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Insulin-induced CARM1 upregulation facilitates hepatocyte proliferation

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ABSTRACT

Previously, we reported that CARM1 undergoes ubiquitination-dependent degradation in renal podocytes. It was also reported that CARM1 is necessary for fasting-induced hepatic gluconeogenesis. Based on these reports, we hypothesized that treatment with insulin, a hormone typically present under the 'fed' condition, would inhibit gluconeogenesis via CARM1 degradation. HepG2 cells, AML-12 cells, and rat primary hepatocytes were treated with insulin to confirm CARM1 downregulation. Surprisingly, insulin treatment increased CARM1 expression in all cell types examined. Furthermore, treatment with insulin increased histone 3 methylation at arginine 17 and 26 in HepG2 cells. To elucidate the role of insulin-induced CARM1 upregulation, the HA-CARM1 plasmid was transfected into HepG2 cells. CARM1 overexpression did not increase the expression of lipogenic proteins generally increased by insulin signaling. Moreover, CARM1 knockdown did not influence insulin sensitivity. Insulin is known to facilitate hepatic proliferation. Like insulin, CARM1 overexpression increased CDK2 and CDK4 expression. In addition, CARM1 knockdown reduced the number of insulin-induced G2/M phase cells. Moreover, GFP-CARM1 overexpression increased the number of G2/M phase cells. Based on these results, we concluded that insulin-induced CARM1 upregulation facilitates hepatocyte proliferation. These observations indicate that CARM1 plays an important role in liver pathophysiology.

1. Introduction

Protein arginine methyltransferases (PRMTs) catalyze the methylation of arginine residues within their substrates, including histone proteins. In mammals, nine PRMTs (PRMT1-9) have been reported; these are subdivided into three groups according to the methylation manner. Briefly, type I PRMTs (PRMT1, 2, 3, 4, 6, and 8) facilitate asymmetric dimethylarginine (ADMA) formation, whereas type II PRMTs (PRMT5 and 9) facilitate symmetric dimethylarginine (SDMA) formation [1,2]. PRMT7 is classified as a type III PRMT because it facilitates monomethylarginine (MMA) [3].

It was recently reported that PRMTs are associated with liver pathophysiology. Choi et al. reported that PRMT1 increases hepatic gluconeogenesis by inhibiting protein kinase B (Akt)-mediated FoxO1 phosphorylation [4]. Han et al. reported that PRMT6 promotes fasting-induced hepatic gluconeogenesis via CREB-regulated transcriptional coactivator 2 (CRTC2) methylation [5]. Moreover, we found that PRMT1 and PRMT3 are involved in hepatic lipogenesis [6,7]. Increasing evidence suggests that PRMTs play important roles in liver metabolism, and impaired PRMT expression or activity may induce metabolic disorders such as type II diabetes and non-alcoholic fatty liver disease (NAFLD).

PRMT4, also known as coactivator-associated arginine methyltransferase 1 (CARM1), promotes epigenetic regulation of transcription via methylation of histone 3 at arginine 17 and 26. CARM1 also methylates various non-histone proteins and acts as a transcriptional cofactor [1,8]. Due to its various functions, CARM1 is believed to be important in cellular pathophysiology.

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Krones-Herzig et al. reported that the interaction between CARM1 and CREB in hepatocytes is increased following treatment with forskolin, which increases intracellular cAMP. Moreover, this interaction promotes gluconeogenic gene expression via histone 3 arginine 17 methylation [9]. This result suggests that CARM1 is important in fasting-induced gluconeogenesis as well.

In a previous study, we reported that CARM1 undergoes ubiquitination-dependent degradation under high-glucose conditions in renal podocytes [10]. Based on our results as well as those from Krones-Herzig, we hypothesized that CARM1 undergoes ubiquitination-dependent degradation to inhibit gluconeogenesis in the fed state. To evaluate this hypothesis, HepG2 cells, AML-12 cells and rat primary hepatocytes were treated with insulin to mimic the feeding-mediated inhibition of gluconeogenesis, and the effect of insulin on CARM1 expression was examined.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), Ham's nutrient mixture F-12, and fetal bovine serum (FBS) were purchased from Life Technologies (Gibco BRL, Grand Island, NY, USA). Human recombinant insulin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Propidium iodide (PI) staining solution (51-66211E) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). CARM1 antibody (A300-421A) was purchased from Bethyl Laboratories (TX, USA). H3 (ab1791), H3R17 (ab8284), SREBP1c (ab63991), FAS (ab22759), CDK2 (ab6538), and CDK4 (ab7955) antibodies were purchased from Abcam (Cambridge, UK). ACC (#3676), p-Akt (#9271), and t-Akt (#9272) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin (sc-1616), lamin B (sc-6216), and α-tubulin (sc-5546) antibodies and human CARM1 siRNA (sc-44875) were purchased from Santa Cruz Biotechnology (CA, USA). H3R26 antibody (#07–215) was obtained from Millipore (Billerica, MA, USA). All reagents were of the highest purity commercially available.

2.2. Cell culture

HepG2 and AML-12 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2 cells were maintained in DMEM supplemented with 10% FBS. Cells were grown to 60–70% confluence in 60-mm dishes in DMEM with 15 mM HEPES buffer, 10% FBS, 25 mM glucose, 0.35% additional sodium bicarbonate, 2.5 mM L-glutamine, streptomycin (100 μg/ml), and penicillin-streptomycin (100 U/ml) at 37 °C in 5% CO_2. AML-12 cells were maintained in DMEM (5.5 mM glucose) and Ham's F12 1:1 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone supplemented with 10% FBS, streptomycin (100 μg/ml), and penicillin-streptomycin (100 U/ml) at 37 °C in 5% CO_2. Media were changed every other day. Passaged cells were plated to yield near-confluent cultures at the end of the experiments. Rat hepatocytes were isolated from 200 to 300-g male Sprague–Dawley rats by a two-step collagenase perfusion method. Isolated hepatocytes were plated onto collagen-coated six-well dishes using DMEM (20 mmol/L glucose) and Ham's F12 1:1 medium with the same supplements used for the growth of AML12 cells. Cells were incubated with fresh medium every day. Two days after plating, cells were treated with insulin. Experiments were performed in accordance with National Institutes of Health Animal Research Standards.

2.3. Protein extraction and western blot analysis

Cell pellets were lysed in NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo, IL, USA) or M-PER Mammalian Protein Extraction Reagent (Thermo, IL, USA) containing protease inhibitor cocktail (Sigma, MO, USA) and phosphatase inhibitor cocktail I + II (Sigma, MO, USA). Each fractional protein was extracted according to the manufacturer's instructions. Protein levels were quantified using the Bradford procedure. Whole cell extracts, cytoplasmic extracts, or nuclear extracts (30 μg each) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto enhanced nitrocellulose membranes. Blots were then washed with TBST (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20), blocked with 5% skim milk for 1 h and incubated overnight at 4 °C with primary antibodies at the dilutions recommended by the supplier. Membranes were then washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized using EZ-Western Lumi Pico Western Blotting Detection Reagent (Daeillab Service Co, Seoul, Korea) on a luminescent image analyzer (Image Quant LAS4000 mini, GE Healthcare Life Sciences).

2.4. Plasmid and DNA transfection

HA and HA-CARM1 were described previously [10]. GFP and GFP-CARM1 were kindly provided by Mark T. Bedford (University of Texas, M.D. Anderson Cancer Center, Smithville, TX). Transient transfections were performed using Lipofectamine® 3000 (Invitrogen) as instructed by the manufacturer.

2.5. siRNA transfection

For reverse transfection, siRNA targeting either human scramble or CARM1 was transfected into HepG2 cells using Lipofectamine® RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 40 nM of scramble or siCARM1 was diluted in 300 μl Opti-MEM® (Gibco, NY, USA) in a six-well plate, and 5 μl RNAiMAX was then added. After incubation for 10 min at room temperature, cells (diluted in 1.5 ml complete growth media) were seeded into wells containing the siRNA and RNAiMAX complex. siRNA-transfected cells were incubated for 24 h and used for subsequent experiments.

2.6. Cell cycle analysis (PI staining)

HepG2 cells were seeded onto six-well plates and incubated with or without insulin for 24 h. Cells were then detached with 0.05% trypsin/EDTA (Gibco). Detached cells were collected in tubes and centrifuged at 450 × *g* for 3 min at 4 °C. After two washes with cold phosphate-buffered saline (PBS), cells were resuspended in cold 70% EtOH and incubated overnight at 4 °C. After two additional washes with cold PBS, cells were resuspended in FACS buffer (0.1% sodium azide, 0.1% FBS in PBS) and incubated with 20 μg/μl RNase A at room temperature. After 15 min, 50 μg/μl of PI was added, and stained cells were analyzed by flow cytometry (Accuri C6, BD Biosciences).

2.7. Immunofluorescence

HepG2 cells were transfected with the GFP-PRMT4 construct. After 24 h, nuclei were stained with Hoechst 33342 (Life Technologies, NY, USA) for 20 min. Cells were imaged using a fluorescence microscope (Nikon Te-300; Nikon, Melville, NY, USA) at the following fluorescence excitation and emission settings: excitation

between 460 and 500 and emission between 515 and 550 nm for GFP-CARM1, excitation between 325 and 375 and emission above 420 nm for Hoechst staining.

2.8. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Values are representative of three to four independent experiments. All experiments were analyzed by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc, IL, USA). A P value <0.05 was considered to indicate statistical significance.

3. Results

3.1. Effect of insulin on CARM1 expression and histone 3 methylation in hepatocytes

To examine potential alterations in CARM1 expression, HepG2 cells were treated with insulin for the indicated times. Unexpectedly, CARM1 expression increased significantly following treatment with insulin (Fig. 1A). HepG2 cells represent both human hepatocytes and hepatocellular carcinoma cells. To exclude the possibilities raised by the carcinogenic characteristics of HepG2 cells, we also used AML-12 cells, which represent a normal mouse hepatocyte cell line, and rat primary hepatocytes. As shown in Fig. 1B and C, CARM1 expression was increased in both additional cell types. These results suggest that insulin treatment increases CARM1 expression in hepatocytes rather than hepatocellular carcinoma cells. Next, to determine the localization of CARM1 in HepG2 cells, cells were first treated with insulin and cytosolic, and nuclear fractions were then isolated separately. Increased CARM1 expression was observed in both cytosolic and nuclear fractions (Fig. 1D). Moreover, an insulin-induced increase in histone 3 methylation at arginine 17 and 26 was detected in HepG2 cells (Fig. 1E).

3.2. Effects of CAMR1 expression on lipogenesis and insulin sensitivity

Insulin promotes hepatic lipogenesis through activation of sterol-regulatory element-binding protein (SREBP1c) [11]. To reveal whether increased CARM1 expression by insulin is associated with increased hepatic lipogenesis, hemagglutinin (HA)-tagged CARM1 was transfected into HepG2 cells. Surprisingly, CARM1 overexpression did not increase the cleavage of SREBP1c (activated SREBP1c) or the expression of lipogenic proteins, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). Next, we examined whether CARM1 expression is required to maintain insulin sensitivity. CARM1 expression was silenced by *carm1* siRNA transfection (Fig. 2B), and Akt phosphorylation was detected after insulin stimulation. Interestingly, CARM1 knockdown did not influence insulin-stimulated Akt phosphorylation in HepG2 cells.

3.3. Effect of CARM1 expression on cell cycle progression

Insulin *per se* is not a mitogen. However, the presence of insulin accelerates hepatocyte proliferation [12]. Consistent with the insulin-induced effects on hepatocyte proliferation, CARM1 overexpression increased the expression of cyclin-dependent kinase 2 (CDK2) and CDK4 in HepG2 cells (Fig. 3A). In addition, insulin-induced increases in G2/M phase cells were reduced by CARM1 knockdown (Fig. 3B; scramble 26.2% vs. scramble + insulin 32.0% vs. siCARM1 + insulin 27.9%).

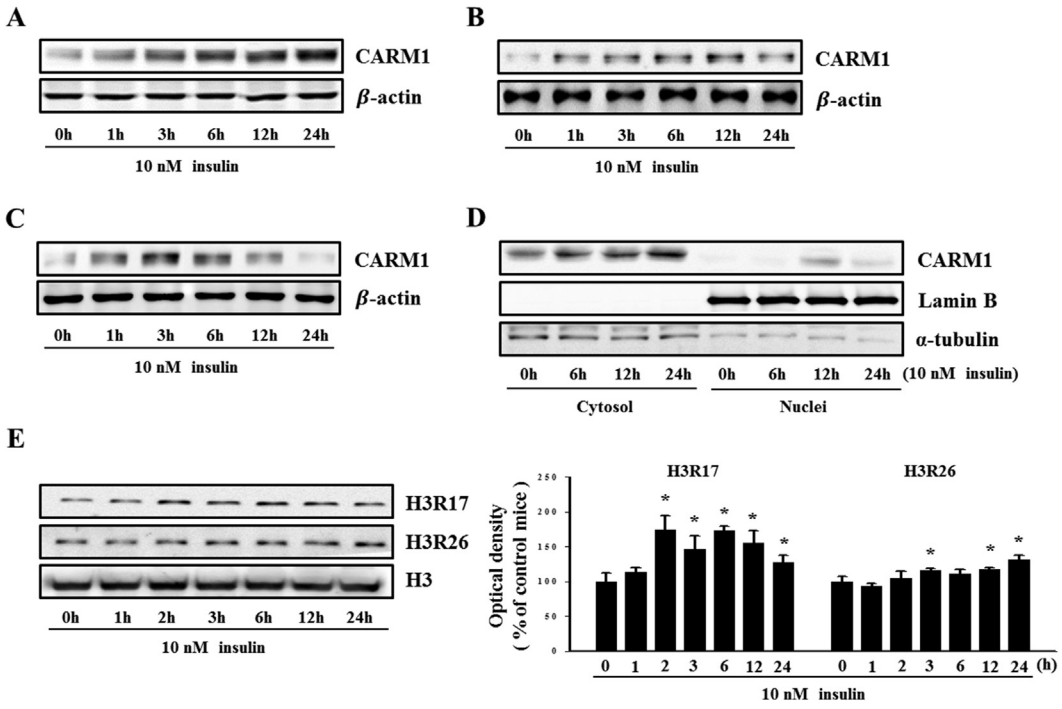

Fig. 1. Effects of insulin on CARM1 expression and histone 3 methylation in hepatocyte (A–C) Cells (A: HepG2 cells, B: AML-12 cells and C: rat primary hepatocyte) were treated with 10 nM insulin for various time intervals. CARM1 expressions were assayed by western blot analysis. β-actin was used as a loading control. Representative immunoblots were from at least three independent experiments. (D) HepG2 cells were treated with 10 nM insulin for various time intervals. The cytosolic and nuclear fractions were extracted and CARM1 expressions were assayed by western blot analysis. Lamin B was used as a loading control for a nuclear fraction and α-tubulin was for a cytosolic fraction. Representative immunoblots were from at least three independent experiments. (E) HepG2 cells were treated with 10 nM insulin for various time intervals. H3R17 and H3R26 levels were assayed by western blot analysis and and normalized to the H3 level. Data represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$ *vs*. 0 h.

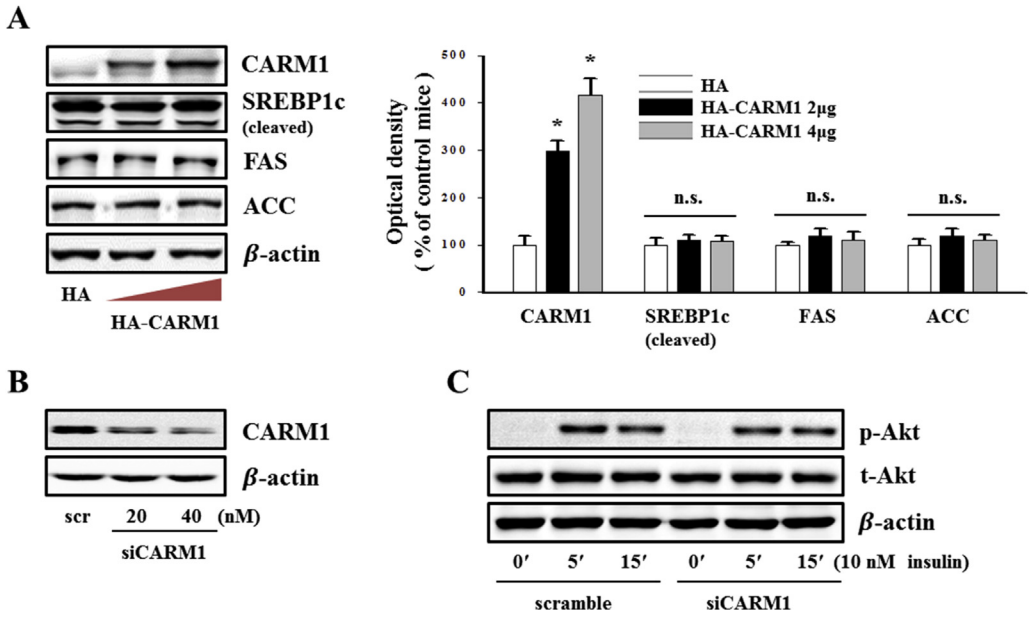

Fig. 2. Effects of CAMR1 expression on lipogenesis and insulin sensitivity (A) HepG2 cells were transfected with HA or HA-CARM1 plasmid. After 24 h, Cell extracts were subjected to western blot analysis with indicated antibodies. Data represent the means ± SEM of four independent experiments. $^{*}p < 0.05$ vs. HA (n.s. = not significant). (B) HepG2 cells were transfected with scramble or *carm1* siRNA according to reverse transfection method. After 24 h, cell extracts were subjected to western blot analysis with indicated antibodies. Representative immunoblots were from at least three independent experiments. (C) HepG2 cells were transfected with 40 nM scramble or *carm1* siRNA according to reverse transfection method. After 24 h, the cells were stimulated with 10 nM insulin for 5 and 15 min. Cell extracts were subjected to western blot analysis with indicated antibodies. Representative immunoblots were from at least three independent experiments.

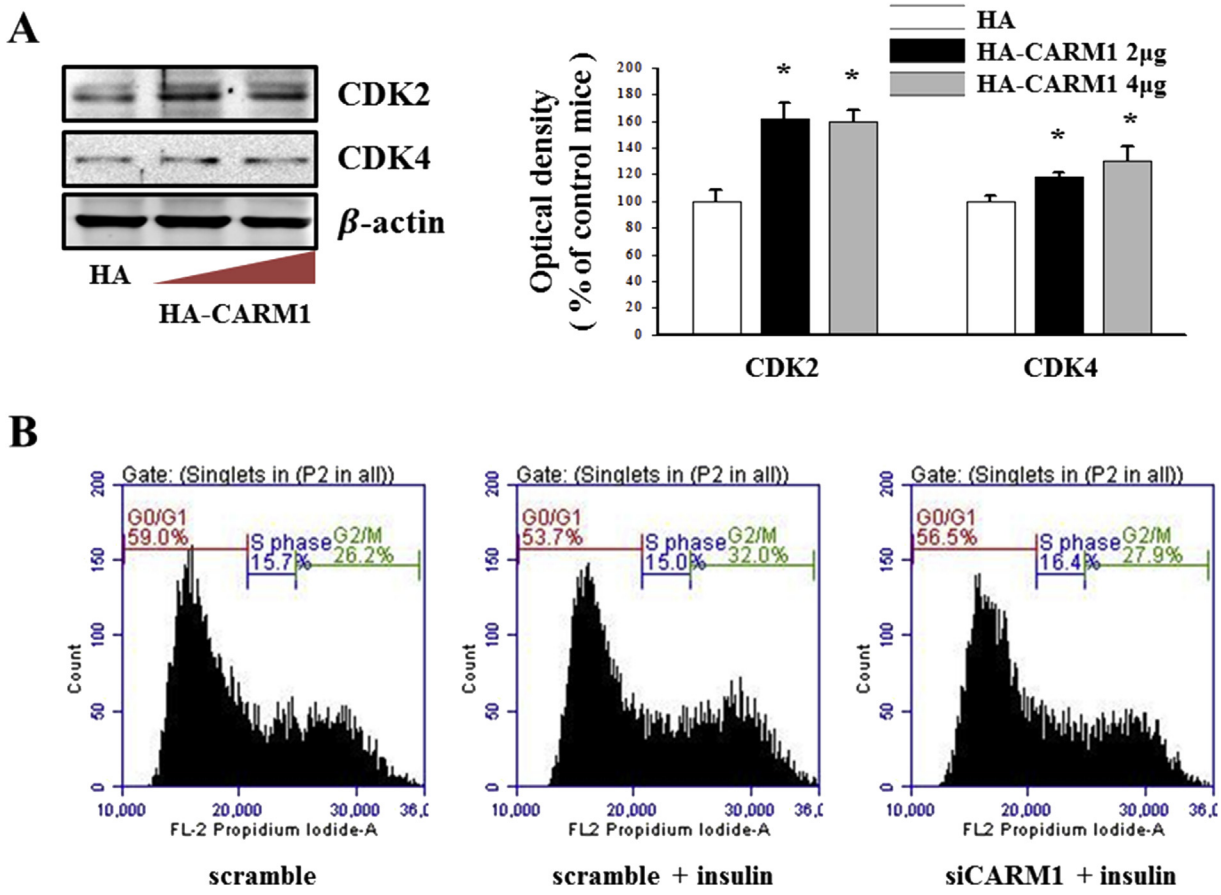

Fig. 3. Effect of CARM1 expression on cell cycle progression (A) HepG2 cells were transfected with HA or HA-CARM1 plasmid. After 24 h, Cell extracts were subjected to western blot analysis with indicated antibodies. Data represent the means ± SEM of four independent experiments. $^{*}p < 0.05$ vs. HA. (B) HepG2 cells were transfected with 40 nM scramble or *carm1* siRNA according to reverse transfection method. After 24 h, media was changed to fresh media containing 10% FBS and the cells were treated with 10 nM insulin for 12 h. Cell cycle distribution was determined by PI staining. Representative images were from at least four independent experiments.

3.4. Effect of CARM1 overexpression on cell cycle progression

To analyze the cell cycle phase upon CARM1 overexpression, green fluorescent protein (GFP)-tagged CARM1 was transfected into HepG2 cells. GFP-CARM1 expression was detected in both the cytosol and nuclei (Fig. 4A). GFP-CARM1 overexpression increased G2/M phase cells compared with GFP-alone transfected cells (Fig. 4B, left histogram; GFP 14.5% vs. GFP-CARM1 20.4%). Interestingly, few GFP-positive cells were observed in GFP-CARM1 transfected cells but not in GFP-alone transfected cells (Fig. 4B, middle histogram; M4 region). In addition, we determined that these cells were highly distributed in the G2/M (46.4%) and S phases (34.3%) rather than in the G0/G1 phase (20.6%) (Fig. 4B, right histogram).

4. Discussion

We initially expected CARM1 expression to be reduced by insulin treatment. However, CARM1 expression was significantly increased in three hepatocyte cell lines upon insulin stimulation: HepG2 cells, AML-12 cells, and rat primary hepatocytes. Although

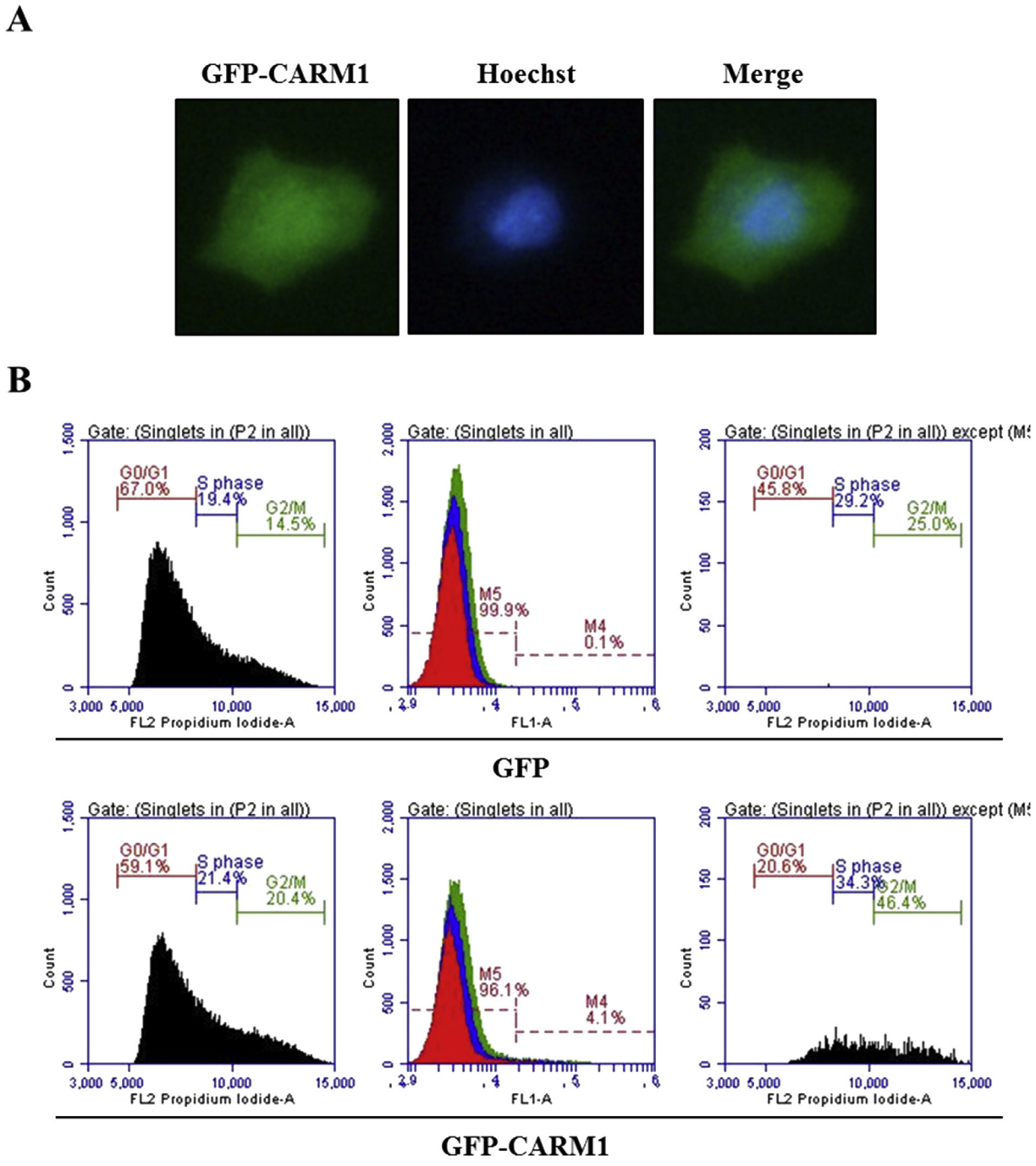

Fig. 4. Effect of CARM1 overexpression on cell cycle progression (A) HepG2 cells were transfected with GFP-CARM1 plasmid. After 24 h, the nuclei were stained with Hoechst for 20 min. GFP and nuclear signals were detected under fluorescence microscope. (B) HepG2 cells were transfected with GFP or GFP-CARM1 plasmid. After 24 h, media was changed to fresh media containing 10% FBS. After 12 h, cell cycle distribution was determined by PI staining. Representative images were from at least four independent experiments.

these results were unexpected, we further explored the roles of increased CARM1 expression in hepatocytes, as the increases were highly significant in all cell types examined. For additional studies, we used HepG2 cells because: 1) they are derived from humans (the insulin used in these experiments was human recombinant), 2) the inhibitory effects of insulin on gluconeogenic gene expression have been well-observed in these cells, and 3) the newly commercially available transfection reagent has higher efficacy than existing reagents for HepG2 cell transfection.

Insulin is a highly important regulator of hepatocyte function. Metabolically, insulin induces hepatic lipogenesis via SREBP1c activation, which subsequently promotes the transcription of FAS and ACC. In addition, an insulin response sequence (IRS) was found in the FAS promoter region where it is activated upon by upstream stimulatory factor-1 (USF-1) [13]. Recently, it was also revealed that histone deacetylase 9 (HDAC9)-mediated USF-1 deacetylation blocks FAS promoter activity under fasting conditions [14]. Interestingly, CARM1 overexpression reduced endogenous HDAC9 expression in human adipogenic stem cells (hASC) [15]. These results suggested that insulin-induced CARM1 upregulation reduces HDAC9 expression, which subsequently activates USF-1-mediated FAS promoter activity. However, in the current study, CARM1 overexpression did not influence the expression of FAS or other lipogenic proteins. This discrepancy may be due to differences in cell types (hepatocytes vs. hASC).

In skeletal L6 myotubes, insulin treatment increased PRMT1 in nuclear and membrane fractions, and these increases were associated with insulin sensitivity [16]. In this study, insulin treatment increased CARM1 expression in both the cytosol and nuclei. However, CARM1 knockdown by siRNA did not influence insulin sensitivity, as determined by Akt serine 473 phosphorylation. Therefore, the increased CARM1 expression in HepG2 cells does not affect lipogenesis or insulin sensitivity.

Aside from its metabolic effects, insulin facilitates hepatocyte proliferation. CDK2 and CDK4 expression is increased when hepatocytes are cultured in insulin-containing media [17]. Moreover, mitogen-induced hepatocyte proliferation was reduced in the absence of insulin [18]. Moreover, our results revealed that insulin-induced CARM1 expression is important in facilitating hepatocyte proliferation based on the following reasons: (1) CDK2 and CDK4 were increased by CARM1 overexpression; (2) the increase in G2/M phase cells by insulin was reduced by CARM1 knockdown; (3) G2/M phase cells were increased by GFP-CARM1 overexpression; and (4) GFP-positive cells, observed only in GFP-CARM1 transfected cells, were distributed mainly in G2/M and S phases. Consistent with our results, El Messaoudi et al. reported that CARM1 expression positively regulates the expression of cyclin E1, an essential component of hepatocyte proliferation [19,20]. Fujiwara et al. reported that CARM1 induces PC12 cell proliferation through promoting the degradation of p21 mRNA, which blocks the activities of cyclin-CDK complexes [21]. This evidence strongly supports our results that insulin–induced CARM1 expression facilitates hepatocyte proliferation.

Insulin is not a direct mitogen. Indeed, based upon flow cytometric analysis, insulin treatment did not induce cell cycle progression in the absence of FBS (data not shown). However, in the presence of FBS, insulin treatment significantly increased G2/M phase cells. Moreover, CARM1 overexpression-induced increases in G2/M phase cells were observed only in the presence of FBS. These results suggest that CARM1 *per se* is unable to induce hepatocyte proliferation in the absence of mitogens. However, CARM1 facilitates hepatocyte proliferation, aiding the signals induced by mitogens.

Interestingly, similar to CARM1 upregulation, insulin treatment also increased asymmetric demethylation of histone 3 at arginine 17 and 26. Methylation of these sites is the activating marker of transcription [22]. We therefore hypothesized that the proliferation of some genes associated with proliferation may be increased by these methylations rather than that of genes related to lipogenesis and insulin sensitivity. Further studies should be performed to unveil the precise target genes of insulin-induced histone methylation in hepatocytes.

In conclusion, we found that CARM1 is upregulated by insulin treatment in hepatocytes. In addition, this increase facilitates hepatocyte proliferation. These observations indicate that CARM1 plays an important role in liver pathophysiology.

Conflict of interest

None of the authors have any potential conflicts of interest to disclose.

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Transparency document

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