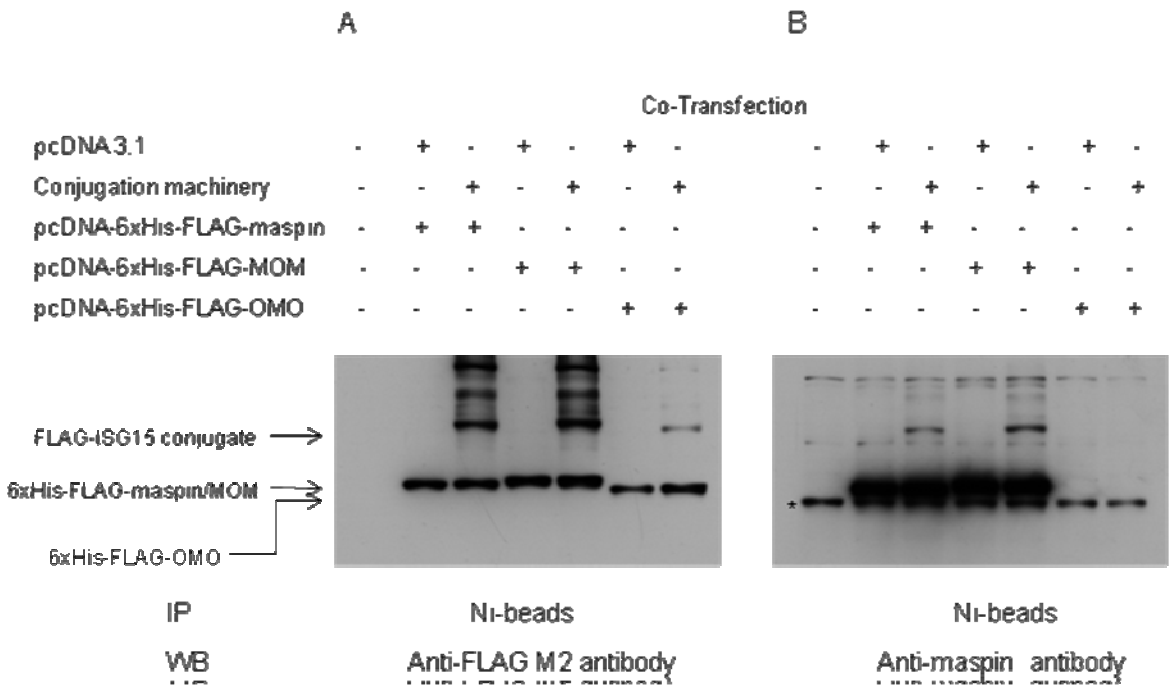

Figure 14 Conjugation of ISG15 to Recombinant Maspin or Maspin/Ovalbumin Chimeric Mutants (MOM or OMO). HeLa cells were co-transfected with plasmids encoding ISG15 conjugation machinery (pFLAG-ISG15, pFLAG-UBE1L, and pcDNA-UbcH8) and pcDNA-6xHis-FLAG-maspin or two maspin/ovalbumin chimeric mutants (MOM and OMO). Maspin-ISG15 conjugates were isolated by a nickel pull-down assay. Bound fractions were collected and subjected to Western blotting using anti-FLAG M2 (A) and anti-maspin antibodies (B).

Human maspin contains 37 total lysine residues. However, the crystal structure of maspin reveals approximately 9 lysine residues exposed on the surface molecule. Interestingly, K47 and K311 are conserved between maspin and serpin 2a (a member of the serine protease inhibitor which is also conjugated to ISG15). K345 resides closely to the reactive center loop which is required for inhibition of cell invasion. Therefore, K47, K311, and K345 were initially mutated to arginine and subjected to test for ISG15 conjugation.

Either 6xHis-FLAG-Maspin wild type, K47R, K311R, or K345R was co-transfected with ISG15 conjugation machinery (FLAG-ISG15, FLAG-UBE1L, and UbcH8) into HeLa cells. As described above, free 6xHis-FLAG-Maspin and conjugates were precipitated by Ni pull down assay. As shown in Figure 15, all mutants were able to form a conjugate to FLAG-ISG15 in a comparable amount to wild type as confirmed by Western blot analysis using anti-FLAG M2 (Figure 15A) and anti-maspin (Figure 15B) antibodies. The results indicated that K47, K311, and K345 are not the ISG15 conjugation site on maspin proteins. Screening of other surface lysines for the ISG15 conjugation site is still under investigated.

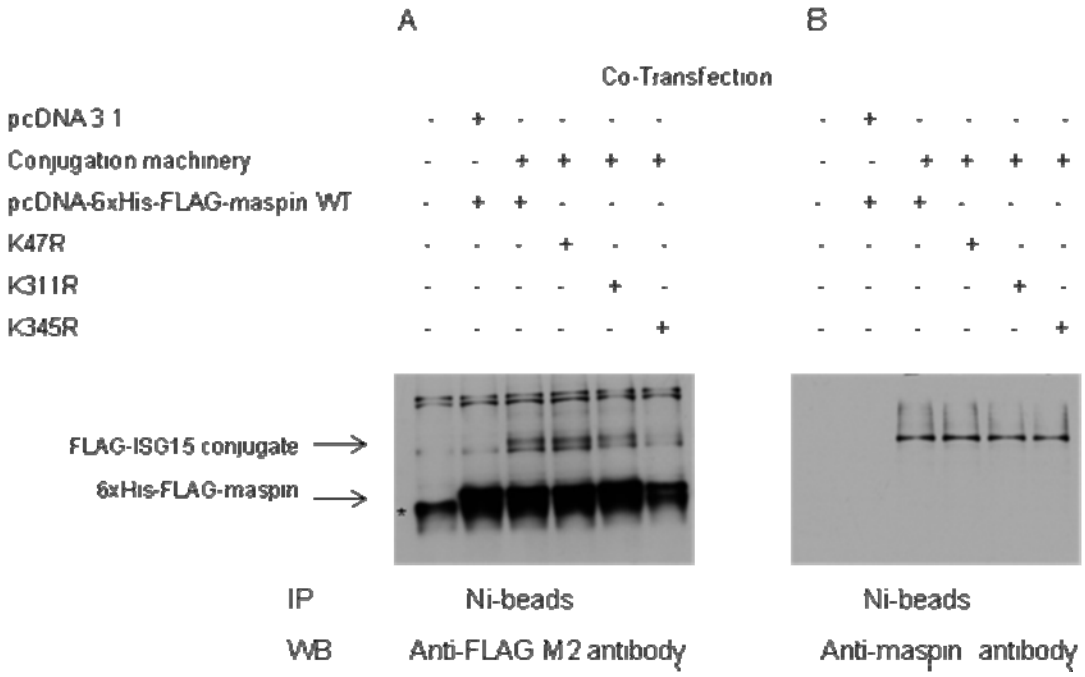

Figure 15 Identification of Lysine on Maspin for ISG15 Conjugation site. HeLa cells were co-transfected with plasmids encoding ISG15 conjugation machinery (pFLAG-ISG15, pFLAG-UBE1L, and pcDNA-UbcH8) and pcDNA-6xHis-FLAG-maspin or three maspin mutants (K47R, K311R, and K345R). Maspin-ISG15 conjugates were isolated by a nickel pull-down assay. Bound fractions were collected and subjected to Western blotting using anti-FLAG M2 (A) and anti-maspin antibodies (B).

In summary, maspin has been shown to inhibit carcinoma cell invasion by altering cellular proteome via the 26S proteasome inhibition. In this study, a known functional domain, the reactive center loop (RCL), of maspin is sufficient, though not required, for this inhibitory action. Notably, inhibition of proteasome activity in turn increases the accumulation of high molecular weight ubiquitin-proteins conjugates. Furthermore, post-translation of maspin

by ISG15 conjugation system is effectively demonstrated and also has a remarkable effect on the ubiquitin conjugation system; however, the ISG15conjugation site(s) are under investigated.

References

Abd El-Wahed MM. Expression and subcellular localization of maspin in human ovarian epithelial neoplasms: correlation with clinicopathologic features. J Egypt Natl Canc Inst. 2005 Sep;17(3):173-83.

Akiyama Y, Maesawa C, Ogasawara S, Terashima M, Masuda T. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. Am J Pathol. 2003 Nov;163(5):1911-9.

Bailey CM, Khalkhali-Ellis Z, Kondo S, Margaryan NV, Seftor RE, Wheaton WW, et al. Mammary serine protease inhibitor (Maspin) binds directly to interferon regulatory factor 6: identification of a novel serpin partnership. J Biol Chem. 2005 Oct 7;280(40):34210-7.

Bailey CM, Khalkhali-Ellis Z, Seftor EA, Hendrix MJ. Biological functions of maspin. J Cell Physiol. 2006 Dec;209(3):617-24.

Bass R, Fernandez AM, Ellis V. Maspin inhibits cell migration in the absence of protease inhibitory activity. J Biol Chem. 2002 Dec 6;277(49):46845-8.

Bettstetter M, Woenckhaus M, Wild PJ, Rummele P, Blaszyk H, Hartmann A, et al. Elevated nuclear maspin expression is associated with microsatellite instability and high tumour grade in colorectal cancer. J Pathol. 2005 Apr;205(5):606-14.

Boltze C, Schneider-Stock R, Quednow C, Hinze R, Mawrin C, Hribaschek A, et al. Silencing of the maspin gene by promoter hypermethylation in thyroid cancer. Int J Mol Med. 2003 Oct;12(4):479-84.

Ciechanover A. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Cell Death Differ. 2005 Sep;12(9):1178-90.

Chen EI, Florens L, Axelrod FT, Monosov E, Barbas CF, 3rd, Yates JR, 3rd, et al. Maspin alters the carcinoma proteome. Faseb J. 2005;19(9):1123-4.

Desai SD, Haas AL, Wood LM, Tsai YC, Pestka S, Rubin EH, et al. Elevated expression of ISG15 in tumor cells interferes with the ubiquitin/26S proteasome pathway. Cancer Res. 2006 Jan 15;66(2):921-8.

Dye BT, Schulman BA. Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. Annu Rev Biophys Biomol Struct. 2007;36:131-50.

Futscher BW, Oshiro MM, Wozniak RJ, Holtan N, Hanigan CL, Duan H, et al. Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet. 2002 Jun;31(2):175-9.

Giannakopoulos NV, Luo JK, Papov V, Zou W, Lenschow DJ, Jacobs BS, et al. Proteomic identification of proteins conjugated to ISG15 in mouse and human cells. Biochem Biophys Res Commun. 2005;336(2):496-506.

Goldstein G, Scheid M, Hammerling U, Boyse EA, Schlesinger DH, Niall HD. Isolation of a Polypeptide that has Lymphocyte-Differentiating Properties

and is Probably Represented Universally in Living Cells. Proc Natl Acad Sci U S A. 1975;72:11-5.

Gregori L, Poosch MS, Cousins G, Chau V. A uniform isopeptide-linked multiubiquitin chain is sufficient to target substrate for degradation in ubiquitin-mediated proteolysis. J Biol Chem. 1990;265:8354-7.

Hamerman JA, Hayashi F, Schroeder LA, Gygi SP, Haas AL, Hampson L, et al. Serpin 2a is induced in activated macrophages and conjugates to a ubiquitin homolog. J Immunol. 2002 Mar 1;168(5):2415-23.

Haas AL, Siepmann TJ. Pathways of ubiquitin conjugation. Faseb J. 1997;11:1257-68.

Haas AL, Warms JV, Hershko A, Rose IA. Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. J Biol Chem. 1982;257(2543-2548).

Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, Kolli N, et al. Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. Mol Cell Biol. 2004 Jan;24(1):84-95.

Hershko A, Ciechanover A. The ubiquitin system for protein degradation. Ann Rev Biochem. 1992;61:761-807.

Hojo T, Akiyama Y, Nagasaki K, Maruyama K, Kikuchi K, Ikeda T, et al. Association of maspin expression with the malignancy grade and tumor vascularization in breast cancer tissues. Cancer Lett. 2001;171(1):103-10.

Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res. 2000;10:1845-64.

Ito R, Nakayama H, Yoshida K, Oda N, Yasui W. Loss of maspin expression is associated with development and progression of gastric carcinoma with p53 abnormality. Oncol Rep. 2004 Nov;12(5):985-90.

Jentsch S, Seufert W, Sommer T, Reins HA. Ubiquitin-conjugating enzymes: novel regulators of eukaryotic cells. Trends Biochem Sci. 1990;15:195-8.

Jentsch S, Pyrowolakis G. Ubiquitin and its kin: how close are the family ties? Trends Cell Biol. 2000 Aug;10(8):335-42.

Jung V, Pestka SB, Pestka S. Cloning of polymerase chain reaction-generated DNA containing terminal restriction endonuclease recognition sites. Methods Enzymol. 1993;218:357-62.

Khalkhali-Ellis Z. Maspin: the new frontier. Clin Cancer Res. 2006;12(24):7279-83.

Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. 1998 Oct;8(10):397-403.

Loeb KR, Haas AL. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J Biol Chem. 1992 Apr 15;267(11):7806-13.

Maass N, Hojo T, Zhang M, Sager R, Jonat W, Nagasaki K. Maspin--a novel protease inhibitor with tumor-suppressing activity in breast cancer. Acta Oncol. 2000;39(8):931-4.

Maass N, Hojo T, Rosel F, Ikeda T, Jonat W, Nagasaki K. Down regulation of the tumor suppressor gene maspin in breast carcinoma is associated with a higher risk of distant metastasis. Clin Biochem. 2001 Jun;34(4):303-7.

Maass N, Biallek M, Rosel F, Schem C, Ohike N, Zhang M, et al. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer. Biochem Biophys Res Commun. 2002 Sep 13;297(1):125-8.

Malakhov MP, Kim KI, Malakhova OA, Jacobs BS, Borden EC, Zhang DE. High-throughput immunoblotting. Ubiquitiin-like protein ISG15 modifies key regulators of signal transduction. J Biol Chem. 2003;278(19):16608-13.

Marioni G, D'Alessandro E, Giacomelli L, De Filippis C, Calgaro N, Sari M, et al. Maspin nuclear localization is related to reduced density of tumour-associated micro-vessels in laryngeal carcinoma. Anticancer Res. 2006 Nov-Dec;26(6C):4927-32.

Narasimhan J, Potter JL, Haas AL. Conjugation of the 15-kDa interferon-induced ubiquitin homolog is distinct from that of ubiquitin. J Biol Chem. 1996 Jan 5;271(1):324-30.

Ngamkitidechakul C, Warejcka DJ, Burke JM, O'Brien WJ, Twining SS. Sufficiency of the reactive site loop of maspin for induction of cell-matrix

adhesion and inhibition of cell invasion. Conversion of ovalbumin to a maspin-like molecule. J Biol Chem. 2003 Aug 22;278(34):31796-806.

Nyman TA, Matikainen S, Sareneva T, Julkunen I, Kalkkinen N. Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-alpha-regulated genes. Eur J Biochem. 2000;267(13):4011-9.

Odero-Marah VA, Khalkhali-Ellis Z, Schneider GB, Seftor EA, Seftor RE, Koland JG, et al. Tyrosine phosphorylation of maspin in normal mammary epithelia and breast cancer cells. Biochem Biophys Res Commun. 2002 Jul 26;295(4):800-5.

Pemberton PA, Wong DT, Gibson HL, Kiefer MC, Fitzpatrick PA, Sager R, et al. The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. J Biol Chem. 1995 Jun 30;270(26):15832-7.

Pemberton PA, Tipton AR, Pavloff N, Smith J, Erickson JR, Mouchabeck ZM, et al. Maspin is an intracellular serpin that partitions into secretory vesicles and is present at the cell surface. J Histochem Cytochem. 1997 Dec;45(12):1697-706.

Pickart CM. Mechanism underlying ubiquitination. Annu Rev Biochem. 2001;70:503-33.

Pitha-Rowe I, Hassel BA, Dmitrovsky E. Involvement of UBE1L in ISG15 conjugation during retinoid-induced differentiation of acute promyelocytic leukemia. J Biol Chem. 2004 Apr 30;279(18):18178-87.

Primeau M, Gagnon J, Momparler RL. Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer. 2003 Jan 10;103(2):177-84.

Sager R, Sheng S, Pemberton P, Hendrix MJ. Maspin. A tumor suppressing serpin. Adv Exp Med Biol. 1997;425:77-88.

Seftor RE, Seftor EA, Sheng S, Pemberton PA, Sager R, Hendrix MJ. maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res. 1998 Dec 15;58(24):5681-5.

Sheng S, Carey J, Seftor EA, Dias L, Hendrix MJ, Sager R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci U S A. 1996 Oct 15;93(21):11669-74.

Sheng S. The promise and challenge toward the clinical application of maspin in cancer. Front Biosci. 2004 Sep 1;9:2733-45.

Shi HY, Zhang W, Liang R, Abraham S, Kittrell FS, Medina D, et al. Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res. 2001 Sep 15;61(18):6945-51.

Streuli CH. Maspin is a tumour suppressor that inhibits breast cancer tumour metastasis in vivo. Breast Cancer Res. 2002;4(4):137-40.

Takeuchi T, Yokosawa H. ISG15 modification of Ubc13 suppresses its ubiquitin-conjugating activity. Biochem Biophys Res Commun. 2005 Oct 14;336(1):9-13.

Takeuchi T, Kobayashi T, Tamura S, Yokosawa H. Negative regulation of protein phosphatase 2Cbeta by ISG15 conjugation. FEBS Lett. 2006 Aug 7;580(18):4521-6.

Wada K, Maesawa C, Akasaka T, Masuda T. Aberrant expression of the maspin gene associated with epigenetic modification in melanoma cells. J Invest Dermatol. 2004 Mar;122(3):805-11.

Walters KJ, Goh AM, Wang Q, Wagner G, Howley PM. Ubiquitin family proteins and their relationship to the proteasome: a structural perspective. Biochim Biophys Acta. 2004 Nov 29;1695(1-3):73-87.

Yin S, Li X, Meng Y, Finley RL, Jr., Sakr W, Yang H, et al. Tumor-suppressive maspin regulates cell response to oxidative stress by direct interaction with glutathione S-transferase. J Biol Chem. 2005;280(41):34985-96.

Yuan Q, An J, Liu DG, Sun L, Ge YZ, Huang YL, et al. Proteomic analysis of differential protein expression in a human hepatoma revertant cell line by using an improved two-dimensional electrophoresis procedure combined with matrix assisted laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis. 2004 Apr;25(7-8):1160-8.

Zhang M, Sheng S, Maass N, Sager R. mMaspin: the mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. Mol Med. 1997 Jan;3(1):49-59.

Zhao C, Beaudenon SL, Kelley ML, Waddell MB, Yuan W, Schulman BA, et al. The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an

IFN-alpha/beta-induced ubiquitin-like protein. Proc Natl Acad Sci U S A. 2004 May 18;101(20):7578-82.

Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. Proc Natl Acad Sci U S A. 2005 Jul 19;102(29):10200-5.

Zou W, Papov V, Malakhova O, Kim KI, Dao C, Li J, et al. ISG15 modification of ubiquitin E2 Ubc13 disrupts its ability to form thioester bond with ubiquitin. Biochem Biophys Res Commun. 2005 Oct 14;336(1):61-8.

Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, et al. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science. 1994 Jan 28;263(5146):526-9.

Zou Z, Gao C, Nagaich AK, Connell T, Saito S, Moul JW, et al. p53 regulates the expression of the tumor suppressor gene maspin. J Biol Chem. 2000 Mar 3;275(9):6051-4.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

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