Transcription factor C/EBPβ promotes the transcription of the porcine GPR120 gene

Kun Chen¹, Ji-Dan Zhou¹, Feng Zhang¹, Fang Zhang¹, Rui-Rui Zhang¹, Meng-Si Zhan¹, Xiao-Yin Tang¹, Bing Deng², Ming-Gang Lei¹ and Yuan-Zhu Xiong¹

¹Key Laboratory of Swine Genetics and Breeding of Agricultural Ministry, and Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan 430070, People’s Republic of China
²Wuhan Institute of Animal Science and Veterinary Medicine, Wuhan Academy of Agricultural Science and Technology, Wuhan, Hubei, People’s Republic of China

Correspondence should be addressed to M-G Lei or Y-Z Xiong
Emails
leiminggang@aliyun.com or xiongyuanzhu2008@163.com

Abstract
G protein-coupled receptor 120 (GPR120), an adipogenic receptor critical for the differentiation and maturation of adipocytes, plays an important role in controlling obesity in both humans and rodents and, thus, is an attractive target of obesity treatment studies. However, the mechanisms that regulate the expression of porcine GPR120 remain unclear. In this study, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) techniques were used to analyze and identify the binding of C/EBPβ (transcription factor CCAAT/enhancer binding protein beta) to the GPR120 promoter. C/EBPβ overexpression and RNA interference studies showed that C/EBPβ regulated GPR120 promoter activity and endogenous GPR120 expression. The binding site of C/EBPβ in the GPR120 promoter region from −101 to −87 was identified by promoter deletion analysis and site-directed mutagenesis. Overexpression of C/EBPβ increased endogenous GPR120 expression in pig kidney cells (PK). Furthermore, when endogenous C/EBPβ was knocked down, GPR120 mRNA and protein levels were decreased. The stimulatory effect of C/EBPβ on GPR120 transcription and its ability to bind the transcription factor-binding site were confirmed by luciferase, ChIP, and EMSA. Moreover, the mRNA and protein expression levels of C/EBPβ were induced by high fat diet feeding. Taken together, it can be concluded that C/EBPβ plays a vital role in regulating GPR120 transcription and suggests HFD-feeding induces GPR120 transcription by influencing C/EBPβ expression.

Key Words
- porcine
- GPR120
- C/EBPβ
- ChIP

Introduction
Adipose tissue dysfunction, the deviation from the main and normal function that controls fat storage and release in adipose tissue, causes metabolic diseases, such as type 2 diabetes mellitus, insulin resistance, and cardiovascular disease (Haber et al. 2003, Haslam & James 2005, Cao et al. 2008, Woodhouse 2008, Beltran-Sanchez et al. 2013, Zhang & Leung 2014). Therefore, researchers are working to study the mechanism of energy metabolism.
Unesterified fatty acids (FFAs), which act as endogenous signaling molecules, play various roles in the physiological regulation through FFA receptors (FFARs) such as FFAR4 (Habet et al. 2003, Itoh et al. 2003, Cao et al. 2008, Miyauchi et al. 2009, Oh et al. 2010). FFAR4, a receptor for unsaturated long-chain FFAs, is also known as G protein-coupled receptor 120 (GPR120) (Miyauchi et al. 2009, Oh et al. 2010). Furthermore, it has been shown to play an important role in various physiological homeostasis mechanisms, such as adipogenesis, food preference, and appetite regulation (Fredriksson et al. 2003, Stoneberg et al. 2005, Gotoh et al. 2007, Tanaka et al. 2008, Miyauchi et al. 2009). GPR120 has been reported to be abundantly expressed in the adipose tissue as well as in the intestine and lung (Hirasawa et al. 2005, Katsuma et al. 2005, Gotoh et al. 2007, Zhao et al. 2013).

GPR120 is abundantly expressed in adipose tissues and adipocytes (Gotoh et al. 2007, Ichimura et al. 2012). Moreover, the expression of GPR120 is enhanced with the lipid accumulation within the cells during the induction of adipocyte differentiation in 3T3-L1 cells (Gotoh et al. 2007, Miyauchi et al. 2009). Furthermore, GPR120 mRNA expression can be increased significantly in white adipose tissue of high fat diet-fed mice (Gotoh et al. 2007). In line with this, it has been reported that siRNA against GPR120 reduces the expression of peroxisome proliferator-activated receptor γ2 (PPAR-γ2) and the lipid droplet accumulation in 3T3-L1 adipocytes (Gotoh et al. 2007). PPAR-γ2 mRNA expression in mouse adipose tissue is also up-regulated with high fat diet feeding (Gotoh et al. 2007). These findings suggest GPR120 is involved in adipogenesis and lipid metabolism. In pig, GPR120 is also identified as a fat taste receptors and is present in the gastrointestinal tract. It plays an important role in the regulation of food intake (Colombo et al. 2012, van der Wielen et al. 2014). Moreover, compared to the rodent model, due to the close similarity to human metabolic features, cardiovascular systems, proportional organ sizes, pathologic reaction to high-energy intake and genetic modulation of fat deposition, the pig is used as a better biomedical model for energy metabolism (Bellinger et al. 2006, Spurlock & Gabler 2008, Swindle et al. 2012). Despite the available information, little is known about the regulatory mechanism of GPR120.

To better understand the transcriptional regulation of GPR120, we examined the mutations and cis regulation elements of the porcine GPR120 gene by analyzing its 5' upstream sequence. Our results demonstrate that transcription factor C/EBPβ binds to the promoter of GPR120 and enhances its expression.

### Materials and methods

#### Animals

The mice used were C57BL/6J mice purchased from Huafukang (Beijing, China). These mice were weighed and divided into two groups with equal mean body weights. One group was fed a high fat diet (HFD) (36% kcal from carbohydrate, 41% from fat, 23% from protein) for 12 weeks (4–16 weeks of age). The other group was fed the standard chow diet. The procedures were conducted in accordance with the ethics provided by Hubei Province Committee on Laboratory Animal Care.

#### In silico sequence analysis

Transcription factor binding sites and CpG islands in the GPR120 promoter were predicted with the T searchable v.1.3 (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) and MethPrimer (http://www.urogene.org/methprimer/) respectively.

#### Plasmid construction, cell culture and transient transfection

Seven GPR120 promoter deletion fragments were amplified from pig genomic DNA using primer pairs (Supplement 1, see section on supplementary data given at the end of this article) and inserted into the Kpn I/Xho I sites of pGL3-Basic vector (Fermentas, Promega). The mutants of binding sites were generated using a MutanBEST Kit (Takara, Otsu, Japan) and mutagenic primers (Supplement 1).

All cell lines used were purchased from the China Center for Type Culture Collection (CTCC). 3T3-L1 and pig kidney (PK) cells were fed again with DMEM (Gibco) supplied with 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% CO2. The cells were seeded at a density of 1.5–2.0×10^5/ml by DMEM supplemented with 10% FBS. After 24 h, they were transfected with plasmid DNA or small interfering RNAs (siRNA) utilizing Lipofectamine 2000 Reagent (Invitrogen).

#### Luciferase reporter assay

3T3-L1 and PK cells were cultured into 24-well plates and co-transfected with 0.2 μg of the constructed reporter plasmids DNA and 0.05 μg of prL-TK vector (Promega) or 2 μl of siRNA using Lipofectamine 2000 Reagent. Luciferase enzymatic activities were analyzed with a PerkinElmer 2030 Multilabel Reader (PerkinElmer) using the dual-luciferase reporter assay system (Promega) 24 h after the transfection. Assays were performed in triplicate, and the data were expressed as mean ± S.D.
Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed with SYBR Green I Real-time PCR Master Mix (Toyobo) on a LightCycler 480 Real-time System (Roche). β-actin was used as a housekeeping gene. The primers sequences used in the qRT-PCR are listed in Supplement 1, see section on supplementary data given at the end of this article. qRT-PCR amplified conditions were at 95 °C for 2 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 15 s. The expression of target genes was normalized according to the expression of β-actin; relative gene expression was calculated by 2^−ΔΔCt method (Livak & Schmittgen 2001, Schmittgen & Livak 2008). Student’s t-test was used for statistical analysis. A P value <0.05 was deemed to indicate statistical significance.

Electrophoretic mobility shift assays

Nuclear protein of pig abdominal fat was extracted with Nucleoprotein Extraction Kit (Promega). Corresponding to the C/EBPβ binding sites in the GPR120 promoter, double-stranded oligonucleotides (Sangon, Shanghai, China) were designed and synthesized. The DNA binding activity of C/EBPβ protein was detected by LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA, USA). Ten microgram pig abdominal fat nuclear extract was added to 20 fmol biotin-labeled double-stranded oligonucleotides, 2.5% glycerol, 50 ng poly (dI:dC), 1× binding buffer, 5 mM MgCl2, 0.05% NP-40, and 0.1 mM EDTA. In addition, the control group was added with 2 pmol unlabeled competitor oligonucleotides, and the supershift group was added with 10 μg C/EBPβ antibody (Abcam, Cambridge, UK). The mixtures were then incubated at room temperature for 20 min. The reaction was analyzed by electrophoresis in 6.5% polyacrylamide gels at 180 V for 70 min and then transferred to a nylon membrane. The dried nylon was scanned with GE ImageQuant LAS4000 mini (GE Healthcare, Shanghai, China).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) was performed using EZ-ChIP Chromatin immunoprecipitation Kit-17-371 (Millipore, Billerica, MA, USA). After adding 1% formaldehyde to growth media to crosslink for 10 min and adding glycine to neutralize for 5 min at room temperature, PK cells were treated with SDS lysis buffer, and cell pellets were sonicated to shear crosslinked chromatin with an average size of 200–600 bp. The ChIP dilution buffer containing protease inhibitor cocktail II was added into sheared crosslinked chromatin. Fragmented chromatin were incubated with normal mouse IgG (Millipore) and antibody anti-C/EBPβ (Abcam) at 4 °C overnight. Chromatin complexes were precipitated with protein G agarose beads. Carefully, the protein/DNA complexes were eluted by elution buffer. To reverse protein-DNA crosslink, 5 mM NaCl was added to all samples and incubated at 65 °C for 5 h. The fragmented chromatin was incubated with proteinase K (Millipore) at 45 °C for 2 h. Precipitated DNA was purified by Spin Columns (Millipore) and used as the template following PCR reaction program as follows: 94 °C for 4 min, followed by 32 cycles at 94 °C for 20 s, 59.6 °C for 30 s, and 72 °C for 20 s, with primers provided in Supplement 1, see section on supplementary data given at the end of this article.

RNA interference

siRNAs targeting C/EBPβ were obtained from genePharma. 3T3-L1 cells were co-transfected with 2 μl of siRNA and 0.2 μg of plasmid DNA by Lipofectamine 2000 reagent. Finally, the enzymatic activity of luciferase was measured with PerkinElmer 2030 Multilabel Reader at 24 h.

Western blotting

Western blotting was performed as reported previously (Steneberg et al. 2005). Proteins were heated with 5×SDS buffer at 95 °C for 10 min. For immunoblotting, proteins subjected to SDS–PAGE were transferred to PVDF membranes (Millipore). Membranes were then probed with anti-GPR120 (Santa Cruz). β-actin (Santa Cruz) was used for a loading control.

Statistical analysis

All of the experiments were repeated at least three times. All results were presented as means ± s.d. Statistical significance was assessed using Student’s t-test. A P value <0.05 was deemed to indicate statistical significance.

Results

Identification of the promoter region and regulatory elements of the porcine GPR120 gene

A 1777 bp 5′-flanking region sequence (−1612 to +165 bp) of the porcine GPR120 gene was cloned from
porcine genomic DNA (Table 1). To determine the promoter region, six deletion fragments were constructed into pGL3-Basic luciferase reporter vector based on the predicted CpG islands (Fig. 1A). As no stable adipocyte cells are available in pigs, PK cell line and mouse 3T3-L1 preadipocyte cell line were chosen as research subjects. The luciferase reporter vectors harboring different deletion fragments of the GPR120 promoter were transiently transfected into 3T3-L1/ PK cells. Surprisingly, five deletion fragments (P1, P2, P3, P4, and P5) significantly enhanced the luciferase activities in both cells (P<0.05) (Fig. 1B), while 5'-flanking region sequence P6 displayed insignificant increases in luciferase activities (Fig. 1B). The data of the luciferase activity assay showed that the 5'-flanking sequence P5 (−323 to +165 bp) was important to its transcriptional activity.

We predicted one potential binding site (TGTCG) for C/EBPβ in the P5 region with the TFSEARCH v.1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html) (Fig. 2A). Further analysis of a putative C/EBPβ binding site suggested that it is highly conserved in human, mouse and pig (Fig. 2B). Site-directed mutagenesis was performed using a WT pGL3-P5 construct as a template, aiming to functionally determine the importance of C/EBPβ

### Table 1  Primers and oligos

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>qRT-PCR-GPR120-F</td>
<td>GATGGAGGCCAGGGTACAAA</td>
</tr>
<tr>
<td>qRT-PCR-GPR120-R</td>
<td>GCCAGGACACCAAAATAGA</td>
</tr>
<tr>
<td>C/EBPβ-F</td>
<td>CCGGGTTACCGCCACCATGCAACGCTTGCG</td>
</tr>
<tr>
<td>C/EBPβ-R</td>
<td>CCGCCTGAGCTAGCTGGCCAGGGGGAGG</td>
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<tr>
<td>qRT-PCR-C/EBPβ-F</td>
<td>GAAACTTCTGGGATGTGCG</td>
</tr>
<tr>
<td>qRT-PCR-C/EBPβ-R</td>
<td>ATCTGTTGGCTCTCGGAGTA</td>
</tr>
<tr>
<td>PPARγ-F</td>
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</tr>
<tr>
<td>PPARγ-R</td>
<td>AGGTCGCTGTATCAATTT</td>
</tr>
<tr>
<td>GPR120-P1-F</td>
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</tr>
<tr>
<td>GPR120-P2-F</td>
<td>CCGGAGAAGGCAGGCTGGCAACGCTTGCG</td>
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<td>GPR120-P3-F</td>
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<tr>
<td>GPR120-P4-F</td>
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</tr>
<tr>
<td>GPR120-P5-F</td>
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<td>GPR120-P6-F</td>
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</tr>
<tr>
<td>GPR120-P-R</td>
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<tr>
<td>GPR120-mut-F</td>
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<tr>
<td>GPR120-mut-R</td>
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<td>C/EBPβ-wt-F-bio</td>
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<td>si/C/EBPβ-3 antisense</td>
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</tr>
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</tr>
<tr>
<td>Mus GPR120-R</td>
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</tr>
<tr>
<td>Mus C/EBPβ-F</td>
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</tr>
<tr>
<td>Mus C/EBPβ-R</td>
<td>TGGGCACTTCATTGGCTGTA</td>
</tr>
<tr>
<td>Mus PPARγ-F</td>
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<td>Mus PPARγ-R</td>
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<tr>
<td>Mus C/EBPβ-ChIP-F</td>
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<tr>
<td>Mus C/EBPβ-ChIP-R</td>
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</table>

Note: the part italicised is enzyme site induced.
binding sites. We mutated the C/EBPβ transcription factor binding site in pGL3-P5 plasmid (Fig. 2C). The pGL3-P5-wt and pGL3-P5-mut were transfected into PK/3T3-L1 cells respectively. The modification of TGTC to GACT in the K101 to K87 bp region significantly suppressed the promoter activities in both cells (P<0.01) (Fig. 2D). These results indicate that the C/EBPβ binding site is important for GPR120 promoter activity.

![Diagram](https://example.com/diagram.png)

**Figure 2**
Site-directed mutagenesis of C/EBPβ binding site in the GPR120 promoter. (A) Analysis of transcription factor site within the GPR120 promoter. (B) The sequences of C/EBPβ binding sites of the GPR120 gene promoter are conserved. The aligned sequences are conserved within mouse, pig, and human. (C) The schematic diagram of site-directed mutagenesis in the predicted C/EBPβ binding site in the GPR120 promoter. (D) Site-directed mutagenesis in the C/EBPβ binding site of the GPR120 gene by luciferase activity assay. WT and mutant of the GPR120 promoter were transfected into pig kidney (PK)/3T3-L1 cells, respectively. The CMV-Renilla luciferase reporter vector was applied as an internal control. Data were presented as mean ± S.D. of three replicates. **P<0.01; NS, not significant.
Transcription factor C/EBPβ binds to the GPR120 promoter both in vitro and in vivo

In vitro, electrophoretic mobility shift assay (EMSA) was used to determine that the GPR120 promoter contained the C/EBPβ binding site (Fig. 3A). Incubation of nuclear extracts from pig abdominal fat with C/EBPβ probe resulted in the detection of a specific DNA-protein complex (Fig. 3A, Lane 2). The specific DNA-protein complex was not detected with 100-fold molar excess of a series of competitor probes (ChIP). (A) The probe was incubated with nuclear extract in the presence or absence of 100-fold molar excess of a series of competitor probes (unlabeled or mutant probe) or anti-C/EBPβ antibody (anti-IgG). As described above, pGL3-P5 (−323/+165) includes putative C/EBPβ binding sites, whereas pGL3-P6 (−65/+165) and pGL3-Basic do not. The porcine C/EBPβ cDNA was inserted into the eukaryotic expression vector using the primers shown in Supplementary 1, see section on supplementary data given at the end of this article. These luciferase reporter vectors were co-transfected with pc-C/EBPβ plasmid into 3T3-L1 cells. These results showed that C/EBPβ affected the promoter activity as the luciferase reporter vectors harbored its binding sites and the effects of C/EBPβ were suppressed as the corresponding binding sites were deleted (p<0.05; Fig. 4A). The luciferase activity assay indicated that C/EBPβ stimulated GPR120 transcription; however, it was unclear whether it was the same case in pig cells. Thus, the pc-C/EBPβ plasmid was transfected into PK cells. The results indicate that over-expression of C/EBPβ significantly enhances endogenous GPR120 expression by western blotting analyses and qRT-PCR (5.34-fold, P = 0.007) (Fig. 4B and C) and increases PPARγ gene expression by qRT-PCR (Fig. 4B). Study in vivo demonstrated that HFD feeding induced the expression of C/EBPβ, up-regulated GPR120 and PPARγ-2 in adipose tissue (Fig. 4D and E) and increased the binding of C/EBPβ on GPR120 promoter (Fig. 4F).

Inhibition of C/EBPβ suppresses the expression of GPR120

To verify whether C/EBPβ knockdown influences GPR120 gene expression, we used siRNAs to separately knock down C/EBPβ by transfecting individual siRNAs to PK cells. The knockdown efficiency of siRNAs was shown in Fig. 5A. The siC/EBPβ-3 for C/EBPβ was effective in knocking down C/EBPβ expression (Fig. 5A). 3T3-L1 cells were co-transfected with 2 μl siC/EBPβ-3 and 0.2 μg pGL3-P5 plasmid using Lipofetamine 2000 reagent for 24 h. The inhibition
of C/EBPβ expression significantly suppressed pGL3-P5 promoter activity ($P < 0.05$; Fig. 5B) and the expression of GPR120 protein (Fig. 5C). These findings indicate that C/EBPβ plays a critical role in GPR120 expression.

**Discussion**

Adipose tissue is a metabolical organ that is the primary site of storage for excess energy, and it is important in metabolic homeostasis as it releases adipokines to regulate insulin sensitivity in other organs (Coelho et al. 2013). Unbalanced production of proinflammatory cytokines and adipokines in visceral obesity contributes greatly to various aspects of metabolic syndromes (Rocha & Libby 2009). Hence, the molecular mechanisms of adipogenesis and adipocyte development are significant for energy metabolism.

Previous studies showed that GPR120 has been shown to play an important role in various physiological processes such as lipogenesis, inflammation, adipogenesis, and glucose intolerance (Gotoh et al. 2007, Tanaka et al. 2008, Oh et al. 2010, Ichimura et al. 2012) In the present study, we amplified the 5' upstream sequence of the porcine GPR120 gene and analyzed its promoter region and regulatory elements. Our results indicated that C/EBPβ significantly enhanced the transcription of GPR120, and the binding site (TGTCG) of C/EBPβ in the GPR120 promoter was identified. These results
be evolutionarily conserved motifs (Fig. 2B). Further, the promoter of human genes also contain the putative binding sites of regulator accumulation and the expression of the adipogenic key gene was knocked down by siRNA-suppressed lipid droplet (Gotoh et al., 2007). Similarly, C/EBP is a crucial member of C/EBPs family (Ramji & Foka, 2002), is highly expressed in the adipose, intestine, lung, and liver (Garvey et al., 2011). Furthermore, C/EBP mRNA was previously reported to be transiently induced at early stages of adipogenesis in human preadipocytes (Tomlinson et al., 2006, Fuentes et al., 2010). Moreover, C/EBP is activated by glycogen synthase kinase 3β (Tang et al., 2005) and then triggers transcription of PPARγ and C/EBPα to promote adipogenic differentiation (Tang & Lane, 2012). The bioinformatics analysis showed that there is a binding site (TGTCG) for C/EBP in the P5 region (−323 to +165 bp). The promoters of the homologous mouse and human genes also contain the putative binding sites of C/EBP, indicating that the binding sites of C/EBP might be evolutionarily conserved motifs (Fig. 2B). Further studies revealed that C/EBP could bind to the core promoter of GPR120 in vitro and in vivo (Fig. 3A and B).

In the previous study, the gene deficiency of GPR120 was knocked down by siRNA-suppressed lipid droplet accumulation and the expression of the adipogenic key regulator PPAR-γ2 in mouse embryonic fibroblast and 3T3-L1 respectively (Gotoh et al., 2007, Ichimura et al., 2012). In the present research, over-expression of C/EBP significantly promoted GPR120 and PPARγ gene expression in PK cells (Fig. 4A, B, and C). In line with this, inhibition of C/EBP expression significantly suppressed GPR120 gene expression (Fig. 5). Furthermore, GPR120 mRNA expression can be increased significantly in white adipose tissue of high fat diet-fed mice, and PPAR-γ2 mRNA expression in mouse adipose tissue is also up-regulated with high fat diet feeding (Gotoh et al., 2007). Additionally, transcription factor C/EBPβ is a crucial regulator of adipocyte differentiation, macrophage activation, and metabolism, and it has been proven to directly control a series of the metabolic and gene regulatory changes associated with HFD-feeding or fatty acid-induced insulin resistance and inflammation in adipocytes and macrophages (Rahman et al., 2012). Identifying the regulatory factor of GPR120 is important for understanding its biological roles. In the present study, we demonstrate that C/EBPβ stimulates GPR120 transcription. Moreover,
our data confirmed that GPR120, C/EBPβ, and PPAR-γ2 expression was induced with HFD feeding (Fig. 4D and E) and revealed an increased binding of C/EBPβ on the GPR120 promoter (Fig. 4F). The data indicated that C/EBPβ plays a vital role in regulating GPR120 transcription with HFD feeding.

Members of the C/EBP and PPAR families of transcription factors, particularly C/EBPβ and PPAR-γ2, have long been considered master regulators of adipocyte differentiation and adipogenesis (Lane et al. 1999, Rosen & MacDougald 2006). C/EBPβ is also essential for sustained expression of PPARγ, and transcription factor C/EBPβ binds to the promoter of PPAR-γ2 and induces its expression (Wu et al. 1995). These factors influence systemic metabolic functions synergistically under pathophysiological and physiological conditions and may help prevent obesity. The transcription factor C/EBPβ could bind to the GPR120 core promoter, stimulate the GPR120 expression, and up-regulate the expression of PPAR-γ2. We propose the possibility that the C/EBPβ-GPR120-PPAR-γ2 pathway might be able to influence the process of energy metabolism (Fig. 6). Therefore, further studies of targeting the C/EBPβ/GPR120/PPAR-γ2 axis may provide novel implications for the mechanism of energy metabolism. Further studies will be needed to address the possibility.

In conclusion, we discovered that C/EBPβ is a crucial regulatory factor for GPR120 transcription and identified the binding site of C/EBPβ and we suggest that HFD feeding induces GPR120 transcription by influencing C/EBPβ expression.

References

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-15-0200.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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