Supplementary Information

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Title: MiR-183-5p induced by saturated fatty acids regulates the myogenic differentiation by directly targeting FHL1 in C2C12 myoblasts.

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Running Title: Implication of miR-183-5p in myogenic differentiation

Keywords: microRNA; miR-183-5p; FHL1; differentiation; palmitic acid

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MATERIALS AND METHODS

Cell culture, differentiation, and PA treatment

C2C12 myoblasts, an immortalized mouse cell line (ATCC), were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (Gibco) in a humidified incubator containing 5% CO₂ at 37 °C. Cells were seeded in 6-well plates at a density of 1.3 \times 10^5 cells/well and incubated for 24 hours before transfection. After reaching 80 - 90% confluence, the cells were switched from 10% FBS to 2% horse serum (Gibco) to induce differentiation. When necessary, the cells were treated with BSA-conjugated PA (0.1 mM) for 24 h in a growth medium and harvested in a differentiation medium.

Transfection of miRNA mimic and plasmids

C2C12 myoblasts were transfected with oligonucleotides (Genolution, Seoul, Korea), such as scrambled control miRNA (scRNA), FHL1 siRNA, miR-183-5p mimic or an inhibitor of miR-183-5p (antimiR-183-5p; a 2'-O-methyl-modified antisense oligonucleotide against mature miR-183-5p), using Lipofectamine 2000 (Invitrogen). For the Dual-luciferase reporter assay, the C2C12 cells were cotransfected with 100 nM of the pmirGLO luciferase vector containing the luciferase reporter genes (Promega).

RNA preparation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated from C2C12 cells using Qiazol (Qiagen) and purified with a miRNeasy Mini Kit (Qiagen). The cDNAs were synthesized using a miScript II RT Kit (Qiagen). qRT-PCR was carried out using an SYBR Green I and iTaq polymerase (Promega) in a LightCycler 480 (Roche Applied Science). The primers used for RT-PCR or qRT-PCR and reaction conditions are listed in Supplementary Table 1. Relative expression levels of indicated genes were calculated using the 2^{-ΔΔCt} method.
**Dual-luciferase reporter assay**

Using the primer sets described in Supplementary Table 1, the segment of murine FHL1 3’UTR was amplified by RT-PCR from C2C12 myoblasts and subcloned into pmirGLO (Promega). The miR-183-5p binding sites were mutated by site-directed mutagenesis. The C2C12 cells were cotransfected with 100 nM scRNA or miR-183-5p mimic and pmirGLO luciferase vector containing the FHL1 3’UTRs (wild-type or mutant) using Lipofectamine 2000. Dual-luciferase reporter gene assays were performed 24 h after transfection according to the manufacturer’s protocol (Promega).

**Immunoblot analysis**

The cells were lysed using a lysis buffer containing a protease inhibitor/phosphatase inhibitor. SDS-gel electrophoresis and immunoblotting analysis were conducted as described elsewhere (17) using specific antibodies (Supplementary Table 2). All immunoblots were visualized using a Femto reagent (Thermofisher Scientific) and quantified by densitometry using an analytical scanning system (Fusion Solo, Vilber Lourmat, Paris, France).

**Immunofluorescence analysis**

For immunocytochemistry, C2C12 cells were fixed in 4% paraformaldehyde for 10 min, treated with 0.5% Triton X-100 at 37 °C for 15 min, blocked with 3% BSA for two hr at room temperature, and incubated with primary antibody against myosin heavy chain (MyHC, 1: 100 dilution) at 4°C overnight. They were then incubated with Alexa488-conjugated secondary antibody (Thermofisher Scientific) at room temperature for another 1.5 hrs. The nuclei were stained with Hoechst for 15 min. Images from at least five different areas chosen randomly in more than three independent experiments were taken and analyzed under a fluorescence
microscope (Leica, Germany). The differentiation index was calculated as the percentage of nuclei in MyHC-positive myotubes divided by the total number of nuclei in a field. The fusion index was calculated by the ratio of the number of nuclei in myotubes with more than three nuclei divided by the total number of nuclei. The myotube area and width of myotubes were measured using Image J Software.

**Database and statistical analysis**

The target genes and sites of the miRNAs were analyzed computationally using publicly available algorithms (TargetScan: www.targetscan.org, Pictar: pictar.mdc-berlin.de). The values of all experiments are expressed as the mean ± SEM from at least three independent experiments. Where applicable, the significance of the difference was analyzed using the Student's t-test for unpaired data.
### Supplementary Table 1. Primer lists and PCR conditions

(A) Mouse primer lists for qRT-PCR and RT-PCR

| Gene          | Primer sequence (5'-3')                  | Product size | Annealing Temperature | Concentration | Cycle |
|---------------|------------------------------------------|--------------|-----------------------|---------------|-------|
| miR-183       | F.P TATGGCACTGGTAGAATTCACT                | 90           | 55                    | 2 ng/µl       | 40    |
|               | R.P AGTGAATTCTACCAGTGCCATA               |              |                       | 0.5 nM        |       |
| miRNA         | R.P miScript universal primer (Qiagen)   |              |                       | (qRT-PCR)     |       |
| universal Primer |                                      |              |                       | (RT-PCR)      |       |
| U6            | F.P CTCGCTTCGGCAGCACAA                  | 94           |                       | 2 ng/µl       |       |
|               | R.P AACGCTTCAGGAATTGGGT                 |              |                       | 0.5 nM        |       |
| FHL1          | F.P CTGAAGTGCGTTGACAAGTTC                | 102          | 58                    |                |       |
|               | R.P GTGCCAGTAGCCGATTCTTAT               |              |                       |               |       |
| GAPDH         | F.P AACATCAAATGOGGTAGGGCC               | 252          | 58                    |                |       |
|               | R.P GTTGTCATGGATGACCCTGOC              |              |                       |               |       |

(B) Primer lists for the cloning of FHL1 3'UTRs

| Gene           | Primer sequence (5'-3')                  | Product size | Annealing Temperature | Concentration | Cycle |
|----------------|------------------------------------------|--------------|-----------------------|---------------|-------|
| wild-type FHL1 3’UTR | F.P ATCTGGCCAAACACACGCT                  | 303          | 58                    | 2 ng/µl       | 35    |
|                 | R.P AATTGACGCGGACGAGA                    |              |                       | 0.5 nM        |       |
| mutant FHL1 3’UTR | F.P ATCTGGCCAAACACACGCT                  | 65           | 58                    | 2 ng/µl       |       |
|                 | R.P CTTTTCTGCGTGATCAGGGGA                |              |                       | 0.5 nM        |       |
|                 | F.P TGGCAGTGCGGACAAGAA                   | 257          |                       |               |       |
|                 | R.P AATTGACGCGGACGAGA                    |              |                       |               |       |
### Supplementary Table 2. Antibodies list

| Antibody       | Manufacturer                      | Cat. No.   | Dilution ratio* |
|----------------|-----------------------------------|------------|-----------------|
| FHL1           | Santa Cruz Biotechnology, USA     | sc-374246  | 1:2,000         |
| MyHC           | DSHB                              | MF20       | 1:1,000         |
| Myod           | Santa Cruz Biotechnology, USA     | sc-377460  | 1:1,000         |
| MyoG           | Santa Cruz Biotechnology, USA     | sc-12712   | 1:1,000         |
| MEF2C          | Invitrogen, Thermofisher Scientific, USA | PA5-28247 | 1:10,000       |
| β-actin        | Sigma, USA                        | A2066      | 1:10,000        |
| Antibodies HRP-linked anti-rabbit IgG | Cell Signaling, USA     | #7074     | 1:10,000        |
| Goat anti-mouse(H+L) | Invitrogen, Thermofisher Scientific, USA | #32430  | 1:2,000         |