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

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SIGNAL TRANSDUCTION

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 adipose 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 thermoneutral 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 (NRIP1), 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).

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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 beigeing 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 PRMD16 (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/beigeing 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 transfect 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; isoproterenol, 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 antagomirs 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 antagomir to activate or repress, respectively, the expression of miR-30b/c in vivo. Briefly, 8-week-old male C57BJ6 mice received agomir (10 nmol) or antagomir-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 O_2 concentration in the cells was measured using an XF24 extracellular flux analyzer (Seahorse Bioscience,

North Billerica, MA). Briefly, cells were seeded in a 24-well cell culture microplate (Seahorse Bioscience). The mitochondrial basal respiration in untreated cells was assessed. The cells then were treated with oligomycin (Sigma-Aldrich) to measure the ATP turnover. The maximum respiratory capacity was assessed by adding carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP; Sigma-Aldrich), a chemical uncoupler of electron transport and oxidative phosphorylation; in the end, mitochondrial respiration was blocked by rotenone and antimysin A (Sigma-Aldrich). The oxygen consumption rate (OCR) was calculated by plotting the O_2 tension of the medium in the microenvironment above the cells as a function of time (picomoles per minute).

To determine the effect of miR-30b/c suppression or upregulation in vivo, adipose tissues were isolated from mice treated with miRNA agomirs/antagomirs or respective scrambled controls. OCR was determined according to the procedure reported by Kiefer et al. (27). In brief, freshly isolated BAT was rinsed with Krebs Henseleit buffer, and the tissues in the absence of large vessels were cut into small pieces and washed extensively. Approximately 10 mg of tissue was placed in each well of an XF24-well Islet Flux plate (Seahorse Bioscience) and covered with a customized screen that allows free perfusion while minimizing tissue movement. Basal OCRs were measured in all wells by a XF24 extracellular flux analyzer, and the data were normalized to tissue weight.

Construction of RIP140 Expression Vectors

Complementary DNAs for the mouse RIP140 gene were obtained from total RNAs prepared from mouse tissues by an RT-PCR technique using a Reverse Aid First Strand cDNA Synthesis Kit (Thermo Scientific). The forward primer sequence was 5′-CGAGGTA CCGAACGGAAGCCG AGCCCCTGTGAG-3′ and the reverse sequence 5′-GCG GATATCC CAAACTGGACTGGCAGGTACAC-3′. The PCR products were digested with Kpn I and *Eco*R V (Takara Bio, Shiga, Japan), purified from agarose gels, and subcloned into pcDNA3.1$^+$ (Invitrogen). The PmeI (NEB) and In-Fusion HD Liquid kits (Clontech, Takara Biotechnology, Dalian, China) were used to construct the pWPI + RIP140 and pWPI + RIP140 + 3′ UTR plasmids (Addgene, Cambridge, MA). All constructs were confirmed by DNA sequencing. The cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Luciferase Reporter Assay

The 3′ UTR sequence for the RIP140 gene was amplified by PCR with specific primers: forward, 5′-AGCTTTGTT TAAACATGTGTACCTGCCATACCACTTTG-3′; reverse, 5′-GAATGCGGCCGCCTGGTGAAGTTGTGCATTT-3′. The PCR product was cloned into a pmiR-RB-REPORT vector (RiboBio) between Xhol and Notl sites downstream of the *Renilla* luciferase gene. To generate the mutant construct, four nucleotides in the target site (TGTTTAC) of the miRNA-30b/c seed region were mutated (TCTATTG). HEK293T cells were transfected with RIP140 3′ UTR luciferase reporter or mutant or blank vector alone with miR-30b/c mimic mixture or mimic controls using Lipofectamine 2000. After cotransfection (48 h), cells were collected and firefly and *Renilla* luciferase activities were measured with a Dual-Glo luciferase assay system (Promega), according to the manufacturer's instructions. Luciferase activities were calculated as the ratio of firefly to *Renilla* luminescence and normalized to the average ratio of the blank control.

RNA Isolation and Real-Time RT-PCR

Total RNAs were isolated and prepared using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription of mRNA was conducted using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCRs using the FastStart Universal SYBR Green Master (Roche) were carried out on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The primer sequences for the genes tested were as follows: UCP1 forward, 5′-TACCAAGCTGTGCGATGTCCA-3′, reverse, 5′- GCACACAAACATGATGACGTTCC-3′; RIP140 forward, 5′-TAAACAGCCCTCTGC TCTCAA-3′, reverse, 5′-TTCTTCTCATTCTTCCCTTTGC-3′; Cidea forward, 5′-AAAGGGACAGAAATGGACACC-3′, reverse, 5′-TACATCG TGGCTTTGACATTG-3′; and β-actin forward, 5′-CAACGAGC GGTTCCGATG-3′, reverse, 5′-GCCACAGGATTCCATACCCA-3′. The relative levels of mRNA transcripts to control β-actin were determined by $2^{-\Delta\Delta CT}$. For miRNA analysis, cDNAs were synthesized from 1 μg of RNA with the One-Step PrimeScript miRNA cDNA Synthesis Kit (Takara Bio) and subjected to real-time PCR using the SYBR Premix Ex Taq II (Takara Bio) following the manufacturer's protocols. The mature miRNA sequence for mouse miR-30b-5p (MIMAT0000130) was UGUAAACAUCCUACACUCAGCU and for mouse miR-30c-5p (MIMAT0000514) was UGUA AACAUCCUACACUCUCAGC. The quantitative PCR primers against mature miRNAs (cat. nos. MmiRQP0393, MmiRQP0396) were purchased from GeneCopoeia (Rockville, MD), and the expression levels of microRNAs were normalized to that of U6 expression.

Western Blotting

To determine protein concentrations, cells or tissues were lysed in RIPA lysis buffer containing a protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated at 4°C overnight with one of the following primary antibodies: anti-β-actin (Cell Signaling Technology), anti-UCP1 (Santa Cruz Biotechnology), or anti-RIP140 (Abcam), followed by incubation with horseradish peroxidase–conjugated secondary antibodies. Signals were detected using ChemiDOCTMXRS+ and the Image LabTM system (Bio-Rad).

Statistical Analysis

Statistical analysis was performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL). All data were expressed as means ± SEM. Statistical significance was determined using the Student *t* test when two groups

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. 1*A*), which was in parallel with the expression pattern of UCP1 (Fig. 1*B*). 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 induced during primary brown adipocyte differentiation (Fig. 1*C*), 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. 1*D*). 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. 1*E*).

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 oilgonucleotide controls. Overexpression of the miR-30b/c mimics dramatically upregulated miR-30b and -30c (Fig. 2*A*) 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. 2*A*). 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. 2*B*). 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. 2*C*). In addition, treatment of brown adipocytes with an ATP synthase inhibitor (oligomycin) or a chemical uncoupler (FCCP) significantly lowered OCRs (Fig. 2*C*), 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 beigeing 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. 3*A*). In addition, overexpression of miR-30b and -30c significantly induced UCP1 and Cidea expression in SVFs derived from sWAT (Fig. 3*B*), indicating that miR-30b/c could induce the beigeing effect in sWAT. Consistent with this, the basal and oligomycin-treated OCRs were significantly high in miR-30b/c overexpressed sWAT (Fig. 3*C*), 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. 4*A*) and in sWAT (Fig. 4*B*) 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. 4*C*), 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. 4*D* 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/) predicated a potential miR-30

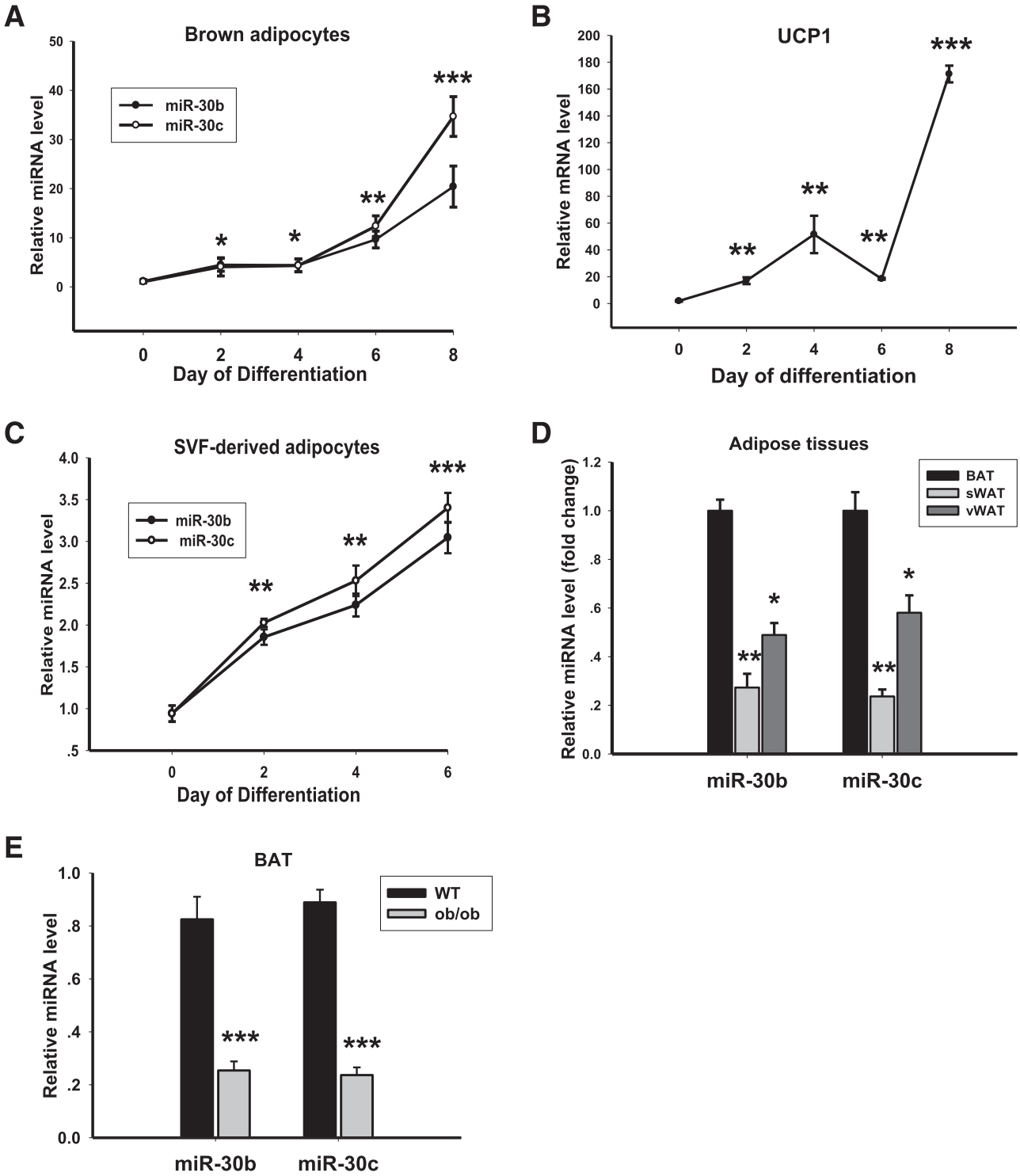

Figure 1—Expression profiles of miR-30 family members in brown adipocytes and fat tissues. *A*: Relative expression levels of miR-30b and -30c during brown adipocyte differentiation (n = 3 independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001). *B*: Relative UCP1 mRNA levels during brown adipocyte differentiation (n = 3; **P < 0.01; ***P < 0.001 vs. day 0). *C*: Relative expression levels of miR-30b and -30c during the differentiation of SVF-derived brown adipocytes (n = 3; **P < 0.01; ***P < 0.001). *D*: Relative expression levels of miR-30b and -30c in fat tissues of C57BL/6 mice (n = 4; *P < 0.05; **P < 0.01). *E*: Expression levels of miR-30b and -30c in BAT of ob/ob and WT mice (n = 6; ***P < 0.001 vs. WT mice). vWAT, visceral white adipose tissue.

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. 5*A*). RT-PCR

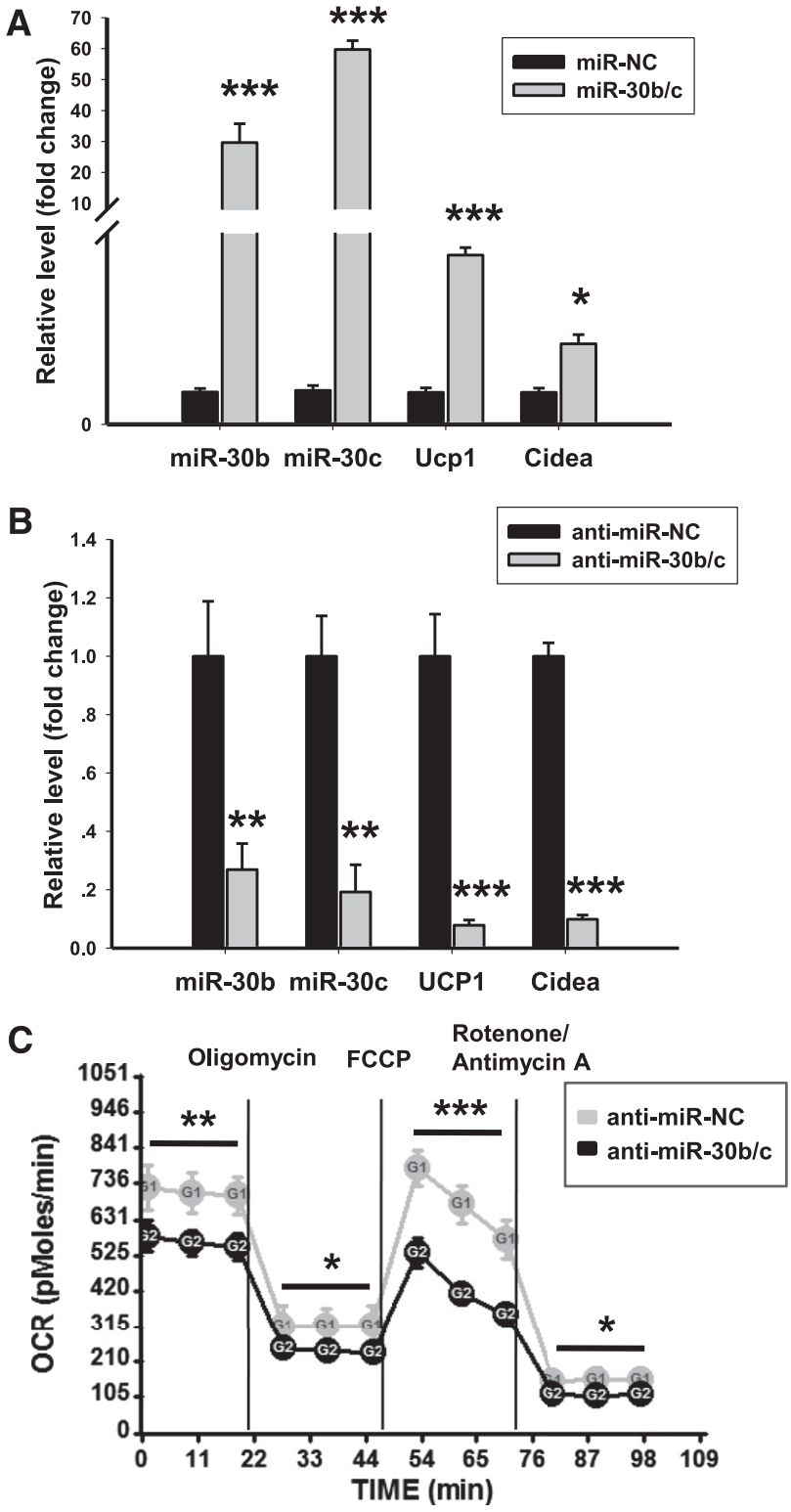

Figure 2—miR-30b/c modulates the expression of thermogenic genes and mitochondrial respiration in brown adipocytes. *A*: Brown preadipocytes were transfected with miR-30b and -30c mimics (miR-30b/c) or nonspecific controls (miR-NC) for 48 h, followed by induction of differentiation for 3 days. At day 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; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

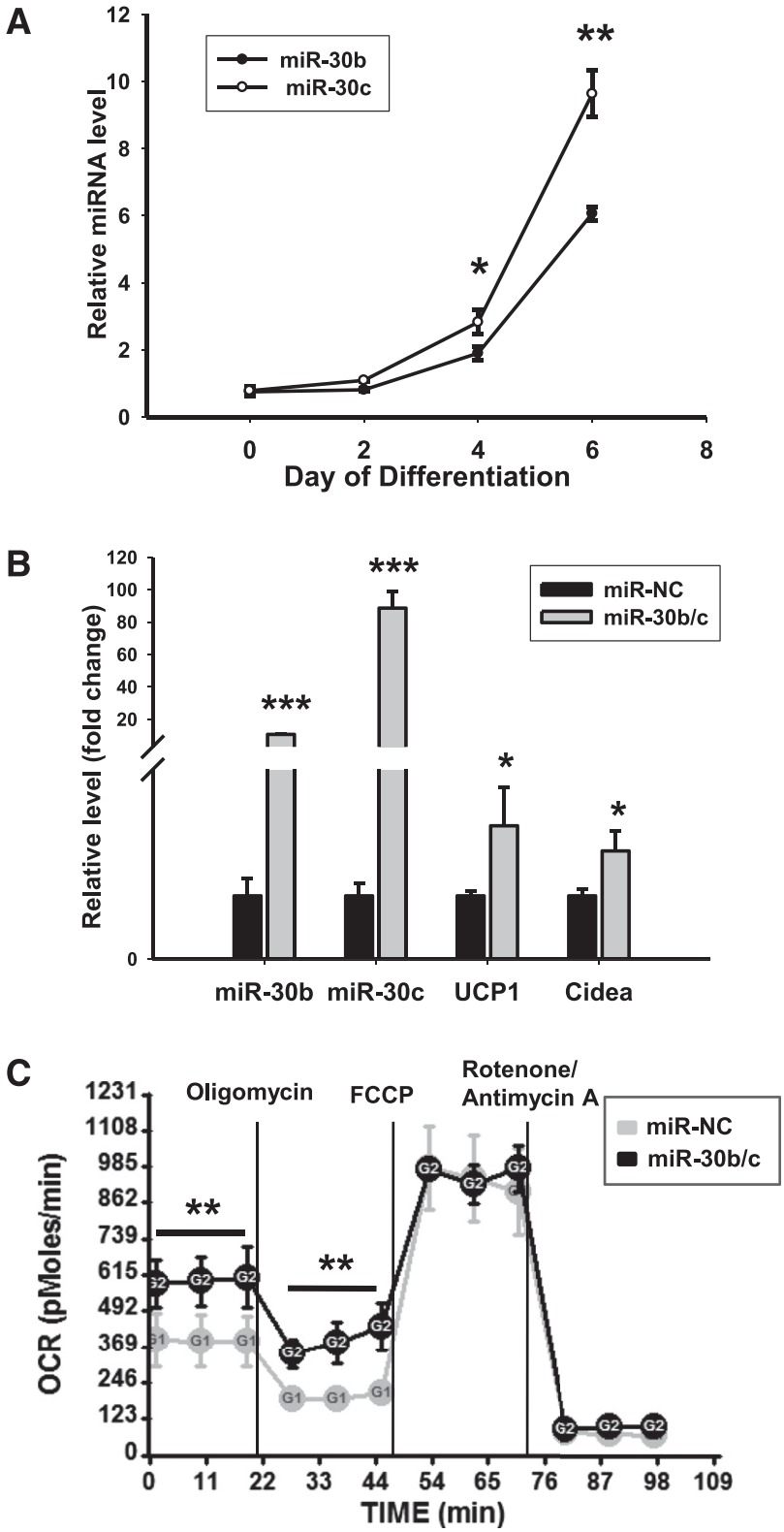

Figure 3—Overexpression of miR-30b and -30c induces thermogenic gene expression and mitochondrial respiration in white adipocytes. *A*: Relative expression levels of miR-30b and -30c during the differentiation of SVF-derived white adipocytes ($n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$). *B*: SVF-derived 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).

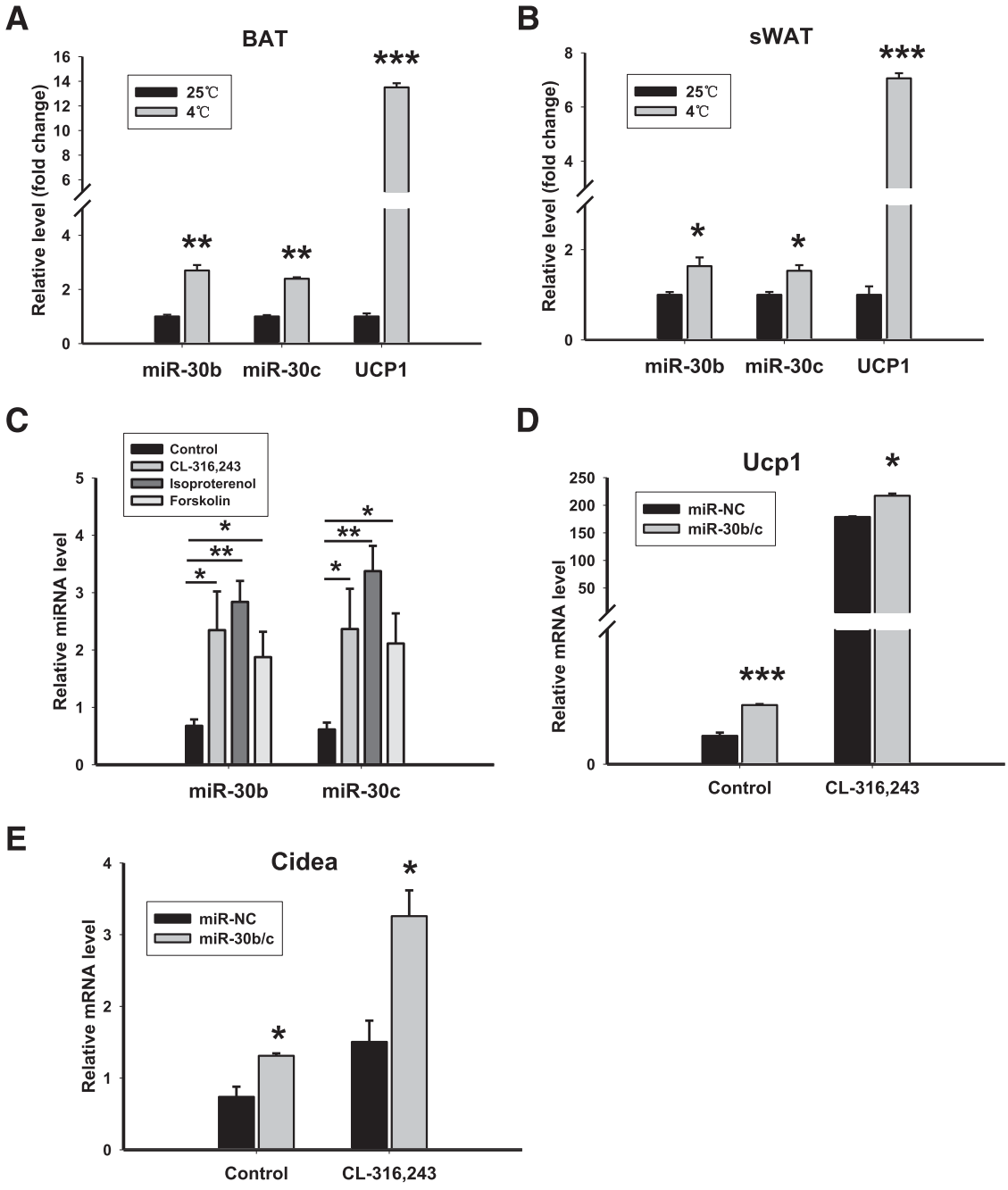

Figure 4—Upregulation of miR-30b and -30c by cold exposure and by β-adrenergic receptor signaling. C57BL/6 mice were maintained at room temperature (25°C) or exposed to cold (4°C) for 1 week. The relative levels of miRNAs and UCP1 in BAT (*A*) and sWAT (*B*) of the mice were analyzed (n = 4–5; *P < 0.05; **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).

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. 5*B*). The expression pattern of RIP140 is negatively correlated with those of miR-30b and -30c and UCP1 during brown adipocyte differentiation (Fig. 1*A* and *B*). Based on luciferase report assays, we found that overexpression of the miR-30 mimics significantly reduced RIP140 3′ UTR

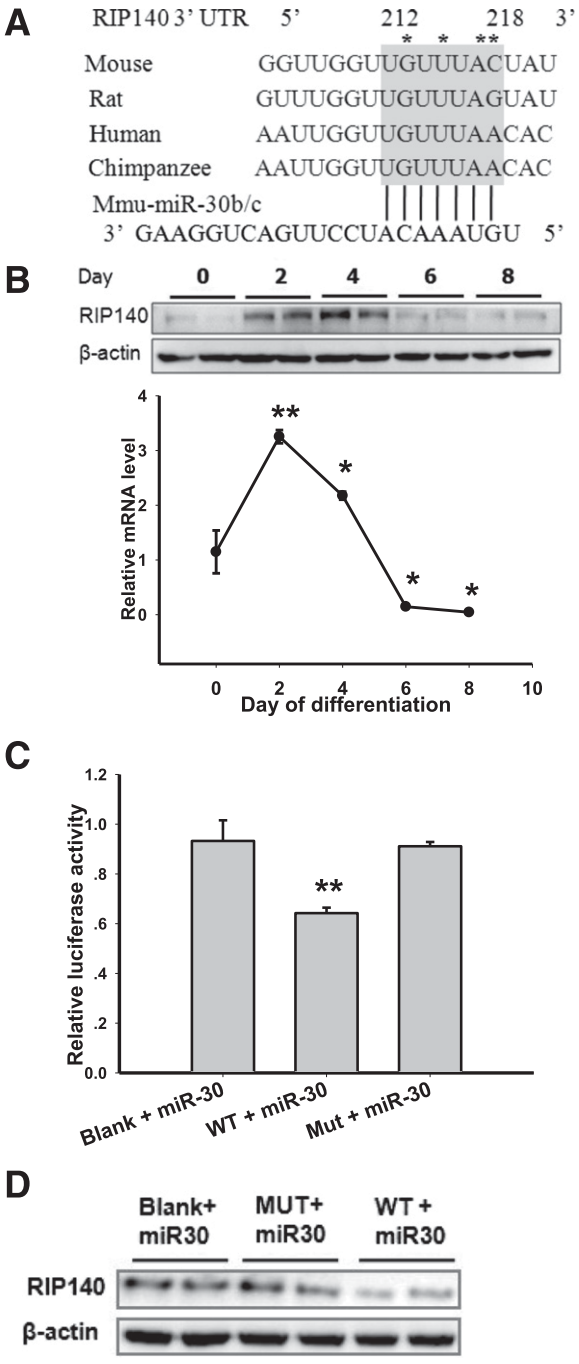

Figure 5—miR-30b and -30c target RIP140. *A*: The 3′ UTR sequence of the *RIP140* gene is conserved in vertebrates, including mice, humans, chimpanzees, and rats. The gray block demonstrates the miR-30 targeting sites of the 3′ UTR, and asterisks show the mutant points. *B*: Expression profiles of RIP140 mRNA (lower graph) and protein (upper blots) during the differentiation of brown adipocytes (n = 3; *P < 0.05; **P < 0.01 vs. day 0). *C*: HEK293T cells were cotransfected with or without miR-30 mimics, together with WT or mutant (Mut) RIP140 3′ UTR inserted downstream of the firefly luciferase gene construct. After transfection (48 h), cells were collected and relative luciferase activity was determined using a Dual-Glo luciferase assay system (n = 4; **P < 0.01 vs. blank and Mut). *D*: HEK293T cells were cotransfected with miR-30b/c mimics (miR-30) and blank or 3′ UTR–RIP140 expression construct (WT) or 3′ UTR mutant–RIP140 construct (MUT). After transfection (48 h), cells were collected and RIP140 expression was analyzed by Western blot.

reported gene activity (Fig. 5*C*), 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. 5*D*), 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. 6*A*) and protein levels (Fig. 6*B*) of RIP140. On the other hand, inhibition of miR-30b/c significantly increased the mRNA (Fig. 6*C*) and protein levels (Fig. 6*D*) 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. 6*E*). The stimulatory effect of miR-30b/c, however, was significantly suppressed by coexpression of RIP140 (Fig. 6*E*), 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 antagomir, 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 antagomir, respectively (Fig. 7*A* and *C*). Consistent with the findings observed in cultured cells, administration of the miR-30b/c antagomir significantly upregulated the expression of RIP140 and greatly downregulated UCP1 expression in the BAT (Fig. 7*D*), Suppressing miR-30b/c in vivo also significantly decreased tissue respiration (Fig. 7*E*), further demonstrating an important role for miR-30b/c in the regulation of thermogenic gene expression in vivo. Agomir injection had no obvious effects on the expression of UCP1 (Fig. 7*B*), 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

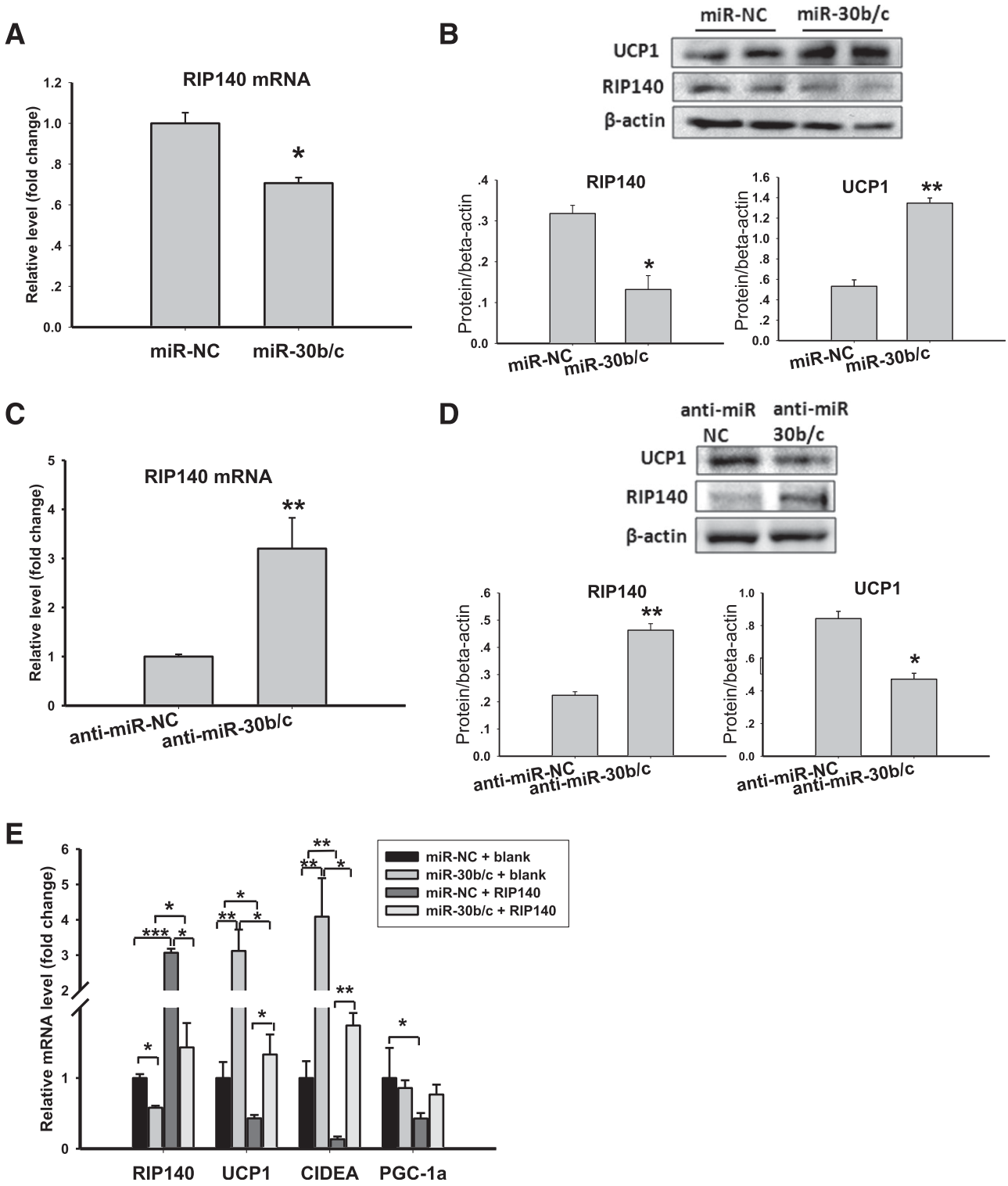

Figure 6—RIP140 mediates the effects of miR-30 on thermogenic gene expression in the brown adipocytes. Preadipocytes were transfected with miR-30 mimics (*A* and *B*) or inhibitors (*C* and *D*) and stimulated to undergo differentiation. Differentiating adipocytes were collected for either RT-PCR or 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 ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

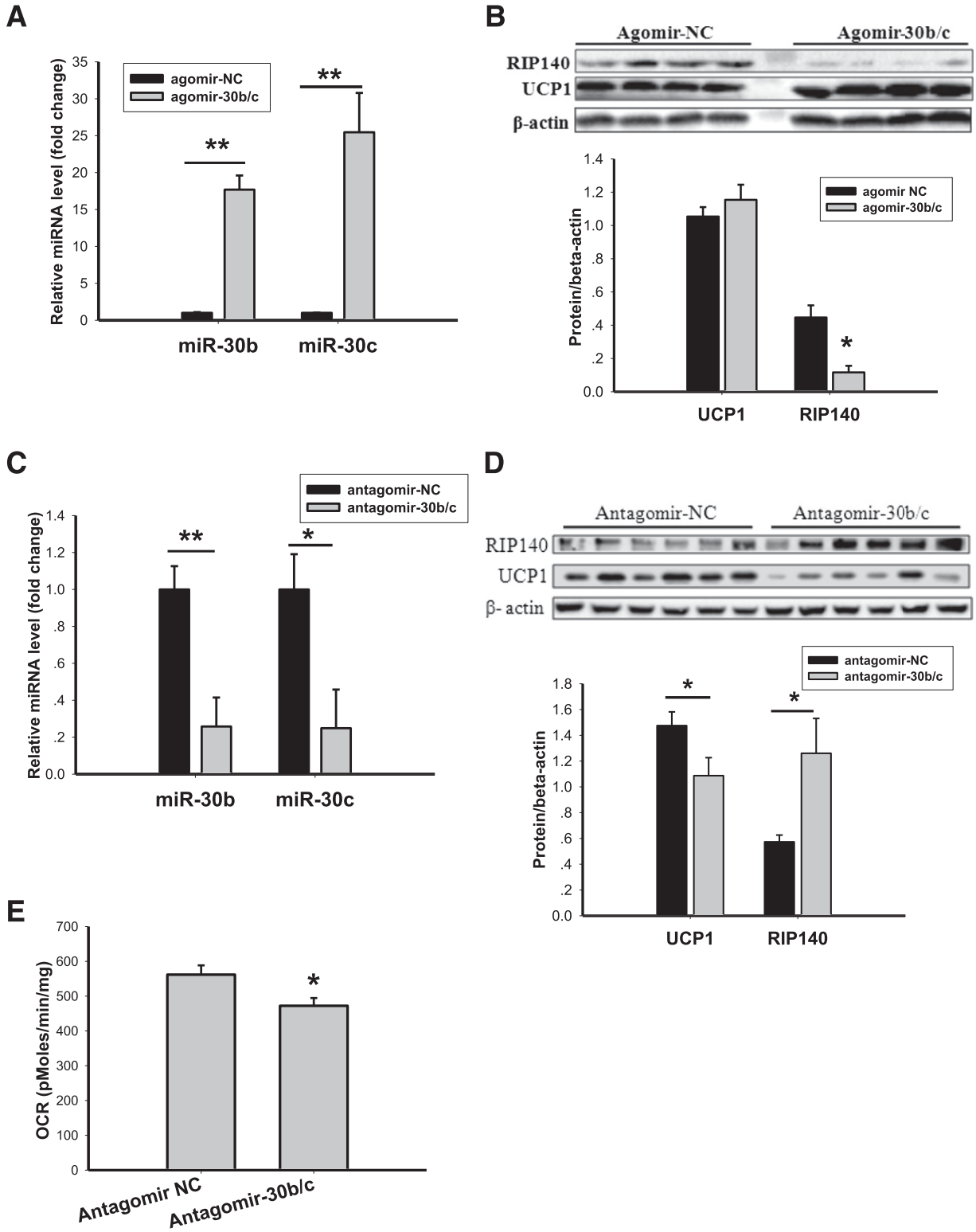

Figure 7—Knockdown of miR-30b/c decreases UCP1 expression and mitochondrial respiration in mouse BAT in vivo. Eight-week-old male C57BJ6 mice received agomir, antagomir-30b/c, or their respective scrambled negative control (NC) through subcutaneous injection. Three days after injection, BATs were collected 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 miRNA–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.

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