In Vitro Evidence Suggests That miR-133a-mediated Regulation of Uncoupling Protein 2 (UCP2) Is an Indispensable Step in Myogenic Differentiation*3

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Xi Chen1, Kehui Wang1, Jiangning Chen1, Jigang Guo, Yuan Yin, Xing Cai, Xing Guo, Guoqiang Wang, Rong Yang, Lingyun Zhu, Yan Zhang, Jin Wang, Yang Xiang, Chunyue Weng, Ke Zen3, Junfeng Zhang3, and Chen-Yu Zhang3

From the Jiangsu Diabetes Center, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 22 Hankou Road, Nanjing, Jiangsu 210093, China

UCP2 and UCP3, two novel uncoupling proteins, are important regulators of energy expenditure and thermogenesis in various organisms. The striking disparity between UCP2 mRNA and protein levels in muscle tissues prompted initial speculation that microRNAs are implicated in the regulatory pathway of UCP2. We found, for the first time, that the repression of UCP2 expression in cardiac and skeletal muscle resulted from its targeting by a muscle-specific microRNA, miR-133a. Moreover, our findings illustrate a novel function of UCP2 as a brake for muscle development. We also show that MyoD can remove the braking role of UCP2 via direct up-regulation of miR-133a during myogenic differentiation. Taken together, our current work delineates a novel regulatory network employing MyoD, microRNA, and uncoupling proteins to fine-tune the balance between muscle differentiation and proliferation during myogenesis.

UCP2 is a member of the uncoupling proteins (UCPs)1, 2. UCPs are mitochondrial inner membrane transporters that uncouple ATP synthesis by dissipating the proton gradient, a process that generates heat and decreases ATP production (1, 2). UCPs have been described across various animal and plant species, and they are prominent in the fields of thermogenesis, obesity, diabetes, and free radical biology (1, 2). In contrast to UCP1, the archetypal uncoupling protein, the exact physiological functions of UCP2 remain obscure (1, 2). One of the main puzzles encountered in the studies of UCP2 is the ambiguous regulatory mechanisms controlling its expression. Previous study has suggested that the expression of UCP2 mRNA in a specific tissue does not necessarily correlate with the expression at the protein level. For example, although UCP2 mRNA is found in mouse heart and skeletal muscle, no UCP2 protein could be detected in these tissues (3). Given the disparity between UCP2 mRNA and protein in mouse muscle tissues, it is quite likely that a post-transcriptional regulatory mechanism exists. Recently, a class of RNA regulatory genes known as microRNAs (miRNAs) has been found to introduce a whole new layer of protein regulation after transcription (4). miRNAs act as endogenous sequence-specific suppressors of translation and thus can block target protein expression without affecting mRNA stability (4). Their widespread and important role in eukaryotes is highlighted by recent discoveries that they control a range of physiological processes including development, differentiation, proliferation, apoptosis, and metabolism (4). In the present study, we identified UCP2 as a target of the muscle-specific miR-133a and demonstrated that UCP2 plays a role in inhibiting muscle differentiation and promoting myoblast proliferation. Therefore, we suggested that down-regulation of UCP2 might be an indispensable step during myogenic differentiation. The specific mechanism through which miR-133a and UCPs regulate the myogenic pathway has also been well described in this study.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Mouse lung fibroblast cells L929, mouse Lewis lung carcinoma cells LLC, and mouse mesenchymal stem cells C2C12 were obtained from China Cell Culture Center (Shanghai, China). L929 and LLC cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. C2C12 myoblasts were maintained at subconfluent densities in growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum). When cells reached ~75% confluence, the medium was switched to differentiation medium (Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum). Medium was changed every other day. After incubation for several days, cells were harvested and subjected to various experiments. Antibodies, including anti-UCP2 (C-20), anti-UCP3 (C-20), anti-MyoD (M-318), anti-cytochrome c (A-8), and anti-glyceraldehyde-3-phosphate dehydrogenase (6C5), were purchased from Santa Cruz Biotechnology. Precursor oligonucleotides (pre-miR-133a, pre-miR-1, and pre-miR-210), antisense

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oligonucleotides (anti-miR-133a), and scrambled negative control oligonucleotides were purchased from Ambion.

PCR Analysis—Assays to quantify mature miRNAs were conducted as described previously (5). For real-time PCR analysis, the relative amount of each gene to internal control was calculated by using the equation $2^{-\Delta CT}$, in which $\Delta CT = C_{T\text{gene}} - C_{T\text{control}}$. For semiquantitative RT-PCR analysis, 20–30 PCR cycles were performed. All primers used are listed in supplemental Table 1.

Overexpression or Knockdown of miR-133a—L929, LLC, or C2C12 cells were seeded on 6-well plates or 60-mm dishes and were transfected the following day by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For each well, equal doses of precursor oligonucleotides, antisense oligonucleotides, or scrambled negative control RNA were used. Cells were harvested 48 h after transfection for semiquantitative RT-PCR, real-time PCR analysis, and Western blotting.

Plasmid Construction and Luciferase Assay—The entire mouse UCP2 3′-untranslated region (UTR) segment was amplified by PCR using mouse genomic DNA as a template. The PCR products were inserted into the p-MIR-report plasmid (Ambion). Efficient insertion was confirmed by sequencing. For luciferase reporter assays, 1 μg of firefly luciferase reporter plasmid, 0.5 μg of β-galactosidase expression vector (Ambion), and equal amounts (200 pmol) of pre-miR-133a, anti-miR-133a, or scrambled negative control RNA were transfected into cells in 6-well plates. The β-galactosidase vector was used as a transfection control. At 24 h after transfection, cells were assayed using luciferase assay kits (Promega).

Cloning of miR-133a—The genomic fragment of pre-miR-133a-1 from mouse chromosomes 18 was amplified by PCR using mouse genomic DNA as a template. The PCR products were cloned into the pcDNA(-)3.1 vector (Invitrogen), and effective overexpression was qualified by semiquantitative RT-PCR analysis after transfecting the expression vector into L929 cells.

Isolation of Mitochondria and Western Blotting—Mitochondria of UCP2 knock-out mice (gift from Bradford B. Lowell, Harvard University), wild type mice and culture cells were isolated as described previously (6). UCP2 or UCP3 protein levels were quantified by Western blot analysis of 50 μg of mitochondrial proteins with antibodies against UCP2 or UCP3. Normalization was performed by blotting the same samples with an antibody to cytochrome c. The MyoD protein level was quantified by Western blot analysis of 100 μg of whole cell extracts with antibodies against MyoD, and the normalization was performed by blotting the same sample with an anti-glyceraldehyde-3-phosphate dehydrogenase antibody.

ChIP Experiments—Chromatin immunoprecipitation experiments were performed as described previously (7). Briefly, C2C12 cells were maintained in differentiation medium (DM) for 3 days and then cross-linked with formaldehyde. Cell extracts were sonicated to generate 200–1000-bp DNA fragments. Rabbit polyclonal anti-MyoD antibody and rabbit IgG were used to immunoprecipitate chromatin fragments. The DNA-protein cross-links were reversed by heating at 65 °C for 4 h, and the recovered DNA was PCR-amplified. In the PCR analysis, 26, 20, and 48 PCR amplicons roughly 200 bp in length were designed to assay for binding sites in the 5-kb sequence upstream of pre-miR-133a-1, the 5-kb sequence upstream of pre-miR-133a-2, and the 10-kb sequence upstream of UCP3. Control amplification was carried out on input chromatin before immunoprecipitation and on mock-immunoprecipitated chromatin.

Statistical Analysis—All photo images of Western blotting, semiquantitative RT-PCR, and chromatin immunoprecipitation (ChIP) experiments were representatives of at least three independent experiments. Real-time PCR and luciferase reporter assay were performed in triplicate, and the entire experiment was repeated several times. Data shown were presented as means ± S.E. of three or more independent experiments, and the differences were considered statistically significant at $p < 0.05$ by using the Student’s t test.

RESULTS

Regulation of UCP2 Expression by miR-133a—We first investigated the distribution of UCP2 mRNA and protein in mouse tissues. Western blot analysis revealed the presence of UCP2 protein in spleen and lung (Fig. 1A). This detection was specific...
Regulation of UCP2 by miR-133a

because the 33-kDa band detected by the antibody disappeared in ucp2−/− mice (Fig. 1A). Surprisingly, we were unable to detect UCP2 protein in skeletal muscle and heart (Fig. 1A) despite a clear identification of UCP2 mRNA in these tissues (Fig. 1B). Moreover, this disparity between protein and mRNA in mouse muscle tissues seemed specific for UCP2; both the protein and the mRNA of UCP3, the most homologous of UCP2 in UCPs, were normally expressed in mouse muscle tissues (Fig. 1, B and C).

Because UCP2 protein is absent from mouse muscle tissues but is highly expressed in other tissues, it is intriguing to speculate that muscle-specific miRNAs modulate the expression of UCP2. Overall, four muscle-specific miRNAs have been identified: miR-1, miR-133a, miR-206, and miR-208 (8–10). Previous work has reported that miR-1 and miR-133a are specifically expressed in cardiac and skeletal muscle (8), whereas miR-206 and miR-208 are expressed only in skeletal muscle or cardiac muscle, respectively (9, 10). Given the absence of miR-206 from heart and miR-208 from skeletal muscle, we then focused on miR-1 and miR-133a. As expected, our PCR analysis revealed that mature miR-1 and miR-133a were strictly limited to cardiac and skeletal muscle (Fig. 1, B and D).

The correlation between miRNAs and UCP2 was further examined by evaluating the expression of UCP2 after overexpression or knockdown of miRNAs. In these experiments, overexpression of miRNAs was achieved by transfecting cells with pre-miRNAs (synthetic RNA oligonucleotides mimicking miRNA precursors), whereas knockdown was achieved by transfecting cells with anti-miRNAs (chemically modified antisense oligonucleotides designed to specifically target against mature miRNAs). As shown in Fig. 2A, three exogenous pre-miRNAs, pre-miR-1, pre-miR-133a, and an unrelated miRNA precursor pre-miR-210, were introduced into the mouse lung fibroblast cell line L929. UCP2 levels were assayed with mitochondrial proteins isolated on day 2 after transfection. Control experiments were performed in the same manner except using cells transfected with scrambled oligonucleotides (negative control) or UCP2 siRNA (positive control). L929 cells were selected for this experiment because they expressed low levels of endogenous miR-1 and miR-133a but a high level of UCP2 protein (supplemental Fig. 1). The expression of UCP2 was significantly abolished by the introduction of pre-miR-133a or UCP2 siRNA, whereas cells treated with pre-miR-1, pre-miR-210, or scrambled oligonucleotides maintained a considerable protein level of UCP2 (Fig. 2A). Moreover, anti-miR-133a significantly delayed the down-regulation of UCP2 by pre-miR-133a. As shown in Fig. 2B, although pre-miR-133a alone was sufficient to reduce UCP2 protein level, cells treated with pre-miR-133a plus anti-miR-133a expressed constant UCP2 protein when compared with control cells treated with scrambled oligonucleotides. To determine at which level miR-133a influenced UCP2 expression, UCP2 transcript levels were evaluated after pre-miR-133a or anti-miR-133a treatment. Although miR-133a intracellular level was altered significantly after pre-miR-133a or anti-miR-133a treatment (Fig. 2C), overexpression or knockdown of miR-133a did not affect UCP2 mRNA stability (Fig. 2, C and D). Taken together, these results confirm that miR-133a blocks UCP2 translation without affecting its mRNA abundance, a hallmark of miRNA-mediated post-transcriptional regulation. Subsequently, the entire UCP2 3′-UTR was fused into a luciferase reporter plasmid, and the resulting plasmid was introduced into L929 cells combined with a transfection control plasmid (β-galactosidase) and pre-miR-133a, pre-miR-1, pre-miR-210, or scrambled oligonucleotides. As expected, overexpression of miR-133a resulted in a ~60% reduction of firefly luciferase reporter activity (normalized against β-galactosidase activity) when compared with the scrambled oligonucleotides transfected cells (Fig. 2E). In contrast, pre-miR-1 and pre-miR-210 did not significantly affect the luciferase reporter activity (Fig. 2E). The observed alteration of luciferase activity was specific because co-transfection of L929 cells with the parental luciferase plasmid (without the UCP2 3′-UTR) and pre-miR-133a did not affect luciferase activity (Fig. 2E). To narrow down the miR-133a-responsive element, our in silico analysis further identified an ~0.5-kb region that is most likely responsible for the down-modulation of UCP2 in muscle tissues. As shown in Fig. 2F, three hybrids in this region were observed. The predicted free energy values of these hybrids (~23.7, ~21.9, and ~21.6 kcal/mol) were all well within the range of true miRNA-target pairs. Subsequently, we constructed an equivalent luciferase reporter with these three putative miR-133a binding sites mutated (the sequence that interacts with the 2–4 bases of miR-133a were mutated). Mutant luciferase reporter was unaffected by overexpression of miR-133a (Fig. 2E). Thus, this region is likely the core sequence through which miR-133a recognizes UCP2 transcripts. Besides L929, the mouse lung cancer cell line LLC, which expressed neither endogenous UCP2 nor endogenous miR-133a (supplemental Fig. 1), was also used in the present study. As anticipated, the effect of overexpression of miR-133a in LLC cells was concordant with that in L929 cells. Overexpression of miR-133a significantly affected luciferase reporter activity, whereas anti-miR-133a could relieve the suppression of pre-miR-133a on UCP2 (Fig. 2G). These results unequivocally demonstrate that miR-133a directly recognizes the 3′-UTR of UCP2 transcripts. In addition to a chemically synthetic miRNA mimic, an expression plasmid of miR-133a was also constructed and transfected into L929 and LLC cells along with luciferase reporters. As shown in Fig. 2H, luciferase activity was markedly repressed by overexpression of miR-133a. In conclusion, these experiments unequivocally demonstrate that miR-133a directly represses UCP2 translation through a post-transcriptional mechanism.

The Braking Role of UCP2 in Myogenesis Differentiation—Because miR-133a is capable of regulating UCP2 expression in vitro, we next used the C2C12 model system to mimic muscle maturation and investigate the effect of endogenous miR-133a on UCP2 during myogenesis. C2C12 cells were propagated as undifferentiated, mononucleated myoblasts under high serum conditions. Upon serum depletion, mononucleated myoblasts fused to each other to form multinucleate myotubes (11). The differentiation in this model system was very efficient, as indicated by marked induction of myogenic markers including myogenin, myosin heavy chain (Fig. 3A, MHC), MyoD, MEF2, and skeletal α-actin (Fig. 3A, s.α-actin). Accelerated myogenic
differentiation was accompanied by a decrease in cell proliferation, as indicated by a significant decrease in expression of Ki-67 and proliferating cell nuclear antigen (Fig. 3A). MiR-133a was also robustly induced during the myoblast-myotube transition. As shown in Fig. 3, A and B, the undifferentiated myoblasts in proliferative condition did not express miR-133a. Upon differentiation into myotubes, a strong up-regulation of miR-133a was observed. UCP2 transcripts were detectable in undifferentiated primary myoblasts, but their steady-state levels increased many folds and then remained constant during the myoblast-myotube transition (Fig. 3, A and C). However, UCP2 proteins were present in proliferative myoblasts but nearly completely eliminated in differentiated myotubes (Fig. 3D) despite an increase in mRNA expression. In sharp contrast, both UCP3 transcripts and proteins were undetectable in myoblasts but strongly induced upon differentiation (Fig. 3, A and D). Hence, miR-133a and UCP3 levels were inversely related to UCP2 levels in a manner consistent with muscle development.

FIGURE 2. Regulation of UCP2 expression by miR-133a. A, L929 cells were treated with the precursor oligonucleotides (pre-miR-133a, pre-miR-1, and pre-miR-210), UCP2 siRNA, or scrambled oligonucleotides. Mouse spleen mitochondrial protein was loaded to indicate the UCP2 band. UCP2 protein levels were determined by Western blot analysis of mitochondrial proteins isolated on day 2 after transfection. cyto. C, cytochrome c. B–D, L929 cells were transfected with scrambled control oligonucleotides, pre-miR-133a, or pre-miR-133a in addition to anti-miR-133a, respectively. Cells were cultured for 48 h before being subjected to Western blot analysis of UCP2 proteins (B), semiquantitative RT-PCR analysis for miR-133a and UCP2 transcripts (C), or real-time RT-PCR analysis for UCP2 transcripts (D). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. E, firefly luciferase reporters containing either wild type (WT) or mutant (Mut) UCP2 3′-UTR were co-transfected into L929 cells with the indicated precursor oligonucleotides. The parental luciferase plasmid was also transfected as a control. Luciferase activity was determined 24 h after transfection. F, schematic description of the hypothesized duplexes formed by interactions between miR-133a-responsive elements and miR-133a. The predicted free energy of each hybrid is indicated. G, firefly luciferase reporters containing miR-133a complementary sites were co-transfected into LLC cells with the indicated oligonucleotides. At 24 h after transfection, luciferase activity was measured. H, repression of UCP2 3′-UTR by miR-133a expression vectors in L929 and LLC cells. *, p < 0.05; **, p < 0.01.
To further investigate the suppression of elevated endogenous miR-133a on UCP2 during myogenic differentiation, we transfected proliferative myoblasts with wild type or mutant luciferase reporter plasmids. Because the luciferase activity generally wanes after a long time of cell culture, reporter activity continued to be reduced throughout the differentiation process (Fig. 3E). However, the accelerated reduction of wild type plasmid when compared with mutant plasmid (Fig. 3E) suggests that endogenous miR-133a may partially contribute to the drop of luciferase activity. Furthermore, anti-miR-133a oligonucleotides significantly delayed the reduction of luciferase activity during myogenesis, whereas pre-miR-133a hastened the drop of luciferase activity when compared with scrambled negative control oligonucleotide treatment (Fig. 3E). These results indicate that exogenous antisense oligonucleotides could partially rescue the suppression of endogenous miR-133a on UCP2. In summary, both the loss of UCP2 protein upon myotube formation without a similar reduction in mRNA and the constant inhibition of a luciferase reporter carrying the UCP2 3’-UTR during myogenic differentiation demonstrate a miRNA-mediated UCP2 translational repression mechanism.

The continuous suppression of UCP2 and induction of UCP3 during myogenesis prompt the speculation that the down-regulation of UCP2 and up-regulation of UCP3 might be indispensable to myotube formation. To verify this hypothesis, a UCP2 expression plasmid designed to specially express the UCP2 open reading frame (ORF) without the miR-133a-responsive 3’-UTR was constructed. We transfected this plasmid into C2C12 cells to rescue the suppressive effect of miR-133a on UCP2. Strikingly, miR-133a-resistant UCP2 strongly inhibited
Regulation of UCP2 by miR-133a

amplification was performed using double distilled H2O as template. Control PCR was performed using rabbit IgG-immunoprecipitated samples (Fig. 4A and B). No enrichment was observed in mock-immunoprecipitated samples (without antibody) or rabbit IgG-immunoprecipitated samples (Fig. 4A). Furthermore, ChIPs experiments using pre-miR-210 promoter as a negative control for MyoD binding were performed. No binding was detected at the pre-miR-210 regulatory regions (Fig. 4A). Likewise, pre-miR-133a-1 and pre-miR-133a-2 have already been shown to be direct targets of MyoD (16). In sum, a novel MyoD-regulated signaling pathway controlling myogenesis and development could be established (Fig. 4C). As shown in this figure, differentiation cues stimulate the expression of MyoD in muscle tissues. This stimulation allows them to activate the muscle-specific gene UCP3 and simultaneously induce miR-133a to repress UCP2 expression. Down-regulation of UCP2 then removes the differentiation suppression exerted by UCP2. In other MyoD-negative tissues (e.g. spleen, lung), UCP2 is normally expressed, whereas UCP3 is silenced. UCP2 and UCP3 are generally expressed in a non-overlapping manner. Taken together, these results not only identify miRNAs as a novel link between MyoD and myogenesis but more importantly reveal a critical role for UCP2 in muscle differentiation and development. In particular, such a positive feedback loop will be expected to push the balance between muscle cell proliferation and differentiation toward the differentiation direction and maintain muscle cells in a permanently differentiated state.
Regulation of UCP2 by miR-133a

DISCUSSION

The lack of reliable and specific methods for biological target validation hampers the full understanding of the mechanisms by which miRNAs execute their functions. Only a few miRNAs have thus far been assigned target mRNAs, and the conventional methodologies are still labor-intensive (4). Nevertheless, a novel approach for target identification, which employs a reverse strategy searching for miRNAs that are attached to the mRNA of a predefined target, might greatly improve the current knowledge. Using this approach, we have unambiguously addressed a key role for miR-133a in mediating UCP2 expression (Fig. 2). According to our current results, UCP2 might serve as a brake for myogenic differentiation (Figs. 3, 4, A–C). MyoD can remove the braking role of UCP2 by directly up-regulating miR-133a (Fig. 4, A–C), which in turn represses UCP2 expression. Thus, specific repression of UCP2 by miRNA-133a might be an indispensable step in the myoblast-myoctube transition.

Although UCP2 and UCP3 share high identity with UCP1 and all three UCPs have activity in uncoupling, the primary physiological function of UCP2 and UCP3 remains unknown. A possible explanation of why UCP2 is unique in preventing muscle differentiation may be the expression pattern of UCPs. UCP1 is specifically expressed in brown fat, and UCP3 is also restricted in certain muscle tissues (1, 2). By contrast, UCP2 has been found in various tissues including skeletal muscle, smooth muscle, cardiac stem cells, and neurons (1, 2). The mild uncoupling caused by UCP2 may have a signaling role (2). It is well established that UCP2 attenuates mitochondrial reactive oxygen species (ROS) production, which has long been thought to have deleterious effects on cells (1, 2). Small amounts of ROS, however, can function as intracellular second messenger and activate signaling cascades involved in growth and differentiation of many cell types (17–19). For example, the p38 mitogen-activated protein kinase (MAPK) signaling pathway, which plays a role in accelerating myogenesis, is sensitive to ROS (20). Sublethal levels of ROS have been shown to mediate differentiation in skeletal muscle (21), smooth muscle (22), cardiac stem cells (23), and neurons (24). Although UCP3 can also reduce ROS production, its restricted expression pattern makes it a less likely candidate for being predominant antagonist of ROS (25). For example, UCP3 does not act as a protective agent against palmitate-dependent induction of ROS production in differentiated skeletal muscle cells (26).

Besides mediating normal physiological processes, miR-133a targeting UCP2 may also be implicated in pathological processes. Here, we speculate that UCP2 and miR-133a may play important roles in regulating heart failure development. This postulate is supported by several pieces of evidence. Enhanced UCP2 expression has recently been reported to play an important role in the development of heart failure (27–30). Patients with heart failure have low myocardial phosphocreatine-to-ATP ratios, suggesting a cardiac energy deficit (31). UCP2, by virtue of its proton leak activity, decreases the generation of ATP and therefore causes myocardial energy deficiency. Interestingly, decreased miR-133a expression has been found in murine models and human disease states of cardiac hypertrophy and heart failure (32, 33). Together, these clues point to a role for miR-133a targeting UCP2 as a heretofore hidden disease regulator of heart failure.

Taken together, our findings illustrate a novel function of UCP2 in fine-tuning MyoD-mediated myogenic differentiation. Precisely understanding the role of UCP2 and its underlying mechanisms may open up an exciting new method of treatment for diseases such as heart failure and skeletal muscle atrophy.

REFERENCES

1. Krauss, S., Zhang, C. Y., and Lowell, B. B. (2005) Nat. Rev. Mol. Cell Biol. 6, 248–261
2. Brand, M. D., and Esteves, T. C. (2005) Cell Metab. 2, 85–93
3. Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillault, F., and Miroux, B. (2001) J. Biol. Chem. 276, 8705–8719
4. Bartel, D. P. (2004) Cell 116, 281–297
5. Chen, C. F., Ridzon, D. A., Broomer, A. I., Zhou, Z. H., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. I. (2005) Nucleic Acids Res. 33, e179
6. Krauss, S., Zhang, C. Y., Scorrano, L., Dalgaard, L. T., St-Pierre, J., Grey, S. T., and Lowell, B. B. (2003) J. Clin. Investig. 112, 1831–1842
7. O’Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005) Nature 435, 839–843
8. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q. L., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) Nat. Genet. 38, 228–233
9. Anderson, C., Catoe, H., and Werner, R. (2006) Nucleic Acids Res. 34, 5863–5871
10. van Rooij, E., Sutherland, L. B., Qi, X. X., Richardson, J. A., Hill, J., and Olson, E. N. (2007) Science 316, 575–579
11. Soulez, M., Rouviere, C. G., Chafey, P., Hentzen, D., Vandromme, M., Noma, T., Tsuji, T., Kimura, S., and Kohno, M. (2002) Lancet 359, 1786–1788
12. Clempus, R. E., and Griendling, K. K. (2006) Circ. Res. 98, 991–1001
13. Anderson, C., Catoe, H., and Werner, R. (2006) Nucleic Acids Res. 34, 5863–5871
14. van Rooij, E., Sutherland, L. B., Qi, X. X., Richardson, J. A., Hill, J., and Olson, E. N. (2007) Science 316, 575–579
15. Berkes, C. A., and Tapscott, S. J. (2005) Semin. Cell Dev. Biol. 16, 585–595
16. Rao, P. K., Kumar, R. M., Farahmand, M., Baskerville, S., and Lodish, H. F. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 8721–8726
17. Clempus, R. E., and Griendling, K. K. (2006) Cardiovasc. Res. 71, 216–225
18. Torres, M., and Forman, H. J. (2005) Biofactors 17, 287–296
19. Lalloi, C., Apel, K., and Danon, A. (2004) Curr. Opin. Plant Biol. 7, 323–328
20. Cnop, M., and Forman, H. J. (2005) Biofactors 21, 309–313
21. Schrauwen, P., and Hesselink, M. (2002) J. Exp. Biol. 205, 2275–2285
22. Duval, C., Camara, Y., Hondares, E., Sibille, B., and Villarroya, F. (2007) FEBS Lett. 581, 955–961
23. Murray, A. J., Anderson, R. E., Watson, G. C., Radda, G. K., and Clarke, K. (2006) Mol. Cell. Neurosci. 33, 345–357
24. Krauss, S., Zhang, C. Y., and Lowell, B. B. (2005) Cell Metab. 2, 85–93
25. Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillault, F., and Miroux, B. (2001) J. Biol. Chem. 276, 8705–8719
26. Bartel, D. P. (2004) Cell 116, 281–297
27. Chen, C. F., Ridzon, D. A., Broomer, A. I., Zhou, Z. H., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. I. (2005) Nucleic Acids Res. 33, e179
28. Krauss, S., Zhang, C. Y., Scorrano, L., Dalgaard, L. T., St-Pierre, J., Grey, S. T., and Lowell, B. B. (2003) J. Clin. Investig. 112, 1831–1842
29. O’Donnell, K. A., Wentzel, E. A., Zeller, K. I., Dang, C. V., and Mendell, J. T. (2005) Nature 435, 839–843
30. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q. L., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) Nat. Genet. 38, 228–233
31. Anderson, C., Catoe, H., and Werner, R. (2006) Nucleic Acids Res. 34, 5863–5871
30. Bodyak, N., Rigor, D. L., Chen, Y. S., Han, Y., Bisping, E., Pu, W. T., and Kang, P. M. (2007) *Am. J. Physiol.* **293**, H829–H835
31. Neubauer, S., Horn, M., Cramer, M., Harre, K., Newell, J. B., Peters, W., Pabst, T., Ertl, G., Hahn, D., Ingwall, J. S., and Kochsieck, K. (1997) *Circulation* **96**, 2190–2196
32. van Rooij, E., Sutherland, L. B., Liu, N., Williams, A. H., McAnally, J., Gerard, R. D., Richardson, J. A., and Olson, E. N. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 18255–18260
33. Care, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M. L., Segnalini, P., Gu, Y. S., Dalton, N. D., Elia, L., Latronico, M. V. G., Hoydal, M., Autore, C., Russo, M. A., Dorn, G. W., Ellingsen, O., Ruiz-Lozano, P., Peterson, K. L., Croce, C. M., Peschle, C., and Condorelli, G. (2007) *Nat. Med.* **13**, 613–618