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Transcriptional and Post-Transcriptional Control of DNA Methyltransferase 3B Is Regulated by Phosphatidylinositol 3 Kinase/Akt Pathway in Human Hepatocellular Carcinoma Cell Lines

Chuanzhong Mei,[1] Lidong Sun,[1] Yonglei Liu,[1] Yong Yang,[1] Xiumei Cai,[1,2] Mingzhu Liu,[1] Wantong Yao,[1] Can Wang,[1] Xin Li,[1] Liying Wang,[1,2] Zengxia Li,[1,2] Yinghong Shi,[3] Shuangjian Qiu,[3] Jia Fan,[3] and Xiliang Zha[1,2*]

[1] *Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China*

[2] *Key Laboratory of Molecular Medicine, Ministry of Education, Shanghai 200032, China*

[3] *Zhongshan Hospital, Fudan University, Shanghai 200032, China*

ABSTRACT

DNA methyltransferases (DNMTs) are essential for maintenance of aberrant methylation in cancer cells and play important roles in the development of cancers. Unregulated activation of PI3K/Akt pathway is a prominent feature of many human cancers including human hepatocellular carcinoma (HCC). In present study, we found that DNMT3B mRNA and protein levels were decreased in a dose- and time-dependent manner in HCC cell lines with LY294002 treatment. However, we detected that LY294002 treatment did not induce increase of the degradation of DNMT3B protein using protein decay assay. Moreover we found that Akt induced alteration of the expression of DNMT3B in cells transfected with myristylated variants of Akt2 or cells transfected with small interfering RNA respectively. Based on DNMT3B promoter dual-luciferase reporter assay, we found PI3K pathway regulates DNMT3B expression at transcriptional level. And DNMT3B mRNA decay analysis suggested that down-regulation of DNMT3B by LY294002 is also post-transcriptional control. Furthermore, we demonstrated that LY294002 down-regulated HuR expression in a time-dependent manner in BEL-7404. In summary, we have, for the first time, demonstrate that PI3K/Akt pathway regulates the expression of DNMT3B at transcriptional and post-transcriptional levels, which is particularly important to understand the effects of PI3K/Akt and DNMT3B on hepatocarcinogenesis. J. Cell. Biochem. 111: 158–167, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: DNA METHYLTRANSFERASE (DNMT); PI3K/Akt; HUMAN HEPATOCELLULAR CARCINOMA

DNA methylation is the best-known epigenetic marker [Esteller, 2008] and controlled by DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group from the methyl donor *S*-adenosyl methionine onto the 5 position on the cytosine ring. To date, three catalytically active DNMTs have been identified in human, DNMT1, DNMT3A, and DNMT3B, their encoding gene respectively located in 19p13.2., 2p23 and 20q11.2. DNMT1 prefers hemimethylated DNA substrates during DNA replication; Dnmt3a and Dnmt3b are necessary for de novo methylation and for the establishment of new methylation patterns in mammalian cells [Okano et al., 1998, 1999; Xie et al., 1999]. Aberrant DNA methylation in cancer cells includes global DNA hypomethylation and specific promoter hypermethylation of genes. Hypermethylation of promoters can affect genes involved in the cell cycle, DNA repair, the metabolism of carcinogens, cell-to-cell interaction, apoptosis, angiogenesis and tumor-suppressor, all of

Abbreviations used: DNMT, methyltransferase; PI3K, phosphatidylinositol 3 kinase; HCC, human hepatocellular carcinoma; Myr-Akt2, myristylated variants of Akt2; Act D, actinomycin D; 5-AZA, 5-aza-2′-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide; UTR, untranslated region.

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*Correspondence to: Dr. Xiliang Zha, Department of Biochemistry and Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China. E-mail: xlzha@shmu.edu.cn

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which are also involved in the development of cancer cells [Herman and Baylin, 2003; Esteller, 2007; Esteller, 2008]. The high expression of DNMTs can be seen in human hepatocellular carcinoma (HCC), prostate, colorectal, breast, and endometrial tumors [Eads et al., 1999; Beaulieu et al., 2002; Patra et al., 2002; Girault et al., 2003; Robert et al., 2003; Saito et al., 2003; Jin et al., 2005]; and also associated with transition from a precancerous to a malignant state [Kanai and Hirohashi, 2007].

The activation of phosphatidylinositol 3 kinase (PI3K)/Akt signaling to promote cell proliferation is of equal significance in tumor development and progression [Wymann and Marone, 2005]. Reduced expression of phosphatase and tensin homologue (PTEN), an important negative regulator for the PI3K/Akt signaling pathway, is associated with PTEN promoter hypermethylation in the tumor [Hartmann et al., 2006; Montiel-Duarte et al., 2008]. And IL-6 increases the rate of nuclear translocation of DNMT1 from the cytosolic compartment via Akt inducing the phosphorylation of DNMT1 [Hodge et al., 2007]. In our previous study we found that PI3K/Akt pathway stabilizes DNMT1 protein [Sun et al., 2007]. However, the relationship between PI3K/Akt pathway and DNMT3B is unknown. Here the authors demonstrate a link between epigenetic silencing and PI3K/Akt signaling pathway and demonstrate that the PI3K/Akt pathway can regulate DNMT3B expression.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

PI3K inhibitor LY294002 was purchased from Cell Signaling Technology (CST). DNMT inhibitor 5-aza-2′-deoxycytidine (5-AZA), transcription inhibitor Actinomycin D (Act D), translation inhibitor cycloheximide (CHX), were obtained from Sigma–Aldrich. Anti-DNMT3B monoclonal antibody (mAb) was obtained from Abcam. Anti-total-Akt mAb, anti-phospho-Akt Ser473 mAb, and anti-phospho-GSK3β Ser9 mAb were ordered from CST. Anti-GAPDH mAb and secondary antibody conjugated with HRP were purchased from Kang-Chen Biotech (Shanghai, China).

CELL CULTURE

HCC cell lines (QGY-7703, SMMC-7721) and human liver immortal cell line L02, purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), were routinely maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) in a 37°C incubator with 5% CO_2. HCC cell lines (BEL-7402, BEL-7404, BEL-7405) and HepG2 (human hepatoblastoma) kindly provided by Shanghai Cancer Institute (Shanghai, China) and Myr-Akt2 mouse embryonic fibroblasts (MEFs) cell lines (stably transfected with Myr-Akt2) and MSCV-MEFs (stably transfected with empty murine stem cell virus vector) established previously by ourselves (unpublished work with Sun) were maintained in the Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2.

IMMUNOHISTOCHEMICAL STAINING AND SCORING

Tissue microarrays consisting of 20 cases of HCC and corresponding matched adjacent non-tumorous liver tissues were kindly provided by Prof. Yinkun Liu (Liver Cancer Institute of Fudan University), All experiments done with these tissues conformed to the stipulations of the Ethics Committee of Zhongshan hospital, Fudan University. The slides were deparaffinized and rehydrated, and then immersed in boiled EDTA buffer (pH 9), heated in a microwave oven for 10 min for antigen retrieval, cooled down for another 10 min at room temperature and washed in PBS buffer. The slides were subsequently blocked for 10 min and then incubated with anti-DNMT3B mAb at 47°C for 60 min. After being washed in PBS, slides were incubated in DAKO EnVision reagent for 30 min at room temperature. Sections were then developed using diaminobenzidine stain reagent (Sino-American, Shanghai) as the chromogen, then counterstained with hematoxylin and coverslipped. Staining results were assessed and scored by two pathologists using a computer-aided manipulator (MPIAS-500, Carl Zeiss Vision). The judgment scale is as follows: ±, <5% of immunopositive cells; +, ≤25% of immunopositive cells; ++, <50% of immunopositive cells; +++, ≥50% of immunopositive cells; in ten randomly selected fields (a final magnification: 300×), which was not overlaid was chosen.

REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was isolated using the Trizol system (Invitrogen) according to the manufacturer's guidelines. Oligo$(dT)_{18}$ primer and M-MLV reverse transcriptase were used for the first strand synthesis. Real-time RT-PCR was performed with Real-time RT-PCR Master Mix containing SYB Green I and hotstart Taq DNA polymerase (Toyobo). The threshold cycle (C_t) values were determined by using an iCycler thermal cycler (Bio-Rad, Hercules, CA) performed according to the manufacturer's instructions and the quantitative data were analyzed following the $2^{-\Delta\Delta C_t}$ (DDCT) method using GAPDH mRNA or PCNA mRNA as control respectively. The primers for human DNMT3B3 and DNMT3B4 were as previously described [Saito et al., 2002; de Silanes et al., 2009]. The primers for mouse DNMT3B were used according to the reference [Phillips et al., 2009]. The remaining primers for real-time RT-PCR designed using Primer Premier software (version 5.0, PREMIER Biosoft International; Table I). RT-PCR reactions were performed in triplicate for each sample-primer set, and the mean of the three experiments was used as the relative quantification value. At the end point of PCR cycles, melt curves were made to check product purity.

WESTERN BLOTTING ANALYSIS

Cells were washed with PBS and lysed in 1× sodium dodecyl sulfate (SDS) lysis buffer containing 50 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, phosphatase inhibitors (100 mM Na_3VO_4) and protease inhibitor (1 mM PMSF). Protein concentration of cells lysates was determined using the modified Lowry protein assay. Equal amounts of total protein (80 μg/well) were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Corp.). The membranes were blocked in 3% non-fat milk in PBST (containing 0.03% Tween-20) for 2 h, and then were incubated with specific primary antibodies, followed by horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized by fluorography using an enhanced

TABLE I. Sequences of Primers for RT-PCR

Gene	Forward primer	Reversed primer	Amplicon (bp)
Human			
DNMT3B (for all isoforms)	gagtccattgctgttggaaccg	atgtccctcttgtcgccaacct	305
DNMT3B3	gatgaacaggatctttggcttt	gcctggctggaactattcaca	163
DNMT3B4	cgggatgaacagttaaagaaagtac	ccaaagatccttttcgagctc	182
Akt1	ctgggcaagggcactttcgg	aggcggtcgtgggtctggaa	203
Akt2	atgccttccagacccacgacc	cccgtcactgatgccctcttt	263
PCNA	caacttggaattccagaacaggagtacagc	gggtacatctgcagacatactgagtgtca	330
GAPDH	aaggctgagaacgggaagc	gagggatctcgctcctgga	68
p3b-1226(−1028/+198)	ccgaggggtaagaatctggtcc	cgccgagctaggtttacttgggg	1226
p3b-900 (−696/+204)	gtgctgttttccagtggttcaatggt	gccgatcgccgagctaggttta	900
p3b-501 (−296/+205)	gccccagaaggagtctggttttgga	cgccgatcgccgagctaggttta	501
p3b-246 (−40/+206)	gggccggggctacaaggggagt	gcgccgatcgccgagctaggttta	246
Mouse			
DNMT3B	gaagcacatcgttcccctta	ctccagtgatgcccctaaaa	133
GAPDH	caacgacccccttcattgac	tccacgacatactcagcac	191

chemiluminescence system (Tiangen). The bands were scanned and quantified by TotalLab2.01 (Nonlinear Dynamics Ltd.) and normalized by that of GAPDH.

PLASMID CONSTRUCTS

To amplify a series of DNMT3B promoters, we designed the following sets of specific primers using Primer Premier software (version 5.0) for p3b-1226 (−1028/+198), p3b-900 (−696/+204), p3b-501 (−296/+205) and p3b-246 (−40/+206) and added the *Xho*I and *Hin*dIII restriction enzymes recognized site at 5′ terminal of each primer respectively. The PCR was performed with PrimeSTAR HS DNA Polymerase Kit (TaKaRa, Kyoto, Japan). The conditions for PCR were as follows: cycle 1, 2 min at 95°C; cycles 2–35, 45 s at 94°C, 20 s at 58°C and 3 min at 72°C; cycle 36, 5 min at 72°C. The specific products for DNMT3B promoters were subcloned into a luciferase expression plasmid pGL3-Basic (Promega) using the *Xho*I and *Hin*dIII restriction enzymes site upstream from 3′-end of luciferase and sequenced by Invitrogen Trading Company Limited (Shanghai, China) and aligned with the GenBank databases.

TRANSFECTION AND LUCIFERASE REPORTER ASSAY AND siRNA

BEL-7404 cells were grown in OPTI-MEM Reduced Serum Medium (Invitrogen, CA), supplemented with 5% fetal bovine serum, and plated on 24-well plates the day before transfection so that cells were approximately 60% confluent by the next day. Cells were co-transfected with 490 ng of pGL3 plasmids and 10 ng of pRL-SV40 vector (Promega), using the Lipofectamine™2000 Transfection Reagent (Invitrogen) according to the manufacturer's directions. The pGL3-Basic vector was used as a negative control, and the pGL3-Control vector (Promega) containing the SV40 early promoter was used as a positive control. The pRL-SV40 vector that had the SV40 early promoter upstream of renilla luciferase was used as an internal control. At 16 h after transfection, cells were treated with 50 μM LY294002 or DMSO for another 8 h, the luciferase activity was assayed using Dual-Luciferase Reporter Assay System (Promega) and measured using Lumat LB 9507 (Berthold Technologies, Germany). The relative luciferase activity was expressed as the ratio of firefly to renilla luciferase activity of the same lysate sample. All the luciferase assays were carried out in 10 independent reactions performed in three separate experiments.

For siRNA knockdown experiment, BEL-7404 cells were transfected with Akt1/2 shRNA human lentiviral particles (sc-43609-V, Santa Cruz, CA) or control shRNA lentiviral particles (sc-108080, Santa Cruz, CA), using Lipofectamine™2000 Transfection Reagent according to the manufacturer's directions.

CELL CYCLE ASSAY

For cell cycle assay, cells were digested with 2 mM EDTA in PBS and rinsed twice with ice-cold PBS solution, then fixed by adding them dropwise into 75% ice-cold ethanol while vortexing, followed by incubation in ice for 60 min. The fixed cells were washed with ice-cold PBS and incubated at 37°C for 30 min in 0.5 ml PBS solution containing 20 μg/ml RNase A, 0.2% Triton X-100, 0.2 mM EDTA and 20 μg/ml of propidium iodide. DNA content was determined by FACS analysis (Becton Dickinson). The percentage of cells in G0/G1, S, and G2/M phases were determined using the ModFit program.

STATISTICAL ANALYSIS

For all experimental groups that satisfied the initial ANOVA criterion, individual comparisons were performed applying post hoc Bonferroni *t*-test with the assumption of two-tail distribution and two samples with equal variance. A value of $P < 0.05$ was taken to indicate a significant difference between the mean values.

RESULTS

Akt (Akt1 OR Akt2) MRNA LEVEL ARE ASSOCIATED WITH DNMT3B mRNA LEVEL IN HCC CELL LINES

Kanai and Hirohashi [2007] and Kondo et al. [2007] had shown the presence of DNA hypermethylation in both precancerous conditions and progressed HCC. To know the DNMT3B protein level in HCC tissue, we analyzed 20 pairs of primary HCC and adjacent non-tumorous liver tissue arranged in a tissue microarray slide used by immunohistochemical staining technique (Fig. 1), and found that DNMT3B protein strongly expressed in HCC tissue, with a positive distribution in the nucleus of liver cell. The expression of DNMT3B (++ ~ +++) in HCC tissue (50.0%) was significantly higher ($P < 0.01$) than in each of adjacent noncancerous tissue (10.0%; Table II).

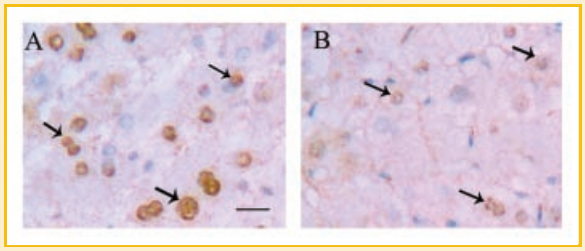

Fig. 1. Immunohistochemical localization of DNA methyltransferase 3B (DNMT3B) in human hepatocellular carcinoma (HCC) tissues. A: Strong expression of DNMT3B in the nucleus of liver cell in HCC tissues. B: Expression of DNMT3B in corresponding matched adjacent non-tumorous tissues. Straight arrows point to DNMT3B expressed in the nucleus of liver cell. Scale bar, 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE II. Comparison of DNMT3B in HCC Tissue With in Adjacent Non-Tumorous Tissue

		DNMT3B expression			
Tissue	Cases	+	++	+++	*P*-value
HCC tissue	20	10	2	8	<0.01
Adjacent non-tumorous tissue	20	18	2	0	

Pearson chi-square test, chi-square = 10.4, $P < 0.01$ from tumor tissue and adjacent non-tumorous tissue.

With investigating the DNMT3B mRNA and protein levels in 6 HCC cell lines and human liver immortal cell line L02. We found inhibition of DNMT3B protein by 0.25 μM 5-AZA treatment for 24 h accompanying decrease of Akt phosphorylation but no obviously change of total Akt levels (Fig. 2A,B). To further analyzed the relationship between Akt and DNMT3B, we detected the mRNA levels of DNMT3Bs (total DNMT3B isoforms, DNMT3B3 and DNMT3B4), Akt1 and Akt2 in seven liver cell lines using real-time RT-PCR, normalized with proliferating cell nuclear antigen (PCNA) mRNA; the relative amount of mRNA of L02 cell line is regarded as 1.0. The DNMT3B mRNA and protein levels in BEL-7402, 7404, 7405 cell lines are higher than that in L02 cell line (Fig. 2B,C and Table III). We found that Akt (Akt1 or Akt2) transcripts are associated with the total DNMT3B transcripts in HCC cell lines, and the total DNMT3B transcripts of 5 HCC cell lines are higher than L02 cell line (Fig. 2C and Table III), the ratio of DNMT3B3 to DNMT3B4 of 6 HCC cell lines varies from 1.1 to 3.0 (Fig. 2C).

REDUCED ACTIVITY OF PI3K PATHWAY DOWN-REGULATES THE DNMT3B mRNA AND PROTEIN LEVELS IN HCC CELL LINES

To investigate the contribution of PI3K signaling in DNMT3B expression, we treated BEL-7404 cells with the different amount of LY294002 (a PI3K pathway inhibitor) and found that LY294002 down-regulated DNMT3B protein (Fig. 3A) level in a dose-dependent manner, 50 μM LY294002 decreased about 50% DNMT3B expression and was used throughout the study. In

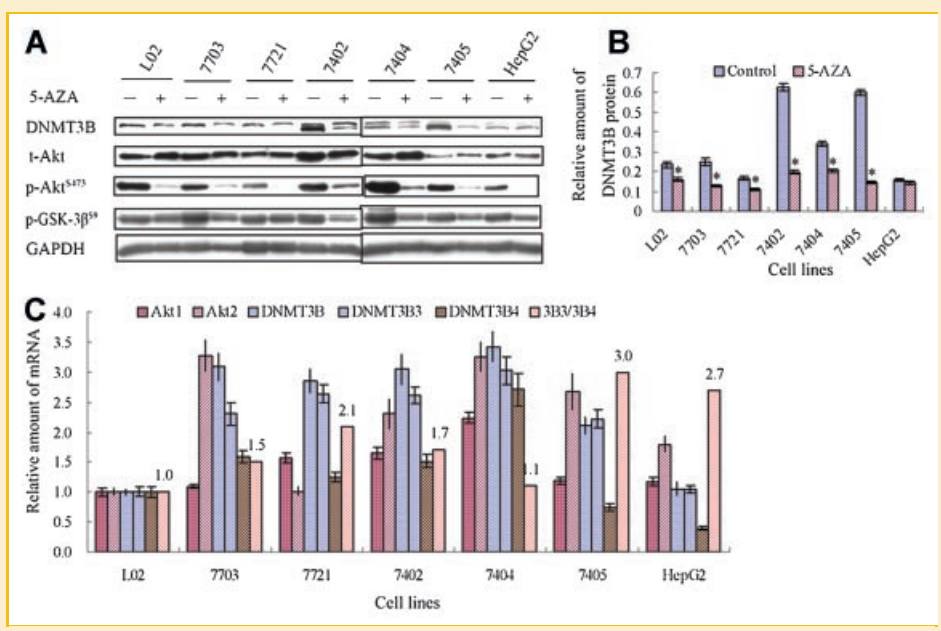

Fig. 2. Akt (Akt1 and Akt2) mRNA level are associated with DNMT3B mRNA level in HCC cell lines. A: Total cell lysates from seven liver cell lines treated with 0.25 μM 5-aza-2′-deoxycytidine (5-AZA) for 24 h were examined by Western blotting analysis. 5-AZA decreases DNMT3B protein level of liver cell lines except for HepG2, total Akt (t-Akt), p-Akt-Ser473 and p-GSK3β-Ser9 (a substrate of Akt) were used to assay the activity of Akt, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as a loading control. B: Quantitative representation of DNMT3B protein in (A) (shown as the mean ± standard deviation (SD) from three experiments; $^{*}P < 0.005$). The data were normalized to GAPDH protein. C: mRNA levels of DNMT3Bs, Akt1 and Akt2 of seven liver cell lines were assessed by real-time RT-PCR with special primers; the data were normalized with proliferating cell nuclear antigen (PCNA) mRNA; the relative amount of mRNA of L02 cell line is regarded as 1.0; the histogram shows the mean ± SD from three independent analyses. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III. Akt (Akt1 or Akt2) mRNA Level Are Associated With DNMT3B mRNA Level in HCC Cell Lines

	Liver cell lines							*P*-value of
mRNA	L02	7703	7721	7402	7404	7405	HepG2	ANOVA
Akt1	1.00 ± 0.07	1.09 ± 0.03	$1.57 \pm 0.08^{*}$	$1.65 \pm 0.10^{*}$	$2.24 \pm 0.09^{*}$	$1.19 \pm 0.06^{\#}$	$1.17 \pm 0.07^{\#}$	<0.001
Akt2	1.00 ± 0.06	$3.28 \pm 0.25^{*}$	1.01 ± 0.07	$2.31 \pm 0.25^{*}$	$3.25 \pm 0.24^{*}$	$2.67 \pm 0.30^{*}$	$1.80 \pm 0.13^{*}$	<0.001
DNMT3B	1.00 ± 0.05	$3.09 \pm 0.24^{*}$	$2.86 \pm 0.20^{*}$	$3.05 \pm 0.25^{*}$	$3.42 \pm 0.25^{*}$	$2.12 \pm 0.14^{*}$	1.05 ± 0.12	<0.001

Individual comparisons were performed applying post hoc Bonferroni *t*-test with the assumption of two-tail distribution: $^{\#}P < 0.05$, $^{*}P < 0.01$; compared with L02 cell.

addition, we performed the time course experiment to evaluate the 50 μM LY294002 effect on DNMT3B mRNA and protein levels. Both DNMT3B protein (Fig. 3B) and mRNA (Fig. 3C) levels were down-regulated by LY294002 in a time-dependent manner, and the DNMT3B mRNA is reduced to about 50% at 4 h time point, prior to the reduction in protein. Here we also found that down-regulation of DNMT3B by LY294002 was also existed in other HCC cell lines (Fig. 3D). To further evaluate the effects of LY294002 on DNMT3B, we performed a CHX chase protein decay assay between LY294002 treated and -untreated cells (Fig. 4). In LY294002-untreated cells or in LY294002-treated cells, the half-life of DNMT3B levels were all about 6 h. Taken together, these findings indicated that PI3K pathway regulated the expression of DNMT3B but did not induce the accelerating degradation of DNMT3B protein in HCC cell lines.

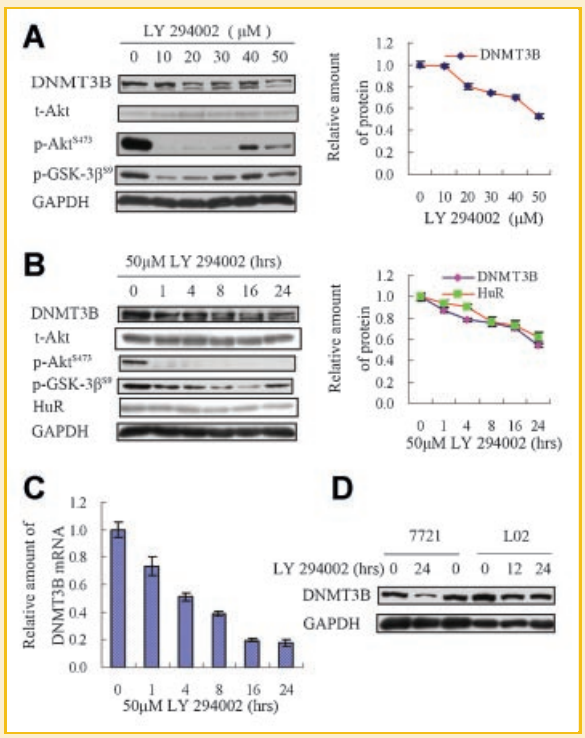

Fig. 3. Reduced activity of PI3K pathway down-regulates the DNMT3B protein and protein levels in HCC cell lines BEL-7404. Total cell lysates were analyzed by Western blotting with antibodies indicated to the left of each panel. A: Dose-dependent regulation of DNMT3B protein level by LY294002 (a specific inhibitor of PI3K pathway) in BEL-7404 cells. Cells were treated with LY294002 or DMSO (control) at indicated concentrations for 24 h. (Left panel) Western blotting, (Right panel) quantitative representation of DNMT3B protein normalized to GAPDH protein. B: Time-dependent regulation of DNMT3B or HuR proteins level by 50 μM LY294002 in BEL-7404 cells. Cells were treated with 50 μM LY294002 or DMSO (control) for the time periods indicated. (Left panel) Western blotting, (Right panel) quantitative representation of DNMT3B protein normalized to GAPDH protein. C: 50 μM LY294002 down-regulates the DNMT3B mRNA level in BEL-7404 cells. Cells were incubated with 50 μM LY294002 or DMSO (control) for the time periods indicated and total RNA was subjected to real-time RT-PCR analysis, the DNMT3B mRNA were normalized to GAPDH mRNA. The data represent the mean ± SD from three independent experiments ($P < 0.01$, compared with the control). D: 50μM LY294002 down-regulates the DNMT3B protien level in SMMC-7721 or L02 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Akt REGULATES THE DNMT3B mRNA AND PROTEIN LEVELS

Akt is an important downstream target of PI3K pathway. To explore whether the high p-Akt-Ser473 protein level was involved in regulating the expression of DNMT3B, we observed the constitutively active Akt2 in Myr-Akt2-MEFs, which has no apparently change of the total Akt protein. We found higher DNMT3B mRNA and protein levels in Myr-Akt2-MEFs than in MSCV-MEFs (Fig. 5A–C). Moreover we evaluated the expression of DNMT3B

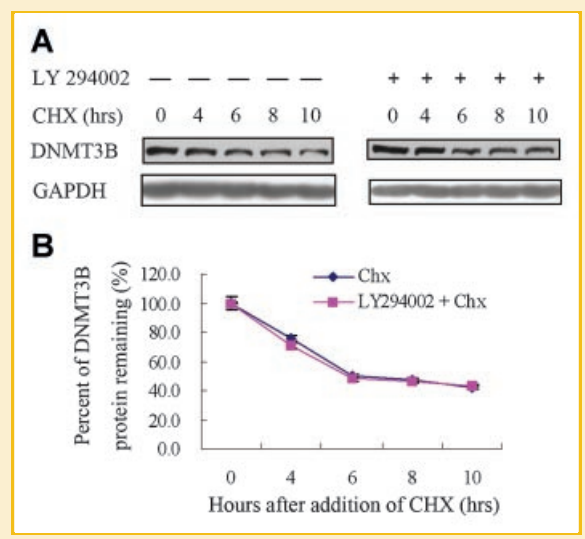

Fig. 4. Reduced activity of PI3K pathway does not induce the accelerating degradation of DNMT3B protein in BEL-7404 cells. DNMT3B protein decay was studied by incubating cells with 50 μM LY294002 or DMSO for 2 h, then adding 50 μg/ml cycloheximide (CHX). Total cell lysates were collected at the indicated intervals after CHX treatment, DNMT3B proteins were assessed by Western blotting. A: Western blotting. B: Quantitative representation of DNMT3B protein normalized to GAPDH. The data represent the mean ± SD from three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in BEL-7404 stably transfected with Akt1/2 shRNA human lentiviral particles (Akt1/2 siRNA), found that the knockdown of Akt1 and Akt2 caused the decrease of DNMT3B mRNA and protein levels (Fig. 5D–F). All these findings suggest that Akt modulates the DNMT3B expression.

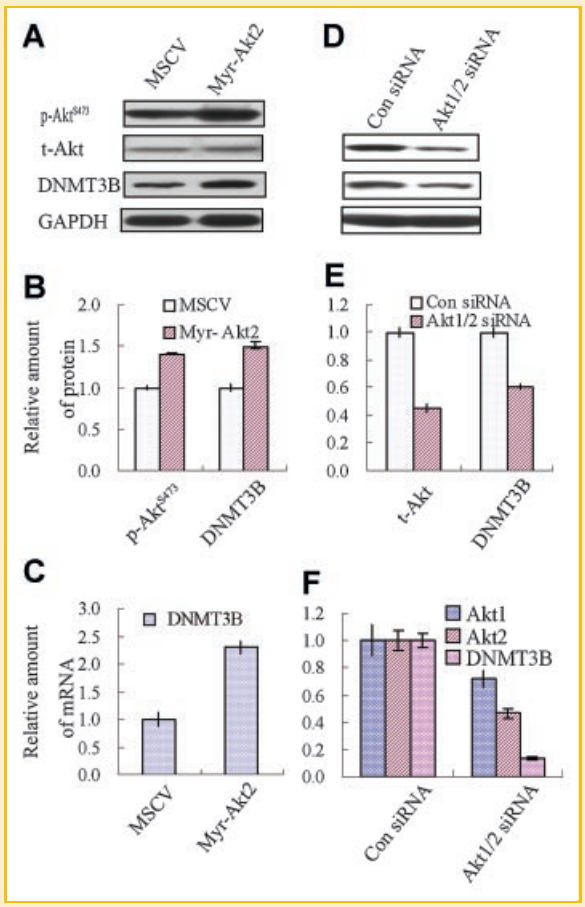

Fig. 5. Akt regulates the DNMT3B mRNA and protein levels. A–C: Expression of a constitutively active Akt2 up-regulates DNMT3B mRNA and protein levels in the myristylated variants of Akt2 (Myr-Akt2) mouse embryonic fibroblasts (MEFs) stably transfected with Myr-Akt2; the murine stem cell virus vector (MSCV) MEFs stably transfected with empty MSCV was regarded as control; the data show the mean ± SD from three independent experiments ($P < 0.01$, compared with the control). A: Total cell lysates were assessed by Western blotting. B: Quantitative representation of p-Akt-Ser473 and DNMT3B protein in (A) normalized to GAPDH. C: mouse DNMT3B mRNA was determined by real-time RT-PCR, normalized to mouse GAPDH mRNA. D–F: Human DNMT3B mRNA and protein levels were down-regulated in stably transfected BEL-7404 cells with Akt1/2 shRNA human lentiviral particles (Akt1/2 siRNA). BEL-7404 cells were stably transfected with human Akt1/2 siRNA or control shRNA lentiviral particles (con siRNA) using Lipofectamine™2000 Transfection Reagent according to the manufacturer's directions. The data show the mean ± SD from three independent analyses ($P < 0.01$, compared with the control). D: Whole cell lysates were assessed by Western blotting. E: Quantitative representation of t-Akt and DNMT3B protein in (D) normalized to GAPDH. F: Human Akt1, Akt2 and DNMT3B mRNAs were assessed by real-time RT-PCR, normalized to human GAPDH mRNA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

REDUCED ACTIVITY OF PI3K PATHWAY DOWN-REGULATES DNMT3B AT TRANSCRIPTION-CONTROL

In order to determine whether LY294002 regulate the expression of human DNMT3B at transcriptional control, we amplified four 5′-flanking genomic regions fragments (Fig. 6A) which contain some regions flanking the defined first exon of DNMT3B1 according to Yanagisawa's study [Yanagisawa et al., 2002]. The DNMT3B1 specific fragments were sequenced and inserted upstream of the firefly luciferase gene in the pGL3-Basic vector and assayed for their luciferase activities (Fig. 6B). With transiently transfection experiments, we found p3b-900 (−696/+204) had the highest activity while p3b-246 (−40/+206) tended to the similar activity of the

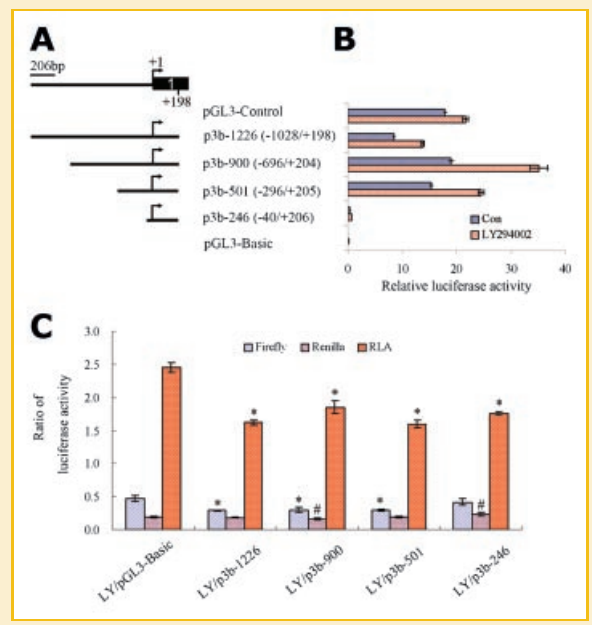

Fig. 6. Reduced activity of PI3K pathway down-regulates DNMT3B in a transcription-control. A: Schematic representation of a series of DNMT3B promoter-luciferase reporter constructs. The transcription start site is denoted by +1 and indicated by a horizontal arrow. Upstream genomic regions of various sizes, all containing part of the first exon, were linked to the 5′-end of cDNA encoding firefly luciferase (*Luc*) in the expression vector pGL3-Basic. B: Dual-luciferase activity assay of DNMT3B promoter-luciferase activity in LY294002 treated or untreated BEL-7404 cells. BEL-7404 cells were transfected with the luciferase reporter constructs or pGL3-Basic vector (as negative control) and pGL3-Control vector (as positive control) in the presence of renilla luciferase vector pRL-SV40, at 16 h after transfection, cells were treated with 50 μM LY294002 or DMSO for 8 h, both luciferase activities were then assessed by measuring luminescence. The DNMT3B promoter-driven firefly luciferase activity was normalized by the Renilla luciferase activity to correct for variation due to transfection-efficiency differences. The data show the mean ± SD from 10 independent reactions performed in three separate experiments. C: LY294002 inhibits DNMT3B promoter-luciferase activity. The data respectively represent the ratio of firefly luciferase activity of pGL3 plasmids, renilla luciferase of pRL-SV40 plasmids and relative luciferase activity of pGL3 plasmids in LY294002 treated or untreated BEL-7404 cells transfected with plasmids. The data show the mean ± SD from 10 independent reactions performed in three separate experiments ($^{\#}P < 0.05$, $^{*}P < 0.01$; compared with BEL-7404 cells transfected with pGL3-Basic plasmid). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

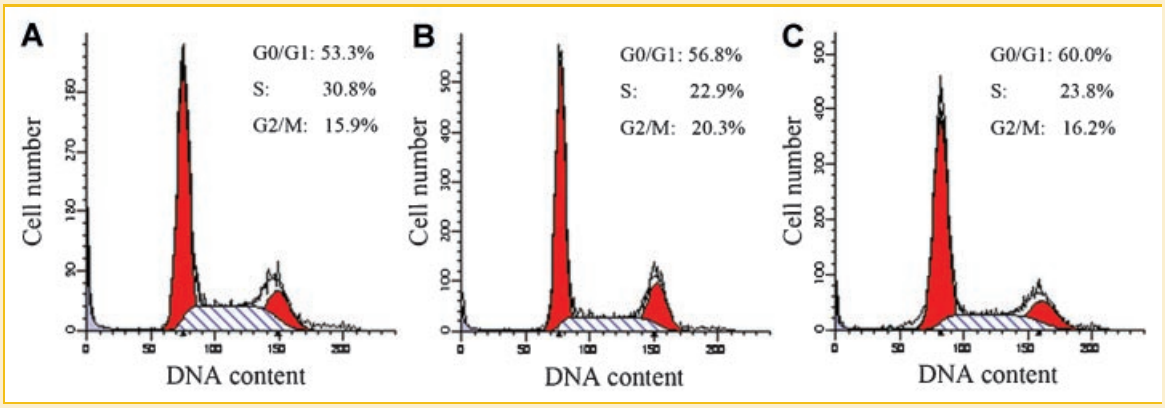

Fig. 7. LY294002 induced a slight arrest of phases G1. The numbers in the frame represent the mean of three independent experiments ($P < 0.05$, compared with DMSO control group, Fisher's exact test). Cells were treated with: A: DMSO only; (B) 50 μM LY294002 for 8 h; (C) 50 μM LY294002 for 24 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pGL3-Basic Vector, however p3b-1226 (−1028/+204) plasmid containing longer fragments than the p3b-900 (−696/+204) showed decreased activity (Fig. 6B). Moreover we found the relative luciferase activities of the luciferase reporter constructs treated with 50 μM LY294002 was higher than those of untreated group (Fig. 6B). Meanwhile we investigated that both the luciferase activities of firefly and renilla were decreased in BEL-7404 cells transiently transfected with these pGL3 plasmids under LY294002 treatment, which were partly consistent with the data of cell cycle assay that represented cycle arrest of the G1 phase in BEL-7404 cell treated with 50 μM LY294002 for 8 h (Fig. 7). In order to eliminate the interference of pGL3-Basic vector treated with LY294002, we evaluated the ratio of relative luciferase activity of pGL3 plasmids in LY294002 treated or untreated BEL-7404 cells transfected with plasmids (Fig. 6C). All the ratio of the DNMT3B1 promoter constructs is lower than the ratio of the pGL3-Basic ($^{*}P < 0.01$, compared with BEL-7404 cells transfected with pGL3-Basic plasmid), which suggested that reduced activity of PI3K pathway inhibited the DNMT3B transcription.

REDUCED ACTIVITY OF PI3K PATHWAY REDUCES DNMT3B mRNA STABILITY

In view of the steady-state levels of DNMT3B mRNA were reduced by 50% in BEL-7404 cell treated with LY294002 for 4 h compared with untreated cells, we evaluated if the reduction in the DNMT3B mRNA levels was due to changes in mRNA stability, the DNMT3B mRNA stability ($t_{1/2}$) was analyzed following treatment with Act D to inhibit de novo transcription. The levels of DNMT3B mRNA (using primers that particularly amplify all DNMT3B isoforms) and the housekeeping control GAPDH mRNA were monitored by real-time RT-PCR As shown, DNMT3B mRNA was found to be significantly more stable in the control group, with an estimated $t_{1/2} = 4.64$ h, while DNMT3B mRNA stability was markedly reduced in the LY294002 group, with $t_{1/2} = 3.63$ h (Fig. 8). de Silanes et al. [2009] had found that the RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3B mRNA, so we assessed the HuR expression in BEL-7404 treated with LY294002, and found PI3K pathway was also involved in regulating HuR expression in a time-dependent manner (Fig. 3B). Together, these data implicates that reduced activity of PI3K pathway reduces DNMT3B mRNA stability.

DISCUSSION

HCC is the fifth most common malignancy and the third leading cause of cancer deaths worldwide [Jain et al., 2010]. Hepatocarcinogenesis is strongly linked to multiple risk factors in humans, such as increases in allelic losses, chromosomal changes, gene mutations, epigenetic alterations, and alterations in molecular cellular pathways [Huynh et al., 2003; Alexia et al., 2006; Boyault et al., 2007]. There are many studies about PI3K/Akt pathway activation occur in

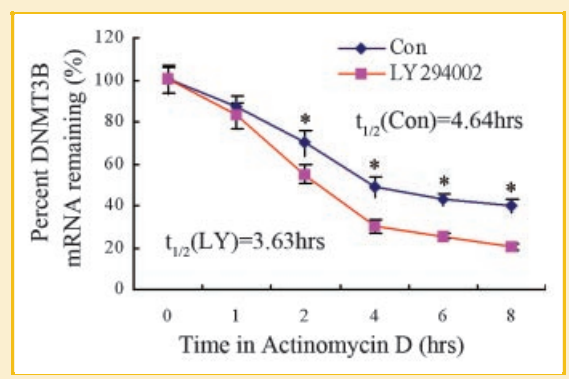

Fig. 8. Reduced activity of PI3K pathway reduces DNMT3B mRNA stability in BEL-7404 cells. DNMT3B mRNA stability was studied by incubating cells with 50 μM LY294002 or DMSO for 2 h, then adding 5 μg/ml actinomycin D (ActD). Total RNA was collected at various time periods after Act D treatment, DNMT3B mRNA levels were analyzed by real-time RT-PCR and normalized to GAPDH mRNA. The data show the mean ± SD from three independent experiments ($^{*}P < 0.01$, compared with the control at the corresponding point in time). The half-lives ($t_{1/2}$) of DNMT3B mRNA was calculated using linear regression analysis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the course of HCC pathogenesis [Cotler et al., 2008; Chen et al., 2009; Dai et al., 2009]. Aberrant DNA methylation, one of the best important epigenetic alterations, consisting of DNA hypomethylation and/or promoter gene CpG hypermethylation, is also implicated in the development of a variety of solid tumors, including HCC [Calvisi et al., 2007]. The relationship between PI3K/Akt pathway and aberrant DNA methylation is unknown.

In this study, we demonstrated that higher DNMT3B protein in HCC tissues than in the matched adjacent non-tumorous tissues, which is similar to that reported by Kanai and Hirohashi [2007] and Kondo et al. [2007]. Meanwhile we found that Akt (Akt1 or Akt2) transcripts are associated with the total DNMT3B transcripts in HCC cell lines, the ratio of DNMT3B3 to DNMT3B4 in 6 HCC cell lines which varies from 1.1 to 3.0. Whereas Saito et al., 2002 and Kanai and Hirohashi [2007] shown overexpression of DNMT3B4 associated with human hepatocarcinogenesis, we hypothesized that difference in the ratio of DNMT3B3 to DNMT3B4 in HCC resulted from complexity of hepatocarcinogenesis. The expression of DNMT3B down-regulated by LY294002 in a dose- and time-dependent manner, suggests that PI3K pathway is involved in regulating the expression of DNMT3B. With regard to the myristylated variants of Akt2 was strongly enhanced oncogenic potential and increased kinase activities in vitro and in vivo, while wild type Akt2 was not oncogenic in vivo [Mende et al., 2001], we constructed the Myr-Akt2-MEFs (the expressed constitutively active Akt2) and the MSCV-MEFs (containing empty MSCV), and determined the significantly high DNMT3B mRNA and protein levels resulted from high p-Akt-Ser473 protein. Although we could not inhibit DNMT3B protein level by using Akt1/2 siRNA completely, a significantly decrease in DNMT3B mRNA was also found. Taken together, our results support the hypothesis that Akt regulate the expression of DNMT3B in vivo.

The DNMT3B mRNA reduction prior to the change of DNMT3B protein (Fig. 3B,C), implied that there is transcriptional control regulation of DNMT3B by PI3K pathway. To determine whether DNMT3B mRNA transcription is modulated by PI3K/Akt pathway, we constructed four DNMT3B1 promoter-driven luciferase plasmids which had different promoter activity and included core promoter and base promoter according to Yanagisawa's study [Yanagisawa et al., 2002]. Whereas the six alternatively spliced the human DNMT3B gene transcripts have been identified, and DNMT3B1 isoform is the longest transcript, we took the promoter sequence of DNMT3B1 as our study object. Another reason of selecting DNMT3B1 promoter in our study was 5′-UTR of sequences of three mainly expressed DNMT3B isoforms (DNMT3B1, -2, -3) are identical though they have different transcriptional start point and different sequences of first exon. With Daul-Luciferase reporter Assay, we found that the p3b-900 (−696/+198) had the highest relative luciferase activity (RLU) and the p3b-246 (−40/+206) had the lowest RLU in BEL-7404 cell, these results are similar to that reported by Yanagisawa in NT2 cells [Yanagisawa et al., 2002]. But p3b-1226 (−1028/+198) represented lower RLU than p3b-501 (−296/+205) in BEL-7404 cells, which showed difference with that reported by Yanagisawa et al. [2002] in NT2 cells. This discrepancy is probably attributable to different host cells used or difference in the promoter fragment length. Transcriptional regulation under LY294002 stimulation had been found in Aurora A kinase gene [Liu et al., 2008]. In our study we did not directly use RLU as the criterion to judging LY294002 effects on DNMT3B transcriptional regulation, as we found that replication of pGL3 plasmids were all inhibited under with LY294002 treatment. In order to eliminate the interference, some of which maybe resulted from a slight G1 arrest, we evaluated the ratio of relative luciferase activity of pGL3 plasmids in LY294002 treatment or untreatment BEL-7404 cells transfected with plasmids. Furthermore we took the ratio of relative luciferase activity of the pGL3-Basic (occupying base transcription) under LY294002 treated or untreated to be the criterion, and found the ratio of the DNMT3B promoter constructs were lower than the criterion. These results indicated that PI3K/Akt pathway increase the expression of DNMT3B at transcription control. To date, there are not much data concerning the transcriptional regulation of DNMT3B. Yanagisawa et al. [2002] firstly reported the promoters of human DNMT3B contain several Sp-binding sites, and Hervouet et al. [2009a] and Jinawath et al. [2005] further found that Sp proteins, particularly Sp3, were essential for the expression of DNMT3B. In addition, Hervouet et al. [2009b] further identified more than 42 transcription factors interacting with DNMT3B (such as Sp, C/EBPalpha, NFkappaB-p65, CREB and FOS) by monitoring transcription factor arrays spotted with 103 transcription factors, but they did not report the roles of those transcription factors in regulating DNMT3B. Although some stimuli such as folate supplementation [Hervouet et al., 2009a], estrogen [Cui et al., 2009], and tamoxifen [Tryndyak et al., 2006; Kubarek et al., 2009] could affect the expression of DNMT3B, the mechanism of regulating DNMT3B is still unknown. Signaling molecules of PI3K/Akt pathway such as Akt could regulate expression of c-met, vascular endothelial growth factor (VEGF), Redd1, and matrix metalloproteinase-2 (MMP-2) via SP [Zhang et al., 2003; Pore et al., 2004; Qureshi et al., 2007; Lin et al., 2009], moreover the expression of MMP-2 is also modulated by Akt via NF-kappaB. Whether PI3K/Akt pathway regulates the expression of DNMT3B via SP or NF-kappaB and how these transcriptional factors modulate the DNMT3B transcription need to be further investigated.

DNMT3B mRNA decay assay with Act D indicated DNMT3B mRNA be regulated by PI3K pathway in the post-transcription level. This hypothesis was further supported with HuR expression down-regulated with LY294002 treatment in a time-dependent manner in BEL-7404. HuR, an RNA binding protein that modulates the translation of target mRNAs, can stabilize the expression of DNMT3B in post-transcription level by binding to the 3′-UTR of the DNMT3B mRNA [de Silanes et al., 2009]. Taken together, we hypothesize that HuR is a mediator of regulating DNMT3B mRNA stability by PI3K pathway. DNMT3B transcripts stability is also regulated by miR-29s [Fabbri et al., 2007; Garzon et al., 2009] which bind the 3′-UTR of DNMT3B and miR-148 [Duursma et al., 2008] which binds the coding regions of DNMT3B transcripts. Whether PI3K/Akt pathway affects DNMT3B expression by regulating miR-29s and miR-148 will be further evaluated.

In summary, we have, for the first time, demonstrated that PI3K/Akt pathway regulates the expression of DNMT3B at transcriptional and post-transcriptional levels. These results are particularly

important to understand the effects of PI3K/Akt and DNMT3B on hepatocarcinogenesis.

REFERENCES

Alexia C, Bras M, Fallot G, Vadrot N, Daniel F, Lasfer M, Tamouza H, Groyer A. 2006. Pleiotropic effects of PI-3′ kinase/Akt signaling in human hepatoma cell proliferation and drug-induced apoptosis. Ann NY Acad Sci 1090: 1–17.

Beaulieu N, Morin S, Chute IC, Robert MF, Nguyen H, MacLeod AR. 2002. An essential role for DNA methyltransferase DNMT3B in cancer cell survival. J Biol Chem 277:28176–28181.

Boyault S, Rickman DS, de Reynies A, Balabaud C, Rebouissou S, Jeannot E, Herault A, Saric J, Belghiti J, Franco D, Bioulac-Sage P, Laurent-Puig P, Zucman-Rossi J. 2007. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 45:42–52.

Calvisi DF, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, Schroeder I, Factor VM, Thorgeirsson SS. 2007. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. J Clin Invest 117:2713–2722.

Chen JS, Wang Q, Fu XH, Huang XH, Chen XL, Cao LQ, Chen LZ, Tan HX, Li W, Bi J, Zhang LJ. 2009. Involvement of PI3K/PTEN/AKT/mTOR pathway in invasion and metastasis in hepatocellular carcinoma: Association with MMP-9. Hepatol Res 39:177–186.

Cotler SJ, Hay N, Xie H, Chen ML, Xu PZ, Layden TJ, Guzman G. 2008. Immunohistochemical expression of components of the Akt-mTORC1 pathway is associated with hepatocellular carcinoma in patients with chronic liver disease. Dig Dis Sci 53:844–849.

Cui M, Wen Z, Yang Z, Chen J, Wang F. 2009. Estrogen regulates DNA methyltransferase 3B expression in Ishikawa endometrial adenocarcinoma cells. Mol Biol Rep 36:2201–2207.

Dai R, Chen R, Li H. 2009. Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells. Int J Oncol 34:1749–1757.

de Silanes IL, Gorospe M, Taniguchi H, Abdelmohsen K, Srikantan S, Alaminos M, Berdasco M, Urdinguio RG, Fraga MF, Jacinto FV, Esteller M. 2009. The RNA-binding protein HuR regulates DNA methylation through stabilization of DNMT3b mRNA. Nucleic Acids Res 37:2658–2671.

Duursma AM, Kedde M, Schrier M, le Sage C, Agami R. 2008. miR-148 targets human DNMT3b protein coding region. RNA 14:872–877.

Eads CA, Danenberg KD, Kawakami K, Saltz LB, Danenberg PV, Laird PW. 1999. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression. Cancer Res 59:2302–2306.

Esteller M. 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 8:286–298.

Esteller M. 2008. Epigenetics in cancer. N Engl J Med 358:1148–1159.

Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM. 2007. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104:15805–15810.

Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G. 2009. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418.

Girault I, Tozlu S, Lidereau R, Bieche I. 2003. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res 9:4415–4422.

Hartmann W, Digon-Sontgerath B, Koch A, Waha A, Endl E, Dani I, Denkhaus D, Goodyer CG, Sorensen N, Wiestler OD, Pietsch T. 2006. Phosphatidylinositol 3'-kinase/AKT signaling is activated in medulloblastoma cell proliferation and is associated with reduced expression of PTEN. Clin Cancer Res 12:3019–3027.

Herman JG, Baylin SB. 2003. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349:2042–2054.

Hervouet E, Debien E, Campion L, Charbord J, Menanteau J, Vallette FM, Cartron PF. 2009a. Folate supplementation limits the aggressiveness of glioma via the remethylation of DNA repeats element and genes governing apoptosis and proliferation. Clin Cancer Res 15:3519–3529.

Hervouet E, Vallette FM, Cartron PF. 2009b. Dnmt3/transcription factor interactions as crucial players in targeted DNA methylation. Epigenetics 4:487–499.

Hodge DR, Cho E, Copeland TD, Guszczynski T, Yang E, Seth AK, Farrar WL. 2007. IL-6 enhances the nuclear translocation of DNA cytosine-5-methyltransferase 1 (DNMT1) via phosphorylation of the nuclear localization sequence by the AKT kinase. Cancer Genomics Proteomics 4:387–398.

Huynh H, Nguyen TT, Chow KH, Tan PH, Soo KC, Tran E. 2003. Over-expression of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK in hepatocellular carcinoma: Its role in tumor progression and apoptosis. BMC Gastroenterol 3:19.

Jain S, Singhal S, Lee P, Xu R. 2010. Molecular genetics of hepatocellular neoplasia. Am J Transl Res 2:105–118.

Jin F, Dowdy SC, Xiong Y, Eberhardt NL, Podratz KC, Jiang SW. 2005. Up-regulation of DNA methyltransferase 3B expression in endometrial cancers. Gynecol Oncol 96:531–538.

Jinawath A, Miyake S, Yanagisawa Y, Akiyama Y, Yuasa Y. 2005. Transcriptional regulation of the human DNA methyltransferase 3A and 3B genes by Sp3 and Sp1 zinc finger proteins. Biochem J 385:557–564.

Kanai Y, Hirohashi S. 2007. Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state. Carcinogenesis 28:2434–2442.

Kondo Y, Shen L, Suzuki S, Kurokawa T, Masuko K, Tanaka Y, Kato H, Mizuno Y, Yokoe M, Sugauchi F, Hirashima N, Orito E, Osada H, Ueda R, Guo Y, Chen X, Issa JP, Sekido Y. 2007. Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatol Res 37:974–983.

Kubarek L, Kozlowska A, Przybylski M, Lianeri M, Jagodzinski PP. 2009. Down-regulation of CXCR4 expression by tamoxifen is associated with DNA methyltransferase 3B up-regulation in MCF-7 breast cancer cells. Biomed Pharmacother 63:586–591.

Lin HC, Song TY, Hu ML. 2009. S-Adenosylhomocysteine promotes the invasion of C6 glioma cells via increased secretion of matrix metalloproteinase-2 in murine microglial BV2 cells. Toxicol Sci 112:322–330.

Liu X, Shi Y, Woods KW, Hessler P, Kroeger P, Wilsbacher J, Wang J, Wang JY, Li C, Li Q, Rosenberg SH, Giranda VL, Luo Y. 2008. Akt inhibitor a-443654 interferes with mitotic progression by regulating aurora a kinase expression. Neoplasia 10:828–837.

Mende I, Malstrom S, Tsichlis PN, Vogt PK, Aoki M. 2001. Oncogenic transformation induced by membrane-targeted Akt2 and Akt3. Oncogene 20:4419–4423.

Montiel-Duarte C, Cordeu L, Agirre X, Roman-Gomez J, Jimenez-Velasco A, Jose-Eneriz ES, Garate L, Andreu EJ, Calasanz MJ, Heiniger A, Torres A, Prosper F. 2008. Resistance to Imatinib Mesylate-induced apoptosis in acute lymphoblastic leukemia is associated with PTEN down-regulation due to promoter hypermethylation. Leuk Res 32:709–716.

Okano M, Xie S, Li E. 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet 19:219–220.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247–257.

Patra SK, Patra A, Zhao H, Dahiya R. 2002. DNA methyltransferase and demethylase in human prostate cancer. Mol Carcinog 33:163–171.

Phillips JM, Burgoon LD, Goodman JI. 2009. Phenobarbital elicits unique, early changes in the expression of hepatic genes that affect critical pathways in tumor-prone B6C3F1 mice. Toxicol Sci 109:193–205.

Pore N, Liu S, Shu HK, Li B, Haas-Kogan D, Stokoe D, Milanini-Mongiat J, Pages G, O'Rourke DM, Bernhard E, Maity A. 2004. Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism. Mol Biol Cell 15:4841–4853.

Qureshi HY, Ahmad R, Sylvester J, Zafarullah M. 2007. Requirement of phosphatidylinositol 3-kinase/Akt signaling pathway for regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-beta in human chondrocytes. Cell Signal 19:1643–1651.

Robert MF, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR. 2003. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 33:61–65.

Saito Y, Kanai Y, Sakamoto M, Saito H, Ishii H, Hirohashi S. 2002. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci USA 99:10060–10065.

Saito Y, Kanai Y, Nakagawa T, Sakamoto M, Saito H, Ishii H, Hirohashi S. 2003. Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas. Int J Cancer 105:527–532.

Sun L, Zhao H, Xu Z, Liu Q, Liang Y, Wang L, Cai X, Zhang L, Hu L, Wang G, Zha X. 2007. Phosphatidylinositol 3-kinase/protein kinase B pathway stabilizes DNA methyltransferase I protein and maintains DNA methylation. Cell Signal 19:2255–2263.

Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Montgomery B, Churchwell MI, Ross SA, Beland FA, Pogribny IP. 2006. Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: Implications for tamoxifen-induced hepatocarcinogenesis. Carcinogenesis 27:1713–1720.

Wymann MP, Marone R. 2005. Phosphoinositide 3-kinase in disease: Timing, location, and scaffolding. Curr Opin Cell Biol 17:141–149.

Xie S, Wang Z, Okano M, Nogami M, Li Y, He WW, Okumura K, Li E. 1999. Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236:87–95.

Yanagisawa Y, Ito E, Yuasa Y, Maruyama K. 2002. The human DNA methyltransferases DNMT3A and DNMT3B have two types of promoters with different CpG contents. Biochim Biophys Acta 1577:457–465.

Zhang X, Li Y, Dai C, Yang J, Mundel P, Liu Y. 2003. Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney. Am J Physiol Renal Physiol 284:F82–F94.