

# Synergic chemoprevention with dietary carbohydrate restriction and supplementation of AMPK-activating phytochemicals: the role of SIRT1

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Calorie restriction or a low-carbohydrate diet (LCD) can increase life span in normal cells while inhibiting carcinogenesis. Various phytochemicals also have calorie restriction-mimetic anticancer properties. We investigated whether an isocaloric carbohydrate-restriction diet and AMP-activated protein kinase (AMPK)-activating phytochemicals induce synergic tumor suppression. We used a mixture of AMPK-activating phytochemical extracts including curcumin, quercetin, catechins, and resveratrol. Survival analysis was carried out in a B16F10 melanoma model fed a control diet (62.14% kcal carbohydrate, 24.65% kcal protein and 13.2% kcal fat), a control diet with multiple phytochemicals (MP), LCD (16.5, 55.2, and 28.3% kcal, respectively), LCD with multiple phytochemicals (LCDmp), a moderate-carbohydrate diet (MCD, 31.9, 62.4, and 5.7% kcal, respectively), or MCD with phytochemicals (MCDmp). Compared with the control group, MP, LCD, or MCD intervention did not produce survival benefit, but LCDmp (22.80 ± 1.58 vs. 28.00 ± 1.64 days,  $P = 0.040$ ) and MCDmp (23.80 ± 1.08 vs. 30.13 ± 2.29 days,  $P = 0.008$ ) increased the median survival time significantly. Suppression of the IGF-1R/PI3K/Akt/mTOR signaling, activation of the AMPK/SIRT1/LKB1 pathway, and NF-κB suppression were the critical tumor-suppression mechanisms. In addition, SIRT1 suppressed proliferation of the B16F10 and A375SM cells under a low-glucose condition. Alterations in histone methylation within *Pten* and *FoxO3a* were observed after the MCDmp intervention. In the

transgenic liver cancer model developed by hydrodynamic transfection of the *HrasG12V* and *shp53*, MCDmp and LCDmp interventions induced significant cancer-prevention effects. Microarray analysis showed that *PPARα* increased with decreased *IL-6* and *NF-κB* within the hepatocytes after an MCDmp intervention. In conclusion, an isocaloric carbohydrate-restriction diet and natural AMPK-activating agents induce synergistic anticancer effects. SIRT1 acts as a tumor suppressor under a low-glucose condition. *European Journal of Cancer Prevention* 25:54–64 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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## Introduction

Calorie restriction (CR), defined as 20–40% restriction of the daily energy intake, extends the longevity of normal cells, but suppresses tumor growth (Colman *et al.*, 2009). Inhibition of the insulin-like growth factor-1 receptor (IGF-1R)/phosphatidylinositol-3-kinase (PI3K)/Akt and mitogen-activated protein kinase pathways have been proposed as underlying tumor-suppression mechanisms

(Dunn *et al.*, 1997; Mjijad *et al.*, 2011). In particular, activation of the AMP-activated protein kinase (AMPK) pathway plays a crucial role through increased cellular NAD<sup>+</sup>/NADH ratio, which in turn activates NAD<sup>+</sup>-dependent class III histone deacetylase, silent mating-type information regulation 2 homolog 1 (SIRT1), and liver kinase B1 (LKB1). This signaling pathway results in a closed-loop AMPK/SIRT1/LKB1 activation process with tumor growth suppression (Lan *et al.*, 2008; Shackelford and Shaw, 2009; Ruderman *et al.*, 2010).

However, CR has major limitations including weight loss, especially in patients with cancer in the advanced stage. Recently, an isocaloric carbohydrate-free ketogenic diet or

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a low-carbohydrate diet (LCD, carbohydrate intake <20% of the daily caloric intake) has been introduced as a supportive dietary intervention (Last and Wilson, 2006; Masko *et al.*, 2010; Ho *et al.*, 2011; Seyfried *et al.*, 2012). These interventions might also produce metabolic disturbances such as ketoacidosis or even weight loss (Bravata *et al.*, 2003). Alternatively, a synthetic antidiabetic AMPK agonist, metformin, has been proposed as a CR-mimetic anticancer agent (Bost *et al.*, 2012).

Various plant-derived bioactive phytochemicals including curcumin, quercetin, (-)-epigallocatechin-3-gallate (EGCG) in green tea extract, and resveratrol are known to be CR-mimetic AMPK activators (Dasgupta and Milbrandt, 2007; Pan *et al.*, 2008; Park *et al.*, 2009; Jung *et al.*, 2010) and SIRT1 activators as well (Chung *et al.*, 2010). Nevertheless, the anticancer effects of a single phytochemical are minimal because of its poor bioavailability, poor intestinal absorption, and rapid metabolism despite strong anticancer activities in-vitro studies. A single agent is only effective at an extremely high-dose oral administration (Scott *et al.*, 2009). In contrast, a mixture of bioactive compounds exerts biological effects at a much lower dose, with synergistic enhancement of each individual compound (Liu, 2004; Ghosh *et al.*, 2009; Ricciardiello *et al.*, 2011).

Previous studies have reported that metformin induced massive apoptosis of cancer cells with glucose restriction, whereas these anticancer effects were attenuated under a high-glucose condition. Synergic tumor growth suppression has also been observed in mice when metformin was used in combination with a glucose uptake inhibitor (Cheong *et al.*, 2011; Menendez *et al.*, 2012). These data suggest that energy restriction in combination with AMPK-activating agents could enhance tumor suppression synergistically.

In this study, we tested whether an isocaloric carbohydrate-restriction diet supplemented with natural AMPK-activating agents would extend the survival time in an animal model using C57BL6 mice bearing B16F10 melanoma. We also explored whether CR-induced SIRT1 activation is oncogenic or acts as a tumor suppressor as the role of SIRT1 in malignant tumors is still controversial (Liu *et al.*, 2009; Herranz and Serrano, 2010; Roth and Chen, 2013). In addition, we evaluated the chemoprevention effects of our dietary modification in a transgenic liver cancer model.

## Materials and methods

### Dietary formulas

The Picolab Rodent Diet 5053 formula (LabDiet, Brentwood, Missouri, USA) consisting of 62.14% kcal carbohydrate, 24.65% kcal protein, and 13.2% kcal fat with 3.07 kcal/g of metabolizable fuel value was used as the control formula. We purchased the TestDiet 9G1X formula (TestDiet, Brentwood, Missouri, USA) consisting

of 16.5% kcal carbohydrate, 55.2% kcal protein, and 28.3% fat with a 4.13 kcal/g fuel value for the LCD intervention. We used green tea, curcumin, quercetin, and resveratrol extracts for CR-mimetic AMPK-activating agents. Approximately 5 mg of each phytochemical extract/g was added to the control and to the LCD formula to prepare the multiple phytochemical (MP) and LCDmp formulas, respectively. We designed a moderate-carbohydrate diet (MCD, <50% of the daily carbohydrate intake) formula consisting of 31.9% kcal carbohydrate, 62.4% kcal protein, and 5.7% kcal fat with 3.22 kcal/g of fuel value, and prepared an MCDmp formula by adding phytochemical extracts.

### Survival analyses in the malignant melanoma model

B16F10 melanoma cells ( $1 \times 10^6$  cells) were injected subcutaneously into the back of 6-week-old C57BL6 male mice (Orient Bio Inc., Seongnam, Korea) after preliminary feeding of each dietary formula *ad libitum* for 1 week. We divided the mice randomly into six groups: control, MP, LCD, LCDmp, MCD, or MCDmp formula ( $n = 15/\text{group}$ ).

We carried out survival analysis using the Kaplan–Meier method, and diet-induced tumor suppression mechanisms were investigated using tumor tissues removed from the control ( $n = 6$ ) and MCDmp ( $n = 5$ ) groups.

### Evaluation of energy-dependent signaling pathways

Western-blot analyses were carried out using antibodies against proteins involved in energy-dependent signaling pathways.

### Evaluation of the role of SIRT1 under a carbohydrate restriction condition

B16F10 mouse or human A375SM melanoma cells were treated with various concentrations of SIRT1 Activator 3 (SA3), which is an allosteric SIRT1 activator (Nayagam *et al.*, 2006), under standard glucose (11 mmol/l) or low-glucose (5.5 mmol/l) conditions in RPMI-1640 medium. A viability assay was performed and we evaluated the protein expression of p-AMPK, SIRT1, acetyl-p53 (Lys 382), acetyl-NF- $\kappa$ B, and nuclear fraction of the NF- $\kappa$ B p65 subunit in the B16F10 melanoma cells cultured in a standard medium or a low-glucose medium (LGM).

### ChIP-on-chip microarray

Chromatin immunoprecipitation (ChIP) assay was performed with the EpiQuik™ ChIP Kit (Epigentek, Brooklyn, New York, USA) using randomly selected melanoma tissue samples ( $n = 3/\text{group}$  from the control and MCDmp groups). Chromatin preparation was performed using an anti-H3K4me3 antibody as H3 methylation precedes acetylation (Wang *et al.*, 2001). An Agilent microarray chip containing 97 651 oligonucleotide probes (Agilent Technology, Palo Alto, California, USA) was used for ChIP-on-chip analysis.

### cDNA microarray

Total RNA fraction was extracted from the melanoma tissues removed from the control ( $n=7$ ) and MCDmp groups ( $n=6$ ), and cDNA microarray was performed.

### Confocal microscopy

Frozen tissue sections from the control and MCDmp groups were prepared using a cryostat (micron HM-525; Thermo Scientific Inc., Pittsburgh, Pennsylvania, USA) at a 6- $\mu$ m thickness. Immunohistochemistry to evaluate cellular polarity and tyrosinase-related protein-1 (TYRP1) was performed.

### Cancer-prevention effects in a transgenic liver cancer model

We evaluated the cancer-prevention effects of each dietary intervention in a transgenic liver cancer model produced by transfection of the *HrasG12V* and *shp53* into C57BL6 male mice as described previously (Ju *et al.*, 2013).

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols of animal experiments were approved by our Institutional Animal Care and Use Committee (IACUC) (Number: 09-199-1 and 2012-0116).

### Statistical analysis

Kaplan–Meier survival data were evaluated using the log-rank (Mantel–Cox) test, and a  $P$  value less than 0.05 was considered to be statistically significant. The statistical analysis of gene expression on cDNA microarray was carried out at the threshold of  $P$  less than 0.05 with more than two fold-change of gene expression. The protein or mRNA expression levels were evaluated as the percentage of control signals (% control).

Detailed materials and methods are described in Supplemental digital content 1.

## Results

### Survival analyses in the B16F10 melanoma model

Supplementation of a mixture of natural AMPK activators under a normal control diet (MP intervention) did not increase the survival time (Fig. 1a), although the mean tumor volume was slightly smaller than that of the control group (Supplemental digital content 2). Smaller tumor volume with a slower tumor growth rate was also observed in both LCD and LCDmp groups (Supplemental digital content 3,  $P<0.05$ ). The median survival time was significantly increased in the LCDmp (Fig. 1b) and the MCDmp groups (Fig. 1c, Supplemental digital content 4) compared with the control group. The MCD intervention did not produce significant survival benefit (Fig. 1c).  $^{18}\text{F}$ -FDG MicroPET imaging showed a smaller tumor size with lower intratumoral  $^{18}\text{F}$ -FDG uptake in the MCDmp group compared with the control group (Fig. 2a). Protein

expression of the hexokinase type 2 (HK2) and pyruvate kinase type M2 (PKM2) within tumor tissues was decreased in the MCDmp group (Fig. 2b).

### Alterations in energy-dependent signaling pathways

Serum glucose level was significantly lower in the MCDmp group ( $106.1\pm 12.19$  mg/dl) than in the control group ( $148\pm 13.36$  mg/dl) ( $P<0.04$ ), as also the serum insulin level ( $0.10\pm 0.02$  and  $0.18\pm 0.02$  ng/ml, respectively,  $P<0.02$ ). Protein levels of the IGF-1R, PI3K, and p-Akt at Thr<sup>308</sup>, phospho-ribosomal protein S6 kinase (p-S6K) at Ser<sup>411</sup>, and the phospho-eukaryotic initiation factor 4E-binding protein 1 (p-4E-BP1) at Thr<sup>37/46</sup> were decreased after the MCDmp intervention (Supplemental digital content 5).

In the MCDmp group, the NAD<sup>+</sup>/NADH ratio was increased (>3-fold difference) (Fig. 2c). The phosphorylated-liver kinase B1 at Ser<sup>428</sup> (p-LKB1-Ser<sup>428</sup>), phosphorylated-AMPK at Thr<sup>172</sup> (p-AMPK-Thr<sup>172</sup>), and SIRT1 were increased independent of nicotinamide phosphoribosyltransferase (NAMPT) expression. NF- $\kappa$ B in the nuclear fraction was decreased (Fig. 2d). On confocal microscopy of melanoma tissues removed from mice, peripheral tumor cells showed restoration of cell polarity (Supplemental digital content 6).

### Effects of SIRT1 on tumor cell proliferation

In-vitro MTT analyses showed that SA3 treatment did not affect cellular proliferation of both B16F10 and human melanoma cells in a standard glucose medium, but decreased proliferation under a low-glucose condition along with increasing p-AMPK in a dose-dependent manner (Fig. 3a, Supplemental digital contents 7 and 8). The level of acetyl-p53 was not decreased in the LGM despite increased SIRT1 expression (Fig. 3b). The NF- $\kappa$ B p65 subunit level in the nucleus fraction was decreased in the LGM and further suppressed after SA3 treatment (Fig. 3b and c).

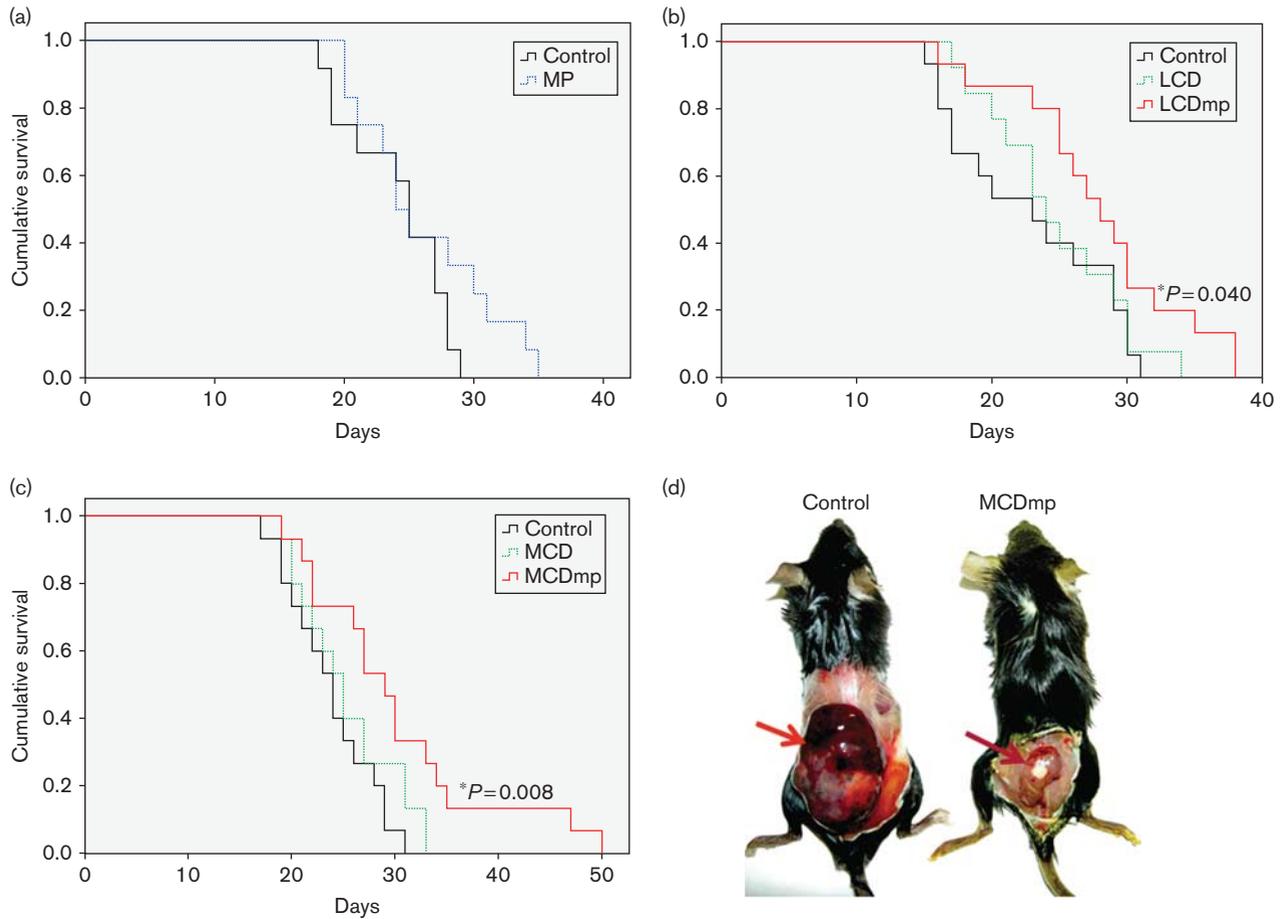
### Histone modification

The ChIP-on-chip study showed alterations in histone methylation (H3K4me3) in the MCDmp group. In particular, H3K4me3 was increased within the promoter region of the *Pten* and *FoxO3a*. ChIP-PCR analysis confirmed increased H3K4me3 within the *Pten* and *FoxO3a* promoter regions (Fig. 4a) and increased expression of these proteins as well as downstream Bim and BAX (Fig. 4b). PTEN, FOXO3a, and Bim were also increased in B16F10 melanoma cells cultured in a LGM (Fig. 4c).

### cDNA microarray results

Forty-three genes were downregulated and two genes were upregulated at the threshold of false discovery rate corrected ( $P<0.05$ ) (Fig. 5a) in melanoma tumor tissues. In particular, *Mif* and *Tyrp1* were decreased, along with decreased vascular endothelial growth factor (VEGF) and DEK oncogene mRNAs. In contrast, expression of the serine peptidase inhibitor F member 1 (*Serpinf1*), which

Fig. 1



Dietary effects on survival. (a) Kaplan–Meier survival analysis showed that AMPK-activating phytochemicals alone do not produce survival benefit in a melanoma model. The median survival time was not significantly increased after the multiple phytochemical (MP) intervention. (b) Survival benefit was not observed after the low-carbohydrate diet (LCD) intervention either. However, LCD supplemented with a mixture of phytochemicals (LCDmp) significantly increased the median survival time. The median survival time of the control, LCD, and LCDmp groups was  $22.80 \pm 1.58$ ,  $24.69 \pm 1.42$ , and  $28.00 \pm 1.64$  days after inoculation of melanoma cells, respectively. (c) The median survival time of the MCDmp group was also significantly increased compared with that of the MCD and control groups ( $30.13 \pm 2.29$ ,  $25.40 \pm 1.24$ , and  $23.80 \pm 1.08$  days, respectively). (d) Tumor size of the MCDmp group was smaller than that of the control group (arrow).

is known as the pigment epithelium derived factor was increased (Supplemental digital content 9). Western-blot analysis confirmed their protein expression patterns (Fig. 5b). Confocal microscopy of tumor tissue samples showed lower TYRP1 expression within the cytoplasm of tumor cells with less pigmentation in the MCDmp group (Fig. 5c and d). Compared with the MCD intervention, MCDmp produced stronger anticancer effects including suppression of the MITF, mTOR signaling, and mitogen-activated protein kinase pathways (Fig. 5e).

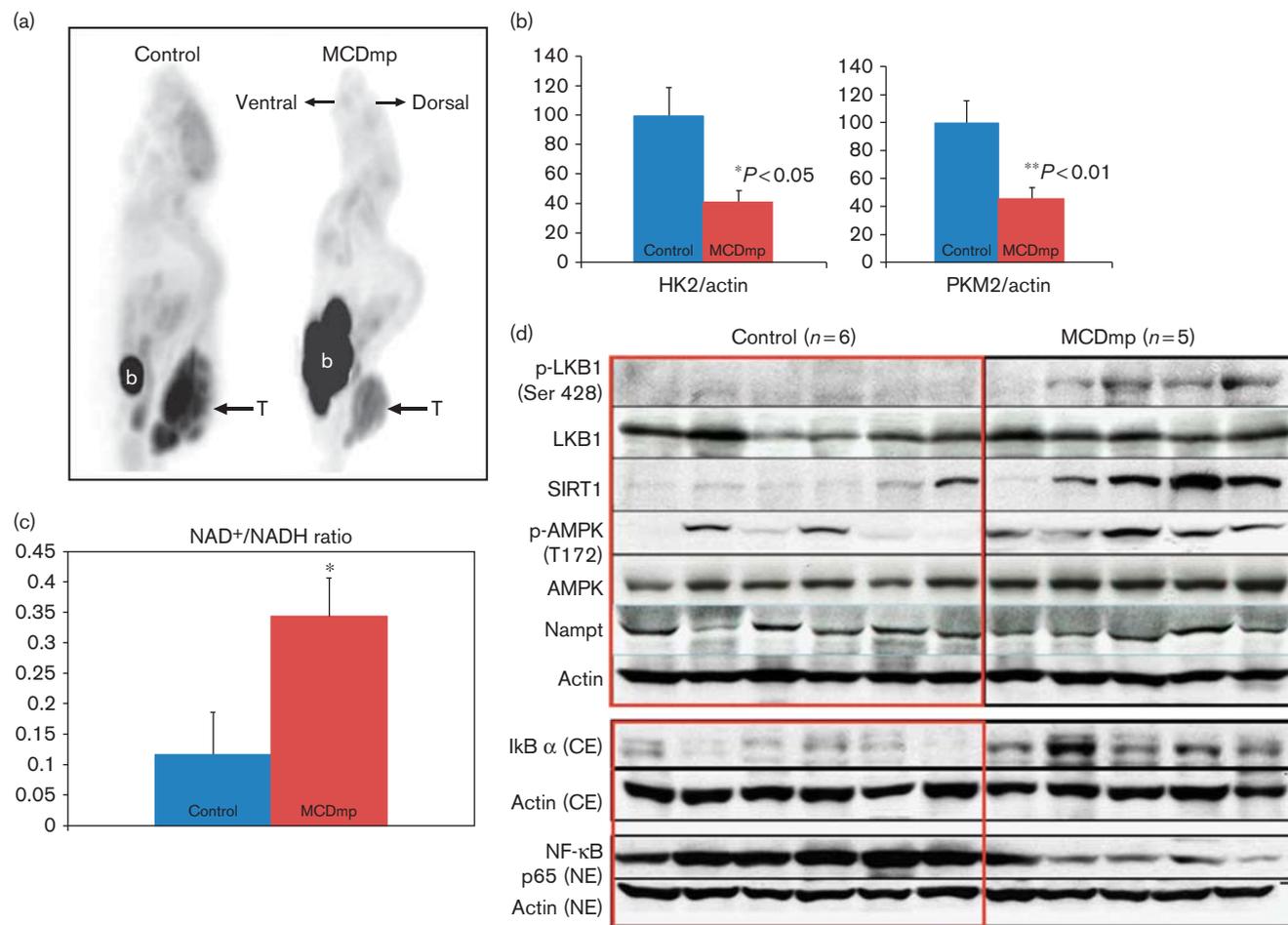
#### Cancer-prevention effects in a transgenic liver cancer model

Numerous tumor nodules developed throughout the entire liver at 4 weeks after transfection of both HrasG12V and shp53 DNAs. The nodules were proven to be poorly differentiated aggressive tumors on H&E staining. Fewer tumor nodules were observed only in the MCDmp and

LCDmp groups compared with the control group (Supplemental digital content 10). There was no significant difference in cancer-prevention effects in the group in which MCDmp was started on the day of DNA transfection (Group 2) however, fewer and smaller tumor nodules developed when the MCDmp intervention was started 2 weeks before DNA transfection (Group 3) (Fig. 6a). Finally, postmortem liver tissues showed fewer tumor nodules in Group 3 (Fig. 6b and c). On Kaplan–Meier survival analysis, the overall survival was significantly prolonged only in Group 3 ( $P < 3.5 \times 10^{-7}$ , Fig. 6d).

cDNA microarray analysis of the tumor-free liver tissues showed upregulation of the peroxisome proliferator-activated receptor  $\alpha$  (*PPAR $\alpha$* ) and downregulation of numerous genes that regulated energy metabolism, tumor initiation, and proliferation in Group 3 (Fig. 7a,

Fig. 2



MCDmp-induced alterations in energy-dependent signaling pathways. (a)  $^{18}\text{F}$ -FDG PET scan shows decreased  $^{18}\text{F}$ -FDG uptake within tumors in the MCDmp group. T, tumor; b, urinary bladder. (b) The expression of the key enzymes for glycolysis (HK2 and PKM2) decreased significantly in the MCDmp group. (c) NAD<sup>+</sup>/NADH ratio was significantly increased (>3-fold) in melanoma tissues removed from the MCDmp group compared with the control group ( $*P = 0.044$ ,  $n = 15$  in each group). (d) Western-blot analysis showed activation of the LKB1/SIRT1/AMPK loop in the MCDmp group. There was no significant alteration in Nampt expression. Therefore, the NAD<sup>+</sup>/NADH ratio could be increased in the MCDmp group, independent of Nampt expression. In addition, the MCDmp intervention suppressed NF- $\kappa$ B p65 expression in the nuclear fraction of cells along with increased cytosolic I $\kappa$ B.

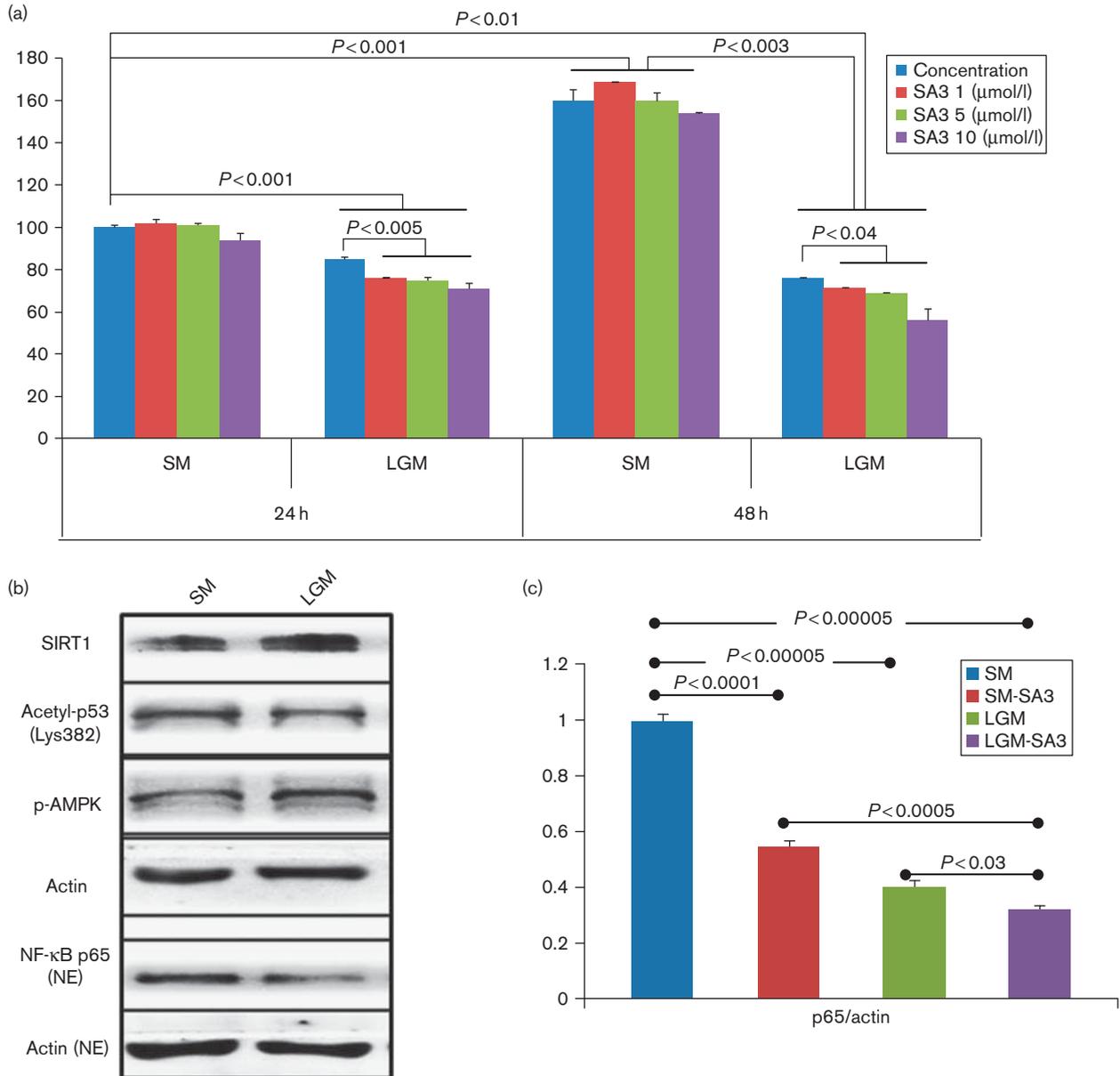
Supplemental digital content 11). Among these, we confirmed decreased mRNA expression of the *IL-6* and *NF- $\kappa$ B* in Group 3 compared with Group 1 using quantitative real-time PCR (Fig. 7b). H&E and TRITC-phalloidin immunostaining of the tumor-free liver tissues obtained 4 weeks after DNA transfection showed distorted lobular arrangement and bile canaliculi in Group 1, whereas normal liver architecture was grossly preserved in Group 3 (Fig. 7c). SIRT1 expression was increased in Group 3 compared with Group 1 (Supplemental digital content 12).

## Discussion

Glucose is a major energy source in both normal and cancer cells. The glycolytic rate is increased in malignant tumors, and the normal metabolic control mechanisms are modified by numerous oncogenes to enhance the glycolytic rate (Levine and Puzio-Kuter, 2010). Our study confirmed that the inhibition of diet-induced hyperinsulinemia and the

IGF-1R/PI3K/Akt pathway along with downregulation of the mTOR signaling could be important tumor-suppression mechanisms of the MCDmp intervention. HK2 and PKM2, which are the key enzymes that increase aerobic glycolysis in cancer cells (Sebastian and Kenkare, 1997; Sun *et al.*, 2011), were also suppressed after the MCDmp intervention. This resulted in reduced glucose uptake with a lower intracellular glucose concentration (Fig. 2a and b), and this process could generate a vicious cycle in tumor cell-proliferation processes. Although amino acids, especially glutamine, can potentiate cellular proliferation, glucose restriction might inhibit glutamine uptake and cellular proliferation despite abundant amino acids (Lopez-Lazaro, 2008; Wellen *et al.*, 2010).

AMPK/SIRT1/LKB1 closed-loop activation after energy restriction plays an important role in tumor suppression. SIRT1 activation might enhance tumor cell proliferation

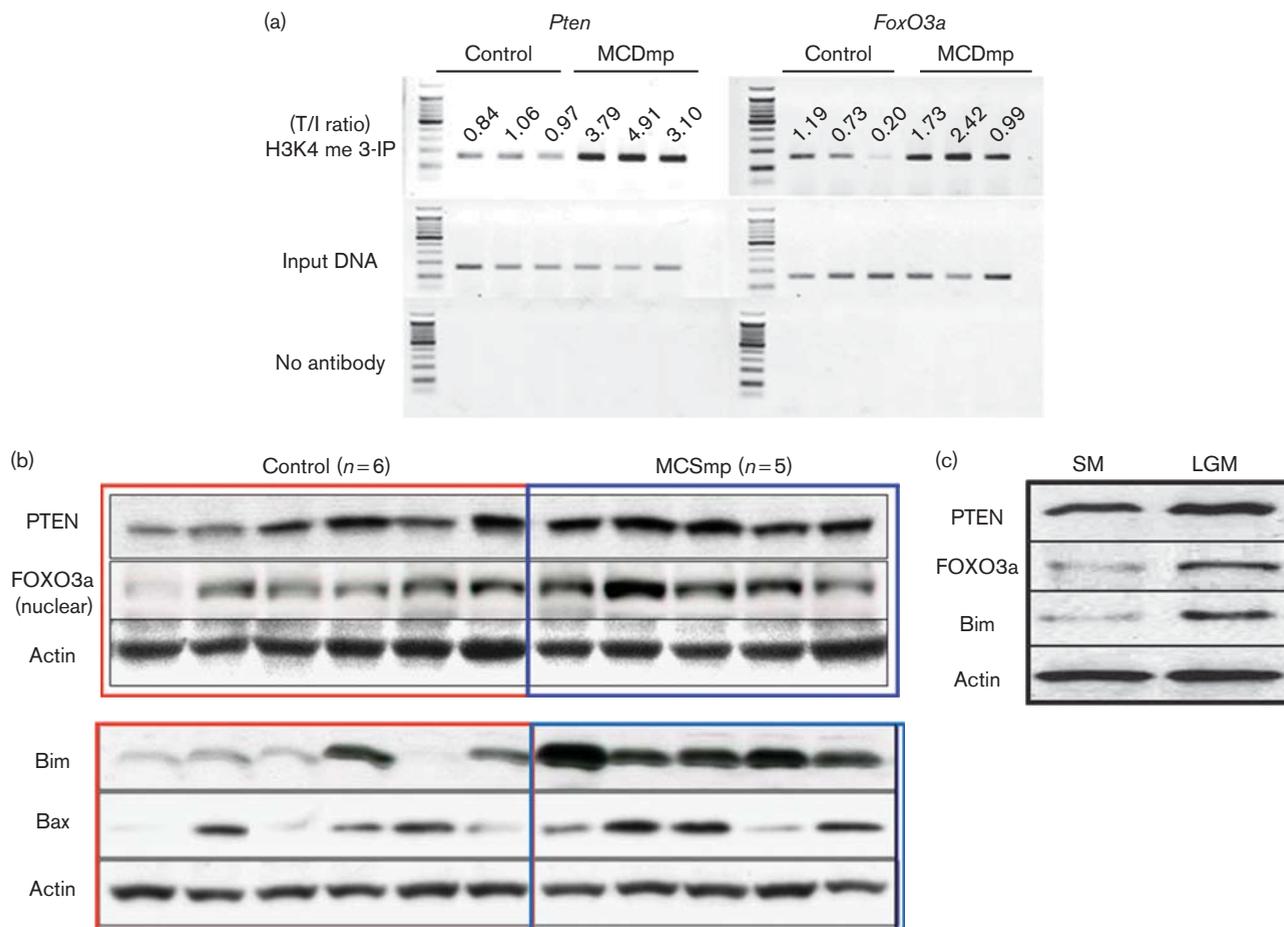
**Fig. 3**


Tumor-suppressive role of SIRT1 under an energy restriction condition. (a) MTT assay of B16F10 melanoma cells cultured in a standard medium (SM, 11 mmol/l of glucose in RPMI-1640 medium) showed no significant cell proliferation at 24 or 48 h after treatment of SA3. However, cell proliferation was significantly suppressed after SA3 treatment when melanoma cells were culture in a low-glucose medium (LGM, 5.5 mmol/l of glucose). (b) SIRT1 and p-AMPK were increased in melanoma cells cultured in an LGM. However, the acetyl-p53 level was not significantly decreased despite an increased SIRT1 level. NF-κB p65 in the nuclear fraction was decreased under a low-glucose condition. (c) NF-κB level was decreased after SA3 treatment under both standard and low-glucose conditions.

through deacetylation of p53; however, AMPK counteracts the deacetylase activity of SIRT1 on p53, but enhances p53 stability (Lee *et al.*, 2012). Our study also showed that the level of acetyl-p53 in B16F10 melanoma cells did not decrease in a LGM even with increased SIRT1 expression. In-vitro study showed that melanoma cell proliferation was suppressed after treatment with an allosteric SIRT1 activator, SA3, along with AMPK activation in a dose-dependent manner when tumor cells were cultured in a

LGM. Melanoma tissues from the MCDmp group showed activation of the AMPK/SIRT1/LKB1 signaling with apparent tumor growth suppression with restored cell polarity compared with the control group. As cell polarity is abolished during the carcinogenesis process (Lee and Vasioukhin, 2008; Luo *et al.*, 2010), our data clearly show that our dietary modification inhibited tumor growth effectively through the activation of AMPK/SIRT1/LKB1 signaling, in particular, SIRT1 activation.

Fig. 4



Epigenetic control of the MCDmp intervention. (a) CHIP-PCR assay of the *Pten* and *FoxO3a* using the anti-H3K4me3 antibody confirmed increased H3K4 methylation after the MCDmp intervention. The numbers indicate the target/input DNA ratios. (b) Western-blot analysis showed increased PTEN and FOXO3a as well as downstream target proteins, Bim and Bax, in the MCDmp group. (c) The low-glucose condition without AMPK-activating agents also induced increased expression of PTEN, FOXO3a, and downstream target Bim.

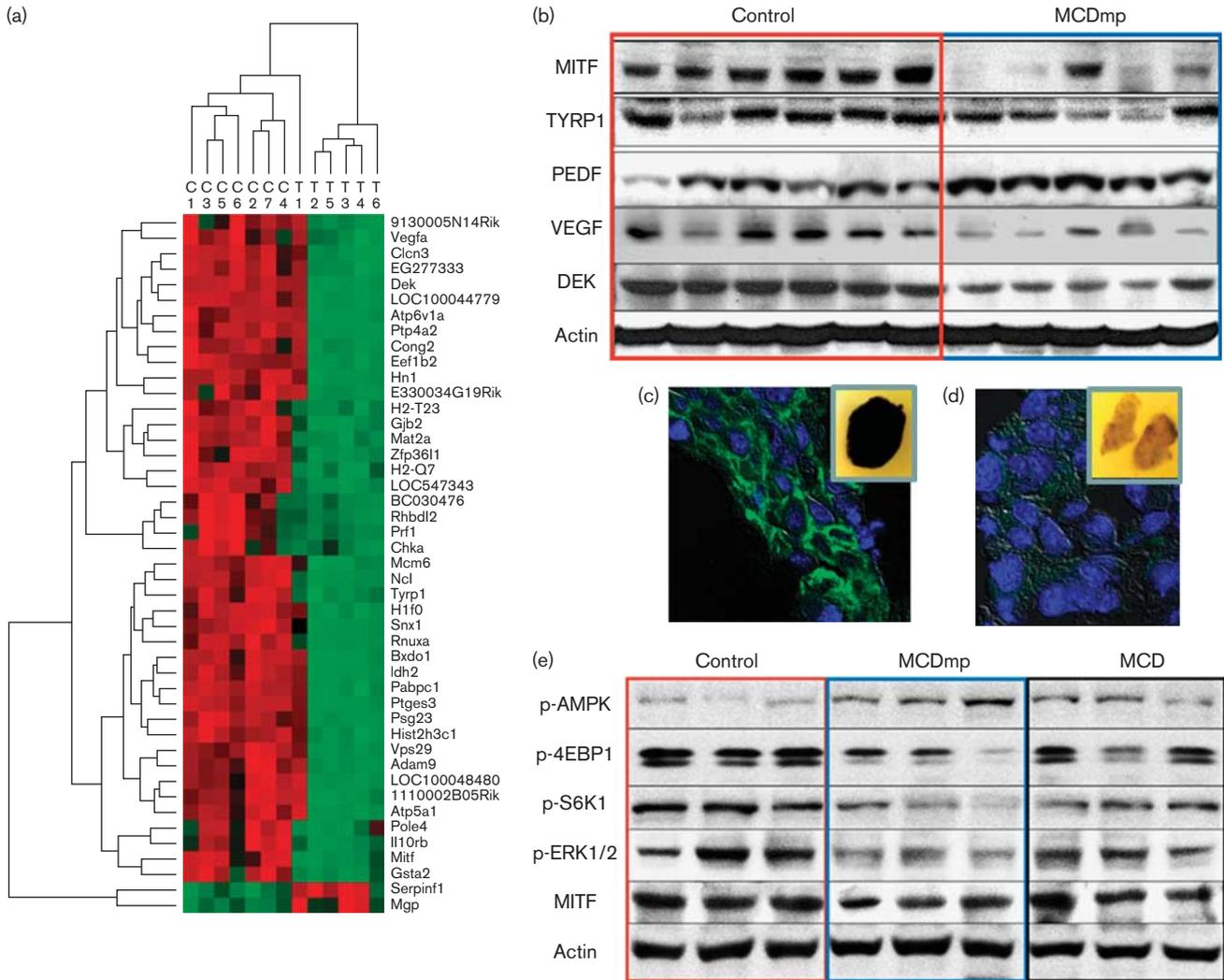
NF- $\kappa$ B is an important downstream target of SIRT1 that deacetylates the RelA/p65 subunit of NF- $\kappa$ B, thereby decreasing NF- $\kappa$ B-induced transcription of the genes encoding antiapoptotic proteins after SIRT1 activation (Yeung *et al.*, 2004). Our study showed decreased NF- $\kappa$ B expression in tumor tissues removed from the MCDmp group as well as in B16F10 melanoma cells cultured in a LGM, especially after the treatment with SA3. Taken together, our data clearly show that energy restriction-induced SIRT1 activation is linked to tumor suppression.

FOXO3a is another target of SIRT1. Transcriptional activity of the FOXO3a can be activated within the nucleus by SIRT1-induced deacetylation of its lysine residues or AMPK-induced phosphorylation (Greer *et al.*, 2007; Canto *et al.*, 2009). Nutrient restriction could further enhance FOXO3a activity through enhanced translocation of the FOXO3a into the nucleus following inhibition of the PI3K/Akt signaling cascade (Calnan and

Brunet, 2008). Nuclear FOXO3a can act as a tumor suppressor after binding to the promoters of various genes including Bim (Czabotar *et al.*, 2009) or removing p53 from the SIRT1-binding sites (Nemoto *et al.*, 2004). In fact, our study showed increased nuclear FOXO3a levels in both melanoma tissues from the MCDmp group and B16F10 melanoma cells cultured in a LGM.

Epigenetic modification of tumor-related genes could be involved in diet-induced tumor suppression. Li *et al.* (2010) have already reported increased longevity in normal cells after glucose restriction, but it resulted in growth inhibition of cancer cells by epigenetic modification. In this study, histone modification of the *Pten* and *FoxO3a* was observed in the MCDmp group. Although carbohydrate restriction alone can increase PTEN and FOXO3a expression, natural AMPK-activating phytochemicals could synergistically enhance tumor-suppression activities through various anticancer activities including histone modification,

Fig. 5



Diet-induced melanoma growth-suppression mechanism. (a) cDNA microarray analysis using melanoma tissues removed from the control and the MCDmp groups showed significant alterations in gene expression. Red represents upregulated mRNAs and green represents downregulated mRNAs. (b) The melanoma-specific proliferation markers, MITF and TYRP1, were decreased in the MCDmp group along with inhibition of VEGF, activation of the endogenous angiogenesis inhibitor (PEDF), and downregulation of the DEK oncogene. (c) Confocal analysis confirmed significantly decreased TYRP1 expression in the MCDmp group (green; anti-TYRP1, blue; DAPI). Inset images represent gross cut-sections of paraffin-embedded tumor tissue blocks showing decreased pigmentation in the MCDmp group. (d) Compared with MCD intervention, the MCDmp group showed higher expression of p-AMPK with suppression of the 4-EBP1, S6K1, ERK1/2, and MITF.

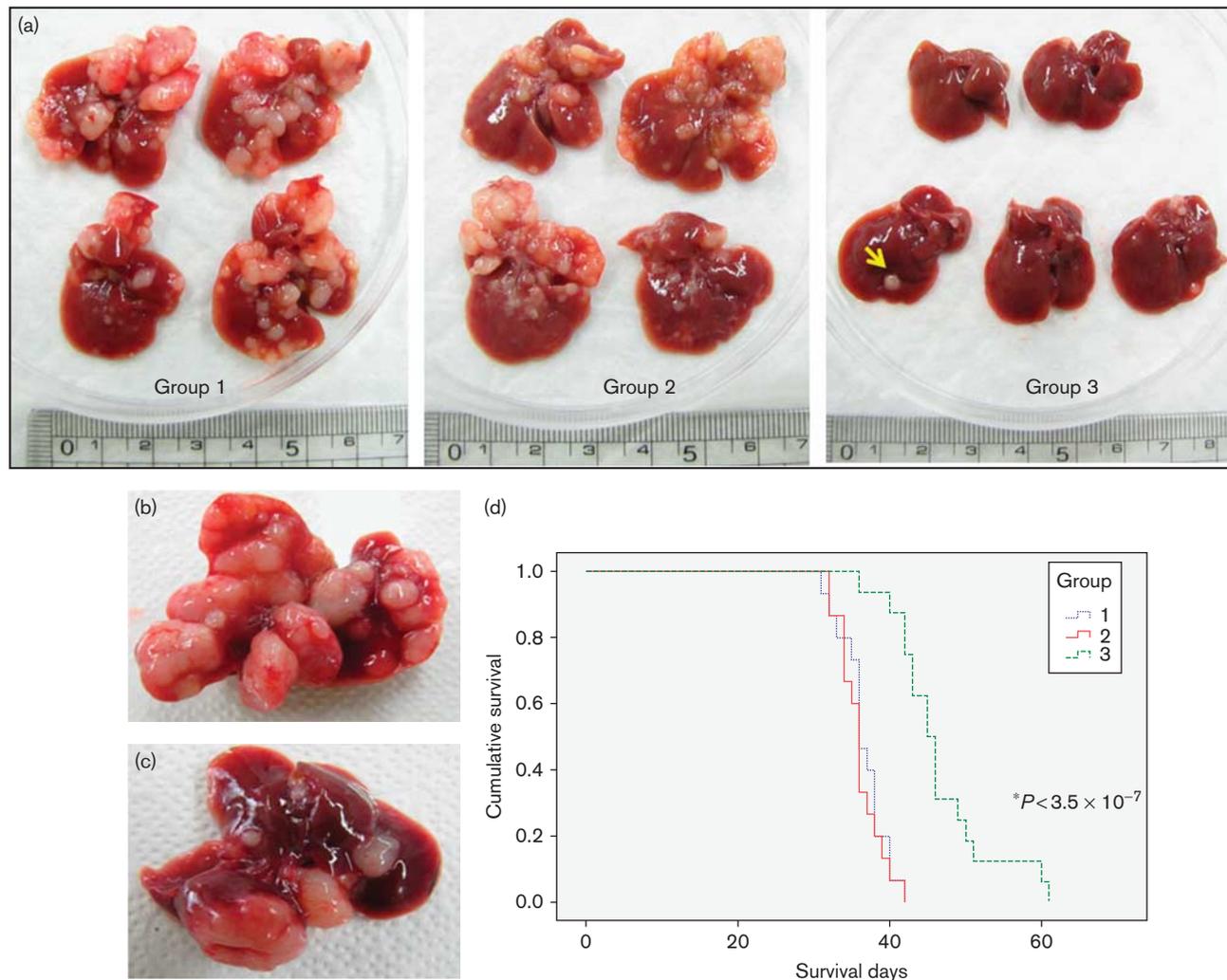
suppression of the energy-dependent signaling cascade, and cancer-related inflammation (Surh, 2003; Link *et al.*, 2010).

cDNA microarray data showed final signaling pathways that are specific for melanoma growth suppression. In particular, MITF, which is the master transcription factor regulating melanocyte differentiation, cell cycle progression, and survival, was suppressed with decreased *Tyrp1*, which encodes tyrosinase interacting protein within melanoma tissue after the MCDmp intervention (Levy *et al.*, 2006). Increased expression of pigment epithelium derived factor, which is an endogenous VEGF inhibitor, along with decreased VEGF, and DEK

oncoprotein suppression could play critical roles in melanoma growth suppression by inhibiting angiogenesis and antiapoptotic activities, respectively (Khodadoust *et al.*, 2009; Konson *et al.*, 2010). Notably, the MCDmp intervention produced stronger anticancer effects than the MCD intervention. Therefore, the AMPK activation induced from energy restriction and natural AMPK-activating agents synergistically inhibit tumor growth, although carbohydrate restriction or phytochemical supplementation alone may not be effective in increasing the survival time.

Dietary modification not only suppresses tumor cell proliferation but also inhibits the development of cancer.

Fig. 6



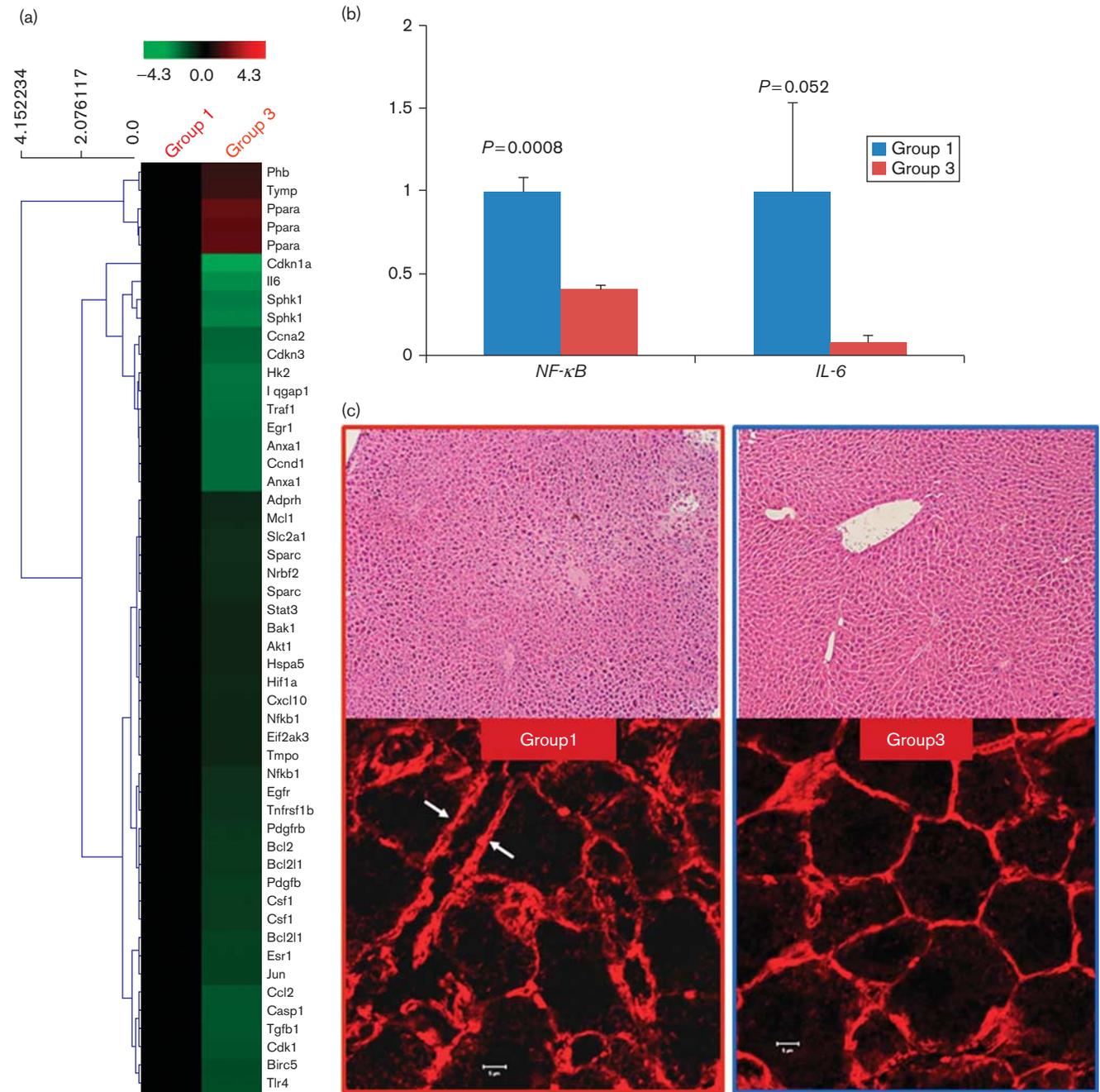
Dietary effects on cancer prevention. (a) Numerous tumor nodules developed within the liver after transfection of the HrasG12V and shP53 genes (Group 1). MCDmp intervention started after DNA transfection did not produce significant tumor-suppression activity (Group 2). However, fewer tumor nodules (arrow) developed when MCDmp was started 2 weeks before DNA transfection (Group 3). Post-mortem liver tissues also showed numerous tumor nodules throughout the liver in Group 1 (b), but fewer tumor nodules in Group 3 (c). (d) Kaplan–Meier survival analysis showed significant survival benefit only in Group 3. The median survival time of Group 1, Group 2, and Group 3 was  $36 \pm 0.77$ ,  $36 \pm 0.45$ , and  $45 \pm 1.20$  days, respectively.

In our transgenic hepatic tumor model, fewer tumor nodules developed after LCDmp and MCDmp interventions. Our data suggest that carbohydrate restriction with AMPK-activating phytochemicals controls malignant transformation of cells synergistically despite cells already acquired oncogenic properties through the suppression of the cancer-related inflammatory process including inhibition of the NF- $\kappa$ B/IL-6/STAT3 pathway (Yang *et al.*, 2010). Increased PPAR $\alpha$  expression could also enhance chemoprevention effects as CR or CR-mimetic phytochemicals might induce PPAR $\alpha$  expression directly or indirectly by AMPK activation and inhibits various transcription factors, particularly NF- $\kappa$ B (Seymour *et al.*, 2010; Peters *et al.*, 2012). Increased SIRT1 activity could negatively regulate NF- $\kappa$ B-dependent STAT3 expression (Bernier *et al.*, 2011). However, carbohydrate restriction or supplementation of AMPK-activating

agents alone did not produce significant cancer-prevention effects. Notably, cancer-prevention effects were not apparent when dietary intervention was started after DNA transfection. This suggests that a diet-induced metabolic reprogramming period is necessary before malignant transformation of cells for cancer prevention. Indeed, previous data showed that the incidence of cancer was significantly low in rhesus monkeys when CR was started at a young age (Mattison *et al.*, 2012).

In conclusion, we confirmed that isocaloric carbohydrate restriction in combination with a mixture of natural AMPK-activating agents could be an effective anticancer approach through synergic activities of each dietary intervention in animal models. SIRT1 enhances tumor suppression under an energy restriction condition.

Fig. 7



MCDmp-induced chemoprevention mechanism. (a) cDNA microarray analysis of the tumor-free liver tissues showed that numerous genes regulating glycolysis, tumorigenesis, and inflammation were downregulated in Group 3. (b) *NF-κB* and *IL-6* expression was markedly decreased in Group 3 on real-time PCR. (c) H&E staining ( $\times 100$ , upper panel) of the tumor-free liver tissues obtained from Group 1 mice at 4 weeks after transfection shows a distorted lobular arrangement, but liver architecture is grossly preserved in Group 3. TRITC-phalloidin immunostaining (lower panel) shows severe dilatation and distortion of the bile canaliculi (arrows) in Group 1. However, the polyhedral architecture of the bile canaliculi is grossly preserved in Group 3. Scale bar = 5  $\mu\text{m}$ .

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## Conflicts of interest

There are no conflicts of interest.

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