Reduced susceptibility of muscle-specific insulin receptor knockout mice to colon carcinogenesis

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Ealey KN, Lu S, Lau D, Archer MC. Reduced susceptibility of muscle-specific insulin receptor knockout mice to colon carcinogenesis. Am J Physiol Gastrointest Liver Physiol 294: G679–G686, 2008. First published January 3, 2008; doi:10.1152/ajpgi.00526.2007.—Insulin resistance is a risk factor for colon cancer, but it is not clear which of its metabolic sequelae are involved. The objective of this study was to determine whether increased adiposity and elevated circulating lipids commonly seen in insulin resistant promote colon carcinogenesis independent of changes in insulin. We made use of muscle-specific insulin receptor knockout (MIRKO) mice that exhibit elevated serum triglycerides (TG), free fatty acids (FFA), and fat mass but have similar body weights, circulating glucose, and insulin and insulin sensitivity to their wild-type littermates used as controls. Seven-week-old male MIRKO mice and controls received four weekly intraperitoneal injections of either 5 mg/kg azoxymethane (AOM) to induce aberrant crypt foci (ACF) or 10 mg/kg AOM to induce tumors and were killed at 24 or 40 wk of age, respectively. The MIRKO mice displayed hyperinsulinemia at 7 wk of age and reduced insulin sensitivity at 16 wk of age compared with controls. The previously reported MIRKO phenotype developed between 16 and 24 wk of age. By 40 wk of age, however, MIRKO mice were again insulin resistant. ACF development did not differ between MIRKO mice and controls, but MIRKO mice developed significantly fewer colon tumors. Our results suggest that circulating TG and FFA are not promoters of colon tumor development. Indeed, we show that the cumulative effects of the metabolic changes that occur with knockout of the insulin receptor in muscle are associated with reduced susceptibility to colon tumorigenesis.

IXULIN RESISTANCE, A MAJOR cause of Type 2 diabetes mellitus (T2DM), is characterized by impaired biological response to the action of insulin (31). The metabolic consequences of insulin resistance include hyperinsulinemia, hyperglycemia, and/or glucose intolerance, as well as elevated circulating levels of triglycerides (TG) and free fatty acids (FFA) (6). Over a decade ago, it was noted that the diet and lifestyle risk factors for the development of colorectal cancer (CRC) are very similar to those for insulin resistance (19, 30). On the basis of these observations, it was hypothesized that hyperinsulinemia or some factor(s) associated with insulin resistance promotes colon carcinogenesis and stimulates the growth of colorectal tumors (19, 30). Evidence from both epidemiological and animal studies supports this association. Risk of CRC is increased threefold in men and women with elevated plasma C-peptide, a surrogate measure of insulin secretion (22, 28). T2DM was associated with a 40–50% increase in the risk of colon cancer in the Nurses’ Health Study and in another large cohort of postmenopausal women (21, 26). Furthermore, chronic injections of insulin in rats treated with azoxymethane (AOM), a colon-specific carcinogen, promote the growth of putative preneoplastic aberrant crypt foci (ACF) (11) and colon tumors (46).

Obesity, particularly visceral adiposity, is an independent risk factor for T2DM, insulin resistance, and hyperinsulinemia (18, 48) and is associated with elevated risk of CRC (25, 39). Excess abdominal tissue is associated with increased production of FFA, increased hepatic production of triglycerides (TG), decreased glucose uptake, and impaired insulin signaling (31). McKeown-Eyssen (30) hypothesized that serum TG and glucose that are elevated by dietary and lifestyle risk factors for CRC may themselves be positively associated with CRC. In case-control studies, elevated circulating TG levels have been associated with an increased risk of the development of colorectal adenomatous polyps (40, 41, 44) and carcinomas in situ (52). In addition, higher serum TG levels were observed in patients with familial adenomatous polyposis who developed CRC compared with those who did not (34). Similar findings were reported in ApoC3Min/+ mice that lack the adenomatous polyposis coli (Apc) gene and spontaneously develop numerous polyps in the intestinal tract (33, 42). These mice have significantly higher serum TG, FFA, and cholesterol compared with wild-type mice because of a reduced expression of lipoprotein lipase (LPL) (36). Pharmacological activation of LPL in Min mice resulted in suppression of serum lipid levels and a significant reduction in the development of intestinal polyps (35–37).

Studies that have investigated the relationship between insulin resistance and colon cancer have not separated the action of insulin from the other effects caused by hyperinsulinemia such as increased hepatic TG release and elevated circulating FFA (11, 46). The objective of the present study was to test the hypothesis that increased adiposity and elevated circulating lipids commonly seen in insulin resistance promote colon carcinogenesis independent of changes in insulin. We made use of a tissue-specific knockout mouse model in which the insulin receptor (IR) gene has been inactivated in the muscle using the Cre-loxP-mediated gene recombination technique (43). MIRKO (muscle-specific insulin receptor knockout) mice exhibit a >95% reduction in IR content in muscle whereas IR expression in other tissues is not affected (7). This results in reduced insulin-stimulated signaling and glucose uptake into skeletal muscle, giving rise to elevated serum TG and FFA and

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increased fat mass (7). MIRKO mice, however, have been reported to exhibit similar body weights, circulating levels of glucose, and insulin and glucose tolerance compared with their wild-type littermates (7). We show that MIRKO mice have a reduced susceptibility to the development of colon tumors compared with controls, and present evidence to suggest that elevated adiposity in MIRKO mice may have inhibited carcinogenesis.

MATERIALS AND METHODS

Animals. The generation of MIRKO mice that are homozygous for the IR gene flanked by two loxP sites and also carry the MCK-Cre transgene [IR(loxp/loxp); MCK-Cre (+/−)] has been described previously (7). A breeding pair of these mice was a generous gift from Dr. Ronald Kahn (Joslin Diabetes Center and Harvard Medical School, Boston, MA). All genotyping was performed by PCR using genomic DNA isolated from the tails of weaned mice as previously described (7). The animals were housed in the Department of Comparative Medicine at the University of Toronto. All protocols for animal use and treatment were reviewed and approved by the University of Toronto Animal Care Committee and were in compliance with the guidelines of the Canadian Council on Animal Care. Mice were housed at 22°C and 50% humidity on a 12:12-h light-dark cycle and fed a standard rodent chow diet and water ad libitum. Male MIRKO mice were used in all of the studies described, and age-matched littermates that were homozygous for the floxed IR gene [IR(loxp/loxp)] but did not express Cre were used as controls. In all of the experiments, we used offspring from a number of individual breeding pairs to obtain sufficient male MIRKO and control mice that were of the same age. To minimize potential variability between litters from different breeding pairs, MIRKO mice and controls from each of the breeding pairs were usually included in each experiment.

Analytical procedures. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed on animals at various ages throughout the study. For the GTT, mice (n = 7–13 per group) were injected with 2 g/kg body wt of dextrose after an overnight fast and blood samples were obtained from the tail vein immediately before and 15, 30, 60, and 120 min after injection as previously described (7). For the ITT, fed animals (n = 7–12 per group) were injected with 0.75 U/kg of human regular insulin (Sigma Chemicals, Oakville, Ontario, Canada) and blood was collected at 0, 15, 30, 60, and 90 min after the injection as in previously described protocols (5, 29). Glucose values were determined from whole venous blood via an automatic glucose analyzer (Ascensia Elite XL, Bayer, Toronto, Ontario, Canada).

At the time of euthanasia, blood was obtained from mice that had either been fasted or fed for the determination of serum FFA, TG, insulin, leptin, and adiponectin. Epididymal fat pads were removed and weighed as previously described (29). Insulin, leptin, and adiponectin were measured in serum by radioimmunoassay (Linco Research, St. Charles, MO). FFA and TG in serum were measured by colorimetric enzyme assays (Wako Chemicals, Richmond, VA and Teco Diagnostics, Anaheim, CA, respectively).

Carcinogenesis studies. For the induction of ACF, 7-wk-old male MIRKO mice and littermate controls (n = 12 per group) received 4 weekly intraperitoneal injections of 10 mg/kg AOM (Midwest Research Institute, Kansas City, MO) according to a previously published protocol (2). Body weights were monitored weekly. One hundred days after the last injection, the animals were killed and colons were removed for the analysis of ACF. The entire colon was flushed with phosphate-buffered saline, cut open longitudinally, and fixed flat between filter papers in 10% phosphate-buffered formalin for ≥24 h. Colons were stained with 0.05% methylene blue (Sigma Chemicals, Oakville, Canada) and the mucosal surface was assessed for ACF with a light microscope at ×40 magnification. ACF were distinguished from normal crypts on the basis of their enlarged size and pericryptal zone, their thicker epithelial lining, and the slitlike shape of the lumen (3). ACF analysis was carried out by a single operator who was blinded to the identity of the samples.

To assess tumorigenesis, 7-wk-old male MIRKO mice and controls (n = 23–27 per group) were treated with four weekly intraperitoneal injections of 10 mg/kg AOM (4). Most animals were killed 30 wk after the last AOM injection (40 wk of age), although a few mice were killed 26–29 wk after the last AOM injection because of rectal bleeding. Colons were removed from each animal, flushed with PBS, and cut open longitudinally. The total number of tumors per colon, their location along the length of the colon (mm from the anus), and their size (largest diameter) were recorded. The tumors were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin for histological examination. Slides were coded and read by a pathologist blinded to the analysis.

Tissue lysis, Western blotting, and immunoprecipitation. Colonic mucosal scrapings were homogenized in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerocephosphate, 1 mM Na3VO4, protease inhibitor cocktail (Sigma Chemicals)] with a hand-held motorized tissue grinder. Tissue homogenates were centrifuged at 26,000 rpm for 30 min in a Beckman ultracentrifuge at 4°C and the resulting supernatants were centrifuged again at 26,000 rpm for 30 min. The supernatants were collected and stored at −80°C. Protein concentrations were determined by the Bio-Rad protein assay.

For Western blotting analysis, 12.5–25 μg of total mucosal protein was solubilized in Laemmli buffer, heated for 5 min at 95°C, electrophoresed by SDS-PAGE on 10% gels, and transferred to polyvinylidene difluoride membranes. After blocking for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), membranes were probed overnight at 4°C with antibodies to phosphorylated MAPK (pMAPK), phosphorylated Akt (Ser473), phosphorylated AMPKα (pAMPKα) (Cell Signaling Technology, Beverly, MA), and peroxisome proliferator-activated receptor-γ (PPARγ) (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed in TBS-T and incubated for 1 h with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Signals were detected by enhanced chemiluminescence (Millipore, Billerica, MA) on Biomax MR Kodak film by autoradiography and protein bands were quantified by densitometry. The expression pattern of β-actin was used to ensure equal loading. For immunoprecipitation, 500 μg of protein from colon mucosal lysates were immunoprecipitated overnight at 4°C with anti-insulin receptor substrate-1 (IRS-1) (Cell Signaling Technology). Immunocomplexes were captured by incubation with protein A agarose beads for 1 h at 4°C. Washed immunoprecipitates were resuspended in Laemmli buffer, boiled at 95°C, and subjected to Western blotting on a 7.5% gel as described above. Membranes were probed with a rabbit polyclonal antibody to the p85 regulatory subunit of phosphatidylinositol 3-kinase (Upstate Biotechnology, Lake Placid, NY).

Statistical analyses. Analysis of the incidence of early deaths, overall tumor incidence, and incidence of adenocarcinomas and adenomas were compared by the Fisher’s exact probability test. All other data were analyzed by unpaired Student’s t-test. Results are given as means ± SE. Statistical significance was accepted at P < 0.05.

RESULTS

Serum parameters. We measured fasting serum levels of TG, FFA, glucose, and insulin as well as body weights in groups of untreated 7-, 16-, 24-, and 40-wk-old MIRKO mice and littermate controls (n = 8–10 per group) and in AOM-treated mice at the time they were killed for analysis of ACF and tumors at 24 and 40 wk of age, respectively. As shown in Table 1, at 7 wk of age, there were no significant differences in TG, FFA, or glucose between MIRKO mice and controls,
were somewhat higher in the MIRKO mice but did not reach significance in any of the serum parameters in 16-wk-old MIRKO mice compared with controls, although TG levels differed in overall body weight (Table 1). There were no significant differences in any of the serum parameters in 16-wk-old MIRKO mice and controls did not reach statistical significance (P = 0.07, Table 1). Sixteen-week-old MIRKO mice had a small but significantly lower body weight compared with controls (Table 1).

By 24 wk of age, MIRKO mice had a distinctly different metabolic phenotype compared with controls. This MIRKO phenotype was very similar to that reported by others for these mice at this age (29). Serum levels of TG and FFA were significantly higher in MIRKO mice and there were no differences in insulin or glucose compared with controls (Table 1). MIRKO mice had a small but significantly lower overall body weight compared with control littermates (Table 1).

Interestingly, by 40 wk of age, with the exception of FFA, there were no significant differences in any of the serum parameters between fasted MIRKO mice and their controls (Table 1) and no significant differences in body weight between the two groups. We also measured serum parameters in a subset of animals that were not fasted when killed and found no differences between the two groups (data not shown). The differences in serum parameters that we observed between MIRKO mice and controls at 24 and 40 wk of age were not affected by AOM treatment.

**GTT and ITT.** To determine whether MIRKO mice displayed changes in insulin resistance as they aged, we assessed glucose tolerance in non-AOM treated MIRKO mice and controls at 7, 16, 24, and 40 wk of age and insulin tolerance in the three older groups. Figure 1A shows that 7-wk-old MIRKO mice displayed similar glucose tolerance curves to controls with no significant differences in the area under the curve (AUC), although glucose levels were significantly lower in MIRKO mice 120 min after the glucose challenge. MIRKO mice and controls exhibited similar levels of glucose tolerance and AUCs following injection of glucose (Fig. 1, B and C) at both 16 and 24 wk of age. During the ITT, however, 16-wk-old MIRKO mice initially had reduced sensitivity to injected insulin, resulting in significantly higher blood glucose levels after 15 min (Fig. 2A). By 24 wk of age, MIRKO mice were equally sensitive to injected insulin and there were no differences in glucose levels throughout the ITT (Fig. 2B). We did not measure glucose at 90 min after insulin injection in 24-wk-old mice because they were exhibiting signs of hypoglycemia.

At 40 wk of age, MIRKO mice showed evidence of impaired glucose tolerance and reduced insulin sensitivity compared with controls. During the GTT, MIRKO mice had significantly higher blood glucose values at 15, 30, and 60 min, and the AUCs were significantly higher (19.54 ± 1.9 vs. 12.3 ± 1.3 mg·dl⁻¹·min⁻¹·1,000⁻¹, MIRKO vs. control, P < 0.05), suggesting reduced ability to clear a glucose load (Fig. 1D). This was confirmed by the reduced insulin sensitivity following the ITT resulting in significantly higher glucose levels 15, 30, and 90 min after insulin injection (Fig. 2C).

**Adiposity, adiponectin, and leptin.** We measured epididymal fat pad weight as a percentage of body weight in groups of untreated MIRKO mice and controls at 16, 24, and 40 wk of age (n = 8–23 per group). In addition, circulating levels of adiponectin were assessed in 16- and 24-wk-old MIRKO mice and controls. There were no significant differences in epididymal fat pad weight between MIRKO mice and controls at 16 and 24 wk of age, but at 40 wk of age MIRKO mice had a significantly greater amount of epididymal fat compared with the control group (Table 2). We observed no significant differences in adiponectin between 16-wk-old MIRKO mice and controls but significantly higher adiponectin levels in 24-wk-old MIRKO mice relative to controls, concomitant with the development of the MIRKO phenotype (Table 2). Furthermore, 24-wk-old control mice had significantly lower adiponectin levels than the controls at 16 wk of age, whereas this decrease in adiponectin levels was not observed between 16- and 24-wk-old MIRKO mice. In addition, there were no significant differences in serum leptin between 24-wk-old MIRKO mice and controls (0.79 ± 0.1 vs. 0.63 ± 0.2 ng/ml, MIRKO vs. controls).

**ACF study.** For analysis of ACF, animals were killed at 24 wk of age, 100 days after treatment with four weekly doses of 5 mg/kg AOM. The average number of ACF per colon did not differ between MIRKO mice and controls (8.2 ± 1.0 vs. 7.7 ± 0.8, MIRKO vs. control). Similarly, there was no significant difference in crypt multiplicity (number of crypts per focus) between the two groups (1.38 ± 0.1 vs. 1.46 ± 0.1, MIRKO vs. control).

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Body Weight, g</th>
<th>TG, mg/dl</th>
<th>FFA, mEq/l</th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Control 25.4±0.4</td>
<td>TG 104.±9.2</td>
<td>FFA 1.24±0.12</td>
<td>Glucose 157.4±22</td>
<td>Insulin 0.29±0.04</td>
</tr>
<tr>
<td></td>
<td>MIRKO 24.3±0.6</td>
<td>106.4±16.5</td>
<td>1.46±0.22</td>
<td>193.9±15.2</td>
<td>0.53±0.15*</td>
</tr>
<tr>
<td>16</td>
<td>Control 26.6±0.7</td>
<td>60.9±7.0</td>
<td>1.11±0.22</td>
<td>105.8±16.3</td>
<td>0.43±0.09</td>
</tr>
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<td></td>
<td>MIRKO 24.2±0.4*</td>
<td>89.6±11.9</td>
<td>1.32±0.08</td>
<td>95.2±16.3</td>
<td>0.37±0.08</td>
</tr>
<tr>
<td>24</td>
<td>Control 30.3±0.9</td>
<td>87.0±6.0</td>
<td>1.00±0.08</td>
<td>132.3±15</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td></td>
<td>MIRKO 27.3±0.8*</td>
<td>124.7±9.4*</td>
<td>2.22±0.45*</td>
<td>109.6±16.7</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td>40</td>
<td>Control 30.0±1.0</td>
<td>131.1±52.8</td>
<td>0.77±0.04</td>
<td>127.1±35.3</td>
<td>0.53±0.30</td>
</tr>
<tr>
<td></td>
<td>MIRKO 27.9±0.7</td>
<td>80.3±19.3</td>
<td>1.44±0.2*</td>
<td>71.3±13.0</td>
<td>0.61±0.30</td>
</tr>
</tbody>
</table>

Values are means ± SE. Metabolic measures were obtained from untreated 7- and 16-wk-old mice and from azoxymethane-treated mice at 24 and 40 wk of age that were part of the carcinogenesis studies. MIRKO, muscle-specific insulin receptor knockout. *P < 0.05 vs. control.
Tumor study. Colon tumorigenesis was initiated in 7-wk-old male MIRKO and control mice by four weekly injections of 10 mg/kg AOM according to previously published protocols (4). Most of the mice were killed 30 wk after the last injection of AOM (40 wk of age). A few mice were killed 26–29 wk after AOM because of rectal bleeding. Furthermore, a number of mice particularly in the control group developed rectal prolapse between 18 and 23 wk after AOM and had to be killed or they died prematurely. These animals were not included in any of the analyses.

All macroscopic polypoid lesions were excised for histological examination. Although the difference in tumor incidence

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**Fig. 1.** Glucose tolerance tests (GTT) in muscle-specific insulin receptor knockout (MIRKO) mice and controls. GTTs were performed on 7- to 8-wk-old (A), 16-wk-old (B), 24-wk-old (C), and 40-wk-old (D) male control (○) and MIRKO (●) mice that were not treated with azoxymethane (AOM). Fasting mice were injected with 2 g/kg body wt ip of dextrose, and blood glucose was measured at various time points. Data are expressed as means ± SE, *P < 0.05.

**Fig. 2.** Insulin tolerance tests (ITT) in MIRKO mice and controls. ITTs were performed on 16-wk-old (A), 24-wk-old (B), and 40-wk-old (C) male control (○ or △) and MIRKO (●) mice that were not treated with AOM. Fed mice were injected intraperitoneally with 0.75 U/kg of human regular insulin, and blood glucose was measured at various time points. Data are expressed as means ± SE, *P < 0.05.
did not reach statistical significance, there was a large, highly significant difference in tumor multiplicity (Table 3), with the controls having three times more tumors per mouse than MIRKO mice. Analysis of tumor histology showed that the control group had a higher ratio of adenocarcinomas to adenomas (2.5) than MIRKO mice (1.6). The adenocarcinomas in the MIRKO mice, however, were significantly larger than those in the controls. Figure 3 illustrates that the difference in overall tumor multiplicity shown in Table 3 was primarily due to the significantly fewer carcinomas per mouse in the MIRKO mice compared with controls.

Immunoblot analysis. Expression levels of key intermediates of the adiponectin and insulin signaling pathways were analyzed by Western blot in colon mucosal cell lysates of untreated 24-wk-old MIRKO mice and controls. There were no significant differences in the levels of pAMPK, p44/42 pMAPK, pAkt, or IRS-1 bound to p85 between MIRKO mice and controls (data not shown). Furthermore, levels of PPARγ were also not different between the two groups.

DISCUSSION

The objective of this study was to determine whether increased adiposity and elevated circulating lipids commonly seen in insulin resistance promote colon carcinogenesis, independent of changes in insulin. The rationale for choosing to assess susceptibility to colon carcinogenesis in MIRKO mice was based on the initial report by Bruning et al. (7) characterizing these mice as having elevated FFA, TG, and fat mass but no differences in body weight, blood glucose, and insulin as well as no impairments in glucose tolerance or insulin sensitivity compared with littermate controls. Unexpectedly, however, we observed that the MIRKO phenotype was not stable and changed with age. Circulating insulin levels were significantly elevated in fasting 7-wk-old MIRKO mice but there were no differences in other serum parameters compared with controls. Elevated serum insulin may indicate that these animals were insulin resistant, although they had normal glucose tolerance and lower glucose levels by the end of the GTT. By 16 wk of age, the MIRKO mice still did not display the reported MIRKO phenotype. There were no differences in serum parameters and they displayed evidence of insulin resistance compared with controls. At 24 wk of age when groups were killed for ACF analysis, we observed the previously reported MIRKO phenotype of significantly elevated FFA and TG with insulin and glucose levels and glucose tolerance and insulin sensitivity similar to controls. These phenotypic characteristics may have developed earlier, however, since we did not make any measurements between 16 and 24 wk of age. By 40 wk of age, when the remaining mice were killed for analysis of tumors, the MIRKO mice displayed impaired glucose and insulin tolerance compared with controls. FFA levels were significantly higher in MIRKO mice at this time point but there were no differences in any other serum parameters compared with controls.

Our observations differ from those of Bruning et al. (7), who initially described the phenotype of the MIRKO mouse at 16 wk of age. Some studies, however, suggest that the age at which the full MIRKO phenotype is expressed may vary between 8 and 24 wk (7, 24, 29, 50). It is possible that the changing phenotype of these mice results from changes in insulin sensitivity that occur with age. Furthermore, there may also be age-specific compensatory mechanisms in other tissues that allow the mice to adapt to metabolic changes resulting from reduced expression of insulin receptor in muscle, as has

Table 2. Epididymal fat pad weight and serum adiponectin in MIRKO mice and controls

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Epididymal Fat</th>
<th>Adiponectin, μg/ml</th>
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<tbody>
<tr>
<td>16</td>
<td>0.71 ± 0.4</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0.56 ± 0.1</td>
<td>4.6 ± 1.0</td>
</tr>
<tr>
<td>24</td>
<td>0.55 ± 0.1</td>
<td>1.9 ± 0.3†</td>
</tr>
<tr>
<td></td>
<td>0.71 ± 0.1</td>
<td>3.4 ± 0.5*</td>
</tr>
<tr>
<td>40</td>
<td>0.69 ± 0.1*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1.09 ± 0.1†</td>
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</table>

Values are means ± SE. Epididymal fat pad weight is expressed as % of body weight. ND, not done. *P < 0.05 vs. control, †P < 0.05 vs. 16-wk-old control mice.

Table 3. Characteristics of tumors in MIRKO mice and controls

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Tumor Size, mm</th>
<th>Ac-to-Ad Ratio</th>
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<tbody>
<tr>
<td></td>
<td>Ad</td>
<td>Ac</td>
</tr>
<tr>
<td>Controls</td>
<td>14</td>
<td>11/14 (79%)</td>
</tr>
<tr>
<td>MIRKO</td>
<td>23</td>
<td>12/23 (52%)</td>
</tr>
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</table>

Multiplicity values are means ± SE of total number of tumors per mouse; tumor type values are total number of adenomas (Ad) and adenocarcinomas (Ac) in surviving mice at approximately 40 wk of age as described in the text; tumor size values are means ± SE of largest diameter of tumor (in mm). *P < 0.05, †P = 0.005.
been reported in other tissue-specific knockout models of insulin resistance and diabetes (27, 45).

To determine the susceptibility of MIRKO mice to the early stages of colon carcinogenesis, we treated 7-wk-old mice with AOM and analyzed ACF at 24 wk of age. There were no significant differences in the total number of ACF or in ACF size between MIRKO mice and controls. The changes in phenotype that occurred in MIRKO mice between 7 and 24 wk of age, however, limit the interpretation of this result. We were concerned that hyperinsulinemia in the 7-wk-old MIRKO mice may have interfered with metabolic activation of AOM, since insulin has been shown to downregulate hepatic CYP2E1, the major enzyme of AOM metabolism (51). We observed no differences, however, in hepatic CYP2E1 activity between MIRKO and controls at 7 wk of age (data not shown). Insulin has also been shown to be a promoter of ACF development (11), although its transient elevation between 7 and 16 wk of age clearly produced no observable effect on ACF. In addition, we observed that blood lipids were not elevated in the MIRKO mice relative to controls until after 16 wk of age, which may have been too late to see a possible promotional effect on ACF.

Although there were no differences in ACF formation between MIRKO mice and controls, by 40 wk of age the MIRKO mice developed significantly fewer colon tumors, particularly adenocarcinomas, compared with controls. As discussed above, MIRKO mice had higher FFA and TG at 24 wk of age, but by 40 wk of age only FFA remained elevated compared with controls. This suggests that FFA are not promoters of colon carcinogenesis, but because of the normalization of TG between 24 and 40 wk of age in MIRKO mice, we cannot make any conclusions about the role of TG in promoting the growth of ACF into tumors. A study by Tran et al. (47), however, provides indirect support for the notion that neither FFA nor TG promote colon carcinogenesis. They observed in rats that acute intravenous infusion of intralipid giving rise to elevated circulating TG and FFA did not promote colonic epithelial cell proliferation, whereas infusion with insulin led to a significant dose-dependent increase in proliferation (47). Infusion of intralipid with insulin had no additional effects on cell proliferation, suggesting that even within a hyperinsulinemic environment, the lipids play no role in colonocyte proliferation (47).

Although MIRKO mice developed significantly fewer tumors than controls, the size of the tumors, particularly adenocarcinomas, was larger in MIRKO mice. The reasons for this are unclear but may relate to the poorer insulin sensitivity and glucose tolerance of 40-wk-old MIRKO mice relative to controls. It is possible that the previously mentioned compensatory mechanisms that occur in younger MIRKO mice gradually overwhelm the tissues, eventually resulting in an insulin-resistant metabolic milieu that promotes increased growth of established tumors in the older mice.

Our results suggest that there is some factor(s) in MIRKO mice that acts to inhibit the promotion of ACF into colonic tumors. MIRKO mice have been reported to have significantly larger fat depots than controls despite having no differences in body weight (7, 9). This results from a shift in utilization of energy substrates and a redistribution of these substrates to adipose tissue. Indeed, we observed that MIRKO mice accumulated significantly more epididymal fat than controls between 24 and 40 wk of age, the time period during which promotion of ACF into tumors occurred. In contrast to these observations, a positive association between increased adiposity and CRC has been clearly established from human and animal studies (12, 25). It has been proposed that this association results in part from obesity-induced insulin resistance and/or increased secretion of proinflammatory cytokines from adipose tissue (20, 39). The morphology and biology of adipose tissue in MIRKO mice, however, differ from other rodent models of obesity and/or insulin resistance (9). MIRKO mice exhibit increased adipose-specific insulin sensitivity as a result of increased differentiation of small, insulin-sensitive adipocytes. In contrast, enlarged insulin-resistant cells often seen in diet-induced obesity are associated with reduced insulin responsiveness (5, 9). It is possible, therefore, that the increased levels of insulin-sensitive adipose tissue in MIRKO mice may have protected them from colon tumor development.

Two recent studies give support to this notion. One study reported that feeding a high-calcium diet to ApcMin/+ mice that are highly susceptible to spontaneous intestinal tumorigenesis resulted in a significant loss of adipose tissue and increased tumor formation compared with ApcMin/+ mice on a low-calcium diet (15). When the ApcMin/+ mice were crossed with obesity-prone Aβ1a/α lethal yellow agouti mice, the resulting “fat“Aβ1ApcMin/+ mice exhibited loss of adipose tissue when fed the high-calcium diet but were protected from increased intestinal tumorigenesis (15). The authors proposed that there is a critical level of adipose tissue required to maintain a protective effect against intestinal tumorigenesis and that this protective effect may result from the secretion of adipokines that directly or indirectly suppress tumor formation (15). Another study assessed cancer development in lipodystrophic A-ZIP/F-1 mice that are diabetic and insulin resistant and have elevated circulating levels of glucose, insulin, lipids, and proinflammatory cytokines but lack white adipose tissue and have undetectable levels of circulating adipokines (32, 38). Compared with lean controls that have normal amounts of body fat, the fatless A-ZIP/F-1 mice were shown to be more susceptible to skin and mammary carcinogenesis (38). The A-ZIP/F-1 mice also developed more skin tumors than obese ob/ob mice that are insulin resistant but have significant levels of circulating adiponectin (1). The authors suggested that adiposity or some “carcinogenesis-suppressing factor” produced by adipose tissue such as adiponectin may be antitumorogenic (1). We observed significantly higher serum adiponectin, a potent insulin sensitizer (10), in 24-wk-old MIRKO mice compared with controls. Elevated circulating adiponectin has been associated with reduced risk for CRC in human studies (39, 49) and is thought to act either indirectly by altering circulating levels of cytokines and hormones associated with insulin resistance (13, 23) or directly through effects on signaling pathways in cancer cells (8, 14). However, we did not observe any differences in the levels of pAMPK or its downstream metabolites in the colons of untreated 24-wk-old MIRKO mice and controls. AMPK is a key signaling protein activated by adiponectin (53). It is possible, however, that adiponectin affects cell signaling pathways in preneoplastic but not normal colonic epithelial cells, as has been observed for leptin (16, 17).

In conclusion, we observed that the MIRKO phenotype, displaying elevated TG and FFA but no differences in insulin and glucose levels and glucose tolerance and insulin sensitivity compared with controls, developed between 16 and 24 wk of...
age. ACF development did not differ between MIRKO mice and controls. MIRKO mice, however, developed significantly fewer colon tumors than controls, and this may be related to an inhibitory effect of an increased accumulation of insulin-sensitive adipose tissue on the growth of ACF into tumors in MIRKO mice compared with controls. Our results suggest that circulating TG and FFA are not promoters of colon tumor development. Indeed, we show that the cumulative effects of the metabolic changes that occur with knockout of the insulin receptor in muscle are associated with reduced susceptibility to colon tumorigenesis.

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GRANTS

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