CHAPTER II

PART I: PRKAR1A IS OVEREXPRESSED AND REPRESENTS A THERAPEUTIC TARGET IN HUMAN CHOLANGIOCARCINOMA

2.1 Introduction

Protein kinase A (PKA) is a serine/threonine kinase which is activated by the second messenger, cAMP. Phosphorylation mediated by the cAMP/PKA signaling pathway can be triggered by various ligands in cells and is involved in controlling a variety of cellular processes, such as cell proliferation, differentiation, and apoptosis (Cho-Chung *et al.*, 1995; Francis and Corbin, 1999). PKA (Figure 2.1) is a heterodimer composed of two types of subunits including:

1) Catalytic (C) subunit: This subunit contains the substrate binding site, a domain that binds ATP (the source of phosphate) and a domain that binds the regulatory subunit.

2) Regulatory (R) subunit: This subunit contains an amino terminal dimerization

domain, an auto-inhibitory domain that is a region responsible for interaction with catalytic subunit to serves as a substrate or pseudosubstrate for the catalytic subunit and two tandem cAMP binding domains at the carboxy terminus in which region the amino acids sequences are highly conserved. Regulatory subunits may also have biologic activity distinct from their role in modulating catalytic subunit activity.

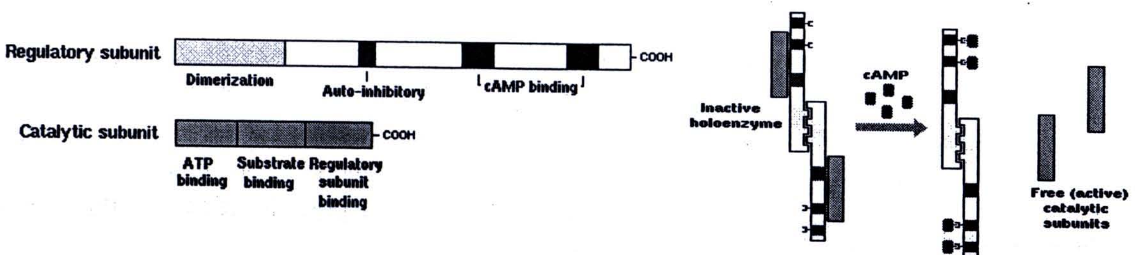

Figure 2.1 Structure of regulatory and catalytic subunits of protein kinase A (Adapted from Scott, 1991).

There are two types of PKA, they are PKAI and PKAII, which share a common C subunit but contain different R subunits, RI and RII, respectively (Beebe SJ and Corbin, 1986). Later four R subunit isoforms (RIα/PRKAR1A, RIβ/PRKAR1B, RIIα/PRKAR2A and RIIβ/PRKAR2B) and three isoforms of the C subunit, (Cα, Cβ and Cγ) have been identified (Taylor *et al.*, 1990). The self-regulation of the PKA system has been described. Role of R subunit in maintaining the C subunit under cAMP control was confirmed by overexpression of Cα or Cβ in cell culture resulted in significant compensate by an increase in RIα/PRKAR1A protein whereas no compensation by RIIα/PRKAR2A was observed, suggesting that this phenomena was specific to RI subunit (Uhler and McKnight, 1987). Moreover, overexpression of RIα/PRKAR1A in prostate carcinoma cells model resulted in an increase of Cα protein level (Neary *et al.*, 2004). The possible mechanism of the compensation was protein stabilization resulting from holoenzyme formation (Amieux and McKnight, 2002). RIα/PRKAR1A protein had longer half-life when incorporated into holoenzyme compared with its stability as free subunit. Uncomplexed RI subunit was rapidly degraded in the absence of C subunit which necessary for formation of stable holoenzyme (Uhler and McKnight, 1987).

PKAI and PKAII also consist in their distinct subcellular locations. The different isotypes have different distributions within cells and among tissues. PKAI localizes in cytoplasmic whereas PKAII tends to associate with nuclei, nucleoli, Golgi and the microtubule-organizing center (Rohlff *et al.*, 1993). In addition, the four types of regulatory subunits have different expression patterns in mammals. PRKAR1A has a nearly ubiquitous distribution whereas PRKAR2B is expressed primarily in brain, testis, B and T lymphocytes (Clegg *et al.*, 1988; Scott, 1991). Similarly, PRKAR2A has a wide expression, whereas PRKAR2B is expressed in brain, adipose, and some endocrine tissues (Skalhegg and Tasken, 2000). The difference in distinct subcellular locations of PKAI and PKAII is mainly due to A-kinase anchoring proteins (AKAPs) that enable to target them close to specific sites. Compartmentalization of PKA holoenzymes is an important aspect of PKA response specificity. The holoenzymes can be anchored to specific compartments via interaction of their R subunits with specific AKAPs through it, cAMP messaging is targeted to specific subcellular location such as plasma membrane, nucleus, Golgi

apparatus, endoplasmic reticulum, and other organelles (Faux and Scott, 1996; Scott, 1991).

In the absence of its activating ligand cAMP, PKA exists as an inactive holoenzyme of two R and two C subunits. Following an increase in intracellular cAMP, two cAMP molecules bind to each R subunit resulting in the dissociation of the holoenzyme and the release of two free active C subunits. Active C subunits phosphorylate target substrate proteins on serine and threonine residues, creating a cellular response to the extracellular stimulus (Figure 2.2). At present, the cAMP/PKA signaling pathway is known to be activated by a number of different receptors that upon binding of their respective ligands, transducer their signals over the cell membrane by coupling to G-proteins. These G-proteins interact with adenylyl cyclase on the inner membrane surface either to activate or to inhibit the production of cAMP. Receptors that activate cAMP/PKA signaling pathway regulate many cellular processes including metabolism (Krebs, 1972), gene regulation (Roesler *et al.*, 1988), cell growth (Dumont *et al.*, 1989), cell differentiation (Liu, 1982; Schwartz and Rubin, 1983) as well as ion channel conductivity (Li *et al.*, 1993).

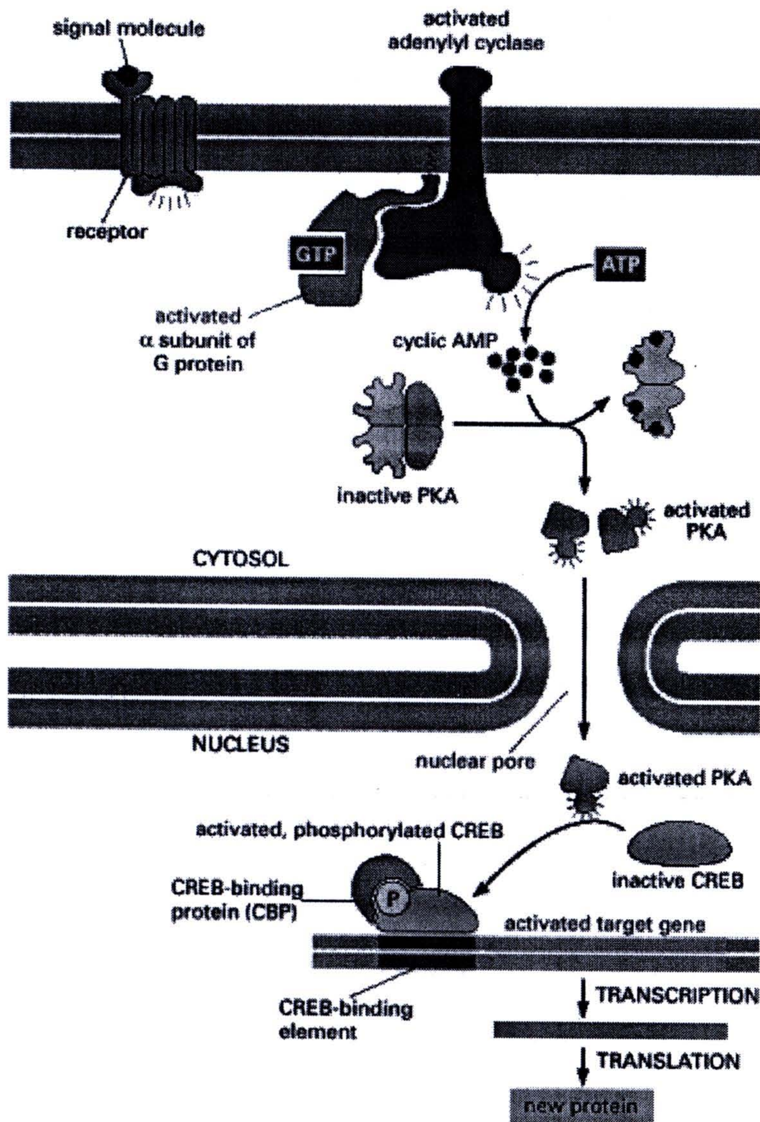

Figure 2.2 A cAMP/PKA signaling pathway. PKA consists of two catalytic subunits and two regulatory subunits. cAMP binds to two sites on each regulatory subunit. Binding of four cAMP molecules causes the release of free and active catalytic subunits, which may phosphorylate serine and threonine residues on target proteins. In this figure, the active subunit phosphorylates a CREB protein, resulting in transcription. (Alberts *et al.*, 2002).

Interestingly, differential expression of PKAI and PKAII has been correlated with cell differentiation and neoplastic transformation. PRKAR2B/PKAII is preferentially expressed in normal nonproliferating tissues and in growth-arrested cells, whereas PRKAR1A/PKAI is overexpressed in growth stimulated cells (Cho-Chung *et al.*, 1995; Tortora and Ciardiello, 2002). The PRKAR1A/PKAI is transiently overexpressed in normal cells following exposure to specific mitogenic stimuli. Moreover, they are constitutively overexpressed in human cancer cell lines, primary tumors, and in association with poor prognosis in patients, and upon transformation *via* chemical and viral carcinogens and growth factors or oncogenes (Bradbury *et al.*, 1994; Cho-Chung, 1990; Cho-Chung *et al.*, 1995; Miller *et al.*, 1993; Tortora and Ciardiello, 2002). Overexpression of PRKAR1A/PKAI has been described in ovarian (McDaid *et al.*, 1999), lung (Young *et al.*, 1995), colon (Bold *et al.*, 1994) and melanoma (Mantovani *et al.*, 2008). In contrast, constitutive overexpression of PRKAR2B/PKAII, following infection with a recombinant retrovirus containing the human *PRKAR2B* gene, induced growth inhibition of human cancer cells and reverted the transformed phenotype of *ras*- transformed mouse fibroblasts (Tortora *et al.*, 1994). These evidences indicate that PKAI and PKAII have different functions in the control of cell growth and differentiation.

Dramatic changes in the proportion of the two PKA regulatory subunits, PRKAR1A/PKAI and PRKAR2B/PKAII, occur during ontogenic development, differentiation processes and neoplastic transformation, indicating distinct roles for these isoenzymes not only in growth control, but also depending on the cell system (Cho-Chung, 1990; Cho-Chung *et al.*, 1995). In particular, several studies support the view that PRKAR1A/PKAI is related to cell proliferation whereas PRKAR2B/PKAII is involved in tissue differentiation (Cho-Chung *et al.*, 1995; Tortora and Cho-Chung, 1990). There are many studies reported about PRKAR1A/PKAI and cancer. Increased expression of PRKAR1A/PKAI has been shown to be associated with both chemical and viral carcinogenesis and oncogene-induced cell transformation. The initiation stage of the dimethylbenz (a) anthracene–induced mammary carcinogenesis in rats (Cho-Chung *et al.*, 1983), and the incidence of gastric adenocarcinoma in rats by N-methyl-N'-nitrosoguanidine and the trophic action of gastrin on gastric carcinoma production (Yasui and Tahara, 1985) which were correlated with an increase in

PRKAR1A and PKAI activity. Ledinko et al. (Ledinko and Chan, 1984) also observed a 3- to 6-fold increase in PKAI activity in rat 3Y1 cells transformed by human adenovirus type 12 compared to untransformed 3Y1 cells. Little or no change occurred in the PKAII following transformation. These reports suggested that PRKAR1A/PKAI may act as a mediator of various mitogenic stimuli and thus represents a potential target for the pharmacological control of cell proliferation.

According to the different roles of PKA isozymes in the initiation and progression of many cancers, several lines of attempts were made to suppress transformed phenotype of cancer cells by inhibiting PRKAR1A/PKAI. There are several approaches for inhibition of PRKAR1A/PKAI expression and function as described below.

1. A group of site-selective cAMP analogues (8-Cl-cAMP, 8-Br-cAMP). The evidence have been demonstrated their ability to inhibit cell growth and to induce apoptosis in several cancer cells such as colorectal cancer, neuroblastoma, breast cancer, thyroid cancer and esophageal cancer has been reported (Carlson *et al.*, 2000; Kim *et al.*, 2000; Ramage *et al.*, 1995; Robinson-White *et al.*, 2008; Wang *et al.*, 2005).

2. PKA inhibitor, H89 that could bind to and inhibit catalytic subunits, also induces apoptosis and inhibits growth in pancreatic cancer and human colon cancer cell lines, which is most consistent with inhibition of PKAI activity (Bockmann and Nebe, 2003; Farrow *et al.*, 2003).

3. Direct approach to inhibit the synthesis and function of PKAI has been developed By the use of antisense oligonucleotide (Nesterova and Cho-Chung, 1995; Tortora and Ciardiello, 2000), and small interfering RNA (siRNA) (Farrow *et al.*, 2003) which revealed that targeted against PRKAR1A could successfully induce growth inhibition in a variety of cancer cell lines both *in vitro* and *in vivo*. Administration of antisense PRKAR1A oligodeoxynucleotide (ODN) targeted against PRKAR1A possessed the anticarcinogenic effect on 7,12-dimethylbenz(α) anthracene (DMBA)-induced mammary carcinogenesis in rats (Nesterova and Cho-Chung, 2004). Furthermore, genes that defined the proliferation-transformation signature were down-regulated, whereas those that defined the differentiation-reverse

transformation signature were up-regulated in antisense treated prostate cancer cells, but not in untreated control cells demonstrated by DNA microarray (Cho *et al.*, 2001).

Importantly, both antisense PRKAR1A (GEM 231^{TM}) and 8-Cl-cAMP have been evaluated in clinical trial. Antisense PRKAR1A (GEM 231^{TM}), given by intravenous administration has successfully completed a phase I study in cancer patients with minor toxicity (Chen *et al.*, 2000). 8-Cl-cAMP was performed a clinical trial in patients with recurrent or refractory multiple myeloma phase II study and colorectal cancer phase I study (http://clinicaltrials.gov/). These evidences supported that PRKAR1A is a potential target for cancer therapy.

Our previous work by Loilome and coworkers (Loilome *et al.*, 2006) found overexpression of PRKAR1A in the liver fluke (*Opisthorchis viverrini*, Ov) and *N*-nitrosodimethylamine (NDMA) induced hamster CCA. Our recent study also demonstrated that PKA isozyme switching from PRKAR2B/PKAII to be PRKAR1A/PKAI during Ov and NDMA induced hamster CCA development in correlation with transformation and proliferation of bile duct epithelia from normal through preneoplastic to malignant stages (Loilome *et al.*, submitted to J Biomed Science, under revision), suggesting that this particular protein might play role(s) in Ov-associated CCA and may serve as a potential drug target for CCA treatment. However, the molecular mechanism by which PRKAR1A/PKAI contributes in CCA development is still unclear. Therefore, this study aimed to investigate the molecular mechanism by which PRKAR1A/PKAI associated with human CCA development as well as to elucidate whether PRKAR1A/PKAI is suitable to be the potential target for inhibiting CCA cell growth.

2.2 Materials and methods

2.2.1 Cell Culture

Four respective CCA cell lines, KKU-100, KKU-M214, KKU-OCA17, and KKU-M156 were developed from primary tumor of patients, who were admitted to Srinagarind Hospital with the primary diagnostic of CCA. CCA cell lines were established by Associate Professor Dr. Banchob Sripa; Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University.

All cell lines were cultured in HAM-F12 (Gibco/BRL, Grand Island, NY) supplemented with 10% inactivated fetal bovine serum, 2 mg/ml sodium bicarbonate and 1% antibiotic-antimycotic solution (Life Technologies, Inc., Gaithersburg, MD). All cultured cell lines were incubated at 37°C in a humidified incubator maintained with an atmosphere of 5% CO_2. Subculture was done when the cell reached the confluent stage and the media were changed once every two to three days.

2.2.2 Sequence-verified shRNA lentiviral plasmid vectors against PRKAR1A

A Mission™ TRC-Hs (Human) clone set of sequence-verified shRNA lentiviral plasmid vectors against PRKAR1A were obtained from the Johns Hopkins University (JHU) High Throughput Biology Center as listed in Table 2.1.

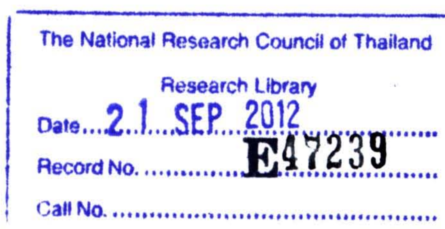

Table 2.1 Mission™ TRC-Hs (Human) clone sets of sequence-verified shRNA lentiviral plasmid vectors against PRKAR1A.

shPRKAR1A	TRC No.	Sequence
shPRKAR1A-C9	TRCN0000039938	CCGGCCTCTGCTCATTAAACTGATTCTCGAGAATCAGTTTAATGAGCAGAGGTTTTTG Clone ID: NM_002734.3-2211s1c1 Accession Number(s): NM_212471.1, NM_212472.1, NM_002734.3 Region: 3UTR
shPRKAR1A-C10	TRCN0000039939	CCGGGCGGAAGATGTATGAGGAATTCTCGAGAATTCCTCATACATCTTCCGCTTTTTG Clone ID: NM_002734.3-879s1c1 Accession Number(s): NM_212471.1, NM_212472.1, NM_002734.3 Region: CDS
shPRKAR1A-C11	TRCN0000039940	CCGGCGACCTAGATTTGAACGTGTTCTCGAGAACACGTTCAAATCTAGGTCGTTTTTG Clone ID: NM_002734.3-1207s1c1 Accession Number(s): NM_212471.1, NM_212472.1, NM_002734.3 Region: CDS
shPRKAR1A-C12	TRCN0000039941	CCGGGCATCCTATGTTAGAAAGGTTCTCGAGAACCTTTCTAACATAGGATGCTTTTTG Clone ID: NM_002734.3-484s1c1 Accession Number(s): NM_212471.1, NM_212472.1, NM_002734.3 Region: CDS
shPRKAR1A-D1	TRCN0000039942	CCGGGCGCTGCTCAAAGATTCTATTCTCGAGAATAGAATCTTTGAGCAGCGCTTTTTG Clone ID: NM_002734.3-238s1c1 Accession Number(s): NM_212471.1, NM_212472.1, NM_002734.3 Region: CDS

2.2.3 Inhibitors and antibodies

The isoquinoline H89 that inhibits PKA and the site-selective cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP were purchased from Calbiochem (Calbiochem, La Jolla, CA). Inhibitors were dissolved in water at a stock concentration of 10 mM and stored at -20 °C until used. Antibodies used for western blotting were as follows: PRKAR1A (BD Transduction Laboratories™, San Jose, CA), PRKAR2B (BD Transduction Laboratories™, San Jose, CA), cyclin D1 (Cell Signaling Technology, Danvers, MA), cdk4 (Cell Signaling Technology, Danvers, MA), ApoptoPak™ Miniature Set (anti-Bcl-2, anti-Bak, anti-Bax) (Millipore, Billerica, MA), Bcl-X (BD Transduction Laboratories™, San Jose, CA) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.2.4 RNA extraction and cDNA synthesis

Total RNA was extracted by Trizol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Reverse transcription reaction consisted of 2.5 µg total RNA and random hexamers (2.5 µM) which were mixed together and then heated at 70°C for 10 min. After that, reaction mixture containing the first strand cDNA synthesis buffer (1x; 75 mM KCl, 50 mM Tris-Cl pH 8.3, 3mM $MgCl_2$), 10 mM DTT, 0.5 mM each dNTPs and 200 units reverse transcriptase (Promega Corp., Medison, WI). Reverse transcription was carried using a DNA thermal cycler (GeneAmp PCR system 2400, Perkin-Elmer Applied Biosystems, Waltham, MA). The thermal conditions were 25 °C for 10 min, 37°C for 1 h, and 95°C for 5 min.

2.2.5 Real time PCR and relative quantification of PRKAR1A and PRKAR2B expression

Real time PCR was performed using TaqMan® Gene Expression assay kit and the ABI 7500 real time PCR system (Applied Biosystems, Foster City, CA). PCR reactions were performed in the relative quantification of PRKAR1A and PRKAR2B gene expression was done using the comparative cycle threshold (C_T) method and GAPDH expression as the endogenous control.

2.2.6 Western blot analysis

2.2.6.1 Protein extraction and determination

Cells were washed with PBS and then lysed with radioimmuno-precipitation assay (RIPA) buffer containing Protease K inhibitor cocktail, 0.5 M

NaF, 0.2 M $NaVO_4$, 1M Tris-HCl pH 7.5, 0.5 M EDTA, 2.5 M NaCl, 10% NP-40, 10% SDS and deionized water. Cell lysate was then centrifuged at 13,000 xg for 10 min at 4°C. The supernatant was transferred to a new tube and determined for protein content using the Pierce BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Five µl of each cell lysate was mixed with 200 µl of BCA working reagent, and then incubated at 37 °C for 30 min. The absorbance was read at 562 nm against blank. The concentration of protein in the sample was estimated from a standard curve that was constructed by using serial dilution starting from 2 mg/ml of standard bovine serum albumin.

2.2.6.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and western blot analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins extract from the cell lysates were solubilized in 4X SDS buffer containing DTT and boiled at 95 °C for 10 min, then cooled down on ice. Fifty µg proteins were loaded into an individual well of NuPage® Novex 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). The gel was electrophoresed in MES running buffer (Invitrogen, Carlsbad, CA) at 80 volt until the tracking dye front reached the bottom of the gel. Then, proteins in electrophoresed polyacrylamide gel were transferred onto a polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) using transferring buffer. Prior to transferring, PVDF membrane was soaked and agitated in 100% methanol for 15 sec then soaked in the transferring buffer at room temperature for at least 5 min with gentle agitating. The electrotransferring of proteins onto a PVDF membrane was performed at 30 Volt for 2 h. Membranes were blocked with 5% skim milk in phosphate buffered saline (PBS) containing 0.03% Tween 20 at room temperature for 30 min and then stained with primary antibody at 4 °C overnight. After rinsing membranes with PBS containing 0.3% Tween 20, membranes were incubated with secondary antibody at room temperature for 1 h and then rinsing with PBS containing 0.3% Tween 20. Finally, membranes were exposed to SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) for chemiluminescent detection. In this study, GAPDH antibody was used as an internal loading control.

2.2.7 Stable knocking down of PRKAR1A using shRNA

Stable transfection of shRNA to CCA cell lines was performed for silencing expression of *PRKAR1A*. A Mission™ TRC-Hs (Human) clone set of sequence-verified shRNA lentiviral plasmid vectors against *PRKAR1A* were obtained from the JHU High Throughput Biology Center according to the sequence in Table 2.1. VSV-G pseudotyped virus was produced by co-transfecting 293T cells with a shRNA transducing vector and two packaging vectors: psPAX2 and PMD2.G using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Infectious virus was harvested at 36 and 48 h after transfection and filtered through 0.22 micron pore size cellulose acetate filter.

The procedure of stable transfection of shRNA to CCA cell lines was performed in a 6-well plate as follow: M156 and OCA17 cells (1×10^5) were plated into 6-well plates (BD Falcon™, San Jose, CA) for 24 h before transfection. After 24 h, 100 µl of non-concentrated virus was added in HAM-F12 containing 8 µg/ml polybrene to get a total volume of 1,000 µl and mixed gently. Then, cells were incubated at 37°C and 5% CO_2. Growth media was replaced 24 h post-transduction. PRKAR1A knockdown cells were selected with 50 µg/ml puromycin containing media and incubated for 24 h. Cells were then replaced with new media and cultured until cells got confluent. In this study, cells treated with empty virus vector under identical conditions were used as control. The efficiency of shRNA transfection was determined by western blot analysis. The stable knockdown CCA cell lines were used for further analysis.

2.2.8 Protein kinase A assay

PKA activity of stable PRKAR1A knockdown CCA cell lines were assessed by using non-radioactive PKA activity assay kit (PepTag® assay, Promega, Madison, WI) according to the manufacturer's instructions. The principle of this assay is the PepTag® Peptide substrate (Kemptide) of PKA is tagged with fluorescent dye molecule. Phosphorylation by PKA of its specific substrate alters the peptide's net charge from +1 to be –1. The different net charge allows the phosphorylated and non-phosphorylated forms of the substrate to be rapidly separated on an agarose gel under electric current. The phosphorylated specie migrates toward the positive electrode, while the non-phosphorylated specie migrates toward the negative electrode.

Cells were washed with PBS and then lysed with PKA extraction buffer containing 25 mM Tris- HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Cell lysates were then centrifuged at 14,000 xg for 5 min at 4°C and collected the supernatant for kinase assay. Cell lysates (80 μg) were mixed with PepTag® PKA reaction 5X buffer, PepTag® A1 peptide, PKA activator 5X solution and deionized water to get final volume of 25 μl and incubated for 30 min at room temperature then reaction was stopped at 95 °C for 10 min. The migration of phosphorylated peptide toward the cathode (+) was detected using 0.8% agarose gel 100 Volt for 15 min. Gel was then photographed under UV light. Two μg/ml of catalytic subunit of PKA was used as a positive control in this experiment.

2.2.9 Cell proliferation assay

Cell proliferation was determined by using alamarBlue® assay (Invitrogen, Carlsbad, CA). Cells (5×10^2) were seeded in black clear bottom 96 well plates (Becton Dickinson, Franklin Lakes, NJ) and incubated overnight. Then, 20 μl of 1X alamar blue was added and the volume in each well was made up to 200 μl with the growth medium. After 72 h incubation, fluorescence was measured on a Perkin Elmer Wallac 1420 Multilable counter (Perkin Elmer, Turku) with a 540 nm excitation filter and a 590 nm emission filter. The effect of PKA inhibitor, cAMP analogues and combination treatment on CCA cell proliferation was also determined as described above with adding the drugs at the designated concentration. Experiments were performed as three independent experiments, with six replicates per experiment.

2.2.10 Apoptosis assay

Apoptosis assays were performed using the caspase-Glo® 3/7 assay (Promega, Madison, WI) according to manufacturer's instructions. Cells (5×10^3) were plated in 100 μl media and incubated overnight. Fifty μl of caspase-Glo® 3/7 reagent was added and mixed to each well, followed by incubation for 1 h at room temperature. Luminescence was measured on a Victor multiwell plate reader (Perkin Elmer, Turku) per the manufacturer instructions. Experiments were performed twice with three replicates per experiment.

2.2.11 Phospho-kinase array assay

The profiles of kinases and their protein substrates phosphorylation were analyzed by using Human Phospho-Kinase Array Kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions using protein extracts from stable PRKAR1A knockdown CCA cell lines (M156 and OCA17) in comparison with their empty viral transfection control. Cell lysates (750 μg per membrane) were incubated overnight with nitrocellulose membrane containing 46 kinase phosphorylation sites and 7 different controls printed in duplicate. Then membranes were incubated first with detection antibody cocktail and later with streptavidin-horseradish peroxidase. The signals were detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). The array images were analyzed and quantified using ImageQuant™ Imager (GE Healthcare UK Ltd., UK).

2.2.12 Statistical analysis

Results from cell proliferation and apoptotic induction experiments were reported as mean ± SD and the differences between control and test groups were tested using Student's *t* test. Results were considered statistically significant between control and test treatment at the level of $P < 0.05$.

2.3 Results

2.3.1 Expression of PKA subunits mRNA and protein in CCA cell lines

Quantitative real time RT-PCR and western blot analysis were performed to determine mRNA and protein level of PRKAR1A and PRKAR2B in four CCA cell lines, M156, OCA17, KKU100 and M214. Real time PCR showed high PRKAR1A/PRKAR2B mRNA ratio in all cell lines studied (Figure 2.3A). In accordance with mRNA level, PRKAR1A protein level was overexpressed in all CCA cell lines, whereas no expression of PRKAR2B was observed (Figure 2.3B). F9, Mouse embryonic carcinoma cell line, which expresses high level of PRKAR2B was used as a control for immunobloting of PRKAR2B. We selected OCA17 and M156 as the representatives of papillary and non-papillary CCA respectively, for further investigations.

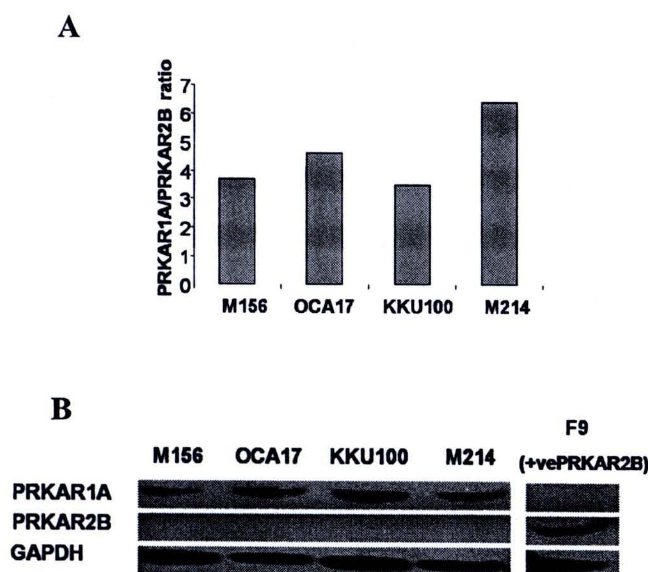

Figure 2.3 Expression of PKA subunits mRNA and protein in CCA cell lines. (A) Real time PCR was performed to determine PRKAR1A and PRKAR2B mRNA level in CCA cell lines. (A) Result showed the high PRKAR1A/ PRKAR2B mRNA ratio and (B) western blot analysis of PRKAR1A and PRKAR2B protein in CCA cell lines showed that PRKAR1A protein was abundant in CCA cell, but did not express the PRKAR2B protein. GAPDH was used to check for equal loading of the gel.

2.3.2 Different expression levels of PRKAR1A obtained using different sequences of shPRKAR1A

To address the functional importance of PRKAR1A, RNAi was performed to stably deplete the expression of PRKAR1A in two CCA cell lines which had high expression level of PRKAR1A (M156 and OCA17). Empty viral vector was used as a control to ensure that the observed action of shPRKAR1A was not resulted from the transfecting protocol. The efficiency of shRNA transfection was determined by western blot analysis using GAPDH for monitoring to the equal amount of protein loading.

Five shRNAs of a hairpin of 21 base pair sense and antisense stem and a 6 base pair loop targeting PRKAR1A: shPRKAR1A-C9, shPRKAR1A-C10, shPRKAR1A-C11, shPRKAR1A-C12 and shPRKAR1A-D1 (Table 2.1) were designed

to suppress PRKAR1A expression. Cells transfected with shPRKAR1A-D1 showed highest suppression of PRKAR1A expression in both CCA cell lines when compared with the other sequences (Figure 2.4A and 2.4B). Therefore, we selected shPRKAR1A-D1 transfection for further analysis. However, we have done the second transfection in OCA17 in order to get completely PRKAR1A knockdown (Figure 2.4C). PRKAR1A protein level of cells transfected with shPRKAR1A-D1 was reduced dramatically without phenotypic change in M156 (Figure 2.5A, B) whereas partial suppression and apoptotic cell characteristic were observed in OCA17 (Figure 2.4C and 2.5C, D) when compared with empty viral vector treated cells. Therefore control cells treated with empty viral vector did not affect the expression levels of PRKAR1A or GAPDH in CCA cell lines.

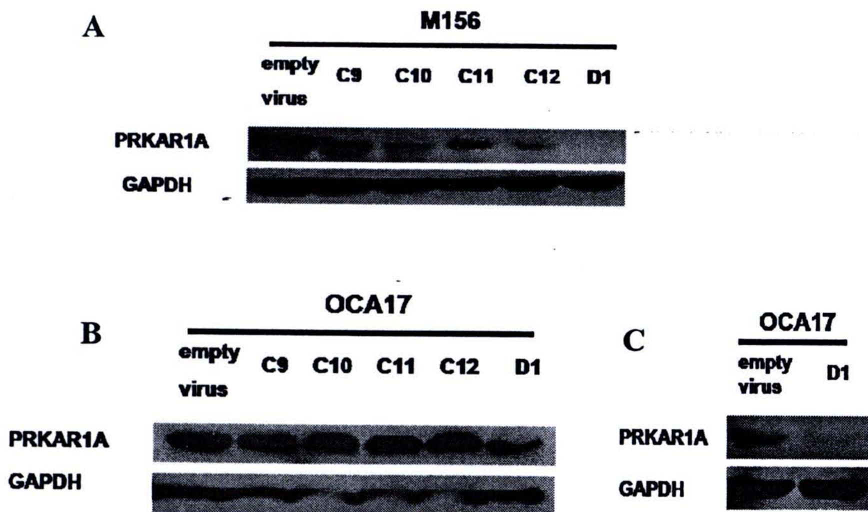

Figure 2.4 PRKAR1A suppression in CCA cell lines by shPRKAR1A. Western blot analysis of PRKAR1A after different shPRKAR1A transfection in (A) M156 and (B) OCA17. (C) The second transfection with shPRKAR1A-D1 in OCA17 completely decreased PRKAR1A protein expression.

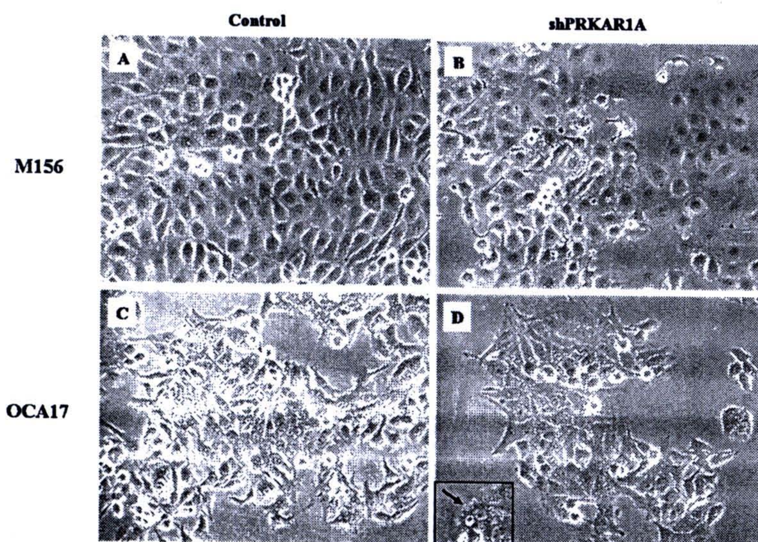

Figure 2.5 Cell morphology of PRKAR1A stable knockdown. Upper panel shows PRKAR1A knockdown in M156 (A) Control, (B) shPRKAR1A treated cells. Lower panel shows PRKAR1A knockdown in OCA17 (C) Control, (D) shPRKAR1A treated cells; arrow indicates apoptotic cells.

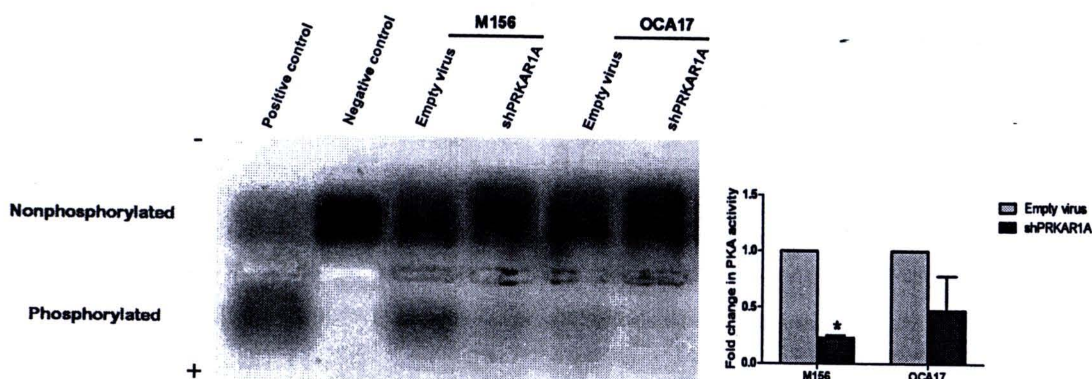

Figure 2.6 Effect of PRKAR1A silencing on PKA activity. Stable PRKAR1A knockdown decreased PKA activity in M156 and OCA17. The phosphorylated peptide migrated toward the cathode (+) whereas nonphosphorylated peptide migrated toward the anode (-). Positive and negative controls were catalytic subunit of PKA and deionized water, respectively. *, $P<0.05$ compared with control cells.

2.3.3 Profiling of kinases and their protein substrates phosphorylation in stable PRKAR1A silencing CCA cell lines

We hypothesized that PRKAR1A/PKAI contributes its function to the growth of CCA cells. To provide insights into how PRKAR1A/PKAI regulates CCA cell growth, the molecular mechanism of PRKAR1A/PKAI was elucidated in this study. We investigated the kinases and their protein substrate phosphorylation profile in PRKAR1A stable knockdown CCA cell lines both in M156 and OCA17 compared with their empty viral transfection control. The differential activated proteins were summarized in Table 2.2. The kinase profiles in 2 cell lines studied showed common phosphorylation activation pattern as following; in M156 cell line (Figure 2.7A), the phospho-kinase array analysis showed a decrease in MAPKs signaling cascade's phosphorylation, including MEK1/2, ERK1/2, p38α, MSK1/2, RSK1/2/3 and HSP27 as well as the reduction of Akt (S473) and β-catenin phosphorylation. Phosphorylated CREB (cAMP response element binding protein) which is responsible for modulating PKA, was not significantly reduced. Conversely, an induction of Akt (T308), Src, c-Jun, STAT4, Chk-2 and e-Nos phosphorylation was found. In OCA17 cell lines (Figure 2.7B), a reduction of MAPKs (MEK1/2, ERK1/2, p38α, MSK1/2 and RSK1/2/3), CREB, Src, Akt (Akt, TOR and p70 S6 kinase) phosphorylation was seen. In contrast to M156, JAK-STAT (STAT2, STAT3 and STAT6) phosphorylation was also observed in OCA17. In addition, a decreased in 5' adenosine monophosphate-activated protein kinase (AMPKα1 and AMPKα2), which plays role in cellular energy homeostasis was seen. As similar as M156 cell line, there was an induction of c-Jun and e-Nos phosphorylation in OCA17 cell line. Based on these findings, we provided a schematic illustration to describe the possible mechanism of PKAI in CCA as shown in Figure 2.8 and 2.9. Our results indicated that PRKAR1A/PKAI may control CCA cell growth mainly via MAPKs as well as in crosstalk with the PI3K/AKT, JAK/STAT and Wnt/β-catenin signaling pathways.

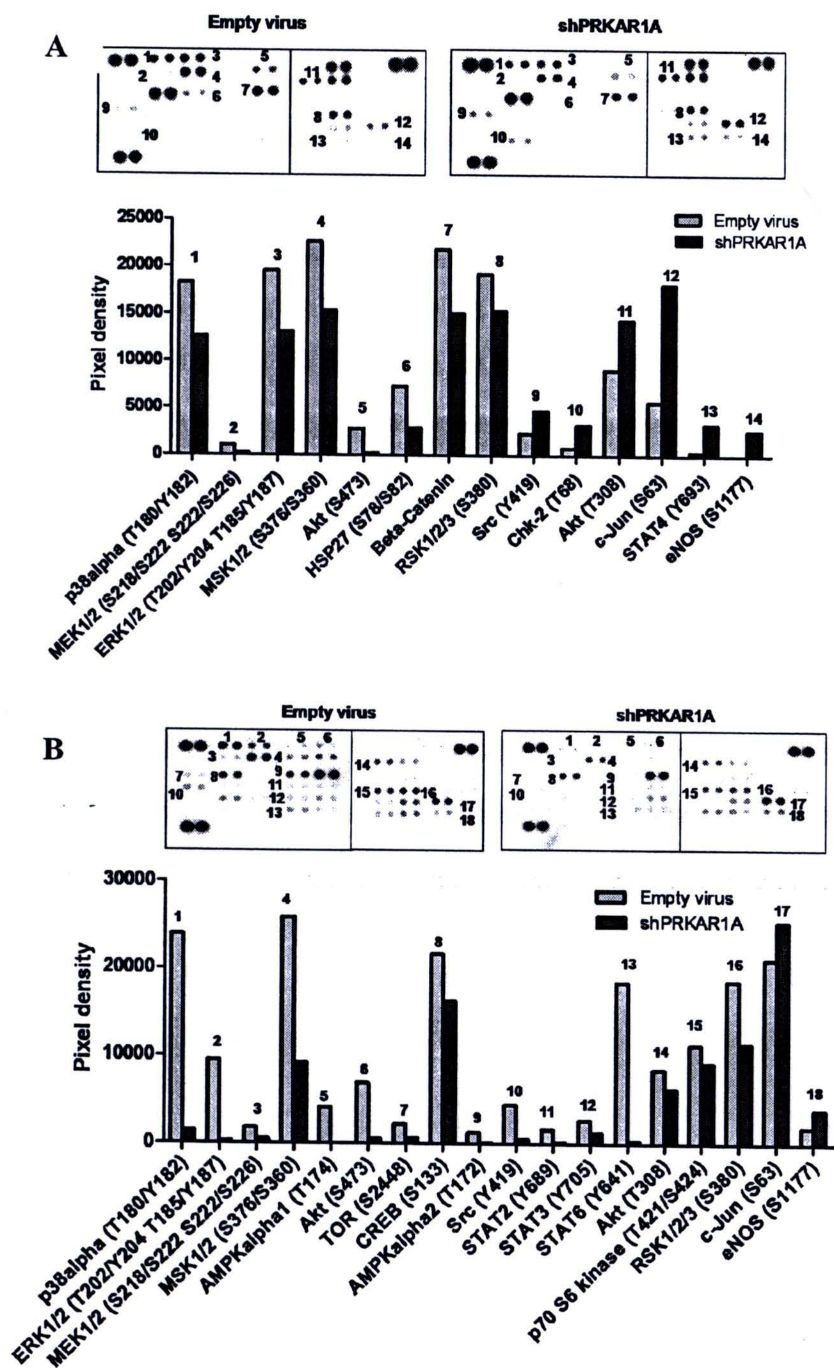

Figure 2.7 The phospho-kinase array of PRKAR1A silencing cell, (A) M156 and (B) OCA17. The lower panels are the profiles created by quantifying the mean spot pixel densities. Array signal from scanned X-ray films images were analyzed using ImageQuant™ Imager.

Table 2.2 List of differential expressed proteins related to PRKAR1A suppression in CCA cell lines.

CCA cell lines		***Decrease in phosphorylated proteins***				***Increase in phosphorylated proteins***
	PI3K-Akt pathway	**Ras-MAPK pathway**	**Wnt-β-catennin pathway**	**JAK-STAT pathway**	**AMPK pathway**	
M156	AKT, HSP27	p38 α, ERK1/2, MEK1/2, MSK1/2, RSK1/2/3	β-catennin			Chk-2, STAT4, Src , eNOS, c-JUN
OCA17	AKT, TOR, p70 S6 kinase	p38 α, ERK1/2, MEK1/2, MSK1/2, AMPKα1, AMPKα2, CREB, Src, RSK1/2/3		STAT2, STAT3, STAT6	AMPKα1, AMPKα2	eNOS, c-JUN

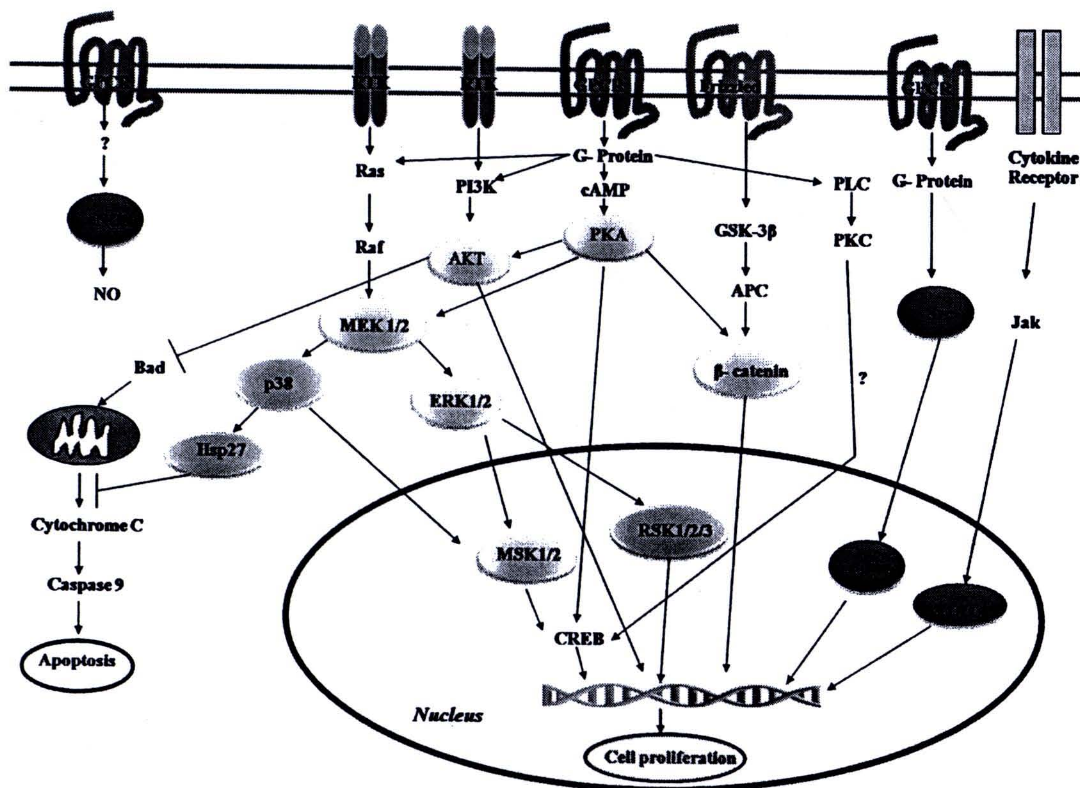

Figure 2.8 The possible molecular mechanism of PKAI in M156. The differential activated proteins were mapped onto networks base on known protein-protein interactions. Color indicates decrease in phosphorylated (green) and increase in phosphorylated (red) proteins when PRKAR1A was suppressed. Proteins in uncolored notes were not identified as differentially activated/suppressed in our experiment and were integrated into the generated networks on the basis of the evidence indicating a relevance to this network.

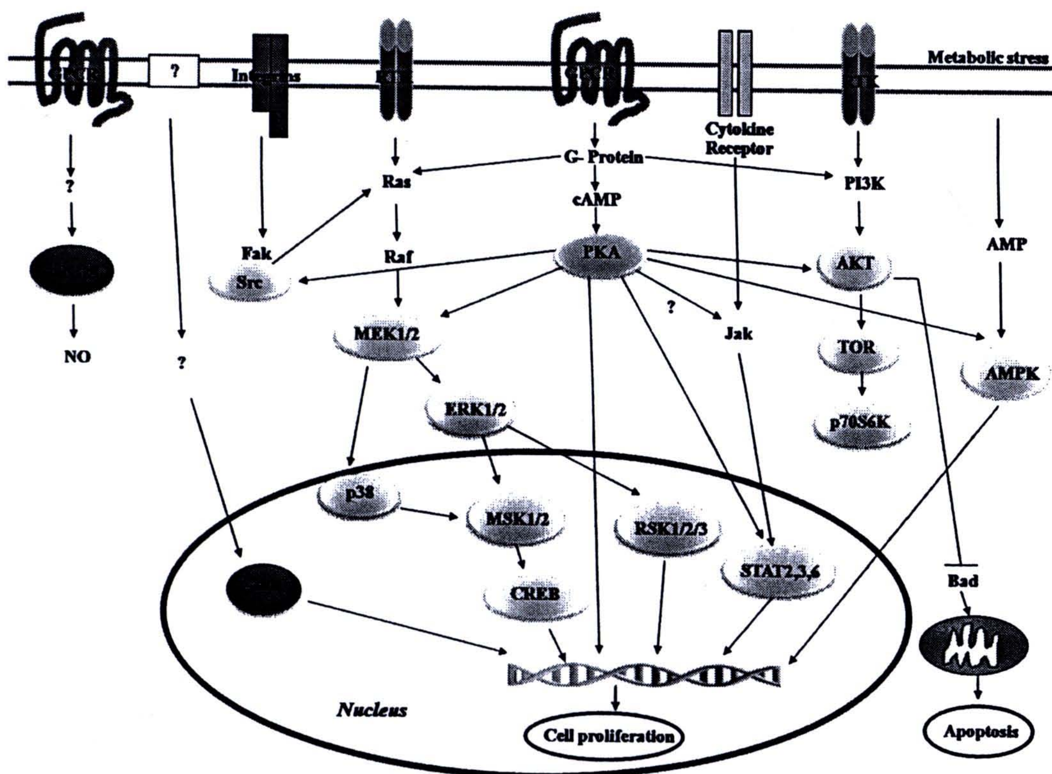

Figure 2.9 The possible molecular mechanism of PKAI in OCA17. The differential activated proteins were mapped onto networks base on known protein-protein interactions. Color indicates decrease in phosphorylated (green) and increase in phosphorylated (red) proteins when PRKAR1A was suppressed. Proteins in uncolored notes were not identified as differentially activated/suppressed in our experiment and were integrated into the generated networks on the basis of the evidence indicating a relevance to this network.

2.3.4 Abrogate PRKAR1A expression reduced CCA cell growth

The involvement of PRKAR1A/PKAI in CCA cell proliferation was examined in two CCA cell lines including M156 and OCA17. The reduction of PRKAR1A expression by shPRKAR1A resulted in a significant inhibition of proliferation approximately 23% and 25% in M156 and OCA17, respectively (Figure 2.10A). Moreover, we further characterized effect of PRKAR1A suppression on the cell cycle, we performed western blot analysis to reveal the underlying molecular mechanism. The down regulation of PRKAR1A expression appeared to have resulted

in decrease expression of cell cycle regulation proteins including cyclin D1 and cdk4 which is an essential promoter for transition from G1- to the S- phase (Figure 2.10B).

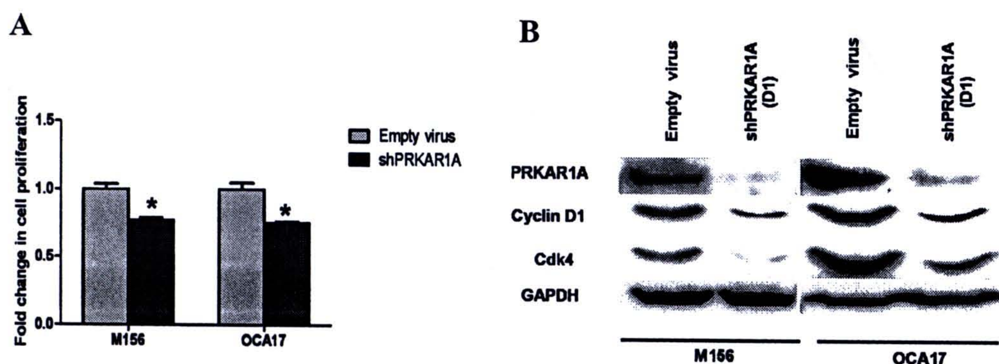

Figure 2.10 Effect of shPRKAR1A on cell proliferation and cell cycle regulation proteins. Cell proliferation was determined by alamarBlue® assay (A) PRKAR1A knockdown using lentiviral infection significantly reduced CCA cell growth. Relative cell proliferation was compared between empty viral transfection control cells and shPRKAR1A-treated CCA cells. Results shown are the mean of six replicates in 3 independent experiments and error bars show 95% confidence interval of mean. *, $P<0.05$ compared with control cells. (B) Suppression of PRKAR1A decreased expression of cell cycle regulation proteins including cyclin D1 and CDK4, respectively.

2.3.5 Suppression of PRKAR1A induced apoptosis

Since apoptosis is closely related to cell proliferation, we tested whether PRKAR1A knockdown inhibited cell growth at least in part by apoptosis induction. The mediators of apoptosis, caspase 3/7, were determined. As shown in Figure 2.11A, decreased PRKAR1A protein expression was significantly increased caspase 3/7 activity about 2.3 and 1.7-fold differences in M156 and OCA17, respectively in comparison with that from the control cells. We also examined the effect of PRKAR1A knockdown on the expression of anti-apoptotic, Bcl-2 and Bcl-X proteins as well as pro-apoptotic proteins, Bak and Bax (Figure 2.11B). Induction of Bcl-2 expression was observed in M156 cell line but not in OCA17 cell line whereas no expression of Bcl-X was found in both lines (data not shown). Conversely, Bax

protein was markedly up-regulated in M156 cell line while increased Bak protein expression was observed in OCA17 cell line. The results confirmed the above finding that apoptosis induction by silencing PRKAR1A expression also contributes to CCA cell growth inhibition.

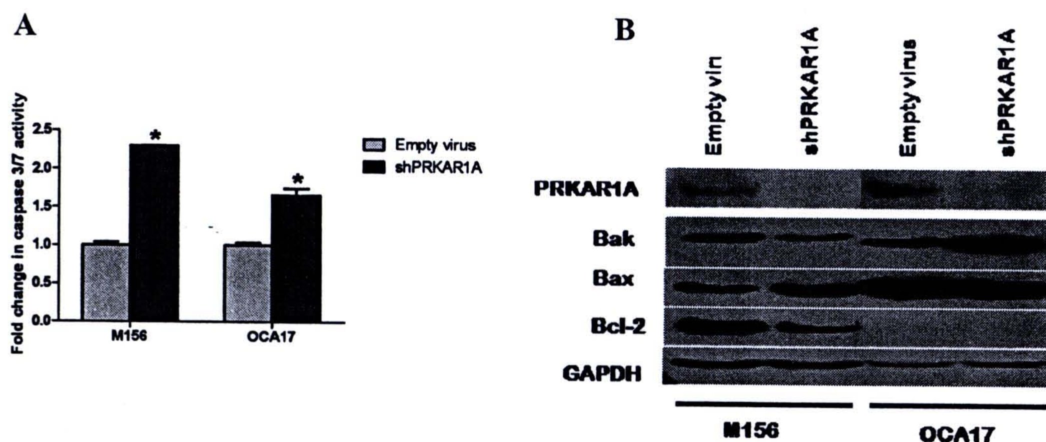

Figure 2.11 Effect of shPRKAR1A on (A) caspase 3/7 activity and (B) apoptotic proteins. Relative caspase level was assayed in comparison to empty viral transfection control. Results shown are the mean of six replicates in 3 independent experiments and error bars show 95% confidence interval of mean. *, $P<0.05$ compared with control cells.

2.3.6 Small molecules inhibitors of PKA inhibited CCA cell growth

To further examine the importance of PRKAR1A/PKAI in the growth of CCA cells, we used a specific PKA inhibitors, H89 and cAMP analogues including 8-Cl-cAMP and 8-Br-cAMP. Four CCA cell lines (M156, OCA17, KKU100 and M214) were treated with different concentrations of test substances and assessed for proliferation by alamarBlue® assay. The IC_{10} values of PKA inhibitors were shown in Table 2.3. The results showed that PKA inhibitor, H89 as well as the cAMP analogues 8-Cl-cAMP and 8-Br-cAMP inhibited CCA cell proliferation as dose dependent manner (Figure 2.12). This finding supports the RNAi experiments and further implicates PRKAR1A/PKAI in the survival of CCA cell lines.

Table 2.3 IC_{10} values of PKA inhibitors in four CCA cell lines.

CCA cell lines	IC_{10} values (μM) of PKA inhibitors		
	H89	8-Cl-cAMP	8-Br-cAMP
M156	0.27 (±0.2)	1.045 (±0.5)	12.33 (±2.4)
OCA17	1.33(±0.0)	90 (±0.0)	4.9 (±2.3)
KKU100	0.7 (±0.4)	8.2 (±6.5)	3.0 (±2.8)
M214	2.39(±0.9)	6.51(±5.2)	<0.1

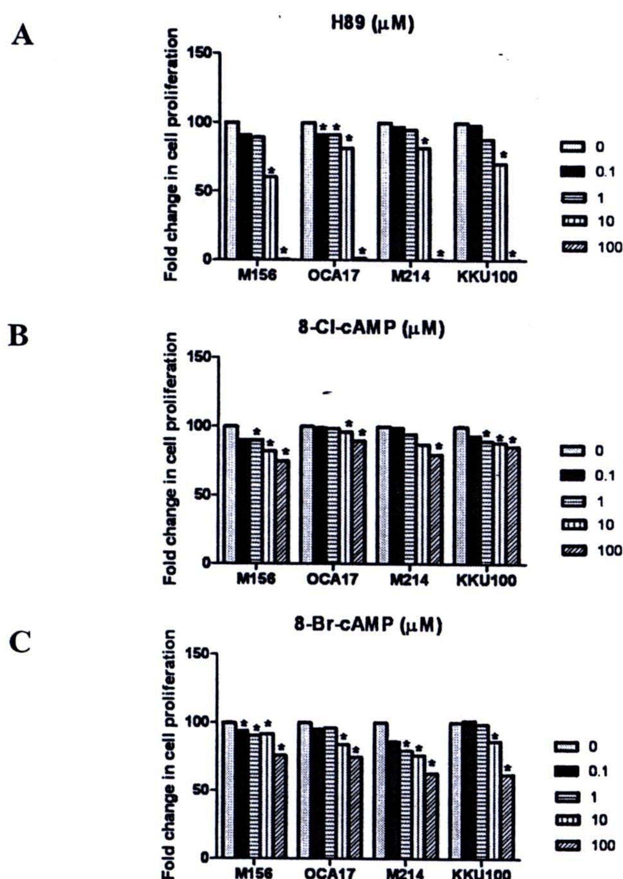

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Figure 2.12 The PKA inhibitor and cAMP analogues reduce CCA cell proliferation. Four CCA cell lines were treated with H89 (A), 8-Cl-cAMP (B) and 8-Br-cAMP (C) with the indicated concentration and cell proliferation was assessed using alamarBlue® assay as described above. Relative cell proliferation was assayed in comparison to untreated cells. Results shown are the mean of six replicates in 3 independent experiments and error bars show 95% confidence interval of mean. *, $P<0.05$ compared with untreated cells.

2.4 Discussion

The involvement of PKA pathway in neoplastic transformation and tumor growth is implicated in many types of cancer. Detection of PRKAR1A/PKAI overexpression correlates with a worse prognosis in patient affected by different types of cancer (Bradbury *et al.*, 1994; Miller *et al.*, 1993). Moreover, PKAI has been directly implicated in the acquisition of the multidrug-resistant (MDR) phenotype (Scala *et al.*, 1995). We also have found the overexpression of PRKAR1A in the tumor tissues of Ov and NDMA induced hamster CCA (Loilome *et al.*, 2006) as well as PKA isozyme switching and PRKAR1A/PKAI pathway might contribute to the induction of biliary cells transformation and proliferation in Ov and NDMA-induced progressive cholangiocarcinogenesis. Others have reported that PKA isozyme switching, with dramatic changes in the proportion of the two PKA regulatory subunits PRKAR1A and PRKAR2B, has been associated with many tumor types, a poorer prognosis, and as a target for therapy (Bradbury *et al.*, 1994; Cho-Chung, 1990; Miller *et al.*, 1993; Tortora and Ciardiello, 2002). A recent study indicated that high expression of PRKAR1A was associated with a diminished response to radiation therapy in prostate cancer, suggesting that such patients would be benefit from therapy patients that reduces PRKAR1A levels (Pollack *et al.*, 2009). For all the above reasons, PRKAR1A/PKAI has been proposed as a potential target for cancer therapy. This has led us to investigate the pattern of expression of the different subtypes of PKA subunits in human cholangiocarcnoma as well as to elucidate the intracellular signaling of PKA and identify their potential role in the control of CCA cell growth.

The ratios of expression for the two PKA regulatory subunits; PRKAR1A and PRKAR2B in human cholangiocarcinoma cell lines were characterized. Real time PCR showed the increased PRKAR1A/PRKAR2B mRNA ratio in four human CCA cell lines. Moreover, PRKAR1A protein was highly expressed in the all human CCA cell lines studied as shown by western blot analysis. In contrast, no expression of PRKAR2B was detected. However, molecular mechanism of PRKAR1A/PKAI underlying CCA progression was still unclear. This study is the first description of the possible molecular networks related to PRKAR1A/PKAI in regulation of CCA cell growth. The interrelation and cross-talk between PRKAR1A/PKAI and the other

intracellular signaling pathways were elucidated by phospho- kinase array. We found that proteins in mitogen-activated protein kinase (MAPKs) and their downstream signaling molecules were reduced. Indeed, the control of cell proliferation by a signal transduction networks between the PKA and MAPKs pathways in many cell types including biliary epithelium is largely known (Bhola and Grandis, 2008; Francis *et al.*, 2004). Therefore, our results suggest that the interplay between PKA and MAPKs signaling pathway may be important in the regulation of CCA cell growth and survival. Moreover, CREB (cAMP responsive element binding protein) phosphorylation was reduced in the OCA17 cell line, whereas no change was observed in the M156 cell line. However, CREB can be activated by PKC pathway signaling (Mao *et al.*, 2007; Roberson *et al.*, 1999). Gonzalez et al. (Gonzalez *et al.*, 1989) reported that the N-terminal fragment of CREB contains consensus recognition sequence for PKC.

The decrease of the kinase and substrate proteins of the PI3K/AKT, JAK/STAT and Wnt/β-catenin pathways was found secondary to PRKAR1A silencing in CCA cell lines. This indicates for the first time the interaction between PKA and those signaling pathways in regulating CCA cells growth and survival. The serine/threonine kinase Akt (or protein kinase B), controls key cellular processes such as glucose metabolism, cell cycle progression, and apoptosis (Lawlor and Alessi, 2001), and activated Akt can contribute to tumorigenesis *in vivo* in lymphoid, breast, ovarian, prostate, brain tissues and CCA (Chung *et al.*, 2009; Scheid and Woodgett, 2001). There was a reduction of Akt phosphorylation at both sites (S473 and T308) in OCA17 cell line whereas decreased phosphorylation at S473 was found but it was increased at T308 in M156 cell line. Nevertheless, full activation of Akt requires phosphorylation at two sites (West *et al.*, 2003). Moreover, reduction of kinase phosphorylation in the Akt downstream targets, TOR and p70 S6 kinase, was seen in the OCA17 cell line.

The JAK/STAT signaling participates in the regulation of cell proliferation, differentiation, survival, motility, and apoptosis in different organs including liver (Hebenstreit *et al.*, 2005). During the last decades, the data on cytokine/growth factor receptors expression and functions in different liver cell types (hepatocytes, cholangiocytes, Kupffer and stellate cells) have rapidly grown. It highlights the

importance of JAK-STAT signaling in normal liver physiology and pathology. Moreover, deregulation of this pathway is closely associated with tumorigenesis. Though the role of the JAK-STAT cascade is poorly deciphered both in normal cholangiocytes and in cholangiocarcinoma cells, a number of papers have been published recently where the role of prolactin (Prl), growth hormone (GH) and interleukin-6 (IL-6) and expression of corresponding receptors in cholangiocytes has been shown (Alvaro *et al.*, 2006; Bogorad *et al.*, 2006; Yokomuro *et al.*, 2000). In the present work, decrease of STAT2, STAT3 and STAT6 phosphorylation in the OCA17 cell line, whereas increase of STAT4 phosphorylation in the M156 cell line were found. Since there are a lot of members belonging to the STAT protein family, the exact role of the JAK-STAT pathway should be further investigated in CCA.

Phosphorylation of β-catenin, the chief downstream effector of the canonical Wnt signaling pathway was down-regulated in M156 whereas no change was found in OCA17 cell line. Recent evidence suggests that alteration of Wnt/β-catenin pathway is implicated in cholangiocarcinogenesis (Ashida *et al.*, 1998; Settakorn *et al.*, 2005; Sugimachi *et al.*, 2001; Tokumoto *et al.*, 2005).

Upon PRKAR1A silencing, eNos (endothelial nitric oxide synthase) and proto-oncoprotein c-JUN were commonly found to be up-regulated in both CCA cell lines. Jadeski et al. reported that eNOS promoted tumor growth and metastasis by stimulation of tumor cell migration and angiogenesis (Jadeski *et al.*, 2000). In addition, c-Jun is a component of mammalian transcription factor activator protein-1 (AP- 1). The current studies demonstrated that c-Jun is involved in mammary epithelial tumor cell growth and invasion (Jiao *et al.*, 2010, in press). Our finding is the first report on pathways related to eNOS and c-JUN that are activated in CCA. This phenomenon may be a consequence of compensatory activities among pathways in the cells to maintain homeostasis of cell survival.

To confirm the involvement of PRKAR1A/PKAI in CCA cell growth and apoptosis, we have performed cell proliferation assay and caspase 3/7 activity determination in stable PRKAR1A silencing CCA cell lines. Cell proliferation was significantly reduced in both CCA cell lines as compared with those transfected with empty viral control. Moreover, this may lead to a decrease in expression of G1–S transition regulatory proteins that include cyclin D1 and cdk4. Our results are

consistent with the previous studies that suppressed PRKAR1A expression by antisense oligodeoxynucleotides resulting in growth inhibition in human cancer cells both *in vitro* and *in vivo* (Cho-Chung *et al.*, 1999; Mantovani *et al.*, 2008; Nesterova and Cho-Chung, 1995; Nesterova and Cho-Chung, 2004). Moreover, PRKAR1A silencing induced CCA cell apoptosis as indicated by phenotypic changes, i.e. activation of caspase 3/7 activity, as well as modulation of Bcl-2 family protein expression by increase of pro- apoptotic protein expression and decrease of anti-apoptotic protein expression.

We further investigated if the small molecule inhibitors of PKA would inhibit CCA cell growth. Site-selective cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP are able to induce degradation of PRKAR1A protein and upregulation of PRKAR2B expression (Cho-Chung *et al.*, 1995; Rohlff *et al.*, 1993). H89 binds to the ATP-binding site of the catalytic subunit and can stop the phosphorylation process directly (Lochner and Moolman, 2006). Proliferation of CCA cell lines was significantly suppressed in the presence of the small molecule inhibitor of PKA, the isoquinoline H89 and the site-selective cAMP analogues, 8-Cl-cAMP and 8-Br-cAMP in a dose-dependent manner. However, high concentrations of cAMP analogues were needed. Our results demonstrated that H89 exhibited more potent growth inhibition than 8-Cl-cAMP and 8-Br-cAMP in all cancer cell lines tested. It may due to H89 can inhibit other kinases and have a relatively large number of PKA-independent effects (Lochner and Moolman, 2006). The study by Davies et al. in 2000 (Davies *et al.*, 2000) showed that at 10 μM of H89 inhibits at least 8 others kinase including MAPKAP-K1b, MSK1, KBα, SGK, S6K1, ROCK II, AMPK, and CHK1. In addition, two of these kinases, MSK1 and S6K1, were inhibited by H89 at IC_{50} 120 nM and 80 nM, respectively which showed potency greater than that for inhibition of PKA (IC_{50} 130 nM). Additionally, 8-Br-cAMP showed better effect than that of 8-Cl-cAMP. Although, 8-Cl-cAMP has less effect in CCA but it shows potent inhibitory effects on a wide variety of human cancer cell lines, with an IC_{50} ranging from 0.1 to 20 μM (Cho-Chung *et al.*, 1989). Moreover, 8-Cl-cAMP trial of several Phase I clinical studies has been completed and recently entered Phase II as an anticancer agent (Tortora and Ciardiello, 2002). The high concentration of drug used for inhibiting

CCA cell growth suggests combining 8-Cl-cAMP with other antineoplastic drugs, in order to get a better therapeutic response.

Our results demonstrated that suppression of PRKAR1A/PKAI had little effect on CCA cell growth inhibition. It implies that inhibition only PRKAR1A/PKAI may not be enough for growth inhibition. This was consistent with our kinase profile result which revealed that PKAI crosstalk with other signaling pathways in the regulation of CCA cell growth. Therefore, blocking only one of these pathways may result in activation of an alternative pathway giving rise to some advantage to cell growth so blocking two or more pathway would be more effective therapeutic approach to treatment of CCA.

In conclusion, our data demonstrates that overexpression of PRKAR1A is found in CCA and provides the signaling pathways by which PRKAR1A/PKAI is involved in cholangiocarcinogeneis. This work also indicates that abrogation of PRKAR1A expression leads to induce CCA cells growth inhibition and apoptosis. Importantly, we showed that a PKA inhibitor as well as cAMP analogues potentiates the growth inhibitory effect of antitumor drugs and should be further investigated as a therapeutic strategy against CCA, either as single-drug or in combination with other antitumor drugs.