The thesis committee for Shih-Hao Yeh certifies that this is the approved version of the following thesis:

DEGRADATIONAL AND TRANSCRIPTIONAL INVESTIGATIONS OF THE RAF KINASE INHIBITORY PROTEIN

Committee:

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Mark R. Hellmich, Ph.D., Supervisor

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Celia Chao, M.D.

_____

Owen P. Hamill, Ph.D.

_____

Dean, Graduate School

DEGRADATIONAL AND TRANSCRIPTIONAL INVESTIGATIONS OF THE RAF KINASE INHIBITORY PROTEIN

by

Shih-Hao Yeh, M.S.

Thesis

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Dedication

This thesis is dedicated to my parents, Hsian-Liang Yeh, and Huang-Yi Huang, and my family, Yi-Fang Ho and Isaac Yeh, as well as every friend who supported me at Galveston.

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I am truly grateful to my mentor, Dr. Hellmich, for supporting and training me to be a scientist. Without his optimism and generosity, this research thesis could not be finished. I am especially thankful for his great patience, thoughtfulness and encouragement while I was severely bothered by my family problems and losing my courage. His enthusiasm and indomitableness of striving for science under tough situation especially give me a precious lesson of life.

I also want to give special thanks to my committee members, Dr. Chao and Dr. Hamill, for sharing their precious knowledge and comments for revision of this thesis and especially their generous help for correcting my grammar with patience.

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List of Abbreviation

EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular signal-Regulated Kinase
GPCR	G Protein-Coupled Receptors
GRK-2	G-Protein-Coupled Receptor Kinase 2
HCNP	Hippocampal Cholinergic Neurostimulatory Peptide
IB	Immunoblotting
IP	Immunoprecipitation
NF-κB	Nuclear Factor κB
PEBP	PhosphatidylEthanolamine-Binding Protein
PKC	Protein kinase C
RKIP	Raf-1 Kinase Inhibitory Protein
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TF	Transcription Factor

DEGRADATIONAL AND TRANSCRIPTIONAL INVESTIGATIONS OF THE RAF KINASE INHIBITORY PROTEIN

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RKIP (Raf-1 kinase inhibitory protein) is a novel potent metastasis suppressor by its function of inhibiting ERK pathway, NF-κB pathway, and GRK-2. RKIP expression level is reported to be critical in the differentiation process and the pathogenesis of many different cancers and inflammatory diseases. However, the understanding of how RKIP expression level is regulated is poor.

In this thesis, I endeavored to investigate whether RKIP is regulated by degradational mechanisms using ubiquitination and cycloheximide blocking experiments or transcriptional mechanisms other than by Snail repression using luciferase reporter assays. My results indicate that RKIP is a relatively stable protein, which has a half-life around 1 to 2 days and is not subject to rapid degradation by ubiquitin-proteosomal pathway in either human HEK293 cells or rat AR42J cells. I found that the RKIP promoter reporter was not significantly repressed by Snail in transfected HEK293 cells but was subject to repression by the GSK-3 inhibitor BIO compound in both E-box (putative Snail binding site)-dependent and -independent manner. My results indicate that there are other Snail-independent transcriptional regulations involved in the regulation of RKIP expression.

I. Introduction

1.1 Raf-1 Kinase Inhibitory Protein (RKIP) and Its Function

RKIP (Raf-1 kinase inhibitory protein) is a novel potent metastasis suppressor, which has been reported absent or deficient in different kinds of melanoma, breast, metastatic prostate, colorectal, and pancreatic cancers (Schuierer *et al.*, 2004; Hagan *et al.*, 2005; Fu *et al.*, 2006; Minoo *et al.*, 2007; Kim *et al.*, 2010). RKIP is expressed in varying degrees in normal breast epithelial cells and primary breast cancers but its levels are significantly reduced in metastatic lymph nodes. Importantly, re-expression of RKIP inhibits/reverses the metastatic phenotype (Hagan *et al.*, 2005). Therefore, investigating the mechanisms of how RKIP is down-regulated should become an important for understanding the metastatic process.

Besides metastasis, the loss of RKIP is reported to cause deficient sperm capacitation, reduced reproduction rates (Moffit *et al.*, 2007), and pulmonary hypertension in RKIP knockout mice (Morecroft *et al.*, 2011). In addition, the facts that RKIP regulates the mitotic spindle checkpoint (Rosner, 2007), RKIP is up-regulated during macrophage differentiation (Schuierer *et al.*, 2006), and RKIP enhances neuronal differentiation (Hellmann *et al.*, 2010) indicate the critical role of RKIP in determining cell fate.

RKIP, also known as PEBP-1 (phosphatidylethanolamine-binding protein) is a 187 amino acid, 23kDa globular cytosolic protein that belongs to the highly conserved PEBP

family. The PEBP family is a group of more than 400 proteins with a variety of functions but with an evolutionarily conserved structure. The three-dimensional structure as well as possession of the conserved ligand-binding pocket and the coupled cleavage site of human RKIP/PEBP-1 (hPEBP1) has been determined by X-ray crystallography (Benfield *et al.*, 1998). RKIP and its mammalian homologues are widely expressed in most tissues where it participates in the regulation of several ubiquitous signaling pathways involved in cell proliferation, apoptosis, and cell survival. RKIP is the precursor of the Hippocampal cholinergic neurostimulatory peptide (HCNP) that enhances acetylcholine synthesis in the central nervous system (Ojika *et al.*, 2000). Besides been enzymatically cleft into HCNP, the major function of the RKIP is to bind and inhibit signaling kinases.

RKIP was firstly reported and named by its function of being an inhibitor of the proliferation-promoting Ras-Raf-1-MEK1/2-ERK1/2 pathway. The binding of RKIP to the Raf-1 with its ligand-binding pocket competitively blocks the interaction between Raf-1 and MEK1/2 and thereby inhibits the activation of ERK1/2-mediated cascades (Yeung *et al.*, 1999). Subsequently, it was also shown to be an inhibitor of the G-protein-coupled receptor kinase 2 (GRK-2) (Lorenz *et al.*, 2003) and the nuclear factor κB (NF-κB) signaling pathway (Yeung *et al.*, 2001, Tang *et al.*, 2010). GRK-2 is a negative feedback regulator of the G protein-coupled receptor (GPCR) signaling pathway, which phosphorylates GPCR and induces receptor desensitization and internalization. Binding of phosphorylated RKIP to

GRK-2 inhibits its function and therefore desensitization of GPCR signaling (Lorenz *et al.*, 2003). The transcription factor NF-κB is well known to regulate a large number of genes that regulate apoptosis, immune and inflammatory responses, cell proliferation, apoptosis, and cell survival (May & Ghosh, 1997). RKIP is proposed to be a physiological inhibitor of NF-κB based on its ability to inhibit NF-κB transactivity by binding and inhibiting the upstream kinases TNF-receptor-associated factor 6 (TRAF6), TAK1, NIK, IKKα and IKKβ of the canonical NF-κB activating pathway (Yeung *et al.*, 2001). The inhibition of NF-κB thereby inhibits Snail transcription, which is a key regulator of the epithelial to mesenchymal transition (EMT).

Depletion of RKIP enhances the stimuli-induced Ras-Raf-1-MEK1/2-ERK1/2 signaling which regulates cell proliferation and differentiation. GRK-2 dysregulation due to the absence of RKIP desensitize cells to environmental stimuli. Repression of RKIP also activates NFκB-activated anti-apoptotic genes and enhances Snail-dependent EMT, thereby promoting metastasis as well as drug- and apoptotic resistance. The dysregulation of these universal pathways due to absence or depletion of RKIP is thought to be a key factor underlying the abnormal properties of cancerous cells; namely high survival rate, resistance to apoptosis, and metastasis (Granovsky &Rosner, 2008; Wu & Bonavida, 2009). The fact that restoration of RKIP in highly metastatic cell lines of prostate and breast cancer sensitizes them to apoptosis (Chatterjee *et al.*, 2004) indicates that RKIP is an important modulator involved in the

maintenance of normal cell properties. Therefore, RKIP must be considered a potent target for inhibiting metastasis and reversing apoptosis resistance.

1.2 Regulations of RKIP

RKIP is an intermediate modulator of signal transduction pathways and their crosstalk via phosphorylation. Protein kinase C (PKC)-driven phosphorylation changes affinity and specificity of the ligand-binding pocket of RKIP to different kinases. Nonphosphorylated RKIP binds Raf-1 directly and inhibits its function by interrupting the interaction between MEK1/2 and Raf-1. However, PKC-driven RKIP phosphorylation at the Serine 153 (S153) site of RKIP interrupts the binding between RKIP and Raf-1. The released phospho-RKIP from Raf-1 alternatively binds and inhibits GRK-2, reducing the GPCR desensitization (Corbit *et al.*, 2003; Lorenz *et al.*, 2003). Phosphorylation at Serine 99 (S99) site of RKIP is also reported to be responsible for ERK1/2-mediated feedback regulation (Shin *et al.*, 2009). Association between RKIP and NF-κB signaling complex is also reported to be ligand-dependent but the mechanism is not yet known (Tang *et al.*, 2010). The switching of RKIP between its different tasks seems to occur mainly through post-translational modification especially phosphorylation.

The other known regulation of RKIP is by transcriptional regulation. It has been reported that the transcription factor Snail is a repressor of RKIP transcription in metastatic prostate cancer cells (Beach *et al.*, 2008). It is thought that Snail binds to the distant E-box

cis-elements upstream to the RKIP gene, interrupting the RKIP transcription. This together with the facts that Snail is a key regulator of the epithelial to mesenchymal transition (EMT) which can be activated by NF-κB signaling and that RKIP inhibits transactivity of NF-κB, the NF-κB-Snail-RKIP circuitry is considered critical for cancer metastasis (Wu & Bonavida, 2009).

1.3 Specific Aim

Besides phosphorylation and Snail-dependent transcriptional control of RKIP, other possible mechanisms of RKIP regulation remain unexplored. The findings of the relation between RKIP expression levels and cancer metastases, as well as between RKIP level and cell differentiation have indicated that the change of cytosolic content of RKIP is critical for cell fate. Therefore, metabolic regulation of RKIP synthesis and degradation may also be important in cell physiology.

One of our lab interests is in the role(s) of RKIP in the pathogenesis of pancreatic cancers and pancreatitis. Preliminary data show that RKIP is also lost in pancreatic acinar cells during pancreatitis. Besides, ethanol-induced PKC activity phosphorylates RKIP in both human acinar cells and rat pancreatic AR42J cells. In AR42J cells, ethanol treatment (100 mM for 10 min) reduced the amount of phosphorylated RKIP in affinity-purified phosphoproteins compared to untreated group (data not shown). It is not clear whether the RKIP depletion in acinar and AR42J cells is regulated by IκB-like

phosphorylation-modulated degradation or not. We thereby hypothesize that ethanol-induced RKIP depletion is via phosphorylation-driven ubiquitin-proteosome pathway. Moreover, besides E-box and the Snail suppression, there are still many potential transcription factors binding sites such as AP-1, SP-1, YY-1, or others, locating within the RKIP promoter region (Odabaei *et al.*, 2004). Therefore, our central hypothesis is that cytosolic RKIP expression level is regulated by ubiquitin-dependent degradational and Snail-independent transcriptional mechanisms. We propose two specific aims to test our central hypothesis:

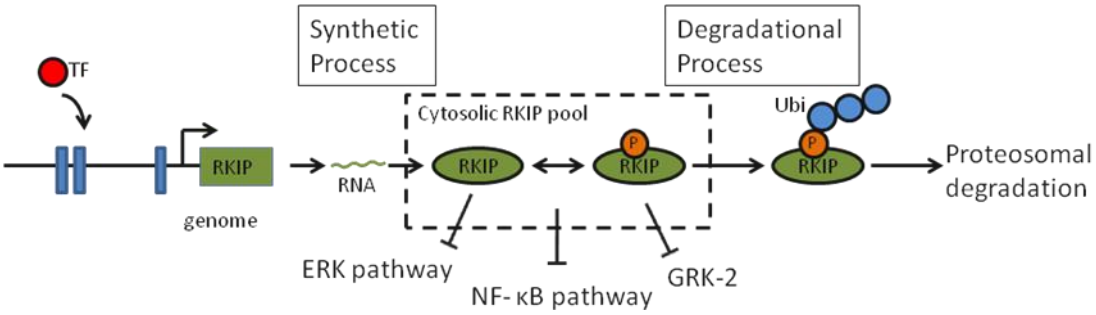

Figure 1. Illustration of transcriptional and degradational regulation of RKIP.
TF, transcription factors; Ubi, ubiquitin.

1. To determine if ethanol-induced RKIP phosphorylation causes RKIP degradation via ubiquitin proteosome pathway. Our working hypothesis is that RKIP can be degraded quickly with ethanol treatment via IκB-like serial reactions of phosphorylation, ubiquitination, and proteosome-operated degradation.
2. To identify novel mechanisms regulating RKIP transcription. Based on the existence of putative transcription factors binding sites within the RKIP promoter region, our working hypothesis is that besides Snail, RKIP expression is also regulated by other transcription factors.

II. Materials and Methods

2.1 Materials

The pcDNA3.1(+) plasmid with wild-type RKIP tagged with Flag at the N-terminus (RKIP-Flag) was generously provided by Dr. Walter Kolch at the University of Glasgow. The *firefly* pGL3-basic and *renilla* pGL4.73[hRluc/SV40] Luciferase Reporter Vectors were bought from Promega (Medison, MI, USA). The CMV-Tag2B with wild-type Snail and long-lived Snail 6SA were kindly provided by Dr. Binhua P. Zhou at the University of Kentucky.

Absolute ethanol (BP2818-100) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The MG-132 (proteosome inhibitor, PI-102) was purchased from Enzo Life Science (Farmingdale, NY, USA). The de-ubiquitination enzyme inhibitor NEM (N-ethylmaleimide, E3876), the Tumor Necrosis Factor-α (TNF-α, H8916), the protein synthesis inhibitor cycloheximide (CHX, C1988) and the mouse anti-Flag M2 antibody (F3165) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-STAT1 (#9172) was bought from Cell Signaling Technology (Danvers, MA, USA). The GSK-3 inhibitor IX (BIO, sc-202634), the primary antibodies rabbit anti-RKIP FL-187 (sc-28837), rabbit anti-HA probe Y-11 (sc-805) rabbit anti-IκBα C-21 (sc-371) and rabbit anti-SNA1 H130 (sc-28199), the secondary antibodies donkey anti-rabbit IgG (sc-2089) and chicken

anti-mouse IgG (sc-2985), and the Protein A/G Plus Agarose Immunoprecipitation Reagent (sc-2003) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell Cultures and Transfection

Human embryonic kidney 293 (HEK293) cells and human pancreatic cells MIA PaCa-2 were grown in DMEM (Dulbelcco's Modified Eagle's Medium, Cellgro® Mediatech, Manassas, VA, USA) media with 10% heat-inactivated fetal bovine serum (FBS, Cellgro® Mediatech). Human pancreatic cells PANC-1, AsPC-1 and BxPC-3 were grown in RPMI 1640 Medium (Gibco® Invitrogen) media with 10% heat-inactivated FBS. Rat pancreatic AR42J cells were grown in F12K Medium (Gibco® Invitrogen) with 20% heat-inactivated FBS. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Transient transfection of plasmids of RKIP-Flag, HA-Ubiquitin, Snail, and luciferase reporters were carried on by Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction.

2.3 Co-immunoprecipitation of Ubiquitination Experiments

The immunoprecipitation procedure of ubiquitination experiments was modified from Marchese A and Benovic JL, 2004. HEK293 cells were grown onto 25 cm^2 T-flasks for 24 hours and transiently co-transfected (1:1) with plasmids with Flag-tagged RKIP (RKIP-Flag) gene and HA-tagged Ubiquitin (HA-Ubi) gene or control plasmids by Lipofectamine 2000.

24 hours later, cells were split into new 25 cm^2 T-flasks for different treatment conditions. 2 days after transfection, cells were pretreated with 50μM MG-132 or DMSO for 1 hour, treated with 100 mM ethanol for 10 minute, 10ng/ml TNF-α for 15 minute or control DMEM media before cell lysis. The media were then removed by vacuum suction and cells were washed by iced PBS on ice. Then, 1ml of Lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% Non-idet P-40, 0.5 mM sodium vanadate, 2 mM sodium fluoride, 2 mM sodium pyrophosphate, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, 1 mM PMSF) with additional 50 μM MG-132 and 5 mM NEM was added into the flasks. Cell debris were then scrapped up, transferred to 1.5 ml eppendorf tubes and incubated for 30 min on ice, followed by sonication (200 ms pulse × 5, output 3) and then centrifugation at 13,000 rpm (~15,000g) for 20 min. The cell extracts in supernatants were transferred to new 1.5 ml eppendorf tubes and were determined the total protein concentration. For western blotting, 10 μg the each cell extracts were fractionated by SDS-PAGE as standard western blotting procedure.

For co-immunoprecipitation, we transferred cell extracts with 1 mg of total proteins each to 2 new 1.5 ml eppendorf tubes for anti-Flag and anti-HA immunoprecipitations. Three μg of mouse Anti-Flag M2 and rabbit anti-HA probe antibodies were added respectively into cell extracts and agitated at 4°C for 1 hour. For each reaction, 50 μl of the prepared Protein A/G Plus Agarose Beads (diluting 1:1 [w/v] with lysis buffer) was added into the tube and

agitated at 4°C overnight. On the next day, beads-protein complex was washed by repeating 4 times of spinning down by centrifugation at 13,000 rpm for 1 min, removing supernatant, adding back 750 µl fresh lysis buffer, mixing, and agitating at 4°C for 10 min. After the final spin down, supernatant was removed and 30 µl 2X Sample Buffer (37.5 mM Tris-HCl, pH 6.5, 8% SDS, 10% glycerol and 0.003% bromophenol blue) was added and the reaction was incubated at 37°C for 1 hour to elute the immunoprecipitated ubiquitinated RKIP. At last, the sample was centrifuged 13,000 rpm for 1 min to sediment beads and the supernatant was collected. The eluted proteins were resolved in SDS-PAGE and standard western blotting protocol except that the PVDF membrane was incubated in 40 ml denaturation solution (62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS) for 30 min at 60°C before primary antibody incubation.

2.4 Western Blotting and densitometry

The cells grown in cultural plates were washed out by ice cold PBS to remove residual media then lysed with cold radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5% sodium deoxycholate, 1 mM sodium vanadate, 1% Non-idet P-40, 0.1% SDS, 5 µg/ml chymostatin, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 µg/ml pepstatin A, 1 mM PMSF). Cell debris were then scrapped up, transferred to 1.5 ml eppendorf tubes and incubated for 30 min on ice, followed by sonication (200 ms pulse × 5, output 3) and

then centrifugation at 13,000 rpm (~15,000g) for 10 min. The cell extracts in supernatants were transferred to new 1.5 ml eppendorf tubes and determined the total protein concentration by Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA, USA). Each cell extracts (10 μg) were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electrically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Milford, MA, USA). The PVDF membrane was then blocked with 5% skimmed milk in Tris buffered saline with tween 20 (TBST, 50 mM Tris-HCl pH 7.4, 135 mM NaCl, 0.1% tween 20) for 1 hour at room temperature (RT) and then agitated with indicated primary antibody diluted in 5% skimmed milk/TBST overnight at 4°C. After three times wash by TBST, the PVDF membrane was then agitated with secondary antibody diluted in TBST. After three times wash steps, the specific immunostaining on the membrane was detected by Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Bioscience, Piscataway, NJ, USA). The intensity of specific bands was measured using the ImageJ v.1.45 software (NIH) and the data were analyzed by GraphPad Prism 5 software (GraphPad).

2.5 Plasmids construction

The fragments with different lengths from the 5'-untranslated region (5'-UTR) of RKIP gene was selected according to the article of Okita *et al.*, 2009. Fragments were amplified by Polymerase chain reaction (PCR) from the genomic DNA extracted from normal human

pancreatic tissue using primers in Table 1. The fragments were then inserted into the molecular cloning site of pGL3-basic reporter vector by Mlu I and Bgl II restriction sites. The fidelity of the sequence was double-checked by electrophoresis and DNA sequencing.

Table 1. Constructs of RKIP promoter fragments

Plasmids	Primers	Insert Length	Putative elements
pGL3-(-97/+64)	5'-atataaacgcgtttgcatggacccaggag -3' 5'-attattagatctacactggctcgggaaga-3'	161 bp	promoter
pGL3-(-596/+64)	5'-atataaacgcgttccagccgttgcaacaca -3' 5'-attattagatctacactggctcgggaaga-3'	660 bp	Promoter + enhancer
pGL3-(-1651/+64)	5'-attattacgcgtgaggaattatcaatgtc -3' 5'-attattagatctacactggctcgggaaga-3'	1715 bp	Promoter + enhancer +1 E-box*

*****E-box: CANNTG, potential Snail-binding consensus site

2.6 Luciferase Assay

Luciferase assay was executed with Dual-Luciferase Reporter Assay System kit purchased from Promega (Medison, MI, USA). One day before transfection, cells were grown in 24-well plates to be about 40%~60% confluent at the time of transfection depending on cell types. The *firefly* pGL3 reporter plasmids and *renilla* pGL4-SV40 plasmids were transfected in 100:1 ratio by Lipofectamine 2000. For co-transfection experiments with additional plasmids, the ratio of pGL3 reporters: pGL4-SV40: tested plasmid was 100:1:100.

Two days after transfection, the growth medium was removed and the cells were washed by PBS once. For each reaction, cells were lysed and agitated with 100 μl 1× passive lysis buffered (PLB) of the kit at room temperature for 30 min. After passive lysis, 20 μl of cell lysate was loaded and mixed together with 100μl LAR II solution into new tube, which was then immediately loaded into the luminometer for the first 10-second measurement of the

intensity of *firefly* luciferase. After the first reading, the tube with cell lysate was taken out from the luminometer and added with 100 μl Stop & Glo Reagent. The tube was then loaded into the luminometer again for the second 10-second measurement of the intensity of *renilla* luciferase. The results were analyzed and plotted by GraphPad Prism 5 software (GraphPad).

III. Results and Discussion

3.1 RKIP Was Not Regulated by a Degradational Mechanism in Response to Ethanol Treatment in human HEK293 Cells and rat AR42J Cells

Our preliminary data indicated that the phospho-RKIP level in purified phosphoproteins from rat pancreatic AR42J cells is reduced in response to a 10 minute, 100 mM ethanol treatment (data not shown). The facts that ethanol can activate specific PKC isoforms in acinar and other pancreatic cells (Gorelick *et al.*, 2008) and that PKC modulates RKIP phosphorylation (Gorelick *et al.*, 2008; Park, 2009) suggest a possibility that ethanol-induced RKIP depletion is mediated by PKC phosphorylation. IκB degradation through ubiquitin-proteosome pathway is a notable example of phosphorylation-induced degradation (Chen *et al.*, 1995). Therefore, we hypothesized that ethanol-induced phospho-RKIP depletion is regulated by ubiquitination.

3.1.1 Ethanol did not induce RKIP depletion in transfected human HEK293 cells by the ubiquitin-proteosome pathway.

To determine whether ethanol-induced RKIP depletion is regulated by ubiquitin-proteosome pathway, I first attempted doing immunoblotting and co-immunoprecipitation experiments to study RKIP ubiquitination in human HEK293 cells. The HEK 293 cell is a well-studied cell model that is easily transfected whereas AR42J cell is relatively small and

does not easily uptake exogenous plasmid. The two questions to be addressed by our experiments were 1) whether RKIP is also depleted in HEK293 cells by ethanol and 2) if so whether it is driven by the ubiquitin-proteosome pathway. We transiently transfected both plasmids with Flag-tagged RKIP gene and HA-tagged Ubiquitin gene into HEK293 cells to maximize the visibility of RKIP ubiquitination. Flag and HA tags were used to ensure the specificity of immunodetection. 50μM proteosome inhibitor MG-132 was pretreated before harvesting cells to stop protein degradation and accumulate the ubiquitinated proteins. The RKIP-Flag and HA-Ubiquitin transfected cells were treated with either 100 mM ethanol for 10 minutes or 10ng/ml TNF-α for 15 minutes to induce IκBα degradation as a positive control.

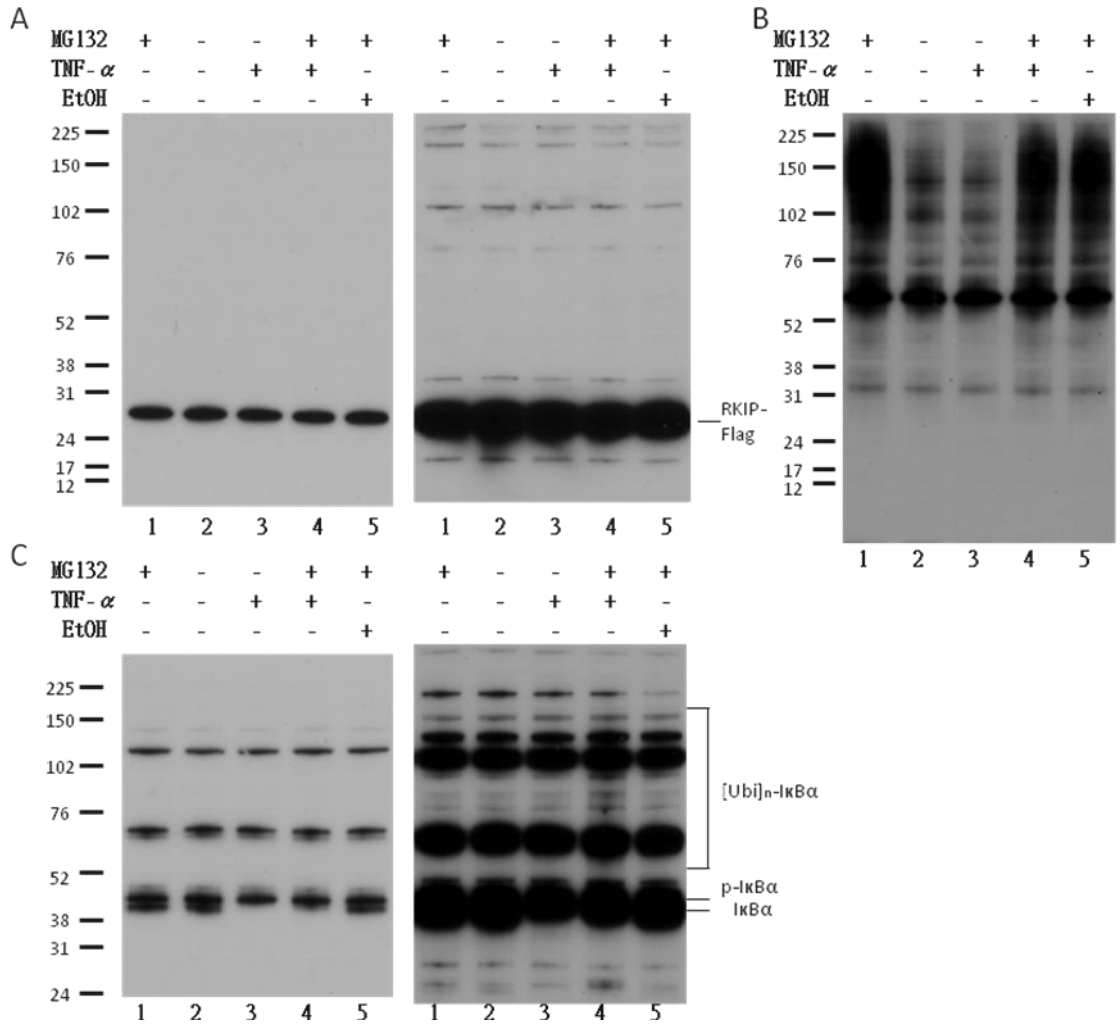

Figure 2. No RKIP ubiquitination or degradation was detectable in total cell lysates of transfected HEK293 cells by western blotting. The RKIP-Flag and HA-Ubiquitin transfected HEK293 cells were pretreated with 50μM MG-132 (lane 1, 2, 5) or DMSO (lane 3, 4) for 1 hour before incubation of cells with 10 ng/ml TNF-α (lane 3, 4) for 15 min, 100 mM ethanol for 10 min (lane 5) and vehicle DMEM media (lane 1, 2). The total cell lysates were fractionated by SDS-PAGE and analyzed by western blotting. (A) Cell lysates were blotted with mouse anti-Flag M2 antibody to locate the RKIP-Flag protein. Left panel is short exposure for 5 sec and right panel is long exposure for 5 min. (B) The same cell lysates as (A) were blotted with rabbit anti-HA probe (Y-11) antibody to visualize all ubiquitinated proteins and (C) with rabbit anti-IκBα (C-21) to detect TNF-α-induced IκBα degradation events as positive control.

Because it is possible that the proportion of ubiquitinated RKIP is too low to be detected by western blotting, I also performed co-immunoprecipitation experiments to amplify the proportion of ubiquitinated RKIP (as shown in Fig.3). The results of

co-immunoprecipitation were complicated because the specificity of antibodies was problematic and the conditions of lysis buffer needed to be further optimized. Non-specific bands in Figure 3 suggest that unknown non-specific proteins in cell lysates and IgG antibodies for immunoprecipitation were not successfully removed from samples. However, regardless of the complexity or our result, one consistency was that the smear bands in the ethanol-treated group never significantly outstripped those in the non-treated control groups (lane 8 and lane 4, Fig. 3, n>3), suggesting that 10 minute treatment of 100 mM ethanol did not have significant effect on accumulation of ubiquitinated RKIP in HEK293 cells. The results of western blotting and co-immunoprecipitation does not support the hypothesis that ethanol induces RKIP depletion driven by the ubiquitin-proteosome pathway in transfected human HEK293 cells. RKIP is a wide-expressed protein and the ubiquitin-proteosome pathway is a universal mechanism. We cannot rule out the possibility that ethanol did not induce the same effect on HEK293 cells as in AR42J cells either by specific pathways or by nonspecific physical-chemical mechanisms on cell membrane.

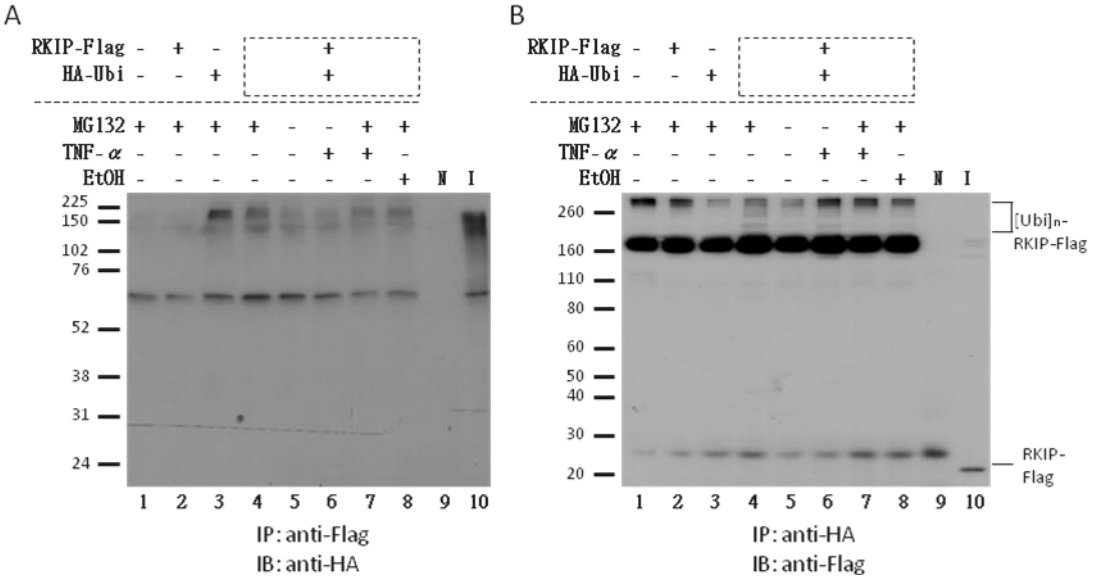

Figure 3. Ethanol does not enhance the accumulation of ubiquitinated RKIP. Co-immunoprecipitation experiments were performed on the same whole cell extracts of HEK293 cells in Figure 2, together with the technical control groups for immunoprecipitation. Cells of lane 1, 2, 3 were transfected with control plasmids pcDNA3.1(+) only, RKIP-Flag and control plasmids, HA-Ubi and control plasmids, respectively. Lane 4~lane 8 were exactly the same cell lysates from lane 1~5, Fig.2, respectively. Lane 9 (N) was the negative control, which was performed through the immunoprecipitation but contained only lysis buffer without cell lysate, and lane 10 (I) was the cell lysates of lane 4 before immunoprecipitation. 50μM MG-132 was used for detection of accumulation of ubiquitinated RKIP. (A) Cell lysates were immunoprecipitated by mouse anti-Flag M2 and immunoblotted by rabbit anti-HA probe. The band around 60kDa was nonspecific band, which also appeared in Fig 2B. (B) Cell lysates were immunoprecipitated by rabbit anti-HA probe and immunoblotted by mouse anti-Flag M2.

3.1.2 RKIP was a relatively stable protein with long half-life compared to IκBα

During the above experiments, I also endeavored to identify the half-life of RKIP in rat AR42J cells. The ubiquitin-proteosome pathway is an efficient mechanism that degrades protein very quickly. Notable examples include p53, IκBα and Snail, which all have short half-lives limited to minutes (Van Antwerp and Verma, 1996; Zhou *et al.*, 2004; Reich, *et al.*, 2008). RKIP is readily phosphorylated and if it was degraded by the ubiquitin-proteosome

pathway, its half-life should also be short and regulated by similar mechanisms. Therefore, we hypothesized that RKIP has a short half-life in AR42J cells.

The cycloheximide blocking method was used to determine the half-life of RKIP in AR42J cells. AR42J cells were grown onto the 6-well plate and treated with 100 μM cycloheximide (CHX) or water vehicle at time zero, and were lysed and harvested at the indicated times. The cell lysates were then fractionated by SDS-PAGE and analyzed by immunoblotting with anti-RKIP and anti-IκBα as a control (Fig. 4A). IκBα degraded quickly in the CHX-treated group while RKIP degraded much slower than IκBα. Figure 4B show the results of densitometric analysis of the bands' intensity normalized to the peak condition at the 1_{st} hour time point. The data were not well fitted by a first order process (i.e., exponential decay), but it was still clear that the half-life of IκBα in our AR42J model system was around 4 hours compared with the half-life of RKIP in AR42J was around 24 hours. The half-life of RKIP in another comparable experiment with different time points was even longer (36~48 hours, Fig. 5B) indicating that RKIP is relatively more stable than IκBα.

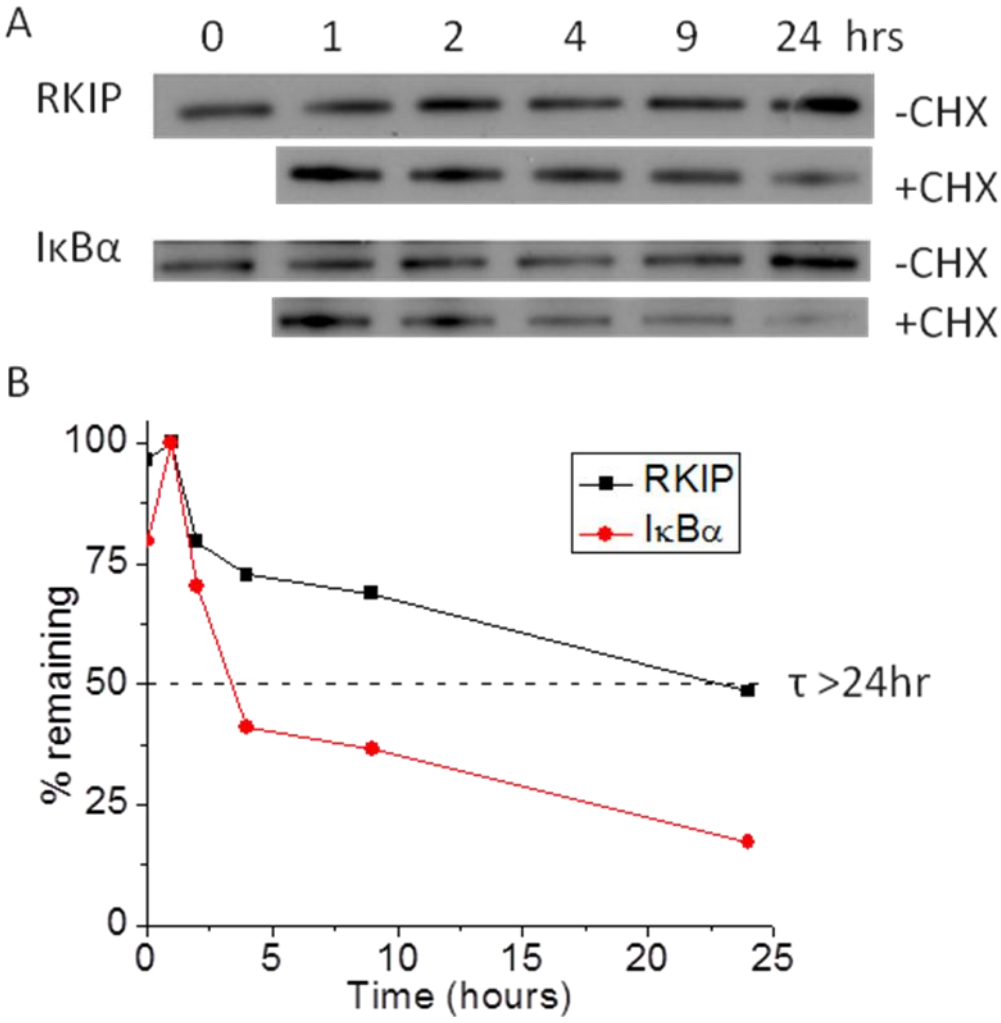

Figure 4. RKIP has a longer half-life than IκBα. (A) AR42J cells were treated with 100μM cycloheximide (CHX) or water vehicle for 0, 1, 2, 4, 9, 24 hours respectively and the cell lysates were resolved by western blotting with anti-RKIP and anti-IκBα antibodies. (B) The time course of remaining RKIP (black) and IκBα (red) which were normalized by the peak values of the 1_{st} hour time point. τ: half-life.

3.1.3 100 mM ethanol treatment did not decrease the half-life of RKIP significantly

We next tested the hypothesis that the ethanol-induced RKIP depletion reduces the half-life of RKIP. The rationale here is that the overall pool of RKIP should be decreased quicker if the ethanol-induced RKIP depletion contributes to the overall RKIP degradation.

To determine whether the ethanol-induced RKIP depletion affects the half-life of RKIP, we performed similar cycloheximide-blocking experiments like Fig. 4. AR42J cells were treated with 100 mM ethanol and/or 100μM cycloheximide for the indicated periods and the time-course of RKIP decay was measured (Fig. 5). The effect of cycloheximide was significant in both decreasing the total protein yield (red and purple line, Fig. 5B) as well as the residual RKIP (red and purple line, Fig. 5C). However, up to 48 hours treatment of 100 mM ethanol neither reduced the RKIP intensity (blue line) nor shifted the decay curve leftward (purple line). These results indicate that 100 mM ethanol treatment does not actually deplete the RKIP pool in a long- term manner in AR42J cells. There are slight decreases of RKIP amounts in the 3_{rd} and 9_{th} hour time points that were probably the result of short-term ethanol-induced depletion, which the recovery of the RKIP pool was blocked by cycloheximide. However, the changes were small and inconsistent. We cannot rule out the possibility that ethanol transiently decreases RKIP but the RKIP pool is capable of rapid recovery. Further studies will be required to test this possibility. Nevertheless, we can conclude that 100 mM ethanol treatment is insufficient to decrease the cytosolic RKIP pool permanently in AR42J cells. The result suggests that the ethanol-induced RKIP depletion may not be the mechanism that contributes to the steady RKIP deficiency in metastatic cancerous cells.

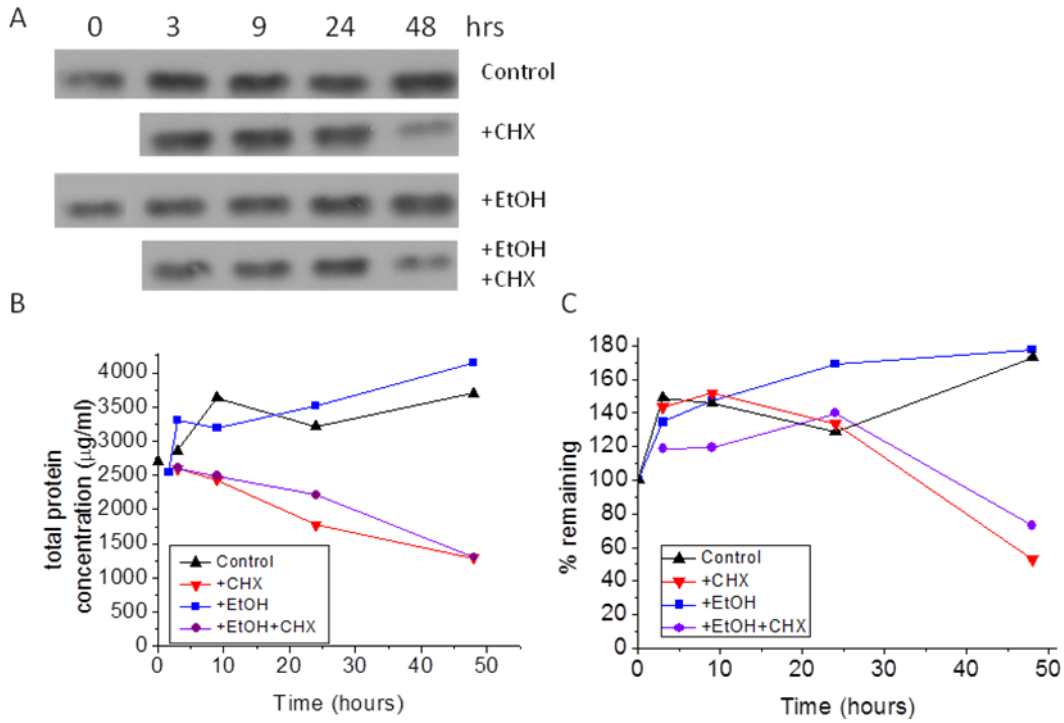

Figure 5. 100 mM ethanol treatment does not reduce the half-life of RKIP. (A) AR42J cells were treated with 100 mM ethanol and/or 100μM cycloheximide (CHX) for indicated 0, 3, 9, 24, 48 hours and the cell lysates were resolved by western blot with anti-RKIP. (B) The time course of remaining RKIP of control (black), CHX only (red), ethanol only (blue), and both (purple) which were normalized by the intensity of the 0 time point.

Taking together, the results of my studies indicate that RKIP degradation is not enhanced by ethanol treatment in either HEK293 cells or AR42J cells, and RKIP is relatively stable. In addition, the recent finding of our lab that RKIP is regulated by translocation rather than degradation by 10 min, 100 mM ethanol treatment (data not shown), seems to invalidate the claim that RKIP regulation is via a degradational mechanism in response to ethanol treatment.

3.2 Investigations of Transcriptional Regulation of RKIP

The best-known transcriptional regulation of RKIP is the repression by the Snail

transcription factor (Beach *et al.*, 2008). NF-κB and Yin Yang 1 (YY1) can regulate RKIP through modulating Snail (Bonavida & Baritaki, 2011). However, there are still many potential binding sites for transcription factors, such as AP-1, SP-1, YY-1, or others, locating within the RKIP promoter region (Odabaei *et al.*, 2004). The rationale is that expression level of RKIP affects the cell fate so that the RKIP expression should be tightly regulated. Therefore, our aim was to identify a novel transcriptional modulator of RKIP.

3.2.1 The transcriptional activities of RKIP promoter reporters in pancreatic cancerous cell lines.

In order to investigate the regulation of RKIP promoter, we subcloned three 5'-UTR fragments of RKIP into *firefly* pGL3-basic luciferase reporter plasmids (Fig. 6A, see Okita *et al.*, 2009). The first question we addressed was whether the transcription activity patterns of RKIP promoter reporters were correlated to the RKIP expression level in different pancreatic cell lines. The RKIP promoter reporter plasmids along with *renilla* pGL4-SV40 reporter plasmid were co-transfected into HEK293 cells and several pancreatic cell lines at the ratio of 100:1 for luciferase assay (Fig. 6B). Transfection efficiency was low in AR42J and BxPC-3 cells, which made the result of BxPC-3 unreliable. Unlike Okita's 2009 results, the shortest fragment (-97/+64) of RKIP promoter had contributed to most of the activities in these cell lines. In HEK293, AR42J, PANC-1, and AsPC-1, the repression pattern was shown in the group of longest fragment (-1651/+64), suggesting negative regulation, whereas the

repression did not occur in MIA PaCa-2 cells. Our preliminary data indicates that RKIP is expressed high in both AR42J and AsPC-1 but the transcription activity patterns are different. Snail expression level is lower in PANC-1 and AsPC-1. Our results on AR42J and AsPC-1 suggest that the transcription activity pattern is not necessarily correlated with the RKIP expression level. Snail, the transcription suppressor of RKIP, migrates into doublet as unphosphorylated and phosphorylated forms by western blotting (Yook *et al.*, 2005). Our preliminary data also indicates that the proportion of the phosphorylation band in the AR42J and PANC-1 cells are lower than the other three cell lines (data not shown). Interestingly, the suppress patterns of RKIP promoter reporters of AR42J and PANC-1 cells shares more significant inhibition on reporters pGL3-(-596/+64) and pGL3-(-1651/+64). It requires further investigation whether the lower phosphorylation ratio of Snail in these two cell lines indicates less Snail degradation and longer-lived Snail activity on inhibiting RKIP promoter. One thing that should be noted is that the ratios of *firefly/renilla* luciferase activities between cell lines are not comparable because the two reporters were regulated differently in different cellular environments, even though they were transfected at constant amounts and ratios. For example, MIA PaCa-2 exhibited much higher *renilla* luciferase activity than the other cell lines.

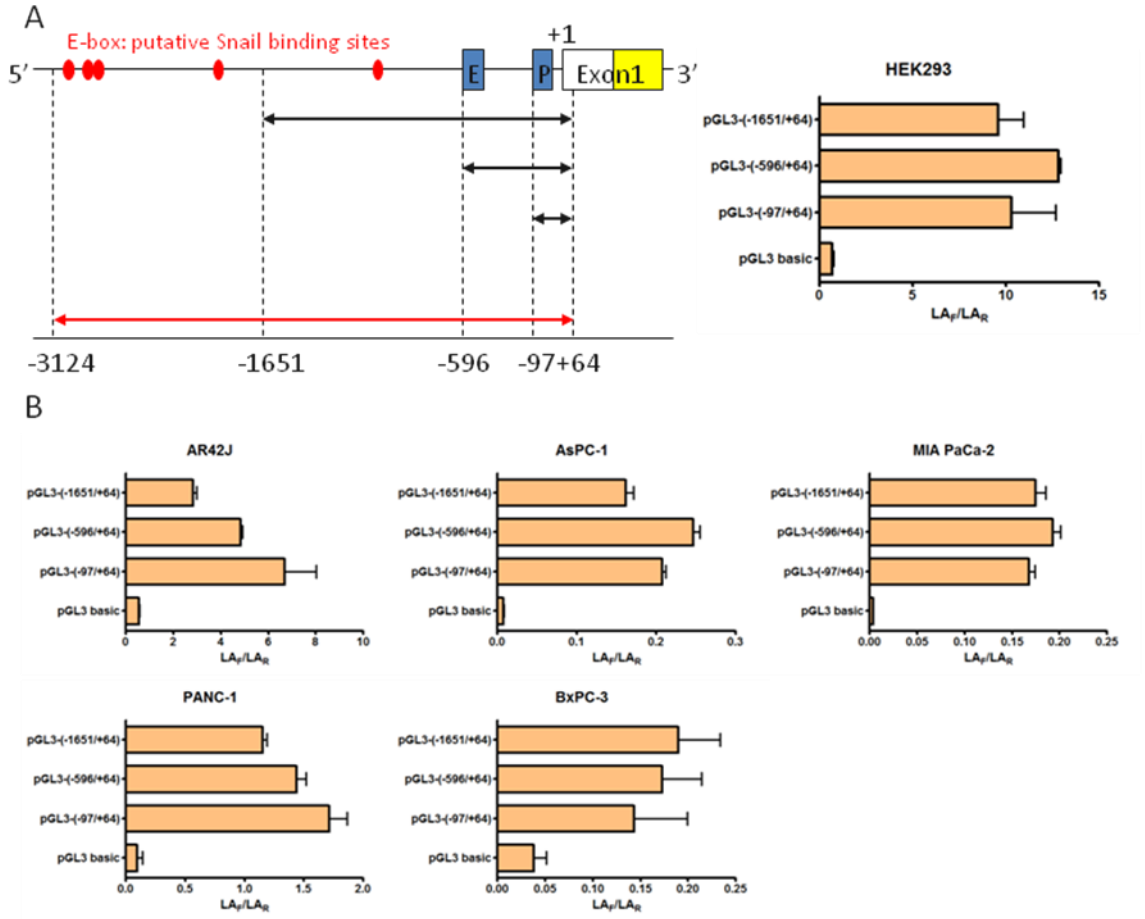

Figure 6. The transcriptional activities of RKIP promoter reporter plasmids in pancreatic cell lines. (A) The pGL3-(-97/+64) plasmid contains inserted fragment with the putative promoter reported in Okita 2009; the pGL3-(-596/+64) plasmid contains an additional putative enhancer; and the pGL3-(-1651/+64) plasmid contains additional one of E-boxes (CANNTG, potential Snail-binding consensus site consensus). The red line indicates the fragment we are still cloning which contains most of E-boxes. The yellow square indicates the RKIP coding region. P: promoter, E: Enhancer, +1 is the beginning nucleotide of human RKIP mRNA (NCBI Reference Sequence: NM_002567.2). (Right panel) The ratio of *firefly*/*renilla* luciferase activities of different lengths of reporters expressed in the HEK293 cells. (B) The ratio of luciferase activities of RKIP promoter reporter plasmids in pancreatic cell lines. The diagrams are arranged in the order of RKIP expression level from high to low: AR42J > AsPC-1 > MIA PaCa-2 > PANC-1 > BxPC-3.

3.2.2 Snail-1 did not significantly repress the activities of our RKIP promoter reporters in HEK293 cells by luciferase assay

I next tested the role of Snail inhibition using the RKIP promoter reporters and the

Snail-expressing plasmids. Our working hypothesis was that the presence of E-box enhances the Snail repression on RKIP expression. According to Beach *et al.* 2008, the reporter RKIP0.5 containing the most proximal E-box site (no specified location) was repressed by forced expression of Snail in the MCF7 cells. However, we did not observe any E-Box consensus site within our (-596/+64) fragment. Instead, our most proximal E-box is located at (-1080/-1075) within pGL3-(-1651/+64) reporter (Fig.6A). We co-transfected RKIP and *renilla* reporters, as well as plasmids containing wild-type Snail-1, long half-life Snail-1 6SA or Slug (Snail-2) genes respectively into HEK293 cells. The expression of transfected Snail was confirmed by western blots and the results are shown in Figure 7. The Snail expression was originally low which could be proven in Slug transfected group (lane 3, 6 in Fig. 7A). Although we did not have the control plasmid CMV-Tag2B as a negative control, we were able to compare the activity pattern of wild-type Snail-1 group, Snail-1 6SA group with parental HEK293 cells in Figure 6A. The Snail repression on pGL3-(-1651/+64) reporter was not significantly enhanced after Snail transfection after normalization of the value of pGL3-(-1651/+64) to the value of pGL3-(-596/+64) ($P>0.05$). Therefore, our results indicate that Snail-1 does not significantly repress the activities of our RKIP promoter reporters in HEK293 cells. However, the difference of luciferase expression pattern between Snail and Slug does indicate the existence of differential regulation.

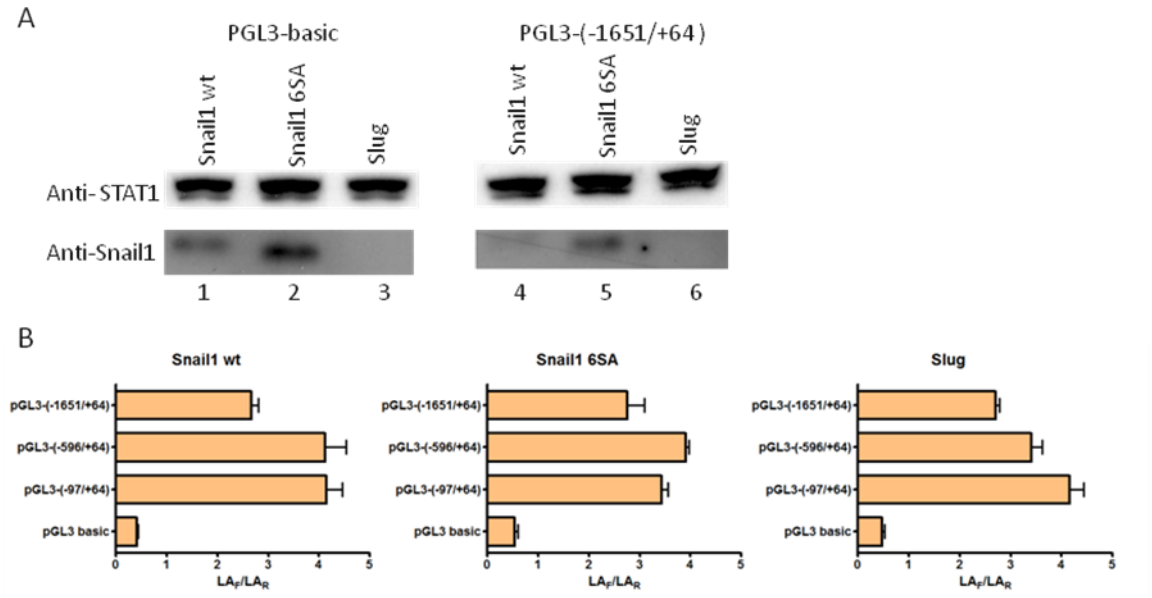

Figure 7. Snail-1 does not significantly repress the activities of our RKIP promoter reporters in HEK293 cells by Luciferase Assay. (A) HEK 293 Cells were co-transfected with pGL3 RKIP promoter reporters, *renilla* pGL4-SV40 reporter, and either wild-type Snail1, Snail 6SA or Slug (Snail 2) in 100:1:100 ratios. The expression level of Snail-1 in transfected HEK293 cells were verified by western blot with anti-SNA1 (Snail-1) antibody. The blot by anti-STAT1 was used as an internal control. (B) The ratio of luciferase activities of RKIP promoter reporter plasmids in transfected HEK293 cells.

3.2.3 BIO compound, a GSK-3 inhibitor, inhibited the activities of RKIP promoter reporters by luciferase assay

Snail is a substrate of GSK-3β phosphorylation and suppression of GSK-3β stabilizes the Snail in the cytosol (Zhou *et al.*, 2004). Therefore, we hypothesized that inhibition of GSK-3β will enhance the repression of RKIP promoter by stabilizing Snail. To test this hypothesis, I treated HEK293 cells with BIO compound, a GSK-3 inhibitor, or DMSO vehicle for 8 hours two days after RKIP reporter plasmid transfection. However, we found no significant difference between the BIO-treated group and the control group. Nevertheless, in

a parallel experiment with MIA PaCa-2 cells under the same treatment conditions, the difference of the activities of pGL3-(-1651/+64) and pGL3-(-97/+64) between the BIO-treated group and the control group were significant (Fig. 8). The repression of the activity of pGL3-(-1651/+64) by BIO compound indicates an enhanced Snail repression because pGL3-(-1651/+64) contains one E-box (putative Snail binding site). However, the results of repression of the activity of pGL3-(-97/+64) by inhibition of GSK-3 and the difference in repression between HEK293 cells and MIA PaCa-2 cells indicate the possibility that RKIP promoter is regulated by another unknown regulatory mechanism not involving Snail repression. Further investigation will be required to address the question whether this regulation is mediated by GSK-3 regulated Wnt or Hedgehog pathways (Zhou and Hung, 2005).

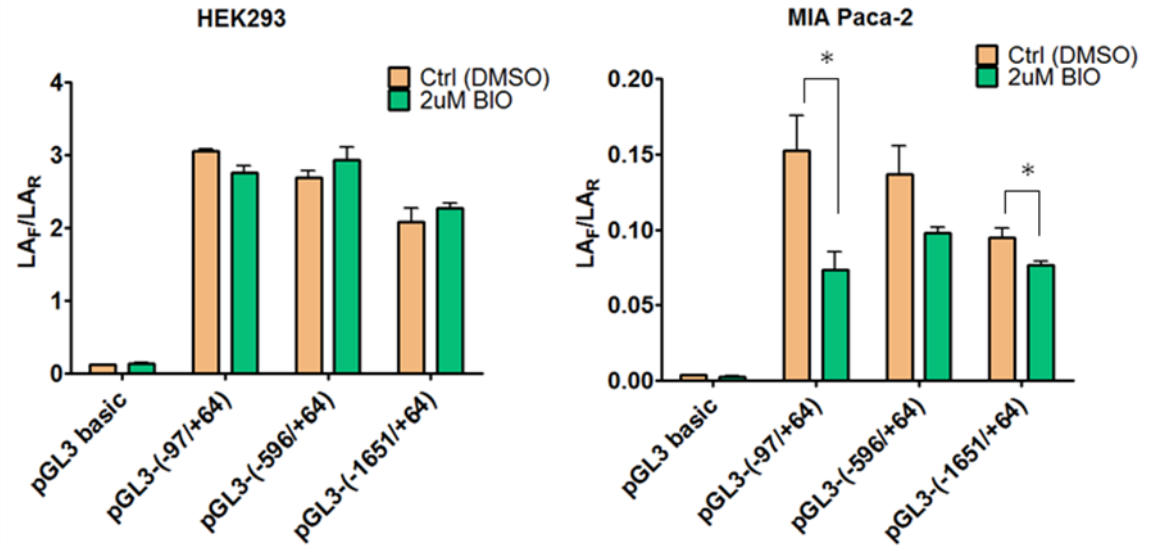

Figure 8. BIO compound inhibited the activities of RKIP promoter reporters by luciferase assay in MIA PaCa-2 cells. BIO compound (green) or DMSO vehicle (skin color) were treated for 8 hours at one day after RKIP promoter reporter transfection. * $p < 0.05$.

IV. Conclusions and Future Direction

The results of my degradation experiments and the recent finding of RKIP translocation in our lab indicate that RKIP is a relatively stable protein that is not regulated by quick ubiquitin-proteosome-mediated degradation in either HEK293 cells or AR42J cells within short time. However, our recent findings also show that RKIP is phosphorylated in less than 10 min and destroyed 30~60 min after ethanol treatment. I have not ruled out the possibility that RKIP is degraded and recovered with fast kinetics. Further time course studies of ubiquitination and cycloheximide blocking experiments within two hours are required to determine whether phospho-RKIP is degraded by ubiquitination or by another mechanism, and recovered by s quick synthesis. Moreover, to address the question of whether long-term exposure of ethanol induces long-term RKIP degradation, there remains the possibility that the ethanol added to the cell culture at the beginning of experiments might have evaporated over the longer incubation period so that its effect to induce RKIP degradation was lost. Therefore, further cycloheximide blocking or pulse-chase experiments with repetitive ethanol treatments will be required to determine if long-term exposure of ethanol still does not have effects on RKIP degradation.

The results of my RKIP promoter reporter assays indicate that some unknown regulatory mechanism(s) other than Snail repression is operating on the RKIP transcription. The question whether the stronger Snail suppression on RKIP promoter reporters in AR42J

and PANC-1 cells is the result from less phosphorylation of Snail remains interesting. In order to address this question, further luciferase reporter assays with manipulation of Snail phosphorylation level in AR42J and PANC-1 cells will be required. Furthermore, the finding that inhibition of GSK-3 suppresses the RKIP promoter is novel, although the specificity of the GSK-3 inhibition by BIO compound still needs to be verified either by other GSK-3 inhibitors, GSK-3 siRNA, or by confirmation of Snail phosphorylation. My result of pGL3-(-97/+64) repression in MIA PaCa-2 cells also indicates cell-specific, Snail-independent regulatory mechanism(s) are involved. Investigations on what specific pathway(s) are involved specifically in MIA PaCa-2 cells versus HEK293 cells, and whether malfunction in this pathway(s) contributes to the long-term repression of RKIP in cancerous tissues could fill an important gap in our knowledge regarding RKIP regulation. Furthermore, on the key role of RKIP in determining the cell fate, and how RKIP dysregulation contributes to the GSK-3-dependent neuronal cell development, and body pattern formation, as well as GSK-3-relevant diseases such as Parkinson disease and Alzheimer disease also warrants further investigation.

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VITA

Shih-Hao Yeh was born in 1977 in Chia-Yi City, Taiwan (R.O.C.) to Hsiang-Liang Yeh and Huang-Yi-Huang. After graduating from the Chia-Yi Senior High School, Shih-Hao earned his Bachelor of Science degree of Life Science in 2000 and Master of Science of Life Science in 2002 from the National Tsing Hua University. During 2003 to 2007, he was recruited in the Institute of Biomedical Science, Academia Sinica for military service of science and technology. At the summer of 2007, he was enrolled into the Graduate School of Biomedical Science at University of Texas Medical Branch. After 4-year research works in Dr. Mark Hellmich's lab, he is graduating at the end of 2011 with a Master of Science degree in the Human Pathophysiology and Translational Medicine Program.

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Chang HK, Yeh SH, Shieh RC. (2007) Charges in the cytoplasmic pore control intrinsic inward rectification and single-channel properties in Kir1.1 and Kir2.1 channels. *J Membr Biol.* **215**(2-3):181-193.

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Permanent address: No. 39, Ln. 375, Fang-An Rd., East Dist.,

Chiayi City, 60068 Taiwan (R.O.C.)

This dissertation was typed by Shih-Hao Yeh.