MicroRNA-27 (miR-27) Targets Prohibitin and Impairs Adipocyte Differentiation and Mitochondrial Function in Human Adipose-derived Stem Cells*

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Prohibitin (PHB) has been reported to play a crucial role in adipocyte differentiation and mitochondrial function. However, the regulative mechanism of PHB during adipogenesis remains unclear. In this study, we determined that the levels of both miR-27a and miR-27b were down-regulated following adipogenic induction of human adipose-derived stem cells, whereas the mRNA level of PHB was up-regulated. Overexpression of miR-27a or miR-27b inhibited PHB expression and adipocyte differentiation. Using PHB 3′-UTR luciferase reporter assay, we observed that miR-27a and miR-27b directly targeted PHB in human adipose-derived stem cells. A compensation of PHB partially restored the adipogenesis inhibited by miR-27. Moreover, we demonstrated the novel finding that ectopic expression of miR-27a or miR-27b impaired mitochondrial biogenesis, structure integrity, and complex I activity accompanied by excessive reactive oxygen species production. Our data suggest that miR-27 is an anti-adipogenic microRNA partly by targeting PHB and impairing mitochondrial function. Pharmacological modulation of miR-27 function may provide a new therapeutic strategy for the treatment of obesity.

Background: Prohibitin is essential in adipocyte differentiation and mitochondrial functions, but the regulative mechanisms of prohibitin by microRNA remain unclear.

Results: miR-27 negatively regulates adipogenesis by targeting prohibitin and impairing mitochondrial biogenesis, structure, and activity.

Conclusion: miR-27 targets prohibitin and suppresses adipocyte differentiation.

Significance: Manipulation of miR-27 may offer opportunities for the therapeutic modulation of adipogenesis in obesity.

The recent discovery of microRNAs (miRNAs)2 has introduced a novel type of regulatory control over gene expression during plant and animal development (1, 2). miRNAs comprise a large family of ~22-nucleotide single-stranded RNAs that decrease gene expression by binding to target miRNAs and leading to translational repression. miRNAs can therefore cause partial or full silencing of respective target genes. Furthermore, it seems that miRNAs can form extensive regulatory networks with a complexity comparable to that of transcription factors (7, 8). Hyperplasia and hypertrophy of adipocytes can cause the formation of and increase in adipose tissues and may then result in obesity (9). It is widely recognized that proliferation, differentiation, and apoptosis of preadipocytes are all related to the hyperplasia of adipose tissue (10). Elucidating the mechanisms involved in preadipocyte differentiation will be critical in the study of therapies for obesity. Our previous studies have demonstrated that miRNAs are involved in stem cell differentiation (11, 12). To profile the global changes in miRNA expression during adipogenesis, a miRNA expression array study was performed using total RNA extracted from human-derived preadipocytes and adipocytes (13). Several miRNAs, including miR-27, have been implicated in the process of accelerating or inhibiting preadipocyte differentiation (3, 14–18).

Prohibitin (PHB) is highly expressed in cells that rely heavily on mitochondrial function (19). Mitochondrial biogenesis and remodeling are considered to be necessary adjustments during adipogenesis as the cells become increasingly active in metabolism (20). A recent publication has shown that PHB deficiency in nematodes markedly reduces mitochondrial membrane potential and fat content early in adulthood (21). Our group has revealed that PHB silencing by synthetic siRNA induces down-regulation of peroxisome proliferator-activated receptor γ (PPARγ), reduction of adipogenesis, and dysfunction of mitochondria in the mouse 3T3-L1 cell line (22). PHB has been predicted to be a broadly conserved target of miR-27 by using
the miRNA prediction database. Indeed, miR-27 has been observed to target PHB to promote proliferation in cancer cells (23, 24). In this study, we demonstrate that miR-27 suppresses adipogenesis by targeting PHB and impairing mitochondrial biogenesis and function in human ASC.

**EXPERIMENTAL PROCEDURES**

**Human ASC Culture and Adipocyte Differentiation**—Human ASC and their culture medium were purchased from Invitrogen. The cells were cultured in ASC growth medium containing basal medium, growth supplement, and 2 mmol/liter l-glutamine. The cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. The medium was replaced every 2–3 days. Passages 5–6 were used for all experiments. To initiate adipocyte differentiation, overconfluent ASC (day 0) were treated with an adipogenic medium containing ASC basal medium, 10% FBS, 2 mmol/liter l-glutamine, 1 μmol/liter dexamethasone (Sigma), 10 μmol/liter insulin (Sigma), 0.5 mmol/liter isobutylmethylxanthine (Sigma), 200 μmol/liter indomethacin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. The differentiation medium was changed every 3 days thereafter until the indicated times (22).

For certain adipogenic experiments, 1 μM rosiglitazone (Thermo Fisher Scientific), a PPARγ agonist, was added to the adipogenic medium.

**TaqMan miRNA Assay**—Total RNA, including miRNA, was extracted from ASC by using a mirVana isolation kit (Invitrogen) according to the instructions of the manufacturer. Reverse transcription was performed using a TaqMan microRNA reverse transcription kit (Invitrogen) and reverse transcriptase transcription was performed using a TaqMan microRNA gen) according to the instructions of the manufacturer. Reverse transcription was performed as described previously (22). Total RNA was isolated from ASC by using a mirVana isolation kit (Invitrogen, CA) and reverse transcription was performed using a TaqMan microRNA assay (Invitrogen), 1 μM of RNase inhibitor, and specific miRNA reverse transcriptase primers in a total volume of 15 μl at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min.

Real-time PCR for miRNA assay was then conducted on a LightCycler 480 system (Roche Applied Science) at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each PCR contained 0.2 μl of the reverse transcription reaction product, 10 μl of TaqMan Universal PCR Master Mix (Invitrogen), 1 μl of a mixture of PCR primer/probe from the specific TaqMan MicroRNA assays, and 8.8 μl of nuclease-free water in a total volume of 20 μl. The relative miRNA levels were normalized to endogenous U6 snRNA expression for each sample (25).

**Real-time PCR Analysis**—Real-time PCR for mRNA analysis was performed as described previously (22). Total RNA was isolated from ASC at the indicated times using an RNeasy mini kit (Qiagen). Isolated RNA was reverse-transcribed with an oligo(dT) primer using an Advantage RT-for-PCR kit (Clontech). Real-time PCR was performed using a LightCycler Fast-Start DNA Master SYBR Green I kit (Roche Applied Science) and a LightCycler real-time thermal cycler (Roche Applied Science). The primer pairs used were 5′-agtgggatgggtggaggtct-3′ and 5′-gatggctggcagagttctgaggctg-3′ for human PPARγ, 5′-gaggggaggctggctggctggctg-3′ and 5′-tcgcaaacctggctgctggctg-3′ for human PPARγ, and 5′-ggaagggcaccacccaggagt-3′ and 5′-tcgacgcaggcaggctg-3′ for human 18S rRNA (used as an internal control). The amplified products were analyzed by electrophoresis on 2% E-Gel agarose (Invitrogen) to verify the primer specificity and PCR product size.

**Creation and Transduction of Lentivirus**—Lentiviral plasmids pLenti/miR-Control, pLenti/miR-27a, and pLenti/miR-27b were purchased from System Biosciences (Mountain View, CA); pLenti/Luc-UTR/Blank, pLenti/Luc-UTR/PHB, and pLenti/Luc-UTR/PHBmut were purchased from Applied Biological Materials (Richmond, British Columbia, Canada); pLenti/GFP was purchased from Invitrogen; and pLenti/PHB was created in our laboratory. The creation, concentration, titration, and ASC transduction of lentivirus were described previously (22). ASC transduction efficiency of lentivirus was evaluated by determining GFP expression of Lenti/miR-Control, Lenti/miR-27a, and Lenti/miR-27b by flow cytometry. Briefly, ASC were harvested after transduction of lentivirus for 3 days and analyzed on a Guava EasyCyte flow cytometer (EMD Millipore). The green fluorescence of the measured cell population (events = 5000) was gated using the Guava Express Plus program.

**Immunoblotting**—Cells were lysed in mammalian protein extraction reagent (Thermo Fisher Scientific) supplemented with protease inhibitor mixture (Sigma). The cell lysates were resolved by electrophoresis on 10% or 4–12% precast BisTris gel (Invitrogen). Proteins were transferred from the gel to a nitrocellulose membrane using an iBlot dry blotting system (Invitrogen). Specific proteins were detected using anti-PHB (BioLegend, San Diego, CA), anti-C/EBPβ (Cell Signaling, Danvers, MA), anti-PPARγ (Cell Signaling), anti-aP2 (Abcam, Cambridge, MA), and anti-HSP90 (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody. Blots were revealed with enhanced chemiluminescent reagents (Thermo Fisher Scientific).

**Oil Red O Staining**—Oil Red O (Sigma) staining was performed as described previously (22). Forty-eight hours after the induction of adipocyte differentiation, ASC in 35-mm dishes were washed with PBS and fixed with 10% formalin. The cells were washed once with 60% isopropl alcohol and left to dry completely. The cells were then stained with 0.2% Oil Red O for 10 min, rinsed once with 60% isopropl alcohol, and thoroughly washed four times with water. The dishes were scanned to obtain the pictures. The dye was then extracted with 100% isopropl alcohol and quantified on a spectrophotometer (Molecular Devices, Sunnyvale, CA) by reading the absorbance at a wavelength of 510 nm.

**Luciferase Reporter Assay**—Luciferase activities in ASC were determined using a luciferase assay kit (Applied Biological Materials) and an Lmax microplate luminometer with SoftMax Pro software (Molecular Devices) according to the instructions of the manufacturers. In brief, ASC were transduced with Lenti/miR-Control, Lenti/miR-27a, or Lenti/miR-27b at a multiplicity of infection (m.o.i.) of 2. The following day, the cells were washed with Lenti/Luc-UTR/Blank, Lenti/Luc-UTR/PHB, or Lenti/Luc-UTR/PHBmut at m.o.i. = 1. After 2 days of...
miR-27 Targets Prohibitin and Inhibits Adipogenesis

additional incubation, the cells were washed once with PBS. Cell lysis buffer, included in the kit, was added to the cells, followed by incubation at room temperature for 25 min. An equal volume of luciferase assay reagent was added to the cells. The luminescence was measured using a 10-s integration time between 30 min and 1 h after the addition of the assay reagent.

Detection of Mitochondrial DNA Content—Total DNA in ASC was isolated with a DNeasy DNA isolation kit (Qiagen). The DNA levels of the human mitochondrial ND1 (NADH dehydrogenase 1) gene and nuclear 18 S rRNA were determined by real-time PCR quantification. The relative mtDNA content was reflected by the ratio of DNA levels between mitochondrial ND1 and nuclear 18 S rRNA as described previously (26).

Mitochondrial Membrane Potential Assay—The mitochondrial membrane potential of ASC was measured by detecting the accumulation of tetramethylrhodamine ethyl ester (TMRE), a red fluorescent dye, in active mitochondria by flow cytometry. Briefly, ASC were incubated in 200 nmol/liter TMRE at 37 °C and 5% CO2 for 20 min. The cells were then washed with PBS once and trypsinized. The red fluorescence of the cell population (events = 10,000) was gated using the Guava Express Plus program in a Guava EasyCyte system.

MitoTracker Staining and Confocal Microscopy—ASC in Lab-Tek chamber slides (Thermo Fisher Scientific) were stained with 250 nmol/liter MitoTracker (Invitrogen) in serum-free DMEM for 15 min at 37 °C according to the manufacturer’s instructions. Images were captured and analyzed using a Leica TCS SP5 confocal microscopy system (Leica Microsystems, Bannockburn, IL) as described previously (22).

Measurement of ATP Concentration—Three days post-transduction of ASC in a 96-well plate with Lenti/miR-Control, Lenti/miR-27a, or Lenti/miR-27b, the ATP concentration was measured using an ATP assay system bioluminescence detection kit (Promega, Madison, WI) and the Lmax microplate luminometer with SoftMax Pro software (22).

Reactive Oxygen Species (ROS) Detection—ROS were detected with the cell-permeable, peroxide-sensitive fluorophore CellROX Orange reagent (Invitrogen) according to the manufacturer’s instructions. The dye is non-fluorescent while in a reduced state and exhibits bright orange fluorescence upon oxidation by ROS. ASC in a 96-well plate were transduced with Lenti/miR-Control, Lenti/miR-27a, or Lenti/miR-27b and cultured for 3 days. The cells were then incubated in 5 μmol/liter CellROX Orange reagent at 37 °C for 30 min, followed by washing twice with prewarmed PBS. Afterward, the plate was read on a GENios Plus microplate reader with universal reader control and data analysis software (Magellan V3.11, Tecan, San Jose, CA). To ensure that the CellROX Orange reagent was detecting hydrogen peroxide, cells were preincubated with 250 units/ml cell-permeable PEG-catalase (Sigma) at 37 °C for 2 h.

Detection of Mitochondrial Complex I/IV Activities—The activities of mitochondrial complex I and IV were determined in whole cell lysates of ASC with complexes I and IV enzyme activity dipstick assay kits (Abcam), respectively, according to our previous description (22) and the manufacturer’s instructions.

Results

miR-27a and miR-27b Are Predicted to Target Prohibitin and Are Down-regulated during Adipogenesis—Our previous studies have revealed that PHB is essential in adipocyte differentiation (22). To further investigate the regulation of PHB, computational prediction of miRNA families targeting PHB was performed using the TargetScan Database (version Human 6.0). Two miRNAs, hsa-miR-27 and hsa-miR-128, were predicted to be broadly conserved miRNA families among vertebrates targeting human PHB (Fig. 1A). The mRNA level of PHB and the levels of miR-27 and miR-128 were therefore examined in ASC during adipogenesis. The induced expression of adipogenic markers PPARγ and aP2 indicated that an adipogenic model was successfully established. An increase in the mRNA level of PHB was observed (Fig. 1B), which confirmed our previous observation at the protein level of PHB in ASC during adipogenesis (22). In addition, our data demonstrated that both miR-27a and miR-27b were down-regulated in ASC during adipogenesis (Fig. 1C), which was in accordance with the observation in mouse 3T3-L1 preadipocytes (15, 17, 27) and human ASC (16). miR-128 was not detected in ASC before or after adipogenic induction. Similar to our results, miR-128 has been previously reported to be expressed at very low levels or not at all in human ASC using miRNA profiling analysis (28, 29). These results suggest that miR-27 is associated with adipogenesis and may be a candidate miRNA family targeting PHB.

miR-27a and miR-27b Repress Adipocyte Differentiation—To investigate the potential effects of miR-27 during adipogenesis, miR-27a and miR-27b were overexpressed using lentiviral constructs (m.o.i. = 2). The transduction efficiency of concentrated lentivirus in ASC was evaluated by detecting GFP expressed by the lentiviral construct using flow cytometry. Because of the high transduction efficiency (always in excess of 85%), no antibiotic selection was required (Fig. 2A). An increase of 3–4-fold in miR-27a/b in transduced ASC confirmed overexpression of miR-27a/b (Fig. 2B). In the adipogenic induction experiments, the protein levels of PHB and adipogenic markers C/EBPβ, PPARγ, and aP2 were decreased upon overexpression of miR-27a or miR-27b (Fig. 2C). Likewise, the mRNA of PHB in ASC was significantly reduced as well (Fig. 2D). The lipid accumulation in ASC was also remarkably attenuated at day 14 (Fig. 2E). These data indicate that miR-27 down-regulates PHB expression and suppresses adipocyte differentiation in ASC.

Prohibitin Targets miR-27a and miR-27b in ASC—Although hsa-miR-27a and hsa-miR-27b are located at chromosomes 19 and 9, respectively, their mature sequences exhibit a single-nucleotide difference, whereas the seed site sequence is identical. Both are predicted to target the same site of the human PHB 3′-UTR (Fig. 3A). To experimentally validate the targeting effect, a luciferase reporter construct expressing the 3′-UTR of human PHB (Lenti/Luc-UTR/PHB) was created. Another con-
miR-27 Targets Prohibitin and Inhibits Adipogenesis

FIGURE 1. Expression of prohibitin and miR-27 during adipogenesis. A, the mature sequences of miR-27a/b and miR-128 and the partial sequences of the 3′-UTR of PHB from various species are illustrated. The seed sites of miR-27a/b and its binding sites at the 3′-UTR of PHB are shown in green and by the green rectangle, respectively. The seed site of miR-128 and its binding sites at the 3′-UTR of PHB are shown in red and by the red rectangle, respectively. The minimum free energy (mfe) was calculated using the PicTar open-source software. hsa, Homo sapiens; tbe, Tupaias belangeri; mmu, Mus musculus; rno, Rattus norvegicus; oco, Oryctolagus cuniculus; dno, Dasyus novemcinctus; oan, Ornithorhynchus anatinus. B and C, overconfluent ASC were treated with adipoctye differentiation medium for the indicated times (d, days). Total RNA, including miRNA, was then isolated. B, the relative mRNA expression levels of PHB, PPARγ, and aP2 were analyzed by RT-PCR. 18 S rRNA was used as an internal control. The relative levels of each mRNA at 0 h were set to 1. *, p < 0.05; **, p < 0.01 compared with corresponding mRNA level at 0 h. C, the levels of miR-27a, miR-27b, and miR-128 were determined by RT-PCR. U6 snRNA was used as an internal control. The relative levels of miR-27a and miR-27b at 0 h were set to 1. miR-27b was not detected. *, p < 0.05; **, p < 0.01 compared with corresponding miR-27 level at 0 h.

Data are means ± S.D.

Results

struct expressing the mutated 3′-UTR of PHB (Lenti/Luc-UTR/PHBmut), with a deletion of six nucleotides at the predicted miR-27 target site, was used as a control. Lenti/Luc-UTR/Blank was used as a negative control. Results from co-transduction experiments indicated that the relative luciferase activities in Lenti/Luc-UTR/PHB-treated ASC were significantly inhibited by either miR-27a or miR-27b, whereas the luciferase activities in Lenti/Luc-UTR/PHBmut-treated ASC were unaffected (Fig. 3B). These results suggest that PHB is a direct target of miR-27a and miR-27b in ASC.

Replenishment of PHB Restores the Adipogenesis Attenuated by miR-27—It has been reported that miR-27 directly targets PPARγ and represses adipogenesis (16, 17). Our previous study revealed that PHB silencing inhibits PPARγ expression and adipocyte differentiation; additionally, an increase in PHB occurs earlier than that in PPARγ during adipogenesis, implying that PHB is located upstream of PPARγ in the signaling transduction pathway of adipogenesis (22). In an analysis using a miRNA target prediction website (PicTar), the PicTar scores for miR-27a and miR-27b binding to PHB (5.3307 and 5.2602, respectively) were higher than those for binding to PPARγ (4.2331 and 4.2280, respectively). We therefore tested the effect of replenishment of PHB by co-transducing Lenti/PHB and the effect of supplementation of rosiglitazone, a PPARγ agonist, on the adipogenesis inhibited by overexpression of miR-27. Our results indicated that PHB levels were increased by the transduction of Lenti/PHB, but not by the administration of rosiglitazone (Fig. 4, A and C). The levels of the adipogenic marker aP2 and lipid accumulation were slightly decreased by a gain of function of PHB, which was in agreement with our previous observations (22) and a recent report in 3T3-L1 cells with uncertain mechanisms (30). Of particular note was that the reduction of the levels of aP2 and lipid by miR-27a/b was partially restored by overexpression of PHB (Fig. 4, A and B). However, the reduction in the levels of aP2 and lipid by miR-27a/b was not restored upon administration of rosiglitazone compared with vehicle (Fig. 4, C and D). These data suggest that the anti-adipogenic activity of miR-27 is more tendentious via targeting PHB. The decrease in the PPARγ level may be partially due to an indirect effect of PHB silencing by miR-27.

Effects of miR-27 on Mitochondria in ASC—Our previous studies revealed the essential roles of PHB in mitochondrial biogenesis and morphology during adipogenesis (22), as well as in stabilizing the mitochondrial membrane potential in granulosa cells (31). Because PHB is a direct target of miR-27, as shown above, we therefore examined the mitochondrial content, mitochondrial membrane potential, and mitochondrial morphology during adipogenesis upon overexpression of miR-27a or miR-27b. Our results demonstrated that the content of relative mtDNA was doubled in ASC subject to adipogenic induction. The amount of mtDNA was partially suppressed upon overexpression of miR-27a or miR-27b whether or not the cells were subject to adipogenic induction (Fig. 5A). The mitochondrial membrane potential was examined using TMRE to label the active mitochondria. TMRE is a cell-permeant, positively charged dye that readily accumulates in active mitochondria due to their relative negative charge. Our data revealed that the mitochondrial membrane potential of ASC, with or without adipocyte differentiation, was significantly impaired by miR-27, as indicated by rotenone, an inhibitor of mitochondrial respiratory complex I, used as a positive control (Fig. 5B). MitoTracker analysis revealed that instead of normal tubular mitochondria, nearly 20% of the miR-27a- or miR-27b-overexpressing ASC consisted of fragmented mitochondria before or after cellular adipogenesis (Fig. 5D).
The lack of PHB reduces mitochondrial membrane integrity, disrupts oxidative phosphorylation, and results in an augmentation of ROS levels (32–35). Our recent study illustrated that ROS formation and mitochondrial complex I activity are increased upon PHB silencing in mouse 3T3-L1 preadipocytes (22). In light of this, we investigated the role of miR-27 in mitochondrial function in ASC. Our results showed that ROS levels increased by nearly 3-fold in either miR-27a- or miR-27b-overexpressing ASC, whereas the content of ATP was unaffected. The increase in ROS could be ablated when the cells were preincubated with PEG-catalase, a hydrogen peroxide scavenger, indicating the specificity of the oxidation by ROS (Fig. 6, A and B). These results suggest that the function of the mitochondrial oxidative phosphorylation system is affected upon miR-27 forced expression in ASC. To further investigate the underlying mechanisms of the extra ROS generation, the activities of mitochondrial complex I were examined. Our data demonstrated a reduction in complex I activity in miR-27a- or miR-27b-overexpressing ASC before or after adipogenesis (Fig. 6C). Taken together, our findings highlight that the impairment of mitochondrial electron transport in the oxidative phosphorylation system may be a mediator for the anti-adipogenic effect of miR-27.

**DISCUSSION**

Identifying functional miRNA–gene regulatory modules is a challenging task because one gene can be regulated by multiple
miRNAs, and one miRNA can regulate a large number of genes (36, 37). miR-27 has been experimentally validated to target PHB in cancer cells (23, 24). Our group and others have recently reported that PHB deficiency reduces adipocyte differentiation and adipose accumulation and impairs mitochondrial structure and function (21, 22). Here, we have further demonstrated that miR-27 inhibits adipogenesis and impairs mitochondria by targeting PHB in human ASC.

As PHB silencing by artificial siRNA inhibits adipogenesis in the mouse 3T3-L1 preadipocyte cell line (22), we therefore hypothesized that PHB silencing by natural miRNA can inhibit adipogenesis in human ASC. miRNAs are short (21–23 nucleotides) RNAs that bind to the 3′-UTR of target genes. Computational prediction of miRNA targeting human PHB with the TargetScan Database results in two broadly conserved miRNA families, hsa-miR-27 and hsa-miR-128. Although it is more likely for miRNAs in animals to have only a partially complementary sequence of nucleotides to bond with the target mRNA, nucleotides 2–7 of miRNA (its “seed region”) nevertheless have to be perfectly complementary (38). Both miR-27 and miR-128 have an exact match at positions 2–8 of the mature miRNA (the seed region + position 8), followed by an adenosine to the 3′-UTR of PHB. miR-128 has been implicated to be an anti-onco-miRNA that represses cell growth, motility, and invasiveness and induces chemotherapeutic sensitivity and apoptosis (39–42). Our data show that both the mRNA and protein levels of PHB are decreased by miR-27a/b before and after adipogenesis because miR-27a/b has an exact match with the target sequence at the 3′-UTR of PHB.

Mature miRNA is part of an active RNA-induced silencing complex that contains Argonaute proteins and many other associated proteins. Gene silencing may occur either through mRNA degradation or by preventing mRNA from being translated. It has been demonstrated that if there is a complete complementation between miRNA and the target mRNA sequence, Argonaute-2 (Ago2) can cleave the mRNA and lead to direct mRNA degradation. Otherwise, silencing is achieved only through preventing translation (37). Indeed, our data reveal that both the mRNA and protein levels of PHB are decreased by miR-27a/b before and after adipogenesis because miR-27a/b has an exact match with the target sequence at the 3′-UTR of PHB. miRNAs have now been implicated in the control of a wide range of biological functions, including development, differentiation, metabolism, growth, and apoptosis. In this study, we showed that both miR-27a and miR-27b attenuate adipocyte differentiation in human ASC, which is similar to the previous observations in the mouse 3T3-L1 cell line and human ASC (16, 17). Although miR-27 has been implicated to target PPARγ in HeLa cells and HEK293 cells (16, 17) and to target PHB in human cancer cell lines (23, 24), it is still important to experimentally validate here that both miR-27a and miR-27b directly target PHB in human ASC because the miRNA-mRNA target relationships differ among tissues, cells, and conditions (36).
Partial restoration of adipogenesis resulting from the compensation of PHB further confirms the function of miR-27a and miR-27b as negative modulators of adipogenesis via targeting PHB in ASC. In contrast, administration of rosiglitazone, an agonist of PPARγ, has not been observed to significantly restore the anti-adipogenic effect of miR-27a or miR-27b. Our data imply that the decrease in PPARγ levels may be due partially to the direct effect of miR-27 and/or the indirect effect of PHB silencing by miR-27.

Numerous studies have consistently demonstrated that mitochondrial biogenesis is markedly enhanced during adipogenesis as cellular metabolism becomes more active. The enhancement is possibly the result of activation or promoted expression of nuclear encoded mitochondrial genes that are under the control of adipogenic transcription factors (20, 22, 43, 44). PHB is an evolutionarily conserved protein that is located mainly at the inner mitochondrial membrane and has been implicated in diverse cellular processes (45, 46). Studies from our group and others have demonstrated that a loss of PHB results in reduced adipocyte differentiation in vitro and fat accumulation in vivo; meanwhile, fragmented mitochondrial morphology, accompanied by decreases in mitochondrial biogenesis, membrane potential, and complex I activity and an increase in ROS production, has been observed (21, 22, 33). In this study, we observed repression of adipogenesis and impairment of mitochondrial biogenesis, structure integrity, and functional performance upon ectopic expression of miR-27a or miR-27b in ASC, which are similar to the effects of PHB deficiency. This novel finding of the role of miR-27 in mitochondria is most likely to be observed in other areas of study due to the multifunctional character of the mitochondrion. Stable ATP production in this situation may be the consequence of compensatory mechanisms at play in mitochondria, involving an increase in electron flow through complex II and/or III (33). Although down-regulation of miR-27a, accompanied by a reduction in the mitochondrial membrane potential and induction of ROS, has been observed in cancer cells treated with certain anticancer drugs, there is no evidence showing the

FIGURE 5. Effects of miR-27 on mitochondrial content, membrane potential, and morphology. ASC were transduced with Lenti/miR-Control (miR-Cont), Lenti/miR-27a (miR-27a), or Lenti/miR-27b (miR-27b). Three days later, the cells were induced to adipocyte differentiation with adipogenic inducers for a period from days 0 to 14. A, the relative mtDNA content was evaluated by a ratio of the DNA level of mitochondrial ND1 to that of nuclear 18S rRNA. The relative mtDNA content in ASC overexpressing Lenti/miR-Control at day 0 was set to 1. B and C, the mitochondrial membrane potential of ASC was measured by detecting the accumulation of TMRE, a red fluorophore, in active mitochondria by flow cytometry. The cells without the addition of TMRE were used to facilitate the gating of red events. A statistical analysis of the percentage of TMRE-positive cells was shown in C. D, ASC at days 0 and 14 were stained with MitoTracker Red. The mitochondrial morphology was analyzed by a confocal microscopy system. Scale bar = 10 μm. The bar graph represents the percentage of cell populations with fragmented mitochondria. *, p < 0.05; **, p < 0.01 compared with Lenti/miR-Control at day 0. *, p < 0.05; **#, p < 0.01 compared with Lenti/miR-Control at day 14.
miR-27 Targets Prohibitin and Inhibits Adipogenesis

direct effect of miR-27 on mitochondria thus far (47, 48). Interestingly, miR-128, a brain-enriched miRNA that targets PHB at the same position as miR-27, has been recently described to cause the loss of mitochondrial membrane potential and enhancement of ROS production (49). To further investigate the role of PHB as a mediator in mitochondrial impairment induced by miR-27 in ASC, the possible restoration of mitochondrial function may be examined upon a compensation of PHB.

Our findings provide evidence that miR-27 is an anti-adipogenic miRNA partly by targeting PHB and impairing mitochondrial functions and therefore may be a therapeutic candidate for the treatment of obesity. Interestingly, miR-27 has been recently reported to be pro-angiogenic as well. Thus, administration of miR-27 may also be beneficial to patients suffering from ischemic cardio-cerebrovascular diseases, which are often associated with obesity (50). However, a reduction in adipocyte differentiation and fat storage may lead to lipids being stored in the liver with detrimental side effects such as insulin resistance or steatosis (51). In addition, miR-27 has been implicated to facilitate the growth and survival of human cancer cells (24, 52). Therefore, the therapeutic effectiveness of miR-27 needs to be stringently evaluated in in vivo studies.

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FIGURE 6. Effects of miR-27 on mitochondrial function. ASC were transduced with Lenti/miR-Control (miR-Cont), Lenti/miR-27a (miR-27a), or Lenti/miR-27b (miR-27b) and incubated for 3 days. A, the ATP concentration in the cells was detected by luminescence assay and was normalized to the protein concentration. B, ROS formation in ASC was determined using CellROX Orange reagent staining and flow cytometry, followed by preincubation with or without 250 units/ml catalase for 2 h. **, p < 0.01 compared with Lenti/miR-Control without catalase preincubation; ##, p < 0.01 compared with miR-27b without catalase preincubation; #, p < 0.05 compared with miR-27b without catalase preincubation. C, ASC were induced to adipocyte differentiation from days 0 to 14. The activities of complexes I (Cox I) and IV (Cox IV) were determined in whole cell lysates of ASC with mitochondrial dipstick assay kits. The activity of complex I was normalized to the activity of complex IV. The complex I/complex IV activity in the cells transduced with Lenti/miR-Control at day 0 was set to 1.* p < 0.05 compared with Lenti/miR-Control at day 0; **, p < 0.01 compared with Lenti/miR-Control at day 14.
miR-27 Targets Prohibitin and Inhibits Adipogenesis

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