Sustained of mir-499-5p delivery from injection alters the muscle metabolomic profiles in broiler chicken

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Research

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Abstract

Background

Myogenic miRNAs (myomiRs) which dramatically increased during myogenesis have been shown to play critical roles in many aspects of muscle function. As a myomiR, miR-499-5p, has been identified to be highly expressed in cardiac and skeletal muscle. The study focused on the effects of miR-499-5p on muscle metabolism in broiler chicken.

Methods

In the current study, we assigned 16 broiler chicks to control group and treatment group and then monitored the effects using metabolomics. Chicks were fed basal diets without or with miR-499-5p delivery. Muscle samples were collected and analyzed by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).

Results

Our findings revealed that miR-499-5p injection altered the concentrations of a variety of metabolites in the muscle. Thereinto, a total of 46 metabolites were identified at higher ($P < 0.05$) concentrations and 30 metabolites were identified at lower ($P < 0.05$) concentrations in the treatment group as compared with the control group. These metabolites were primarily involved with the regulation of lipid and carbohydrate metabolism. Further metabolic pathway analysis revealed that fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism and terpenoid backbone biosynthesis were the most important and critical pathway which may partially interpret the effects of miR-499-5p.

Conclusions

To our knowledge, this research is the first report of metabolic signatures and related metabolic pathways in the skeletal muscle for miR-499-5p injection and provide new insight into the effect of miRNA on growth performance.

Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs of about 22 nucleotides (nt) derived from 70-nt long stem-loop precursors (pre-miRNAs) through a sequential processing by two RNase III enzymes, Drosha and Dicer[1–3]. MiRNAs are posttranscriptional regulators that bind to the target messenger RNA's (mRNA) 3'-untranslated region (3'-UTR), usually resulting in translational repression in mammals[4, 5]. It is estimated that each miRNA regulates on average 200 target genes through an interaction between the seed sequence and the complementary target sites[6].
A number of miRNAs seem to be expressed in a muscle-specific manner and are as a group often referred to as myogenic miRNAs (myomiRs)\cite{7}. The expression of myomiRs is dramatically increased during myogenesis\cite{8}. MyomiRs have been shown to play critical roles in many aspects of muscle function, including muscle development, satellite cell activity, muscle fiber specification\cite{9–11}. MiR-499-5p, as a myomiR, highly expressed in cardiac and skeletal muscle and encoded by myosin heavy chain 7b (MyHC7b) which is a member of the MyHC family,\cite{12} has been identified to be an important regulator of muscle fiber type transition\cite{13, 14}. It was reported that miR-499-5p and miR-208b are functionally redundant, and play a dominant role in the specification of muscle fiber identity by activating slow and repressing fast myofiber genes\cite{12}. Several transcriptional repressors such as Sox6 and Purβ, which have been determined to inhibit MyHC7b transcriptional activity, were identified as miR-499-5p target genes\cite{12, 14, 15}.

Metabolomics provides a powerful platform for identifying small molecular metabolites in biological samples (biofluids or tissues) using high-throughput approaches. The identification and integrative analysis of these metabolites can facilitate the characterization of metabolism at the molecular and cellular levels under a given set of physiological conditions\cite{16}. Metabolomic analysis can provide novel insights into alterations of the metabolic status of biological systems affected by internal or external stimulating conditions\cite{17}. Thereby, these two technologies are expected to provide novel insight into the effects of miR-499-5p on the metabolism of broilers.

Though the importance of miR-499-5p in muscle development has been documented, the underlying physiological and metabolic mechanisms remained largely unknown. The current study was thereby conducted to identify the metabolic phenotype associated with external injection of miR-499-5p that could be linked to the growth and development of muscle through ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).

**Materials And Methods**

**Animals and Experimental Design**

A total of 16 14-day-old male Da Heng broiler chicks were randomly divided into 2 groups, with 8 replicates in each group. Birds were fed basal diets. All birds were housed in wired cages and offered free access to feed and water, with a lighting schedule of 20 h light and 4 h dark. AgomiRs of miR-499-5p, synthesized from Ribobio, were chemically engineered and cholesterol-modified oligonucleotides to mimic miRNA expression, and injected intramuscularly into gastrocnemius muscle at a dose of 5 nmol. A scramble miRNA agomiR was used as the negative control. The injections were repeated every 72 h and given 6 times to ensure the efficacy. All the 16 chicks were slaughtered. Gastrocnemius muscles were taken from each chick a week after the last injection, all fresh tissue samples were washed briefly with Phosphate Buffered Saline (PBS) and divided into 2.0 mL plastic centrifuge tubes (each sample weighing approximately 200 mg) and then immediately frozen in liquid nitrogen.

**Histological Examination of Gastrocnemius Muscle**
Gastrocnemius muscles fixed in 4% paraformaldehyde were cut into 10-μm thick sections using a cryosectioning machine (CM1900, Leica), and stained with haematoxylin and eosin for morphological analysis. Three images from each bird were analyzed. Micrographs were obtained using a digital camera system (BA200Digital, Motic) and analyzed using Image Pro Plus software.

**Statistical Analysis**

Data were expressed in mean ± SD. Statistical analysis was carried out using One-Way Analysis of Variance ANOVA using the SPSS Software (Version 20.0). The significant value between groups was set at $P < 0.05$.

**Metabolites Extraction**

The 16 tissues of gastrocnemius muscle were individually grounded with liquid nitrogen and the homogenate was resuspended with prechilled 80% methanol and 0.1% formic acid by well vortexing. The samples were incubated on ice for 5 min and then were centrifuged at 15000 rpm, 4°C for 5 min. A some of supernatant was diluted to final concentration containing 60% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube with 0.22 μm filter and then were centrifuged at 15000 g, 4°C for 10 min. Finally, the filtrate was injected into the LC-MS/MS system analysis. Quality control (QC) samples were also prepared by mixing equal volumes of each sample; the samples were aliquoted for analysis prior to sample preparation. The QC samples were used to monitor deviations of the analytical results from the pooled mixtures and compare to the errors caused by the analytical instrument itself.

**Metabolomic Analysis of Muscle Samples**

LC-MS/MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher). Samples were injected onto an Hyperil Gold column (100×2.1 mm, 1.9μm) using a 16-min linear gradient at a flow rate of 0.2 mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 16 min. Q Exactive HF-X mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and aux gas flow rate of 10 arb.

**Data Processing and Analysis**

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.0 (CD 3.0, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100000. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the
molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) database to obtained the accurate qualitative and relative quantitative results.

For multivariate statistical analysis, both principal component analysis (PCA) and orthogonal projections to latent structures discriminant analyses (OPLS-DA) were performed to visualize the differences between groups. PCA and OPLS-DA were both performed using the program SIMCA-P Software (Version 13.0). PCA was firstly employed to visualize the sample clustering, trends and outliers among the observations. Then OPLS-DA was performed to highlight the difference between groups. The OPLS-DA model was validated by 200 random permutations test for avoiding overfitting. Afterwards, loading plots were constructed, which showed the contribution of variables to the difference between the two groups. It also showed the important variables which were situated far from the origin, but the loading plot is complex because of many variables. To refine this analysis, the first principal component of variable importance in the projection (VIP) was obtained through OPLS-DA. Metabolites were annotated and identified on the basis of accurate mass and MS information by searching through the Database. Metabolites were finally verified by comparing retention times and fragmentation patterns with standards. The fold change (FC) value of each metabolite was calculated by comparing mean peak values obtained from the treatment group (TG) to that from the control group (CG). Differential metabolites were selected based on the basis of VIP value (>1.0), FC value (FC > 1.2 or FC < 0.833) and Student’s t-test ($P < 0.05$). Pearson's product-moment correlation was performed to calculate the correlation. Corresponding $P$-values and false discovery rate (FDR) of each correlation were also calculated using “cor.test function” in R software. Differential metabolites were further mapped onto general biochemical pathways according to annotation in Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results

Effect of miR-499-5p Overexpression on Body Weight and Muscle Fiber Diameter

Body weights of chicks in two groups were monitored at the beginning and end of the experiment period. As shown in Fig. 1, the body weights of chicks in two groups at Day-1 and Day-18 were presented. There were no significant differences in the initial body weights of each group on Day-1 (Fig. 1A). After intramuscular injection of agomiRs of miR-499-5p and negative control for 5 times, there were still no significant differences in the body and leg muscle weights on Day-18 (Fig. 1B and C). Different from the chicks in the CG, a dramatic decrease in the diameter of muscle fiber can be found in the TG in Fig. 2 ($P < 0.05$).

Characterization of LC-MS/MS Data

PCA mainly shows the distribution of the original data, which reduces the dimensionality of data and summarizes the similarities and differences between multiple MS spectra using score plots. In the present study, PCA was performed and the result revealed that most of the muscle samples in the score
plots were inside the 95% Hotelling $T^2$ ellipse (Fig. 3A). The correlation of three QC samples was calculated by “Pearson” correlation coefficient, and the results showed the correlation of all the QC samples exceeds 99% (Fig. 3B). As a supervised multivariate classification tool, OPLS-DA model was constructed following PCA for obtaining an improved separation and gain a better understanding of the variables responsible for the classification. As shown in Fig. 3C, all the samples in the OPLS-DA score plots were within the 95% Hotelling $T^2$ ellipse. The $R^2_Y$ value of the OPLS-DA model that represents the explained variance was 0.94. The cross-validation indicated good predictive ability of this model, with a relatively high $Q^2$ value of 0.48. OPLS-DA model exhibited a clear separation between the TG and CG. Furthermore, a permutation test was applied to assess the robustness and predictive ability of the OPLS-DA model (Fig. 3D). The corresponding $R^2_Y$ and $Q^2$ intercept values were 0.93 and -0.56, respectively, indicating a satisfactory effectiveness of the OPLS-DA model.

**Differential Metabolites in Gastrocnemius**

An obvious separation can be observed between the treatment and control group in the OPLS-DA model, indicating that there was a significant difference in the metabolome of gastrocnemius muscle of two groups. We determined those differentially expressed metabolites that played important roles in separating the treatment and control group. Differential metabolites between the two groups were selected when the P values of the Student’s t-test were less than 0.05 and the VIP values were more than 1.0. The profile of differential metabolites between the TG and CG was visualized by a volcano plot (Fig. 4). A total of 76 differential metabolites were testified using MS/MS analysis (Tables 1 and 2) based on these criteria. Of the identified metabolites, 46 metabolites were found at higher levels whereas 30 metabolites were found at lower levels in the TG compared with that in the CG. These metabolites are primarily involved in the metabolic processes of carbohydrates, nucleotides and lipids. On the basis of the FC value, several metabolites were determined including 7alpha-Hydroxy-3-oxochol-4-en-24-oic acid (FC = 2.74), 5alpha-cholane-3alpha,7alpha,12alpha,24-tetrol (FC = 1.72), O-heptanoylcarnitine (FC = 2.59), stearoylcarnitine (FC = 2.04), Linoleyl carnitine (FC = 2.11), Propionylcarnitine (FC = 2.81), Palmitoylcarnitine (FC = 1.84), trans-2-Tetradecenoyl carnitine (FC = 1.80), O-oleoylcarnitine (FC = 2.07), O-pentadecanoyl carnitine (FC = 1.86), (2E)-hexadecenoylcarnitine (FC = 1.63), O-heptadecanoyl carnitine (FC = 2.27), and Hexanoylcarnitine (FC = 1.60) along with Taurochenodeoxycholic acid (FC = 0.13), Palmitelaidic acid (FC = 0.68), Phloionolic acid (FC = 0.61), and Lauric acid (FC = 0.31).

**Metabolic Pathway Enrichment Analysis**

The differential metabolites detected in gastrocnemius in the present study were pinpointed the involved pathways. As shown in Table 3, a total of 13 pathways were obtained when the differential metabolites between the two groups were imported into the KEGG database. These metabolites were distributed among the metabolic pathways of fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism, terpenoid backbone biosynthesis, glycolysis / Gluconeogenesis, caffeine metabolism, vitamin B6 metabolism, primary bile acid biosynthesis, thiamine metabolism, pentose phosphate pathway, fatty acid biosynthesis, biosynthesis of unsaturated fatty acids and purine...
metabolism. Among them, fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism and terpenoid backbone biosynthesis exhibited significant differences ($P < 0.05$), so these four metabolic pathways were thus characterized as the significantly relevant pathways associated with the metabolic changes of chicks due to miR-499-5p injection.

**Discussion**

Recently, miRNAs have been shown to regulate gene expression and be involved in the proliferation and differentiation of skeletal muscle[18]. Previous evidence has indicated that miR-499-5p regulated skeletal myofiber specification by targeting Sox6[15, 19], Rod1[19], Thrap1[9], and TGF\(\beta\)R1[20]. It was found in our previous study that miR-499-5p levels in skeletal muscle were decreased accompanied by increasing age. The present study demonstrated that miR-499-5p injection significantly decreased the diameter of muscle fiber. The result was consistent with the previous studies because the diameter of slow-twitch muscle fiber was smaller than fast-twitch muscle fiber and miR-499-5p could regulate skeletal myofiber specification[9, 15, 19, 20]. However, little is known about the metabolic change of miR-499-5p involvement in the process.

To gain better insight into the significant changes caused by miR-499-5p injection, we developed a UHPLC-MS/MS method to analyze the endogenous metabolites in broiler muscle. To our knowledge, this is the first study to systematically identify metabolites that are expressed differentially in the muscle of broilers that have been injected by miR-499-5p. The results of PCA and OPLS-DA indicated that there were significant differences in the muscle metabolites of the TG and CG and the levels of 76 metabolites were altered by miR-499-5p, many of which are involved in pathways for metabolizing carbohydrates and lipids.

It was shown that the accumulation of lipids in non-adipose tissues elevates the cellular levels of bioactive lipids that inhibit the signaling pathways implicated in metabolic regulation together with activated inflammatory response[21]. Specifically, sterol lipids have been shown to influence fluidity and permeability of membranes[22, 23], and produce different signaling molecules such as sterol-derived hormones, Other sterol-derived signaling molecules include Vitamin D, bile acids, and oxysterols[24, 25]. It may be therefore that the elevated levels of 7alpha-Hydroxy-3-oxochol-4-en-24-oic acid and 5alpha-cholane-3alpha,7alpha,12alpha,24-tetrol in the TG may be beneficial for the functions mentioned above. However, taurochenodeoxycholic acid, as a sterol, was annotated to the pathway of primary bile acid biosynthesis. As a consequence, there might be potential disadvantages of certain functions and the metabolism of host cells responded to miR-499-5p in consideration of the decreased levels of taurochenodeoxycholic acid about which further research remain to be conducted. Carnitine is a conditionally essential nutrient that acts as an essential factor in fatty acid oxidation in mammals and performs the metabolic function of transporting activated fatty acids into the mitochondria of muscle cells, including those in the heart, for oxidation. It was indicated that miR-499-5p regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1[26]. Carnitine binds fatty acids, generating various acyl-carnitines with different chain lengths[27]. As shown in Table 2, The levels of 11
long-chain (≥ 10 carbons) acyl carnitines were all found to be elevated in the TG. These changes indicated there were different patterns in fatty acid oxidation between the two groups. Muscle is one of the most active tissues for fatty acid oxidation mainly by the catabolic process of β-Oxidation. Fatty acid molecules are broken by the process of β-Oxidation in the mitochondria to generate Acetyl coenzyme A (acetyl-coA). Long-chain acyl-carnitines were produced by the reaction of long-chain fatty acyl-CoA and carnitine after long-chain fatty acids are first bound to CoA, and then long-chain acyl-carnitines could be transported across the inner mitochondrial membrane[28]. The decreased levels of 3 long-chain fatty acids may be closely associated with increased consumption of long-chain acyl-carnitines in skeletal muscle. Carnitine palmitoyltransferase (CPT) deficiencies are common disorders of mitochondrial fatty acid oxidation[29]. It is indicated that the inhibition of CPT1 activity was sufficient to substantially diminish food intake and endogenous glucose production[30]. This is by virtue of the unique sensitivity of the outer membrane CPT 1 to the simple molecule, malonyl-CoA[31]. Increased consumption of long-chain acyl-carnitines in muscle may have a relationship with the food intake and endogenous glucose production.

Glyceraldehyde 3-phosphate (GAP) is an essential intermediate metabolite in several central pathways of all organisms. GAP can be reversely catalyzed by Glyceraldehyde-3-phosphate dehydrogenase (GADPH) into nicotinamide adenine dinucleotide (NADH) and 1, 3-bisphosphoglycerate. The increased GAP levels in the TG evidenced the activation of the fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism, and terpenoid backbone biosynthesis in response to miR-499-5p injection. NADH is a ubiquitous biological molecule that participates in many metabolic reactions in cellular metabolism and energy production. Recent studies showed that NADH played important roles in transcriptional regulation, longevity, calorie-restriction-mediated life-span extension and age-associated diseases[32–35]. Collectively, considering the influential roles of GAP within the body, we speculated that the activation of fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism, and terpenoid backbone biosynthesis could be, at least partially, responsible for the effects of miR-499-5p.

Conclusions

In summary, metabolomics analysis revealed substantial and significant changes in the skeletal muscle metabolite profiles of broilers in response to miR-499-5p injection. The differential metabolites induced by miR-499-5p were predominantly connected with lipid and carbohydrate metabolism. The results of our study uncovered the complex metabolic effects of miR-499-5p injection, which elucidate fructose and mannose metabolism, galactose metabolism, inositol phosphate metabolism and terpenoid backbone biosynthesis associated with miR-499-5p, offering a new insight into the effect of miR-499-5p on growth performance of broiler chicken.

Abbreviations
MiRNAs: MicroRNA; 3'-UTR: 3'-untranslated region; MyomiR: myogenic miRNA; MyHC7b: Myosin heavy chain 7b; UHPLC-MS/MS: ultra-high-performance liquid chromatography–tandem mass spectrometry; PBS: Phosphate Buffered Saline; QC: Quality control; PCA: Principal component analysis; OPLS-DA: Orthogonal projections to latent structures discriminant analyses; VIP: Variable importance in the projection; FC: Fold change; FDR: False discovery rate; KEGG: Kyoto Encyclopedia of Genes and Genomes; CG: Control group; TG: Treatment group; Acetyl-coA: Acetyl coenzyme A; CPT: Carnitine palmitoyltransferase; GAP: Glyceraldehyde 3-phosphate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Declarations

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Authors’ contributions

Zhixiong Li and Chaowu Yang conceived the experiments. Zhixiong Li and Mao Yuan performed the experiments. Zhixiong Li analyzed the data. Yaqiu Lin, Xiaosong Jiang, Chaowu Yang, Chunlin Yu, and Ling Chen wrote and prepared the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data may be provided following request to the corresponding author.

Ethics approval and consent to participate

All animal procedures used in the study were approved by the committee for the Care and Experimental Animal at Southwest Minzu University (Chengdu, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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**Tables**

Table 1 Increased metabolites for agomiR-499-treated group compared with control
| Metabolite name                        | Molecular formula | Retention time | FC<sup>a</sup> | P-value  | VIP<sup>b</sup> |
|---------------------------------------|-------------------|----------------|----------------|----------|-----------------|
| Joro toxin                            | C<sub>27</sub>H<sub>47</sub>N<sub>7</sub>O<sub>6</sub> | 14.54          | 2.72           | <0.001   | 3.08            |
| palmatine                             | C<sub>21</sub>H<sub>22</sub>N O<sub>4</sub>   | 13.93          | 1.77           | <0.001   | 1.82            |
| O-heptanoylcarnitine                  | C<sub>14</sub>H<sub>27</sub>N O<sub>4</sub> | 9.88           | 2.59           | <0.001   | 3.07            |
| stearoylcarnitine                     | C<sub>25</sub>H<sub>49</sub>N O<sub>4</sub> | 15.23          | 2.04           | <0.001   | 2.24            |
| 4,6-Henicosanedione                    | C<sub>21</sub>H<sub>40</sub>O<sub>2</sub>  | 15.44          | 14.63          | <0.001   | 7.37            |
| Valeric acid                          | C<sub>5</sub>H<sub>10</sub>O<sub>2</sub>   | 6.68           | 1.93           | 0.001    | 2.03            |
| Methyl 9-octadecenoate                | C<sub>19</sub>H<sub>36</sub>O<sub>2</sub> | 15.22          | 5.48           | 0.001    | 4.93            |
| Reduced Vitamin K                     | C<sub>31</sub>H<sub>46</sub>O<sub>2</sub> | 14.00          | 1.92           | 0.001    | 2.02            |
| 7alpha-Hydroxy-3-oxochol-4-en-24-oic acid | C<sub>24</sub>H<sub>36</sub>O<sub>4</sub> | 13.54          | 2.74           | 0.001    | 2.98            |
| 5-O-Mycaminosyprotylonolide           | C<sub>31</sub>H<sub>53</sub>N O<sub>8</sub> | 14.85          | 2.57           | 0.001    | 2.86            |
| (2Z)-4-(Octadecyloxy)-4-oxo-2-butenolic acid | C<sub>22</sub>H<sub>40</sub>O<sub>4</sub> | 14.00          | 2.01           | 0.002    | 2.17            |
| N-Stearoyl-L-tyrosine                 | C<sub>27</sub>H<sub>45</sub>N O<sub>4</sub> | 13.54          | 2.24           | 0.002    | 2.42            |
| Oleoyl tyrosine                       | C<sub>27</sub>H<sub>43</sub>N O<sub>4</sub> | 13.33          | 3.26           | 0.002    | 3.58            |
| cis-2-Carboxycyclohexyl-acetic acid   | C<sub>9</sub>H<sub>14</sub>O<sub>4</sub>  | 7.14           | 2.57           | 0.002    | 2.75            |
| Linoleyl carnitine                    | C<sub>25</sub>H<sub>45</sub>N O<sub>4</sub> | 13.56          | 2.11           | 0.002    | 2.17            |
| hydroprene                            | C<sub>17</sub>H<sub>30</sub>O<sub>2</sub> | 14.12          | 6.25           | 0.002    | 4.89            |
| 16,16-Dimethyl prostaglandin A1       | C<sub>22</sub>H<sub>36</sub>O<sub>4</sub> | 13.55          | 3.16           | 0.002    | 3.29            |
| promolote                             | C<sub>16</sub>H<sub>23</sub>N O<sub>4</sub> | 8.58           | 2.96           | 0.003    | 3.56            |
| Propionylcarnitine                    | C<sub>10</sub>H<sub>19</sub>N O<sub>4</sub> | 2.07           | 2.81           | 0.003    | 3.37            |
| Benzamide                             | C<sub>7</sub>H<sub>7</sub>N O   | 13.79          | 1.65           | 0.003    | 1.47            |
| Pregnane-3,3-diol                      | C<sub>21</sub>H<sub>36</sub>O<sub>2</sub> | 14.99          | 5.38           | 0.004    | 5.07            |
| Decylubiquinone                       | C<sub>19</sub>H<sub>30</sub>O<sub>4</sub> | 13.80          | 1.68           | 0.004    | 1.51            |
| Chemical Name                          | Molecular Formula | MW   | LogP  | IC50  |
|---------------------------------------|-------------------|------|-------|-------|
| Methyl stearate                       | C₁₉H₃₈O₂          | 15.34| 2.80  | 0.005 |
| Cassaidine                            | C₂₄H₴₁NO₄         | 12.99| 6.80  | 0.005 |
| Iminoctadine                          | C₁₈H₴₁N₇          | 14.72| 2.46  | 0.006 |
| DO0750000                             | C₁₅H₂₄O₂          | 13.97| 4.53  | 0.006 |
| Palmitoylcarnitine                    | C₂₃H₴₅NO₄         | 13.69| 1.84  | 0.007 |
| glyceraldehyde 3-phosphate            | C₃H₇O₆P           | 1.18 | 2.02  | 0.008 |
| 11-Deoxy prostaglandin F1α            | C₂₀H₃₆O₄          | 13.69| 2.53  | 0.011 |
| trans-2-Tetradecenoylcarnitine        | C₂₁H₃₉NO₄         | 12.98| 1.80  | 0.012 |
| O-oleoylcarnitine                     | C₂₅H₄₇NO₄         | 13.77| 2.07  | 0.012 |
| Xanthine                              | C₅H₄N₄O₂          | 1.72 | 1.71  | 0.013 |
| Erucic acid                           | C₂₂H₄₂O₂          | 15.67| 1.77  | 0.015 |
| 8,9-DiHETrE                           | C₂₀H₃₄O₄          | 13.42| 2.20  | 0.019 |
| clominorex                            | C₉H₉ClN₂O         | 4.99 | 1.51  | 0.024 |
| 1-hexadecyl-sn-glycerol 3-phosphate   | C₁₉H₴₁O₆P         | 14.60| 1.47  | 0.026 |
| O-pentadecanoylcarnitine              | C₂₂H₴₃NO₄         | 13.50| 1.86  | 0.029 |
| 5alpha-cholane-3alpha,7alpha,12alpha,24-tetrol | C₂₄H₄₂O₄ | 14.07| 1.72  | 0.030 |
| 10-Deoxymethymycin                    | C₂₅H₴₃NO₆         | 13.61| 2.02  | 0.031 |
| (2E)-hexadecenoylcarnitine            | C₂₃H₴₃NO₄         | 13.42| 1.63  | 0.034 |
| 16-Acetoxy-17-methoxy-17-oxokauran-18-oic acid | C₂₃H₳₄O₆ | 11.71| 1.77  | 0.034 |
| (+/-)-Camphoric acid                  | C₁₀H₁₆O₄          | 8.80 | 1.59  | 0.034 |
| PD-128042                             | C₂₃H₹₃NO₄         | 12.86| 2.25  | 0.041 |
| Lersivirine                           | C₁₇H₁₈N₄O₂        | 15.03| 1.56  | 0.042 |
| O-heptadecanoylcarnitine              | C₂₄H₴₇NO₄         | 13.81| 2.27  | 0.043 |
| Hexanoylcarnitine                     | C₁₃H₂₅NO₄         | 8.80 | 1.60  | 0.045 |
a FC, fold change for the treatment group to control, b VIP, variable importance in the projection.

Table 2 Decreased metabolites for agomiR-499-treated group compared with control
| Metabolite name                                                      | Molecular formula | Retention time | FC<sup>a</sup> | P<sup>b</sup> value | VIP<sup>b</sup> |
|--------------------------------------------------------------------|-------------------|----------------|----------------|---------------------|----------------|
| Cortisol, 9-fluoro-16. alpha.-hydroxy-                            | C<sub>21</sub> H<sub>29</sub> F O<sub>6</sub> | 12.74          | 0.65           | 0.002               | 1.41           |
| Diosgenin                                                          | C<sub>27</sub> H<sub>42</sub> O<sub>3</sub> | 15.11          | 0.70           | 0.004               | 1.15           |
| Triamciol one diacetate                                           | C<sub>25</sub> H<sub>31</sub> F O<sub>8</sub> | 14.75          | 0.47           | 0.004               | 2.36           |
| Geranylacetone                                                     | C<sub>13</sub> H<sub>22</sub> O       | 13.73          | 0.71           | 0.005               | 1.09           |
| KJ98000000                                                        | C<sub>18</sub> H<sub>39</sub> O<sub>7</sub> P | 15.31          | 0.58           | 0.007               | 1.94           |
| Lauric acid                                                        | C<sub>12</sub> H<sub>24</sub> O<sub>2</sub> | 13.29          | 0.31           | 0.014               | 3.15           |
| dihydroconiferyl alcohol glucoside                                | C<sub>16</sub> H<sub>24</sub> O<sub>8</sub> | 8.00           | 0.30           | 0.016               | 2.92           |
| 3-Dehydro-2-deoxyecdysone                                         | C<sub>27</sub> H<sub>42</sub> O<sub>5</sub> | 15.07          | 0.72           | 0.017               | 1.13           |
| ibufenac                                                           | C<sub>12</sub> H<sub>16</sub> O<sub>2</sub> | 12.14          | 0.70           | 0.020               | 1.09           |
| Palmitelaidic acid                                                | C<sub>16</sub> H<sub>30</sub> O<sub>2</sub> | 13.25          | 0.68           | 0.020               | 1.19           |
| Phloionolic acid                                                  | C<sub>18</sub> H<sub>36</sub> O<sub>5</sub> | 12.38          | 0.61           | 0.026               | 1.36           |
| spiro[3H-indole-3,5'(4'H)-thiazol]-2-ol, 2'- (methylthio)-        | C<sub>11</sub> H<sub>10</sub> N<sub>2</sub> O S<sub>2</sub> | 1.25          | 0.69           | 0.028               | 1.16           |
| MFCDO0010043                                                      | C<sub>16</sub> H<sub>10</sub> S       | 1.25           | 0.68           | 0.029               | 1.13           |
| (17beta)-4-(Acetylsulfanyl)-3-oxoandrost-4-en-17-yl propionate    | C<sub>24</sub> H<sub>34</sub> O<sub>4</sub> S<sub>2</sub> | 9.65          | 0.47           | 0.030               | 2.07           |
| Quinagolide                                                       | C<sub>20</sub> H<sub>33</sub> N<sub>3</sub> O<sub>3</sub> S | 15.55       | 0.72           | 0.030               | 1.09           |
| Buclizine                                                         | C<sub>28</sub> H<sub>33</sub> Cl N<sub>2</sub> | 10.15         | 0.52           | 0.035               | 1.75           |
| Sulbutiamine                                                      | C<sub>32</sub> H<sub>46</sub> N<sub>8</sub> O<sub>6</sub> S<sub>2</sub> | 13.99       | 0.68           | 0.036               | 1.49           |
| (2S)-2-Piperazinecarboxamide                                      | C<sub>5</sub> H<sub>11</sub> N<sub>3</sub> O | 1.29          | 0.68           | 0.036               | 1.15           |
| Probucol                                                          | C<sub>31</sub> H<sub>48</sub> O<sub>2</sub> S<sub>2</sub> | 13.45         | 0.35           | 0.037               | 2.28           |
| (-)-Prostaglandin E1                                              | C<sub>20</sub> H<sub>34</sub> O<sub>5</sub> | 12.50          | 0.74           | 0.040               | 1.01           |
| NK77550000                                                        | C<sub>11</sub> H<sub>11</sub> Cl       | 0.10           | 0.68           | 0.040               | 1.14           |
| Metabolite                                                                 | Formula          | N2O2  | FC  | VIP  | VIF  |
|----------------------------------------------------------------------------|------------------|-------|-----|------|------|
| Toborinone                                                                | C21 H24 N2 O5    | 13.23 | 0.62| 0.040| 1.54 |
| Bardoxolone methyl                                                         | C32 H43 N O4     | 14.92 | 0.58| 0.042| 1.44 |
| Taurochenodeoxycholic acid                                                | C26 H45 N O6 S   | 12.82 | 0.13| 0.044| 3.60 |
| 1-Palmitoyl-2-(5-keto-6-octendioyl)-sn-glycero-3-phosphatidylcholine       | C32 H58 N O11 P  | 14.92 | 0.72| 0.045| 1.02 |
| persin                                                                    | C23 H40 O4       | 14.96 | 0.60| 0.047| 1.58 |
| 3-Hydroxybutyric acid                                                     | C4 H8 O3         | 1.60  | 0.58| 0.048| 1.83 |
| n-Butyl lactate                                                           | C7 H14 O3        | 7.68  | 0.68| 0.049| 1.21 |
| 12-Hydroxydodecanoic acid                                                 | C12 H24 O3       | 12.70 | 0.59| 0.049| 1.46 |
| Avasimibe                                                                 | C29 H43 N O4 S   | 12.82 | 0.15| 0.049| 3.44 |

a FC, fold change for the treatment group to control, if the FC value is less than 1, it means that the metabolites were lesser in the treatment group than those in the control group. b VIP, variable importance in the projection.

Table 3 Annotation of differential metabolites between agomiR-499-treated group and control
| Pathway name                        | Differential metabolites | $P$-value |
|------------------------------------|--------------------------|-----------|
| Fructose and mannose metabolism    | glyceraldehyde 3-phosphate | 0.04      |
| Galactose metabolism               | glyceraldehyde 3-phosphate | 0.04      |
| Inositol phosphate metabolism      | glyceraldehyde 3-phosphate | 0.04      |
| Terpenoid backbone biosynthesis    | glyceraldehyde 3-phosphate | 0.04      |
| Glycolysis / Gluconeogenesis       | glyceraldehyde 3-phosphate | 0.08      |
| Caffeine metabolism                | Xanthine                 | 0.08      |
| Vitamin B6 metabolism              | glyceraldehyde 3-phosphate | 0.08      |
| Primary bile acid biosynthesis     | Taurochenodeoxycholic acid | 0.12      |
| Thiamine metabolism                | glyceraldehyde 3-phosphate | 0.12      |
| Pentose phosphate pathway          | glyceraldehyde 3-phosphate | 0.16      |
| Fatty acid biosynthesis            | Lauric acid              | 0.16      |
| Biosynthesis of unsaturated fatty acids | Erucic acid         | 0.20      |
| Purine metabolism                  | Xanthine                 | 0.37      |

**Figures**

**Figure 1**

The body weights of chicks in control group (CG) and treatment group (TG). (A) The body weights of chicks in two groups at Day-1. (B) The body weights of chicks in two groups at Day-18. agomiR-NC and agomiR-499-5p represent the control and treatment groups, respectively.
Figure 1

The body weights of chicks in control group (CG) and treatment group (TG). (A) The body weights of chicks in two groups at Day-1. (B) The body weights of chicks in two groups at Day-18. agomiR-NC and agomiR-499-5p represent the control and treatment groups, respectively.
Figure 2

The diameter of muscle fiber in control group (CG) and treatment group (TG). Statistical significances are indicated by *P < 0.05. agomiR-NC and agomiR-499-5p represent the control and treatment groups, respectively.
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The diameter of muscle fiber in control group (CG) and treatment group (TG). Statistical significances are indicated by *P < 0.05. agomiR-NC and agomiR-499-5p represent the control and treatment groups, respectively.
Figure 3

PCA and OPLS-DA score plots. (A) PCA score plots for consecutively analyzed quality control (QC) samples. (B) The Pearson correlation coefficient of three QC samples. (C) OPLS-DA score plots discriminating control group (CG) and treatment group (TG). (D) Permutation test for the OPLS-DA model C.
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PCA and OPLS-DA score plots. (A) PCA score plots for consecutively analyzed quality control (QC) samples. (B) The Pearson correlation coefficient of three QC samples. (C) OPLS-DA score plots discriminating control group (CG) and treatment group (TG). (D) Permutation test for the OPLS-DA model.
Figure 4

Volcano plots of metabolites in muscle between control groups and treating group. Each dot represents a metabolite. The larger dots indicate higher variable importance in the projection (VIP) values. The abscissa and ordinate represent the fold change and P-value of metabolites, respectively. The increased and decreased (P < 0.05) metabolites in the treatment group (TG) are represented by the red and blue dots, respectively, and the black dots represent the unchanged metabolites (P > 0.05) between two groups.
**Figure 4**

Volcano plots of metabolites in muscle between control groups and treating group. Each dot represents a metabolite. The larger dots indicate higher variable importance in the projection (VIP) values. The abscissa and ordinate represent the fold change and P-value of metabolites, respectively. The increased and decreased (P < 0.05) metabolites in the treatment group (TG) are represented by the red and blue dots, respectively, and the black dots represent the unchanged metabolites (P > 0.05) between two groups.