Caveolin-1 Increases Aerobic Glycolysis in Colorectal Cancers by Stimulating HMGA1-Mediated GLUT3 Transcription

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Abstract

Caveolin-1 (CAV1) acts as a growth suppressor in various human malignancies, but its expression is elevated in many advanced cancers, suggesting the oncogenic switch of its role during tumor progression. To understand the molecular basis for the growth-promoting function of CAV1, we characterized its expression status, differential roles for tumor growth, and effect on glucose metabolism in colorectal cancers. Abnormal elevation of CAV1 was detected in a substantial fraction of primary tumors and cell lines and tightly correlated with promoter CpG sites hypomethylation. Depletion of elevated CAV1 led to AMPK activation followed by a p53-dependent G1 cell-cycle arrest and autophagy, suggesting that elevated CAV1 may contribute to ATP generation. Furthermore, CAV1 depletion downregulated glucose uptake, lactate accumulation, and intracellular ATP level, supporting that aerobic glycolysis is enhanced by CAV1. Consistently, CAV1 was shown to stimulate GLUT3 transcription via an HMGA1-binding site within the GLUT3 promoter. HMGA1 was found to interact with and activate the GLUT3 promoter and CAV1 increased the HMGA1 activity by enhancing its nuclear localization. Ectopic expression of HMGA1 increased glucose uptake, whereas its knockdown caused AMPK activation. In addition, GLUT3 expression was strongly induced by cotransfection of CAV1 and HMGA1, and its overexpression was observed predominantly in tumors harboring high levels of CAV1 and HMGA1. Together, these data show that elevated CAV1 upregulates glucose uptake and ATP production through HMGA1-mediated GLUT3 transcription, suggesting that CAV1 may render tumor cells growth advantages by enhancing aerobic glycolysis. Cancer Res; 72(16); 4097–109. ©2012 AACR.

Introduction

Caveolin-1 (CAV1) is a structural component of caveolae and involved in diverse cellular functions such as vesicular transport, cholesterol homeostasis, and signal transduction (1). Despite a growing body of evidence on CAV1 implication in tumorigenesis, its role in cancer development and progression is still contentious. Both tumor suppression and promotion roles of CAV1 have been proposed on the basis of its expression levels detected in cancers. CAV1 expression is reduced in many human cancers and also downregulated in cells transformed by oncopgenes, such as v-Abl, Bcr-Abl, and H-RasG12V (1, 2). Moreover, CAV1 null mice are more susceptible to carcinogen-induced skin tumor formation, and a mutation in CAV1 has been identified in 16% of scirrhous breast carcinoma, leading to the proposal that CAV1 may function as a tumor suppressor (3, 4). Nevertheless, accumulating evidence argues that CAV1 has tumor-promoting functions. CAV1 is increased in several cancers, including prostate and breast carcinomas, and its elevation is associated with enhanced tumor progression, multidrug resistance, and metastatic activity (5, 6). However, the molecular basis for its oncogenic effects remained largely undefined.

Cancer cells often take up high amounts of glucose and rely on glycolysis rather than mitochondrial respiration for ATP generation despite the presence of oxygen, a phenomenon known as the Warburg effect (7). This metabolic shift toward aerobic glycolysis enables cancer cells to convert glucose more efficiently into macromolecules, which are needed for rapid cell growth. Glucose is a hydrophilic molecule that cannot enter the cell by simple diffusion and thus expression of specific facilitative transporters, named as GLUTs, is commonly elevated in cancer cells. GLUT3, one of the 14 members of the SLC2 family of GLUTs, is highly expressed in various cancer cells including colon carcinoma (8, 9). Previous studies showed that CAV1 is associated with glucose metabolism (10–12). It was also reported that insulin receptors are localized in caveolae microdomains and the structure of caveolae is important in glucose uptake (11). Furthermore, a recent study...
revealed that downregulation of CAV1 in stromal fibroblasts undergo aerobic glycolysis and generate high levels of glycolytic end products and promote adjacent cancer cell growth by the paracrine secretion of these energy-rich glycolytic bioproducts (12).

AMPK is a cellular energy sensor that is activated by an increasing cellular AMP:ATP ratio caused by metabolic stresses that interfere with ATP production or that accelerate ATP consumption (13). AMPK activation turns on catabolic pathways that generate ATP although turning off ATP-consuming pathways. Given its role as metabolic sensor and modulator, AMPK has been implicated as the initiator of cell-cycle arrest under low-glucose conditions and a connection to cell-cycle regulators. Consistently, AMPK acts as an upstream activator of p53, and this AMPK–p53 signaling plays an important role in metabolic changes during cancer development (14). It was also shown that p53 transactivates Sestrin1 and Sestrin2, resulting in AMPK phosphorylation, which subsequently inhibits mTORC1 activity leading to growth arrest and autophagy (15). These findings thus suggest that tumor cells lacking a functional AMPK or p53 continue to proliferate under energetically stressful conditions.

We report here that overexpressed CAV1 in colorectal cancer cells enhances aerobic glycolysis through the HMGA1-mediated GLUT3 transcription. We found that CAV1 depletion downregulates glucose uptake and ATP production and causes autophagy via activation of AMPK-p53 signaling. Therefore, our data suggest that elevated CAV1 may provide tumor cells growth advantages by upregulating GLUT3-mediated glucose uptake and aerobic glycolysis.

Materials and Methods

**Human colorectal tissues and cancer cell lines**

A total of 100 primary colorectal tumor specimens and their adjacent normal tissues were obtained by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Signed informed consent was obtained from each patient. Tumor specimens composed of at least 70% carcinoma cells and adjacent tissues found not to contain tumor cells were chosen for molecular analysis. Nineteen human colorectal cancer cell lines were obtained from Korea Cell Line Bank (Seoul National University, Seoul, Korea) or American Type Culture Collection. The cells were maintained in Dulbecco’s modified Eagle’s medium medium supplemented with 10% FBS (GIBCO BRL) at 37°C in a humidified atmosphere with 5% CO₂.

**Semiquantitative reverse transcriptase PCR, immunoblot, and promoter methylation assays**

Our strategies for the semiquantitative reverse transcriptase PCR (RT-PCR) and bisulfite DNA sequencing analyses were previously described (16, 17). Briefly, 1 μg of DNAse I-treated RNA was converted to cDNA by reverse transcription using random hexamer primers and MoMuLV reverse transcriptase (Life Technologies, Inc.). Primer sequences are available upon request. Quantitation was achieved by densitometric scanning of the ethidium bromide–stained gels, and analysis was done using the Molecular Analyst software program (Bio-Rad). For bisulfite sequencing analysis, 50 ng of bisulfite-modified DNA was subjected to PCR amplification of the CAV1 promoter region using primers P2 (5'-AGGTTAAGAGTTTATTTTAATT-3') and P5 (5'-CTATATTTTTACCCCCAACACT-3'). The PCR products were cloned into pcDNA3 vectors (Invitrogen), and 5 clones of each specimen were sequenced by automated fluorescence-based DNA sequencing to determine the methylation status. Western analyses were carried out using antibodies specific CAV1 (Santa Cruz Biotechnology), LC3-I/II (Cell Signaling), AMPK (Cell Signaling), p70S6K (Cell Signaling), p53 (Cell Signaling), GLUT3 (Santa Cruz Biotechnology), HMGI/Y (Santa Cruz Biotechnology), U1 snRN70 (Santa Cruz Biotechnology), and β-Actin (Santa Cruz Biotechnology).

**Expression plasmids, siRNA, and transfection**

CAV1 expression vector was constructed using a PCR-based approach. The PCR products were cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen). Transfection was done using Lipofectamine 2000 (Invitrogen), siRNA duplex against CAV1 (5'-CCGAGAGGACACAGAUCU-3' and 5'-GCAUUGGAGG-GCCAGCUU-3'), AMPK-α1 (5'-GCAGAAGUAGAGCAGCA-3'), p53 (5'-CACUAAACUAAUGUGUUA-3') were synthesized by Drhaman Research. Transfection of siRNA was done using siRNA-Oligofectamine mixture or electroporation (Neon transfection system; Invitrogen).

**Cell-cycle and colony formation assays**

Cells were seeded at the density of 1 x 10⁵ cells and transfected with expression vector or siRNA. Cell numbers were counted using a hemocytometer for 5 days at 24-hour intervals. For cell-cycle analysis, cells were fixed with 70% ethanol and resuspended in 1 mL of PBS containing 100 μg/mL RNase and 50 mg/mL propidium iodide. Cell-cycle assay was carried out using a FACScan flow cytometer (Becton Dickenson) and MultiCycle software (Phoenix Flow Systems). For colony formation assay, RKO cells (1 x 10⁵) were transfected with 2 μg of WT-CAV1 vectors and maintained in the presence of hygromycin B (200 μg/mL) for 12 days. HCT116 cells were transfected with 1 to 10 pmol of si-CAV1. Colonies were fixed with methanol for 15 minutes and stained with 0.05% crystal violet in 20% ethanol.

**Reporter constructs and luciferase assay**

Human GLUT3 promoter regions were cloned into the pGL3-basic vector (Promega). The putative HMGA1-binding sites (−435/−415 and −138/−123), GATA3-binding sites (−339/−334 and −280/−275), and Sp1-binding site (−44/−35) were mutated (5'-CTTTAAAAAAATTATAA-3' and 5'-TTTTAAAAAAAATTATAA-3') for HMGA1; 5'-TGCCAG-3' and 5'-AGGGCG-3' for GATA3; 5'-GGCCGGC-3' for Sp1 using site-directed mutagenesis. Cells were transfected with 500 ng of the promoter constructs using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured by using Luciferase assay system (Promega).

**Chromatin immunoprecipitation assay**

Cells were incubated in 1% formaldehyde solution for 20 minutes. The cells were lysed and the pellet was resuspended in
nuclei lysis buffer and sonicated. Immunoprecipitation was carried out with HMGA1 antibody (Santa Cruz Biotechnology). PCR was done using the following primer pairs: GLUT3-P2 (5′-CTGATTTCCTTCTCAGGTCTGTC-3′) and GLUT3-P3 (5′-CCATACAAAACGAAGTCAAGTGG-3′).

**Measurement of intracellular ATP and lactate levels and glucose uptake**

Intracellular ATP levels were measured using ATP Bioluminescence Assay Kit HSI (Roche Applied Science). Briefly, 6 × 10⁵ cells were lysed with boiling lysis reagent and supernatant was collected. Fifty microliters of diluted sample was mixed with 50 μL of luciferin/luciferase reagents. Luminescence was measured using Luminoskan Ascent (Thermo Scientific). Intracellular L(+)-lactate level was measured using lactate assay kit (BioVision). Cells were suspended with lactate assay buffer and incubated in the presence of reaction mixture for 30 minutes. Lactate amount was quantified by measuring the absorbance at 570 nm using a Bio-Rad 680 microplate reader (Bio-Rad). For glucose uptake assay, 3 × 10⁵ cells were incubated in the presence of 20 μmol/L of 2-NBDG (2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose; N13195; Invitrogen) for 2 hours. The cells were resuspended in cold growth medium and stained with propidium iodide. Samples were maintained on the ice and analyzed by flow cytometry (Becton Dickinson).

**Immunofluorescence assay**

Cells were seeded on glass chamber slides (Nalge Nunc). After 48 hours, the cells were fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 2% bovine serum albumin-PBS for 30 minutes. Slides were incubated with anti-CAV1, anti-IC3, or anti-GLUT3 antibody for overnight. Fluorescent imaging was obtained with a confocal laser scanning microscope (Carl Zeiss MicroImaging, Inc.).

**Animal studies**

Four-week-old immunodeficient female nude mice (nu/nu; Orient Bio Inc.) were maintained in pressurized ventilated cages. For xenograft assay, HCT116 p53⁻/⁻ and p53⁻/⁻ cells (5 × 10⁶) transfected with si-CAV1 or si-Control were injected subcutaneously into 8 mice for each group. Tumor growth was monitored periodically for 24 days and volume (V) was calculated using the modified ellipsoidal formula $V = \frac{1}{2} \times length \times width^2$. All animal studies were conducted with the approval of Korea University Institutional Animal Care and Use Committee and Korea Animal Protection Law.

**Statistical analysis**

Student t test was used to determine the statistical significance of the difference. The $\chi^2$ test was used to determine the statistical significance of expression and methylation status between tumor and normal tissues. P value of less than 0.05 was considered significant. Pearson correlation coefficient ($r$) was used to measure the strength of the association between CAV1, GLUT3, and HMGA1 expression levels.

**Results**

**Oncogenic conversion of CAV1 function in colorectal cancers**

To explore CAV1 role for colorectal tumorigenesis, we initially characterized its expression status in 19 cancer cell lines and 100 primary tumors. CAV1 expression was detected in all cell lines and tissues, but its levels were highly variable among specimens (Fig. 1A). CAV1 mRNA levels in normal tissues, primary tumors, and cell lines were observed in a range of 0.60–1.64 (mean 1.12), 0.04–2.93 (mean 1.01), and 0.03–3.09 (mean 1.08), respectively (Fig. 1B). However, 30 (30%) and 15 (15%) of 100 primary tumors expressed CAV1 mRNA less than a half (<0.56) and more than 2-folds (>2.24) of normal means (1.12), respectively. Compared with adjacent noncancerous tissues, 37 (37%) and 26 (26%) cancerous tissues displayed substantially low and high expression, respectively, indicating that CAV1 expression is down- or upregulated in colorectal cancers (Supplementary Fig. S1A). Both α and β transcripts of CAV1 and CAV2 showed comparable expression, whereas CAV3 transcript was not detected (data not shown). CAV1 reduction was significantly more frequent in stage I and II tumors (15 of 33, 35.5% and 10 of 23, 43.5%, respectively) compared with stage III tumors (5 of 44, 11.4%) and more common in well and moderately differentiated tumors (10 of 32, 31.3% and 18 of 56, 32.1%, respectively) than poorly differentiated tumors (2 of 12, 16.7%; Supplementary Fig. S2B). In contrast, CAV1 elevation was significantly more frequent in stage III (9 of 44, 23.3%) versus stage I and II tumors (3 of 33, 9.1% and 3 of 23, 13%, respectively) and more common in poorly differentiated (4 of 12, 33.3%) versus well and moderately differentiated tumors (4 of 32, 12.5% and 7 of 56, 12.5%). These results indicated that CAV1 expression is commonly down- and upregulated in early and advanced colorectal cancers, respectively. Next we tested whether this variable expression of CAV1 is caused by genetic and/or epigenetic alteration of the gene. Although allelic deletion, amplification, or sequence mutations of the CAV1 gene were not identified in both cell lines and primary tumors, its mRNA expression was reactivated in all low CAV1 cells following treatment with the demethylation agent 5-aza-dC (Supplementary Fig. S1B). Bisulfite DNA sequencing analysis of 37 CpG sites within the promoter and exon 1 region revealed that methylation status of the 8 CpGs (numbered 30–37; nucleotides −494 to −231) was tightly associated with mRNA expression status (Fig. 1C and Supplementary Fig. S1C). Cell lines and tumor tissues with high CAV1 showed partial methylation only at 1 to 2 CpG sites, whereas tumors with low CAV1 exhibited partial or complete methylation at 5 to 8 CpG sites, suggesting that methylation of these CpGs may play a crucial role for gene transcription.

We explored whether the biphasic regulation of CAV1 expression in cancers is associated with the switch of its function. Whereas restoration of CAV1 expression in RKO and SW480 cells led to approximately 37% to 51% inhibition of *in vitro* cellular growth, knockdown of endogenous CAV1 overexpressed in HCT116 and LoVo cells resulted in 55% to 83% decrease in cellular growth, indicating that CAV1 exerts opposite effects on growth of these cells (Fig. 1D and Supplementary
Figure 1. Expression and function of CAV1 in colorectal cancers. A, expression status of CAV1 in cancer cell lines and primary tumors. P, patient; N, adjacent noncancerous tissue; T, cancer tissue. B, expression levels of CAV1 mRNA. C, a correlation of promoter methylation with mRNA levels. Black, gray, and white squares represent complete methylation, partial methylation, and unmethylation, respectively. D, opposite effects of CAV1 on cellular growth (\( P < 0.05; * P < 0.01 \)). E, differential effects of CAV1 on cell proliferation. Cell-cycle progression was analyzed at 48 hours after transfection using flow cytometry. F, an immunoblot assay of CAV1 effect on p21Waf1 expression. G, opposite effects of CAV1 on colony-forming ability of tumor cells.
CAV1 depletion induces autophagy by activation of AMPK-p53 signaling

To further define the growth-promoting role of CAV1, we examined its effect on cell metabolism using HCT116 cells. Interestingly, microscopic analyses revealed that CAV1 knockdown increases autophagic phenotypes, including formation of autophagosomes (Fig. 2A and B). CAV1 depletion increased several autophagy markers (LC3-II, ATG5/12, and P-ULK) and expression of autophagy-related genes, such as Beclin-1, DRAM, SESTRIN1, and TIGAR (Fig. 2C and D). Consistently, expression of p70S6K, a target of mTOR that inhibits autophagy, was downregulated by CAV1 knockdown (Fig. 2D). Moreover, si-CAV1 transfection caused activation of AMPK, a key kinase that senses intracellular AMP:ATP ratio, and stabilization of p53, a downstream target of AMPK in signaling autophagy (Fig. 2E). Knockdown of either AMPK or p53 abolished CAV1 depletion-induced autophagy (Fig. 2E and F). p53 induction in CAV1-depleted cells was disrupted by AMPK knockdown or pretreatment with an AMPK inhibitor compound C, indicating AMPK-p53 signaling by CAV1 knockdown (Supplementary Fig. S2A and B). Consistently, expression of p70S6K, a target of mTOR that inhibits autophagy, was downregulated by CAV1 knockdown (Supplementary Fig. S2A and B). Autophagy phenotypes and expression of related markers were also induced by different si-CAV1 duplex and si-CAV1-induced autophagy was rescued by cotransfection of WT-CAV1, excluding the possible off-target effect of siRNA (Fig. 2D and Supplementary Fig. S2C–E). In addition, we observed that P-Akt and P-p38 levels are increased by CAV1 depletion, whereas P-Erk1/2, P-JNK, and P-IxB levels are not affected (Supplementary Fig. S2F).

p53 mediates CAV1 depletion–induced autophagy and growth suppression

Next we characterized role for p53 in autophagy induction. As predicted, p53 stability was markedly increased in CAV1-depleted cells (Supplementary Fig. S3A). Whereas phosphorylation at serine 15 and 37 was elevated, serine 46 phosphorylation, which is known to activate apoptotic signaling of p53, was not affected by CAV1 knockdown (Supplementary Fig. S2A and B). Autophagy phenotypes and expression of related markers were also induced by different si-CAV1 duplex and si-CAV1-induced autophagy was rescued by cotransfection of WT-CAV1, excluding the possible off-target effect of siRNA (Fig. 2D and Supplementary Fig. S2C–E). In addition, we observed that P-Akt and P-p38 levels are increased by CAV1 depletion, whereas P-Erk1/2, P-JNK, and P-IxB levels are not affected (Supplementary Fig. S2F).

CAV1 upregulates glucose uptake and aerobic glycolysis by GLUT3 induction

AMPK activation by CAV1 depletion suggests that CAV1 might be involved in ATP metabolism. We observed that CAV1 depletion significantly decreases intracellular ATP level in high CAV1-expressing colon (HCT116 and LoVo) and gastric (MKN1) cancer cells (Fig. 4A and Supplementary Fig. S4A). Cancer cells prefer to use glycolysis to produce ATP despite the presence of oxygen (7). Thus, we tested whether CAV1 affects glucose uptake and aerobic glycolysis. In all 3 cell lines, glucose uptake and lactate accumulation were substantially decreased by si-CAV1 transfection (Fig. 4B and Supplementary Fig. S4B). As predicted, an addition of nonmetabolic glucose analog (2-DG) led to AMPK activation and autophagy induction in HCT116 cells, indicating that ATP production and cell growth are highly relied upon aerobic glycolysis (Supplementary Fig. S4C). We tested whether CAV1 induction of aerobic glycolysis is linked to suppression of mitochondrial respiration. However, CAV1 depletion resulted in the decrease in both mitochondrial membrane potential and intracellular ROS level (Supplementary Fig. S4D and E). These showed that elevated CAV1 upregulates ATP production by enhancing both aerobic glycolysis and mitochondrial respiration.

Next we examined whether CAV1 affects expression of glucose transporters. Among 14 GLUT genes (GLUT1-14) we examined, only GLUT3 showed a substantial reduction of its mRNA level in CAV1-depleted cells (Fig. 4C). GLUT3 was also found to partially colocalize with CAV1 and its level was markedly reduced in CAV1-depleted cells (Fig. 4D and Supplementary Fig. S4F). Although GLUT4 mRNA level was slightly decreased by CAV1 depletion, expression of other 12 GLUT genes was barely detectable or not affected by CAV1 in colon cancer cells we tested (Supplementary Fig. S4G). GLUT3 reduction by CAV1 depletion was observed in both p53+/+ and p53−/− cells, indicating that CAV1 induction GLUT3 occurs in p53-independent fashion (Supplementary Fig. S4H). Knockdown of GLUT3 expression led to a marked reduction of cellular growth, which is comparable with si-CAV1–induced growth inhibition (Supplementary Fig. S4I). Moreover, consistent with effect of si-CAV1, si-GLUT3 evoked growth inhibition effect more significantly in p53+/− cells compared with p53−/− cells (75% vs. 36% reduction). As predicted, GLUT3 depletion resulted in a marked decrease in glucose uptake and intracellular ATP level, AMPK activation, and autophagy induction (Fig. 4E and F).

CAV1 enhances HMGA1-mediated GLUT3 gene transcription

We investigated whether CAV1 affects GLUT3 transcription using promoter reporter assays (Fig. 5A). Compared with empty luciferase vector, the P1007-Luc and P515-Luc reporters, which comprise nucleotides +1 to −1,007 and +1 to −515, led to more than 20- to 22-fold increase in luciferase activity, but this increase was greatly attenuated in CAV1-depleted cells (Fig. 5B). Although P515-Luc activity was reduced by si-CAV1, the activity of the P258-Luc and P188-Luc reporters was not influenced by CAV1 depletion (Supplementary Fig. S5A). This indicates that the sequence region between −515 and −288,
Figure 2. CAV1 depletion induces autophagy through AMPK-p53 signaling. A, microscopic examination of autophagosomes in HCT116 cells. PC, phase contrast; EM, electron microscopy. B, a dose-associated increase in autophagosome by si-CAV1 transfection. C, an immunofluorescence analysis of LC3 expression. D, induction of autophagy-related genes in CAV1-depleted cells. Immunoblot assays were carried out at 48 hours after transfection. E, activation of AMPK-p53 signaling by si-CAV1 transfection. F, blockade of either AMPK or p53 abolishes CAV1 depletion-induced autophagy. DAPI, 4',6-diamidino-2-phenylindole.
Figure 3. A p53-dependent autophagy induction. A, no induction of autophagy in p53-deficient cells. B, no induction of autophagy in CAV1-depleted p53+/− cells. C, comparison of CAV1 effect on autophagy gene expression between p53+/+ and p53−/− sublines. D, a p53 role for CAV1 depletion-induced growth inhibition. Cells were transfected with si-CAV1 (20 pmol). E, a p53 role for CAV1 depletion-induced inhibition of xenograft tumors. Cells transfected with si-CAV1 (20 pmol) were injected subcutaneously into female nude mice (*, P < 0.05; **, P < 0.01). F, comparison of xenograft tumor weight at 24 days after injection.
which contains the putative binding sites for HMGA1, GATA3, and Sp1, plays a critical role for the response to CAV1. Using reporters containing mutated binding sites, we found that the HMGA1-binding site (HMGA1-BS1; C0435 to C0420) but not GATA3- or Sp1-binding sites was required for CAV1 activation of the promoter (Fig. 5B and data not shown). The MT1-P515-Luc reporter containing a mutated HMGA1-BS1 was unable to respond to CAV1 depletion, whereas the MT2-P515-Luc reporter containing a mutated HMGA1-BS2 (C0138 to C0117) showed CAV1 responsiveness, indicating that HMGA1-BS1 plays a critical role in CAV1 activation of GLUT3 transcription (Fig. 5B). The GLUT3 chromatin was specifically immunoprecipitated with antibody against HMGA1, but this interaction was greatly attenuated from CAV1-depleted cells (Fig. 5C).

**Figure 4.** CAV1 upregulates glucose uptake and aerobic glycolysis. A, reduction of intracellular ATP level in CAV1-depleted cells. B, downregulation of glucose uptake and lactate accumulation by CAV1 depletion (Bars, SD; *, P < 0.05; **, P < 0.01). Transfected cells were incubated with 2-NBDG and amount of 2-NBDG uptake was measured by flow cytometry. C, an immunofluorescence assay of GLUT3. D, effect of CAV1 knockdown on GLUT3 expression. E, effect of GLUT3 knockdown on glucose uptake and intracellular ATP level. F, AMPK activation and autophagy induction by GLUT3 knockdown. DAPI, 4',6-diamidino-2-phenylindole.
Moreover, HMGA1 depletion decreased GLUT3 mRNA expression, glucose uptake, and intracellular ATP level, whereas it activated AMPK-p53 signaling (Fig. 5D and Supplementary Fig. S5B). To understand the mechanism underlying CAV1 induction of HMGA1-mediated GLUT3 transcription, we tested whether CAV1 binds to HMGA1, but failed to detect an interaction between 2 proteins (data not shown). Expression of HMGA1 and its phosphorylation and acetylation were also not affected by CAV1 depletion (Fig. 5E). It has been known that activity of HMGA1 as a transcription factor is controlled primarily by its intracellular distribution (18, 19). Thus, we analyzed CAV1 effect on the subcellular distribution of HMGA1. Interestingly, the nuclear HMGA1 was reduced and its cytoplasmic level was concomitantly increased following si-

Figure 5. Identification of GLUT3 as a direct transcription target of HMGA1. A, a putative HMGA1-binding sites in the GLUT3 promoter and construction of reporter plasmids. B, disruption of promoter responsiveness to CAV1 by mutation of the HMGA1-binding site. Data represent means of triplicate assays (bars, SD; *, *P < 0.05; **P < 0.01). C, effect of CAV1 depletion on HMGA1 binding to the GLUT3 chromatin. Cross-linked chromatin was immunoprecipitated with antibodies against HMGA1 or rabbit IgG and analyzed by PCR using primers that flank the HMGA1-binding site (HMGA1-BS1). D, GLUT3 reduction by HMGA1 deletion. E, no effect of CAV1 on expression and modification of HMGA1 protein. HMGA1 proteins were immunoprecipitated and subjected to immunoblot assays using pan-phosphorylation or pan-acetylation antibody. F, effect of CAV1 depletion on subcellular distribution of HMGA1.
CAV1 transfection (Fig. 5F). This finding suggested that CAV1 may upregulate HMGA1 binding to the GLUT3 promoter by enhancing its nuclear localization (Fig. 5F).

**CAV1 cooperates with HMGA1 to activate GLUT3 expression**

We assessed whether GLUT3 expression is related to CAV1 or HMGA1 level in colon cancers. In 19 cancer cell lines, GLUT3 level was strongly correlated with CAV1 level ($r = 0.739; P < 0.01$) and HMGA1 level ($r = 0.62; P < 0.01$; Fig. 6A and B). Given that HMGA1 expression is not upregulated by CAV1, it is conceivable that high GLUT3 expression could be achieved when CAV1 and HMGA1 are simultaneously elevated in the same cells. As predicted, SNU-C2A cells with high CAV1 but low HMGA1 level and RKO cells with high HMGA1 but low CAV1 level exhibited low GLUT3 expression (Fig. 6A). To test this possibility, we compared GLUT3-inducing activity of HMGA1 under high and low CAV1 conditions. In high CAV1-expressing LoVo cells, WT-HMGA1 transfection activated GLUT3 expression but, this effect was diminished by si-CAV1 cotransfection (Fig. 6C). Likewise, although WT-CAV1 transfection exerted no detectable effect on GLUT3 expression in SW480 cells, GLUT3-inducing activity of HMGA1 was greatly enhanced by WT-CAV1 cotransfection (Fig. 6D). Consistently, HMGA1 binding to the GLUT3 chromatin was greatly increased when SW480 cells were cotransfected with WT-CAV1 and WT-HMGA1 (Supplementary Fig. S6A). We next evaluated the relationship between CAV1, HMGA1, and GLUT3 in 20 matched tissue sets, which have markedly lower or higher CAV1 in cancer tissues compared with its adjacent normal tissues. CAV1 protein levels were comparable with its mRNA levels and showed a tight correlation with GLUT3 levels (Fig. 6E). A tumor-specific elevation of GLUT3 was detected from 8 of 10 high CAV1 tumors, whereas none of 10 low CAV1 tumors showed GLUT3 elevation (Fig. 6F). Moreover, all of the 8 high GLUT3 tumors but none of 12 low GLUT3 tumors exhibited high HMGA1 (Fig. 6E). In addition, 6 (66.7%) and 7 (77.8%) of 9 stage III tumors but only 2 (18.2%) and 3 (27.3%) of 11 stage I and II tumors showed high GLUT3 and high CAV1, respectively. Together, GLUT3 expression showed a correlation with expression of CAV1 and HMGA1 in both cancer cell lines and primary tumors, supporting that CAV1 and HMGA1 cooperate to stimulate GLUT3 expression.

**Discussion**

We show here that elevated CAV1 in colorectal cancer cells enhances aerobic glycolysis by upregulating glucose uptake through HMGA1-mediated GLUT3 induction. Our data also suggest that elevated expression of CAV1 may protect cancer cells from metabolic stresses and thus contribute to tumor progression through the regulation of glucose metabolism.

The regulation of CAV1 expression and its biologic significance in colorectal tumorigenesis have been poorly defined (20). In this study, we found that a substantial fraction of primary tumors have markedly reduced or elevated levels of CAV1 compared with adjacent normal tissues. Moreover, this differential regulation of CAV1 expression was associated with hyper- or hypomethylation of promoter CpG sites. In particular, methylation contents of 8 CpGs sites located within nucleotides −494 to −231 were identified to correlate tightly with mRNA levels, suggesting a critical role of this region in gene transcription. Consistent with this biphasic pattern of CAV1 expression, CAV1 exerted a growth-inhibiting and growth-promoting effect on tumor cells with low and high CAV1 level, respectively. Therefore, this finding supports that biphasic regulation of CAV1 expression in colorectal tumorigenesis is associated with the switch of its effect on tumor cell growth.

Although CAV1 elevation has been linked to an aggressive phenotype of cancer cells, however, the molecular mechanism underlying its oncogenic function remained largely undefined (21, 22). It was shown that CAV1 is regulated by c-Myc and suppress c-Myc–induced apoptosis under clinically relevant circumstances (23). Interestingly, prostate cancer cells have been shown to secrete CAV1, which stimulates clonal growth of tumor cells that do not express CAV1 (24). We found that elevated CAV1 in colorectal cancer cells is implicated in enhanced glucose uptake and aerobic glycolysis. Our data show that CAV1 upregulates glucose uptake, lactate accumulation, and intracellular ATP level. CAV1 regulation of ATP generation was also supported by activation of AMPK, which directly senses increases in the intracellular AMP:ATP ratio, by its depletion. These results indicate that elevated CAV1 enhances aerobic glycolysis. Aerobic glycolysis is an inefficient way to produce ATP compared with mitochondrial respiration. Nevertheless, most of cancer cells from diverse origin have a high rate of glycolysis. Moreover, it was shown that the upregulation of glycolytic metabolism correlates with increased tumor aggressiveness and poor patient prognosis (25). It was proposed that the glycolytic shift is beneficial for rapidly proliferating cancer cells that require macromolecules such as nucleotides, amino acids, and lipids (26). It was also proposed that cancer cells prefer glycolysis for ATP generation because active mitochondrial respiration may generate exaggerated levels of ROS, which may have detrimental effects on tumor growth (27).

We observed that both mitochondrial membrane potential and intracellular ROS level are decreased in CAV1-depleted cells, suggesting that elevated CAV1 in cancer cells may increase both aerobic glycolysis and mitochondrial respiration. Interestingly, our study revealed that AMPK activates p53, which subsequently induces cell-cycle arrest and autophagy in CAV1–depleted cells. CAV1 blockade led to AMPK activation in all high CAV1 tumor cells we tested, but cells lacking functional p53 did not exhibit autophagic phenotypes, indicating a strictly p53-dependent induction of autophagy. Autophagy is a catabolic process whereby intracellular components are delivered to lysosomes for degradation and recycled to regenerate metabolic building blocks. Autophagy is induced by diverse stimuli, including nutrient deprivation and hypoxia, and contributes to a diversity of processes, such as cellular homeostasis, developmental cell death, clearance of harmful protein aggregates. Several studies lead to the hypothesis that autophagy has tumor-suppressive functions. However, a series of recent investigations also suggest that autophagy promotes malignant progression (28). Our study revealed that CAV1 knockdown results in a p53-dependent autophagy and attenuates both in vitro and in vivo tumor growth more significantly in...
p53+/− cells versus p53−/− cells. Therefore, CAV1 upregulation might be a survival strategy of tumor cells, which protect themselves from autophagy triggered by AMPK-p53 signaling under metabolic stress conditions. In this context, it is noticeable that CAV1 elevation is more common in wtp53 cancer cells versus mtp53 cancer cells. We observed that 2 (HCT116 and LoVo) of 4 wtp53 cell lines have abnormally high CAV1 expression, whereas only 1 (SNU-C2A) of 15 p53-deficient cell lines have elevated CAV1 level. It is thus plausible that elevated CAV1 may facilitate cell proliferation and prolong cell viability under energetically stressful conditions by escaping autophagy induction.

The role for CAVs in glucose metabolism has been suggested by several studies (10, 11). Interestingly, recent studies showed...
that stromal fibroblasts lacking CAV1 undergo aerobic glycolysis and generate high levels of glycolytic end products and promote tumor growth by the paracrine secretion of these energy-rich glycolytic bioproducts (12). Furthermore, it was suggested that cancer cells use oxidative stress to induce CAV1 downregulation in adjacent fibroblasts via a lysosomal mediated pathway and that aerobic glycolysis in stromal cells is a direct consequence of autophagy promoted by loss of CAV1 (27). This finding is in line with our results from epithelial tumor cells, suggesting that CAV1 effect on glycolysis and autophagy may not be a cell type specific.

HMGA1 is a member of nonhistone DNA-binding proteins, which contain 3 DNA-binding domains and an acidic carboxy-terminal region (17). HMGA1 is involved in diverse cellular processes, including embryonic development and neoplastic transformation and tumor progression. Although HMGA1 has no intrinsic transcriptional activity, it regulates gene transcription by altering chromatin structure via protein–DNA and protein–protein interactions (18). We found that HMGA1 is a transcription factor for GLUT3, and CAV1 induces GLUT3 expression by upregulating HMGA1 interaction with GLUT3 promoter. HMGA1 blockade attenuated glucose uptake and activated AMPK signaling, supporting its implication in glucose metabolism. Furthermore, HMGA1 binding to the GLUT3 promoter, GLUT3 expression, and glucose uptake were increased in low CAV1 cells by cotransfection of CAV1 and HMGA1 but not by CAV1 alone. It was reported that CAV1 is also localized in the nucleus and can bind to promoter of genes, such as cyclin D1 (29). In this study, we could not detect CAV1 binding to the GLUT3 promoter. To elucidate molecular basis for CAV1 regulation of HMGA1, we carried out a series of assay, but failed to detect its interaction with or posttranslational modification of HMGA1. Interestingly, however, it was observed that nuclear amount of HMGA1 is enhanced by CAV1, raising the possibility that CAV1 increases HMGA1 activity as a transcription factor by enhancing its nuclear localization. Further studies are needed to elicit the underlying mechanism for CAV1 regulation of HMGA1.

In conclusion, CAV1 elevation is found in many primary colorectal carcinomas and cell lines and implicated in increased aerobic glycolysis. High CAV1 upregulates glucose uptake through HMGA1-mediated GLUT3 transcription, and its depletion results in autophagy by AMPK-p53 signaling. Collectively, our study suggests that elevated CAV1 expression may contribute to colorectal tumor progression by rendering tumor cells growth and survival advantages by enhancing glycolysis and ATP generation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported in part by grants from National Research Foundation of Korea (2009-0078864 and 2009-0087099) and the National Cancer Center (0820070), Republic of Korea.

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Received February 7, 2012; revised May 3, 2012; accepted May 22, 2012; published OnlineFirst June 15, 2012.

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