miR-27 regulates mitochondrial networks by directly targeting the mitochondrial fission factor

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Mitochondrial morphology is dynamically regulated by forming small, fragmented units or interconnected networks, and this is a pivotal process that is used to maintain mitochondrial homeostasis. Although dysregulation of mitochondrial dynamics is related to the pathogenesis of several human diseases, its molecular mechanism is not fully elucidated. In this study, we demonstrate the potential role of miR-27 in the regulation of mitochondrial dynamics. Mitochondrial fission factor (MFF) mRNA is a direct target of miR-27, whose ectopic expression decreases MFF expression through binding to its 3'-untranslated region. Expression of miR-27 results in the elongation of mitochondria as well as an increased mitochondrial membrane potential and mitochondrial ATP level. Our results suggest that miR-27 is a novel regulator affecting morphological mitochondrial changes by targeting MFF.

INTRODUCTION

MicroRNAs (miRNAs) are a type of small noncoding RNA that regulates numerous cellular activities by suppressing gene expression.1–3 These RNAs are involved in various cellular processes, including cellular proliferation, differentiation, death and development via imperfect base pairing with target mRNAs.4–6 Aberrant expression of miRNAs has been associated with many pathological conditions, such as malignancies and metabolic disorders.7–9

Mitochondria continuously change their morphology by fusing or dividing in response to the different physiological needs of the cells,10 and several studies have shown that the tight regulation of mitochondrial morphology is critical for the maintenance of mitochondrial structures and functions affecting cell fate.11–13 Mitochondrial dynamics is governed by several core proteins, including mitofusin 1 (MFN1), mitofusin 2 (MFN2), dynamin-related protein 1 (DRP1), mitochondrial fission factor (MFF), mitochondrial fission 1, mitochondrial dynamics 51 and optic atrophy protein 1 (OPA1).14–18

Although disruption of the dynamic mitochondrial balance is known to be related to several physiological and pathological conditions such as aging, apoptosis, cancer, neurodegenerative diseases and diabetes, the regulatory mechanisms involved in mitochondrial dynamics remain largely unknown.19–21 Recently, several studies have indicated the involvement of miRNAs in the regulation of mitochondrial dynamics. For example, miR-499 and miR-30 regulate the mitochondrial fission machinery by directly targeting DRP1;22,23 miR-484 and miR-761 are responsible for regulating mitochondrial fission 1 and MFF, respectively;24,25 miR-140 and miR-19b negatively regulate mitochondrial fusion by downregulating MFN1;26,27 and miR-106b is responsible for mitochondrial dysfunction by targeting MFN2.28

Results from this study reveal that miR-27 functions as a novel factor regulating mitochondrial dynamics by suppressing MFF expression. We show that miR-27 suppresses the association of MFF mRNA with polysomes via its 3'-untranslated region (UTR). Ectopic expression of the miR-27 precursor resulted in mitochondrial fusion, thereby increasing the mitochondrial membrane potential as well as the mitochondrial ATP level. Taken together, our data provide experimental evidence, suggesting that miR-27 is involved in negatively regulating mitochondrial fission by directly targeting MFF.
MATERIALS AND METHODS

Cell culture, transfection, plasmids and miRNAs

Human CHANG liver cells stably overexpressing mitochondria-targeted yellow fluorescent protein (mitYFP) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics. Enhanced green fluorescent protein (EGFP) reporters were cloned by inserting 3’-UTR fragments from the MFF mRNA into pEGFP-C1 (BD Bioscience, San Jose, CA, USA). A mutant reporter lacking the binding sites for the miR-27 seed region was generated by site-directed mutagenesis using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). The plasmids, miRNAs [control miRNA (Ctrl)], as well as the precursor and an inhibitor of miR-27 (Bioneer, Daejeon, Korea) were transiently transfected using Lipofectamine 2000 (Invitrogen).

Western blot analysis

Whole-cell lysates were prepared using RIPA buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA and 0.1% sodium dodecyl sulfate) containing 1× protease inhibitor cocktail (Roche, Basel, Switzerland), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). The membranes were incubated with primary antibodies against MFF (Abcam, Cambridge, MA, USA), GFP (Santa Cruz Biotech, Santa Cruz, CA, USA) or β-actin (Abcam) and then further incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech). The signals were detected using enhanced luminescence (Bio-Rad, Hercules, CA, USA).

RNA analysis

Total RNA was prepared from whole-cell lysates using Trizol (Invitrogen). After reverse transcription (RT) using random hexamers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference, cDNA was synthesized as described above. The relative levels of the MFF mRNA were assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis using the SYBR green PCR master mix (Kapa Biosystems, Wilmington, MA, USA) and gene-specific primer sets (Table 1). RT-qPCR analysis was performed using the StepOne Plus system (Life Technologies, Waltham, MA, USA).

Fluorescence microscopy

To visualize the changes in mitochondrial morphology, YFP signals from CHANG liver cells stably expressing mitYFP or cells incubated with 100 nM MitoTracker Red CMXRsos (Invitrogen) for 30 min at 37°C were observed under the fluorescence microscope. Images were acquired using an Axiovertm camera attached to an Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany).

Measurement of the mitochondrial membrane potential and ATP level

The mitochondrial membrane potential was measured using the JC1 Mitochondrial Membrane Potential Assay Kit (Abcam). Cells were incubated with tetraethyl benzimidazoly carbocyanine iodide (JC-1) staining solution (Abcam) for 10 min at 37°C in the dark, and the fluorescence was measured at 535 nm (excitation)/590 nm (emission) using a Victor3 fluorescent plate reader (Perkin-Elmer, Waltham, MA, USA).

The cellular mitochondrial ATP level was measured using the Mitochondrial ToxGlo assay (Promega, Madison, WI, USA) according to the manufacturer’s procedure. Briefly, CHANG liver cells were harvested and suspended by pipetting until they were evenly dispersed. The resuspended cells were then incubated with galactose-containing media at 37°C for 90 min and then further incubated with ATP detection reagent. Luminescence was measured using a Victor3 plate reader (Perkin-Elmer).

Polyosomal analysis

Forty-eight hours after transfection of the precursor of miR-27 or the inhibitor of miR-27 with the control miRNA, CHANG liver cells were preincubated with cycloheximide (100 μg ml⁻¹, 15 min) and then lysed with polyosome extraction buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 0.5% (v/v) Nonidet P-40, 1× protease inhibitor cocktail and RNase inhibitor, followed by centrifugation at 10,000 g for 10 min. The lysates were further fractionated by ultracentrifugation through linear sucrose gradients as described in the previous studies.²⁹,³⁰ RNAs from each fraction were isolated, and cDNA was synthesized as described above. The relative levels of MFF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) miRNAs were analyzed by RT-qPCR using specific primer sets.

RESULTS

Identification of miRNAs targeting MFF

Although MFF is one of the critical factors regulating mitochondrial morphological changes, the mechanisms involved in the regulation of MFF expression are not fully understood.¹۶,¹۷,²۵,⁳¹ To identify miRNAs affecting the morphological changes of mitochondria via MFF, we analyzed the MFF mRNA using TargetScan 4.2 and mircurona.org. Human MFF mRNA (NM_020194) is composed of a 266-bp 5′-UTR, the MFF coding sequence, and an ~ 700-bp 3′-UTR, which harbors miRNA binding sites, as shown in Figure 1a. In silico analysis revealed that several miRNAs can potentially interact with the MFF mRNA 3′-UTR. Among these miRNAs, we examined the effects of miR-141, miR-27b and miR-200c on MFF expression using western blot analysis (Figure 1b). miR-27b and miR-200c resulted in a significant downregulation of MFF expression, whereas miR-141 did not alter the MFF level in CHANG liver cells, despite the positive prediction of binding between miR-141 and the MFF mRNA. Because miR-200c did not show reproducible effects on MFF expression in CHANG liver cells, we decided to further examine the relationship between miR-27b and MFF expression. Although miR-27a and miR-27b are located on different chromosomal

Table 1 Primer list used in this study

| Primer name | Sequences |
|-------------|-----------|
| Human MFF-3U-F | 5′-AAAAAAGATCTTAA CACGTCTGAGCA-3′ |
| Human MFF-3U-R | 5′-AAAGATGACTCTTGACCAGA-3′ |
| Human MFF-3UM-F | 5′-AAGTTGAGACAAAGAAAAACATTATT-3′ |
| Human MFF-3UM-R | 5′-CTCTTTGTGCTACATTTCTGAGAAT-3′ |
| Human MFF-F | 5′-CACCACCTGTGACTTACCG-3′ |
| Human MFF-R | 5′-GTCCTGACAAGCTGCTGAGAT-3′ |
| Human GAPDH-F | 5′-TGACCACACCAGCTTCAGA-3′ |
| Human GAPDH-R | 5′-GCCATGAGACTGTCATGAGA-3′ |

Abbreviations: F, forward; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MFF, mitochondrial fission factor; R, reverse.
miR-27 targets the mitochondrial miR-27 binding sites, but it did not affect the expression of miR-27 expression decreased the expression of EGFP harboring levels were analyzed by western blotting using an anti-EGFP into CHANG liver cells, and miR-27 expression and EGFP Figure 2c. The reporter constructs were sequentially transfected seed region base pairing with the sites (1509–1305 bp) harboring the miR-27 translation sites, whereas the miR-27 precursor repressed the translation of MFF mRNA by miR-27. Because miR-27 negatively regulated MFF expression without significantly changing the MFF mRNA levels, we investigated whether miR-27 was involved in MFF mRNA translational repression. The relative association of the MFF mRNA with polyribosomes (polysomes) was analyzed using polysome fractionation on sucrose gradients, as described in the previous studies.29,30,34 The levels of MFF mRNA in each fraction, that is, untranslated (fractions 1 and 2), ribosome subunits and monoribosomes (fractions 3–5), low-molecular-weight polysomes (fractions 6–8) and high-molecular-weight polysomes (fractions 9–13), were then measured by RT-qPCR. The polysome profiles were not affected by transfection of the miR-27 precursor or inhibitor (Figure 3a). Compared with the distribution of the MFF mRNA in control cells (peaking at fractions 7 and 8), the expression of the miR-27 precursor resulted in a shift of the MFF mRNA distribution to the lower portions of the gradient, with much of the MFF mRNA peaking at fraction 7 (Figure 3b, top). Conversely, expression of the miR-27 inhibitor increased the relative abundance of the MFF mRNA in the highly translated fractions (fractions 8 and 9). In contrast, the distribution of GAPDH mRNA was not affected by miR-27 expression (Figure 3b, bottom). These data revealed that miR-27 altered the MFF mRNA translational status. Taken together, these observations imply that miR-27 represses MFF mRNA translation by interacting with its 3’-UTR.

**Reduction of mitochondrial fission by miR-27**

MFF mediates mitochondrial fission by recruiting DRP1, and MFF downregulation is responsible for mitochondrial elongation.16,17,33 Because miR-27 regulates MFF expression, we further tested whether miR-27 affected changes in mitochondria morphology. The miR-27 precursor or inhibitor with control miRNA were transfected into CHANG liver cells stably

[Image 330x579 to 442x704]
miR-27 results in morphological changes of mitochondria

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Figure 2 miR-27 regulates the mitochondrial fission factor (MFF) expression. After transfection of pre-miR-27, anti-miR-27 and control miRNA, the MFF mRNA and protein levels were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (a) and western blotting (b), respectively. (a) The relative expression of MFF mRNA was analyzed using GAPDH mRNA for normalization. The data represent the mean ± s.e.m. from three independent experiments. (b) The MFF protein level was analyzed by western blotting using an anti-MFF antibody, and the β-actin level is shown as a loading control. Images are representative of three independent experiments, and the numbers represent the mean ± s.e.m. from three independent experiments. (c) Schematic representation of the reporter plasmids pEGFP (control), pEGFP-MFF 3U and pEGFP-MFF 3UM, the last of which bears five mutated nucleotides in the MFF mRNA that correspond to the miR-27 seed region. (d) CHANG liver cells were co-transfected with the plasmids presented in panel c and with the indicated miRNAs. Forty-eight hours after transfection, the EGFP expression levels were assessed by western blotting. The data represent the mean ± s.e.m. of three independent experiments. *P<0.05. EGFP, enhanced green fluorescent protein.

miR-27 expression results in the reduction of mitochondrial morphology induced by miR-27 expression affected the mitochondrial membrane potential, CHANG liver cells were transfected with miR-27 precursor or inhibitor with control miRNA, and the mitochondrial potentials were determined by JC-1 dye staining. As shown in Figure 5a, ectopic miR-27 expression resulted in an increase in the mitochondrial membrane potential, whereas miR-27 inhibition decreased the mitochondrial membrane potential.

To disprove that the increase in mitochondrial activity induced by miR-27 was because of a change in cell number, mitochondrial ATP synthesis after miR-27 expression was analyzed using the ToxGlo assay. The mitochondrial ATP level was increased by ectopic miR-27 expression (P = 0.028) and was decreased by inhibitor expression (P = 0.021) (Figure 5b). However, there were no significant effects on cell number (Figure 5c). These results indicate that the effects of miR-27 on mitochondrial activity are independent of cell viability. Taken together, these results suggest that miR-27 enhances mitochondrial function by downregulating MFF expression.

DISCUSSION

The dynamic control of mitochondrial morphology is a pivotal process for maintaining cellular homeostasis, and its dysregulation is associated with several human diseases such as cancer, diabetes and neurodegenerative diseases.11-13 Although several
efforts have been made to identify novel factors that regulate mitochondrial dynamics, including miRNAs, and to elucidate the regulatory mechanisms governing mitochondrial dynamics, these factors and mechanisms remain largely unknown.19–28

The present study demonstrates that miR-27 negatively regulates mitochondrial fission by inhibiting MFF mRNA translation. Ectopic miR-27 expression resulted in increases in mitochondrial fusion and mitochondrial activity, whereas miR-27 inhibition enhanced mitochondrial fission and reduced the mitochondrial membrane potential. We performed an in silico analysis using TargetScan 4.2 and microrna.org to examine whether miR-27 affected the expression of other factors that regulate mitochondrial morphology, and we confirmed that miR-27 could not regulate DRP1, mitochondrial fission 1 or MFN1/2. To our knowledge, this is the first study demonstrating the direct regulation of the mitochondrial fission machinery and mitochondrial activity by miR-27 via MFF regulation.

MFF is one of the critical regulators promoting mitochondrial fission by recruiting DRP1 to the mitochondria, and fine-tuning of its expression is responsible for mitochondrial dynamics.16,17,31 However, the detailed mechanisms involved in MFF expression are not fully elucidated. It was recently shown that MFF downregulation by miR-761 promotes mitochondrial fusion in rat cardiomyocytes and protects those cells from hydrogen peroxide-induced apoptosis.25 In addition, in this study, we demonstrated a novel function of miR-27 as a MFF mRNA translational suppressor that enhances mitochondrial fusion. Further studies may enable us to identify novel regulators and to understand the molecular axis affecting MFF expression at the transcriptional, posttranscriptional and post-translational levels (Figure 5d).

In contrast to our findings, miR-27 was recently reported to impair adipocyte differentiation and mitochondrial function by targeting prohibitins in human adipose-derived stem cells.41 Prohibitins have a role as protein scaffolds in the mitochondria and are involved in diverse cellular processes, including the processing of OPA1 by m-AAA protease, thereby enhancing mitochondrial fusion.42–43 Previous studies have shown that prohibitin levels increase and that miR-27 levels decrease during adipogenesis.36,41,44–46 The inverse correlation between prohibitins and miR-27 is responsible for the efficient mitochondrial fusion during adipogenesis in response to the cellular energy demand in adipocytes.

The dynamin-like GTPase OPA1, a gene product of human dominant optic atrophy, is involved in mitochondrial fusion and remodeling, and downregulation of OPA1 results in mitochondrial fragmentation.43,47,48 However, a recent
study by Otera et al.\textsuperscript{16} demonstrated that depletion of MFF suppresses OPA1 knockdown-induced mitochondrial fragmentation, indicating that MFF limits OPA1 silencing-induced mitochondrial fission. Another study showed that OPA1 normally counteracts the proapoptotic action of mitochondrial fission 1, which promotes mitochondrial fission.\textsuperscript{48} We did not examine the expression levels of prohibitin or OPA1 in our system; therefore, it is difficult to conclude what would be the predominant mechanism governing mitochondrial dynamics with this limited information. Additionally, \textit{in silico} analysis indicated that miR-27 potentially targets OPA1; however, we did not see significant downregulation of OPA1 after overexpression of miR-27 in our system. The discrepancy between the results of a previous study and our findings showing the effect of miR-27 on mitochondrial morphology may result from the use of various assays and systems to measure the relative levels of MFF, prohibitin and OPA1. Additional studies must be designed to further explore and understand the role of miR-27 in the regulation of mitochondrial dynamics in various systems.

miRNA expression alterations have been reported to be involved in several physiological and pathological processes such as aging, tumorigenesis, metabolism and inflammation,\textsuperscript{8,49–51} and several studies have demonstrated differential expression of miR-27 in various diseases. miR-27 is upregulated in breast cancer, oral cancer, glioma and

\begin{figure}
\centering
\includegraphics[width=\textwidth]{miR-27_inhibits_mitochondrial_fission}
\caption{miR-27 inhibits mitochondrial fission. CHANG liver cells expressing mitochondria-targeted YFP (mtYFP) were transiently transfected with pre-miR-27, anti-miR-27 or control miRNA. Forty-eight hours after transfection, the mtYFP (a) or Mitotracker (b) signals were observed under a fluorescence microscope. The data are representative of three independent experiments. (c) Scoring of mitochondrial morphology for the indicated cells. Forty-eight hours after transfection, each cell was placed into one of three morphological categories, and the percentages of cells with the indicated mitochondrial morphologies (intermediate, elongated or fragmented forms) from 100 cells were calculated from three independent experiments. The data represent the mean±s.e.m. from three independent experiments. \textit{**} \textit{P}<0.01.}
\end{figure}
Peripheral arterial disease\textsuperscript{41,52–55} and is downregulated in prostate cancer and head and neck squamous cancer.\textsuperscript{56,57} Several reports have shown that mitochondrial fusion can inhibit cell death, whereas mitochondrial fission is involved in the promotion of apoptosis.\textsuperscript{58–61} From this interpretation, it would be assumed that the increased expression of miR-27 in various cancers may enhance mitochondrial fusion via targeting MFF, thereby promoting mitochondrial dysfunction and tumor progression. Further studies are necessary to explore the relationship between miR-27 and MFF in health and disease.

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