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**microRNA-200a-3p enhances mitochondrial elongation by targeting mitochondrial fission factor.**

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Running title: miR-200a-3p promotes mitochondrial fusion

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## Abstract

Mitochondria play pivotal roles in the ATP production, apoptosis and generation of reactive oxygen species. Although dynamic regulation of mitochondria morphology is a critical step to maintain cellular homeostasis, the regulatory mechanisms are not yet fully elucidated. In this study, we identified miR-200a-3p as a novel regulator of mitochondrial dynamics by targeting mitochondrial fission factor (MFF). We demonstrated that the ectopic expression of miR-200a-3p enhanced mitochondrial elongation, increased mitochondrial ATP synthesis, mitochondrial membrane potential and oxygen consumption rate. These results indicate that miR-200a-3p positively regulated mitochondrial elongation by downregulating MFF expression.

## Introduction

Mitochondria play essential roles in balancing cellular energy homeostasis as well as regulation of apoptosis (1-3). Tight regulation of mitochondrial morphology in response to various cellular stimuli is critical to maintain mitochondrial function. Mitochondria continuously change their morphologies by dividing (fission) or elongating (fusion) each other. Several key proteins regulating mitochondrial morphology have been identified. Dynamin-related protein (DRP1), mitochondrial fission 1 protein (FIS1), and mitochondrial fission factor (MFF) promote mitochondrial fragmentation, while mitofusin 1/2 (MFN1/2), and optic atrophy 1 (OPA1) lead to mitochondrial elongation (3-6). Relative expression levels or post-translational modifications of key regulatory proteins are responsible for dynamic changes in mitochondrial morphology (3, 4, 6-9). Although recent reports have shown that post-translational regulatory mechanisms to control the quality of key proteins including phosphorylation (10), de-acetylation (11), and ubiquitination (12), detailed mechanism governing mitochondrial morphology is not fully understood.

microRNAs (miRNAs), small non-coding RNAs (18-22 nt long) downregulate gene expression by destabilizing target mRNAs or inhibiting translation, thereby affecting various cellular processes such as cell proliferation, survival, death, and differentiation (13-24). miRNA expression could be regulated in time- and tissue-specific manners, and differential regulation of miRNAs is closely related to the pathogenesis of diseases (14, 19, 25-28). Recent studies have shown that miRNAs regulate dynamic changes of in the mitochondria morphology by regulating the expression of several key proteins governing mitochondrial dynamics. For example, miR-483-5p and miR-484 are responsible for suppressing mitochondrial fission by targeting FIS1 (29, 30). miR-499 affects mitochondrial dynamics by down-regulating DRP1 expression (31). miR-140 and miR-19b have been reported to decrease mitochondrial elongation through targeting MFN1, and miR-106, miR-195, and miR-761 down-regulate MFN2 expression (32-36). miR-27, miR-761, and miR-593 are responsible for mitochondrial dynamics by downregulating MFF expression (37-39).

In this study, we investigated the role of miR-200a-3p as a novel factor governing mitochondrial dynamics by targeting MFF, that function as a Drp1 receptor (40). The results of this study indicate that miR-200a-3p is bound to 3'untranslated region (3'UTR) of *MFF* mRNA and decreased MFF expression. Ectopic expression of miR-200a-3p in Hep3B cells enhanced mitochondria elongation and increased mitochondrial activity without changes of other regulatory proteins including DRP1, MFN1/2, and OPA1. Our results suggest that miR-200a-3p functions as a novel factor regulating mitochondrial dynamics by decreasing MFF expression.

## Results

### **miR-200a-3p is a novel factor regulating MFF expression.**

Mitochondria dynamics is tightly regulated by several key proteins including DRP1, OPA1, MFN1/2 and MFF (3, 6). It has been reported that expression and activity of those key regulators are modulated via multiple steps including transcriptional, translational, post-transcriptional, and post-translational modification. Previous studies have reported that miR-27, miR-593-5p, and miR-761 regulate MFF expression (38, 39, 41). In this study, we identified miR-200a-3p as a novel regulator governing MFF expression. A survey using two different miRNA prediction algorithms, Targetscan and microrna.org, revealed that *MFF* mRNA 3'UTR has a potential binding site for miR-200a-3p (Fig. 1A). To investigate whether miR-200a-3p affects MFF expression, *MFF* mRNA and proteins levels were determined by RT-qPCR and Western blotting after miR-200a-3p transfection. As shown in Figure 1B, *MFF* mRNA level did not change by miR-200-3p. However, miR-200a-3p overexpression decreased MFF protein, and inhibition of miR-200a-3p increased it. To further analyze the regulation of MFF expression by miR-200a-3p, EGFP reporter was constructed by inserting *MFF* 3'UTR (1751-2040 nt) at the 3'UTR of EGFP open reading frame and EGFP levels were assessed after miR-200a-

3p expression. miR-200a-3p downregulated the reporter expression containing *MFF* 3'UTR, but not that of mutant reporter that missing the seed region for miRNA binding (Fig. 1C and D). These results suggest that miR-200a-3p is responsible for MFF downregulation.

#### **miR-200a-3p increases mitochondrial elongation by MFF downregulation.**

To investigate the effect of miR-200a-3p on the morphological changes of mitochondria, we observed mitochondria morphology of CHANG cells expressing mtYFP or Hep3B cells incubated with Mitotracker, after regulation of miR-200a-3p level. As shown in Figures 2A and B, ectopic expression of miR-200a-3p increased the number of cells having elongated mitochondria, whereas miR-200a-3p inhibition increased the portion of cells having fragmented mitochondria in CHANG mtYFP cells. The regulation of mitochondria morphology by miR-200a-3p was further analyzed in Hep3B cells. As shown in Figures 2C and D, miR-200a-3p also increased the number of cells having elongated mitochondria of Hep3B cells.

Next, the effect of miR-200a-3p affected the levels of key proteins governing mitochondrial dynamics was investigated. The levels of DRP1, MFN1/2, and OPA1 did not change after upregulation or inhibition of miR-200a-3p (Fig. 3). Taken together, those results indicate that miR-200a-3p promotes mitochondrial elongation via MFF downregulation.

#### **miR-200a-3p enhances mitochondrial activity.**

Morphological changes of mitochondria directly affect mitochondrial function including cellular respiration, ATP synthesis, reactive oxygen species production, and mitochondrial-mediated apoptosis (42-45). We investigated whether miR-200a-3p changes the mitochondrial activity. Mitochondrial ATP synthesis and membrane potential were assessed by Toxglo assay and JC1 staining after ectopic expression of miR-200a-3p. As shown in Figure 4A, miR-200a-3p overexpression increased mitochondrial ATP synthesis and membrane potential. These results suggest that miR-200a-3p positively regulates the mitochondrial activity. In addition, oxygen consumption rate (OCR) was also

measured in Hep3B cells transfected with miR-200a-3p using a Seahorse FX analyzer. miR-200a-3p increased the basal respiration rate of mitochondria (Fig. 4B). These results indicate that miR-200a-3p has a potential to increase the mitochondrial activity via MFF downregulation.

## Discussion

Fine-tuning of mitochondrial morphology is a critical step to maintain cellular homeostasis, and impaired regulation of mitochondrial dynamics leads to mitochondrial dysfunction that is responsible for the pathogenesis of several diseases such as cancer, neurodegenerative diseases, cardiovascular diseases (7, 46-48). Previous studies have shown that epigenetic and post-translational modifications are important regulatory mechanisms to control the quality of key proteins control mitochondrial dynamics (10-12, 31, 49). In addition, several studies have indicated that miRNAs are one of critical regulators governing the morphological changes of mitochondria (29, 31-36, 38, 41, 50). In this study, we identified miR-200a-3p as a novel regulator of mitochondrial dynamics by targeting MFF.

miR-200a-3p is a member of miR-200 family consisting of miR-200a, miR-200b, miR-200c, miR-141, and miR-429. miR-200 family play a role in the regulation of cancer progression by targeting zinc finger E-box-binding homeobox 1/2 (ZEB1/2) (51-55). miR-200a-3p is differentially expressed in various types of cancers and functions as a potential therapeutic target (56, 57). Besides tumor suppressive roles of miR-200a-3p, functional studies of miR-200a-3p are not fully elucidated. Herein, we found that miR-200a-3p is involved in the mitochondrial quality control by enhancing mitochondrial elongation. Ectopic expression of miR-200a-3p downregulated MFF level (Fig. 1) and promoted mitochondrial elongation thereby increasing mitochondrial membrane potential and basal respiratory rate (Fig. 2 and 4) Although several reports have shown differential expression of miR-200a-3p in some types of disease models (58-62), the correlation between miR-200a-3p and

mitochondrial dynamics in those models has not yet investigated in this study. Further studies are needed to confirm the implication of miR-200a-3p/MFF axis in the pathogenesis of human diseases.

## Materials and Methods

### Cell culture, transfection, plasmids and miRNAs

Human CHANG liver cells that stably express yellow fluorescent protein, targeting mitochondria (CHANG-mtYFP cells) and Hep3B cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) contained with 10% fetal bovine serum and 1% antibiotics. For reporter analysis, Enhanced green fluorescent protein (EGFP) reporter vectors were constructed by inserting 3'UTR region of *MFF* mRNA (1509-1778 bp) into pEGFP-C1 (BD Bioscience) (41). A mutant reporter lacking the binding sites for the miR-200a-3p seed region was generated by site-directed mutagenesis using a KOD-Plus-Mutagenesis Kit (Toyobo). miRNAs (Bioneer) were transiently transfected using Lipofectamine 2000 (Invitrogen).

### Western blot analysis

Cells were lysed in RIPA buffer (10mM Tris-HCl (pH 7.4), 150mM NaCl, 1% NP-40, 1mM EDTA and 0.1% sodium dodecyl sulfate) and analyzed by SDS-PAGE. Transferred membranes were incubated with primary antibodies against MFF (Abcam), GFP (Santa Cruz Biotech), MFN1 (Abcam), MFN2 (Sigma Aldrich), OPA1 (BD Bioscience), or  $\beta$ -actin (Abcam), and further incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech). Chemiluminescent signals were developed using Clarity<sup>TM</sup> Western ECL substrate (Bio-Rad).

### Fluorescence microscopy

Mitochondrial morphologies were observed under a fluorescence microscope, Axiovert 200M microscope (Carl Zeiss). Yellow fluorescence from mtYFP or red fluorescence from MitoTracker Red CMXRos (Invitrogen) was analyzed as described by Tak, et al.(41). Images were acquired using an



Axiovertcam mRM camera attached to Axiovert 200M microscope (Carl Zeiss). Mitochondrial length was determined by analyzing random 100 cells images of the cells transfected with mtYFP or stained with MitoTracker using Image J software.

### **Measurement of the mitochondrial membrane potential and ATP level**

Mitochondrial membrane potential or mitochondrial ATP levels were determined using a JC1 Mitochondrial Membrane Potential Assay Kit (Abcam) or the Mitochondrial ToxGlo assay (Promega) according to the manufacturer's protocol (41).

### **Analysis of oxygen consumption**

Oxygen consumption rate (OCR) was assessed by Seahorse FX24 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer's instruction. The number of cells ( $1 \times 10^3$ ) was used for OCR measurement. Basal OCR was measured for 3 min every 8 min for four points. Small molecule-metabolic modulators oligomycin (3  $\mu$ M), FCCP (1  $\mu$ M), and antimycin A (1  $\mu$ M) were injected sequentially at the indicated time points after baseline OCR measurement.

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Figure legends

**Figure 1. miR-200a-3p down-regulated MFF expression.**

(A) Schematic diagram of *MFF* mRNA having miR-200a-3p binding site. (B) Hep3B cells were transfected with pre-miR-200a-3p, anti-miR-200a-3p, and control miRNA (CTRL). Forty-eight hours after transfection, abundance of *MFF* mRNA and protein were analyzed by RT-qPCR and western blotting, respectively. (C) Schematic diagrams of the reporter plasmids pEGFP-C1 (control), pEGFP-MFF 3U, and pEGFP-MFF 3UM that lack miR-200a-3p binding site in the *MFF* mRNA. (D) After transfection of miRNAs and EGFP reporters, GFP expression levels were analyzed by western blotting. Results are representative of three independent experiments.

**Figure 2. miR-200a-3p inhibited mitochondria fission.**

(A) CHANG-mtYFP cells were transfected with pre-miR-200a-3p, anti-miR-200a-3p, and control miRNA (CTRL). Forty-eight hours after transfection, mitochondrial morphology was observed by tracing YFP signals. (B) The number of cells was counted and grouped into three different categories according to mitochondrial morphology (intermediate, elongated or fragmented forms) from 100 cells. (C) Hep3B cells were transfected with pre-miR-200a-3p, anti-miR-200a-3p, and control miRNA (CTRL). After transfection of miRNAs, mitochondria were stained with MitoTracker and mitochondrial morphology was observed using a fluorescence microscope. (D) The number of cells were analyzed as described in (B). Images are representative of three independent experiments and the data represent the mean $\pm$ SEM from three independent experiments. Arrows indicate elongated form of mitochondria and arrow heads indicated fragmented mitochondria. \* $P$ <0.05

**Figure 3. Expression of DRP1, MFN1/2, and OPA1 were not changed by miR-200a-3p.**



CHANG-mtYFP cells were transfected with pre-miR-200a-3p, anti-miR-200a-3p and control miRNA (CTRL). Forty-eight hours after transfection, MFF, DRP1, MFN1/2, and OPA1 proteins were analyzed by western blotting. Results are representative of three independent experiments.

**Figure 4. miR-200a-3p affected mitochondrial function by regulating MFF expression.**

(A) Hep3B cells were transfected with miR-200a-3p or control miRNA, and stained with ATP detection reagent (*left*) and JC-1 dye (*right*) to determine mitochondrial membrane potential and mitochondrial ATP levels. Change in the relative luminescence was assessed by measuring the fluorescence. Data represent the mean $\pm$ SEM from three independent experiments. (B) Oxygen consumption rates in miRNA transfected cells were analyzed using a Seahorse XF analyzer. Data represent the mean $\pm$ SEM from three independent measurements. \* $P<0.05$

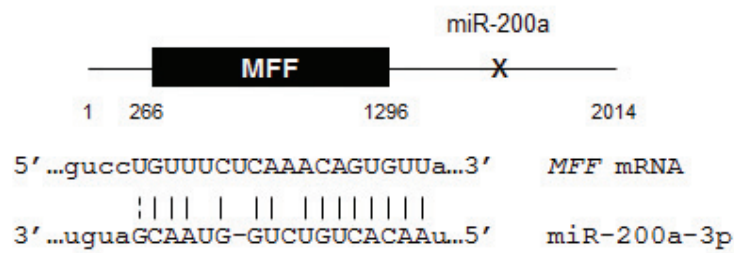
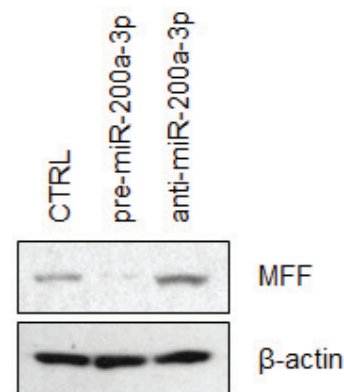
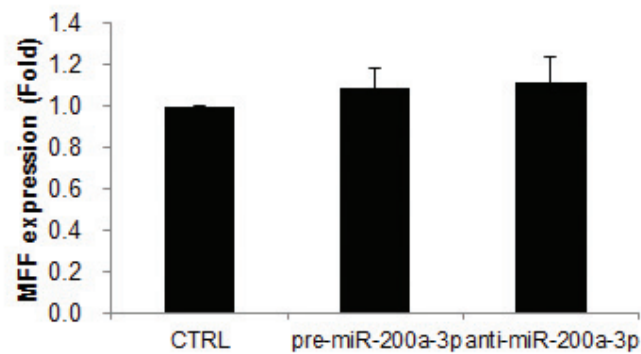
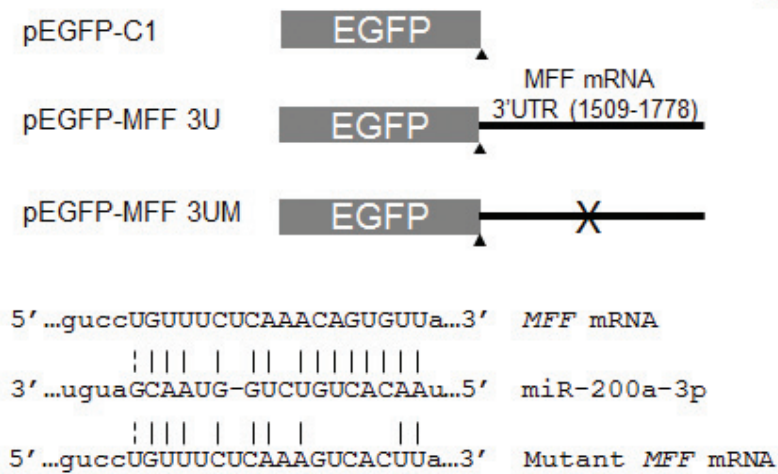
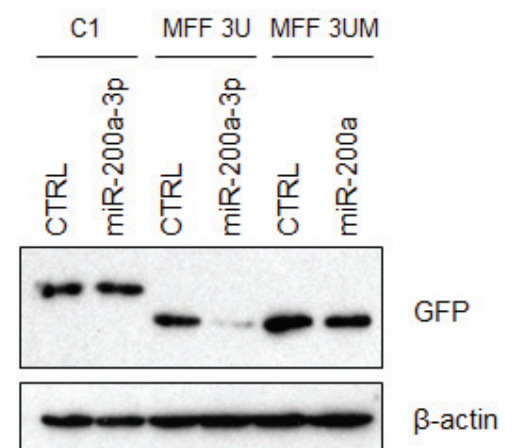
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Fig. 1 Figure 1

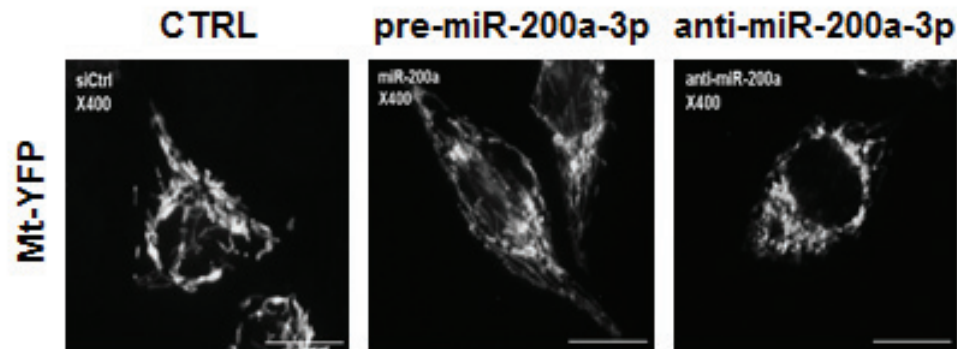
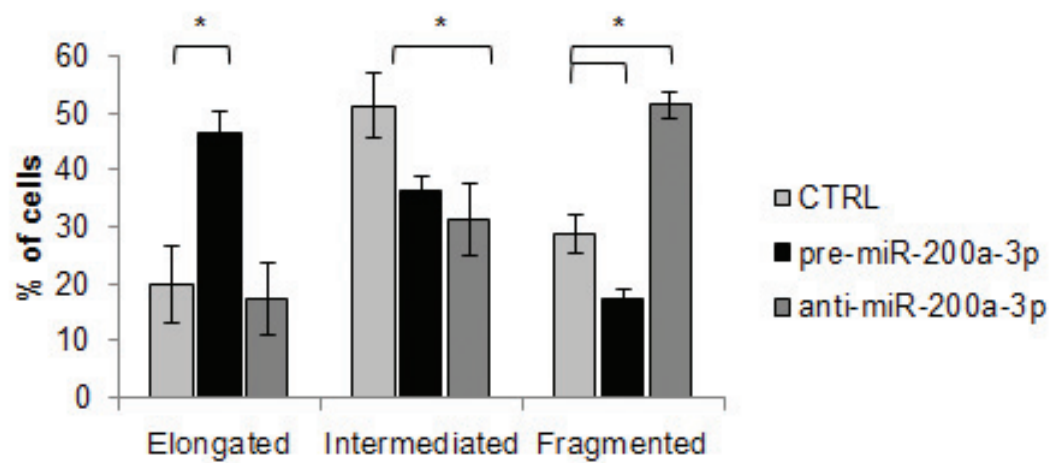
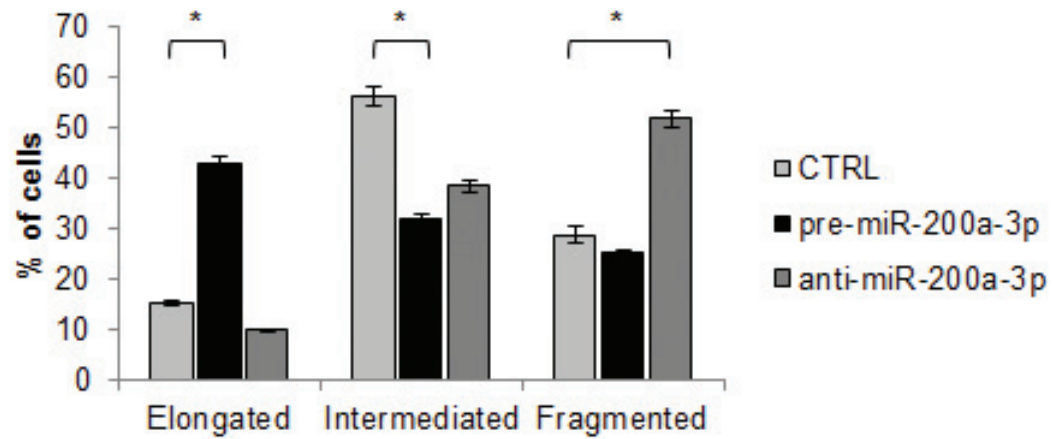
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Fig. 2 Figure 2

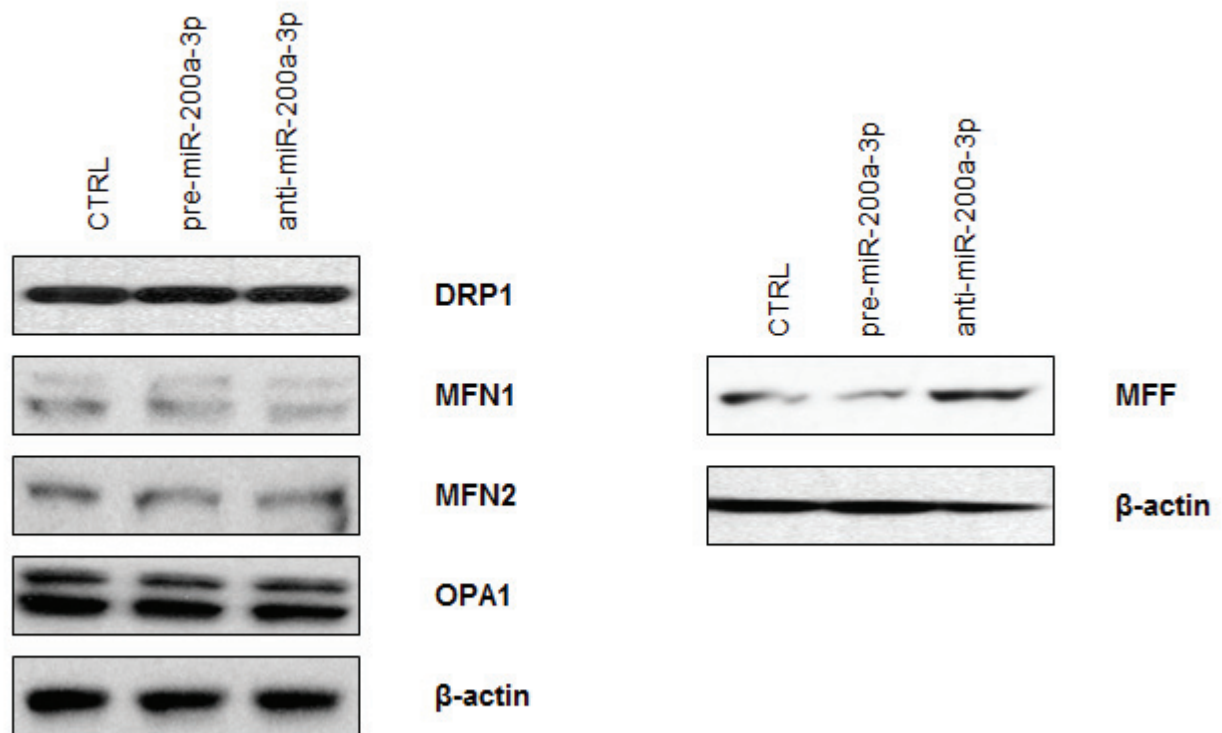


Fig. 3 Figure 3

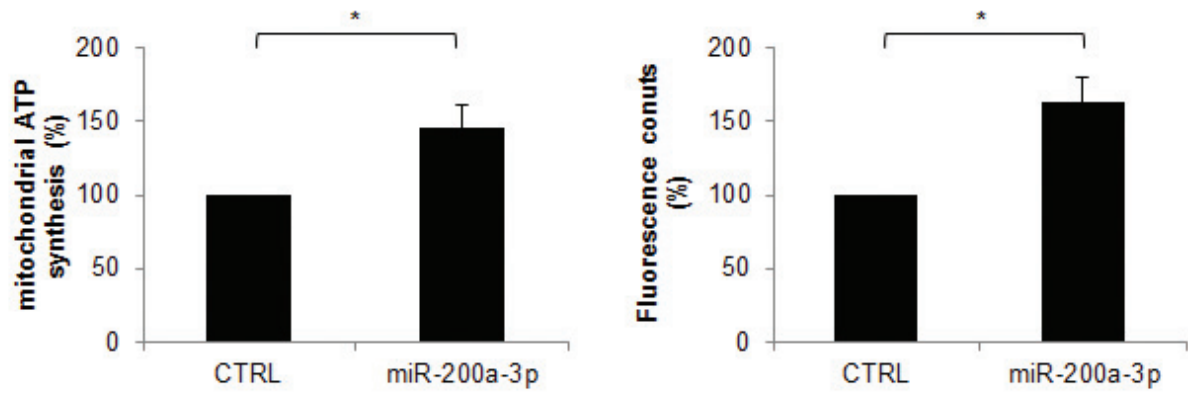
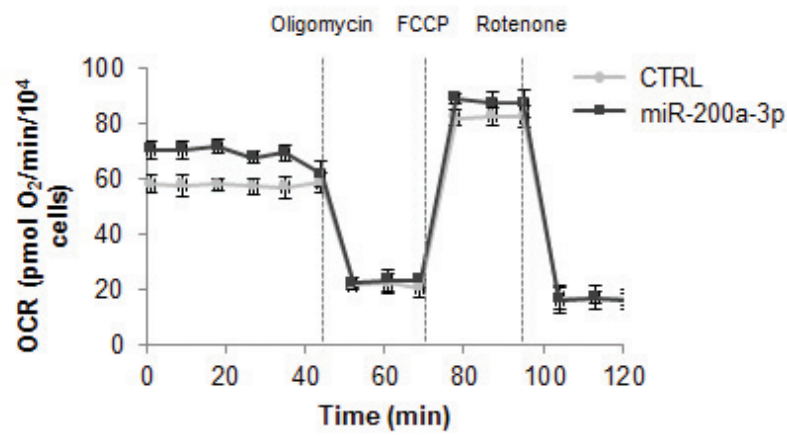
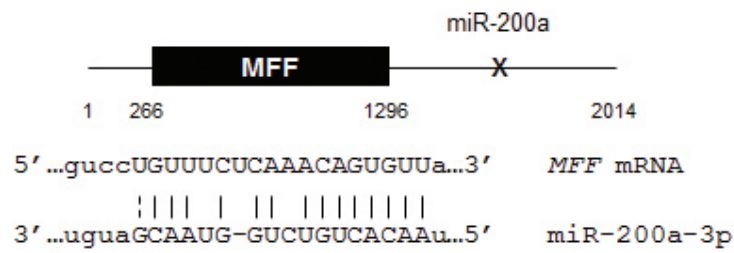
**A****B**

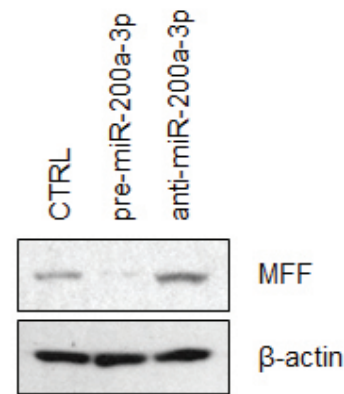
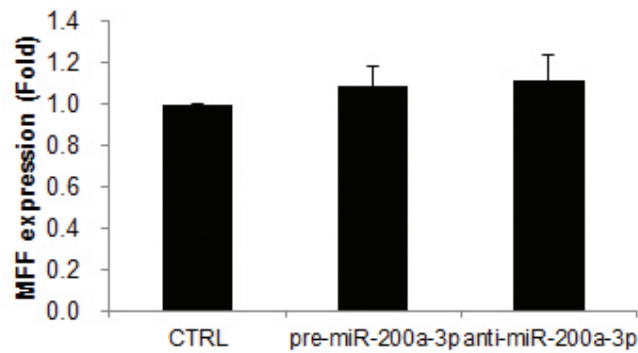
Fig. 4 Figure 4

**A**

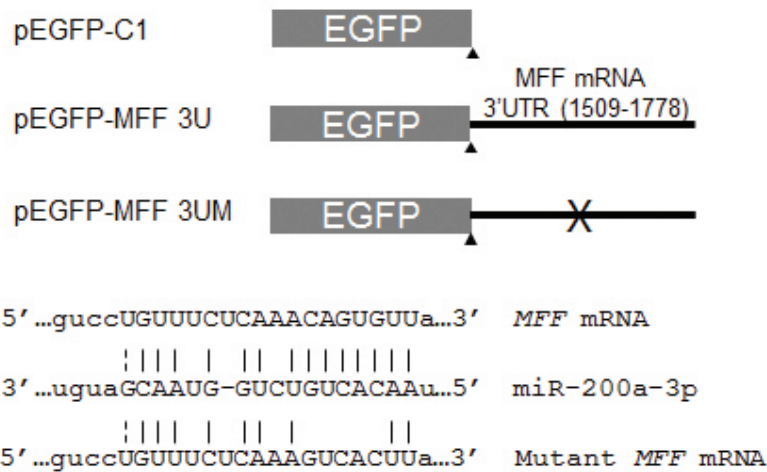
*MFF* mRNA (NM\_020194)



**B**



**C**



**D**

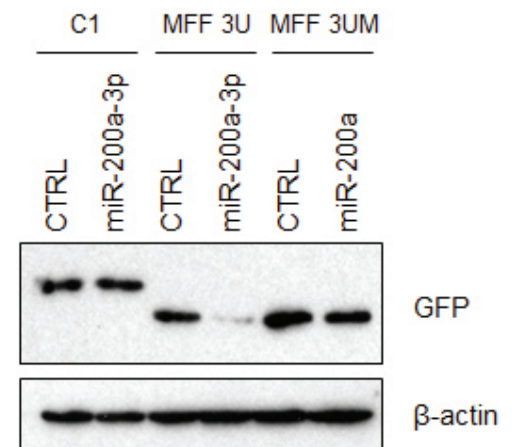


Fig. 5 Figure 1

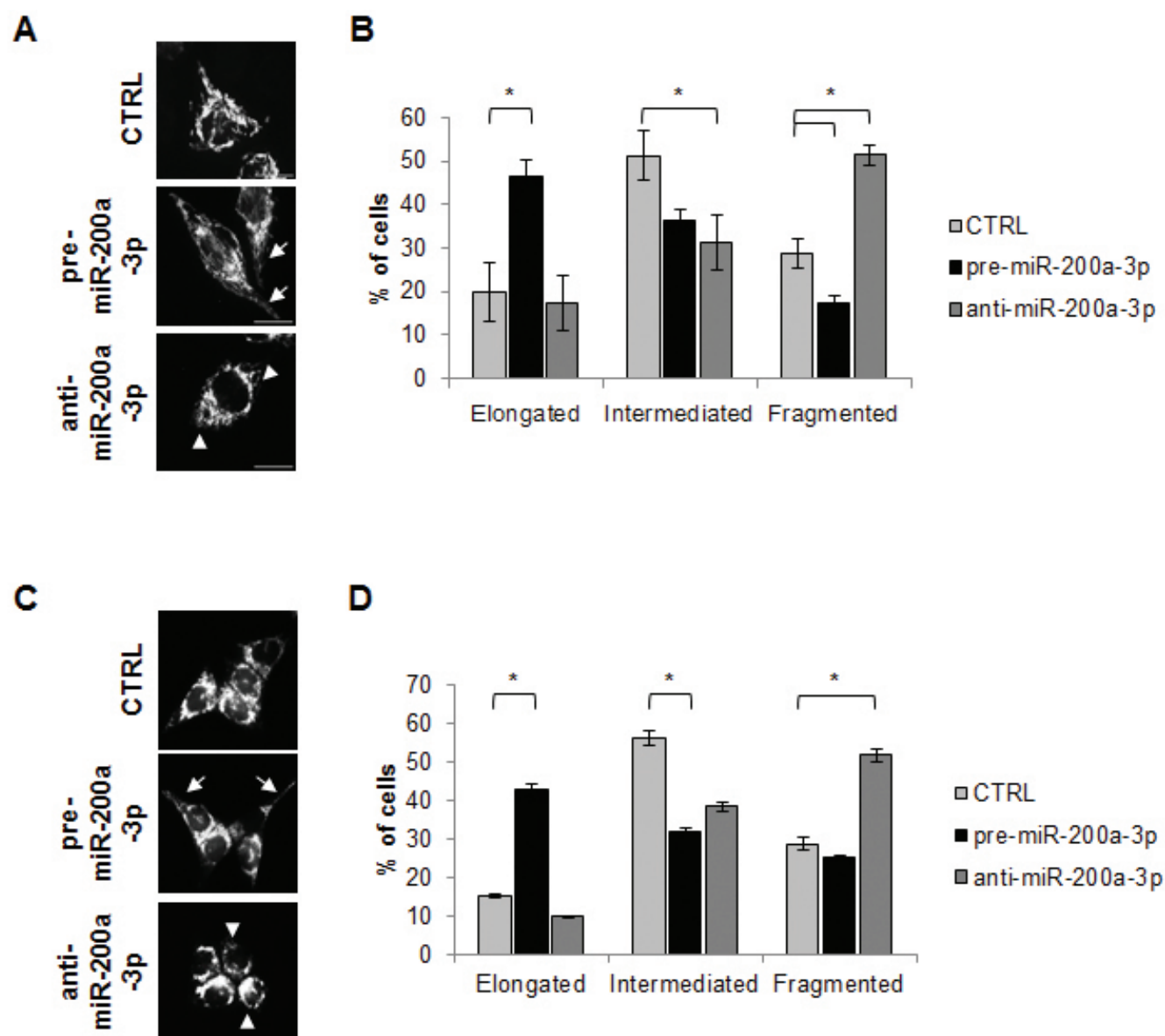


Fig. 6 Figure 2

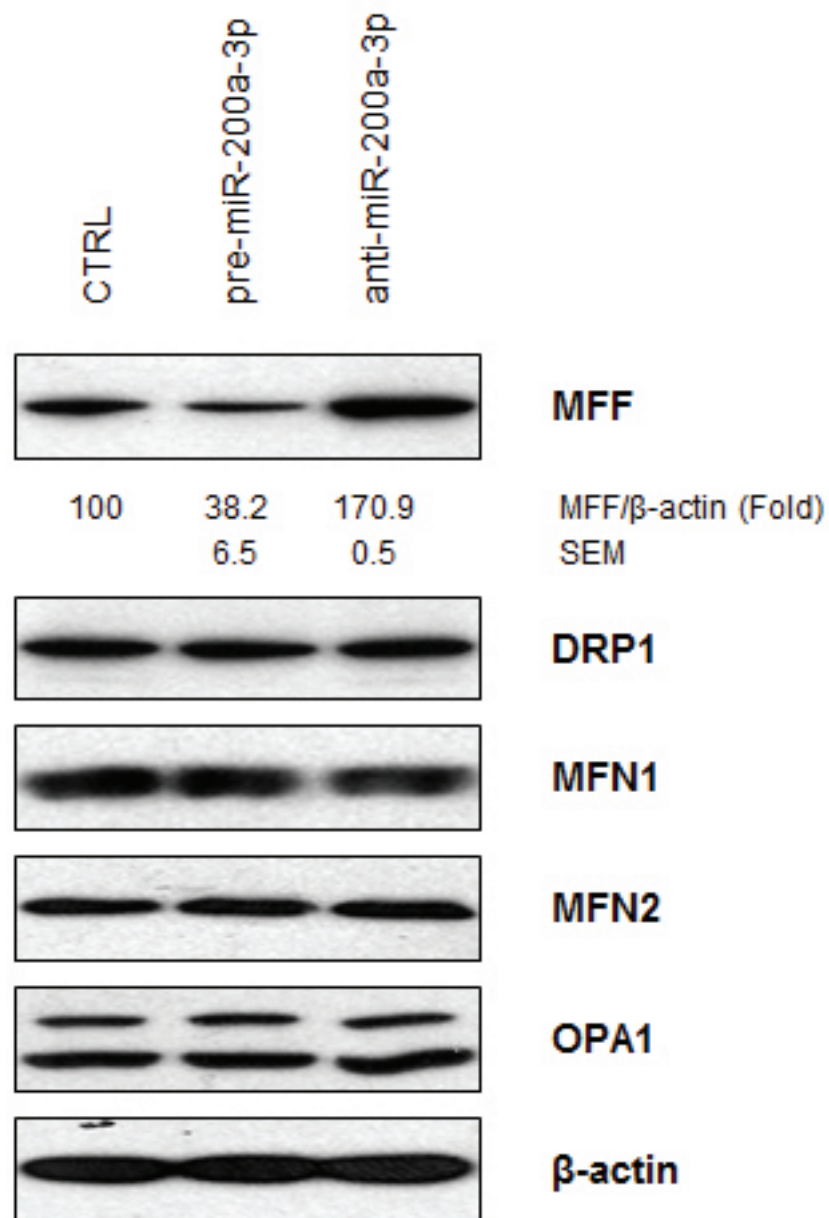


Fig. 7 Figure 3



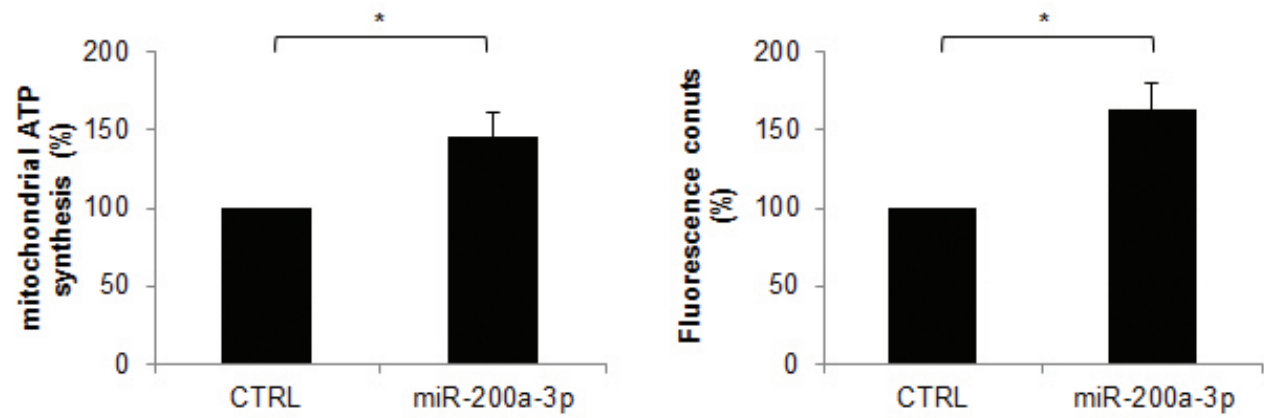
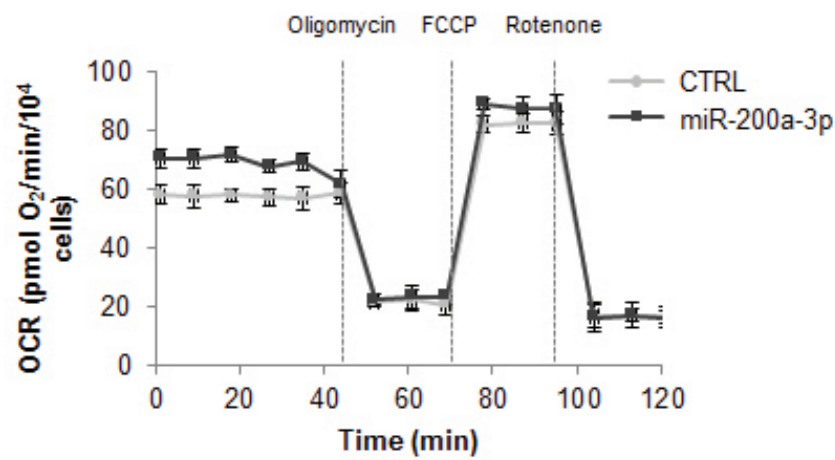
**A****B**

Fig. 8 Figure 4