Irisin Stimulates Browning of White Adipocytes Through Mitogen-Activated Protein Kinase p38 MAP Kinase and ERK MAP Kinase Signaling

The number and activity of brown adipocytes are linked to the ability of mammals to resist body fat accumulation. In some conditions, certain white adipose tissue (WAT) depots are readily convertible to a “brown-like” state, which is associated with weight loss. Irisin, a newly identified hormone, is secreted by skeletal muscles into circulation and promotes WAT “browning” with unknown mechanisms. In the current study, we demonstrated in mice that recombinant irisin decreased the body weight and improved glucose homeostasis. We further showed that irisin upregulated uncoupling protein-1 (UCP-1; a regulator of thermogenic capability of brown fat) expression. This effect was possibly mediated by irisin-induced phosphorylation of the p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal–related kinase (ERK) signaling pathways. Inhibition of the p38 MAPK by SB203580 and ERK by U0126 abolished the upregulatory effect of irisin on UCP-1. In addition, irisin also promoted the expression of betatrophin, another newly identified hormone that promotes pancreatic β-cell proliferation and improves glucose tolerance. In summary, our data suggest that irisin can potentially prevent obesity and associated type 2 diabetes by stimulating expression of WAT browning-specific genes via the p38 MAPK and ERK pathways.

Obesity is the most common metabolic disorder in the world. Obesity develops when energy intake exceeds energy expenditure (1) and is principally characterized by excess adipose tissue (2,3). Obese subjects appear to have a high risk of developing type 2 diabetes (2). Increasing energy expenditure has emerged as a potential and attractive strategy to prevent obesity (4). Although regulators of the energy expenditure are not entirely clear, adipocytes appear to play a central role in modulating energy balance and nutrient flux in vertebrates.

Two different types of adipose tissue including white adipose tissue (WAT) and brown adipose tissue (BAT) have been widely studied. White adipocytes store energy (e.g., triglycerides), whereas brown adipocytes consume energy (5,6). Many studies have shown that the changes
in BAT activity can profoundly affect adaptive thermogenesis and glucose homeostasis (7,8). The thermogenic capability of brown fat is mainly mediated by the presence of a mitochondria uncoupling protein-1 (UCP-1), which uncouples the electron transport chain from energy production and results in the release of potential energy obtained from food as heat. The expression of UCP-1 is regulated by several transcriptional factors, including peroxisome proliferator–activated receptor γ (PPARγ) coactivator-1α (PGC-1α), which can be induced by cold exposure and/or β-adrenergic signaling (9). In several rodent models, browning of WAT depots appears to be protective against diet-induced metabolic disorders, including obesity and diabetes (10,11).

Irisin, secreted by skeletal muscles and increased with exercise, is a small polypeptide hormone containing 111 amino acids with an estimated molecular mass of 22 kDa. It has been shown that PGC-1α and exercise upregulate the expression of fibronectin type III domain containing 5 (Fndc5), a type I transmembrane protein of skeletal muscle. Fndc5 is then proteolysed at amino acid position 30 and 140 to give rise to irisin (12). Overexpression of irisin by adenoviral vector increases total body energy expenditure and results in the release of potential energy from food as heat. The expression of UCP-1 is induced by cold exposure and/or β-adrenergic signaling (9). In several rodent models, browning of WAT depots appears to be protective against diet-induced metabolic disorders, including obesity and diabetes (10,11).

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However, little is known about the molecular mechanism and signaling pathways mediating the induction of the functional “brown-like” adipocyte phenotype by irisin.

In the current study, we established an efficient system for the expression and purification of human recombinant irisin (r-irisin) in Pichia pastoris and delivered the r-irisin in mice through intraperitoneal injection. We also treated primary adipocytes and 3T3-L1-derived adipocytes with r-irisin to detect its direct effects on the fat cell browning process in order to understand the underlying “browning” mechanisms. Our study demonstrated that administration of r-irisin in vivo decreased body weight, promoted brown fat–specific gene expression in subcutaneous white adipose, and improved glucose tolerance, which is consistent with a previous study (12). In addition, from cell culture experiments, we showed that irisin stimulated brown fat–specific gene expression via the activation of the p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal–related kinase (ERK) pathways.

**RESEARCH DESIGN AND METHODS**

**Expression and Purification of Human Irisin From Pichia**

Human irisin cDNA (360 bp) was designed and synthesized by Life Technologies. The synthesized cDNA was cloned into EcoR1/Xba1 sites of the pPICZαA plasmid. *Pichia pastoris* X-33 was transformed with a linearized pPICZαA-irisin plasmid according to the kit manual (*Pichia* Easycomp Transformation Kit; Invitrogen). The culture of yeast and induction of protein expression were performed as previously described (20).

The r-irisin protein in the supernatant was purified by a two-step method (20). First, r-irisin was precipitated with 60% saturated ammonium sulfate. The precipitated proteins were collected and dissolved in buffer A (25 mmol/L HEPES, pH 7.9, 10% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, and 0.5 mmol/L dithiothreitol) and dialyzed against the same buffer. The resulting sample was loaded onto a concanavalin A–agarose column. The column was first washed with 0.2 mol/L KCl in buffer A, and proteins were eluted with 1.5 mol/L KCl in buffer A. The elution fractions were analyzed by Western blotting. The purified irisin was dialyzed against 10% glycerol in PBS and stored at −80°C.

**Site-Directed Mutagenesis of Irisin cDNA**

There are two N-glycosylation sites in human irisin protein (Asn7 and Asn52). To confirm the glycosylation, we constructed two mutant irisin cDNA clones, N7H and N52H, by using a site-directed mutagenesis kit (Stratagene). In brief, the pPICZαA-irisin plasmid was used as a template for site-directed mutagenesis PCR with the point-mutation primers. After *DpnI* digestion, the PCR fragments were transformed into *Escherichia coli* Top10. The cDNA of the clones was purified with a Qiagen plasmid midi-purification kit. Each mutant cDNA was sequenced completely to ensure that no other base changes were produced. The mutation primers were as follows: N7H forward, 5′-ttcatcgcgtcagctgcatctgtgacacttgagggc, and reverse, 5′-tgctcaaatggctctgctcgtgagcggatgagaaattc; N52H forward, 5′-gattttgagaaattcggatgagagagcggatgagaaattc; N52H forward, 5′-gattttgagaaattcggatgagagagcggatgagaaattc. The purified irisin was dialyzed against 10% glycerol in PBS and stored at −80°C.

**Cell Culture**

**Primary Adipocyte Culture**

Male Sprague-Dawley rats (200 g) were used in the study, and the adipocytes were isolated using a method as described previously (21). In brief, subcutaneous fat pads were minced with scissors and digested in type I collagenase (1 mg/mL; Sigma-Aldrich) at 37°C in a water bath shaker. After 90 min, the contents were immediately filtered in mesh and centrifuged at 1,000 rpm for 10 min. The layer of floating cells was then washed three times...
with PBS. Adipocytes were incubated in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with 10% FBS and 100 units/mL penicillin-streptomycin (Sigma-Aldrich) in an atmosphere of 5% CO₂ at 37°C.

**Differentiation of 3T3-L1 Preadipocytes Into Mature Adipocytes**

Murine preadipocyte (3T3-L1) cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and induced to differentiate into mature adipocytes as described previously (22). In brief, preadipocytes were cultured using basic medium (DMEM supplemented with 10% bovine calf serum and 100 units/mL penicillin-streptomycin) at 37°C in a 5% CO₂ incubator. After confluence (day 1), the cells were initiated for the adipocyte differentiation by incubating with induction medium (basic medium supplemented with 0.5 mmol/L isobutylmethylxanthine, 0.25 μmol/L dexamethasone, and 10 μmol/L insulin). Two days after induction (day 3), the medium was changed to basic medium supplemented with insulin only for an additional 2 days. On day 5, the medium was switched back to basic medium for another 2 days. r-irisin was added to the 3T3-L1 cells at day 3 in adipocyte differentiation for a total of 4 days stimulation. On day 7, 3T3-L1 derived adipocytes with or without r-irisin were processed for Oil Red O staining for confirming adipocyte differentiation, RNA extraction for reverse transcription-quantitative PCR (RT-qPCR), immunocytochemistry (ICC) with anti–UCP-1 antibodies, and Western blotting.

**Binding of Irisin on the Cell Membrane of Adipocytes**

On day 7 of differentiation, 3T3-L1–derived adipocytes were incubated with or without irisin for 30 min. After washing, the cells were incubated with anti-irisin rabbit polyclonal antibody (1:1,000; Phoenix Pharmaceuticals, Inc.) for 1 h. Then FITC–anti-rabbit IgG antibody was added and incubated for another hour. After washing, the cells were examined using a confocal fluorescence microscope. Additionally, a luciferase-irisin fusion protein was constructed into pREN2-irisin expression vector and produced as described previously (23). 3T3-L1–derived adipocytes were incubated in culture with either luciferase-irisin or luciferase. ICC was performed using antiluciferase antibody (Santa Cruz Biotechnology) as described above.

**RNA Isolation and Real-Time PCR**

Total RNA was extracted from cells with or without irisin treatment by using Trizol reagent (Invitrogen). Reverse transcription of 2 μg total RNA was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR in triplicate was performed with SYBR Green Master Mix (Applied Biosystems), and gene-specific primers are listed in Table 1. The 2^−ΔΔct method was used to calculate the relative expression of genes using β-actin RNA as an internal control.

**ICC**

3T3-L1–derived adipocytes were fixed with ice-cold methanol for 10 min at 4°C. The cells were then washed three times with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature. The cells were incubated with primary antibody of UCP-1 (1:500; Sigma-Aldrich, Oakville, ONT, Canada) overnight at 4°C. After washing, the cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody for 30 min at room temperature. After washing with PBS, the cells were incubated in avidin-biotinylated HRP for another 30 min. Diaminobenzidine was used for ICC and hematoxylin was used for counterstaining nuclei. Positive and negative expression of protein UCP-1 was represented by dark brown in cytoplasm, respectively.

### Table 1—Primer sequences for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP-1</td>
<td>AGGCTTCGAGTACACATTAGGT</td>
<td>CTGAGTGAAGCAGAGCTGATT</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>CCGTGCAGTTGATGAGCGAC</td>
<td>TGCTGCTGCTGCTGCTGCTG</td>
</tr>
<tr>
<td>COX7α</td>
<td>CTCTGCGGGAAGTTCATGCT</td>
<td>GATCGGGAGAGTTCATGCT</td>
</tr>
<tr>
<td>Elov13</td>
<td>TCTTCAACCAGGTTAATAAGTG</td>
<td>GAGCAACACAGTACAGGACCAC</td>
</tr>
<tr>
<td>PPARα</td>
<td>AGAGCCCATCAGTTCCTCCTC</td>
<td>ACTCGTATCGTACAAACAAA</td>
</tr>
<tr>
<td>TMEM26</td>
<td>TTCTATTTCTGTCCTCTGTC</td>
<td>GCCGGAAGAAGCAGTATT</td>
</tr>
<tr>
<td>Ebf3</td>
<td>TAACCCCTTCTTAAACCTGTA</td>
<td>GTTTCACGTCAAGCATGACAT</td>
</tr>
<tr>
<td>Cidea</td>
<td>TGCTTGGTCTATCGCCAG</td>
<td>GCCGTGTTAAGGATCGT</td>
</tr>
<tr>
<td>Prdm16</td>
<td>CTTGCCACATCGTCTGAT</td>
<td>CTGGAAACTCTGACGTC</td>
</tr>
<tr>
<td>aP2</td>
<td>GGAACACGTGGATGTTACTGAG</td>
<td>GGCAGACCTTTGATGCTT</td>
</tr>
<tr>
<td>Adipoq</td>
<td>GAGACCTGAGGAGAAGGAGACTACA</td>
<td>GTAGGGTAAGAAACAGACCG</td>
</tr>
<tr>
<td>Betatrophin</td>
<td>CAGGATTGCCACACAGGAGGCTTC</td>
<td>TCAGTGGCAGCTTGCAGCT</td>
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*Adipoq, adiponectin; Prdm16, PR domain containing 16.*
**Western Blotting**

An equal amount of proteins from cell lysates was loaded in each well of a 12% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes, blocked for 1 h with 5% fat-free milk at room temperature, and blotted with the indicated primary antibodies overnight at 4°C at 1:1,000 dilution (rabbit anti-irisin, anti–UCP-1 [Sigma-Aldrich], anti-ERK1/2, anti–phospho-ERK1/2, anti-AKT, anti–phospho-AKT, anti–phospho-JNK, anti-phospho-p38 MAPK, and anti-phospho-p38 MAPK antibodies [Cell Signaling Technology, Inc.]); rabbit anti-GAPDH antibody was used at 1:10,000 (Sigma-Aldrich). After washing with Tris-buffered saline with Tween, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Then, immune complexes were detected using the ECL method, and immunoreactive bands were quantified by densitometric analysis using Alpha Imager 2200.

**Animal Studies**

C57BL/6 mice fed with high-fat diet (HFD) (60% fat) for 10 weeks with an initial body weight in the range of 35–43 g were purchased from the animal center of Shandong University. Obese mice were randomly divided into two groups: control ($n = 8$) and r-irisin treated ($n = 8$). The mice were treated daily with purified r-irisin at a dose of 0.5 μg/g body weight/day by intraperitoneal injection for 14 days, and the mice in the control group were given daily saline at the same volume. At the end of r-irisin treatment (day 14), mouse body weights were recorded and some mice were subjected to intraperitoneal glucose tolerance test before being killed. Mouse subcutaneous fat tissues were harvested for immunohistochemistry and total RNA extraction for subsequent RT-qPCR.

**Statistical Analysis**

Results are presented as mean ± SE of at least three independent experiments, and each experiment was conducted in triplicate. Statistical significance among multiple groups was analyzed by one-way ANOVA followed by Student t test for comparison of the results between two groups using the SPSS software package (SPSS 17.0). $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Expression and Characterization of Purified Human Recombinant Irisin**

In order to achieve high-yield expression of human irisin gene in a yeast expression system, we designed and constructed an optimized irisin cDNA coding sequence for yeast codon usage. The synthesized gene and protein sequence of r-irisin are shown in Supplementary Fig. 1. The resulting plasmid pPICZA-irisin was transformed into *P. pastoris*. Eight clones were selected from 50 single colonies that grew on yeast extract peptone dextrose (YPD) containing zeocin (100 μg/mL) for testing the capacity of r-irisin protein expression. To examine the protein expression, the secreted r-irisin at various time points was separated by SDS-PAGE and stained with Coomassie brilliant blue. There were three protein bands present on the gel with a molecular weight of 25, 22, and 15 kDa (Fig. 1A). By Western blotting analysis, all three bands are human irisin protein (Fig. 1B). The protein identity of all three bands was also confirmed by MALDI-TOF mass spectrometry (Supplementary Fig. 2).

Human irisin protein has two putative N-glycosylation sites at asparagine 7 (Asn7) and asparagine 52 (Asn52). The two higher molecular weights (25 and 22 kDa) of the secreted irisin protein compared with the native form of irisin were likely glycosylated. Treatment of the purified r-irisin with PNGase F to remove N-linked glycans and subsequent Western blotting analysis revealed (Fig. 1C) that the enzyme-treated r-irisin exhibited a single band at low molecular weight (15 kDa), confirming that most of the secreted r-irisin expressed by the *P. pastoris* was glycosylated.
Figure 2—Effects of r-irisin and mutants on the expression of brown adipocyte-specific genes in 3T3-L1-derived adipocytes. 3T3-L1-derived adipocytes were treated with r-irisin (20 nmol/L) as described in RESEARCH DESIGN AND METHODS. A: Expression of brown/beige adipocyte marker, betatrophin, and white adipocyte marker (aP2 and adipoc) genes were measured by RT-qPCR. *P < 0.05; **P < 0.01 vs. untreated. B: Western blot and densitometric analysis for UCP-1 protein in cells with indicated treatments. **P < 0.01. C: Oil Red O staining for lipid droplets confirmed adipocyte differentiation (top) and quantification of UCP-1-positive cells in ICC using Imagepro Plus software (bottom). Data were expressed as mean ± SE of three independent experiments. **P < 0.01 vs. adipocytes without irisin. D: Biological function of mutant irisin. 3T3-L1-derived
To confirm the glycosylated sites, we mutated each of the two glycosylation sites at Asn7 or Asn52, individually. The mutant r-irisin protein from each mutant (N7H or N52H) showed only two bands with molecular weight 22 and 15 kDa. The 22-kDa protein likely represents the mutant irisin that was glycosylated on one of the two glycosylated sites (N7H or N52H) since this band was still sensitive to PNGase-F treatment (Fig. 1D). Our experiments confirm that human irisin indeed has two glycosylated sites at Asn7 and Asn52.

R-irisin Stimulates the Browning of Primary Adipocytes and 3T3-L1-Derived Adipocytes

To investigate the browning effect of r-irisin on adipocytes, we used both 3T3-L1-derived adipocytes and primary rat adipocytes as cellular models treated with or without human r-irisin as described in RESEARCH DESIGN AND METHODS. Gene expression studies showed that treating 3T3-L1-derived adipocytes with r-irisin (20 nmol/L) led to a rapid upregulation of brown cell markers: UCP-1, PGC-1α, transmembrane protein 26 (TMEM26), early B cell factor 3 (Ebf3), elongation of very long chain fatty acids-like 3 (Elovl3), cell death-inducing DNA fragmentation factor (Cidea), and cytochrome c oxidase, subunit VII α (Cox7α) (all P value <0.01) (Fig. 2A). UCP-1 is a special mitochondrial protein that dissipates chemical energy to heat. PGC-1α is a powerful transcriptional coactivator that is a master regulator of mitochondrial biogenesis, including UCP-1 and Fndc5 in brown fat cells. The increased expression of beige cell marker TMEM26 (24) and brown cell markers such as Cidea and Cox7α indicated that the r-irisin may exert effects on converting white adipose cells into beige-like adipocytes and brown adipose cells. Interestingly, treatment of adipocytes with r-irisin also significantly increased the expression of the betatrophin (ANGPTL8) gene (3.53 ± 0.97-fold, P < 0.01). Betatrophin, a newly identified pancreatic β-cell growth hormone, is primarily expressed in liver and fat in mice (25). This result suggests that increased betatrophin expression by irisin may, at least in part, explain improved insulin resistance by irisin.

The increased UCP-1 expression at the protein level by r-irisin in 3T3-L1-derived adipocytes was also demonstrated both by Western blotting (Fig. 2B) and ICC study.
Figure 4—Irisin stimulates adipocyte browning via p38/ERK pathways. Primary rat adipocytes and 3T3-L1–derived adipocytes were treated with PBS (control [con]) or r-irisin (20 nmol/L) at indicated time points. Phosphorylated and total p38 and ERK1/2 in cell lysates were analyzed by Western blotting with anti-P-p38 or anti-P-ERK antibodies. Expression levels of P-p38 and total p38 protein in primary adipocytes (A) and 3T3-L1 adipocytes (B) were measured by corresponding densitometric quantification. Expression levels of P-ERK and total ERK in primary (C) and 3T3-L1–derived (D) adipocytes were measured with corresponding densitometric quantification. Densitometric analysis of the related bands was expressed as relative optical density of the bands, corrected using respective total protein as a loading control and normalized against the untreated control. Data were expressed as mean ± SE of three independent experiments. *P < 0.05; **P < 0.01 vs. control. E: 3T3-L1–derived adipocytes were pretreated for 30 min with p38 inhibitor SB023580 or ERK inhibitor U0126 at indicated concentrations followed by r-irisin treatment. Western blotting detected phosphorylated and total p38 and ERK proteins.
(Fig. 2C, bottom) with confirmed adipocyte differentiation (Fig. 2C, Oil Red O staining, top). Quantification of UCP-1 ICC studies in r-irisin–treated 3T3-L1–derived adipocytes revealed a significant increase in UCP-1–positive adipocytes over controls (2.78 ± 0.63–fold, \( P < 0.01 \)) with multilocular lipid droplets compared with those treated with saline (Fig. 2C). Moreover, treatment of primary cultured rat adipocytes with r-irisin over a range of concentrations (2, 20, and 100 nmol/L) (Fig. 3) was able to significantly increase the expression of the UCP-1 and PGC-1α genes. Considering that UCP-1 mRNA was increased fourfold compared with those that were untreated using 20 nmol/L r-irisin, we chose 20 nmol/L r-irisin for the following experiments.

**Effect of Mutated Irisin on Browning**

To determine whether glycosylation in irisin has a significant effect on biological function, we next examined the expression of browning markers in 3T3-L1–derived adipocytes treated with Asn7- or Asn52-mutated irisin protein. As shown in Fig. 2D, the effects of upregulation of UCP-1 and PGC-1α by irisin was significantly reduced by the mutated irisin at either site. This loss of function was also confirmed at the protein level, with significant UCP-1 reduction compared with treatment with the wild-type irisin (Fig. 2E), suggesting that the posttranslational glycosylation of the secreted irisin from yeast enhances the biological function of the browning effect evidenced by increased expression of UCP-1 and PGC-1α. However, we cannot exclude the possibility that the decreased biological activity observed in the N7H and N52H mutant irisin was due to mechanisms other than the altered glycosylation status.

**Identifying Signaling Pathways of Irisin-Mediated Browning**

Although the browning function mediated by irisin has been extensively studied, the molecular signaling pathways that specifically mediate browning are unclear. Here we showed that p38 MAPK and ERK play a key role in

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**Figure 5**—Inhibiting p38 MAPK and ERK prevents the expression of UCP-1. Primary rat (A and C) and 3T3-L1 (B and D) adipocytes were pretreated with SB (10 μmol/L) or U0 (10 μmol/L) and both for 30 min, and then r-irisin (20 nmol/L) was supplemented for an additional 4 days. UCP-1 expression at mRNA level by qPCR analysis (A and B) and at protein levels by Western blotting (C and D) was examined. For C and D, the bar graph represents the ratio of UCP-1/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and is normalized against the control group. Data are expressed as mean ± SE of three independent experiments. *\( P < 0.05 \); **\( P < 0.01 \) vs. control group; ++\( P < 0.01 \); +\( P < 0.05 \) vs. irisin group.
mediating the irisin browning function. As shown in Fig. 4, the phosphorylated p38 MAPK (P-p38) and phosphorylated ERK (P-ERK) were significantly increased at as early as 5 min, peaked between 10 and 20 min, and decreased at 30 min after the r-irisin treatment in both primary rat (Fig. 4A and C) and 3T3-L1 adipocytes (Fig. 4B and D). The increased levels of the P-p38 and P-ERK proteins after the r-irisin treatment were statistically significant, as quantified by densitometry.

To further examine the possible involvements of p38 MAPK and ERK pathways in irisin-induced browning effects, 3T3-L1 adipocytes were treated with or without SB203580 (p-38MAPK inhibitor) or U0126 (ERK inhibitor) individually in the presence of irisin. P38 and ERK phosphorylation induced by irisin was gradually suppressed with increasing concentrations of the p38 and ERK inhibitors (Fig. 4E), whereas there was no reduction in the amount of total p38 or ERK protein. The irisin-induced UCP-1 upregulation was significantly reduced by either SB203580 or U0126. Moreover, the combination of both inhibitors almost completely abolished the effect of irisin-mediated increasing expression of UCP-1 mRNA (Fig. 5A and B) and protein (Fig. 5C and D) levels. These findings indicate that the irisin-induced browning effect marked by enhanced expression of the browning gene UCP-1 was mediated via activation of p38 MAPK and ERK signaling pathways.

A previous study showed that irisin significantly improves glucose tolerance and insulin resistance in the mice fed with HFD (12); the mechanism of this effect, however, was unknown. AKT is an important signaling molecule in the insulin signaling pathway. We therefore investigated the effect of irisin on the activation of AKT using an antibody to phosphorylated serine 473 of AKT. Unlike insulin (100 nmol/L), which can significantly promote the phosphorylation of AKT (Supplementary Fig. 3B), irisin stimulation of 3T3-L1 adipocytes showed no significant effects on the phosphorylation of AKT in all testing time points (Supplementary Fig. 3A). In addition, there was no additive or synergistic effect between insulin and irisin on the level of phosphorylation of AKT (Supplementary Fig. 3B), indicating that the AKT system was not involved in the browning action of irisin. Furthermore, we showed that treatment of adipocytes with irisin had no effect on the phosphorylation of c-Jun NH2-terminal kinases (JNKs), indicating that the JNK signaling pathway is not involved in the irisin-mediated browning process (Supplementary Fig. 3C).

### Detection of Irisin Binding to Cell Membrane of Adipocytes

So far, there is no direct evidence showing a specific membrane receptor for irisin in adipocytes. Based on the results of the activation of P-p38 and P-ERK signaling transduction by irisin, we infer that the adipocyte membrane might have receptors for irisin. Ligand binding to its membrane receptor has historically been determined using a radioactive isotopically labeled ligand. However, the potential hazards of radioisotope handling and the environmental impact of

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**Figure 6**—Binding of irisin to the plasma membrane of 3T3-L1 adipocytes. Confocal microscopy of adipocytes treated with irisin, anti-irisin antibody, and FITC-anti-rabbit IgG antibody (A). Fluorescence microscopy of adipocytes treated with luciferase as control or luciferase-irisin fusion protein (B). The red arrows indicate the cell membrane of the adipocytes bound with irisin fusion protein.
radioisotope disposal make this approach less desirable. Therefore, we used live cell binding methods to observe the binding property of irisin to the cell membrane. The 3T3-L1 adipocytes were briefly incubated with r-irisin. The r-irisin bound on the cell surface membrane was visualized by immunofluorescence after sequential incubation with anti-irisin antibody and FITC–anti-rabbit IgG antibody. As shown in Fig. 6A, the green fluorescence can only be seen on the membrane of irisin-treated cells. To further confirm the irisin binding to the cell membrane, we added luciferase-irisin fusion protein or luciferase alone (as control) into the cell culture medium. Irisin binding to the cell membrane was visualized by antiluciferase antibody. As shown in Fig. 6B, the green fluorescence was observed clearly with increasing intensity along the membrane on luciferase-irisin–incubated cells, whereas no clear membrane signal was observed on luciferase-treated cells. These data suggest that 3T3-L1–derived adipocytes may have yet-to-be-identified irisin receptor on the cell membrane.

**R-irisin Affects the Body Weight and Glucose Homeostasis In Vivo**

To examine potential therapeutic effects of r-irisin, we treated both normal and fat mice with daily injections of r-irisin for 14 days. Figure 7A shows that the mRNA levels of brown adipocyte marker genes, including UCP-1, PGC-1a, Cox7a, Elov3, PPARα, and Cidea, were significantly elevated in the adipose tissues of the normal mice receiving r-irisin (**P < 0.01). Similar gene expression results were also observed in adipose tissue isolated from fat mice fed with HFD (Supplementary Fig. 4). Consistent with 3T3-L1–derived adipocytes, the expression of betatrophin (ANGPTL8) was also significantly increased (*P < 0.05) in the adipose tissue of the mice treated with r-irisin compared with the control mice (Fig. 7B). The changes in browning gene expression in the subcutaneous adipose tissues were accompanied by an increase in the number of UCP-1–positive, multilocular adipocytes (Fig. 7C). The body weights of the r-irisin–treated fat mice were reduced after 14 days compared with the saline-treated control fat mice (Fig. 7D). R-irisin treatment of fat mice significantly improved glucose tolerance, as demonstrated by a quicker recovery curve in intraperitoneal glucose tolerance test (Fig. 7E) and significantly reduced levels of fasting insulin (Fig. 7F) when compared with the control fat mice. These results suggest that in vivo treatment of fat mice with r-irisin can decrease body weight, promotes the expression of brown-specific genes, and improves HFD-induced insulin resistance.
DISCUSSION
PGC-1α–dependent irisin, a novel myokine, is derived from cleaving Fndc5 protein. Irisin promotes brown fat–like development and thermogenesis in WAT both in vitro and in vivo. The discovery of irisin has created an opportunity to further understand the role of adipocytes in obesity, diabetes, and other associated metabolic disorders (12,13,26,27). However, the molecular mechanisms and cellular signaling pathways responsible for the browning effect of irisin have not been elucidated.

In this study, we successfully constructed the yeast expression plasmid containing a synthesized optimal codon usage, human irisin-coding sequence and generated pure recombinant irisin protein in P. pastoris with high yield that is fully biologically functional. The P. pastoris system is widely used for heterogenic protein expression, with the capacity to generate post-translational modified proteins (28). The human recombinant irisin protein expressed in yeast showed a predominant band of ~22 kDa, which is similar to the glycosylated irisin previously reported (12). Using PNGase-F digestion and site-directed mutagenesis analysis, we for the first time confirmed two glycosylation sites in human irisin located at the position of asparagine 7 and asparagine 52. Given the glycosylation, a common feature of secreted protein, of irisin in mice and humans (12), we site directly mutated the two glycosylation sites to detect the biological effect of the mutant irisin. As shown in Fig. 2D and E, posttranslational glycosylation of the secreted irisin enhances the expression of UCP-1 and PGC-1α, compared with mutated irisin, indicating that yeast-produced irisin is superior to that produced in a bacterial expression system.

It is now clear that there are two distinct types of brown adipocytes. One is the classical brown fat that arises from a myf5, muscle-like cell lineage (29,30). Another is the brown adipocytes that are found interspersed within the WAT, in response to chemical or hormonal stimulation, environmental changes, cold exposure, and defined genetic manipulation (31–33). These brown fat cells, UCP-1 positive, are designated as beige or brown-in-white (brite) cells (11,34). In the current study, we demonstrated that r-irisin indeed increased the expression of thermogenic genes, including UCP-1, PGC-1α, Cox7a, Efb3, and Elovl3, in both primary and cell line–derived adipocytes. We also found that the expression of TMEM26, a marker of beige cells, was increased when r-irisin was added to the cultured 3T3-L1 preadipocytes during the differentiation. Wu et al. (24) also showed that beige cell precursors have preferential sensitivity to the browning effect of irisin. Our results showed that irisin significantly elevated the expression of TMEM26, which suggests that irisin may influence the number of beige-like cells.

Irisin functions as a muscle-derived energy expenditure signal that directly communicates with adipose tissue and induces browning. It is therefore important to identify the molecular pathways mediating the browning function by irisin. Boström et al. (12) identified PPARα as a potential factor for the expression of UCP-1, but other pathways may also be involved. PGC-1α, a master regulator of mitochondrial biogenesis and function, acts as a cold-inducible protein that controls adaptive thermogenesis in BAT, and its expression is regulated by the activation of cAMP/PKA/p38 MAPK signaling pathways (35,36). Moreover, other molecules, such as AMPK and PPARβ, have been suggested as exercise mimetics (37,38). Our data show that irisin significantly increased the levels of P-p38 MAPK and P-ERK in primary adipocytes and 3T3-L1–derived adipocytes, and this effect was abolished by inhibiting p38 and ERK phosphorylation (Fig. 5) to prevent irisin-induced UCP-1 expression. We suggest that the p38 and ERK signaling pathways play a central role in the irisin-induced emergence of brown adipocytes.

Our study further suggests a possible existence of an irisin-specific receptor on the membrane of adipocytes. Although more experiments are needed to characterize and validate the existence and specificity of this receptor, our study at least provides the first evidence for its existence.

In conclusion, the recombinant irisin increases the expression of thermogenic genes in vitro and in vivo. It stimulates expression of brown cell–specific genes in adipocytes via p38 and ERK signaling pathways. This effect is likely mediated through a specific membrane receptor on adipocytes. Our findings have provided initial experimental evidence in supporting possible therapeutic usage of recombinant irisin for the treatment of obesity and diabetes as well as associated metabolic disorders.

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