Keratin 8 absence down-regulates colonocyte HMGCS2 and modulates colonic ketogenesis and energy metabolism

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INTRODUCTION

Keratins (Ks), the intermediate filament (IF) proteins of epithelial cells, are divided into acidic type I (K9–K28) and basic type II (K1–K8 and K71–K80) keratins (Coulombe and Omary, 2002; Schweizer et al., 2006) and make up the largest subgroup of IFs. These two types of keratins form noncovalent obligate heteropolymers in a 1:1 ratio. Keratins are accordingly expressed in specific pairs and in a tissue-specific manner (Quinlan et al., 1984). Intestinal simple epithelial cells express K7, K8, K18–K20, and K23, whereas adult hepatocytes express only K8 and K18 (Omary et al., 2009). One established role of keratins is to protect epithelia from mechanical and nonmechanical stresses; keratins participate in signaling events that regulate processes such as cellular architecture, growth, proliferation, and...
apoptosis (Omary et al., 2009; Toivola et al., 2010; Pan et al., 2013). The major type II keratin in intestinal epithelia is K8. K8-knockout (K8+/−) mice, which lack almost all colonocyte keratins, develop colonic epithelial hyperproliferation (Baribault et al., 1994), a Th2-type colitis, and an ulcerative colitis–like state that is reversed after antibiotic therapy (Habtezion et al., 2005). K8−/− mice also develop diarrhea due to colonic Na and Cl ion transporter mistargeting (Toivola, Krishnan, et al., 2004). The colonic hyperproliferation in K8−/− mice is linked to a decreased capacity of K8−/− colonoocytes to undergo normal apoptosis at the upper part of the colonic crypt (Habtezion et al., 2011). Of note, the K8−/− small intestine is spared any major inflammatory response (Baribault et al., 1994; Ameen et al., 2001), which is likely related to the relative lack of bacteria in the small intestine compared to the colon.

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGC2) catalyzes the rate-limiting step of ketogenesis, in which ketone bodies (β-hydroxybutyrate, acetoacetate, and acetone) are produced during ketogenic conditions such as starvation, untreated diabetes, and other conditions in which carbohydrate levels are low (Hegardt, 1999). In the ketogenic pathway, acetyl-CoA, mostly derived from the β-oxidation of fatty acids, is converted into ketone bodies through a cascade of enzymatic reactions (Hegardt, 1999). The expression of HMGC2 is regulated by peroxisome proliferator–activated receptor α (PPARα; Serra et al., 1996a; Meertens et al., 1998), which also regulates many changes in fatty acid and glucose metabolism (Lefebvre et al., 2006) by heterodimerizing with retinoid X receptor and binding to peroxisome proliferator hormone response elements on DNA of target genes (Hegardt, 1999). Ketogenesis occurs mainly in the liver, but also in the colon of adult mammals (Serra et al., 1996a; Hegardt, 1999).

Short-chain fatty acids (SCFAs) are products of intestinal bacterial fermentation of mainly dietary carbohydrates. Butyrate, a major SCFA, constitutes the main energy source for colonoocytes and is involved in the regulation of HMGC2 expression and thus ketone body production (Roediger, 1980; Hegardt, 1999; Cherbuy et al., 2004). Butyrate is thus an important intermediate in maintaining colonic homeostasis and health (Hamre et al., 2008; Bultman, 2014). Absorption of luminal butyrate is mainly mediated by monocarboxylate transporter 1 (MCT1), the expression and function of which are regulated by butyrate (Hadjigapiou et al., 2000; Cuff et al., 2002; Borthakur et al., 2012) and further upstream by PPARα (König, 2008).

Using a proteomic approach, we identified HMGC2 as a major down-regulated protein in colonic crypts isolated from K8−/− mice. The decreased HMGC2 levels correlated with decreased enzyme activity, blunted response to ketogenic conditions, and down-regulation of the upstream MCT1 transporter feeding into the ketogenic pathway, thereby leading to increased luminal SCFA in K8−/− mice.

**RESULTS**

**HMGC2 is a major down-regulated protein in K8−/− colonoocytes**

Proteins isolated from colonic crypt lysates of K8 wild-type (K8+/+) and K8+/− mice were compared using two-dimensional differential in-gel electrophoresis (2D DIGE) coupled with mass spectrometry to identify differentially regulated proteins (Figure 1, A and B). In K8−/− colonoocytes, the major down-regulated proteins were, as expected, K8, K19, and their fragments (unpublished data). Most differentially expressed proteins are mitochondrial, cytoplasmic or endoplasmic reticulum proteins involved in metabolism and oxidative stress (Supplemental Table S1).

One of the most prominently down-regulated proteins in the K8−/− colon was the rate-limiting enzyme of ketogenesis, HMGC2. Its four isoforms were, on average, fourfold decreased in K8−/− colonoocytes (Figure 1, A and B, spots 1032, 1033, 1035, and 1044) when compared with K8+/+. Western blot analysis of HMGC2 in isolated K8−/− and K8−/− colonic crypts (Figure 1C) confirmed the down-regulation of HMGC2 (see also later discussion of Figures 2A, 4, A–F, and 5C and Supplemental Figures S2 and S3 for total colon lysate and purified mitochondrial samples). Similarly, HMGC2 was slightly but consistently down-regulated also in K8 small interfering RNA (siRNA)-treated HT-29 and Caco-2 colorectal cancer cells (Supplemental Figure S1). To determine whether HMGC2 enzyme activity is modulated in K8−/−, we determined HMGC2 activity and quantity in the same batch of mitochondria isolated from colonic epithelial scrapings (Figure 1D and Supplemental Figure S2). HMGC2 activity (K8+/+, 0.98 ± 0.11; K8−/−, 0.54 ± 0.12; p < 0.01) and relative protein quantity (K8+/+, 1.00 ± 0.20; K8−/−, 0.53 ± 0.18; p < 0.05) were both significantly decreased in K8−/− colonoocytes (Figure 1, D and E). The enzyme activity in K8−/− mice correlated with the decrease in enzyme quantity (HMGC2 activity/quantity ratio: K8+/+, 0.92 ± 0.19; K8−/−, 1.0 ± 0.38; Figure 1F), and decreased protein levels thus cause the decreased activity.

**K8-knockout–induced HMGC2 down-regulation is a distinct noninflammatory phenotype unique for the colon and is associated with a decrease in PPARαs**

To investigate whether the K8-related down-regulation of HMGC2 in the colon also occurs in other digestive organs, we analyzed HMGC2 levels in K8−/− distal and proximal colon, small intestine, and liver. HMGC2 down-regulation was clearly observed in both parts of the colon, and the levels of HMGC2 in the proximal colon were in general higher than in the distal colon in both K8+/+ and K8−/− genotypes (Figure 2A and Supplemental Figure S3). This is expected because HMGC2 levels correlate with luminal fermenting bacteria, which are more numerous in the proximal than the distal colon, therefore producing more SCFA (Topping and Clifton, 2001; Hamre et al., 2008). PPARα was also significantly decreased 1.3- to 1.8-fold (p = 0.03 for distal colon) in K8−/− (Figure 2A). Confocal immunofluorescence microscopy analysis confirmed that HMGC2 levels are decreased in K8−/− colonoocytes and that the remaining HMGC2 accumulated in the apical part of the colonoocytes (Figure 2B). HMGC2 was undetectable in the small intestine (Figure 2C), as previously described (Hegardt, 1999). The liver, which is the main ketogenic organ, contains more HMGC2 than the proximal colon (Supplemental Figure S3) but did not reveal any major difference in HMGC2 levels between the genotypes (Figure 2C and Supplemental Figure S3). Our results were confirmed using two independent HMGC2 antibodies (Supplemental Figure S3). Furthermore, HMGC2 down-regulation is likely not caused by colitis in general since HMGC2 levels were unchanged or increased after experimental dextran sulfate sodium (DSS)–induced colitis (Figure 2D).

**Ketogenesis is blunted in the K8−/− colon**

To study whether the decreased levels of HMGC2 in K8−/− colonoocytes are enough to respond to ketogenic conditions, mice were starved for 12 and 24 h or given a ketogenic diet for 3 or 14 d, after which HMGC2, blood glucose, and blood β-hydroxybutyrate levels were assayed. Ketogenesis was readily induced, since K8+/+ and K8−/− blood glucose levels decreased consistently with starvation at 12 h and remained low at 24 h (Figure 3A), whereas blood β-hydroxybutyrate, which is the most prominent ketone body,
FIGURE 1: HMGCS is a major down-regulated protein in K8−/− colon. Colonic crypts from K8−/− and K8+/+ mice were isolated and protein lysates labeled with Cy3 (green) for K8+/+ and Cy5 (red) for K8−/− (A). The mixed lysates were separated by 2D DIGE, and differentially expressed proteins were given individual spot numbers and detected by mass spectrometry (Supplemental Table S1). Unchanged protein spots appear yellow. The major down-regulated protein is HMGCS2 (spots 1032, 1033, 1035, and 1044), as shown separately for K8+/+ and K8−/− and as an overlay (B). Lysates of isolated colonic crypts from 3 K8+/+ and 3 K8−/− were analyzed by Western blotting for HMGCS2, K8, and Hsc70 as a loading control (C). Measured HMGCS2 activity values (Supplemental Figure S1) for K8−/− shown as fold decrease relative to HMGCS2 activity in K8+/+ colonocytes (D). HMGCS2 levels were assayed by immunoblotting of the same mitochondrial preparations that were used for measuring HMGCS2 activity. The results were quantified with ImageJ software and normalized to mitochondrial marker prohibitin, which is not altered in K8−/− (E). K8+/+ to K8−/− HMGCS2 enzyme activity and quantity ratios were calculated, and the correlation between HMGCS2 enzyme activity and quantity was analyzed (F). The results are based on three independent experiments (each experiment involving one K8+/+ and one K8−/− mouse) and represent the average ± SD, with significant differences shown as *p < 0.05 and **p < 0.01.
cholesterol, bicarbonate, and triglycerides were not markedly different between K8\textsuperscript{+/+} and K8\textsuperscript{−/−}, except for a slight decrease of cholesterol in K8\textsuperscript{−/−} 7 d after onset of ketogenic diet (Figure 3E). No differences in food ingestion or weight gain were detected between

**FIGURE 2:** HMGCS2 and PPAR\(\alpha\) are decreased and HMGCS2 is mislocalized in adult colon but not in small intestine or liver of K8\textsuperscript{−/−} mice. Lysates of distal and proximal colon (A) and small intestine and liver (C) were obtained from K8\textsuperscript{+/+} and K8\textsuperscript{−/−} mice, and the lysates were normalized by protein assay. Protein levels of HMGCS2, PPAR\(\alpha\), and K8 were analyzed by immunoblotting, and equal loading was confirmed by Coomassie brilliant blue staining (A, C). Immunostaining of colonic HMGCS2 (B) showed decreased cytoplasmic pools of HMGCS2 (green) in crypts, with the remaining HMGCS2 located apically in the colonocytes (arrows). Scale bars, 100 μm (a, c), 10 μm (b, d); L, lumen; M, muscles. (D) Proximal colon lysates were obtained from 2.5-mo-old BALB/c mice treated with 2% DSS or without DSS (control) for 8 d. Samples were analyzed by immunoblotting for HMGCS2. Equal loading is shown by Hsc70.
FIGURE 3: Blood and serum parameters are similar in K8+/+ and K8−/− mice under baseline and ketogenic conditions. Mice were starved for 24 h (A, B, E) or fed with a control or ketogenic diet (C, D, E) as outlined in Materials and Methods. Blood samples from male and female K8+/+ and K8−/− mice were taken from the submandibular vein 0, 12, and 24 h after onset of starvation (A, B) and after 0 and 14 d of a ketogenic diet (C, D) and were assayed for the levels of blood glucose (A, C), blood β-hydroxybutyrate (B, D), and a panel of blood markers (E). Serum samples from mice subjected to 24 h of starvation or to 3 and 7 d of a ketogenic diet were analyzed for cholesterol, bicarbonate, and triglycerides (E). The results represent the average ± SD; shown as n.s., p > 0.05, not significant; *p < 0.05 and ***p < 0.001, statistically significant.
genotypes (Supplemental Figure S4). Because the liver is the main ketogenic organ controlling blood ketone levels, these baseline data suggest that liver ketogenesis remains unchanged despite K8 deletion and that a ketogenic diet and starvation should be useful models for assessing colonocyte ketogenesis.

Western blot analysis showed, as expected, that both starvation and 3 d of ketogenic diet significantly increased HMGC2 levels in K8−/−, whereas no significant changes in HMGC2 levels were observed in K8+/− at any time after starvation (Figure 4, A and B) or ketogenic diet (Figure 4, C–F). The decrease of K8−/− HMGC2 was, in comparison to K8+/+, on average threefold during normal conditions and fourfold during ketogenic conditions (Figure 4, A–F). The corresponding decrease of HMGC2 mRNA levels was 3.8-fold during normal diet conditions and on average 3.7-fold after starvation and 3 d of ketogenic diet (Figure 4G). The protein levels of PPARα, in general, follow the same pattern as HMGC2 levels (Figure 4, A, C, and E). A slight up-regulation of PPARα was observed after ketogenic conditions in K8+/+ mice, whereas a slight down-regulation in K8−/− was seen after both normal and ketogenic conditions. K8 knockout thus clearly leads to a blunted colonic ketogenic response.

Normal colonic mitochondrial ultrastructure and adenosine monophosphate–activated protein kinase signaling in K8−/− colon

We next assessed whether the K8−/− colon ketogenic phenotype could stem from abnormalities in mitochondria and common energy metabolism pathways. In contrast to K8−/− hepatocyte mitochondria, which are smaller than K8+/+ mitochondria (Tao et al., 2009), no differences in mitochondrial size were noted in K8−/− colonocytes, even if there were fewer cristae per mitochondrial section area compared with K8+/+ (Supplemental Figure S5, B and C). The protein levels of mitochondrial prohibitin and cytochrome c were comparable in K8−/− and K8+/+ (Supplemental Figure S2), suggesting that the mitochondrial function is unaltered in the K8−/− colonocytes. To analyze whether there are additional changes in K8−/− colon energy metabolism, we analyzed a panel of proteins involved in energy metabolism pathways under baseline and ketogenic conditions. Acetyl-CoA carboxylase (ACC), a central enzyme in the biosynthesis of fatty acids, converts acetyl-CoA into malonyl-CoA and functions as an inhibitor of β-oxidation (Lopaschuk et al., 2010). Ketogenic conditions induce β-oxidation of fatty acids into acetyl-CoA (Hegardt, 1999), whereas ACC activity remains low (Lopaschuk et al., 2010) in order to maintain the availability of acetyl-CoA for conversion into ketone bodies through ketogenesis. The levels of ACC in K8+/+ and K8−/− colonocytes were unaltered under basal conditions but remained high under ketogenic conditions in K8−/− but not K8+/+ colon (Supplemental Figure S6, A–C; a significant 2.5-fold decrease in K8+/+ [p = 0.0005], whereas it was only 1.4-fold [not significant] in K8−/− after 3 d of ketogenic diet when quantified from Western blots), thus supporting the notion of a blunted ketogenic pathway after K8 inactivation. No changes were observed in the expression patterns of ACC inhibitors adenosine monophosphate-activated protein kinase α (AMPKα) and AMPKβ and their inactive phosphorylated forms in K8+/+ and K8−/− colonocytes (Supplemental Figure S6, A–C). Independent of basal or active ketogenesis, the expression levels of glucose transporter 4 (GLUT4) and cytochrome c oxidase (COX IV, a mitochondrial enzyme of the respiratory chain; Supplemental Figure S6, A–C) were marginally decreased in total colon lysates of K8−/− mice and unchanged in colonic epithelium isolated from K8−/− mice in comparison to K8+/+ (unpublished data). Analysis of ADP/ATP and NAD/NADH ratios in epithelial scrapings did not reveal any major differences between the genotypes (unpublished data). Together these results confirm that ketogenesis is activated under the ketogenic conditions used here and blunted in K8−/−, and that mitochondrial ultrastructure and energy intermediates are largely unchanged in the absence of K8.

The SCFA transporter MCT1 is down-regulated in K8−/− and luminal SCFA acid levels are increased upstream of HMGC2

Absorption of luminal SCFA is mainly mediated by MCT1 (Hadjigajioiu et al., 2000). To assess whether expression of MCT1, and consequently the absorption of SCFA, could be perturbed in the absence of keratins, we assayed K8+/− colon MCT1 levels by immunoblotting. Significant down-regulation of MCT1 was observed in total lysates of K8−/− (0.59 ± 0.10; p < 0.005) in comparison to K8+/+ (1.04 ± 0.04; Figure 5, A and B) and in isolated K8−/− colonic epithelium (0.40 ± 0.11) compared with K8+/− (1.00 ± 0.11; Figure 5C) under both control and starved conditions. The decrease in MCT1 levels was also confirmed in HT-29 and Caco-2 cells in which K8 has been down-regulated (Supplemental Figure S1). This decrease in MCT1 could also be appreciated in MCT1 immunostainings in K8−/− colon and in Caco-2 cells treated with K8 siRNA (Supplemental Figure S7). However, K8 down-regulation or absence did not lead to any differences in MCT1 localization, which is seen as a tight plasma membrane staining in Caco-2 cells and mostly laterally between the epithelial cells in the colon in vivo (Supplemental Figure S7). Because MCT1 expression and function are up-regulated by butyrate (Cuff et al., 2002), the ratio of butyrate-producing bacteria to total Eubacteria in K8+/+ and K8−/− was assayed. No differences in K8−/− and K8+/+ stool microbial populations were seen, including the two clusters of butyrate-producing bacteria of the Firmicutes phylum, Clostridium clusters XIVa and IV (Pryde et al., 2002; Figure 6A). Therefore the metabolic state of the K8−/− phenotype is not caused by a difference in the microbial balance and butyrate-producing bacteria. The SCFA levels in K8+/± and K8−/− stool were furthermore profiled to determine whether the observed down-regulation of MCT1 could be due to diminished production of butyrate by the microflora in K8−/−. Of interest, a significant increase in the levels of butyric acid (K8+/+, 1.59 ± 0.72 mM; K8−/−, 5.33 ± 0.83 mM; p < 0.005) and isobutyric acid (K8+/+, 0.17 ± 0.062 mM; K8−/−, 0.56 ± 0.12 mM; p < 0.01) was detected in K8−/− stool in comparison to K8+/+ (Figure 6B), indicating that bacterial production of SCFA is not perturbed, whereas the uptake may be compromised due to decreased levels of MCT1.

DISCUSSION

The molecular functions of keratins in the colonic epithelium are largely unclear, even if it is well established that K8 ablation in mice leads to early and chronic colitis (Baribault et al., 1994; Toivola, Krishnan, et al., 2004; Habtezion et al., 2005, 2011). To understand how keratins may help maintain intestinal homeostasis, we used a proteomic approach to identify differentially expressed proteins in K8−/− colonocytes. We show that 1) HMGC2 is decreased and the reduced HMGC2 levels in K8−/− colonocytes correlate with decreased enzyme activity in mitochondria; 2) the HMGC2 down-regulation phenotype is colon-specific and not related to an acute inflammation per se, as determined using the DSS colitis model; and 3) the decrease of HMGC2 leads to blunted ketogenesis in K8−/− colon. In addition, by studying upstream regulation of HMGC2, we find that 4) the HMGC2 transcriptional regulator PPARα and the SCFA (butyrate) transporter MCT1 are down-regulated in the K8−/− colon, whereas 5) luminal butyrate levels are increased 6) without changes in the composition of the butyrate-producing bacteria. 

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FIGURE 4: Onset of ketogenic conditions fails to increase colonic HMGCS2 in K8−/− to the same levels as in K8+/+. Total colon lysates from K8+/+ and K8−/− mice subjected to normal and ketogenic conditions (24 h of starvation (A) or 3 d (C) or 14 d (E) of a ketogenic diet) were analyzed by Western blot for levels of HMGCS2, PPARα, and K8. Equal amounts of proteins were loaded after normalization by protein assay, as shown by tubulin protein levels (A, C, E). HMGCS2 levels (B, D, F) were quantified with ImageJ and normalized to tubulin (A, C, E, respectively; average ± SD, *p < 0.05, **p < 0.01). HMGCS2 mRNA levels (G) were examined by RT-PCR and normalized to actin and are shown as fold change of HMGCS2 mRNA levels between the different genotypes and treatment regimens (average ± SD, ***p < 0.001).
To our knowledge, this is the first study describing IF keratins as modulators of colocyte energy metabolism.

HMGCS2 is an important and unique enzyme in the colonic epithelium, since ketogenesis only occurs in a few other organs apart from the liver (Serra et al., 1996b; Hegardt, 1999). Colonic HMGCS2 is regulated by bacterially produced SCFA (mainly butyrate), which strengthens the colonic defense barrier (Hamer et al., 2010). Because the liver is lacking the microbial component, these findings further suggest the central involvement of microflora and/or SCFA in the down-regulation of K8−/− colonic HMGCS2. Moreover, the modulation of HMGCS2 described here is likely specifically due to the ablation of K8 and not due to the inflammatory status of the K8−/− colon phenotype, since the expression of HMGCS2 was increased rather than decreased in DSS colitis (Naito et al., 2010).

The ramifications of low HMGCS2 levels in K8−/− are noticeable after exposing mice to ketogenic conditions, upon which K8−/− mice were not able to increase colonic HMGCS2, in contrast to wild-type mice. Ketogenic conditions, such as the intake of a high-fat, low-carbohydrate diet or starvation, activates β-oxidation of fatty acids and generates an excess of acetyl-CoA that is converted into ketone bodies (Cullingford, 2004). The blunted ketogenic response in K8−/− was supported by our observation that ACC levels remained relatively unchanged in K8−/− colon under ketogenic conditions. ACC catalyzes the conversion of acetyl-CoA into malonyl-CoA, which is an inhibitor of carnitine palmitoyltransferase I (CPTI), the rate-limiting enzyme of fatty acid β-oxidation (McGarry and Brown, 1997). Because the main function of ACC in liver is to provide malonyl-CoA and therefore inhibit β-oxidation (McGarry and Brown, 1997), lower levels of ACC under ketogenic conditions are expected to decrease levels of acetyl-CoA (Guzman et al., 2000). These results also suggest diminished β-oxidation in K8−/− colon. How keratins modulate this pathway needs further study but could be linked to the observation that K8/K18 are inhibitory of hepatic CPTI activity (Velasco et al., 1998). Changes in proteins involved in ketone body metabolism (decreased acetooacetate-CoA thiase in the first step of ketogenesis) and β-oxidation have also been described in desmin-null mouse muscle (Fountoulakis, Soumaka, Rapti, et al., 2005). In contrast to hepatocyte mitochondria, which are smaller after K8 knockdown (Tao et al., 2009), colon mitochondrial size appears normal, even if alterations in cristae can be seen (Supplemental Figure S5). Hence mitochondrial morphology changes are unrelated to the observed changes in colocyte HMGCS2.

Mice raised in a germ-free environment have diminished metabolic processes, since the butyrate-producing bacteria are non-existent (Donohoe et al., 2011). HMGCS2 expression is dependent on colonic microflora and SCFA (Cherbuy et al., 2004), and the
K8−/− colonic microflora consist of fewer microorganisms than K8+/+ microflora (Habtezion et al., 2011). These data, together with the fact that HMGCS2 is not altered in K8−/− hepatocytes (Figure 2C), suggest that the decreased HMGCS2 in the absence of K8 could be due to diminished availability of luminal SCFA or aberrant transport of SCFA across the cell membrane. SCFAs are transported into colonic enterocytes mainly through MCT1, the expression and function of which are up-regulated by butyrate (Cuff et al., 2002) and PPARα.

**FIGURE 6:** Ratios of the butyrate-producing bacteria from the Firmicutes phylum are unaltered in K8−/− colons, whereas K8−/− stool contains increased levels of butyrate. Colonic contents (fecal stool) were collected from K8+/+ and K8−/− mice and analyzed by quantitative PCR for detection and quantification of the total amount of Eubacteria and a panel of bacterial groups and clusters. (A) No significant differences in the levels of butyrate-producing bacteria in Clostridium clusters XIVa and IV were observed in the colon of K8−/− and K8+/+ mice. Levels are normalized to total eubacteria for both genotypes. (B) Levels of the SCFAs acetate, propionate, isobutyrate, and butyrate were assessed from freshly collected K8+/+ and K8−/− stool by gas chromatography profiling. The results represent the mean ± SD, with significant differences shown as **p < 0.01.
Hypothesis is supported by the generalized mistargeting of membrane proteins in the K8\(^{-/-}\) colon, including ion transporters AE1/2 and ENACy (Toivola, Krishnan, et al., 2004). Lack or mutation of keratins and other intermediate filament proteins leads to altered distribution of macromolecules in several contexts, including GLUT transporters in embryonic epithelia (Vijayaraj, Kröger, et al., 2009) and the endocrine pancreas (Alam et al., 2013) and cholesterol in the adrenal gland (Shen et al., 2012). In this study, however, we could not detect any major mistargeting of MCT1 in the absence of K8, indicating that keratins may affect MCT1 stability in the cell. Of interest, butyrate is an inhibitor of histone deacetylases, and it has been shown that colonic K8 lysines also are acetylated and this is regulated by butyrate levels (Drake, Griffiths, et al., 2009; Kilner et al., 2012).

Taken together, these results suggest a model in which absorption of SCFAs is decreased in K8\(^{-/-}\) colonocytes due to reduced MCT1 levels, leading to elevated levels of butyrate in the lumen, as well as diminish HMGCS2 levels in colonocytes. These perturbations likely contribute to the inflammatory phenotype observed in the K8\(^{-/-}\) mouse (Figure 7).

**MATERIALS AND METHODS**

**Mice**

K8\(^{-/-}\) and K8\(^{+/+}\) mice in the FVB/n background were generated by interbreeding K8 heterozygote (K8\(^{-/+}\)) mice and genotyped as described (Baribault et al., 1994). BALB/c mice were purchased from the Turku Central Animal Facility, where all of the mice were housed. Animals were treated according to the animal study protocol approved by the State Provincial Office of South Finland.

**Antibodies**

Primary antibodies used for Western blotting and immunostaining were chicken anti-HMGCS2 (Genway, San Diego, CA), rabbit anti-HMGCS2 (AVIVA, San Diego, CA; epitope different from the Genway antibody), chicken anti-MCT1 (Chemicon, Temecula, CA), rabbit anti-MCT1, goat anti-PPAR\(\alpha\) (Santa Cruz Biotechnology, Dallas, TX), rabbit anti-α-smooth muscle actin, rabbit anti-prohibitin (Abcam, Cambridge, UK), rabbit anti-cytochrome c, rabbit anti-Glut4, rabbit anti–COX IV, rabbit anti-ACC, rabbit anti-phospho-ACC, rabbit anti–AMPK\(\alpha\) (Abcam, Cambridge, UK), rabbit anti–AMPK\(\beta\)1 (Cell Signaling Technology, Danvers, MA), rat anti-Hsc70 (Enzo Life Sciences, Helsinki, Finland), rat anti-K8 (Troma 1 Developmental Studies Hybridoma Bank, Iowa City, IA), and mouse anti–tubulin (Sigma-Aldrich, Munich, Germany). Secondary antibodies used for Western blotting were anti-mouse immunoglobulin G (IgG)–horseradish peroxidase (HRP), anti-rat IgG-HRP (GE Healthcare, Buckinghamshire, UK), anti-rabbit IgG-HRP (Cell Signaling Technology), anti-chicken IgG-HRP (Genway), and anti-goat IgG-HRP (Cell Signaling Technology). Secondary antibodies used for immunostaining were anti-rabbit Alexa Fluor 488 and anti-rat Alexa Fluor 546 (Life Technologies, State Provincial Office of South Finland.)

(König, 2008). Because butyrate is the primary source of colonocyte energy and inhibits inflammation and carcinogenesis, it is feasible that diminished availability of luminal butyrate or the inability of colonocytes to oxidize butyrate could contribute to the onset of inflammation in the colon (Roediger, 1990; Hamer et al., 2008). We show here that the absence of K8 correlates with lower epithelial levels of MCT1 and higher levels of K8\(^{-/-}\) fecal butyrate, which, however, are not caused by changes in the ratios of butyrate-producing bacterial species in the microbiome (Figures 5 and 6). These results suggest that the K8\(^{-/-}\) microbiome is capable of producing SCFAs, which, however, may not enter colonocytes properly and, hence, affect the fuel for ketogenesis. A similar association between keratins and butyrate levels was also seen in a human colorectal cancer study, in which higher levels of fecal butyrate corresponded to decreased K8 levels (Khan et al., 2011). Besides the down-regulation of MCT1, down-regulation of PPAR\(\alpha\) is also seen when K8 is absent in colonocytes, confirming that PPAR\(\alpha\) up-regulates genes involved in fatty acid oxidation and ketogenesis (König B, 2008).

Keratins could participate in the entry of SCFAs to epithelial cells by allowing proper targeting of MCT1 to the cell membrane. This hypothesis is supported by the generalized mistargeting of membrane proteins in the K8\(^{-/-}\) colon, including ion transporters AE1/2 and ENACy (Toivola, Krishnan, et al., 2004). Lack or mutation of keratins and other intermediate filament proteins leads to altered distribution of macromolecules in several contexts, including GLUT transporters in embryonic epithelia (Vijayaraj, Kröger, et al., 2009) and the endocrine pancreas (Alam et al., 2013) and cholesterol in the adrenal gland (Shen et al., 2012). In this study, however, we could not detect any major mistargeting of MCT1 in the absence of K8, indicating that keratins may affect MCT1 stability in the cell. Of interest, butyrate is an inhibitor of histone deacetylases, and it has been shown that colonic K8 lysines also are acetylated and this is regulated by butyrate levels (Drake, Griffiths, et al., 2009; Kilner et al., 2012).

Taken together, these results suggest a model in which absorption of SCFAs is decreased in K8\(^{-/-}\) colonocytes due to reduced MCT1 levels, leading to elevated levels of butyrate in the lumen, as well as diminish HMGCS2 levels in colonocytes. These perturbations likely contribute to the inflammatory phenotype observed in the K8\(^{-/-}\) mouse (Figure 7).
Ketogenic conditions

K8+/− mice (nine females, five males) and K8−/− mice (nine females, six males) 3 mo of age were subjected to 24 h of starvation with unlimited access to water. Weight, as well as blood glucose and β-hydroxybutyrate levels, was measured at 0, 12, and 24 h after the beginning of food starvation. Blood glucose and β-hydroxybutyrate levels were measured with blood obtained from the submandibular vein using goldenrod lancets (Medipoint, Mineola, NY) and a glucose/β-hydroxybutyrate meter (Precision Xceed; Abbott Diabetes Care, Alameda, CA). Mice were killed by CO2 asphyxiation at the end of 24 h of starvation.

K8+/− mice (three females, two males) and K8−/− mice (three females, three males) 3–6 mo of age were subjected to ketogenic diet (TD.96355; Harlan Teklad Laboratories, Madison, WI) for 3, 7, or 14 d. Corresponding K8+/+ and K8−/− mice were subjected to control diet (TD.00606; Harlan Teklad Laboratories). The macronutrient composition of the ketogenic diet (by weight) was 15.3% protein, 0.5% carbohydrate, and 67.4% fat, with an energy content of 6.7 kcal/g. The corresponding composition of the control diet was 9.2% protein, 70.9% carbohydrate, and 5.1% fat, with an energy content of 3.7 kcal/g. Because the ketogenic diet contains twice as many calories, mice eat less of it than the control diet (manufacturer’s suggestion). Therefore the intake of protein, minerals, and vitamins is halved in the ketogenic diet. Cages, water, and the control diet were changed on day 7, and the ketogenic diet was changed on days 1, 2, 4, 6, 7, 9, and 11. The control diet consisted of pellets administered with normal feeder systems, and the ketogenic diet, which has a butter-like texture, was administered by a feeder designed for powdered food. Daily measurements of mouse and food weight, as well as of blood glucose and β-hydroxybutyrate levels, were done. Blood glucose and blood β-hydroxybutyrate were measured at days 0, 3, 7, and 14 with a glucose/β-hydroxybutyrate meter (Precision Xceed). Mice were killed by CO2 inhalation on day 14. Liver, small intestine, and distal and proximal colon samples were excised and stored in liquid nitrogen for protein analysis. DSS-induced colitis

DSS 2% (40,000 Da; TdB Consultancy, Uppsala, Sweden) was administered in autoclaved drinking water to 2.5-mo-old BALB/c mice for 8 d to induce acute colitis (Wirtz et al., 2007). Control BALB/c mice were given normal drinking water, and mice were killed on day 8 of the experiment. Distal and proximal colon samples were excised and stored in liquid nitrogen for protein analysis.

Mitochondrion isolation, HMGCS2 activity assay, and ADP/ATP and NAD/NADH assays

For HMGCS2 enzyme activity measurements, mitochondria were purified from colonic epithelium scrapings isolated from K8+/+ and K8−/− 5- to 6-mo-old mice starved overnight for 18 h, by a modification of a method described by Nonn et al. (2003). Colonic scrapings were suspended in MSH buffer (250 mM mannitol, 75 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4) with 1 mM EDTA directly after isolation and homogenized manually with a Dounce homogenizer (75 strokes), following by pelleting (10 min, 600 × g, 4°C). The supernatants were recentrifuged for 15 min at 6800 × g and 4°C, and the pellets containing mitochondria were resuspended in ice-cold MSH buffer. The protein content of the isolated mitochondria was determined with a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). HMGCS2 activity in the isolated mitochondria was assayed by a modification of a method described by Patel et al. (2007). The activity of HMGCS2 was determined as the difference in the rate of disappearance of acetoacetate-CoA at 303 nm before and after the addition of acetyl-CoA and phosphotransacetylase (PTA) in a pairwise analysis of K8+/+ and K8−/− samples with equal amounts of mitochondrial proteins as input. Reagents for the enzyme assay were obtained from Sigma-Aldrich (Munich, Germany). Briefly, a 1-ml quartz cuvette containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 0.2 mM dithiothreitol, and 5 mM acetyl phosphate to a total volume of 827 μl was prewarmed to 30°C. A 75-μg amount of isolated mitochondria was lysed by incubation with Triton X-100 (1.4% [vol/vol]) for 4 min at 30°C. After addition of 10 μM acetoacetoyl-CoA (AcAcCoA) and the lysed mitochondria to the cuvette, the absorbance was measured at 303 nm for 1 min after 2 min of incubation. Acetyl-CoA (AcCoA; 100 μM) and PTA (10 U) were added to the cuvette and the change in absorbance was measured. HMGCS2 activity was then calculated as follows:

\[
\text{HMG-CoA synthase activity} = \frac{[\text{activity after addition of AcCoA} + \text{regeneration system} (\text{AcAcCoA hydrolase} + \text{HMG-CoA synthase activity})] - [\text{activity} (2–3 \text{ min}) \text{ before addition of AcCoA} + \text{regeneration system} (\text{AcAcCoA hydrolase activity})]}{
\]

HMGCS2 activities were related to HMGCS2 protein levels, which in turn were determined by immunoblotting of the same mitochondrial preparations, and normalized to the levels of the mitochondrial marker prohibitin.

The intracellular ADP/ATP ratios in colonocytes isolated from K8+/+ and K8−/− mice were measured using a bioluminescent ADP/ATP ratio assay kit (Abnova, Taipei, Taiwan) according to the manufacturer’s instructions. The luminescence readings were performed using a Hidex Sense microplate reader (Hidex, Turku, Finland). The intracellular levels of NAD and NADH were measured using a colorimetric NAD/NADH assay kit (Abcam, Taipei, Taiwan) according to the manufacturer’s instructions. Total NAD and NADH levels were determined through colorimetric detection of absorbance at 450 nm with a Wallac 1420 VICTOR2 multilabel counter (PerkinElmer, Boston, MA).
Immunoblotting and immunostaining

Samples for protein analysis were homogenized in a homogenization buffer (0.187 M Tris-HCl, pH 6.8, 3% SDS, 5 mM EDTA) to obtain total tissue lysates. Equal parts of the central distal and proximal colons were mixed for colon lysates unless otherwise stated. Protein concentrations were measured with a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA); the samples were normalized and separated by SDS–PAGE, transferred to a polyvinylidene fluoride membrane, and analyzed by immunoblotting. Western blot bands were quantified with ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to loading control (tubulin, Hsc70, or prohibitin). Fresh-frozen K8+/− and K8−/− colon samples were cryosectioned (6 μm) and fixed in −20°C acetone for 10 min, Caco-2 cells were fixed in 1% paraformaldehyde for 10 min, and the fixed tissue and cell samples were immunostained as described (Ku et al., 2004). Samples were analyzed with a Leica TCS SP5 (Leica, Mannheim, Germany) confocal microscope.

Transmission electron microscopy and cristae quantification

For transmission electron microscopy, K8+/− and K8−/− mice were starved for 18 h before killing, and the colon was excised and fixed directly in 5% glutaraldehyde in 0.16 M collidine buffer (pH 7.4). After dehydration and embedding, 70-nm sections were cut and stained with 2.5% uranyl acetate in methanol with 0.01% acetic acid and 0.25% lead citrate and analyzed with a JEM-100S transmission electron microscope (Jeol, Tokyo, Japan) and JEM-1400 Plus transmission electron microscope. For quantifying cristae, the number of cristae per mitochondria section area was counted using ImageJ. The fold change with SD was calculated on three mice, with six mitochondria per genotype.

RNA quantification

Samples for RNA quantification were homogenized with an UltraTurrax T8 (IKA-Analysentechnik) homogenizer, and RNA was isolated with a RNeasy kit (Qiagen, Hilden, Germany). After normalization of the RNA concentrations, DNase treatment (Promega, Madison, WI) and cDNA synthesis (Promega) were performed. Real-time (RT) PCR analysis by a Taqman 7900HT (Applied Biosystems, Madison, WI) and cDNA synthesis (Promega) were performed. Primers (forward, CCGAGTGTCCAAGGATGC; reverse, TGGGCAATATTATCTGACACACTAGA; Oligomer, Helsinki, Finland) and probes (#80; Universal Probe Library, Roche, Basel, Switzerland). Actin was used as an endogenous control. Fold change differences were analyzed with RQ Manager (Applied Biosystems) and Excel (Microsoft, Redmond, WA).

Profilin of fecal SCFA and bacterial levels

Approximately 200 mg of fresh mouse stool was collected immediately after defecation from K8+/− and K8−/− mice (three per genotype) and profiled for SCFA by gas chromatography (Alimetrics, Espoo, Finland) and for a panel of butyrate-producing and other bacteria by quantitative PCR (Alimetrics).

K8 and K18 knockdown in HT-29 and Caco-2 cells

HT-29 colon cancer cells were transfected for 8 h and overnight for 24 h with Pre-design Chimera RNAi (Ambnova, Taipei, Taiwan) designed for KRT8 (H00003856-R01), KRT18 (H00003875-R01), and scrambled negative control Naito1 (R0017). Transfections of 30% confluent HT-29 cells were performed with 400 nM RNA interference in Lipofectamine2000 (Invitrogen, Carlsbad, CA) and diluted in Opti-MEM medium (Gibco). Cells were harvested after 72 h.

Caco-2 cells were transfected with siMAX siRNA (Eurofins Genomics, Ebersberg, Germany) designed for KRT8 (clone ID TRCN0000062384) and nontarget (scr) siRNA (5′-CCUACAUCCGUAU-GAUGdTdT-3′) was used as a control. Transfections of 20–30% confluent Caco-2 cells were performed with 50 pmol of siRNA in Lipofectamine2000 and diluted in Opti-MEM. Cells were harvested after 72 h.

Statistical analysis

Statistical calculations were performed using Excel and GraphPad Prism with Student’s t test.

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REFERENCES

Boldface names denote co–first authors.

Bultman SJ (2014). Molecular pathways: gene-environment interactions. Curr Opin Genomics 17, 801–808.

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Borthakur A, Priyamvada S, Kumar A, Natarajan AA, Gill RK, Alrefai WA, Dudeja PK (2012). A novel nutrient sensing mechanism underlies substrate-induced regulation of monocarboxylate transporter-1. Am J Physiol Gastrointest Liver Physiol 303, G112–G113.

Bultman SJ (2014). Molecular pathways: gene-environment interactions regulating dietary fiber induction of proliferation and apoptosis via butyrate for cancer prevention. Clin Cancer Res 20, 799–803.
Camerano N, Mascaro C, Mayordomo C, Vilardell F, Haro D, Marrero PF (2006). Ketogenic HMGC.S2 is a c-Myc target gene expressed in differentiated cells of human colonic epithelium and down-regulated in colon cancer. Mol Cancer Res 4, 645–653.

Cherbuy C, Andreu C, Honvo-Houeto E, Thomas M, Ide C, Druence N, Chaumontet C, Darcy-Villon B, Duee PH (2004). Expression of mitochondrial HMGCoa synthase and glutaminase in the colonic mucosa is modulated by bacterial species. Eur J Biochem 271, 87–95.

Couplonne PA, Omary MB (2002). “Hard” and “soft” principles defining the structure, function and regulation of keratin intermediate filaments. Curr Opin Cell Biol 14, 110–122.

Cuff MA, Lambert DW, Shrazil-Beechey SP (2002). Substrate-induced regulation of the human colonic monocarboxylate transporter. MCT1. J Physiol 539, 361–371.

Cullingford TE (2004). The ketogenic diet; fatty acids, fatty acid-activated receptors and neurological disorders. Prostaglandins Leukot Essent Fatty Acids 70, 253–264.

Donohoe DR, Garge N, Zhang X, Sun W, O’Connell TM, Burger MK, Bultman SJ (2011). The microbiome and pericyte regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 13, 517–526.

Drake PJ, Griffiths GJ, Shaw L, Benson RP, Corfe BM (2009). Application of high-content analysis to the study of post-translational modifications of the cytoskeleton. J Proteome Res 8, 28–34.

Fountoulakis M, Soumaka E, Rapti K, Mavroidis M, Tsangaris G, Maris A, Wesleider N, Capetanaki Y (2005). Alterations in the heart mitochondrial proteome in a desmin null heart failure model. J Mol Cell Cardiol 38, 461–474.

Guzman M, Velasco G, Geelen MJ (2000). Do cytoskeletal control fatty acid translocation into liver mitochondria? Trends Endocrinol Metab 11, 49–53.

Habtezion A, Toivola DM, Asghar MN, Kronmal GS, Brooks JD, Butcher EC, Omary MB (2011). Absence of keratin 8 confers a paradoxical microflora-dependent resistance to apoptosis in the colon. Proc Natl Acad Sci USA 108, 1445–1450.

Habtezion A, Toivola DM, Butcher EC, Omary MB (2005). Keratin-8-deficient mice develop chronic spontaneous Th2 cells amenable to antibiotic treatment. J Cell Sci 118, 1971–1980.

Hadjiajoupou C, Schmidt L, Dudeja PK, Layden TJ, Ramaswamy K (2000). Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. Am J Physiol Gastrointest Liver Physiol 279, G775–780.

Hammer HM, Jonkers D, Venema K, Vannoutven S, Troost FJ, Brummer RJF (2008). Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther 27, 104–119.

Hegardt FG (1999). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. Biochem J 338, 559–582.

Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. J Clin Invest 103, 1489–1498.

Khan AQ, Noirel J, Wright PC, Corfe BM, Kilner J, Waby JS, Chowdry J, Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B, Wahli W (2000). The expanding significance of keratin intermediate filaments in normal and diseased epithelia. Curr Opin Cell Biol 12, 169–174.

Serra D, Bellido D, Asins A, Arias G, Vilaro S, Hegardt FG (1996a). The expression of mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase in neonatal rat intestine and liver is under transcriptional control. J Biol Chem 271, 17529–17534.

Toivola DM, Krishnan S, Binder HJ, Singh SK, Omary MB (2004). Keratins modulate colonic electrolyte transport via protein mistargeting. J Cell Biol 164, 911–921.

Trivedi AD, Patil PR, Pradeepa R, Deshpande SR, Hegardt FG (1996b). Tissue-specific expression and dietary regulation of chimeric mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A synthase/human growth hormone gene in transgenic mice. J Biol Chem 271, 7529–7534.

Shen WJ, Zaidi SK, Patel S, Cortez Y, Ueno M, Azhar R, Azhar S, Kraemer FB (2012). Ablation of vimentin results in defective steroidogenesis. Endocrinology 153, 3249–3257.

Topping DL, Clifton PM (2001). Short-chain fatty acids and human colonic flora-dependent resistance to apoptosis in the colon. Proc Natl Acad Sci USA 98, 14454–14459.

F. Tovar DM, Krishnan S, Binder HJ, Singh SK, Omary MB (2004). Keratins modulate colonic electrolyte transport via protein mistargeting. J Cell Biol 164, 911–921.

Toivola DM, Strnad P, Omary MB (2010). Intermediate filament function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 90, 1251–1288.

Trovato DM, Krishnan S, Binder HJ, Singh SK, Omary MB (2004). Keratins modulate colonic electrolyte transport via protein mistargeting. J Cell Biol 164, 911–921.

Vijayaraj P, Kroger C, Reuter U, Windoffer R, Leube RE, Magin TM (2009). Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. J Cell Biol 187, 175–184.

Wirtz S, Neufert C, Weigmann B, Neurath MF (2007). Chemically induced mouse models of intestinal inflammation. Nat Protoc 2, 541–546.