Research Article

Cancer Prevention Research

Lycopene Attenuated Hepatic Tumorigenesis via Differential Mechanisms Depending on Carotenoid Cleavage Enzyme in Mice

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Abstract

Obesity is associated with increased liver cancer risks and mortality. We recently showed that apo-10′-lycopenoic acid, a lycopene metabolite generated by beta-carotene-9′,10′-oxygenase (BCO2), inhibited carcinogen-initiated, high-fat diet (HFD)–promoted liver inflammation, and hepatic tumorigenesis development. The present investigation examined the outstanding question of whether lycopene could suppress HFD-promoted hepatocellular carcinoma (HCC) progression, and if BCO2 expression is important using BCO2-knockout (BCO2-KO) and wild-type male mice. Results showed that lycopene supplementation (100 mg/kg diet) for 24 weeks resulted in comparable accumulation of hepatic lycopene (19.4 vs. 18.2 nmol/g) and had similar effects on suppressing HFD-promoted HCC incidence (19% vs. 20%) and multiplicity (58% vs. 62%) in wild-type and BCO2-KO mice, respectively. Intriguingly, lycopene chemopreventive effects in wild-type mice were associated with reduced hepatic proinflammatory signaling (phosphorylation of NK-κB p65 and STAT3; IL6 protein) and inflammatory foci. In contrast, the protective effects of lycopene in BCO2-KO but not in wild-type mice were associated with reduced hepatic endoplasmic reticulum stress–mediated unfolded protein response (ER^{UPR}), through decreasing ER^{UPR}-mediated protein kinase RNA-activated like kinase–eukaryotic initiation factor 2α activation, and inositol requiring 1α–X-box–binding protein 1 signaling. Lycopene supplementation in BCO2-KO mice suppressed oncogenic signals, including *Met* mRNA, β-catenin protein, and mTOR complex 1 activation, which was associated with increased hepatic microRNA (miR)-199a/b and miR214 levels. These results provided novel experimental evidence that dietary lycopene can prevent HFD-promoted HCC incidence and multiplicity in mice, and may elicit different mechanisms depending on BCO2 expression. *Cancer Prev Res; 7(12); 1219–27. ©2014 AACR.*

Introduction

Primary liver cancer is the third leading cause of cancer-related deaths worldwide (1, 2), and hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 70% to 85% of cases (1, 2). Non-alcoholic fatty liver disease (NAFLD) is a pathology that is observed in 75% to 100% of overweight and obese adults and children (3), and its rising prevalence parallels closely with HCC's escalating morbidity and mortality trends (3). The prevention of liver cancer progression through dietary means represents an important disease control strategy because HCC has a high mortality rate and a poor prognosis (4, 5).

Previous animal studies demonstrated that high-fat diet (HFD) and obesity promoted liver tumorigenesis by inducing chronic inflammation through the IL6/STAT3 pathway (6), with STAT3-activated tumors being more aggressive in humans (6–8). Metabolic surplus from excess calorie consumption can also elevate synthesis of hepatic enzymes, which creates excess demand on the endoplasmic reticulum (ER) for proper protein folding (9–11). This excess demand on the ER leads to the induction of ER stress–mediated unfolded protein response (ER^{UPR}; refs. 9–11), which was associated with liver cancer development (11).

Observational studies have shown beneficial associations between lycopene-rich foods against various cancers (as reviewed in refs. 12–14), including those of gastrointestinal tract origin (14). Patients with NAFLD have

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significantly reduced plasma lycopene (15), suggesting a potential interactions between low lycopene status and the development of liver diseases. In the rat model, dietary lycopene has been shown to reduce the liver-specific carcinogen diethylnitrosamine (DEN) initiation of hepatic preneoplastic foci and macroscopic nodules (16–18), inhibit hepatic glutathione *S*-transferase placental-form–positive foci in rats that develop spontaneous liver tumors (19), and ameliorate DEN-initiated, HFD-promoted precancerous lesions (20). However, the primary outcomes for these rat studies were hepatic preneoplastic lesions that may develop into tumors. There are currently no published *in vivo* studies to demonstrate whether lycopene can effectively reduce HCC development and progression. Our mechanistic understanding of how lycopene functions against hepatic tumorigenesis is also far from complete (21).

Lycopene can be preferentially metabolized by the enzyme beta-carotene 9′,10′-oxygenase (BCO2), and generate apo-10′-lycopenoids, including apo-10′-lycopenal, -lycopenol and -lycopenoic acid (APO10LA; refs. 22, 23). It is important to understand whether lycopene effects on various cellular functions and signaling pathways are the results of intact lycopene or apo-10′-lycopenoids (13). We have recently shown that APO10LA supplementation significantly reduced hepatic inflammation (decreased inflammatory foci, TNFα, IL6, NF-κB p65 protein expression, and STAT3 activation) and tumorigenesis in HFD-fed mice (24). Therefore, lycopene metabolites such as APO10LA, may exhibit protective effects against obesity-associated hepatic inflammation and tumorigenesis. The outstanding question is whether BCO2 expression is critical for the potential biologic effects of lycopene against HFD-promoted liver tumorigenesis. This information is critically needed because 19 SNPs of BCO2 have been found in humans (25). These BCO2 SNPs in humans are associated with increased circulatory proinflammatory IL18 expression (25), and with reduced circulatory high-density lipoprotein (25), suggesting a gene–diet interaction between the BCO2 enzyme and dietary lycopene on human health outcomes. We hypothesize that lycopene is effective in inhibiting HFD-promoted liver tumorigenesis, and lycopene biologic actions could be different in the absences of BCO2 expression.

Using the BCO2-knockout (BCO2-KO) and wild-type mice in the present study, we investigated the potential inhibitory effects of lycopene against HFD-promoted hepatic tumorigenesis, and elucidated the underlying mechanisms by which lycopene exhibited these chemopreventive effects.

Materials and Methods

Study design

The *in vivo* experimental protocol was adapted from previous publications that studied hepatic tumorigenesis (6, 24, 26). All study protocols were approved by the Institutional Animal Care and Use Committee at the Jean Mayer-USDA Human Nutrition Research Center on Aging at Tufts University. The generation of BCO2-KO mice with BCO2 ablation at the protein level was described in the previous study (27). The respective wild-type control mice with a 129SvJ/SvEvTac F1 generation–mixed genetic background were established by conventional cross-breeding in our animal facility. The rationale for the selected wild-type background was based upon the embryonic stem (ES) cell mouse strain used to generate the BCO2-KO mice (27). Therefore, using mice that share the same genetic background as these ES cells would sufficiently represent the biologic effects of BCO2 enzyme expression. The schematic for the study design is shown in Fig. 1A. Study mice were fed the standard laboratory chow (Harlan Laboratories), maintained on a 12-hour light/dark cycle in a controlled temperature and humidity room, and given water ad libitum. Two-week-old male wild-type and BCO2-KO mice were injected i.p. with a liver-specific carcinogen, DEN (Sigma-Aldrich) at a dosage of 25 mg/kg body weight as previously described (6, 24). At 6 weeks of age, wild-type and BCO2-KO mice were randomized to either an obesogenic HFD

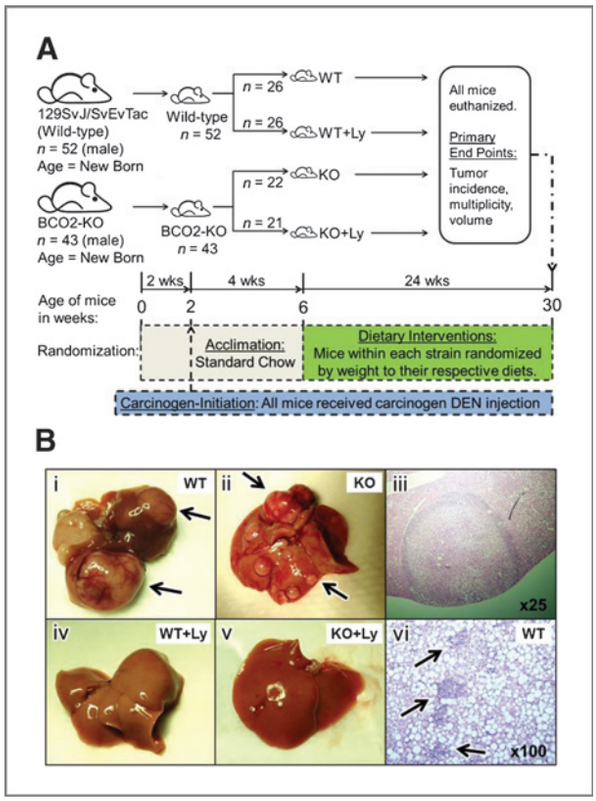

Figure 1. Study design, gross, and histopathology of liver tumor and inflammation. A, schematic for the study design. B, representative picture or light micrograph of livers from WT, WT+Ly, KO or KO+Ly; Bi, liver from WT with tumors (arrows). Bii, liver from KO with tumors (arrows). Biii, H&E-stained liver tumor at ×25. Biv, liver from WT+Ly. Bv, liver from KO+Ly. Bvi, H&E-stained liver from WT with inflammatory cell infiltration (arrows) at ×100. DEN, diethylnitrosamine; KO, knockout on HFD; Ly, lycopene; WT, wild-type on HFD.

(F6635; Bio-Serv; WT, wild-type on HFD; KO, BCO2-KO on HFD), in which 45% of energy was fat derived, or the same HFD supplemented with lycopene (WT+Ly or KO+Ly; 100 mg/kg diet) for 24 weeks (Fig. 1A). All study mice were weighed weekly, given fresh diets every 2 to 3 days, and maintained on their respective diets until the experiment was completed. Mice were euthanized at 30 weeks of age by exsanguination under deep anesthesia without being food deprived.

Lycopene treatment

Both lycopene in the form of a 10% lycopene beadlet or placebo beadlet without lycopene (BASF) was incorporated directly into the HFD to achieve a homogenous diet mixture. Only lycopene but not lycopene metabolites was detectable in the lycopene-supplemented diets using our HPLC (high-performance liquid chromatography) analysis. Both diets were made every 2 to 4 weeks, and were kept at 4°C (<1 week) or −20°C (up to 4 weeks) inside opaque boxes. The rationale for selecting this lycopene dose was based on the assumption that BCO2-KO and wild-type mice have similar carotenoid absorption as other strains of mice on a carotenoid-supplemented semipurified diet (∼1/10 of human absorption; refs. 28–30). An established equation was used to calculate the dosage equivalence for human consumption (31, 32), which indicated that the lycopene-supplemented dose of 100 mg/kg diet is equivalent to approximately 8.1 mg lycopene per day in a 60-kg adult man. The average human dietary lycopene is approximately 8 mg/d (12, 33), and lycopene doses used in dietary supplements are between 15 and 30 mg/d.

Liver tumors quantification and liver tissue processing

Whole livers were removed from study mice after euthanization and processed as previously described (24). Briefly, two investigators unaware of treatment groups counted the surface liver tumors (tumor multiplicity). Livers were weighed and washed with saline for further processing. Surface liver tumors were removed, snap-frozen in liquid nitrogen and stored at −80°C. The left lobe of mouse liver was fixed in 10% buffered formalin solution (Thermo Fisher Scientific), processed and embedded in paraffin for serial sectioning as described in the previous study (24). The remaining sections of liver were divided into smaller portions, snap-frozen in liquid nitrogen and stored at −80°C.

Histopathologic evaluation of liver tissue

Sections of (5 μm) formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) for histopathologic examination. H&E-stained liver slides were examined by two independent investigators blinded to treatment groups under light microscopy (Zeiss). Liver histopathology of nontumor areas was graded in 20 random fields at ×100 magnification, according to the degree of liver inflammation severity as described previously (24, 34). Briefly, inflammatory foci were evaluated by the number of inflammatory cell clusters, which mainly constitute the infiltration of mononuclear inflammatory cells. Mean foci per field were calculated and reported as inflammatory cell clusters per cm^2. The liver tumor was confirmed as HCC by two independent investigators according to the following criteria: (i) the presence of trabecular pattern with 3+ cell-thick hepatocellular plates/cords; (ii) mitotic figure; (iii) enlarged convoluted nuclei or high nuclei/cytoplasmic ratio; (iv) the presence of tumor giant cells with compact growth pattern; and (v) the presence of endothelial cells lining of sinusoids that surround enlarged hepatocellular plates/cords.

HPLC analysis

Lycopene (all-*trans* and 5-, 9-, and 13-*cis*-isomers) and lycopene metabolites, including apo-10′-lycopenal, apo-10′-lycopenol, and apo-10′-lycopenoic acid concentrations in liver tissue and diets, were measured by gradient reverse phase HPLC consisted of a Waters 2695 separations module and a Waters 2996 photodiodearray detector (Waters) as previously described (23, 29). Lycopene and metabolites were quantified relative to the internal standard by determining peak areas calibrated against known amounts of standard.

RNA and microRNA extraction and quantitative real-time PCR

Total RNA was extracted from frozen liver sections with TRIzol reagent (Invitrogen), as previously described (24). cDNA was prepared from the RNA samples using M-MLV (for mRNA; Invitrogen) or M-MuLV [for microRNA (miR); BioLabs) reverse transcriptases and an automated thermal cycler PTC-200 (MJ Research). Quantitative real-time PCR (qRT-PCR) was performed using FastStart Universal SYBR Green Master (ROX; Roche). Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are listed in Supplementary Table S1.

Protein isolation and Western blotting

Both tissue protein preparation and Western blotting analysis were as described previously (24). The following antibodies were used for Western blotting: mTOR, NF-κB p65, eukaryotic initiation factor (eIF) 2α, phosphorylated-eIF2α (Ser51), phosphorylated NF-κB p65 (Ser536), phosphorylated-STAT3 (Tyr705), phosphorylated S6 (Ser235/236), S6, STAT3 (Cell Signaling Technology), IL6 (R&D Systems), CCAAT/enhancer–binding protein homology protein (CHOP), and cyclin D1 (Santa Cruz Biotechnology). Proteins were detected by a horseradish peroxidase–conjugated secondary antibody (Bio-Rad). The specific bands were visualized by the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce) according to the manufacturer's instructions. Dilution series and calibration curve were performed for each of the antibodies used to quantify protein. Antiactin antibody (Sigma-Aldrich) was used to detect β-actin for loading normalization of some proteins. Intensities of protein bands were quantified using GS-710 Calibrated Imaging Densitometer (Bio-Rad).

Statistical analysis

SAS 9.3 software was used to perform the statistical analysis. Two-way ANOVA analysis with Tukey adjustments for multiple comparisons was used to evaluate the effects of BCO2 protein expression, lycopene supplementation, and the potential interactions between these two factors. The χ^2 test was used to examine the effects of mouse strains or dietary lycopene on liver tumor incidence. Student *t* test or Wilcoxon signed-rank test was used to test for the differences between the following comparisons: (i) WT and WT+Ly; (ii) KO and KO+Ly. Statistical significance was $P < 0.05$.

Results

Lycopene supplementation inhibited HCC development in both wild-type and BCO2-KO mice without altering body/liver weights

Food intake by weight was similar among the four groups of mice (Table 1). Lycopene had no significant effect on body or liver weight in either mouse strain (Table 1), although BCO2-KO mice exhibited significantly lower final body and liver weights than wild-type mice (Table 1). DEN initiation resulted in visible and multiple surface liver tumors in HFD-fed BCO2-KO and wild-type mice (Fig. 1Bi–iii). Liver histopathology of H&E-stained, formalin-fixed, and paraffin-embedded liver tumors classified all observed tumors to be either well-, moderate-, or poorly differentiated HCC (Fig. 1Biii). Dietary lycopene supplementation significantly decreased the HCC incidence (17% for wild-type; 20% for BCO2-KO; Table 1) and multiplicity (58% for wild-type; 61% for BCO2-KO; Table 1; Fig. 1Bi–v), irrespective of mouse strain. Hepatic lycopene concentrations were 19.4 and 18.2 nmol/g tissue in WT+Ly and KO+Ly mice, respectively, and were not detectable in mice without supplementation (Table 1). Although there was no significant effect of mouse strain on total hepatic lycopene concentrations with lycopene supplementation (Table 1), BCO2-KO mice accumulated a great proportion of hepatic all-*trans* lycopene (65%), as compared with WT (51% as all-*trans* lycopene, Table 1). Using our HPLC analysis, we did not detect measurable amounts of lycopene metabolites in hepatic tissue from all groups of mice (data not shown).

Lycopene-mediated suppression in hepatic tumorigenesis was associated with reduced hepatic inflammation in wild-type mice

Numerous animal studies suggested that HFD-promoted liver tumorigenesis was associated with an elevated proinflammatory response, by inducing the NF-κB, and the IL6/STAT3 signaling pathway (6, 7, 35). In the present study, H&E staining of liver tissues showed the infiltration of inflammatory cells in wild-type and BCO2-KO mice (Fig. 1Bvi). Lycopene supplementation reduced the number of hepatic inflammatory foci (58%; $P = 0.06$; Table 1) in wild-

Table 1. Body weights, liver weights, food consumption, hepatic lycopene concentration, inflammatory foci, and tumor outcomes of DEN-induced wild-type or BCO2-KO mice with or without lycopene supplementation for 24 weeks[a]

	Wild-type		BCO2-KO		*P* values for two-way ANOVA or Wilcoxon signed-rank test			
Study group	WT	WT+Ly	KO	KO+Ly	Overall	Diet effect	Strain effect	Diet–strain interaction
Animal (*n*)	26	26	22	21				
Food consumption, g/d	2.6 ± 0.1	3.1 ± 0.5	3.0 ± 0.1	2.9 ± 0.1	0.35	0.59	0.54	0.11
Final body weights, g	52.2 ± 1.1	49.1 ± 1.3	43.6 ± 1.9	41.6 ± 1.7	<**0.01**	0.06	<**0.01**	0.71
Liver weight, g	2.4 ± 0.2	2.3 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	<**0.01**	0.14	<**0.01**	0.98
Liver/body weight, %	4.6 ± 0.2	4.6 ± 0.2	3.4 ± 0.1	3.2 ± 0.1	<**0.01**	0.64	<**0.01**	0.55
Liver tumor outcomes								
Incidence, %	88	71	100	80 ($P = 0.10$)	NA	**0.03**	0.48	NA
Multiplicity, *n*	17.8 ± 4.5	7.4 ± 1.8[b]	10.3 ± 2.2	4.0 ± 0.7[b]	**0.05**	**0.01**	0.52	0.98
Hepatic lycopene, nmol/g tissue	ND	19.4 ± 4.1[b]	ND	18.2 ± 3.2[b]	**0.01**	<**0.01**	0.82	0.90
All-*trans*:*cis*-isomers, %	ND	51:49	ND	65:35	NA	NA	<**0.01**	NA
Inflammatory Foci, *n*/cm^2	0.95 ± 0.6	0.55 ± 0.1 ($P = 0.06$)	0.0 ± 0.0	0.1 ± 0.1	NA	0.29	**0.04**	NA

Abbreviations: NA, not applicable; ND, not detected.

[a]Values are means ± SEMs or *n* (%). Two-way ANOVA was used to examine the overall, diet, strain, and diet–strain effects. Student *t* test, χ^2 test, or Wilcoxon signed-rank test was used to compare between WT and WT+Ly or KO and KO+Ly.

[b]Different from WT or KO, $P < 0.05$.

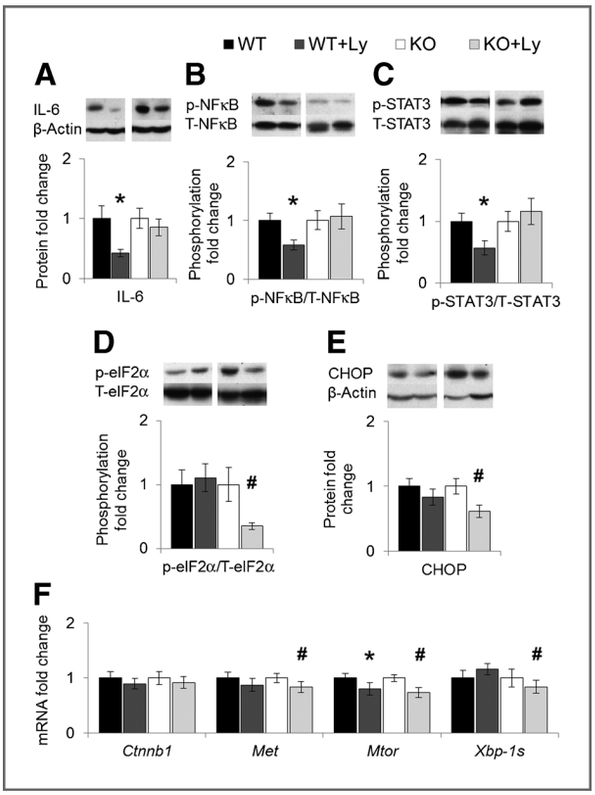

Figure 2. Effects of lycopene supplementation on hepatic proinflammatory and ER stress biomarkers. The study design is described in Fig. 1. Protein or mRNA expressions in liver lysates (WT, WT+Ly, KO, KO+Ly $n = 16–20$) were analyzed by Western blotting or qRT-PCT, and β-actin was used as loading control unless specified otherwise. Graphical representation of fold changes in: A, IL6. B, NF-κB p65 (Ser536) phosphorylation (NF-κB p65 as loading control). C, STAT3 (Tyr705) phosphorylation (STAT3 as loading control). D, eIF2α (Ser51) phosphorylation (eIF2α as loading control). E, CHOP. F, *Ctnnb1*, *Met*, *Mtor*, and *Xbp-1s* mRNA. Representative Western blots with one sample per group are shown. Fold changes normalized to WT or KO; values are means ± SEMs; *, different from WT; and #, different from KO; $P < 0.05$. *Ctnnb1*, β-catenin mRNA; KO, knockout on HFD; Ly, lycopene; p-, phosphorylated; T-, total; WT, wild-type on HFD.

type mice. Lycopene supplementation in wild-type mice significantly reduced hepatic proinflammatory biomarkers, including IL6 (58%; Fig. 2A) protein expression, phosphorylation of NF-κB p65 (Ser536; 42%; Fig. 2B), and STAT3 (Tyr705; 43%; Fig. 2C), as compared with nonsupplemented mice. None of these lycopene-mediated modulations were observed in BCO2-KO mice.

Reduced HCC development by dietary lycopene was associated with attenuated expression of hepatic ER stress markers in BCO2-KO but not wild-type mice

Dietary lycopene significantly suppressed markers of ER^{UPR}-mediated protein kinase RNA-activated like kinase (PERK)–eIF2α signaling in BCO2-KO mice, but not wild-type mice, including the activation of eIF2α by phosphorylation (65%; Fig. 2D), and the expression of CHOP protein (39%; Fig. 2E). Similarly, lycopene supplementation also significantly reduced ER^{UPR}-mediated activation of inositol requiring (IRE) 1α–X-box–binding protein 1 (XBP1) system in BCO2-KO but not wild-type mice, as measured by the splicing of XBP1 mRNA (*Xbp-1s*; 16%; Fig. 2F).

Lycopene supplementation was associated with decreased mTOR activation and protooncogene *Met* expression in BCO2-KO but not wild-type mice

Chronic mTORC1 activation promoted HCC development in mice, through inducing mTOR protein expression and the activation of S6 ribosomal protein by phosphorylation (36). Elevation in protooncogenes *Met* and *β-catenin* is both positively associated with increased hepatocarcinogenesis, partially through promoting cell proliferation (37, 38). Lycopene chemopreventive effects in BCO2-KO mice were associated with reduced *Mtor* mRNA (27%; Fig. 2F), mTOR protein (43%; $P = 0.06$; Fig. 3A), *Met* mRNA (17%; Fig. 2F), cell proliferation marker cyclin D1 protein (44%; Fig. 3B), β-catenin protein (33%; Fig. 3C), but not β-catenin mRNA (*Ctnnb1*; Fig. 2F). In wild-type mice, dietary lycopene also lessened *Mtor* mRNA (20%; Fig. 2F), mTOR protein (42%; Fig. 3A), cyclin D1 protein (44%; Fig. 3B), and β-catenin protein (37%; Fig. 3C) expression. However, lycopene reduced mTOR signaling as measured by S6 ribosomal protein phosphorylation was only observed in BCO2-KO mice (61%, Fig. 3D), but not in wild-type mice. miR199a/b directly targeted *Mtor* and *Met* mRNA, leading to the subsequent downregulation of their protein products (37, 39). miR214 induction was shown to reduce β-catenin protein without altering its mRNA (*Ctnnb1*) expression (38, 40). Decrement in miR199a/b and miR214 has been associated with HCC development (37, 38, 40–42), and linked to ER^{UPR} (41). We observed that the lycopene-mediated reduced ER^{UPR} in BCO2-KO mice coincided with the significant elevation in hepatic miR199a/b (25%; Fig. 3E) and miR214 expression (23%; Fig. 3E), but not in wild-type mice.

Discussion

To the best of our knowledge, the present study provides the first experimental evidence that lycopene supplementation is effective in inhibiting DEN-initiated HCC incidence and multiplicity in two different strains of mice, the BCO2-KO strain and its respective wild-type. The final body weight difference between wild-type and BCO2-KO mice in the present study did not impede the beneficial effects of dietary lycopene against HCC development. This result underscores the potential chemopreventive effects of dietary lycopene against HFD-promoted tumorigenesis in mice, regardless of the amount of body weight gain. Moreover, the hepatic lycopene concentrations in lycopene-supplemented mice (18.2–19.4 nmol/g tissue) were within ranges for humans (0.1–20.7 nmol/g tissue; ref. 43). Therefore, we believe that the lycopene-supplemented dosage used in the present study was physiologically relevant to lycopene biologic effects in human conditions. It should be

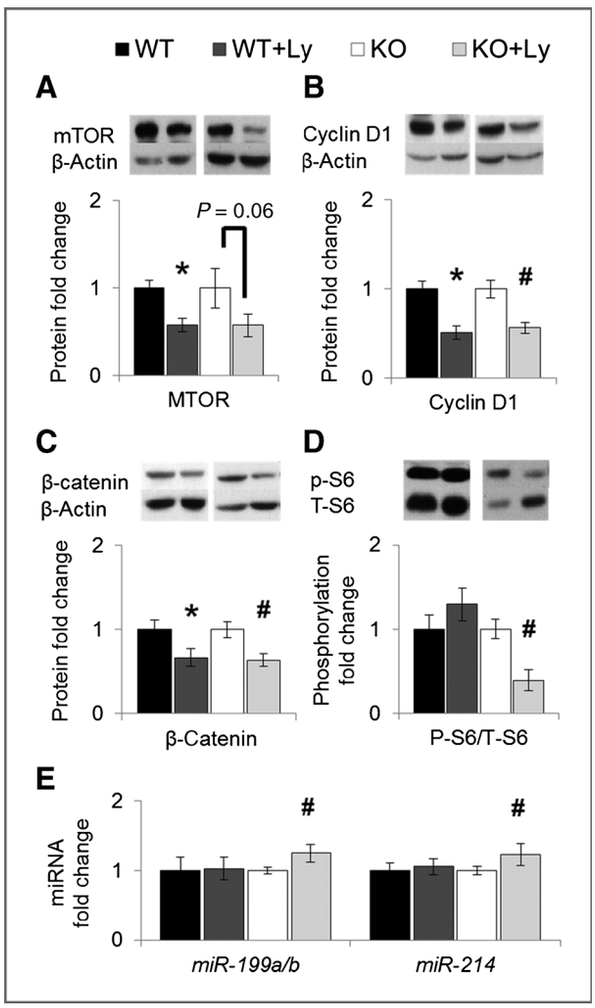

Figure 3. Effects of lycopene supplementation on hepatic tumorigenic biomarkers and miRNA expression. The study design is described in Fig. 1. Protein or miRNA expressions in liver lysates (WT, WT+Ly, KO, KO+Ly $n = 16–20$) were analyzed by Western blotting and β-actin was used as loading control unless specified otherwise. Graphical representation of fold changes in: A, mTOR. B, cyclin D1. C, β-catenin. D, S6 (Ser235/236) phosphorylation (S6 as loading control). E, miR199a/b and miR214 (5S as loading control). Representative Western blots with one sample per group are shown. Fold changes normalized to WT or KO; values are means ± SEMs; *, different from WT; and #, different from KO; $P < 0.05$. KO, knockout on HFD; Ly, lycopene; p-, phosphorylated; T-, total; WT, wild-type on HFD.

pointed out that the *in vivo* study design for the present study was selected to investigate how lycopene can inhibit HFD-promoted hepatic tumorigenesis after the carcinogen initiation (e.g., i.p. injection of DEN to the animals at 2 weeks of age). It was not our intention to evaluate lycopene effects on the initiation stage of hepatocarcinogenesis in this study.

The present study suggests that the molecular mechanisms for lycopene chemopreventive effects may be mouse-strain specific. The lycopene-mediated chemoprevention in wild-type mice was associated with reduced hepatic inflammatory foci, lowered hepatic IL6 protein, as well as with decreased activation of NF-κB p65 (by phosphorylation), and the oncogenic transcription factor STAT3. These lycopene-mediated mechanistic modulations were similar to the chemopreventive effects of the lycopene metabolite APO10LA in C57Bl/6J wild-type mice (24). Intriguingly, our study also revealed that dietary lycopene exhibits chemopreventive effects in the absence of BCO2 expression. In contrast with wild-type mice, lycopene-mediated chemopreventive effects in BCO2-KO mice were associated with reduced ER^{UPR} (IRE1α–XBP1 and PERK–eIF2α) and mTORC1 activation, as well as with suppressed oncogenic *Met* gene and β-catenin protein expression.

Elevated ER^{UPR} is associated with liver cancer development (11). ER^{UPR} consists of three distinct pathways regulated by ER membrane–bound proteins: IRE1α–XBP1 system, PERK–eIF2α signaling, and activating transcription factor (ATF) 6α (9–11). Elevated IRE1α and ATF6α signaling activation in HCC tissue was correlated with increased severity of HCC histologic grading (44), and can induce PERK–eIF2α signaling (44). Induced protooncogene *Met* expression, β-catenin protein and chronic mTORC1 activation through S6 phosphorylation promoted HCC development in mice (36–38). These oncogenic signals can be stimulated by ER^{UPR} through suppressing miR199a/b and miR214 expression (45–48). The miRNA profile or the "miRNome" identified in human liver tumors found that miR199a/b and miR214 are decreased in human HCC (37, 39, 41, 42). Interestingly, we observed in BCO2-KO mice that lycopene-mediated ER^{UPR} inhibition coincided with increased miR199a/b and miR214 expressions. miR199a/b upregulation inhibited proliferation and invasiveness of HCC cell lines (42). miR199a/b can directly degrade protooncogene *Met* and *Mtor* mRNA and reduced their encoded protein products (37, 39), whereas transfection of miR199a/b into HCC-derived cell lines inhibited phosphorylation of S6 (37). Therefore, the present findings suggested that lycopene chemopreventive effects in BCO2-KO mice were associated with reduced mTORC1 activation, potentially through ameliorating ER^{UPR}.

Lycopene supplementation in wild-type mice also reduced mTOR mRNA and protein, but had no effects on S6 phosphorylation. The mTORC1 pathway integrates inputs from at least five cellular and extracellular signaling, and mTORC1 kinase activity can be modulated by modifying mTOR-associated proteins within the mTORC1 complex (49). It is plausible that lycopene supplementation in wild-type mice induced upstream signaling(s) that increased mTORC1 activity and counteracted the suppressive effect on S6 phosphorylation from mTOR protein reduction.

Downregulation of hepatic miR214 was associated with cell growth, cell invasion, stem-like traits, and early recurrence of HCC (38, 40). miR214 overexpression inhibited proliferation of HCC cells *in vitro* (41), reduced HCC tumorigenicity and β-catenin protein *in vivo* (38). We observed in BCO2-KO mice that lycopene-induced hepatic miR214 expression was associated with decreased cell proliferation marker cyclin D1 protein, β-catenin protein but not mRNA. These results from the present study were consistent with previous findings, in which miR214

reduced β-catenin protein via inhibiting β-catenin mRNA translation (38, 40). Future investigation is needed to elucidate how lycopene ameliorated β-catenin and cyclin D1 protein expression in wild-type mice, without altering miR214 expression. It should be noted that lycopene chemopreventive effects may be mediated through multiple mechanisms in addition to those examined in this study. Lycopene is a natural dietary agent that may inhibit tumorigenesis. It may not deliver comparable potent effects as other pharmacologic drugs against tumor progression, as shown by its modest effects on miR199a/b and miR214 induction. During our article preparation, Tan and colleagues (50) showed that lycopene-mediated hepatic gene regulation in mice could be dependent or independent of BCO2 status. The beta-carotene-15,15′-oxygenase (BCO1) is responsible for the central cleavage of carotenoids at the 15,15′ double bond (51–54). It remains controversial to whether lycopene is a potential substrate for BCO1, as numerous studies found no detectable activity of BCO1 towards lycopene (21, 52, 54). Nevertheless, the central cleavage product apo-15-lycopenal (acyclo-retinal) was recently detected when lycopene was incubated in purified recombinant human BCO1 (55), and previously in recombinant murine BCO1 (56). Because we did not see a difference on hepatic lycopene levels between WT and BCO2 KO mice, it is possible that lycopene can be cleaved by both BCO1 and BCO2. Apo-15-lycopenoic acid (acyclo-retinoic acid), an oxidative products of apo-15-lycopenal, is structurally similar to acyclic retinoic acid (21). Treatment with acyclic retinoids was shown to inhibit secondary primary tumors in patients with HCC (57). Further investigations with BCO1/BCO2 double KO mice are currently ongoing in our laboratory to examine whether lycopene metabolites generated by BCO1-mediated cleavage inhibit hepatic tumorigenesis.

We observed greater hepatic all-*trans* lycopene (65%) accumulation in BCO2-KO than WT mice (51%). This observation is different from results published by Tan and colleagues (50), in which they found that BCO2KO mice accumulated marginally higher percentage of *cis* lycopene (68%) than WT mice (63%). The disparities between our results and those published by Tan and colleagues could be due to the difference in lycopene dosage (100 vs. 250 mg/kg diet), length of lycopene supplementation (24 vs. 3 weeks), or the strains of mice selected as the WT (129SvJ/SvEvTac F1 vs. C57BL/6 × 129/SvJ F1). Interestingly, certain members of the carotenoid cleavage enzyme family have intrinsic isomerase activity concurrently with carotenoid cleavage (58–60). For example, BCO1-mediated conversion of 9-*cis*-β-carotene to 9-*cis*-retinal occurred with a suboptimal output, indicating that this enzyme can catalyze *cis* to *trans* isomerization (59). It is plausible that the BCO1 isomerization capacity in our BCO2-KO study mice in conjunction with a long-term lycopene supplementation yielded the observed hepatic lycopene isomers distribution. Future investigation is also required to determine whether BCO2 can function as an isomerase.

In summary, our results demonstrated that lycopene elicited differential mechanism of chemopreventive effects against hepatic tumorigenesis in mice depending on the present or absence of BCO2. The lycopene-mediated chemopreventive effects were associated with reduced hepatic inflammatory responses in wild-type mice, but were associated with inhibition of ER^{UPR} response in BCO2-KO mice. Together with our previous report on APO10LA's efficacy against liver cancer, these findings suggest that both lycopene and lycopene metabolites could be effective dietary agents for preventing liver cancer or reducing cancer risk for patients with NAFLD.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.C. Ip, C. Liu, L.M. Ausman, X.-D. Wang
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