

BMB Reports – Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-17-006

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

Article Type: Article

Keywords: microRNAs; miR-200a-3p; mitochondrial fission factor; mitochondria fragmentation; mitochondria dynamics

Corresponding Author: Eun Kyung Lee

Authors: Heejin Lee^{1,#}, Hyosun Tak^{1,#}, So Jung Park², Yoon Kyung Jo², Dong Hyung Cho^{2,#}, Eun Kyung Lee^{1,*,#}

Institution: ¹Department of biochemistry, The Catholic University of Korea, ²Department of Gerontology, Graduate School of East-West Medical Science, Kyung Hee University,

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

Heejin Lee^{1#}, Hyosun Tak^{1#}, So Jung Park², Yoon Kyung Jo², Dong Hyung Cho^{2*}, and Eun Kyung Lee^{1*}

¹ Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul, South Korea

² Department of Gerontology, Graduate School of East–West Medical Science, Kyung Hee University, Yongin, South Korea

Equal contribution

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

Keywords: microRNAs, miR-200a-3p, mitochondrial fission factor, mitochondria fragmentation, mitochondria dynamics

*Corresponding author's information:

-Eun Kyung Lee, Ph.D. (leeek@catholic.ac.kr)

Department of Biochemistry, College of Medicine, The Catholic University of Korea

222 Banpodaero, Seocho-gu, Seoul 06591, South Korea

-Dong Hung Cho, Ph.D. (dhcho@khu.ac.kr)

Department of Gerontology, Graduate School of East–West Medical Science, Kyung Hee University

1732 Deogyong-daero, Giheung-gu, Yongin 17014, South Korea

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 β -actin (Abcam), and further incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotech). Chemiluminescent signals were developed using ClarityTM 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 (1×10^3) was used for OCR measurement. Basal OCR was measured for 3 min every 8 min for four points. Small molecule-metabolic modulators oligomycin (3 μ M), FCCP (1 μ M), and antimycin A (1 μ M) were injected sequentially at the indicated time points after baseline OCR measurement.

Acknowledgements

This work is supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (2014R1A2A1A11053431, 2012M3A9D1054517, and 2012R1A5A2047939).

References

1. Detmer SA and Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. *Nature reviews Molecular cell biology* 8, 870-879
2. Suen D-F, Norris KL and Youle RJ (2008) Mitochondrial dynamics and apoptosis. *Genes & development* 22, 1577-1590
3. Bereiter-Hahn J and Jendrach M (2010) Mitochondrial dynamics. *Int Rev Cell Mol Biol* 284, 1-65
4. Ni HM, Williams JA and Ding WX (2015) Mitochondrial dynamics and mitochondrial quality control. *Redox Biol* 4, 6-13
5. Zorzano A, Liesa M, Sebastian D, Segales J and Palacin M (2010) Mitochondrial fusion proteins: dual regulators of morphology and metabolism. *Semin Cell Dev Biol* 21, 566-574
6. Hyde BB, Twig G and Shirihai OS (2010) Organellar vs cellular control of mitochondrial dynamics. *Semin Cell Dev Biol* 21, 575-581
7. Kuzmicic J, Del Campo A, Lopez-Crisosto C et al (2011) [Mitochondrial dynamics: a potential new therapeutic target for heart failure]. *Rev Esp Cardiol* 64, 916-923
8. Gomes LC and Scorrano L (2013) Mitochondrial morphology in mitophagy and macroautophagy. *Biochim Biophys Acta* 1833, 205-212
9. Chang CR and Blackstone C (2010) Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. *Annals of the New York Academy of Sciences* 1201, 34-39
10. Kashatus JA, Nascimento A, Myers LJ et al (2015) Erk2 phosphorylation of Drp1 promotes mitochondrial fission and MAPK-driven tumor growth. *Mol Cell* 57, 537-551
11. Samant SA, Zhang HJ, Hong Z et al (2014) SIRT3 deacetylates and activates OPA1 to regulate mitochondrial dynamics during stress. *Mol Cell Biol* 34, 807-819
12. Escobar-Henriques M (2014) Mitofusins: ubiquitylation promotes fusion. *Cell Res* 24, 387-388

13. Ke XS, Liu CM, Liu DP and Liang CC (2003) MicroRNAs: key participants in gene regulatory networks. *Curr Opin Chem Biol* 7, 516-523
14. Ma J, Lin Y, Zhan M, Mann DL, Stass SA and Jiang F (2015) Differential miRNA expressions in peripheral blood mononuclear cells for diagnosis of lung cancer. *Lab Invest* 95, 1197-1206
15. Sun J, Sonstegard TS, Li C et al (2015) Altered microRNA expression in bovine skeletal muscle with age. *Anim Genet* 46, 227-238
16. Kaviani M, Azarpira N, Karimi MH and Al-Abdullah I (2016) The role of microRNAs in islet beta-cell development. *Cell Biol Int*
17. Achkar NP, Cambiagno DA and Manavella PA (2016) miRNA Biogenesis: A Dynamic Pathway. *Trends Plant Sci*
18. Shah MY, Ferrajoli A, Sood AK, Lopez-Berestein G and Calin GA (2016) microRNA Therapeutics in Cancer - An Emerging Concept. *EBioMedicine* 12, 34-42
19. Feng J, Xing W and Xie L (2016) Regulatory Roles of MicroRNAs in Diabetes. *Int J Mol Sci* 17
20. Catalanotto C, Cogoni C and Zardo G (2016) MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *Int J Mol Sci* 17
21. Geiger J and Dalgaard LT (2016) Interplay of mitochondrial metabolism and microRNAs. *Cell Mol Life Sci*
22. Moss EG (2002) MicroRNAs: hidden in the genome. *Curr Biol* 12, R138-140
23. Donzelli S, Cioce M, Muti P, Strano S, Yarden Y and Blandino G (2016) MicroRNAs: Non-coding fine tuners of receptor tyrosine kinase signalling in cancer. *Semin Cell Dev Biol* 50, 133-142
24. Mishra P and Chan DC (2016) Metabolic regulation of mitochondrial dynamics. *J Cell Biol* 212, 379-387

25. Kamiya Y, Kawada J, Kawano Y et al (2015) Serum microRNAs as Potential Biomarkers of Juvenile Idiopathic Arthritis. *Clin Rheumatol* 34, 1705-1712
26. Song MA, Paradis AN, Gay MS, Shin J and Zhang L (2015) Differential expression of microRNAs in ischemic heart disease. *Drug Discov Today* 20, 223-235
27. Irwandi RA and Vacharaksa A (2016) The role of microRNA in periodontal tissue: A review of the literature. *Arch Oral Biol* 72, 66-74
28. Ojha CR, Rodriguez M, Dever SM, Mukhopadhyay R and El-Hage N (2016) Mammalian microRNA: an important modulator of host-pathogen interactions in human viral infections. *J Biomed Sci* 23, 74
29. Fan S, Chen WX, Lv XB et al (2015) miR-483-5p determines mitochondrial fission and cisplatin sensitivity in tongue squamous cell carcinoma by targeting FIS1. *Cancer Lett* 362, 183-191
30. Wang K, Long B, Jiao JQ et al (2012) miR-484 regulates mitochondrial network through targeting Fis1. *Nat Commun* 3, 781
31. Wang JX, Jiao JQ, Li Q et al (2011) miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. *Nat Med* 17, 71-78
32. Guan X, Wang L, Liu Z et al (2016) miR-106a promotes cardiac hypertrophy by targeting mitofusin 2. *J Mol Cell Cardiol* 99, 207-217
33. Joshi SR, Dhagia V, Gairhe S, Edwards JG, McMurtry IF and Gupte SA (2016) MicroRNA-140 is elevated and mitofusin-1 is downregulated in the right ventricle of the Sugen5416/hypoxia/normoxia model of pulmonary arterial hypertension. *Am J Physiol Heart Circ Physiol* 311, H689-698
34. Zhang R, Zhou H, Jiang L et al (2016) MiR-195 dependent roles of mitofusin2 in the mitochondrial dysfunction of hippocampal neurons in SAMP8 mice. *Brain Res* 1652, 135-143
35. Zhou X, Zhang L, Zheng B et al (2016) MicroRNA-761 is upregulated in hepatocellular carcinoma and regulates tumorigenesis by targeting Mitofusin-2. *Cancer Sci* 107, 424-432

36. Li X, Wang FS, Wu ZY, Lin JL, Lan WB and Lin JH (2014) MicroRNA-19b targets Mfn1 to inhibit Mfn1-induced apoptosis in osteosarcoma cells. *Neoplasma* 61, 265-273
37. Zhou X, Zuo S and Xin W (2015) miR-27b overexpression improves mitochondrial function in a Sirt1-dependent manner. *J Physiol Biochem* 71, 753-762
38. Fan S, Liu B, Sun L et al (2015) Mitochondrial fission determines cisplatin sensitivity in tongue squamous cell carcinoma through the BRCA1-miR-593-5p-MFF axis. *Oncotarget* 6, 14885-14904
39. Long B, Wang K, Li N et al (2013) miR-761 regulates the mitochondrial network by targeting mitochondrial fission factor. *Free Radic Biol Med* 65, 371-379
40. Toyama EQ, Herzig S, Courchet J et al (2016) Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science* 351, 275-281
41. Tak H, Kim J, Jayabalan AK et al (2014) miR-27 regulates mitochondrial networks by directly targeting the mitochondrial fission factor. *Exp Mol Med* 46, e123
42. Bose A and Beal MF (2016) Mitochondrial dysfunction in Parkinson's disease. *J Neurochem* 139 Suppl 1, 216-231
43. Lee H and Yoon Y (2016) Mitochondrial fission and fusion. *Biochem Soc Trans* 44, 1725-1735
44. Silva Ramos E, Larsson NG and Mourier A (2016) Bioenergetic roles of mitochondrial fusion. *Biochim Biophys Acta* 1857, 1277-1283
45. Wada J and Nakatsuka A (2016) Mitochondrial Dynamics and Mitochondrial Dysfunction in Diabetes. *Acta Med Okayama* 70, 151-158
46. Chen H and Chan DC (2009) Mitochondrial dynamics—fusion, fission, movement, and mitophagy—in neurodegenerative diseases. *Human molecular genetics* 18, R169-R176
47. Cho DH, Nakamura T and Lipton SA (2010) Mitochondrial dynamics in cell death and neurodegeneration. *Cell Mol Life Sci* 67, 3435-3447

48. Rehman J, Zhang HJ, Toth PT et al (2012) Inhibition of mitochondrial fission prevents cell cycle progression in lung cancer. *Faseb j* 26, 2175-2186
49. Saita S, Ishihara T, Maeda M et al (2016) Distinct types of protease systems are involved in homeostasis regulation of mitochondrial morphology via balanced fusion and fission. *Genes Cells* 21, 408-424
50. Xu Y, Zhao C, Sun X, Liu Z and Zhang J (2015) MicroRNA-761 regulates mitochondrial biogenesis in mouse skeletal muscle in response to exercise. *Biochem Biophys Res Commun* 467, 103-108
51. Brabletz S and Brabletz T (2010) The ZEB/miR-200 feedback loop--a motor of cellular plasticity in development and cancer? *EMBO Rep* 11, 670-677
52. Bracken CP, Gregory PA, Kolesnikoff N et al (2008) A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. *Cancer Res* 68, 7846-7854
53. Hill L, Browne G and Tulchinsky E (2013) ZEB/miR-200 feedback loop: at the crossroads of signal transduction in cancer. *Int J Cancer* 132, 745-754
54. Cong N, Du P, Zhang A et al (2013) Downregulated microRNA-200a promotes EMT and tumor growth through the wnt/beta-catenin pathway by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma. *Oncol Rep* 29, 1579-1587
55. Sulaiman SA, Ab Mutalib NS and Jamal R (2016) miR-200c Regulation of Metastases in Ovarian Cancer: Potential Role in Epithelial and Mesenchymal Transition. *Front Pharmacol* 7, 271
56. Diaz T, Tejero R, Moreno I et al (2014) Role of miR-200 family members in survival of colorectal cancer patients treated with fluoropyrimidines. *J Surg Oncol* 109, 676-683
57. Koutsaki M, Spandidos DA and Zaravinos A (2014) Epithelial-mesenchymal transition-associated miRNAs in ovarian carcinoma, with highlight on the miR-200 family: prognostic value and prospective role in ovarian cancer therapeutics. *Cancer Lett* 351, 173-181

58. Chen C, Yang D, Wang Q and Wang X (2015) Expression and Clinical Pathological Significance of miR-200a in Concurrent Cholangiocarcinoma Associated with Hepatolithiasis. *Med Sci Monit* 21, 3585-3590
59. Dhayat SA, Mardin WA, Kohler G et al (2014) The microRNA-200 family--a potential diagnostic marker in hepatocellular carcinoma? *J Surg Oncol* 110, 430-438
60. Leskela S, Leandro-Garcia LJ, Mendiola M et al (2011) The miR-200 family controls beta-tubulin III expression and is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients. *Endocr Relat Cancer* 18, 85-95
61. Li H, Tang J, Lei H et al (2014) Decreased MiR-200a/141 suppress cell migration and proliferation by targeting PTEN in Hirschsprung's disease. *Cell Physiol Biochem* 34, 543-553
62. Zuberi M, Mir R, Das J et al (2015) Expression of serum miR-200a, miR-200b, and miR-200c as candidate biomarkers in epithelial ovarian cancer and their association with clinicopathological features. *Clin Transl Oncol* 17, 779-787

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.

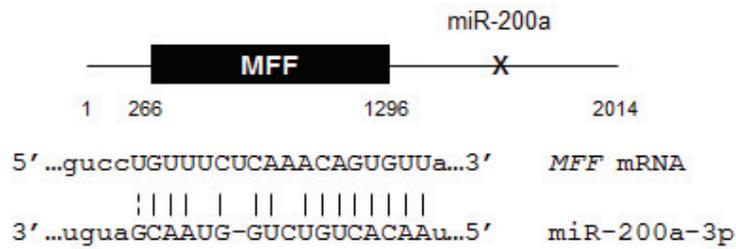
CHANG-mtYFP cells were transfected with pre-miR-200a-3p, anti-miR-200a-3p and control miRNA (CTRL). Forty-eight hours after transfection, MFF, DRP1, MFN1/2, and OPA1 proteins were analyzed by western blotting. Results are representative of three independent experiments.

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

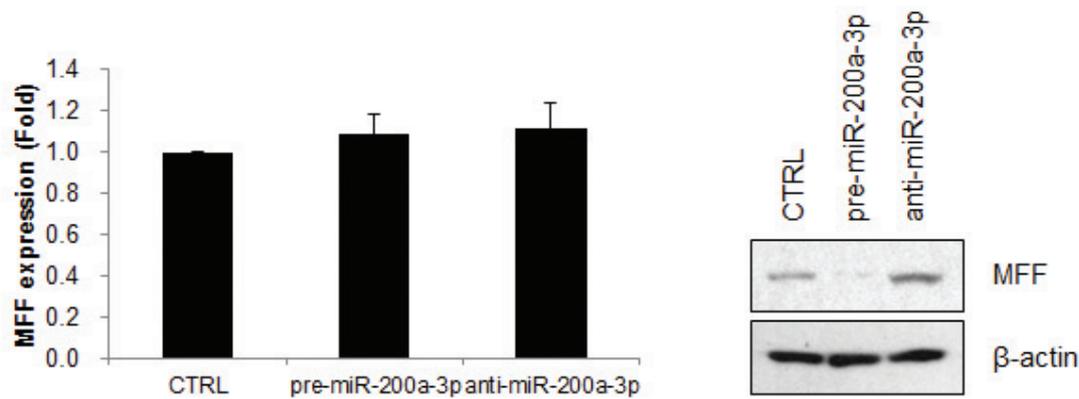
(A) Hep3B cells were transfected with miR-200a-3p or control miRNA, and stained with ATP detection reagent (*left*) and JC-1 dye (*right*) to determine mitochondrial membrane potential and mitochondrial ATP levels. Change in the relative luminescence was assessed by measuring the fluorescence. Data represent 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

A

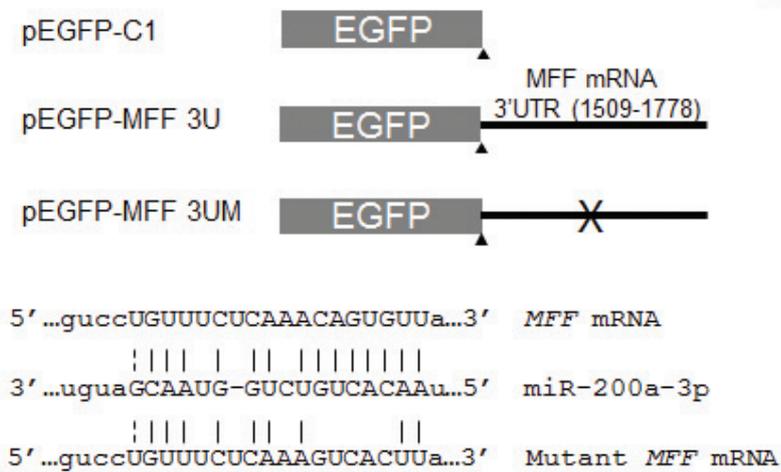
MFF mRNA (NM_020194)



B



C



D

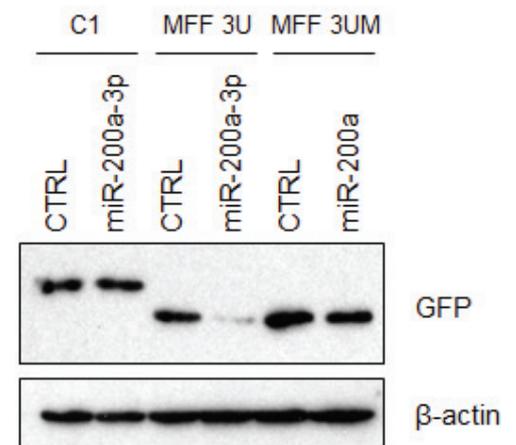


Fig. 1 Figure 1

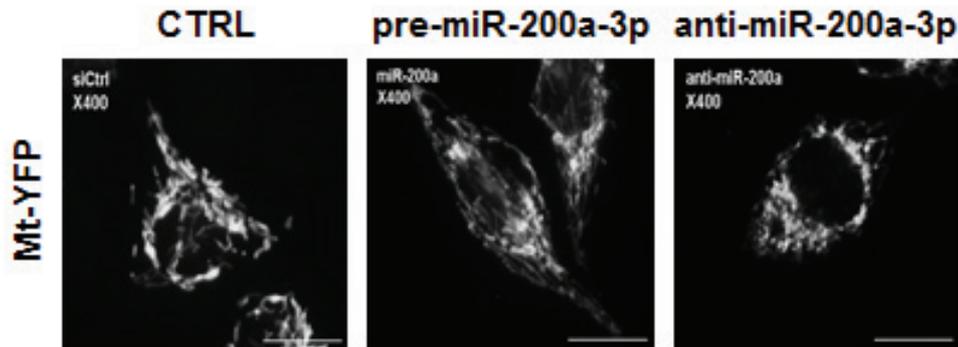
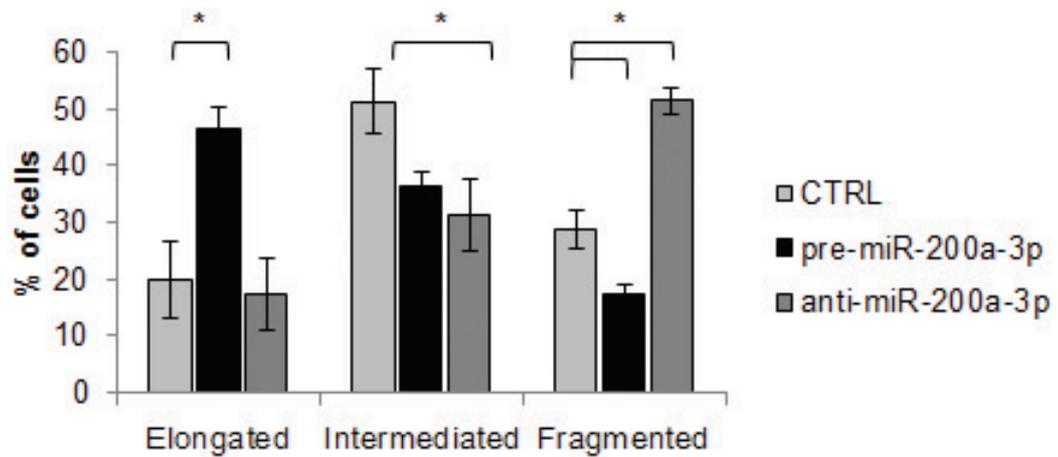
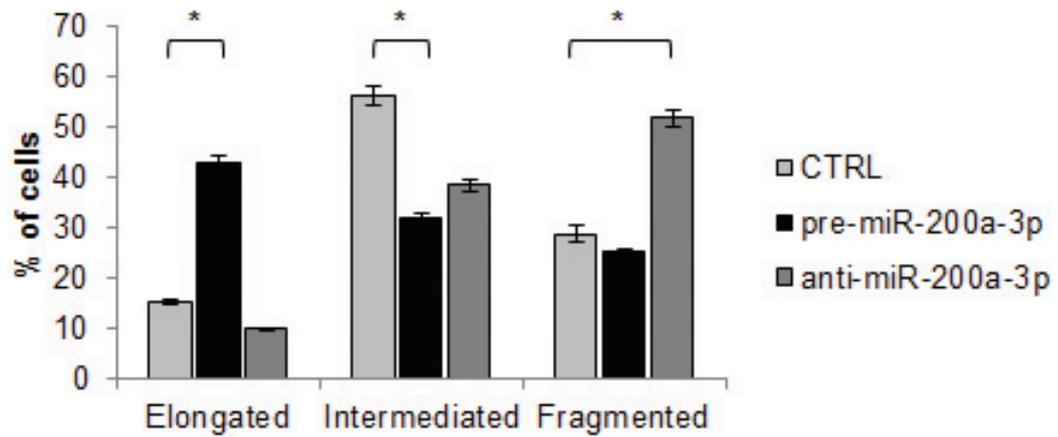
A**B****C**

Fig. 2 Figure 2

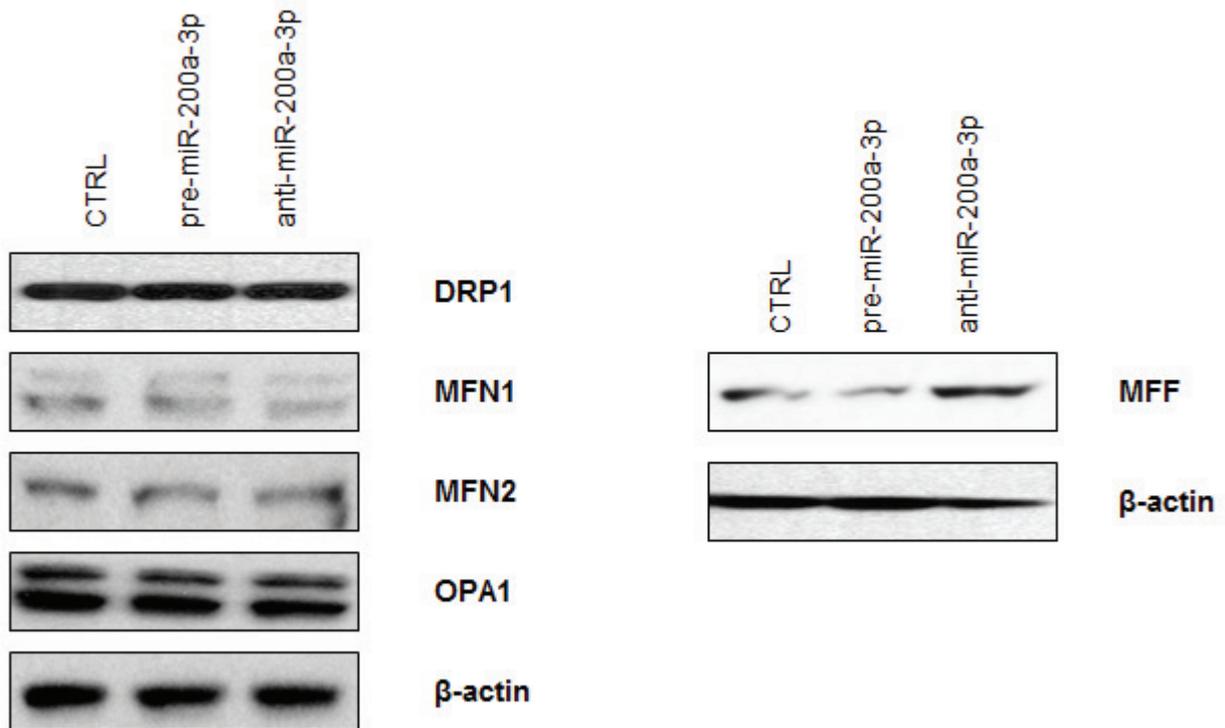


Fig. 3 Figure 3

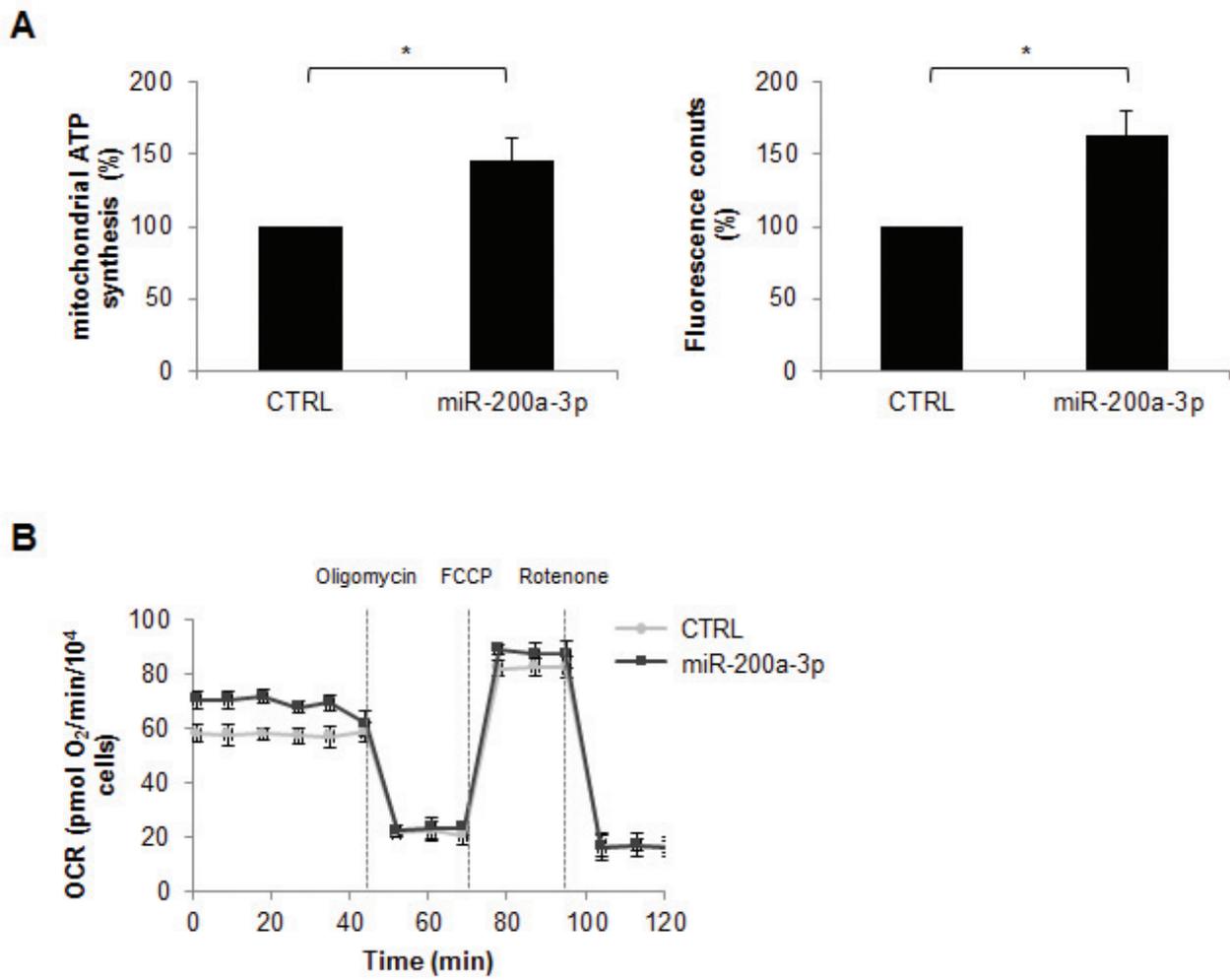
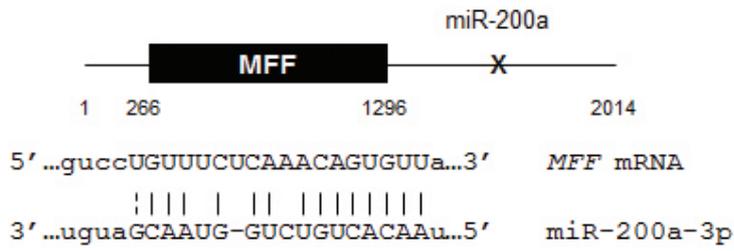


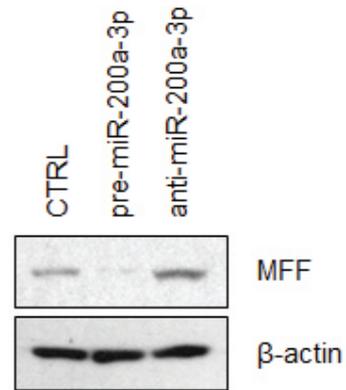
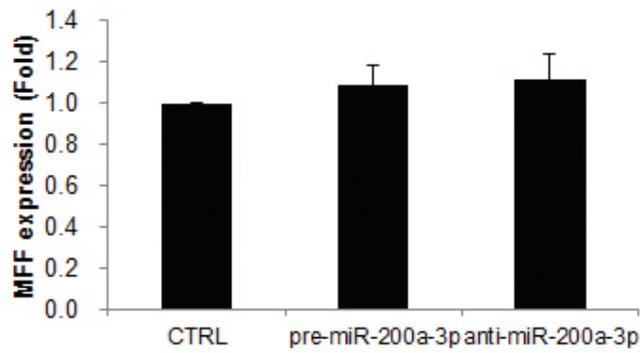
Fig. 4 Figure 4

A

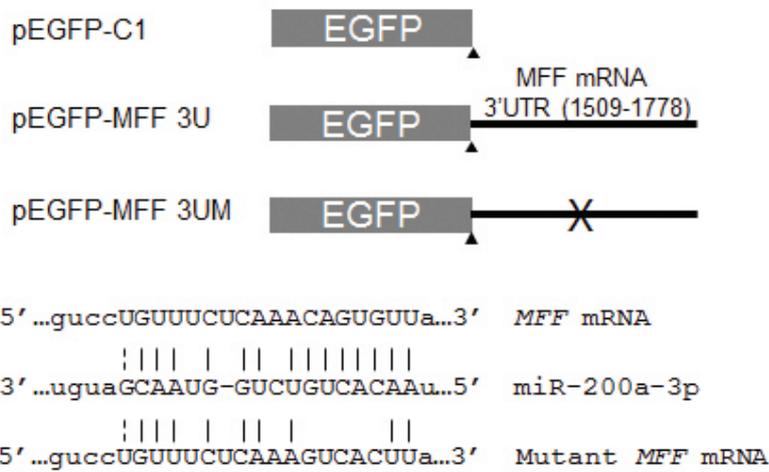
MFF mRNA (NM_020194)



B



C



D

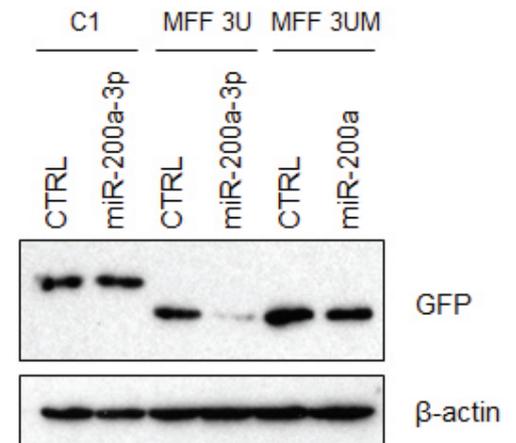


Fig. 5 Figure 1

UNC

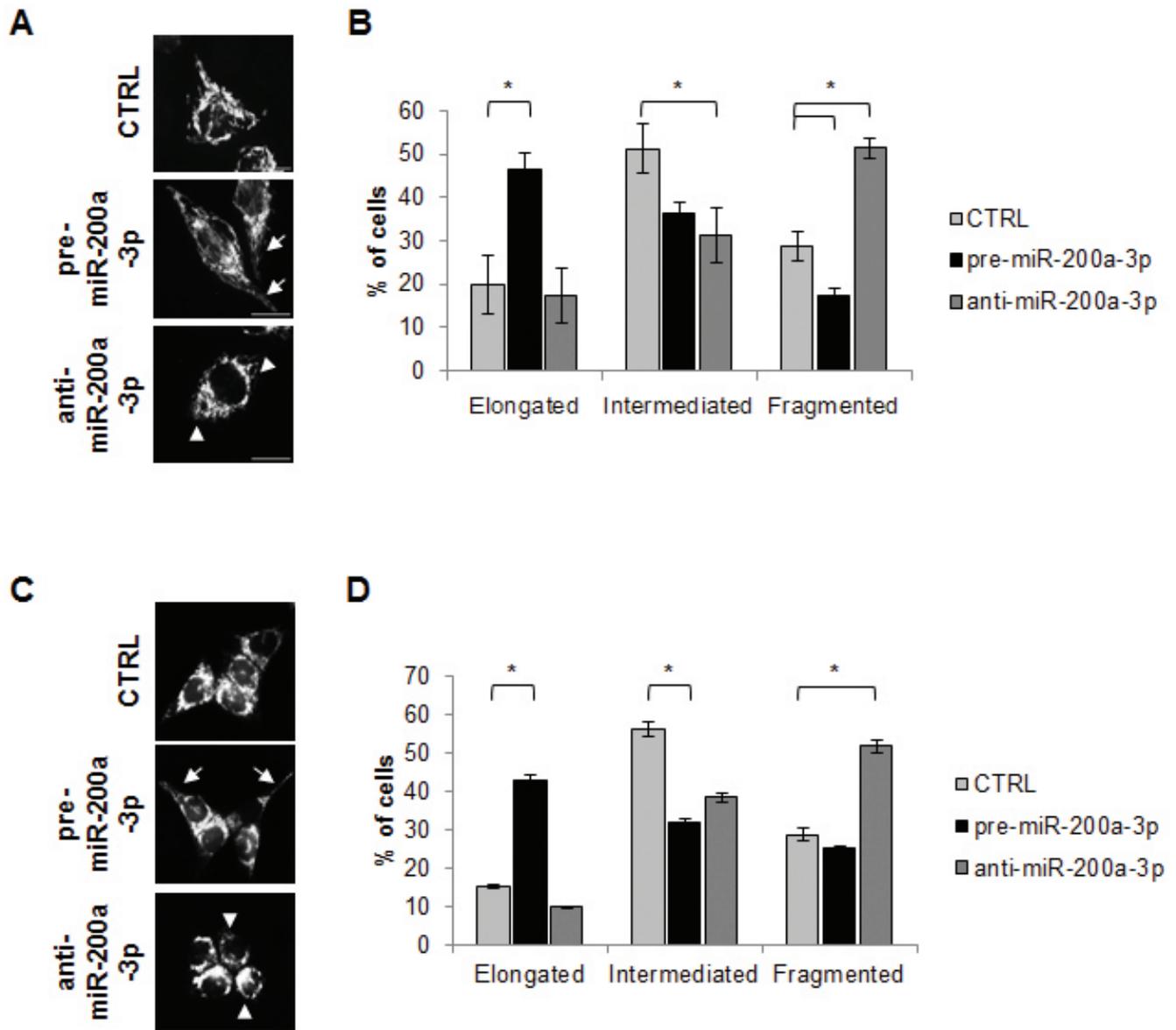


Fig. 6 Figure 2

UNCORRECTED

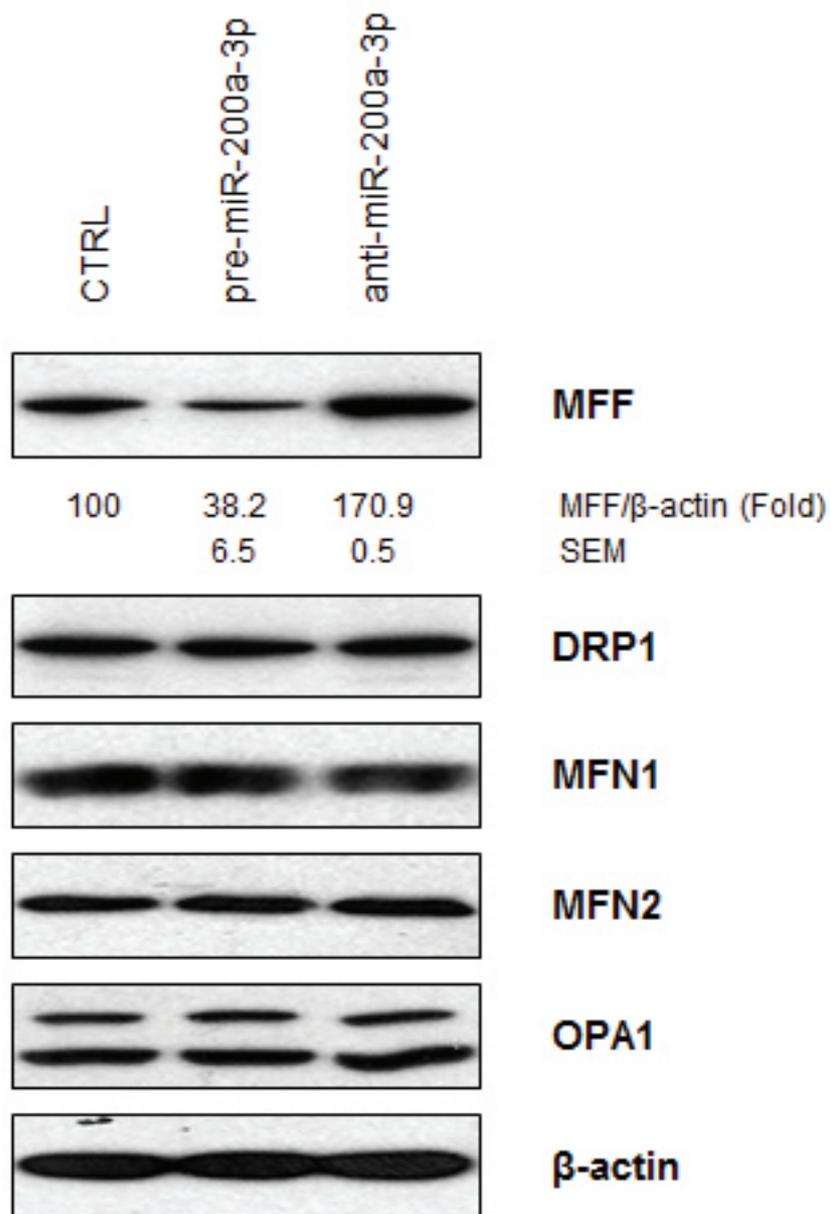


Fig. 7 Figure 3

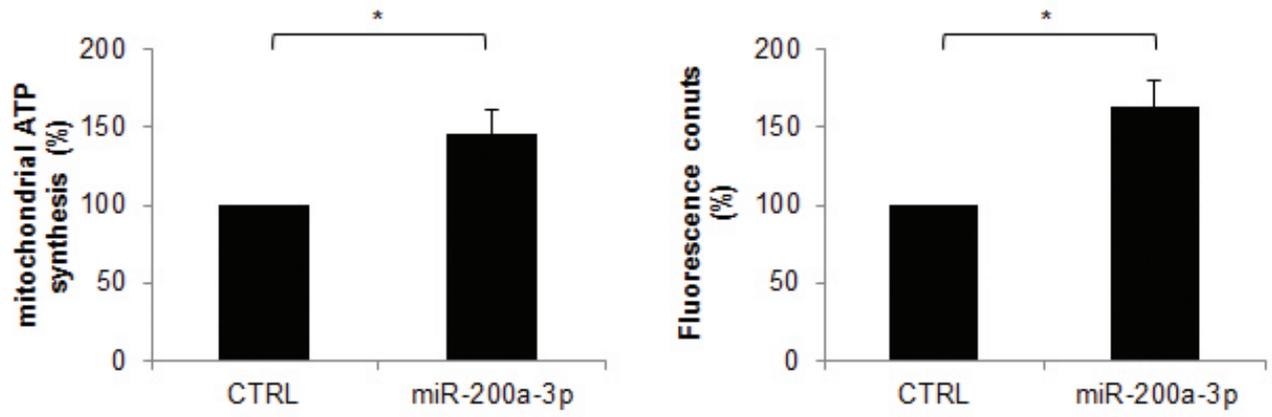
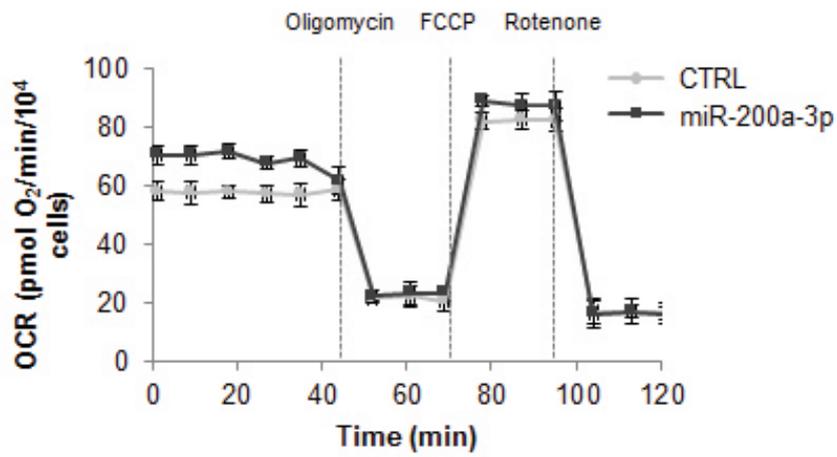
A**B**

Fig. 8 Figure 4