Molecular Medicine

Supplemental Data

Functional Characterization of Glycine *N*-Methyltransferase and Its Interactive Protein DEPDC6/DEPTOR in Hepatocellular Carcinoma

Chia-Hung Yen,[1] Yao-Cheng Lu,[1] Chung-Hsien Li,[1] Cheng-Ming Lee,[1] Chia-Yen Chen,[1] Ming-Yuan Cheng,[2] Shiu-Feng Huang,[3] Kuen-Feng Chen,[4] Ann-Lii Cheng,[5] Li-Ying Liao,[6] Yan-Hwa Wu Lee,[7] and Yi-Ming Arthur Chen[1,8]

Online address: http://www.molmed.org

SUPPLEMENTARY MATERIALS AND METHODS

Plasmids and Lentiviral Constructs

The coding region of *DEPTOR* (*DEPDE6*) was obtained from complementary DNA (cDNA) extracted from HEK293T cells using reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA clone was inserted into a pcDNA3-HA vector (Invitrogen, Carlsbad, Calif., USA). To construct the various different DEPTOR and GNMT expression plasmid segments, pHA-DEPTOR and pGNMT-FLAG plasmids (1) were used as templates for PCR and ligated into pcDNA3-HA and pFLAG-CMV5a, respectively. To generate the lentiviral expression construct for GNMT and DEPTOR, the coding sequences for FLAG-tagged GNMT and HA-tagged DEPTOR were inserted into the lentiviral vector pLKO_AS3w.eGFP.puro (National RNAi Core Facility, Academia Sinica, Taiwan). The resultant lentiviral constructs were designated as pLV-GNMT-FLAG and pLV-HA-DEPTOR, respectively. A plasmid (pGNMT^{N140S}) containing a mutant human *GNMT* cDNA (asparagine-140 was replaced with serine) was generated by site-directed mutagenesis based on the procedures described by Ho et al. (2). The primers used in the PCR reactions mentioned above are listed in the Supplementary Table 2. The plasmids encoding different shRNAs for *DEPTOR* (pLKO.1-shDEPTOR-1 and pLKO.1-shDEPTOR-2) were purchased from Addgene (http://www.addgene.org). pLKO.1-shLuc (National RNAi Core Facility) targeting the luciferase gene was used as a control for the RNA interference experiments. pEGFP-C2 plasmid is kind gift from Dr. Yueh-Hsin Ping, National Yang Ming University, Taipei, Taiwan.

Western Blotting

Cells were washed with PBS and lysed in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Tx-100) supplemented with protease- and phosphatase inhibitors (1 mM PMSF, 10 μg/ml Leupeptin, 50 μg/ml TLCK, 50 μg/ml TPCK, 1 μg/ml Aprotinin, 1 mM NaF, 5 mM NaPPi, and 10 mM Na_3VO_4). The cell lysates were cleared via two 20 min spins at 13,200 rpm, 4°C. Protein concentrations were determined by Bio-Rad Protein Assay. Samples corresponded to 20 μg of protein were used for Western blotting as described previously.(1) The commercial antibodies used were: mouse monoclonal antibodies (mAbs) against HA (Covance, Berkeley, CA); mAb against FLAG (Sigma Co, St. Louis, MO); rabbit polyclonal antibody against DEPTOR (Upstate/Millipore, Billerica, MA); goat polyclonal antibodies against mTOR (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyconcal antibodies against phospho-T389 S6K1, phospho-S473 Akt, S6K1, Akt, Beclin-1 (Cell Signaling Technology, Beverly, MA) and rabbit monocloncal antibodies against phospho-T37/T46 4EBP1, 4EBP1, LC-3B, caspase-3 (Cell Signaling Technology).

SUPPLEMENTARY REFERENCES

1. Chen SY, et al. (2004) Glycine N-methyltransferase tumor susceptibility gene in the benzo(a)pyrene-detoxification pathway. *Cancer Res* 64: 3617-3623.
2. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77: 51-59.

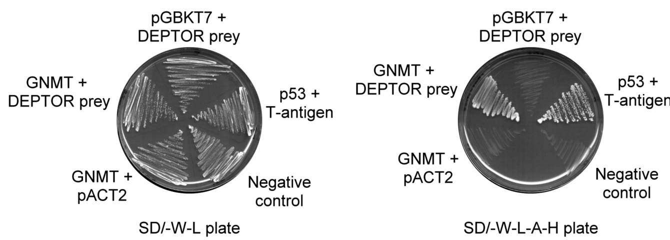

Figure S1. The DEPTOR prey (including its PDZ domain) was tested for interaction with GNMT. The yeast was grown in media lacking tryptophan and leucine (-W-L), which selects for the presence of plasmids. Only those combinations displaying a strong interaction grew under stringent conditions, specifically on medium lacking tryptophan, leucine, adenine and histidine (-W-L-A-H). SV40 T antigen combined with p53 served as a positive control. Lamin C, which neither forms complexes nor interacts with most other proteins, served as a negative control. SD: synthetic dropout medium.

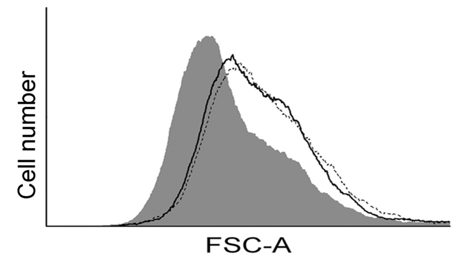

Figure S3. DEPTOR knockdown in HuH-7 cells causes an increase in cell size. Forward scatter measurement of cell size of DEPTOR shRNA knockdowns in HuH-7 (——, shDEPTOR-1; ------, shDEPTOR-2) compared to luciferase shRNA (■, shLuc).

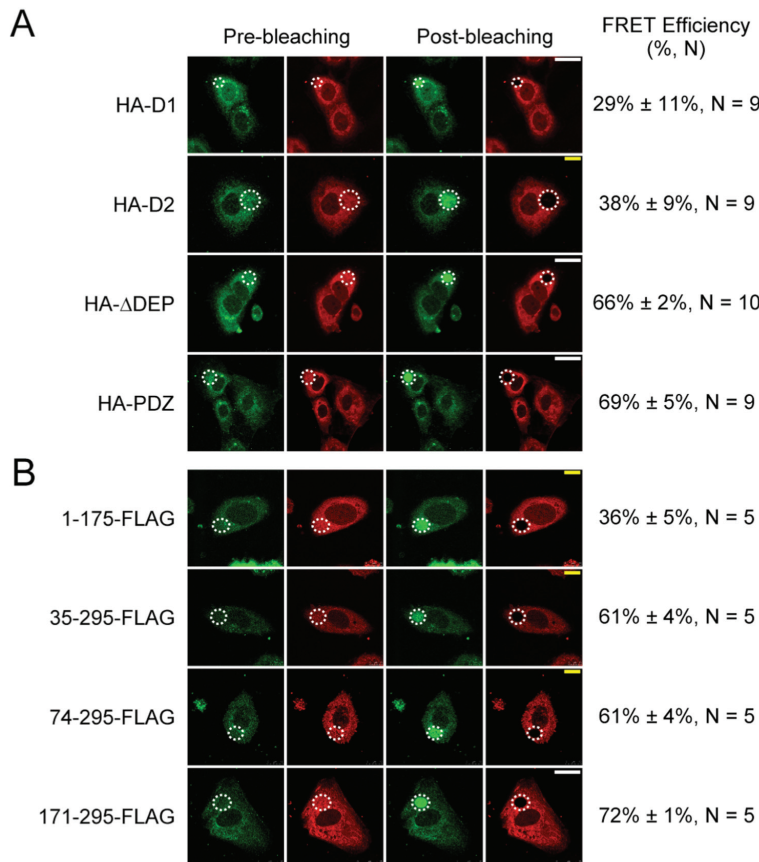

Figure S2. The image results of Fig. 1f. HuH-7 cells were co-transfected with (A) full length GNMT and different segments of DEPTOR or (B) full length DEPTOR and different segments of GNMT. Cells then were fixed and immunofluorescent stained for DEPTOR (FITC, energy donor) and GNMT (Rhodamin, energy acceptor). FRET was measured using a Leica TCS SP5 Confocal Spectral Microscope. White dot circles represent the region of interest (ROI), which is the area of photobleaching in the rhodamine channel. One ROI was selected for bleaching in each cell. The data represents the mean ± S.D. N represents the total numbers of ROIs for the FRET experiments. Yellow bar = 10 μm; white bar = 25 μm.

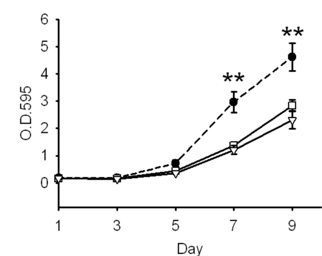

Figure S4. DEPTOR knockdown in HA22T cells led to a decrease in proliferation. HA22T cells bore shRNA against LacZ (---●---, shLacZ) or DEPTOR (—□—, shDEPTOR-1; —▽— shDEPTOR-2) were seeded on 48-well plate and cell number at indicated time point was evaluated by crystal violet staining. Data were normalized against OD595 values on d 1 of each treatment. DEPTOR knockdown in HuH-7 cells led to a decrease in proliferation. Each experiment was performed in triplicate; error bars represent S.D. **, p <0.01.

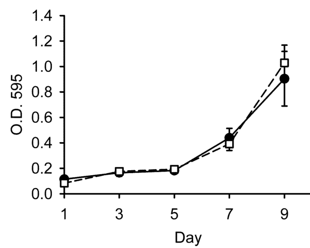

Figure S5. Overexpression of DEPTOR does not enhance growth ability. GFP and DEPTOR stable cells were seeded on 48-well plate and cultured in complete DMEM medium for indicated period. Cell number at indicated time point was evaluated by crystal violet staining. Each experiment was performed in triplicate. —●—, GFP; ---□---, DEPDC6.

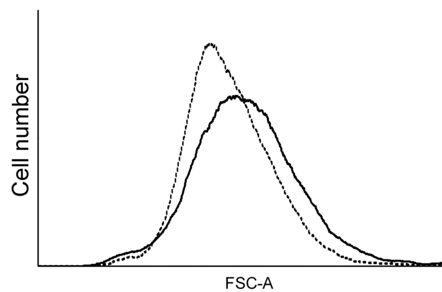

Figure S6. GNMT overexpression in HuH-7 cells causes an increase in cell size. Forward scatter measurement of cell size of a GNMT stable cell line (——) compared to a GFP stable cell line (------).

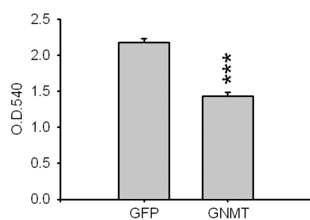

Figure S7. GNMT overexpression in HepG2 cells led to a decrease in proliferation. GFP and GNMT stable cells were seeded on 96-well plate and cultured for 3 d. The cell numbers were evaluated by MTT assay. Each experiment was performed in triplicate; error bars represent S.D. ***, $p < 0.001$.

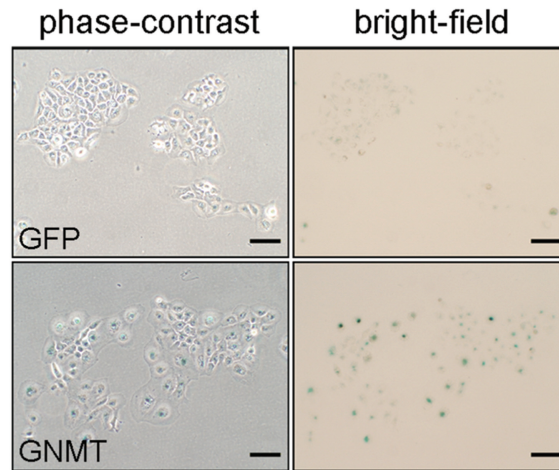

Figure S8. Expression of senescence-associated (SA) β-gal in GFP and GNMT stable cells. GFP and GNMT stable cells were fixed and subjected to SA-β-gal assay as described in Materials and methods. Representative pictures are shown (BAR = 100 μm).

Supplementary Table 1. Death hazard ratios for prognostic factors from Cox proportional hazard regression analysis

Variable	Hazard ratio (95% CI)	*P* value
Gender		
Male vs Female	1.04 (0.40 - 2.69)	0.942
Tumor type		
Multiple vs Solitary	1.79 (0.60 - 5.28)	0.295
TNM stage		
II+III+IV vs I	2.25 (0.73 - 6.90)	0.158
DEPTOR expression		
T>TA vs T≤TA	4.51 (1.60 - 12.74)	0.004

Supplementary Table 2. Primers for construction of different segments of DEPDC6 and GNMT

	No.	Name	Sequence	Position	Enzyme site	Primer usage*											
						pHA-DEPTOR	pHA-D1	pHA-D2	pHA-ΔDEP	pHA-PDZ	pLV-HA-DEPTOR	p1-175-FLAG	p35-295-FLAG	p74-295-FLAG	p171-295-FLAG	pGNMT-N140S	pLV-GNMT-FLAG
	1	DEPTOR(F)	5' CGGC**GGATCC**ATGGAGGAGGGCGGC 3'	1--15	*Bam* H I	●	●	●									
	2	DEPTOR(R)	5' CGGC**CTCGAG**TCAGCACTCTAACTCCTC 3'	1213--1230	*Xho* I	●			●	●							
	3	DEP1(R)	5' CCG**CTCGAG**TCATGGGAAGGTGCC 3'	363--375	*Xho* I		●										
primers for DEPTOR	4	DEP2(R)	5' CCG**CTCGAG**TCATCGCCTCCGCCG 3'	661--675	*Xho* I			●									
	5	DDEP(F)	5' CGC**GGATCC**ATGAGGCGAAGACTGATG 3'	670--681	*Bam* H I				●								
	6	PDZ(F)	5' CGC**GGATCC**ATGACATTCACGATTGTTGG 3'	988--999	*Bam* H I					●							
	7	Lv-DEPTOR-F	5' CG**GCTAGC**ATGTACCCATACGATGTTCC 3'	HA-tag	*Nhe* I						●						
	8	Lv-DEPTOR-R	5' GC**GGCGCGCC**TCAGCACTCTAACTCCTC 3'	1213--1230	*Asc* I						●						
	1	Flag-F	5' GCG**GAATTC**ATGGTGGACAGCGTGTAC 3'	1--18	*Eco* RI							●				●	
	2	Flag-FE35	5' GCG**GAATTC**ATGATCGGAGACACCCGC 3'	103--117	*Eco* RI								●				
	3	Flag-F74	5' GCG**GAATTC**ATGCTGGTGGAAGAGGGC 3'	220--237	*Eco* RI									●			
	4	Flag-FE171	5' GCG**GAATTC**ATGGGCCTACTGGTCATT 3'	511--525	*Eco* RI										●		
primers for GNMT	5	Flag-R175	5' CGC**GGATCC**AATGACCAGTAGGCCCCC 3'	508--525	*Bam* H I							●					
	6	Flag-R295	5' CGC**GGATCC**GTCTGTCCTCTTGAGCAC 3'	868-888	*Bam* H I								●	●	●	●	
	7	GNMT-N140S-F	5' GTCATCTGCCTTGGAAGCAGTTTCGCTCACTT	406-438												●	
	8	GNMT-N140S-R	5' CAAGTGAGCGAAACTGCTTCCAAGGCAGATGA	406-438												●	
	9	Lv-GNMT-F	5' CG**GCTAGC**ATGGTGGACAGCGTG 3'	1--15	*Nhe* I												●
	10	Lv-GNMT-R	5' GC**GGCGCGCC**CTACTTGTCATCGTCGTC 3'	FLAG-tag	*Asc* I												●

* Primer pairs used for construction for each plasmid were indicated by black circle (●)