miR-30 Promotes Thermogenesis and the Development of Beige Fat by Targeting RIP140

Members of the microRNA (miR)-30 family have been reported to promote adipogenesis and inhibit osteogenesis, yet their role in the regulation of thermogenesis remains unknown. In this study, we show that miR-30b/c concentrations are greatly increased during adipocyte differentiation and are stimulated by cold exposure or the β-adrenergic receptor activator. Overexpression and knockdown of miR-30b and -30c induced and suppressed, respectively, the expression of thermogenic genes such as UCP1 and Cidea in brown adipocytes. Forced expression of miR-30b/c also significantly increased thermogenic gene expression and mitochondrial respiration in primary adipocytes derived from subcutaneous white adipose tissue, demonstrating a promoting effect of miRNAs on the development of beige fat. In addition, knockdown of miR-30b/c repressed UCP1 expression in brown adipocyte tissue in vivo. miR-30b/c targets the 3'-untranslated region of the receptor-interacting protein 140 (RIP140), and overexpression of miR-30b/c significantly reduced RIP140 expression. Consistent with RIP140 as a target of miR-30b/c in regulating thermogenic gene expression, overexpression of RIP140 greatly suppressed the promoting effect of miR-30b/c on the expression of UCP1 and Cidea in brown adipocytes. Taken together, the data from our study identify miR-30b/c as a key regulator of thermogenesis and uncover a new mechanism underlying the regulation of brown adipose tissue function and the development of beige fat.

Brown adipose tissue (BAT) plays a major role in energy expenditure and nonshivering thermogenesis, and impaired BAT function is associated with obesity and metabolic disorders (1). Deletion of BAT-specific uncoupling protein 1 (UCP1) causes increased body weight gain under thermo-neutral conditions (2). By contrast, an increase in BAT mass or enhanced BAT function is associated with a lean and healthy phenotype in animals caused by increased energy expenditure (3,4), suggesting that improving BAT function could be a promising therapeutic strategy to treat obesity and related metabolic diseases.

The recent discovery of inducible brown fat cells, known as "beige" cells, in subcutaneous white adipose tissue (sWAT) indicates the existence of a distinct type of thermogenic fat cells (5). Beige cells are capable of triggering a program of respiration and energy expenditure by inducing the expression of UCP1 (6,7). Indeed, the presence of UCP1-positive cells has been found not only in sWAT of rodents but also in the neck and upper-chest region of humans (8). The induction of UCP1 expression and the thermogenic program are under the control of several key positive transcriptional regulators, including peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α), the peroxisome proliferator–activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein β, and PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM16) (9–12).

Receptor-interacting protein 140 (RIP140), also known as nuclear receptor–interacting protein 1 (NIRIP1), is a corepressor of genes implicated in glucose uptake, glycolysis, the tricarboxylic acid cycle, fatty acid oxidation, mitochondrial biogenesis, and oxidative phosphorylation in major metabolic tissues such as fat, muscle, liver, and heart (13,14). RIP140-null mice are leaner and exhibit resistance to obesity induced by a high-fat diet (15).
RIP140 deficiency also leads to increased UCP-1 gene expression in WAT of mice (15). As a transcriptional corepressor of UCP1, RIP140 functions through histone and DNA methylation by recruiting DNA methyltransferase, the COOH-terminal binding protein, histone methyltransferase, and histone deacetylase on the UCP1 promoter (16,17). RIP140 was recently shown to block the browning program in WAT by preventing the expression of brown fat genes and inhibiting a triacylglycerol futile cycle (18). However, how RIP140 is regulated in cells remains elusive.

MicroRNAs (miRNAs) are a class of short noncoding RNAs (22–24 nucleotides) that regulate mRNA translation and stability by binding to the complementary sequences in the 3′ untranslated region (UTR) of target genes. Several miRNAs were recently identified in BAT; these play important roles in regulating the differentiation and metabolism of brown adipocytes (19). MiR-193b-365, a brown, fat-enriched miRNA gene cluster, upregulates the expression of PRDM16 and PPARγ and promotes brown fat differentiation by directly targeting negative regulators of brown adipogenesis (20). MiR-133, on the other hand, negatively regulates brown adipogenesis and thermogenesis by repressing the expression of PRDM16 (21). We recently identified the miR-106b/93 cluster as a negative regulator of brown adipogenesis (20). RIP140, on the other hand, negatively regulates brown adipogenesis and thermogenesis by repressing the expression of PRDM16 (21). We recently identified the miR-106b/93 cluster as a negative regulator of brown adipocyte differentiation (22).

In this study, we investigated the roles of miR-30 family members in the regulation of thermogenesis. We found that the expression of miR-30 family members is greatly increased during brown adipocyte differentiation, and the expression of these miRNAs is induced by cold exposure or the β-adrenergic receptor activator. In addition, overexpression of miR-30b and miR-30c induced thermogenesis in BAT and increased UCP1 expression in sWAT. On the other hand, knockdown miR-30b/c decreased UCP1 expression in BAT in vitro and in vivo. We found that miR-30b/c suppresses the expression levels of RIP140, suggesting the potential involvement of RIP140 in miR-30b/c-mediated regulation of thermogenic gene expression. Our study highlights an important role of miR-30 family members in regulating BAT function and uncovers a potential new mechanism regulating the browning/beiging process in adipose tissues.

RESEARCH DESIGN AND METHODS

Cell Culture and Transfection

Cells from a brown preadipocyte cell line, which was kindly provided by Dr. J. D. Lin (University of Michigan, Ann Arbor, MI [23]), were maintained in DMEM (Gibco) containing FBS and penicillin and streptomycin. To induce preadipocyte differentiation, confluent cells were exposed to a differentiation cocktail containing isobutylmethyl xanthine, dexamethasone, and insulin (Sigma-Aldrich). After 2 days of induction, differentiation culture medium was replaced and cells were incubated in fresh DMEM containing 20 nmol/L insulin and 1 nmol/L 3,5,3′-tri-iodo-L-thyronine for an additional 5–6 days until multiple small lipid droplets accumulated in the cytoplasm.

Stromal vascular fractions (SVFs) from interscapular BAT and inguinal sWAT were isolated and cultured as previously described (24). Briefly, BAT or sWAT from 3-week-old male C57BL/6 mice were quickly removed and minced. The tissue pieces were digested in HEPES buffer containing type II collagenase (Sigma-Aldrich) and BSA with shaking. After centrifugation and washing with PBS, the preadipocytes were seeded on a culture plate and induced to differentiation using the methods described above.

To transfet miRNA mimics or inhibitors, the designed duplex oligonucleotide (miRNA mimics) or single-stain antisense (miRNA inhibitors) for miR-30 family members or nonspecific control oligos purchased from GenePharma, Inc. (Shanghai, China), were transfected into the cells at a final concentration of 100 nmol/L using GM siRNA-mate (GenePharma), according to the manufacturer’s instructions. After transfection (48 h), cells were induced to differentiation using the standard protocol.

For treatment, the preadipocytes incubated with fresh DMEM were treated with or without CL-316,243, a selective β3-adrenergic receptor activator; isoprotrenol, a nonselective β-adrenergic receptor activator; or Forskolin, a cellular cAMP inducer. Cells were collected 24 h after treatment and stored at −80°C for further analyses.

Cold Temperature Exposure

Eight-week-old C57BL/6 male mice were kept at room temperature (25°C) or cold temperature (4°C). In both groups, each mouse was maintained in a single cage on a 12-h light/12-h dark cycle with free access to water and food. After 7 days, interscapular BAT and inguinal sWAT were isolated and subjected to further analyses.

miRNA Agomir/Antagomir Treatment In Vivo

miRNA agomirs and antagonirs are chemically modified and cholesterol-conjugated stable miRNA mimics or inhibitors. In vivo delivery of these molecules has resulted in target gene silencing or upregulation (25,26). We used a mixture of miRNA-30b and -30c agomir or antagonimir to activate or repress, respectively, the expression of miR-30b/c in vivo. Briefly, 8-week-old male C57B6J mice received agomir (10 nmol) or antagonimir-30b/c (20 nmol) or their respective negative controls (RiboBio, Guangzhou, China) through subcutaneous injection. Three days after injection, mice were killed and tissues were collected. The expression of miRNAs was verified by real-time RT-PCR, and the expression of target proteins was determined by Western blot.

Mitochondrial Respiration Assay

To determine the mitochondrial respiration activities, the O2 concentration in the cells was measured using an XF24 extracellular flux analyzer (Seahorse Bioscience,
北威尔明顿，MA）。简言之，细胞被接种在24孔细胞培养板（Seahorse Bioscience）。线粒体基质呼吸在未处理细胞中被评估。细胞随后被用含奥拉明（Sigma-Aldrich）的4-三氟甲氧基苯酚的溶液处理（FCCP；Sigma-Aldrich），一个电子运输的阻断剂，抗氧化磷化物；在短端，线粒体呼吸被被阻断到由核糖核酸和抗核糖核酸A（Sigma-Aldrich）。氧气消耗率（OCR）被计算为在细胞中氧气浓度的二分之一。在该条件下，细胞成为了一个自由流动的微环境，细胞的温度变化被最小化。

由于目的，miR-30b/c抑制或上调在活体中细胞因子和组织因子的表达，使用miRNA agomiRs/antiagomiRs或相应的对照实验。OCR是在分别用miR-30b/c模拟物/抑制物或空白载体与miR-30b/c模拟物的细胞中被计算。

通过测量miRNA的相对表达量来评估miR-30b/c抑制或上调的效应。miRNA的相对表达量通过ΔΔCT法被计算。

Western Blotting

为了确定蛋白质浓度，细胞或组织被使用在RIPA缓冲液中。蛋白质被通过SDS-PAGE和转移到硝酸纤维素膜。质膜在4°C下过夜，与主要的初级抗体：β-抗体（Cell Signaling Technology），anti-UCP1（Santa Cruz Biotechnology），或anti-RIP140（Abcam），随后被用过氧化氢酶-偶联的次级抗体。信号被使用ChemidetectionTM系统（Bio-Rad）来检测。

Statistical Analysis

统计学分析是通过SPSS软件版本19.0（SPSS Inc., Chicago, IL）来执行。所有数据被表示为均值±标准误差。统计学意义被通过Student t test确定。
were compared or one-way ANOVA when more than two groups were compared. \( P < 0.05 \) was considered statistically significant.

RESULTS

miR-30b and miR-30c Are Abundantly Expressed in BAT and Are Upregulated During Brown Adipocyte Differentiation

To study the potential role of miRNAs in regulating brown adipocyte function, we examined miRNA expression during brown adipocyte differentiation by microarray experiments. This study led to the identification of several miRNAs whose expression levels are dynamically altered during adipocyte differentiation, including two members of the miR-30 family, miR-30b and miR-30c. By real-time RT-PCR, we found that both of these miR-30 family members were significantly upregulated during brown adipocyte differentiation (Fig. 1A), which was in parallel with the expression pattern of UCP1 (Fig. 1B). To further confirm this result, we examined the expression of miR-30b and miR-30c in BAT-derived primary adipocytes. The expression levels of both miR-30b and miR-30c were significantly increased during primary brown adipocyte differentiation (Fig. 1C), providing further evidence of a potential role for these miRNAs in regulating adipocyte function. To test this further, we examined the expression levels of miR-30 family members in three different mouse fat depots, including inguinal sWAT, perigonadal visceral white adipose tissue, and interscapular BAT. The expression levels of both miR-30b and miR-30c were significantly higher in BAT compared with those in either sWAT or visceral white adipose tissue (Fig. 1D). Taken together, these results strongly suggest that miR-30b and miR-30c may play critical roles in regulating brown adipocyte function and energy homeostasis. Consistent with this, the expression levels of miR-30b and miR-30c were dramatically decreased in BAT of ob/ob mice compared with wide-type (WT) littermates (Fig. 1E).

miR-30b and miR-30c Are Required for Thermogenic Gene Expression and Mitochondrial Respiration in Brown Adipocytes

To determine the roles of miR-30b and miR-30c in brown adipocytes, we transfected brown preadipocytes with miR-30b/c mimic mixtures or nonspecific oligonucleotide controls. Overexpression of the miR-30b/c mimics dramatically upregulated miR-30b and -30c (Fig. 2A) but not other miR-30 family members, including miR-30a, -30d, -30e, and -384 (data not shown). Treating brown adipocytes with the miR-30b/c mimics dramatically induced mRNA levels of UCP1 and Cidea, two thermogenic genes abundantly expressed in mouse BAT (28) (Fig. 2A). Oil-red O staining showed that treating brown adipocytes with antisense oligonucleotides that specifically target both miR-30b and miR-30c had no significant effects on triglyceride accumulation in brown adipocytes (data not shown), but this significantly suppressed the mRNA levels of UCP1 and Cidea (Fig. 2B). Overexpression or inhibition of miR-30b and miR-30c had no significant effects on the expression of other genes examined, including PRDM16, PGC-1α, PPARγ, C/EBPα, C/EBPβ, ELVOL3, AP2, and adiponectin (data not shown). Interestingly, overexpression or inhibition of miR-30b or -30c alone had no significant effects on UCP1 or Cidea expression (data not shown).

Because UCP1 is a hallmark of brown adipocytes that dissipates mitochondrial proton gradients to generate heat, we evaluated the respiration of cells treated with miR-30b and -30c inhibitors using the Seahorse Bioscience XF24 respirometry analyzer. Suppression of miR-30b/c led to a marked decrease in basal OCR (Fig. 2C). In addition, treatment of brown adipocytes with an ATP synthase inhibitor (oligomycin) or a chemical uncoupler (FCCP) significantly lowered OCRs (Fig. 2C), suggesting a causative role for miR-30b and miR-30c in the suppression of mitochondrial respiration.

miR-30b and miR-30c Induce Thermogenic Gene Expression and Mitochondrial Respiration in sWAT

To investigate the potential role of miR-30b/c in the beiging effect of WAT, we isolated and cultured sWAT-derived SVFs and analyzed the expression pattern of miRNAs during adipocyte differentiation. Consistent with the results found in brown adipocytes, the expression levels of both miR-30b and -30c were upregulated during adipocyte differentiation (Fig. 3A). In addition, overexpression of miR-30b and -30c significantly induced UCP1 and Cidea expression in SVFs derived from sWAT (Fig. 3B), indicating that miR-30b/c could induce the beiging effect in sWAT. Consistent with this, the basal and oligomycin-treated OCRs were significantly high in miR-30b/c overexpressed sWAT (Fig. 3C), suggesting an increase in mitochondrial activity.

Expression of miR-30b and miR-30c Is Induced by Cold Exposure In Vivo and by the β-Adrenergic Receptor Activator and cAMP Inducer in Primary Adipocytes

To provide further evidence of the role of miR-30b/c in thermogenesis, we examined miRNA expression in mice maintained at room temperature (25°C) or exposed to cold (4°C). The expression levels of miR-30b/c were significantly increased in BAT (Fig. 4A) and in sWAT (Fig. 4B) in response to cold exposure. Treatment of preadipocytes with the selective β3-adrenergic receptor activator CL-316,243, nonselective β-adrenergic receptor activator isoproterenol, or the cAMP inducer Forskolin significantly upregulated miR-30b and -30c expression (Fig. 4C), indicating a cell autonomous effect of the β-adrenergic receptor signaling pathway on miR-30b and -30c expression. Overexpression of miR-30b/c further enhanced CL-316,243-induced gene expression of UCP1 and Cidea (Fig. 4D and E), suggesting a β-adrenergic receptor–independent function of miR-30b/c in the regulation of thermogenesis.

Identification of Nuclear Corepressor RIP140 as a Target of miR-30b/c

In silico analysis using online programs including TargetScan 6.2 (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/) predicted a potential miR-30
target, RIP140 (gene Nrip1), which has been shown to function as a corepressor of thermogenic genes including UCP1 and Cidea (13,14). Bioinformatic analysis showed that the miR-30 targeting sites on the RIP140 3' UTR sequence are highly conserved in vertebrates, including mice, humans, chimpanzees, and rats (Fig. 5A).
Figure 2—miR-30b/c modulates the expression of thermogenic genes and mitochondrial respiration in brown adipocytes. A: Brown preadipocytes were transfected with miR-30b and -30c mimics (miR-30b/c) or nonspecific controls (miR-NC) for 48 h, followed by induction of differentiation for 3 days. At day 4 after induction, cells were harvested and the levels of miR-30b and miR-30c, as well as mRNA levels of UCP1 and Cidea, were determined by real-time RT-PCR \((n = 3; * \ P < 0.05; *** \ P < 0.001)\). B: Brown preadipocytes were transfected with miR-30b and -30c inhibitors (anti-miR-30b/c) or nonspecific controls (anti-miR-NC) for 48 h and then induced to differentiation. The relative levels of miR-30b and miR-30c, as well as mRNA levels of UCP1 and Cidea, were analyzed by RT-PCR \((n = 3; ** \ P < 0.01; *** \ P < 0.001)\). C: Brown preadipocytes were transfected with miR-30b and -30c inhibitors or controls for 48 h. The basal levels of cell OCRs and levels in the presence of ATP synthase inhibitor oligomycin, uncoupler FCCP, or rotenone/antimycin A were determined using a Seahorse Bioscience XF24 respirometry analyzer \((n = 4; * \ P < 0.05; ** \ P < 0.01; *** \ P < 0.001)\).
Overexpression of miR-30b and -30c induces thermogenic gene expression and mitochondrial respiration in white adipocytes.

**A**: Relative expression levels of miR-30b and -30c during the differentiation of SVF-derived white adipocytes ($n = 3$; *$P < 0.05$; **$P < 0.01$).

**B**: SVF-derived preadipocytes were transfected with miR-30b and -30c mimics (miR-30b/c) or nonspecific controls (miR-NC) for 48 h and then subjected to differentiation. At day 4 after the induction of differentiation, cells were harvested and relative levels of miRNA (miR-30b/c) and mRNA (UCP1, Cidea) were determined by real-time PCR ($n = 3$; *$P < 0.05$; ***$P < 0.001$).

**C**: SVF-derived preadipocytes were transfected with miR-30b/c or miR-NC for 48 h. The basal levels of cell OCRs and levels in the presence of ATP synthase inhibitor oligomycin, uncoupler FCCP, or rotenone/antimycin A were determined using a Seahorse Bioscience XF24 respirometry analyzer ($n = 4$; **$P < 0.01$ vs. miR-NC).
and Western blot experiments showed that both the mRNA and protein levels of RIP140 were significantly upregulated at days 2 and 4 after differentiation induction but were markedly reduced at the later stage (days 6 and 8) of the brown adipocyte differentiation process (Fig. 5B). The expression pattern of RIP140 is negatively correlated with those of miR-30b and -30c and UCP1 during brown adipocyte differentiation (Fig. 1A and B). Based on luciferase report assays, we found that overexpression of the miR-30 mimics significantly reduced RIP140 3′ UTR

Figure 4—Upregulation of miR-30b and -30c by cold exposure and by β-adrenergic receptor signaling. C57BL/6 mice were maintained at room temperature (25°C) or exposed to cold (4°C) for 1 week. The relative levels of miRNAs and UCP1 in BAT (A) and sWAT (B) of the mice were analyzed (n = 4–5; *P < 0.05; **P < 0.01, ***P < 0.001 vs. 25°C). C: SVF-derived preadipocytes were cultured and induced to differentiation. On day 4 after induction of differentiation, cells were treated with the β3-adrenergic receptor activator CL-316,243, the nonselective β-adrenergic receptor activator isoproterenol, the cAMP inducer Forskolin, or a vehicle control for 24 h. Relative miR-30b and -30c levels were determined by RT-PCR (n = 6; *P < 0.05; **P < 0.01). SVF-derived preadipocytes were transfected with miR-30b and -30c mimics (miR-30b/c) or nonspecific controls (miR-NC) for 48 h and then subjected to differentiation. At day 4 after induction, cells were treated with β-adrenergic receptor activator CL-316,243 or vehicle control for 24 h, and relative mRNA levels of UCP1 (D) and Cidea (E) were determined by real-time PCR (n = 6; *P < 0.05; ***P < 0.001).
reported gene activity (Fig. 5C), indicating that miR-30b/c could directly target the 3’ UTR sequence of RIP140. To determine whether targeting the 3’ UTR influences the protein expression of RIP140, we cotransfected HEK293T cells with a plasmid encoding WT or mutant RIP140 3’ UTR or an empty plasmid, together with the miR-30b and -30c mimics. Overexpression of WT but not the mutant miR-30 targeting sequence significantly reduced RIP140 protein levels (Fig. 5D), providing further evidence that miR-30 family members could downregulate the expression of RIP140 by directly targeting 3’ UTR sequence.

RIP140 Mediates the Effects of miR-30 on UCP1 Expression in Adipocytes

RIP140 could negatively regulate thermogenic gene expression and represses the beigeing program in WAT (13,14,18). To further investigate the role of miR-30 in the regulation of RIP140 expression, we transfected brown adipocytes with mimics or inhibitors of miR-30b/c. RT-PCR and Western blot analyses showed that overexpression of miR-30b/c significantly reduced both the mRNA (Fig. 6A) and protein levels (Fig. 6B) of RIP140. On the other hand, inhibition of miR-30b/c significantly increased the mRNA (Fig. 6C) and protein levels (Fig. 6D) of RIP140. To determine whether RIP140 plays a role in the regulation of thermogenic gene expression by miR-30b/c, we transfected preadipocytes with RIP140 expression plasmid together with or without miR-30b/c mimics. Overexpression of miR-30b/c mimics significantly increased the expression of UCP1 and Cidea (Fig. 6E). The stimulatory effect of miR-30b/c, however, was significantly suppressed by coexpression of RIP140 (Fig. 6E), indicating that RIP140 acts downstream of miR-30b/c. Together, these findings reveal that miR-30b/c regulates thermogenic gene expression by suppressing RIP140 in brown adipocytes.

Knockdown of miR-30b/c Decreases UCP1 Expression and Mitochondrial Respiration in BAT In Vivo

To determine whether miR-30b/c are involved in thermogenesis in vivo, we administrated a miR-30b/c agomir or antagonir, or their respective controls, to mice through subcutaneous injection. The expression of miR-30b/c in mouse BAT was efficiently increased or decreased by an agomir or antagonir, respectively (Fig. 7A and C). Consistent with the findings observed in cultured cells, administration of the miR-30b/c antagonir significantly upregulated the expression of RIP140 and greatly downregulated UCP1 expression in the BAT (Fig. 7D). Suppressing miR-30b/c in vivo also significantly decreased tissue respiration (Fig. 7E), further demonstrating an important role for miR-30b/c in the regulation of thermogenic gene expression in vivo. Antagomir injection had no obvious effects on the expression of UCP1 (Fig. 7B), however, probably because, under this condition, RIP140 is already low and further suppressing its levels will not increase UCP1 expression.

DISCUSSION

In this study, we identified miR-30b and -30c as key regulators of brown adipocyte function. The expression
Figure 6—RIP140 mediates the effects of miR-30 on thermogenic gene expression in the brown adipocytes. Preadipocytes were transfected with miR-30 mimics (A and B) or inhibitors (C and D) and stimulated to undergo differentiation. Differentiating adipocytes were collected for either RT-PCR or Western blot analyses to determine expression levels of mRNAs (A and C) or proteins (B and D), respectively (n = 3; *P < 0.05; **P < 0.01 vs. respective controls). E: Brown adipose cells were transfected with blank or an RIP140 expression construct or cotransfected with or without miR-30 mimics. After transfection (48 h), cells were collected and gene expression was analyzed by RT-PCR (n = 4; *P < 0.05; **P < 0.01; ***P < 0.001).
Figure 7—Knockdown of miR-30b/c decreases UCP1 expression and mitochondrial respiration in mouse BAT in vivo. Eight-week-old male C57BJ6 mice received agomir, antagomir-30b/c, or their respective scrambled negative control (NC) through subcutaneous injection. Three days after injection, BATs were collected and the expression of miRNAs was verified by real-time RT-PCR (A and C; n = 6/group; *P < 0.05; **P < 0.01 vs. respective NCs). B: The expression of RIP140 and UCP1 was determined by Western blot (upper blots) and statistical analysis (lower graph) in BAT of mice injected with an miRNA agomir or control (n = 4; *P < 0.05). D: The expression of RIP140 and UCP1 in BAT of mice injected with an miRNA antagomir or control (n = 6; *P < 0.05). E: The basal levels of OCRs in BAT from mice injected with an miRNA antagomir or control were determined using a Seahorse Bioscience XF24 respirometry analyzer (n = 5; *P < 0.05).
levels of miR-30b/c were greatly increased during brown adipocyte differentiation. In addition, overexpression of miR-30b and -30c dramatically increased UCP1 expression in brown adipocytes and in primary white adipocytes. Furthermore, suppression of miR-30 expression downregulated UCP1 levels both in vitro and in vivo. Finally, we identified RIP140 as a direct target of miR-30b/c, and overexpression of RIP140 alleviated the promoting effects of miR-30b/c on thermogenic gene expression. Taken together, the data from our study demonstrate for the first time that miR-30 family members are key positive regulators of thermogenesis and the beigeing process of WAT.

Although members of the miR-30 family share an identical seed sequence and have common predicted targets (29,30), they exert distinct functions in various cells and tissues. For example, miR-30a is important for kidney development in Xenopus (31), miR-30c promotes adipocyte differentiation (32) and reduces hyperlipidemia and atherosclerosis (33), and miR-30e is involved in the reciprocal regulation of osteoblast and adipocyte differentiation (34). However, miRNAs of the same family also exhibit similar regulatory modes and tend to coordinate to regulate target gene expression. Bridge et al. (35) showed that miR-30b and miR-30c target Δ-like 4, a membrane-bound ligand of Notch signaling, in vitro and in vivo and regulate angiogenesis in endothelial cells. The synergic action of miR-30b and -30c on thermogenic gene expression might be related to the secondary structures of the mRNA, in which the target sites are located and/or required for RNA deadenylation or sequestration, which is important for mRNA–mRNA interactions and gene repression (36).

In the current study, the expression levels of miR-30b and miR-30c were greatly stimulated by cold stress and by the β-adrenergic receptor activators and a cAMP inducer. Nonshivering thermogenesis induced by cold exposure is activated by the sympathetic nervous system, which stimulates the release of norepinephrine and increases intracellular cAMP levels, leading to the activation of protein kinase A and downstream pathways. Recent studies, however, suggest that cold temperature could stimulate UCP1 expression and thermogenesis by activating multiple signaling and β-AR–cAMP/CREB-dependent and -independent pathways (37,38). Increasing miR-30 expression might be one of the responses that coordinate with other factors to regulate UCP1 expression in response to cold. Interestingly, we found that overexpression of miR-30b and -30c potentiated β-adrenergic receptor activator–induced thermogenic gene expression, suggesting a positive feedback loop of miR-30 family members on the β-adrenergic receptor signaling and action.

Our data strongly suggest that suppressing RIP140 is an important mechanism by which miR-30c and -30b regulate thermogenic gene expression. First, luciferase assays showed that miR-30 directly targets the 3′ UTR of the RIP140 gene. Second, overexpression and knockdown of miR-30b and miR-30c decreased and increased, respectively, both mRNA and protein levels of RIP140 in adipocytes as well in BAT in vivo. Third, the promoting effects of miR-30b/c on thermogenic gene expression could be suppressed by overexpression of RIP140. Consistent with these findings, RIP140 has been shown to play essential roles in regulating metabolic function (14), and disrupting the expression of this protein increases resistance against high-fat diet–induced obesity in mice (15). A recent study demonstrated that a large set of brown fat–associated genes was upregulated in the absence of RIP140 in white adipocytes (18). In line with these studies, we found that overexpression of RIP140 downregulated the expression of UCP1, Cidea, and PGC-1α, and the suppressing effects could be reversed by the presence of miR-30b/c. Interestingly, RIP140 may use different modes of action to suppress Cidea and UCP1 expression. RIP140 negatively regulates Cidea expression by suppressing the expression and activity of PGC-1α (39). On the other hand, the protein directly targets the UCP1 promoter, leading to histone and DNA methylation of the promoter and thus silencing UCP1 expression in adipocytes (16). In our study, we found that overexpression or knockdown of miR-30 had no effects on PGC-1α expression (data not shown), indicating that other factors—but not PGC-1α—might be involved in miR-30-regulated thermogenic gene expression. Further investigations are needed to address this question.

In summary, we discovered miR-30 family members as positive regulators of thermogenic gene expression and mitochondrial respiration. In addition, we showed that miR-30b and miR-30c promote thermogenic events by targeting RIP140 transcription. The study provides new insight into the mechanisms regulating thermogenesis and energy metabolism.

Acknowledgments. The authors thank Dr. Yan Wu from the Metabolic Syndrome Research Center of Central South University, Changsha, Hunan, China, for advice about technique and helping with the in vivo studies.

Funding. This work was supported by grants from the National Science Foundation of China (grant no. 31471131 to F.H.), the International Science & Technology Cooperation Program of China (grant no. 2014DFG32490 to F.H.), and the National Basic Research Program of China (grant no. 2014CB910501 to F.L.).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. F.H. designed the experiments, analyzed data, and drafted the manuscript. M.W., T.X., B.Y., and W.M. performed the experiments. L.H. and M.D. prepared the figures. F.L. supervised the experiments, analyzed data, and edited and revised the manuscript. F.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References
1. Lowell BB, S-Susulic V, Hamann A, et al. Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. Nature 1993;366:740–742
2. Feldmann HM, Golozoubova V, Cannon B, Nedergaard J. UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. Cell Metab 2009;9:203–209
3. Kopecky J, Clarke G, Enerbäck S, Spiegelman B, Kozak LP. Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J Clin Invest 1995;96:2914–2923

4. Arch JR. beta(3)-Adrenoreceptor agonists: potential, pitfalls and progress. Eur J Pharmacol 2002;440:99–107

5. Wu J, Boström P, Sparks LM, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell 2012;150:366–376

6. Xue B, Coulter A, Rim JS, Koza RA, Kozak LP. Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. Mol Cell Biol 2005;25:8311–8322

7. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. Cell 2014;159:20–44

8. Jespersen NZ, Larsen TJ, Peijs L, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. Cell Metab 2013;17:798–805

9. Rosen ED, Sarraf P, Troy AE, et al. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell 1999;4:611–617

10. Seale P, Bjork B, Yang W, et al. PRDM16 controls a brown fat/skeletal muscle switch. Nature 2008;454:961–967

11. Kajimura S, Seale P, Kubota K, et al. Initiation of myoblast to brown fat switch by a PRDM16-C/EBP-beta transcriptional complex. Nature 2009;460:1154–1158

12. Seale P, Conroe HM, Estall J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. J Clin Invest 2011;121:96–105

13. Friih A, Christian M, Parker MG. The metabolic coregulator RIP140: an update. Am J Physiol Endocrinol Metab 2010;299:E335–E340

14. Naultiy J, Christian M, Parker MG. Distinct functions for RIP140 in development, inflammation, and metabolism. Trends Endocrinol Metab 2013;24:451–459

15. Leonardsson G, Steel JH, Christian M, et al. Nuclear receptor corepressor RIP140 regulates fat accumulation. Proc Natl Acad Sci U S A 2004;101:8437–8442

16. Kiskinis E, Hallberg M, Christian M, et al. RIP140 directs histone and DNA methylation to silence Ucp1 expression in white adipocytes. EMBO J 2007;26:4831–4840

17. Rytini MM, Palvimo JJ. SUMOylation attenuates the function of PGC-1alpha. J Biol Chem 2009;284:26184–26193

18. Kiskinis E, Chatzellis L, Curry E, et al. RIP140 represses the adipogenic program including a futile cycle of triacylglycerol breakdown and synthesis. Mol Endocrinol 2014;28:344–356

19. Trajkovski M, Lodish H. MicroRNA networks regulate development of brown adipocytes. Trends Endocrinol Metab 2013;24:442–450

20. Sun L, Xie H, Mori MA, et al. Mir193b-365 is essential for brown fat differentiation. Nat Cell Biol 2011;13:958–965

21. Trajkovski M, Ahmed K, Esau CC, Stoffel M. MyomiR-133 regulates brown fat differentiation through Prdm16. Nat Cell Biol 2012;14:1330–1335

22. Wu Y, Zhuo J, Zhang Y, et al. Identification of miR-ten6b-93 as a negative regulator of brown adipocyte differentiation. Biochem Biophys Res Commun 2013;438:575–580

23. Uldry M, Yang W, St-Pierre J, Lin J, Seale P, Spiegelman BM. Complementarity of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. Cell Metab 2006;3:333–341

24. Cotten B, Nedergaard J. Cultures of adipose precursor cells from brown adipose tissue and of clonal brown-adipocyte-like cell lines. Methods Mol Biol 2001;155:213–224

25. Wang X, Guo B, Li Q, et al. miR-214 targets ATF4 to inhibit bone formation. Nat Med 2013;19:93–100

26. Yang M, Wei Y, Jiang F, et al. MicroRNA-133 inhibits behavioral aggregation by controlling dopamine synthesis in locusts. PLoS Genet 2014;10:e1004206

27. Kiefer FW, Vernet A, O’Brien P, et al. Retinaldehyde dehydrogenase 1 regulates a thermogenic program in white adipose tissue. Nat Med 2012;18:918–925

28. Nordstrom EA, Ryden M, Backlund EC, et al. A human-specific role of cell death-inducing DFFA (DNA fragmentation factor-alpha)-like effector A (CIDEA) in adipocyte lipolysis and obesity. Diabetes 2005;54:1726–1734

29. Lewis BP, Shih H, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003;115:787–798

30. Li K, Li Z, Zhao N, et al. Functional analysis of microRNA and transcription factor synergistic regulatory network based on identifying regulatory motifs in non-small cell lung cancer. BMC Syst Biol 2013;7:122

31. Agrawal R, Tran U, Wessely O. The miR-30 miRNA family regulates Xenopus pronephros development and targets the transcription factor Xlim1/Lhx1. Development 2009;136:3927–3936

32. Karbierer M, Neuhold C, Opiessnig P, Prokesch A, Bogner-Strauss JG, Scheideler M. MicroRNA-30c promotes human adipocyte differentiation and comproresses PAI-1 and ALK2. RNA Biol 2011;8:850–860

33. Söh J, Isibai J, Queiroz J, Fernandez-Hernando C, Hussain MM. MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. Nat Med 2013;19:892–900

34. Wang J, Guan X, Guo F, et al. miR-30e reciprocally regulates the differentiation of adipocytes and osteoblasts by directly targeting low-density lipoprotein receptor-related protein 6. Cell Death Dis 2013;4:e845

35. Bridge G, Monteiro R, Henderson S, et al. The microRNA-30 family targets Dicer1 to modulate endothelial cell behavior during angiogenesis. Blood 2012;120:5063–5072

36. Nilsen TW. Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet 2007;23:243–249

37. Wijers SL, Schrauwen P, van Baak MA, Saris WH, van Marken Lichtenbelt WD. Beta-adrenergic receptor blockade does not inhibit cold-induced thermogenesis in humans: possible involvement of brown adipose tissue. J Clin Endocrinol Metab 2011;96:E598–E605

38. Ye L, Wu J, Cohen P, et al. Fat cells directly sense temperature to activate thermogenesis. Proc Natl Acad Sci U S A 2013;110:12480–12485

39. Hallberg M, Morganstein DL, Kiskinis E, et al. A functional interaction between RIP140 and PGC-1alpha regulates the expression of the lipid droplet protein CIDEA. Mol Cell Biol 2008;28:6785–6795