Molecular Cancer Research

Ketogenic *HMGCS2* Is a c-Myc Target Gene Expressed in Differentiated Cells of Human Colonic Epithelium and Down-Regulated in Colon Cancer

Nuria Camarero, Cristina Mascaró, Cristina Mayordomo, et al.

Mol Cancer Res 2006;4:645-653. Published OnlineFirst August 28, 2006.

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

Ketogenic *HMGCS2* Is a c-Myc Target Gene Expressed in Differentiated Cells of Human Colonic Epithelium and Down-Regulated in Colon Cancer

Nuria Camarero,[1] Cristina Mascaró,[1] Cristina Mayordomo,[1] Felip Vilardell,[2] Diego Haro,[1] and Pedro F. Marrero[1]

[1]Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona; and [2]Institut Català d'Oncologia, Bellvitge Colorectal Cancer Study Group, Barcelona, Spain

Abstract

***HMGCS2*, the gene that regulates ketone body production, is expressed in liver and several extrahepatic tissues, such as the colon. In CaCo-2 colonic epithelial cells, the expression of this gene increases with cell differentiation. Accordingly, immunohistochemistry with specific antibodies shows that HMGCS2 is expressed mainly in differentiated cells of human colonic epithelium. Here, we used a chromatin immunoprecipitation assay to study the molecular mechanism responsible for this expression pattern. The assay revealed that *HMGCS2* is a direct target of c-Myc, which represses *HMGCS2* transcriptional activity. c-Myc transrepression is mediated by blockade of the transactivating activity of Miz-1, which occurs mainly through a Sp1-binding site in the proximal promoter of the gene. Accordingly, the expression of human *HMGCS2* is down-regulated in 90% of Myc-dependent colon and rectum tumors. HMGCS2 protein expression is down-regulated preferentially in moderately and poorly differentiated carcinomas. In addition, it is also down-regulated in 80% of small intestine Myc-independent tumors. Based on these findings, we propose that ketogenesis is an undesirable metabolic characteristic of the proliferating cell, which is down-regulated through c-Myc-mediated repression of the key metabolic gene *HMGCS2*.**
(Mol Cancer Res 2006;4(9):645–53)

Introduction

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) is a potential regulatory point in the pathway that converts acetyl-CoA to ketone bodies (1-3). In rats, hepatic ketogenic capacity increases rapidly during postnatal development (4) or fasting (5) when the mRNA, protein, and activity of HMGCS2 increase in liver (6-8). The factors responsible for the postnatal and starvation-associated induction of *HMGCS2* gene expression have been identified and shown to involve changes in circulating hormone and fatty acid levels. Administration of dibutyryl-cyclic AMP to adult rats increases mRNA, protein levels, and enzymatic activity of HMGCS2 in the liver (6, 9). Similar effects are produced by a high-fat diet (10), whereas insulin causes the opposite effect (6).

The rat *HMGCS2* gene contains elements that control its multihormonal regulation and tissue specificity (11-14). To date, three main regulatory sequences have been described in the rat HMGCS2 promoter: (*a*) an insulin-responsive sequence, located at position −211 with respect to the transcriptional start site, which binds proteins from the forkhead family of transcription factors (12); (*b*) a peroxisome proliferator-activated receptor element, located at position −104, which binds retinoid X receptor-peroxisome proliferator-activated receptor heterodimers (13); and (*c*) a Sp1-binding site, located at position −56, which binds proteins from the Sp1 family of transcription factors (14). In the human promoter, the peroxisome proliferator-activated receptor element and the Sp1-binding site are also present in similar positions (14-16). Thus, transcriptional activation of the HMGCS2 promoter, mediated by a network of recruited transcription factors, can partly explain the induction of gene expression observed in liver under physiologic and pathologic conditions.

The *HMGCS2* gene is also expressed in extrahepatic tissues. In rats, *HMGCS2* is expressed in testis, ovary, intestine, kidney, brain, colon, and lymphocytes (6, 7, 17-19). In humans, expression has been reported in liver, skeletal muscle, heart, pancreas, testis, and colon (20). The role of *HMGCS2* expression in several of these tissues remains to be fully elucidated. However, in tissues that oxidize fatty acids, it has been proposed that HMGCS2 expression prevents acetyl-CoA accumulation, which in turn impairs fatty acid oxidation rates (21). In resting lymphocytes, *HMGCS2* expression may provide cell metabolism with a "branch point sensitivity" mechanism by which cells regulate the rate of precursor utilization (acetyl-CoA) during the cell cycle (19). Thus, a high flux through a pathway in which acetyl-CoA is an intermediate buffers the concentration of acetyl-CoA against changes in its rate of utilization in other pathways.

The capacity of the colon for ketogenesis has been correlated with *HMGCS2* expression, which depends on the amount of butyrate produced by intestinal flora (18, 22). The effect of

Received 1/3/06; revised 6/30/06; accepted 7/25/06.
Grant support: Ministerio de Ciencia y Tecnología (Programa General del Conocimiento) grant BMC2001-2891, Ministerio de Educación y Ciencia grants BFU2004-00289 and BFU2004-01925, FIS of the Instituto de Salud Carlos III grant C03/08, Red de Centros RCMN, and Ajut de Suport als Grups de Recerca de Catalunya grant 2001SGR 00432.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Pedro F. Marrero, Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain. Phone: 34-93-403-4500; Fax: 34-93-402-4520. E-mail: pedromarrero@ub.edu

doi:10.1158/1541-7786.MCR-05-0267

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

butyrate on *HMGCS2* expression *in vivo* is exerted at the transcriptional level through a single Sp1-binding site present in the proximal promoter of the gene (14). Butyrate stimulates cell proliferation in healthy colonic epithelial cells. However, it reduces proliferation and induces cell differentiation and apoptosis in cancer cell lines in this tissue (23-25). One of the hypotheses proposed to explain the "butyrate paradox" is that healthy colonocytes metabolize butyrate efficiently, which would result in a reduction in intracellular concentration and therefore a decrease in capacity to inhibit growth (26). The capacity to metabolize butyrate could be lost in colon cancer cell lines (27). In this context, it has recently been described that a lack of induction of HMGCS2 leads to impaired β-oxidation (21).

In the colon, the β-catenin/TCF-4 complex forms the master switch that controls proliferation and differentiation in healthy and malignant intestinal epithelial cells. Thus, disruption of β-catenin/TCF-4 activity induces cell cycle arrest and differentiation (28). One of the target genes of TCF-4 is *Myc*, which encodes a transcription factor, c-Myc (herein called Myc). This factor can activate or repress gene expression. Myc regulates several major cellular functions, such as proliferation, adhesion, and size, which explains the diversity of Myc target genes found in a variety of metabolic pathways. In addition, Myc has been linked to energy metabolism via up-regulation of glycolysis (29), mitochondrial biogenesis (30), and abrogation of hepatic HMGCS2 expression and ketogenesis after the induction of diabetes (31). Myc represses gene transcription by at least two distinct mechanisms. The first is limited to the Inr sequence in the promoter start site and requires the inhibition of Miz-1-mediated transcription activation by the binding of Myc-Max heterodimers (32-34). The second mechanism is Inr independent and involves other promoter sequences in the recruitment of Myc-containing repressor complexes. In this mechanism, Sp1 has been reported to be one of several proteins with the capacity to recruit Myc (35, 36). However, other promoter sequences also participate in the recruitment of this transcription factor (37) or in Miz-1 transactivation (38).

To study the mechanism that regulates *HMGCS2* expression, here we examined the expression of this gene in human colon. We observed a clear pattern of expression in differentiated cells of the colonic epithelium. Using chromatin immunoprecipitation assays, we also show that HMGCS2 is a direct target of Myc, which abrogates Miz-1-mediated transcriptional activation of *HMGCS2* expression. Therefore, transcriptional repression by Myc could play a crucial role in regulating the expression of this gene. Our data led us to analyze the expression of HMGCS2 in several types of human intestinal cancer. A clear down-regulation of gene expression in colon, rectum, and small intestine was observed. In the first two tissues, this down-regulation correlated with Myc up-regulated tumorigenesis. Consistently with its expression in differentiated cells, HMGCS2 protein expression was down-regulated preferentially in moderately and poorly differentiated carcinomas.

Results

HMGCS2 Expression and Promoter-Associated Histone Acetylation Increases during Differentiation

In liver and colon, HMGCS2 is a histone deacetylase inhibitor target gene (14). Because histone acetylation is related to cell differentiation, we studied the expression pattern of a set of genes in proliferating and differentiated CaCo-2 cells. *HMGCS2* expression was lower in proliferating cells (Fig. 1, compare *lanes 1* and *3*). Similar data were obtained when HMGCS2 expression was evaluated in terms of enzymatic activity (0.024 versus 0.25±0.02 mU/mg for proliferating and differentiated cells, respectively). As expected, differentiation induced the expression of intestinal alkaline phosphatase. Miz-1 expression also increased during differentiation, whereas Myc expression was clearly down-regulated (Fig. 1). As the expression of HMGCS2 correlated with acetylation of H4 promoter-associated histones (14), we studied the effect of trichostatin A on proliferating and differentiated cells. This drug induces mRNA expression of HMGCS2 only on the former (Fig. 1, compare *lanes 1* and *2* with *lanes 3* and *4*), which indicates that histone acetylation is involved in cell differentiation-associated induction of HMGCS2. Trichostatin A did not affect mRNA levels of Miz-1 in proliferating or differentiated cells. However, trichostatin A down-regulated Myc expression in the former.

HMGCS2 Is Expressed in Differentiated Cells of the Colonic Epithelium

To study the expression pattern of HMGCS2 in human colon, we raised an antibody against a human HMGCS2 epitope. The antibody recognizes recombinant human HMGCS2 expressed in a stable cell line (Fig. 2A, *lane 1*). To ensure that antibody specificity recognized the human protein in the extracts, we used another stable cell line expressing rat recombinant HMGCS2 (39). The antibody recognized the rat HMGCS2 protein with a much less affinity (Fig. 2A, *lane 2*). HMGCS2 expression was localized mainly in differentiated cells of the colonic epithelium, whereas proliferating cells did not express the protein (Fig. 2B). In addition, no labeling was observed with a preimmune serum in either cell stage (Fig. 2B).

HMGCS2 Is Down-Regulated by Myc via a Miz-1-Dependent Mechanism

The HMGCS2 expression pattern is consistent with the Myc and Miz-1 expression gradients along the axis of the crypt (28). Moreover, expression of Myc in the liver of transgenic mice

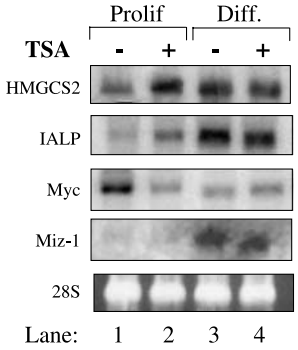

FIGURE 1. Effects of differentiation on trichostatin A–mediated induction of HMGCS2 expression in CaCo-2. Cells were harvested at 5 days (proliferating) or 21 days (differentiated) after a 24-hour treatment with 0.3 μmol/L trichostatin A (*TSA*; +) or DMSO (–) and were then analyzed for HMGCS2, Myc, Miz-1, and intestinal alkaline phosphatase (*IALP*) mRNA expression. Bottom, ethidium bromide staining of 28S rRNA.

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

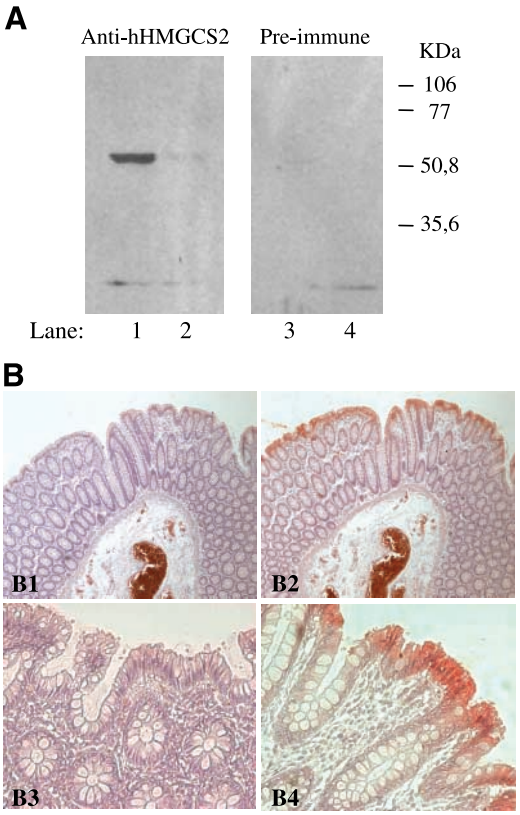

FIGURE 2. Expression of mitochondrial HMGCS2 in human colonic mucosa. **A.** Recombinant human (*lanes 1* and *3*) and rat (*lanes 2* and *4*) HMGCS2 proteins were analyzed by Western blot with preimmune serum (*lanes 3* and *4*) or antibodies against human-HMGCS2 (*lanes 1* and *2*). **B.** Representative HMGCS2 immunohistochemistry of healthy tissue from four patients. Protein was analyzed using the specific anti-human HMGCS2 antibody. B1 and B3, no staining in the presence of preimmune serum; B2 and B4, positive staining (*red*) when incubated with the HMGCS2 antibody. Nuclei were counterstained with hematoxylin. Magnification, ×4 (B1 and B2) and ×20 (B3 and B4).

abolishes the ketosis associated with streptozotocin-induced diabetes and down-regulates HMGCS2 expression (31). Therefore, we studied the role of Myc in the regulation of HMGCS2 expression. Antibodies against Myc effectively precipitated the human HMGCS2 promoter in a chromatin immunoprecipitation assay (Fig. 3A). These data indicate that HMGCS2 is a direct target of Myc. In addition, Myc cotransfection abrogated the expression of a reporter construct driven by the HMGCS2 promoter, indicating a putative role for Myc in HMGCS2 down-regulation in proliferating cells (Fig. 3B). Moreover, Myc overexpression in CaCo-2 cells (Fig. 3D) down-regulated the endogenous HMGCS2 expression by 35% to 40% (Fig. 3C).

Repressor effects of Myc are produced by either Inr-dependent or Inr-independent mechanism. Therefore, we studied the effect of progressive deletions and Sp1-binding site mutation of the rat HMGCS2 promoter (Fig. 4A) on promoter activity and Miz-1-mediated effects (Fig. 4B). The previously characterized peroxisome proliferator-activated receptor element (15) and Sp1-binding sites (14) participated in sustaining transcriptional activity of the HMGCS2 promoter. Miz-1 transactivated all the constructs containing the Sp1-binding site assayed, whereas deletion or mutation of the Sp1 site abrogated

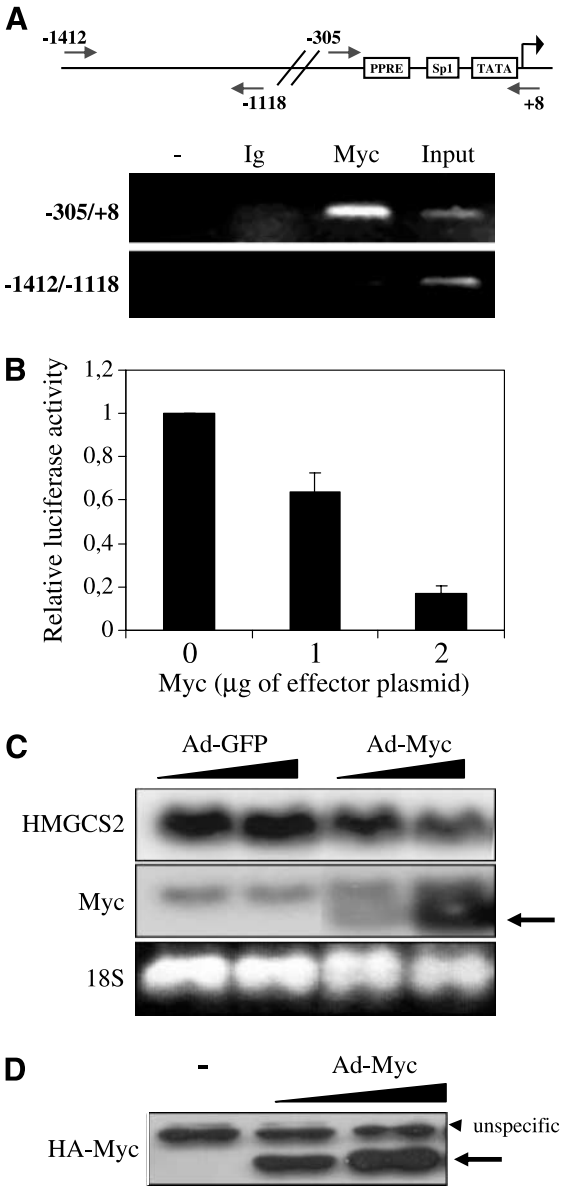

FIGURE 3. HMGCS2 promoter is down-regulated by Myc. **A.** Relative positions of primers used in the chromatin immunoprecipitation assay from CaCo-2 cells to show the recruitment of Myc to the human HMGCS2 proximal promoter. Chromatin preparations were obtained and immunoprecipitated with antibodies against Myc. The immunoprecipitates were then analyzed by PCR (28 cycles) using specific primers for the mitochondrial HMGCS2 promoter (14). Aliquots of chromatin taken before immunoprecipitation were also analyzed (*Input*). **B.** CaCo-2 cells were transiently cotransfected with 2 μg of the reporter plasmid −116pGl3 in combination with increasing amounts of an expression vector for Myc (pME18S-c-Myc). The amount of plasmid was kept constant by using pME18S empty vector. Luciferase activity is depicted relative to the respective *Renilla* activities. The activity in the presence of empty vector was set at 1, and the other data were normalized to this value. Columns, mean of three independent experiments with two plates each; bars, SD. **C.** Fourteen-day-old CaCo-2 cells were infected with adenoviruses expressing either green fluorescent protein (*Ad-GFP*) or green fluorescent protein and recombinant hemagglutinin-tagged c-Myc (*Ad-Myc*) at a multiplicity of infection of 50 and 100, respectively. Forty-eight hours after infection, cells were harvested and analyzed by Northern blot for HMGCS2 and Myc expression. Arrows, expression of recombinant Myc. Bottom, ethidium bromide staining of 18S rRNA. **D.** Whole-cell extracts of uninfected (−) or Ad-Myc-infected (*Ad-Myc*) cells were analyzed for the expression of Myc protein by Western blot using an antibody against hemagglutinin tag.

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

Miz-1-mediated transcriptional activity (Fig. 4B). Conversely, Miz-1-mediated transactivation was not abolished by mutation of the transcription start site (data not shown). As expected, Miz-1-mediated transactivation was blocked by Myc expression (Fig. 4C). In addition, a Myc deletion mutant (pME18S-c-Myc D348-493) lacking the carboxyl end of the protein did not repress Miz-1 transactivation (Fig. 4C).

To assess whether the HMGCS2 Sp1-binding site recruited both transcription factors, we did DNA precipitation assays in which cellular extracts expressing tagged proteins were incubated with biotinylated Sp1 wild-type or mutant probes. Figure 4D shows the binding of Sp1, Myc, and Miz-1 to the Sp1 probe (Sp1-wt). This binding was specific, because a mutated Sp1 probe (Sp1-M) did not recruit any of these proteins.

These data indicate that the Sp1-binding site is involved in Miz-1-dependent transcriptional activity, which can explain, at least in part, Myc transrepression of the HMGCS2 promoter in colon-derived cell lines.

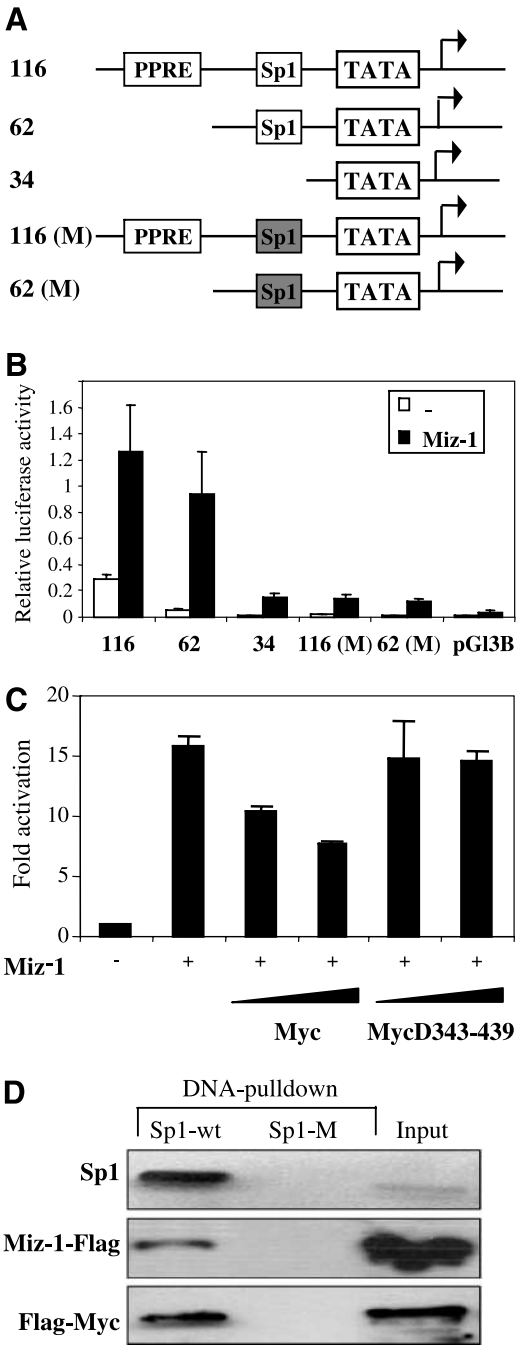

FIGURE 4. Myc overexpression blocks Miz-1-mediated HMGCS2 transactivation. **A.** Promoter constructs used. **B.** Promoter constructs were transiently transfected into CaCo-2 cells and, when indicated (Miz-1), cotransfected with 0.5 μg of an expression vector for Miz-1 (pUHD-Miz-1). Luciferase activities are shown relative to the respective *Renilla* activities. 116 (M) and 62 (M), 116 (M-Sp1) and 62 (M-Sp1) plasmids, respectively. **C.** CaCo-2 cells were transiently cotransfected with 2 μg of the reporter plasmid −62pGl3 in combination with 0.5 μg pUHD-Miz-1 and, when indicated, with 1 and 2 μg pME18S-c-Myc or pME18S-c-Myc D348-493. The amount of expression plasmids was kept constant by the use of empty vectors (pcDNA3 for Miz-1 and pME18S for Myc). The normalized luciferase activity observed in cells transfected with −62pGl3 and empty vectors was set at 1, and the other data were normalized to this value and expressed as fold induction. Columns, mean of three independent experiments with two plates each; bars, SD. **D.** HeLa cells were transfected with either pME18S-c-Myc (*top* and *bottom*) or pcDNA3-Miz-1-Flag (*middle*) plasmids to express Flag recombinant fusion proteins. After 48 hours, cells were harvested and whole-cell extracts were incubated with biotinylated oligonucleotides containing the Sp1 site of human HMGCS2 (*Sp1-wt*) or a scrambled sequence of this site (*Sp1-M*). Avidin-purified oligonucleotides were processed for Western blot analysis with antibodies against Flag or Sp1.

Expression of Human HMGCS2 Is Down-Regulated in Colon Cancer

Myc has been implicated in the genesis of specific human tumors. In all cases, the relative amounts of Myc are increased in the tumor tissue relative to the surrounding healthy tissues, which indicates that elevated expression of Myc contributes to tumorigenesis (40). To examine the putative role of Myc-mediated down-regulation of HMGCS2 expression in distinct types of intestinal cancer, we studied the expression pattern of HMGCS2 (Fig. 5A) and Myc (Fig. 5B) in tumor and healthy matched tissues. HMGCS2 expression was higher in tissues, such as colon and small intestine, whereas the rectum and stomach showed lower levels of expression. In addition, expression differed between samples of healthy small intestine, colon, and rectum from the patient sample tested. Fluctuations between healthy tissues were much lower in the Myc expression pattern (Fig. 5B). This therefore hinders the establishment of a relationship between HMGCS2 and Myc expression in healthy tissues.

Colon HMGCS2 expression was down-regulated in 90% of the tumors (<0.7-fold expression in tumor relative to healthy tissue; Fig. 5A, *right*). In contrast, colon Myc expression was up-regulated in 80% of the tumors (>1.5-fold expression in tumor relative to healthy tissue; Fig. 5B, *right*). These findings indicate that, despite individual fluctuations in gene expression, HMGCS2 is down-regulated in Myc-dependent colon tumors. Similarly, an inverse correlation also occurred between the expressions of these two genes in rectum. In this tissue, HMGCS2 expression was down-regulated in 80% of the tumors (Fig. 5A, *right*), whereas Myc was up-regulated in 70% of tumors (Fig. 5A, *right*). However, this type of correlation was not detected in the small intestine or stomach. In the former, HMGCS2 down-regulation (84% of the tumors) did not correlate with an up-regulation of Myc (14% of the tumors), whereas, in

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

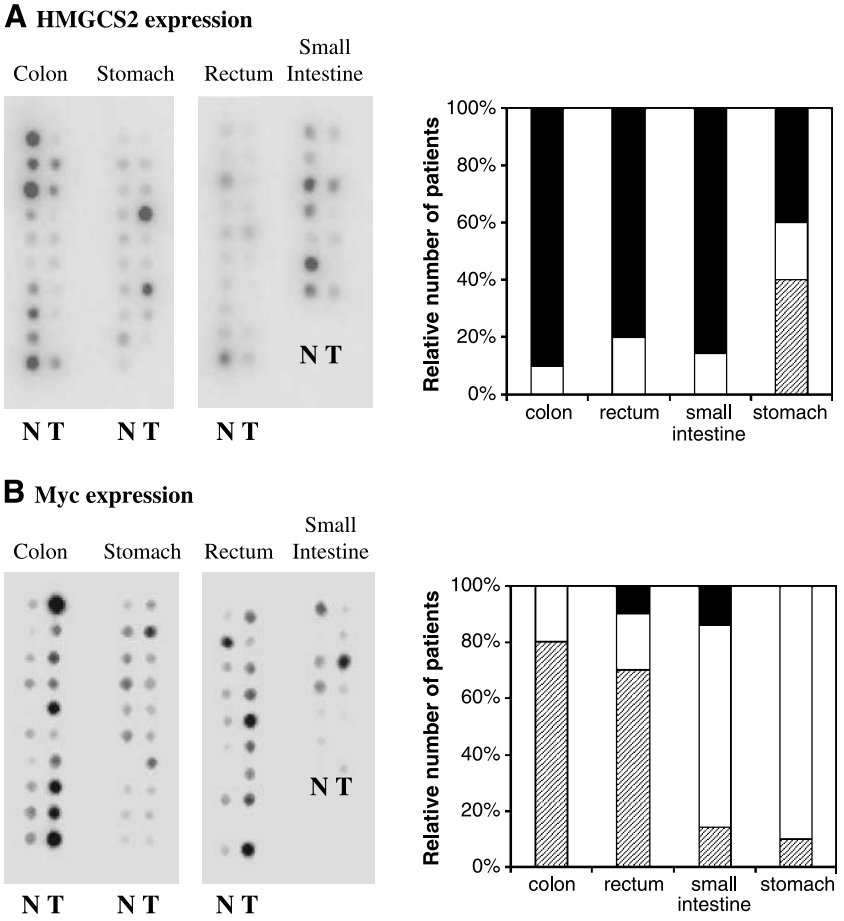

FIGURE 5. HMGCS2 is down-regulated in human colon cancer. Left, ubiquitin-normalized Cancer Profiling Array II was hybridized with human HMGCS2 (**A**) or c-Myc (**B**) cDNA probes. N, healthy; T, tumor. Right, Phosphor-Imager quantification of the dot blot signals from tumor versus healthy tissues represented as a function of down-regulated (<0.7-fold; *black columns*), up-regulated (>1.5-fold; *white columns*), and nonregulated (*shaded columns*).

stomach, no clear down-regulation of *HMGCS2* gene expression was observed (40% of the tumors showed either down-regulation or up-regulation). These data indicate that HMGCS2 is down-regulated in small intestine, colon, and rectum, although only colon and rectum tumors were related to Myc overexpression. Therefore, HMGCS2 down-regulation in tumors may be a characteristic of extending Myc-dependent tumorigenesis.

Expression of HMGCS2 Is Lower in Moderately and Poorly Differentiated Colorectal Adenocarcinoma

To study correlations between differentiation and HMGCS2 down-regulation in colon cancer, immunohistochemistry was done on a tissue array (Colon Tumor Tissue Array from Lab Vision, Freemont, CA) containing samples from 64 colorectal adenocarcinoma specimens (Fig. 6). In 5 cases, no neoplastic tissue was observed. Two cases showed healthy, in addition to tumoral, tissue, whereas marginal adenoma was observed in another 2 cases. In these 4 cases, both the healthy crypts and the epithelium with features of adenoma showed the highest density of HMGCS2-positive cells in comparison with the malignant glands. The grade of differentiation of the adenocarcinomas was scored based on observations of the tumor areas shown in the tissue arrays. Nineteen carcinomata were regarded as well differentiated, 37 as moderately differentiated, and 3 as poorly differentiated. For the purpose of statistical analysis, the small sample of poorly differentiated tumors was grouped with moderately differentiated adenocarcinomas. Staining was categorized based on the intensity and location of staining as well as the percentage of tumor cells staining positive. The percentage of HMGCS2-positive cells was expressed as a ratio of positive cells to the total number of cells counted. The cutoff used was 35%, which was the mean of positive cells among the 59 cases with adenocarcinoma. Thirty-two carcinomas expressed low levels of HMGCS2, whereas the remaining 27 showed high expression. A good correlation was observed between the grade of tumor differentiation and the percentage of cells expressing HMGCS2 ($P = 0.003$; Table 1). Fourteen well-differentiated carcinomas expressed HMGCS2 in >35% of cells, whereas only 5 well-differentiated tumors showed low expression of this protein. In contrast to the well-differentiated tumors, the moderately and poorly differentiated carcinomas tended to show low expression of HMGCS2. Only 13 moderately and poorly differentiated tumors expressed HMGCS2 in >35% of cells.

Discussion

The Myc transcription factor affects the expression of >10% of human genes and is a master switch that couples metabolism to cell growth and proliferation. Myc has been associated with energy metabolism through its up-regulation of glycolysis (29) or mitochondrial biogenesis (30). Here, we have shown that

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

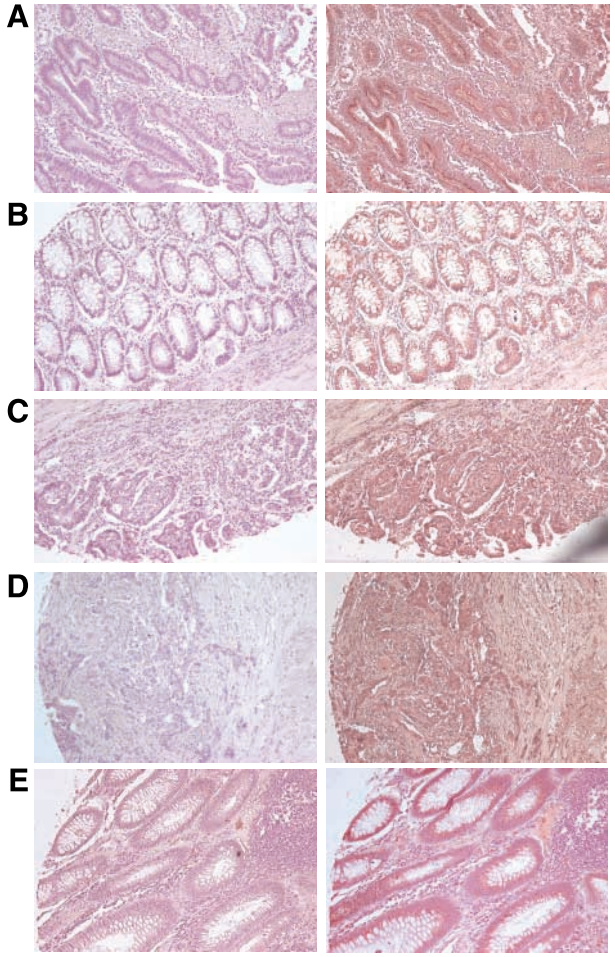

FIGURE 6. Immunohistochemical staining for HMGCS2 in human colon carcinoma (Colon Tumor Tissue Array). Left, negative controls; right, HMGCS2 immunostaining. **A.** Prominent HMGCS2 in a well-differentiated adenocarcinoma. **B.** Intense and diffuse expression of HMGCS2 in normal crypts. **C.** Adenocarcinoma adjacent to the preceding normal crypts, showing a clear diminution of HMGCS2 expression, with only a 30% of tumoral cells expressing the protein. **D.** Low expression of HMGCS2 in a poor differentiated tumor. **E.** Peripheral adenoma showing low expression of HMGCS2.

HMGCS2, the gene that controls the anabolic ketogenic pathway, is a Myc-target gene expressed in differentiated cells of the human colon. This expression pattern is consistent with the Myc and Miz-1 expression gradients along the axis of the crypt (28). The role of Myc in HMGCS2 down-regulation is supported by chromatin immunoprecipitation experiments, which showed occupancy of the HMGCS2 promoter by Myc. In agreement with our results, the overexpression of Myc in the liver of transgenic animals down-regulates HMGCS2 expression and ketogenesis (31). The expression pattern of HMGCS2 in differentiated cells of the colon and its down-regulation by Myc indicates that this gene may be under the control of the Wnt/APC/β-catenin signaling pathway (28, 34). In concordance with this hypothesis, the analysis of gene expression by microarrays shows that HMGCS2 is up-regulated by the expression of a dominant-negative form of TCF-4 in the colon cell line Ls147T (28).

Myc can directly repress gene expression through several mechanisms. The Inr-dependent mechanism implies abrogation of the Miz-1 transactivation effect by Myc/Max heterodimers (34). Alternatively, the Inr-independent mechanism involves Myc interaction with proteins, such as Sp1 (36). However, the Sp1-binding site has also been reported to participate in Miz-1-mediated transactivation (38). The HMGCS2 transcription start site has been established by primer extension, S1 nuclease protection, and rapid amplification of cDNA ends experiments in rat (11), pig (41), and human (15) genes and does not contain a canonical Inr sequence. Mutation of critical elements in the HMGCS2 proximal promoter diminished its transcriptional activity and therefore hinders the study of Myc transrepression. However, the Sp1-binding site, present in the proximal promoter of the gene, recruits Miz-1 and Myc transcription factors (Fig. 4D). This Sp1-binding site is involved in the transactivation mediated by Miz-1 (Fig. 4B), whereas Miz-1-mediated transactivation is blocked by Myc expression (Fig. 4C). Therefore, HMGCS2 expression seems to be under the control of a Inr-independent mechanism that still involves Miz-1 transactivation, which is blocked by Myc. Other redundant indirect mechanisms may also explain HMGCS2 down-regulation by Myc. Thus, the *HMGCS2* gene is a target of HDAC1 (14) and HDAC2 (data not shown). Moreover, HDAC2 may be induced by Myc (42). Interestingly, the effect of histone deacetylase inhibitors is mediated by the same Sp1-binding site that participates in Miz-1 transactivation (14).

HMGCS2 is an inducible gene that controls ketone body production in the liver (1-3) under physiologic conditions, such as those occurring during suckling (4) or the fed-starved transition (5). HMGCS2 regulation occurs mainly at the transcriptional level. The proximal promoter of the gene contains elements that could account for its regulation by hormones, such as insulin (11), and metabolites, such as long-chain fatty acids or butyrate (13, 14). Insulin-mediated regulation of HMGCS2 involves forkhead transcription factors (12). Withdrawals of Foxo1 from the nucleus, as well as down-regulation by Myc, are critical steps in the regulation of cell proliferation. Thus, in addition to hormonal and metabolic signals, HMGCS2 seems to be down-regulated by signals that induce cell proliferation. Consistent with this hypothesis, the expression of this gene is down-regulated in a rat colon cancer model with the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (43), or during liver regeneration after partial hepatectomy (44).

The molecular mechanism proposed to explain the colon cell-specific expression of HMGCS2 observed implies that the expression of this gene may be altered in cancer. The gene was down-regulated in 90% to 80% of colon, small intestine, and rectum tumors. However, only the colon and rectum tumors were Myc dependent. In addition, because of the high variability of HMGCS2 expression, we were unable to establish a straight correlation between Myc and HMGCS2 expression in healthy tissues. This variability in HMGCS2 expression was not related to levels of Myc. Therefore, although HMGCS2 is a direct target of Myc, the expression levels of these two genes depend on additional factors, which may be related to differences in nutritional status (4-6) or the intestinal germ flora (18, 22) of the patients. Down-regulation of HMGCS2 expression in proliferating cells was also observed at the protein level. The analysis of a tissue

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

array-containing samples from 64 colorectal adenocarcinoma specimens indicates that HMGCS2 expression is preferentially down-regulated in moderately and poorly differentiated carcinomas when compared with well-differentiated tumors.

HMGCS2 expression has been associated with the ketogenic capacity of the colon (18). In addition, we have recently proposed that HMGCS2 forms part of a feed-forward mechanism in the colon to facilitate mitochondrial oxidation of butyrate (14). Nevertheless, we also speculate that ketone bodies are cell signaling molecules; therefore, the strict control of HMGCS2 expression is involved in a downstream cell signaling process. In this context, it has been shown that the ketone body acetoacetate, through increased oxidative stress, regulates gene expression (45), inhibits growth inhibition in human endothelial cells (46), and activates the extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling pathways in primary cultured rat hepatocytes (47).

Metabolism is an essential process for life and perturbations may have profound implications. Metabolism is tightly regulated by diet. Thus, there are different dietary manipulations capable to affect HMGCS2 expression (4-6, 18, 22). In this article, we show that HMGCS2 is down-regulated in poorly differentiated carcinomas. The relevance of HMGCS2 expression in tumor progression remains uncovered. However, we propose that some of these specific dietary manipulations can affect cancer incidence if ketone body production, or HMGCS2 expression, plays a role in tumor development/progression.

Materials and Methods

Cell Culture

The human colon cancer cell line CaCo-2, obtained from the American Type Culture Collection (Rockville, MD), was maintained in DMEM supplemented with antibiotics and 10% fetal bovine serum at 37°C and 5% CO_2. CaCo-2 cells were cultured in medium alone for up to 5 and 21 days, with changes of medium every 2 days to obtain proliferating and differentiated cells for Northern blot experiments. Trichostatin A was obtained from Calbiochem (La Jolla, CA) and was used at a concentration of 0.3 μmol/L.

Table 1. Quantitation of HMGCS2 Immunolabeling in Low-Grade and High-Grade Tumors

HMGCS2 expression	% Positive cells	Low-grade tumors, *n* (%)	High-grade tumors, *n* (%)
Low expression	0	1 (5.3)	7 (17.5)
	3		1 (2.5)
	5		4 (10.0)
	8		1 (2.5)
	9		1 (2.5)
	10	1 (5.3)	4 (10.0)
	12	1 (5.3)	1 (2.5)
	15		2 (5.0)
	7		1 (2.5)
	20		1 (2.5)
	30	2 (10.5)	4 (10.0)
High expression	40	2 (10.5)	
	50	2 (10.5)	2 (5.0)
	55	1 (5.3)	
	60	1 (5.3)	2 (5.0)
	65	1 (5.3)	1 (2.5)
	70	4 (21.1)	7 (17.5)
	80	3 (15.8)	1 (2.5)
Total		19 (100.0)	40 (100.0)
Weighed mean % of positive cells		50.6	28.3

Plasmids

The rat HMGCS2 promoter-luciferase fusion plasmids −116pGl3, −62pGl3, and −34pGl3 were constructed by *Hin*dIII subcloning of the −116 to +28 fragment of pSMpCAT6A, the −62 to +28 fragment of pSMpCAT6D, and the −34 to +28 fragment of pSMpCAT7 into the pGL3Basic firefly luciferase reporter gene (Promega, Madison, WI). The plasmids pSMpCAT6A, pSMpCAT6D, and pSMpCAT7 were obtained as described previously (11, 13, 48). The −62 (M-Sp1) construct was generated by PCR amplification from −116pGl3 (M-Sp1) using the oligonucleotides 5′-aagcttTAAGTGGATCTAGAGCTTGCAGAGGCTG-3′ corresponding to coordinates −62 to −35 and 5′-aagcttCCAGCAGTCCACAGCCCCTGCG-3′ corresponding to coordinates +28 and +7. Nucleotides in lowercase were added to provide *Hin*dIII sites at the 5′ and 3′ ends. The PCR product was cloned into the p-GEM-T vector and subsequently subcloned into *Hin*dIII-cut pGL3Basic. The plasmid −116pGl3 (M-Sp1) was obtained as described previously (14). All construction sequences were confirmed by the dideoxynucleotide chain termination method using an automated fluorescence-based system (Perkin-Elmer, Wellesley, MA). Plasmid pcDNA3-hMS was constructed by insertion of the *Eco*RI fragment excised from pBS-MSH (20), corresponding to the cDNA of human HMGCS2, into the *Eco*RI site in the polylinker of the eukaryotic expression vector pcDNA3. The pUHD-Miz-1 and pcDNA3-Miz-1-Flag expression plasmids (32) were a gift from Dr. M. Eilers (Zentrum für Molekulare Biologie Heidelberg, Heidelberg, Germany). The pME18S, pME18S-c-Myc, and pME18S-c-Myc D348-493 (49) were kindly provided by Dr. Javier Leon (Universidad de Cantabria, Cantabria, Spain).

Northern and Southern Blot Analyses

Hybridizations were done following standard methods (50) from extracted total RNA or cDNA immobilized on the Cancer Profiling Array II (Clontech, Palo Alto, CA). Total RNA was extracted from CaCo-2 cell lines with Trizol (Life Technologies, Gaithersburg, MD) and Northern blot analyses were done with 15 μg total RNA/lane using 2.2-kb human HMGCS2 cDNA, obtained by digesting pBS-MSH (20) with *Eco*RI, as a probe. Cancer Profiling Array II included normalized cDNA from tumor and corresponding healthy tissues from individual patients. This array was hybridized with a 3′ cDNA human HMGCS2 probe, a 789-bp fragment obtained by *Hin*dIII digestion of the pcDNA3-hMS plasmid. The probes for Miz-1 and Myc were the 2.4-kb fragment and the 1- to 4-kb fragment of human cDNA prepared by *Eco*RI/*Xba*I digestion of the pUHD-Miz-1 and pME18S-c-Myc plasmids, respectively. The alkaline phosphatase DNA probe corresponds to a 739-bp fragment of the human alkaline phosphatase cDNA and was obtained by reverse transcription-PCR as follows: 1 μg CaCo-2 RNA treated with butyrate was used in the reverse transcriptase reaction. Then, one fourth of the reverse transcriptase reaction was PCR amplified using the oligonucleotides 5′-tctagaCAGCTGAGGAGGAGAACCCGGC-3′ and 5′-ctcgagGTGCTTTGCCAGCCATTCCTGC-3.

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

Nucleotides in lowercase were added to provide *Xba*I/*Xho*I sites at the 5′ and 3′ ends, respectively, for subsequent cloning.

Transient Transfection

CaCo-2 cells were seeded at a density of $2 \times 10^4/cm^2$ 12 hours before transfection. Cells were transfected using the FuGene 6 reagent following the manufacturer's instructions (Roche, Indianapolis, IN) with 2 μg of the reporter 3-hydroxy-3-methylglutaryl-CoA synthase-luciferase gene construct and the amounts of the eukaryotic expression vector indicated in the figures or figure legends. In all cotransfection experiments, the amount of transfected DNA was kept constant by including salmon sperm DNA. After 48 hours, cell lysates were collected for luciferase assay. The pRL-CMV plasmid (Promega) was included in the transfection procedure to correct for variation in transfection efficiency. In the transfection experiments in which Myc was overexpressed, luciferase activity was corrected for the amount of protein assayed (51) because of the regulation of the pRL-CMV control plasmid expression by the expression of Myc.

Stable Transfection

Mev-SM cells, expressing rat HMGCS2, were obtained as described previously (39). Mev-SMh cells, expressing human HMGCS2, were obtained in a similar way by stable transfection of Mev-1 cells with the plasmid pcDNA3-SMh and were selected for their capacity to grow in the absence of mevalonate (39).

Adenovirus Infection

Recombinant adenoviruses expressing both green fluorescent protein and hemagglutinin-tagged c-Myc were purchased from Vector Biolabs (Philadelphia, PA). As a negative control, we generated adenoviruses expressing only green fluorescent protein. Recombinant adenoviruses were generated using the Ad-Easy system. The parent plasmids (pAdEasy-1 and pAd-Track-CMV) were a gift form B. Vogelstein (The Johns Hopkins Oncology Center). All adenovirus vectors were produced in HEK293 cells and purified as described previously (52). Differentiated CaCo-2 cells were infected with recombinant adenoviruses for 4 hours. Cells were left standing for 48 hours.

Luciferase Assays

Cells were harvested using the passive lysis method (Promega) and luciferase assays were done using the Dual Luciferase Reporter Assay System (Promega). Luciferase activity was measured by means of a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

DNA Precipitation Assay

Forty-eight hours after transfection, HeLa cells were lysed by sonication in HKMG [10 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 10% glycerol, 1 mmol/L DTT, 0.5% NP40] containing protease inhibitors. Cellular debris was removed by centrifugation. Cell extracts were precleared with Tetralink Tetrameric Avidin Resin (Promega) for 1 hour and then incubated overnight with 1 μg biotinylated double-stranded oligonucleotides and 10 μg poly(deoxyinosinic-deoxycytidylic acid). DNA-bound proteins were collected with Tetralink Tetrameric Avidin Resin for 2 hours, washed four times in HKMG buffer, separated on a SDS-polyacrylamide gel, and identified by Western blotting. M2 anti-Flag monoclonal antibody (Sigma-Aldrich, St. Louis, MO) was used to detect Flag epitope-tagged proteins. Sp1 was detected using PEP2 antibody from Santa Cruz Biotechnology, Santa Cruz, CA. Biotinylated oligonucleotides comprising the Sp1 site of HMGCS2 (5′-GATCTGTG**GAGGCGGGC**AAGCT-3′: the consensus Sp1-binding site is shown in bold capital letters) and the mutated Sp1 site (5′-GATCTGTG**GAtctaGaGC**AAGCT-3: mutated nucleotides are shown in bold lowercase letters) were synthesized by Sigma.

Chromatin Immunoprecipitation Assay

Formaldehyde cross-linking and chromatin immunoprecipitation assays of CaCo-2 cells were done as described previously (14). Immunoprecipitations were done using 20 μg anti-c-Myc (Santa Cruz Biotechnology) or preimmune serum (Ig).

Antibody Production and Western Blot Analysis

Polyclonal antibodies against human mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase were elicited against a peptide conjugated to keyhole limpet hemocyanin corresponding to the COOH terminus of the protein (sequence YHKVNFSPPGDTNS, amino acids 471-484; Sigma, Genosys). Immunoblotting was carried out using an enhanced chemiluminescence kit following the manufacturer's instructions.

Immunohistochemistry

Human colonic tissues embedded in paraffin were provided by the Banco de Tejidos y Tumores (Institut d'Investigació Biomèdica August Pi i Sunyer), Hospital Clínic de Barcelona (Barcelona, Spain). The Colon Tumor Tissue Array slides were purchased by Lab Vision. The sections for HMGCS2 analysis were antigen retrieved by preheating samples to 60°C for 30 minutes and then allowing them to cool for 5 minutes. Paraffin was removed from the slides using xylene and sections were then rehydrated by ethanol treatment followed by permeabilization in 0.1% Triton X-100, 20 mmol/L glycine, and 100 mmol/L PBS at room temperature for 10 minutes. Blocking was done for 20 minutes in 1% bovine serum albumin, 20 mmol/L glycine, and 100 mmol/L PBS and the sections were then incubated with the specific human HMGCS2 antibody at a dilution of 1:250 for 1 hour at 37°C. After washing the primary antibody with 20 mmol/L glycine and 100 mmol/L PBS (pH 7.4), the slides were incubated with a biotinylated goat anti-rabbit secondary antibody (Sigma) at a dilution of 1:250 for 30 minutes at 37°C. The sections were washed with 20 mmol/L glycine and 100 mmol/L PBS (pH 7.4) and then incubated with horseradish peroxidase-streptavidin (Zymed, San Francisco, CA) at 1:250 dilution for 30 minutes at 37°C. After washing the slides with 20 mmol/L glycine and 100 mmol/L PBS (pH 7.4) again, staining was detected using the AEC kit (Zymed) following the manufacturer's instructions. The slides were counterstained with hematoxylin (Sigma) and mounted with aqueous medium (Sigma).

Statistical Analysis

Statistical correlation between HMGCS2 expression and tumor degree was determined using the two methods with SPSS statistical software (SPSS Worldwide Headquarters, Chicago, IL). *P*s < 0.05 were considered statistically significant.

Published OnlineFirst August 28, 2006; DOI:10.1158/1541-7786.MCR-05-0267

References

1. Williamson DH, Bates MW, Krebs HA. Activity and intracellular distribution of enzymes of ketone-body metabolism in rat liver. Biochem J 1968;108:353–61.

2. Baird GD, Hibbitt KG. Lee J. Enzymes involved in acetoacetate formation in various bovine tissues. Biochem J 1970;117:703–9.

3. Dashti N, Ontko JA. Rate-limiting function of 3-hydroxy-3-methylglutaryl-coenzyme A synthase in ketogenesis. Biochem Med 1979;22:365–74.

4. Girard J, Ferre P, Pegorier JP, Duee PH. Adaptations of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. Physiol Rev 1992;72:507–62.

5. McGarry JD, Foster DW. Regulation of hepatic fatty acid oxidation and ketone body production. Annu Rev Biochem 1980;49:395–420.

6. Hegardt FG. Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. Biochem J 1999;338:569–82.

7. Thumelin S, Forestier M, Girard J, Pegorier JP. Developmental changes in mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene expression in rat liver, intestine and kidney. Biochem J 1993;292:493–6.

8. Quant PA. Activity and expression of hepatic mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase during the starved-to-fed transition. Biochem Soc Trans 1990;18:994–5.

9. Quant PA, Tubbs PK, Brand MD. Glucagon activates mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase *in vivo* by decreasing the extent of succinylation of the enzyme. Eur J Biochem 1990;187:169–74.

10. Hegardt FG, Serra D, Asins G. Influence of etomoxir on the expression of several genes in liver, testis and heart. Gene Pharmacol 1995;26:897–904.

11. Gil-Gómez G, Ayté J, Hegardt FG. The rat mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme-A-synthase gene contains elements that mediate its multihormonal regulation and tissue specificity. Eur J Biochem 1993;213:773–9.

12. Nadal A, Marrero PF, Haro D. Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. Biochem J 2002;366:289–97.

13. Rodriguez JC, Gil-Gomez G, Hegardt FG, Haro D. Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. J Biol Chem 1994;269:18767–72.

14. Camarero N, Nadal A, Barrero MJ, Haro D, Marrero PF. Histone deacetylase inhibitors stimulate mitochondrial HMG-CoA synthase gene expression via a promoter proximal Sp1 site. Nucleic Acids Res 2003;31:1693–703.

15. Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor α in HepG2 cells. J Biol Chem 2001;276:27950–8.

16. Mascaro C, Acosta E, Ortiz JA, et al. Characterization of a response element for peroxisomal proliferator activated receptor (PPRE) in human muscle-type carnitine palmitoyltransferase I. Adv Exp Med Biol 1999;466:79–85.

17. Cullingford TE, Dolphin CT, Bhakoo KK, Peuchen S, Canevari L, Clark JB. Molecular cloning of rat mitochondrial 3-hydroxy-3-methylglutaryl-CoA lyase and detection of the corresponding mRNA and of those encoding the remaining enzymes comprising the ketogenic 3-hydroxy-3-methylglutaryl-CoA cycle in central nervous system of suckling rat. Biochem J 1998;329:373–81.

18. Cherbuy C, Darcy-Vrillon B, Morel MT, Pegorier JP, Duee PH. Effect of germfree state on the capacities of isolated rat colonocytes to metabolize *n*-butyrate, glucose, and glutamine. Gastroenterology 1995;109:1890–9.

19. Curi R, Williams JF, Newsholme EA. Formation of ketone bodies by resting lymphocytes. Int J Biochem 1989;21:1133–6.

20. Mascaro C, Buesa C, Ortiz JA, Haro D, Hegardt FG. Molecular cloning and tissue expression of human mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase. Arch Biochem Biophys 1995;317:385–90.

21. Le May C, Pineau T, Bigot K, Kohl C, Girard J, Pegorier JP. Reduced hepatic fatty acid oxidation in fasting PPARα null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression. FEBS Lett 2000;475:163–6.

22. Cherbuy C, Andrieux C, Honvo-Houeto E, et al. Expression of mitochondrial HMGCoA synthase and glutaminase in the colonic mucosa is modulated by bacterial species. Eur J Biochem 2004;271:87–95.

23. Hass R, Busche R, Luciano L, Reale E, Engelhardt WV. Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig. Gastroenterology 1997;112:875–81.

24. Hague A, Singh B, Paraskeva C. Butyrate acts as a survival factor for colonic epithelial cells: further fuel for the *in vivo* versus *in vitro* debate. Gastroenterology 1997;112:1036–40.

25. Heerdt BG, Houston MA, Augenlicht LH. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. Cancer Res 1994;54:3288–94.

26. Jass JR. Diet, butyric acid and differentiation of gastrointestinal tract tumours. Med Hypotheses 1985;18:113–8.

27. Mariadason JM, Velcich A, Wilson AJ, Augenlicht LH, Gibson PR. Resistance to butyrate-induced cell differentiation and apoptosis during spontaneous Caco-2 cell differentiation. Gastroenterology 2001;120:889–99.

28. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–50.

29. Kim JW, Zeller KI, Wang Y, et al. Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitaion assays. Mol Cell Biol 2004;24:5923–36.

30. Li F, Wang Y, Zeller KI, et al. Myc stimulates nuclearly encoded mitochondrial genes and mictochondrial biogenesis. Mol Cell Biol 2005;25:6225–34.

31. Riu E, Bosch F, Valera A. Prevention of diabetic alterations in transgenic mice overexpressing Myc in the liver. Proc Natl Acad Sci U S A 1996;93:2198–202.

32. Peukert K, Staller P, Schneider A, Carmichael G, Hanel F, Eilers M. An alternative pathway for gene regulation by Myc. EMBO J 1997;16:5672–86.

33. Staller P, Peukert K, Kiermaier A, et al. Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol 2001;3:392–9.

34. Wanzel M, Herold S, Eilers M. Transcriptional repression by Myc. Trends Cell Biol 2003;13:146–50.

35. Gartel AL, Ye X, Goufman E, et al. Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci U S A 2001;98:4510–5.

36. Gartel AL, Shchors K. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. Exp Cell Res 2003;283:17–21.

37. Luo Q, Li J, Cenkci B, Kretzner L. Autorepression of c-myc requires both initiator and E2F-binding site elements and cooperation with the p107 gene product. Oncogene 2004;23:1088–97.

38. Bowen H, Lapham A, Phillips E, et al. Characterization of the murine Nramp1 promoter: requirements for transactivation by Miz-1. J Biol Chem 2003; 278:36017–26.

39. Ortiz JA, Gil-Gomez G, Casaroli-Marano RP, Vilaro S, Hegardt FG, Haro D. Transfection of the ketogenic mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase cDNA into Mev-1 cells corrects their auxotrophy for mevalonate. J Biol Chem 1994;269:28523–6.

40. Adhikary S, Eilers M. Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 2005;6:635–45.

41. Ortiz JA, Mallolas J, Nicot C, et al. Isolation of pig mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene promoter: characterization of a peroxisome proliferator-responsive element. Biochem J 1999;337:329–35.

42. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, Gottlicher M. Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 2004;5:455–63.

43. Fujiwara K, Ochiai M, Ohta T, et al. Global gene expression analysis of rat colon cancers induced by a food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Carcinogenesis 2004;25:1495–505.

44. Asins G, Rosa JL, Serra D, et al. Gene expression of enzymes regulating ketogenesis and fatty acid metabolism in regenerating rat liver. Biochem J 1994; 299:65–9.

45. Uehara Y, Engel T, Li Z, et al. Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1. Diabetes 2002;51:2922–8.

46. Jain SK, Kannan K, Lim G. Ketosis (acetoacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. Free Radic Biol Med 1998;25:1083–8.

47. Abdelmegeed MA, Kim SK, Woodcroft KJ, Novak RF. Acetoacetate activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in primary cultured rat hepatocytes: role of oxidative stress. J Pharmacol Exp Ther 2004;310:728–36.

48. Rodriguez JC, Ortiz JA, Hegardt FG, Haro D. Chicken ovalbumin upstream-promoter transcription factor (COUP-TF) could act as a transcriptional activator or repressor of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene. Biochem J 1997;326:587–92.

49. Vaque JP, Navascues J, Shiio Y, et al. Myc antagonizes Ras-mediated growth arrest in leukemia cells through the inhibition of the Ras-ERK-p21Cip1 pathway. J Biol Chem 2005;280:1112–22.

50. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1989.

51. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

52. Altomonte J, Richter A, Harbaran S, et al. Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. Am J Physiol Endocrinol Metab 2003;285:E718–28.