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Fatty acid binding protein-4 (FABP4) is a hypoxia inducible gene that sensitizes mice to liver ischemia/re-perfusion injury

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Abstract

Background & Aims—Fatty acid binding protein 4 (FABP4) has been known as a mediator of inflammatory response in the macrophages and adipose tissue, but its hepatic function is poorly understood. The goal of this study is to investigate the role of FABP4 in liver ischemia/reperfusion (I/R), a clinical condition involves both hypoxia and inflammation.

Methods—To examine the I/R regulation of FABP4, mice were subjected to I/R surgery before being measured for FABP4 gene expression. Both loss-of-function (by using a pharmacological FABP4 inhibitor) and gain-of-function (by adenoviral overexpression of FABP4) were used to determine the functional relevance of FABP4 expression and its regulation during I/R. To

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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determine the hypoxia responsive regulation of FABP4, primary mouse hepatocytes were exposed to hypoxia. The FABP4 gene promoter was cloned and its regulation by hypoxia inducible factor 1 α (HIF-1 α) was characterized by luciferase reporter gene, electrophoretic mobility shift, and chromatin immunoprecipitation assays.

Results—We found that the hepatic expression of FABP4 was markedly induced by I/R. At the functional level, pharmacological inhibition of FABP4 alleviated the I/R injury, whereas adenoviral overexpression of FABP4 sensitized mice to I/R injury. We also showed that exposure of primary hepatocytes to hypoxia or transgenic overexpression of HIF-1 α in the mouse liver was sufficient to induce the expression of FABP4. Our promoter analysis established FABP4 as a novel transcriptional target of HIF-1 α .

Conclusions—FABP4 is a hypoxia inducible gene that sensitizes mice to liver I/R injury. FABP4 may represent a novel therapeutic target, and FABP4 inhibitors may be used as therapeutic agents to manage hepatic I/R injury.

Keywords

liver injury; ischemia/reperfusion; hypoxia; FABP4; HIF-1 α

Introduction

Liver ischemia/reperfusion (I/R) injury often occurs during liver transplantation. The I/R injury not only contributes to early functional failure, but also increases the prevalence of both acute and chronic rejection, thereby reducing long-term graft survival [1]. Previous studies suggested that the I/R-induced liver injury involves multiple mechanisms, including direct ischemic cellular damage, and indirect cellular injury resulted from inflammatory response [2–4]. Although surgical techniques have improved, I/R-induced liver injury remains an important clinical problem that demands more mechanistic understanding and clinical management.

It has been widely accepted that hypoxic foci are present in the microenvironment during ischemic injury [5]. The hypoxia inducible factor-1 α (HIF-1 α) is a principal regulator of adaptive response to hypoxia by forming a heterodimeric transcriptional complex with the aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1 β [6–12]. Accumulating evidence suggested that activation of HIF-1 α induces the expression of genes involved in glycolysis, glucose metabolism, mitochondrial function, cell survival, apoptosis, and resistance to oxidative stress during I/R [13, 14]. Indeed, HIF-1 α has been reported to protect mice from ischemic damages to the liver [15] and heart [16]. In addition to hypoxia, liver I/R has also been associated with inflammation and apoptosis of the hepatocytes [17]. It is unclear whether and how HIF-1 α may play a role in I/R-associated inflammatory response.

The fatty acid binding protein-4 (FABP4), also known as adipocyte protein 2 (aP2), is a lipid-binding chaperone highly expressed in the macrophages and adipocytes [18, 19]. FABP4 deficient macrophages exhibited a dramatic decrease in the expression of inflammatory cytokines, including TNF- α , IL-6, IL-1 β , and MCP-1 [20, 21]. Also in the

macrophages, FABP4 enhanced lipopolysaccharide (LPS)-induced inflammatory response by forming a positive feedback loop with the c-Jun N-terminal kinase (JNK) and activate protein-1 (AP-1), which can further stimulate the production of pro-inflammatory cytokines [22]. In the adipose tissue, a fat-specific deletion of FABP4 decreased the adipose expression of tumor necrosis factor- α (TNF- α) [23], and treatment of mice with a selective FABP4 inhibitor BMS309403 [24] alleviated type 2 diabetes and atherosclerosis by suppressing inflammation in both adipocytes and macrophages [25, 26]. The role of FABP4 in hepatocytes and liver has been relatively understudied. Whether or not FABP4 plays a role in I/R-induced liver injury and inflammation has not been reported.

In the present study, we showed the expression of FABP4 was markedly induced by liver I/R. Treatment with a FABP4 inhibitor relieved mice from, and a forced expression of FABP4 sensitized mice to, I/R-induced liver injury, respectively. We also showed that FABP4 is a hypoxia inducible gene that is under the direct transcriptional control of HIF-1 α .

Materials and Methods

Animals, drug treatment, and surgical procedures

CD-1 female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The HIF-1 α transgenic mice [27] and the liver/hepatocyte-specific HIF-1 α knockout mice [28] were previously described. Mice were housed in a room under a controlled temperature ($23 \pm 1^\circ\text{C}$) with a 12-h light–dark cycle and with free access to water and diet. When necessary, mice were pre-treated with a daily gavage of vehicle (saline) or BMS309403 (20 mg/kg/day), for 4 consecutive days, i.p. injection of YC-1 (3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole, 25 mg/kg) 2 h before surgery, i.p. injection of *N*-acetylcysteine amide (NACA) (25 mg/kg) 1 h before surgery, or tail vein injection of adenovirus encoding the control luciferase (Ad-Luc) or mouse FABP4 (Ad-FABP4) [29] 7 days before being challenged with I/R. To deplete the Kupffer cells, mice were treated with saline or 20 mg/kg gadolinium chloride (GdCl₃) via tail vein injection. Twenty-four hours later, mice were again given the same dose of GdCl₃ immediately before the I/R. BMS309403 was purchased from Chemrenblock Technology (Jiangsu, China). All other chemicals were purchased from Sigma (St. Louis, MO).

For the liver I/R surgery, mice were anesthetized using ketamine-xylazine cocktail. A midline laparotomy was performed, and 70% hepatic ischemia was accomplished using a microvascular clamp placed across the portal vein, hepatic artery, and bile duct above the branching to the right lateral lobe. Reperfusion was initiated after 60 or 90 min of ischemia by removal of the clamp. Blanching of the liver was used as a positive marker for hepatic ischemia. Reperfusion was ascertained by a return of the reddish color of the liver. Sham-operated mice were subjected to a laparotomy without clamping the liver. The 12-h and 6-h reperfusions were used for the FABP4 inhibitor study and Ad-FABP4 study, respectively. The use of mice in this study complied with relevant federal guidelines and institutional policies

Real-time PCR analysis and Western blotting

Total RNA was extracted and cDNA was prepared by using the High-Capacity cDNA Transcription Kit from Applied Biosystems (Foster City, CA). SYBR Green-based real-time PCR was performed with the ABI 7300 System. Data were normalized against the control cyclophilin. The primer sequences for real-time PCR are shown in the Supplementary Table. Western blotting was performed as we have previously described [30]. The FABP4 antibody (D25B3, Cat #3544) was purchased from Cell Signaling (Danvers, MA).

Serum chemistry and liver histology

The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by using commercial assay kits from Stanbio (Boerne, TX). For H&E staining, liver samples were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. The necrosis was quantified by using the Image J software.

Isolation, culture, and macrophage co-culture of mouse primary hepatocytes

Mouse primary hepatocytes were isolated from female CD-1 mice and plated onto collagen-coated 6-well plates at a density of 2×10^5 cells/well. After cells attached for 2 h, the medium was changed to hepatocyte maintenance medium for overnight. Cells were then exposed to hypoxia condition (1% O₂ and 5% CO₂, balanced with N₂) in a Forma Series II hypoxic incubator (Model 3141) from Thermo Electron Corp (Waltham, MA) with or without the co-culture of RAW264.7 cells using the Transwell Permeable plates from Corning (Corning, NY). When applicable, the HIF-1 α inhibitor (Cat #400092) from CALBIOCHEM (Billerica, MA) was added to the medium. In the macrophage co-culture system, hepatocytes were seeded and cultured overnight. On the next day, the medium was replaced with hypoxic medium (equilibrated with 1% O₂, 5% CO₂, and 94% N₂) and the hepatocytes were overlaid with trans-well filters containing the RAW264.7 macrophages at a density of 1×10^6 cells per well. The co-culture systems were then placed in a hypoxic incubator.

Isolation of mouse primary Kupffer cells

The Kupffer cells were isolated essentially as described [30]. In brief, total liver nonparenchymal cells were collected upon perfusion and by centrifugation. Red blood cells were removed with RBC lysing buffer from BioLegend (San Diego, CA). The cells were then blocked with antibodies against CD16/32 from BioLegend to decrease the nonspecific binding. The resulting cells were stained and sorted for Kupffer cells at the University of Pittsburgh Flow Cytometry Core Facility with phycoerythrin (PE)-labeled F4/80 monoclonal antibody from Invitrogen.

Plasmid construction, transient transfection, and luciferase reporter gene assay

The mouse FABP4 gene promoter sequences of different lengths were PCR-amplified by using cognate forward primers and a common reverse primer: 5' CCGCTCGAG GAGCCTCTGAAGTCCAGATAGCTCA 3'. The mouse liver genomic DNA was used as the PCR template. The PCR products were digested with KpnI and XhoI, and inserted into the same enzyme digested pGL3-basic vector from Promega (Madison, WI). The sequences

of the forward primers are listed in the Supplementary Table. The cloned promoter sequences were verified by DNA sequencing.

HEK293T cells were used for transient transfection and luciferase reporter gene assay. Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, and transfected in 48-well plates. The transfected cells were cultured for an additional 24 h before luciferase assay. Transfection efficiency was normalized against β -gal activity derived from the co-transfected pCMX- β -gal plasmid. Fold inductions were calculated as relative reporter activity compared with empty vector-transfected cells.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assay was performed using ^{32}P -labeled oligonucleotides and HIF-1 α and ARNT proteins prepared by using the TNT kit from Promega. Protein-DNA complexes were resolved by electrophoresis through 5% polyacrylamide gel in 0.5 \times Tris borate-EDTA at 4°C for 3 h. For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction at 50-fold molar excess to the ^{32}P -labeled probes. The EMSA probe sequences are listed in the Supplementary Table.

Chromatin immunoprecipitation (ChIP)

Mouse primary hepatocytes were cultured in normoxia or hypoxia condition for 6 h before formaldehyde cross-linking. Cross-linked DNA was extracted from cells, and incubated overnight with 1 μg of anti-HIF1 α antibody (Cat # ab2185) from Abcam (Cambridge, MA) at 4°C. Parallel samples were incubated with normal IgG as a negative control. The final DNA extracts were amplified by PCR using primers listed in the Supplementary Table.

Statistical analysis

All analyses were performed with the Statistical Package for Social Sciences (SPSS) software version 16.0. Data were expressed as mean \pm SD. Statistical significance was determined by one-way ANOVA. Analysis between two individual groups was determined by Student *t*-test. A *P*value < 0.05 was considered to be statistically significant.

Results

The hepatic expression of FABP4 was induced by I/R

To determine whether I/R injury affected FABP4 gene expression, mice were subjected to 60 min ischemia, followed by reperfusion for 3, 6, or 12 h. Compared to the sham group, reperfusion of 6 h resulted in a significant induction of FABP4 mRNA expression, but a more dramatic induction of FABP4 was observed upon the 12 h reperfusion (Fig. 1A). The I/R induction of FABP4 was also confirmed at the protein level by Western blot analysis (Fig. 1B). The induction appeared to be FABP4-specific, because the expression of FABP5 was not affected after the 12 h reperfusion (Fig. 1C). Ischemia alone did not induce but rather suppressed the expression of FABP4 (Fig. 1D), likely due to the acute ischemic liver tissue damage. We also showed that the I/R-responsive induction of FABP4 remained significant when the Kupffer cells were pharmacological depleted by pre-treating the mice with gadolinium chloride (GdCl_3) (Fig. 1E, left panel). The efficiency of Kupffer cell

depletion was confirmed by the decreased expression of the macrophage marker gene F4/80 (Fig. 1E, right panel). Interestingly, the *in vivo* liver environment appeared to be needed for the I/R responsive induction of FABP4. When primary hepatocytes and Kupffer cells were isolated from mice that were subjected to the 60 min ischemia and 12 h reperfusion, the expression of FABP4 was not induced, but rather suppressed in hepatocytes; whereas the expression of FABP4 in Kupffer cells was unchanged (Fig. 1F, left panel). The purity of the isolated Kupffer cells was verified by the expression of F4/80 (Fig. 1F, right panel). We reasoned the suppression of FABP4 in hepatocytes might have been secondary to the cellular damage. The lack of FABP4 induction in Kupffer cells might be due to the compromised hypoxic condition that was associated with the liver perfusion and cell isolation procedures.

Pharmacological inhibition of FABP4 attenuated the I/R responsive liver injury

To determine the functional relevance of I/R responsive FABP4 induction, we examined the effect of FABP4 inhibitor BMS309403 [24] on I/R-induced liver injury. In this experiment, female mice were orally gavaged with 20 mg/kg BMS309403 or vehicle for 4 consecutive days before being subjected to the 60-min ischemia and 12-h reperfusion protocol. As shown in Fig. 2A, pre-treatment of mice with BMS309403 led to a significant attenuation of I/R-induced elevation of serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The I/R responsive induction of pro-inflammatory cytokines, including IL-1 β , IL-6, TNF- α and MCP-1, was also dramatically attenuated in BMS309403-treated mice (Fig. 2B). At the histological level, the I/R-induced liver necrosis was largely abrogated in mice pre-treated with BMS309403 (Fig. 2C). Quantification of the necrosis showed that the I/R-induced necrotic area reduced from 60% in vehicle-treated mice to approximately 10% in BMS309403-treated mice (Fig. 2D).

Overexpression of FABP4 in the liver sensitized mice to I/R injury

Having shown that the pharmacological inhibition of FABP4 attenuated I/R injury, we went on to determine whether overexpression of FABP4 would sensitize mice to I/R injury. In this experiment, mice were infected with adenovirus encoding the control luciferase (Ad-Luc) or mouse FABP4 (Ad-FABP4) 7 days before being subjected to the 60-min ischemia/6-h reperfusion protocol. The overexpression of FABP4 was confirmed by real-time PCR (Fig. 3A, left panel) and Western blotting (Fig. 3A, right panel). Indeed, compared to their Ad-Luc infected counterparts, the Ad-FABP4 infected mice showed elevated serum levels of AST and ALT (Fig. 3B), increased expression of pro-inflammatory genes (Fig. 3C), and aggravated hepatic necrosis (Fig. 3D).

Overexpression of FABP4 sensitized primary hepatocytes to basal and inducible inflammatory response

The pro-inflammatory effect of FABP4 on macrophages has been documented [21, 22]. To determine whether FABP4 can directly affect the inflammatory response of hepatocytes in the absence of macrophages/Kupffer cells, we isolated mouse primary hepatocytes and infected them with Ad-Luc or Ad-FABP4 for 48 h before measuring the basal and inducible inflammatory response. The success of FABP4 overexpression was confirmed by real-time PCR (Fig. 4A). Overexpression of FABP4 increased the basal expression of IL-1 β (Fig. 4B), IL-6 (Fig. 4C), TNF α (Fig. 4D), but not MCP-1 (Fig. 4E). The FABP4-infected hepatocytes

remained sensitive to the induction of pro-inflammatory cytokines in response to the treatment of LPS (100 ng/ml), TNF α (20 ng/ml), or phorbol 12-myristate 13-acetate (PMA, 20 ng/ml) (Fig. 4B–4E). We also showed that the LPS responsive induction of pro-inflammatory cytokines in primary hepatocytes was attenuated by the FABP4 inhibitor BMS309403 (Fig. 4F), suggesting that FABP4 was required for the optimal inflammatory response.

Hepatic expression of HIF-1 α was induced by I/R, and exposure of primary hepatocytes to hypoxia was sufficient to induce FABP4 expression

It has been known that hypoxia is a microenvironment during ischemic injury [5]. It is also known that HIF-1 α is an important regulator of hypoxic response [6–12]. The expression of HIF-1 α was reported to be induced in a mouse renal I/R model [31]. Consistent with these notions, we found the hepatic expression of HIF-1 α was induced in mice subjected to the 60-min I/R (Fig. 5A), and the pattern of HIF-1 α induction was similar to that of FABP4 (Fig. 1A). The 90-min ischemia had a similar effect in inducing HIF-1 α (data not shown).

The concurrent induction of FABP4 and HIF-1 α by I/R led to our hypothesis that FABP4 might be inducible in response to hypoxia. To test this hypothesis, we exposed mouse primary hepatocytes to normoxia or hypoxia for 6 or 12 h. Indeed, hypoxia induced FABP4 mRNA expression at both time points (Fig. 5B). As a positive control, the expression of vascular endothelial growth factor (VEGF), a HIF-1 α responsive gene, was induced by hypoxia at both time points as expected (Fig. 5C). The hypoxia responsive induction of FABP4 was, at least in part, HIF-1 α dependent, because co-treatment of the cells with a HIF-1 α inhibitor [32] modestly but significantly attenuated the hypoxia responsive induction of both FABP4 (Fig. 5B) and VEGF (Fig. 5C) in a dose-dependent manner.

We also showed that the expression of FABP4 in cultured macrophages was not affected by a 12-h or 24-h hypoxia (data not shown), indicating that the hypoxia responsive induction of FABP4 was hepatocyte-specific. The mechanism for the cell type specificity of the hypoxia inducible expression of FABP4 remains to be understood. However, co-culture of primary hepatocytes with the RAW264.7 macrophages substantially increased the basal expression of FABP4 (Fig. 5D). When co-cultured with macrophages, the hepatocytes remained responsive to hypoxia in inducing FABP4 gene expression (Fig. 5D). The increased basal and hypoxia inducible expression of FABP4 in the co-culture conditions was likely due the secretion of cytokines from the macrophages. These results together suggested that although the macrophages were not responsive to hypoxia to induce FABP4, they were required for the optimal expression of FABP4.

In vivo, pre-treatment of mice with the HIF-1 inhibitor YC-1 [33] failed to attenuate the I/R responsive FABP4 expression (Fig. 5E). The expression of inflammatory genes was also increased in YC-1 treated mice (Fig. 5E), consistent with the reported protective effect of HIF-1 on I/R injury [15]. The intact I/R responsive FABP4 induction in the presence of HIF-1 inhibitor may have been accounted for by the I/R responsive oxidative stress and/or inflammation, because treatment of mice with the anti-oxidative and anti-inflammatory *N*-acetylcysteine amide (NACA) [34, 35] substantially attenuated the I/R responsive expression

of FABP4 (Fig. 5F). NACA also attenuated the I/R responsive expression of IL-6 as expected.

Hepatic expression of FABP4 was induced in HIF-1 α transgenic mice, and FABP4 is a transcriptional target of HIF-1 α

The HIF-1 α responsive induction of FABP4 was also confirmed in HIF-1 α transgenic mice (Fig. 6A), in which the expression of HIF-1 α was targeted to the liver [36]. In the same HIF-1 α transgenic mice, the expression of VEGF was induced as expected. In contrast, the basal expression of FABP4 was reduced in the liver/hepatocyte-specific HIF-1 α knockout mice [28] (Fig. 6B), further suggesting that FABP4 is a HIF-1 α target gene.

To directly test whether FABP4 is a HIF-1 α target gene, we cloned the 3-kb mouse FABP4 gene promoter and examined its regulation by HIF-1 α by transient transfection and reporter gene assay. As shown in Fig. 6C, the 3-kb FABP4 reporter gene was activated by the co-transfection of HIF-1 α and ARNT. Serial deletion analysis showed that the 0.8-kb promoter remained activated by HIF-1 α /ARNT, whereas a further deletion of 200-bp abolished the transactivation. A synthetic reporter gene tk-HRE that contains a hypoxia response element (HRE) was included as a positive control in the reporter gene assay.

Bioinformatic analysis of the 3-kb FABP4 promoter revealed two A/GCGTG motif-containing HREs, HRE1 and HRE2, in the -2.5 kb and -2.0 kb positions. Interestingly, our deletion analysis showed that deletion of HRE1 alone or both HRE1 and HRE2 failed to abolish the transactivation. On the other hand, no typical HRE was found in the 200-bp HIF-1 α responsive region that was essential for the transactivation. The binding of HIF-1 α -ARNT heterodimers to the two HREs was confirmed by electrophoretic mobility shift assay (EMSA), in which the binding of HIF-1 α -ARNT heterodimers to VEGF/HRE was included as a positive control (Fig. 6D). This binding of HIF-1 α -ARNT heterodimers to the two HREs was efficiently competed by unlabeled HREs, but not their mutant variants (Fig. 6D). The lack of typical HRE in the 200-bp HIF-1 α responsive region, although paradoxical, was consistent with a previous report that a promoter construct containing no typical HRE was inducible in cells exposed to hypoxia [37]. Chromatin immunoprecipitation (ChIP) showed efficient hypoxia responsive recruitment of HIF-1 α to both HREs, as well as the 200-bp HIF-1 α responsive region (Fig. 6E).

Discussion

In this study, we showed that the hepatic expression of FABP4 was markedly induced by I/R. The I/R responsive induction of FABP4 was functionally relevant, because pharmacological inhibition of FABP4 alleviated the I/R injury, whereas overexpression of FABP4 in the liver sensitized mice to I/R injury. Hypoxia is a key pathological event associated with I/R. We went on to demonstrate that FABP4 is a hypoxia inducible gene and a direct transcriptional target of HIF-1 α .

The role of FABP4 in I/R injury is a novel finding. FABP4 has been reported to sensitize mice to obesity, carcinogenesis and inflammation. These effects were believed to be due to the activities of FABP4 in the macrophages and adipocytes. In contrast, the role of FABP4 in

hepatocytes is poorly understood. We recently reported that adenoviral over-expression of FABP4 in the mouse liver was sufficient to elevate the serum levels of AST and ALT, increase macrophage marker gene expression, and induce mild steatosis [29]. These results were consistent with the reported clinical association between FABP4 level and the degree of inflammation and fibrosis [38, 39]. In contrast, treatment of mice with the FABP4 inhibitor BMS309403 alleviated LPS-induced acute liver injury and non-alcoholic steatohepatitis [29]. The current study has expanded the function of FABP4 to the liver and hepatocytes. We showed that the I/R-responsive induction of FABP4 remained significant when the Kupffer cells were pharmacological depleted (Fig. 1E), suggested that the macrophages/Kupffer cells are dispensable for this induction. The decreased basal expression of FABP4 in the liver/hepatocyte-specific HIF-1 α knockout mice (Fig. 6B) also suggested the role of hepatocytes in the basal and I/R inducible expression of FABP4.

Our findings are of medical significance, because I/R is a common liver injury following surgical intervention, trauma, and transplantation [40]. As such, understanding the pathophysiology of I/R is important for the clinical management of I/R-associated liver injury. In the current study, we demonstrated that FABP4 inhibitor can efficiently alleviate I/R-induced liver injury, which was supported by decreased AST and ALT levels, inhibition of pro-inflammatory cytokine expression, and decreased histological damage. It is tempting for us to speculate that FABP4 may represent an attractive target to attenuate I/R-induced liver injury.

Another interesting finding of ours is the establishment of FABP4 as a novel target gene of HIF-1 α . Hypoxia is a key pathological event associated with I/R, during which HIF-1 α is accumulated and activated. The hypoxia responsive activation of HIF-1 α may have accounted for the I/R responsive induction of FABP4. The hypoxia responsive induction of FABP4 was confirmed in both in vivo and in isolated primary hepatocytes. Interestingly, our cell culture results showed that although the macrophages were not responsive to hypoxia to induce FABP4, co-culture of macrophages increased the basal and hypoxia inducible expression of FABP4. Moreover, transgenic overexpression of HIF-1 α was sufficient to induce FABP4. Our FABP4 promoter analysis established FABP4 as a direct target gene of HIF-1 α .

Having established FABP4 as a HIF-1 α target gene, it is interesting to note that pre-treatment of mice with a HIF-1 inhibitor failed to attenuate the I/R responsive induction of FABP4 (Fig. 5E). The intact FABP4 induction in the presence of a HIF-1 inhibitor may have been accounted for by the I/R responsive oxidative stress and/or inflammation, because treatment of mice with the anti-oxidative and anti-inflammatory NACA attenuated the I/R responsive induction of FABP4 (Fig. 5F). Based on these results, we concluded that even though FABP4 is a HIF1 target gene, the activation of HIF1 might not be responsible for the I/R induction of FABP4 and liver injury.

In summary, we have demonstrated that fatty acid binding protein FABP4 is a hypoxia inducible gene that sensitized mice to liver I/R injury. Our results suggested a novel function of FABP4 in the liver and in the pathogenesis of I/R-induced liver injury. We propose that FABP4 may represent a novel therapeutic target and FABP4 inhibitors may be used as

therapeutic agents to manage hepatic I/R injury resulting from liver transplantation or other surgical procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALT	alanine aminotransferase
ARNT	aryl hydrocarbon receptor nuclear translocator
AST	aspartate aminotransferase
ChIP	chromatin immunoprecipitation
EMSA	electrophoretic mobility shift assay
FABP4	fatty acid binding protein 4
GdCl₃	gadolinium chloride
HIF-1α	hypoxia inducible factor 1 α
HRE	hypoxia responsive element
I/R	ischemia/reperfusion
LPS	lipopolysaccharide
NACA	<i>N</i> -acetylcysteine amide
VEGF	vascular endothelial growth factor
YC-1	3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole

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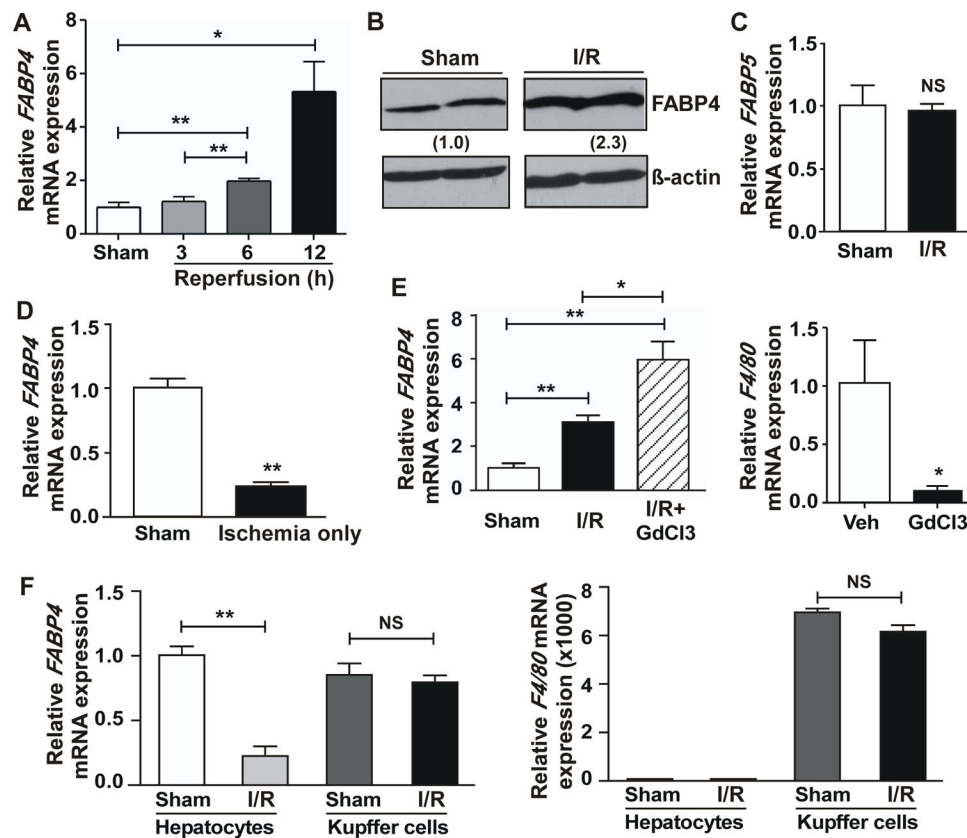


Fig. 1. The hepatic expression of FABP4 was induced by I/R

(A) Female mice were subjected to sham operation or 60-min ischemia followed by 3, 6, or 12 h of reperfusion before tissue harvesting and measuring FABP4 mRNA expression by real-time PCR. *, $P < 0.05$; **, $P < 0.01$; $n = 3-5$. (B) The I/R responsive induction of FABP4 was confirmed by Western blot analysis. Mice were subject to 60-min ischemia and 12-h reperfusion. The quantifications of the fold induction are labeled. (C) The expression of FABP5 mRNA was measured by real-time PCR. The mice were the same as described in (B). NS, statistically not significant. (D) The expression of FABP4 mRNA was measured in mice subjected to sham operation or 60-min ischemia without reperfusion. (E) The mRNA expression of FABP4 (left panel) and F4/80 (right panel) was measured in mice without or with the pre-treatment of GdCl₃ before being subjected to the 60-min ischemia and 12-h reperfusion. (F) The mRNA expression of FABP4 (left panel) and F4/80 (right panel) was measured in primary hepatocytes and Kupffer cells isolated from mice that were subjected to 60-min ischemia and 12-h reperfusion. *, $P < 0.05$; **, $P < 0.01$; $n = 4$.

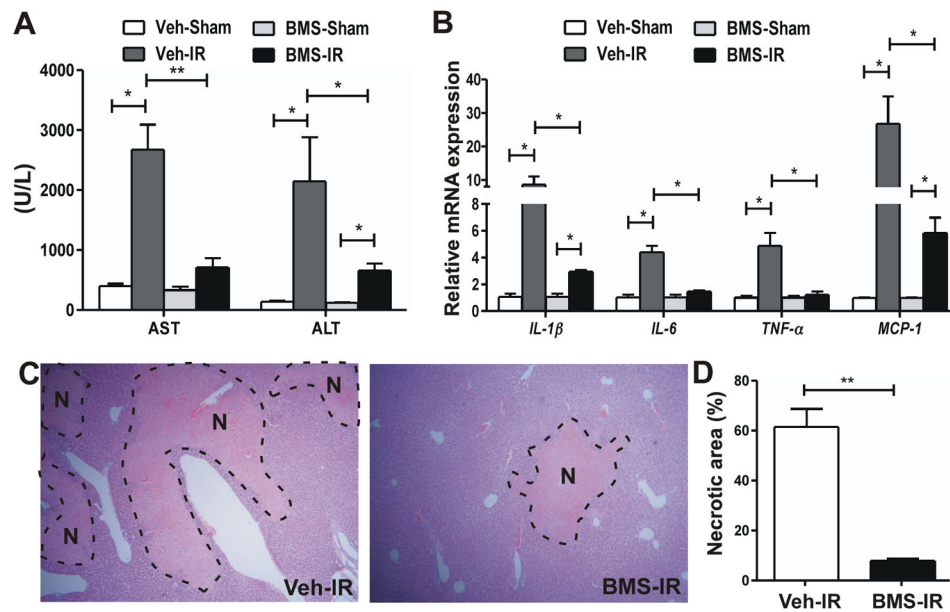


Fig. 2. Pharmacological inhibition of FABP4 attenuated the I/R responsive liver injury
 Fourteen-week-old female mice were orally gavaged with 20 mg/kg BMS309403 or vehicle for 4 consecutive days before being subjected to the 60-min ischemia and 12-h reperfusion protocol. (A) Serum levels of ALT and AST. (B) The mRNA expression pro-inflammatory cytokines IL-1 β , IL-6, TNF- α and MCP-1 in the liver was measured by real-time PCR. (C and D) H&E staining of liver paraffin sections (C) and quantification of the necrotic area (D). The necrotic areas are circled and labeled with “N”. *, $P < 0.05$; **, $P < 0.01$; $n = 3-5$.

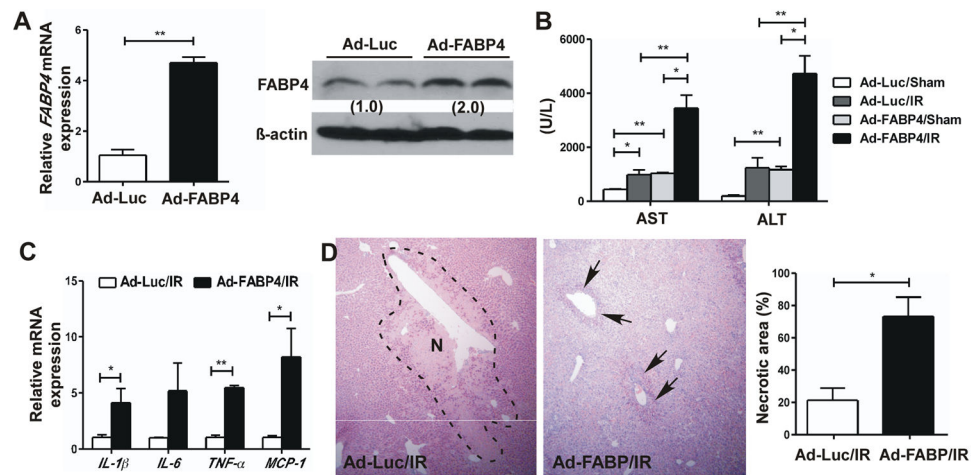


Fig. 3. Overexpression of FABP4 in the liver sensitized mice to I/R injury

Female mice were infected with Ad-Luc or Ad-FABP4 7 days before being subjected to the 60-min ischemia and 6-h reperfusion protocol. (A) The expression of transduced FABP4 was confirmed by real-time PCR (left panel) and Western blotting (right panel). The quantifications of Western blotting are labeled. (B) The serum ALT and AST levels. (C) The mRNA expression pro-inflammatory cytokines IL-1 β , IL-6, TNF- α and MCP-1 in the liver was measured by real-time PCR. (D) H&E staining of liver paraffin sections and quantification of the necrotic area. The necrotic area in the Ad-Luc/IR group is circled, whereas the arrows indicate surviving areas in the Ad-FABP4/IR group. *, $P < 0.05$; **, $P < 0.01$; $n = 3-4$.

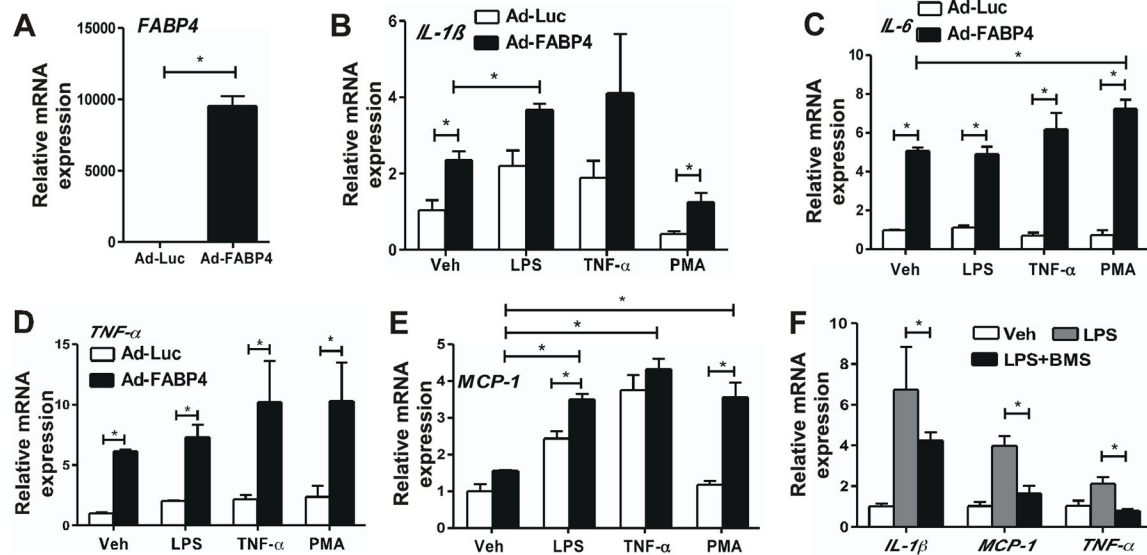


Fig. 4. Overexpression of FABP4 sensitized primary hepatocytes to basal and inducible inflammatory response

(A to E) Primary hepatocytes isolated from female mice were infected with Ad-Luc or Ad-FABP4 for 48 h before being harvested for real-time PCR analysis for the detection of FABP4 (A), IL-1 β (B), IL-6 (C), TNF α (D) and MCP-1 (E). Cells in (B to E) were treated with vehicle, LPS (100 ng/ml), TNF α (20 ng/ml), or PMA (20 ng/ml) as labeled for 3 h before cell harvesting. (F) Primary hepatocytes were treated with LPS in the absence or presence of BMS309403 (10 μ M) before cell harvesting and gene expression analysis by real-time PCR analysis. *, $P < 0.05$.

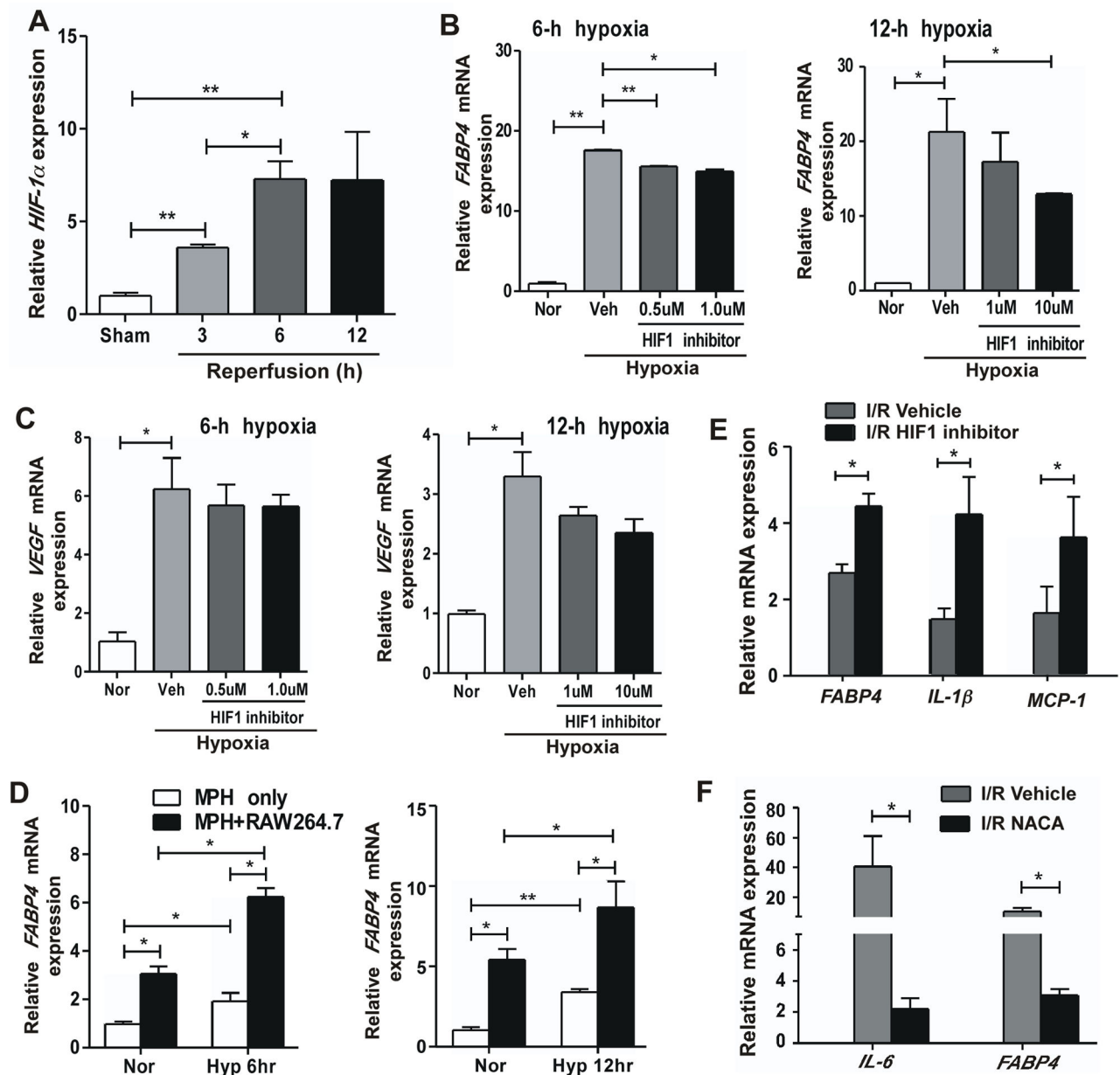


Fig. 5. Hepatic expression of HIF-1α was induced by I/R, and exposure of primary hepatocytes to hypoxia induced FABP4 expression

(A) Female mice were subjected to 60-min ischemia followed by 3, 6, or 12 h of reperfusion before tissue harvesting. HIF-1α gene expression was measured by real-time PCR. *, $P < 0.05$; **, $P < 0.001$; $n = 3-6$. (B) Primary hepatocytes were exposed to hypoxia for 6 h (left panel) or 12 h (right panel) in the presence of vehicle or different concentrations of the HIF1 inhibitor before cell harvesting and measuring the expression of FABP4. (C) Experiments were the same as described in (B) except that the expression of VEGF was measured. (D) Primary hepatocytes were exposed to hypoxia for 6 h (left panel) or 12 h (right panel) in the absence of presence of the co-culture of the RAW264.7 macrophages. *, $P < 0.05$; **, $P < 0.01$; $n = 3$. (E and F) Female mice were subjected to I/R with or without the pre-treatment

of the HIF-1 inhibitor YC-1 (E) or *N*-acetylcysteine amide (NACA) (F). Gene expression was measured by real-time PCR. *, $P < 0.05$; $n = 4-6$.

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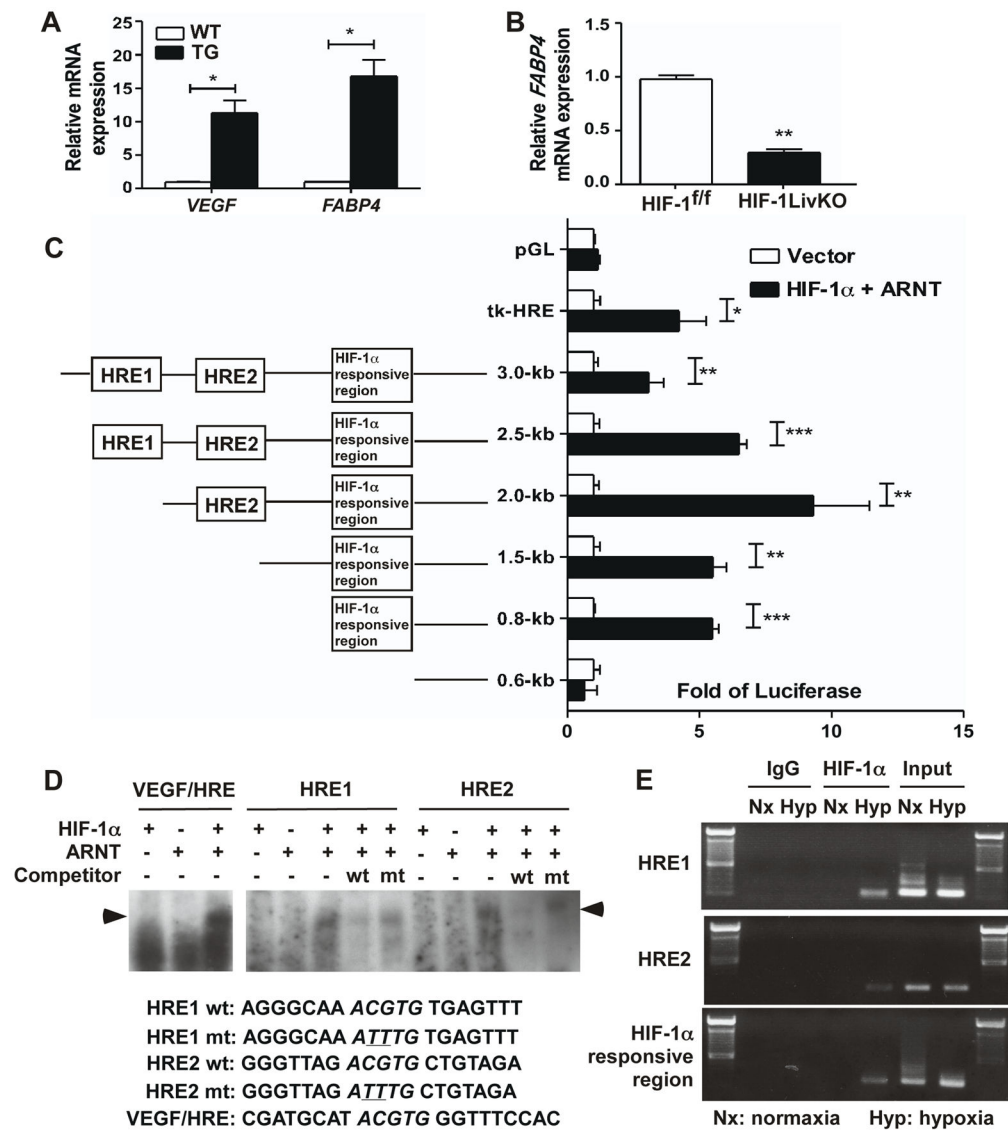


Fig. 6. Hepatic expression of FABP4 was induced in HIF-1 α transgenic mice, and FABP4 is a transcriptional target of HIF-1 α

(A) Hepatic mRNA expression of FABP4 and VEGF was increased in wild-type (WT) and HIF-1 α transgenic (TG) mice as measured by real-time PCR analysis. *, $P < 0.05$, $n = 4-5$.

(B) Hepatic mRNA expression of FABP4 in female HIF-1 α floxed mice and liver/hepatocyte-specific HIF-1 α knockout mice as measured by real-time PCR analysis. **, $P < 0.05$, $n = 4$.

(C) Reporter genes containing various lengths of the mouse FABP4 gene promoter were transfected into HEK293T cells together with the expression vectors for HIF-1 α and ARNT. The transfection efficiency was normalized against the β -gal activity from the co-transfected CMX- β -gal vector. Normalized luciferase activity in cells transfected with empty expression vector (CMX) was arbitrarily set at 1. Results shown represent the averages and standard deviation from triplicate assays. (D) The binding of HIF-1 α -Arnt heterodimers to 32 P-labeled FABP4 HRE1 and HRE2 was demonstrated by electrophoretic mobility shift assay (EMSA). The sequences of two HREs and their mutant

variants are labeled with the mutated nucleotides underlined. In the competition lanes, unlabeled probes were present in 50-fold molar excess relative to the radiolabeled probe. The binding of labeled VEGF/HRE by HIF-1 α -ARNT heterodimers was included as positive controls. Arrowheads indicate the position of the specific binding. (E) Mouse primary hepatocytes exposed to normoxia or hypoxia were subjected to chromatin immunoprecipitation (ChIP) assay to determine the recruitment of HIF-1 α onto the FABP4 gene promoter.