




# Macrophage-specific hypoxia-inducible factor-1 $\alpha$ deletion suppresses the development of liver tumors in high-fat diet-fed obese and diabetic mice

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## Keywords

Hypoxia-inducible factor-1 $\alpha$ , Liver cancer, Macrophage

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## ABSTRACT

**Aims/Introduction:** Chronic inflammation of the liver is often observed with obesity or type 2 diabetes. In these pathological conditions, the immunological cells, such as macrophages, play important roles in the development or growth of liver cancer. Recently, it was reported that hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is a key molecule for the acquisition of inflammatory M1 polarity of macrophages. In the present study, we examined the effects of altered macrophage polarity on obesity- and diabetes-associated liver cancer using macrophage-specific HIF-1 $\alpha$  knockout (KO) mice.

**Materials and Methods:** To induce liver cancer in the mice, diethylnitrosamine, a chemical carcinogen, was used. Both KO mice and wild-type littermates were fed either a high-fat diet (HFD) or normal chow. They were mainly analyzed 6 months after HFD feeding.

**Results:** Development of liver cancer after HFD feeding was 45% less in KO mice than in wild-type littermates mice. Phosphorylation of extracellular signal-regulated kinase 2 was also lower in the liver of KO mice. Those effects of HIF-1 $\alpha$  deletion in macrophages were not observed in normal chow-fed mice. Furthermore, the size of liver tumors did not differ between KO and wild-type littermates mice, even those on a HFD. These results suggest that the activation of macrophage HIF-1 $\alpha$  by HFD is involved not in the growth, but in the development of liver cancer with the enhanced oncogenic extracellular signal-regulated kinase 2 signaling in hepatocytes.

**Conclusions:** The activation of macrophage HIF-1 $\alpha$  might play important roles in the development of liver cancer associated with diet-induced obesity and diabetes.

## INTRODUCTION

Liver cancer is one of the representative cancers whose incidence increases in patients suffering from metabolic diseases, such as obesity and type 2 diabetes<sup>1–3</sup>. The development of liver cancer is considered to be related to chronic inflammation. For example, it develops more frequently in patients with chronic hepatitis or liver cirrhosis induced by hepatitis virus infection<sup>4,5</sup>. Recently, Karin's group showed that the activation

of tumor necrosis factor or interleukin (IL)-6 signaling is involved in the development of obesity- and diabetes-associated liver cancers using mouse models totally lacking these cytokines<sup>6</sup>. In addition, Ohtani *et al.* reported that enhanced IL-6 signaling in stellate cells also plays an important role for obesity- and diabetes-induced liver cancer. Alteration of intestinal bacteria flora by diet-induced obesity and diabetes increases the production of deoxycholate, which is involved in enhanced IL-6 signaling in stellate cells<sup>7</sup>. These results suggest that obesity- and diabetes-associated alterations in the environmental factors possibly affect the functions not only in hepatocytes,

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parenchymal cells in the liver, but also in the stromal cells, thus indirectly increasing the incidence of liver cancer. However, the indirect effects of the environmental factors on stromal cells are less understood than the direct effects on hepatocytes<sup>8</sup>.

Among the stromal cells that are able to affect the functions of cancer, most studies focus on the roles of tumor-associated macrophages (TAMs) existing inside and outside of the cancers. For example, when a large number of TAMs exist in liver cancer, the prognosis of the patients becomes worse<sup>9,10</sup>, suggesting that TAMs possibly affect the development and/or growth of liver cancers. Because macrophages have great variety and plasticity in general, the characteristics of macrophages are expressed between the two polarities of classically activated M1 and alternatively activated M2. Recent studies have presented a simple model of the functional relationship between TAMs and cancer; that is, TAMs show an inflammatory M1 polarity, and they work against the development and growth of cancers in the earlier phases of the carcinogenic process. Then, TAMs develop an anti-inflammatory M2 polarity, and they stimulate the growth and/or metastasis of the cancer in the middle or later phases of the carcinogenesis<sup>11,12</sup>.

Recently, Takeda *et al.*<sup>13</sup> reported that the activation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in macrophages plays a critical role in the induction of M1 polarity, whereas HIF-2 $\alpha$  is important for the acquisition of M2 polarity. We have recently reported that adipose tissue hypoxia observed in obese mice is associated with the induction of M1 polarity of adipose tissue macrophages by activating the HIF-1 $\alpha$  of the cells<sup>14,15</sup>. Because cancers also increase their volume very rapidly and pathologically as adipose tissue of obese animals does, we hypothesized that HIF-1 $\alpha$  in TAMs existing inside or outside of liver cancer might play some role in the induction of macrophage polarity and the development or growth of the cancer.

In the present study, we used diethylnitrosamine (DEN), a chemical carcinogen, to induce liver cancer. Because the incidence of DEN-induced liver cancer in mice is reported to be increased by high-fat diet (HFD)-induced obesity and diabetes<sup>6</sup>, we considered it to be a good model for investigating the mechanisms for obesity- and diabetes-associated carcinogenesis. Using this carcinogenic method, we analyzed macrophage-specific HIF-1 $\alpha$  knockout (KO) mice, in which we recently reported that the deletion efficiency of HIF-1 $\alpha$  protein in myeloid cells was >90%<sup>14,15</sup>. Here, we show that HIF-1 $\alpha$  in macrophages plays an important role in the enhanced development of liver cancer associated with diet-induced obesity and diabetes.

## METHODS

### Generation and maintenance of mice

Homozygous mouse with HIF-1 $\alpha$ -deficient monocytes and macrophages (HIF-1 $\alpha$ <sup>flox/flox</sup>/LysM-Cre; KO) was generated by crossing a C57BL/6J mouse containing loxP sequences on either side of the *Hif1 $\alpha$*  gene with a C57BL/6J mouse expressing Cre recombinase under the lysozyme M (LysM) promoter as

described previously<sup>15,16</sup>. Mice containing the floxed *Hif1 $\alpha$*  allele, which did not express the Cre recombinase (HIF-1 $\alpha$ <sup>flox/flox</sup>), were used as the control. Liver tumor was induced by injecting 15-day-old male mice with 25 mg/kg of DEN (Sigma, St. Louis, MO, USA) intraperitoneally. KO mice and control mice were fed either a normal chow diet (NC) or a HFD containing 60% of its calories from fat (Research Diets Inc., New Brunswick, NJ, USA) for 6 or 8 months (Figure S1). The animal care policies and procedures/protocol used in the experiments were approved by the Animal Experiment Ethics Committee of the University of Toyama (Toyama, Japan).

### Liver tumor analysis

After the liver was removed from the mice, the number of tumors appearing on the surface of the liver that were >1 mm in diameter was counted. The tumor size was determined as the maximal axis of each tumor.

### Magnetic activated cell sorting and quantitative real-time polymerase chain reaction

Magnetic activated cell sorting of liver macrophages was carried out as previously described<sup>14,15,17</sup>, with some modifications. Briefly, liver samples were minced and filtered by using a 40- $\mu$ m nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) to remove cell clusters and debris. After counting cell numbers, F4/80-positive fraction was separated using a MicroBeads Kit with anti-F4/80 microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA, USA). After extracting total ribonucleic acid from F4/80-positive liver macrophages, gene expression was analyzed by using the Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) as previously described<sup>14,15,17</sup>. Messenger ribonucleic acid expressions of the genes were calculated relative to 18S, and they were presented as values relative to those for control mice.

### Immunoblotting

Immunoblotting of liver samples was carried out as described previously<sup>14,15</sup>. Briefly, liver samples were frozen in liquid nitrogen and preserved until they were used for immunoblotting. Tissues were homogenized in lysis buffer with 1% Nonidet P-40 using a Multi-Beads Shocker™ (Yasui Kikai, Osaka-city, Osaka, Japan) cell disrupter. Cell lysate was run on 7.5% separating gel and transferred to polyvinylidene fluoride transfer membrane. Membranes were incubated with anti-extracellular signal-regulated kinase 2 (ERK2), anti-phosphorylated ERK2 and anti- $\beta$ -actin antibodies (Cell Signaling Technology, Danvers, MA, USA). The intensities of the bands were quantified using a LumiVision Analyzer (Aisin, Kariya, Japan).

### Hypoxia probe administration and immunohistochemistry

Immunohistochemistry for the hypoxia probe was carried out using the Hypoxyprobe-1 plus kit (Hypoxyprobe, Burlington, MA, USA) as we described previously<sup>13,14</sup>. Pimonidazole, a hypoxia probe, was intraperitoneally injected at a dose of

60 mg/kg bodyweight at 30 min before tissue collection. Liver samples were processed as histological sections and immunohistochemical analysis with anti-pimonidazole or anti-MAC2 antibody as previously described<sup>14,15,17,18</sup>.

**Statistical analysis**

The statistical analysis was carried out using the Student's *t*-test or a two-factor ANOVA and post-Tukey–Kramer test. Differences were considered statistically significant at *P* < 0.05. The results were presented as the mean  $\pm$  standard error of the mean.

**RESULTS**

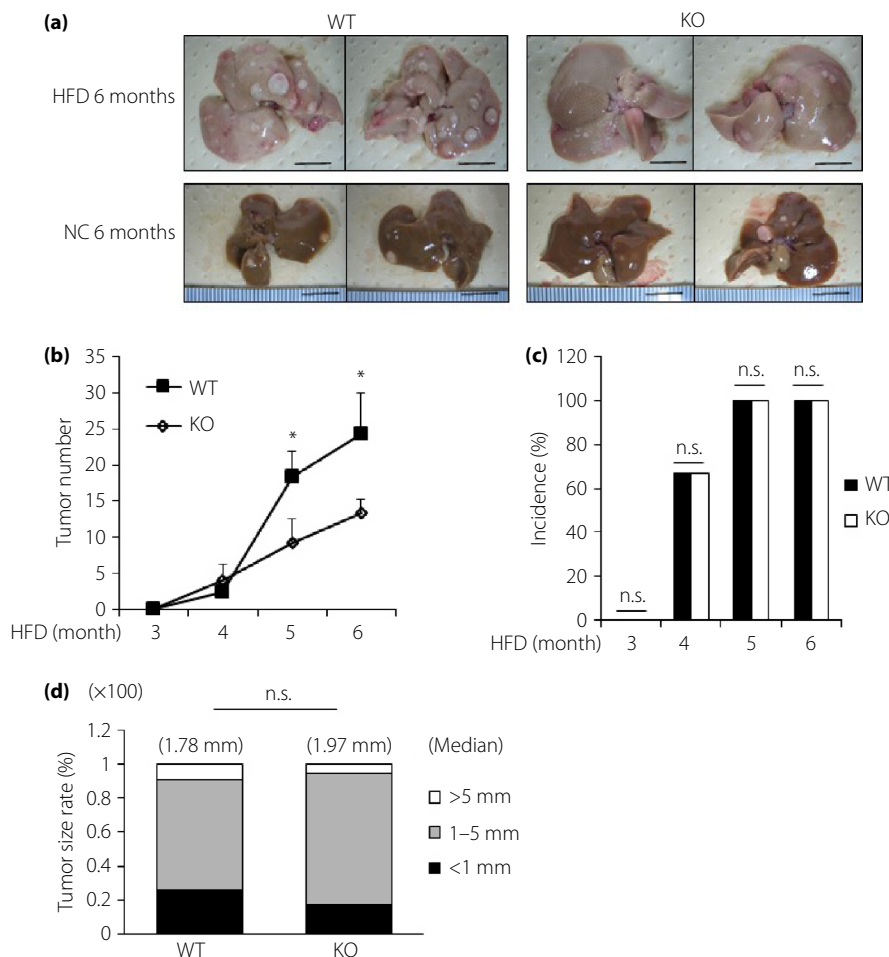
**Myeloid cell-specific HIF-1 $\alpha$  deletion did not affect bodyweight and liver weight**

In the present study, we generated a DEN-induced liver tumor model using myeloid cell-specific HIF-1 $\alpha$  KO mice and their

wild-type littermates (WT) to clarify how HIF-1 $\alpha$  in macrophages affects the development of liver cancer. Both KO mice and WT were fed either HFD or NC for 6 or 8 months from 1.5 months-of-age (Figure S1). Myeloid cell-specific HIF-1 $\alpha$  deletion did not affect bodyweight, liver weight or fasting blood glucose levels both in NC-fed and HFD-fed mice during the time periods (Figure S2).

**Myeloid cell-specific HIF-1 $\alpha$  deletion decreased the number of liver tumors only in HFD-fed mice**

HFD treatment for up to 6 months increased the number, incidence and size of DEN-induced liver tumors compared with NC-fed WT mice (Figure 1a). Myeloid cell-specific HIF-1 $\alpha$  deletion decreased the number of liver tumors (Figure 1a,b), whereas it did not affect the incidence and size of the tumors in HFD-fed mice (Figure 1a,c,d; and Figure S3). Interestingly,

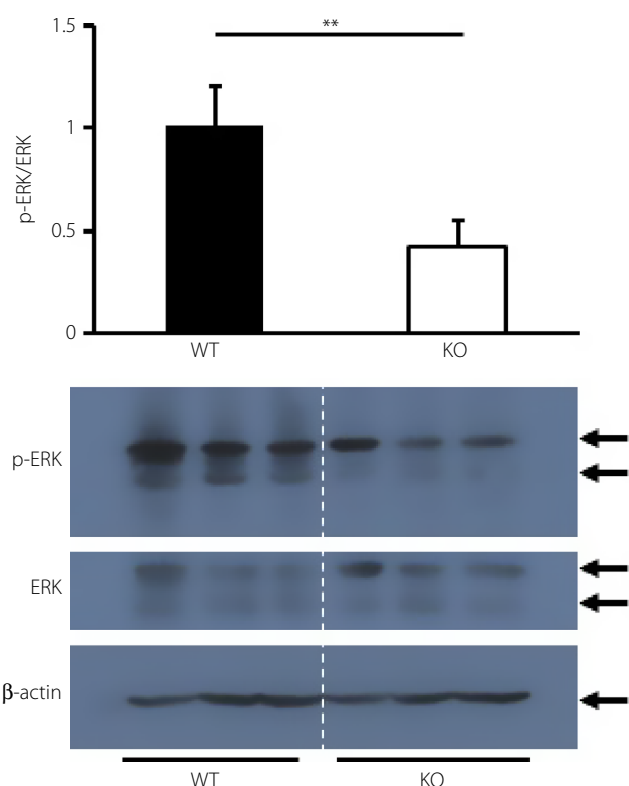


**Figure 1** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  deletion decreased the number of liver tumors only in high-fat diet-fed (HFD) mice. Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  knockout mice (KO) and wild-type littermates (WT) were fed a HFD or normal chow (NC). (a) Representative pictures of the liver from WT and KO mice 6 months after HFD or NC feeding. Scale bar, 1 cm. (b, c) The (b) number and (c) incidence of the tumors were measured after the indicated months of HFD feeding. Open diamond or open column stands for KO, whereas closed square or closed column for WT; *n* = 8. The results are shown as the mean  $\pm$  standard error of the mean. (d) The tumor size was measured 6 months after HFD feeding; *n* = 8. \**P* < 0.05; n.s., not significant compared with the WT control.

when mice were fed NC, there were no differences in the number, as well as the incidence and size of liver tumors, between KO and WT mice (Figure 1a; Figure S4). These results suggest that myeloid cell HIF-1 $\alpha$ , which is possibly activated by HFD treatment, is involved not in the growth, but in the development of liver tumors.

### Myeloid cell-specific HIF-1 $\alpha$ deletion decreased ERK phosphorylation in the liver of HFD-fed mice

To investigate the mechanisms of how HIF-1 $\alpha$  deletion in myeloid cells decreased the development of tumors in HFD-fed mice, we first examined the phosphorylation of ERK in the liver, the activation of which was reported to be critical for DEN-induced carcinogenesis<sup>19</sup>. As expected, phosphorylation of ERK was significantly decreased in KO mice by ~60% compared with WT controls (Figure 2). These results showed that myeloid cell-specific HIF-1 $\alpha$  deletion decreased the development of tumors with suppressing the ERK pathway in the liver of HFD-fed mice.



**Figure 2** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  deletion decreased extracellular signal-regulated kinase (ERK) phosphorylation in the liver of high-fat diet-fed (HFD) mice. After HFD treatment for 6 months, immunoblot analysis was carried out using liver samples from wild-type (WT) and knockout (KO) mice with antibodies against phosphorylated ERK (p-ERK), ERK and  $\beta$ -actin. Representative pictures are shown. The intensities of the bands were quantified using a LumiVision Analyzer. The results are shown as the mean  $\pm$  standard error of the mean;  $n = 4$ . \*\* $P < 0.01$  compared with WT mice.

### Expression of both M1 and M2 marker genes was decreased in HIF-1 $\alpha$ -deleted macrophages in the liver of HFD-fed mice

To understand the mechanisms of how HIF-1 $\alpha$  deletion in myeloid cells decreased the phosphorylation of ERK (Figure 2) and the development of liver cancers (Figure 1), we next examined the polarity of liver macrophages. Macrophages in the liver were separated from the HFD-fed mice. The expressions of HIF-1 $\alpha$  and its downstream genes, such as *glut1* and *VEGFA*, were significantly suppressed with 30~40% residual expression of the WT levels in the KO group (Figure 3a). The expression of M1 macrophage markers, such as CD11c and IL-1 $\beta$ , was inhibited by myeloid cell-specific HIF-1 $\alpha$  deletion in the liver, too (Figure 3b). Interestingly, HIF-1 $\alpha$  deletion also decreased the expression of CD206, a representative M2 macrophage marker (Figure 3c). Some of these marker genes are possibly related to the activation of mitogenic signaling and tumor development in the liver.

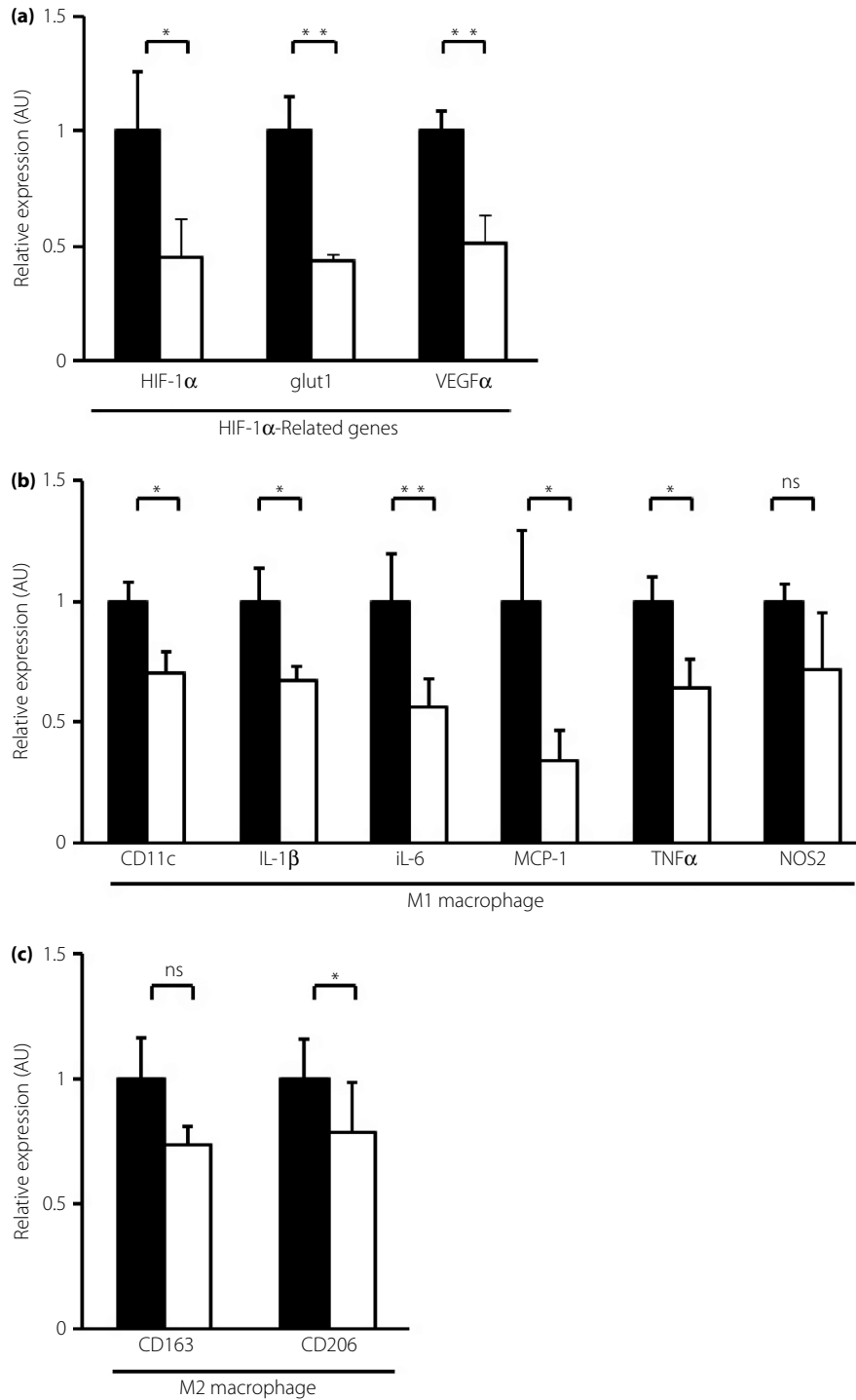
### HIF-1 $\alpha$ in macrophages was not activated by hypoxia in the liver of HFD-fed mice

To examine whether macrophage HIF-1 $\alpha$  was activated by hypoxia in the liver of HFD-fed mice, hypoxia in liver tissue was evaluated by using antibody against pimonidazole, a hypoxic probe. Although large numbers of macrophages were detected with anti-MAC2 antibody both inside and outside of the tumors, only a few cells were faintly stained with anti-pimonidazole antibody in the liver of HFD-fed mice (Figure 4; arrow head). These results suggest that liver macrophages are not exposed to the hypoxic environment, and that HIF-1 $\alpha$  in macrophages might be activated by some other stimuli than hypoxia.

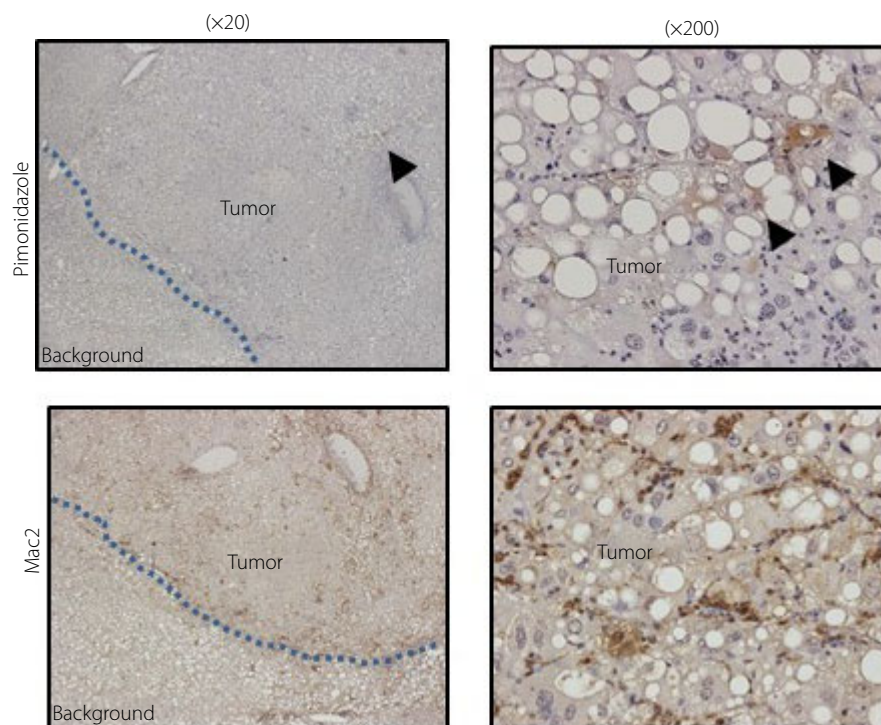
It was recently reported that HIF-1 $\alpha$  protein is stabilized when the cells are stimulated with lipopolysaccharide even in the normoxic condition with accumulating succinate in the cells<sup>20-22</sup>. Contrary to our speculation, succinate was not increased in the liver macrophages in HFD-fed mice, suggesting that the accumulation of succinate is not the mechanism for HIF-1 $\alpha$  activation (Figure S5).

## DISCUSSION

The present study showed that myeloid cell-specific HIF-1 $\alpha$  deletion decreased the number of liver tumors and ERK phosphorylation not in NC-fed mice, but in HFD-fed mice. These results suggest that the activation of macrophage HIF-1 $\alpha$  is involved in those carcinogenic changes in the liver only of obese and diabetic mice, thus, it is very important to clarify the mechanisms of how HFD activates HIF-1 $\alpha$  in myeloid cells. Recently, we reported that inflammatory M1 macrophages are exposed to the most hypoxic area, and they produce inflammatory cytokines in the adipose tissue of HFD-fed obese and diabetic mice<sup>14,15,23</sup>. In the present study, numbers of macrophages invaded both inside and outside of liver cancers after the same HFD treatment as that used in our recent studies (Figure 4)<sup>14,15,23</sup>. Because of the rapid growth and inappropriate



**Figure 3** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) deletion decreased the expression of M1 and M2 marker genes in the liver macrophages of high-fat diet-fed (HFD) mice. After HFD treatment for 6 months, macrophages in the liver were separated from wild-type (WT) and knockout (KO) mice by using magnetic microbeads with anti-F4/80 antibody. Total ribonucleic acid was isolated from the liver macrophages, and real time reverse transcription polymerase chain reaction for the indicated genes was carried out. (a) HIF-1 $\alpha$ -related genes, (b) M1 macrophage marker genes and (c) M2 macrophage marker genes. All data were normalized according to 18S messenger ribonucleic acid level, and presented as a value relative to that for WT. The results are shown as mean  $\pm$  standard error of the mean for five to eight mice per group. \* $P < 0.05$ , \*\* $P < 0.01$ . Glut1, glucose transporter-1; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NOS2, nitric oxide synthase 2; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.



**Figure 4** | Macrophages in the liver of high-fat diet-fed mice was not stained with anti-pimonidazole antibody. After high-fat diet treatment for 6 months, liver tissues of wild type mice were stained with anti-pimonidazole or anti-Mac2 antibody. Representative pictures are shown from four independent experiments.

vasculature, cancers also easily suffer from hypoxia, as well as an expanding white adipose tissue<sup>24,25</sup>. Thus, we originally speculated that HIF-1 $\alpha$  in macrophages at the cancer site was activated by local hypoxia. However, in contrast to the M1 adipose tissue macrophages in white adipose tissue, almost no staining of pimonidazole, a hypoxia probe, could be detected in the macrophages in the liver of HFD-fed mice (Figure 4). These results suggest that HIF-1 $\alpha$  in the macrophages is activated by any mechanisms other than local hypoxia in the liver.

Recent studies have shown that HIF-1 $\alpha$  protein can keep its transcriptional activity even in the normoxic condition when the cells are treated with certain stimuli. For example, when macrophages are stimulated with lipopolysaccharide, some metabolites in the tricarboxylic acid cycle, such as succinate, are accumulated in the cells, which stabilize HIF-1 $\alpha$  protein<sup>20–22</sup>. Thus, we compared the metabolite levels in liver macrophages between NC-fed and HFD-fed mice. Contrary to our speculation, succinate was not increased in the liver macrophages in HFD-fed mice (Figure S5). In this way, the present study could not show the mechanisms of how HIF-1 $\alpha$  in the liver macrophages was activated in HFD-fed mice. This is an important limitation of the present study, and further studies are required to clarify the mechanism that might be different from local hypoxia and altered intracellular metabolites.

As shown in Figures 2 and 3, phosphorylation of ERK2, a well-known growth signal, in the liver (Figure 2), and M1 and

M2 markers in macrophages (Figure 3) was decreased in KO mice after HFD treatment. Interestingly, the number of macrophages in the liver was not significantly different between WT and KO (data not shown). Therefore, we originally hypothesized that any factors, which are secreted from macrophages in a HIF-1 $\alpha$ -dependent manner, induced the development of liver cancer by activating the ERK2 signal in hepatocytes. For example, because some growth factors, such as epidermal growth factor<sup>26,27</sup>, platelet-derived growth factor<sup>28,29</sup> and vascular endothelial growth factor<sup>30–32</sup>, are reported to be associated with the development of liver cancer, we measured their expressions in the liver of KO mice. However, the expressions of these growth factors were not suppressed in KO compared with WT controls (data not shown). Furthermore, deletion of HIF-1 $\alpha$  did not alter insulin levels in fasting conditions (Figure 1), when we examined the phosphorylation of ERK2 (Figure 2). Recently, Ohtani *et al.*<sup>7</sup> reported that inflammatory signaling, such as IL-6 signaling in stellate cells, plays important roles in the occurrence of liver tumor in obese mouse models. We also observed that the expression of inflammatory cytokines was suppressed in the liver macrophages derived from KO mice (Figure 3). From these results, we speculate that the inflammatory cytokines from liver macrophages might be involved in the increased development of liver cancer.

In contrast, it is well-known that the alternative activation of TAMs to M2 polarity is associated with growth, survival,

invasion and/or metastasis of cancers. However, it is not fully understood whether M2 polarity of TAMs is associated with the development of cancers. Generally, M2 polarity of TAMs is acquired following the environmental changes after growing of cancers. In fact, the sizes (growth) of cancers were not altered in KO mice, when M2 markers were decreased in the present study. We believe that the contribution of M2 polarity is less than that of M1 polarity as the mechanism for carcinogenesis, at least in the case of this study.

In the present study, we showed that HIF-1 $\alpha$  in macrophages is involved in the activation of growth signaling in hepatocytes and the development of liver cancer in HFD-fed mice. Activation of HIF-1 $\alpha$  and the acquisition of inflammatory polarity of the macrophages were induced by factors other than local hypoxia in the liver of the obese and diabetic mice. These results raise a possibility that macrophage HIF-1 $\alpha$  might be a target of prevention or treatment of obesity and diabetes-associated liver cancer.

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### DISCLOSURE

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** | Experimental protocol.

**Figure S2** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) deletion did not affect bodyweight, liver weight and fasting blood glucose level in high-fat diet-fed mice.

**Figure S3** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) deletion did not affect the size of liver tumors in high-fat diet-fed mice.

**Figure S4** | Myeloid cell-specific hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) deletion did not affect the number, incidence and size of liver tumors in normal chow-fed mice.

**Figure S5** | High-fat diet did not increase succinate accumulation in liver macrophages.