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## Soy isoflavone ameliorated the alterations in circulating adipokines and microRNAs of mice fed a high-fat diet†

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Soy protein, containing isoflavones and bioactive peptides, is shown to have anti-obesity effects, but the main contributor and underlying mechanisms remain unclear. Recent studies have demonstrated that circulating microRNAs (miRNAs) act as important mediators in obesity and metabolic processes. In this study, we investigated whether soy protein components have distinctive effects on adiposity and circulating miRNA profiles in obese mice. C57BL/6J mice were divided into 4 groups, and each group was fed with a control, high-fat (HF), HF with low-isoflavone soy protein (HF/S), or HF with high-isoflavone soy protein (HF/SI) diet for 16 weeks. In the HF/SI group, changes in the serum adipokine levels, adipocyte diameter, and the number of crown-like structures (CLS) were alleviated compared to those of the HF group. In the HF/S group, the number of CLS was reduced. Decreases in body and adipose tissue weights were not observed in both HF/S and HF/SI groups. Through microarray analysis of serum miRNAs, we identified 23 differentially expressed miRNAs (DEMs) among the groups. The levels of most circulating DEMs were correlated with body weight, serum biochemical parameters, and adipose tissue histology. Functional analysis of predicted target genes of DEMs from both HF vs. CON and HF/S vs. CON comparisons revealed several cancer-related pathways. Only 2 DEMs were identified in the HF/SI vs. CON comparison. In conclusion, the present study confirmed that soy isoflavones are the main contributor to the health-beneficial effects of soy protein in diet-induced obesity. Notably, the extent of serum miRNA dysregulation coincided with obesity and altered the circulating adipokine levels. These findings provide additional insights into the role of soy protein in the regulation of circulating miRNAs in diet-induced obesity. Further work is required to validate the proposed functions of miRNAs in target tissues.

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### 1. Introduction

Obesity is characterized by excessive fat accumulation and chronic inflammation and is frequently associated with the development of various metabolic diseases, including type 2 diabetes, nonalcoholic fatty liver disease, and cancer.<sup>1</sup> It has become evident that adipose tissue is an endocrine organ capable of secreting a number of obesity-related cell signaling adipokines into the bloodstream.<sup>2</sup> Obesity and its related complications are commonly associated with changes in the circulating levels of adipokines, such as reduced levels of adiponectin and increased levels of leptin.<sup>3</sup>

Recent advances in food and nutrition research have been dedicated to identifying food components that exert inhibitory effects on obesity-linked complications. Soybean has attracted

much interest, as it is a good source of protein with a well-balanced amino acid profile. Furthermore, it contains various bioactive components, such as isoflavones and bioactive peptides.<sup>4</sup> Several studies have shown that the intake of soybean is associated with the prevention of hypercholesterolemia, dyslipidemia, hyperglycemia, hepatic steatosis, and hypertension.<sup>5</sup> In addition, the intake of soy products effectively reduced obesity in both humans<sup>6,7</sup> and animals.<sup>8,9</sup> The most beneficial health efficacy of soybean is based on the consumption of isoflavones, mainly genistein and daidzein as aglycone forms and genistin and daidzin as their respective glycosides.<sup>5</sup> Soy isoflavones have been shown to exhibit various biological properties, such as antioxidant, anti-inflammatory, anti-microbial, and anti-cancer activities. Genistein is also known to play an important role in the differentiation of adipocytes by regulating the transcriptional activity of various nuclear receptors, including estrogen receptors<sup>10</sup> and peroxisome proliferator-activated receptor gamma.<sup>11</sup>

In addition to isoflavones, the health beneficial effects of soy have been attributed to bioactive peptides that are produced as a result of fermentation and processing of soybeans,

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or protein digestion in the body.<sup>12</sup> Various bioactive peptides are identified from soy protein, which is mainly composed of two proteins, glycinin (11S) and  $\beta$ -conglycinin (7S). Soy peptides are usually composed of 2 to 20 amino acids, and some of them including soymorphins are shown to play a role in the prevention of multiple chronic diseases.<sup>12</sup> Although consumption of soy  $\beta$ -conglycinin was shown to reduce visceral fat mass in hyperlipidemic humans<sup>6</sup> and high-fat diet-fed mice,<sup>13</sup> most of the studies on soy protein and its bioactive peptides are focused on other bioactivities, such as hypolipidemic, anti-hypertensive, anti-cancer, and anti-inflammatory activities.<sup>12,14,15</sup>

MicroRNA (miRNA), a type of non-coding small RNA, regulates the expression of genes at the posttranscriptional level by translational repression or mRNA degradation in the cytoplasm.<sup>16</sup> Moreover, miRNAs are released from many types of cells such as adipocytes, lymphocytes, macrophages, platelets, endothelial cells, and epithelial cells, into the circulation. Secreted miRNAs are mainly contained in extracellular vesicles to be protected from degradation, suggesting the possible role of circulating miRNAs as a novel cell-to-cell communicator.<sup>17</sup> Adipose tissue is thought to be a major source of circulating miRNAs and several miRNAs in the adipose tissue have been reported to be associated with the development of obesity, suggesting the potential role of circulating miRNA profiles as a biomarker of obesity and metabolic disorders in humans.<sup>18</sup> However, the effects of the soy protein diet on the levels and major source(s) of circulating miRNAs have not yet been studied.

Therefore, in the present study, we compared the effects of low- and high-isoflavone soy protein on obesity to investigate the distinct role of soy protein components, especially bioactive peptides and isoflavones in mice fed a high-fat diet. Also, we not only investigated the anthropometric and serum biochemical changes associated with obesity and adipose tissue inflammation, but also investigated the serum miRNA expression profile to understand the possible role of soy protein components in intercellular communication in a diet-induced obese animal model.

## 2. Materials and methods

### 2.1. Animal and diets

Five-week-old male C57BL/6J mice were obtained from Daehan Bio Link Co. (Korea). After 7 days of acclimation period with a chow diet, mice were randomly divided into four groups and each group was fed with a control diet (CON: 19.5% casein, 12.7% of total calorie from fat;  $n = 11$ ), a high-fat diet (HF: 19.5% casein, 44.4% of total calorie from fat;  $n = 12$ ), a high-fat/low-isoflavone soy protein diet (HF/S: 19.5% soy protein isolate, 44.4% of total calorie from fat, and 35 mg of isoflavone as aglycone equivalents per kg diet;  $n = 12$ ), or a high-fat/high-isoflavone soy protein diet (HF/SI: 19.5% soy protein isolate, 44.4% of total calorie from fat, and 405 mg of isoflavone as aglycone equivalents per kg diet;  $n = 11$ ) for 16 weeks (Fig. S1†). The soy protein isolate used in this study contained

0.037 mg of genistein, 0.147 mg of genistin, 0.014 mg of daidzein, and 0.064 mg of daidzin per g based on a previous study.<sup>19</sup> The soy isoflavone concentrate contained 2.3 mg of genistein, 219 mg of genistin, 3.5 mg of daidzein, and 170 mg of daidzin per g based on a certificate of analysis provided by a supplier. Both the casein and soy protein isolate-based diets were formulated to contain equal amounts of methionine and cysteine. The composition of the diets is shown in Table S1.† The animals were maintained in a temperature ( $22 \pm 2$  °C) and humidity ( $50 \pm 5\%$ )-controlled room with a 12 h dark/light cycle.

At the end of the experiments, all animals were fasted overnight and sacrificed by CO<sub>2</sub> gas inhalation, followed by cardiac blood collection. The blood samples were collected in serum separating tubes (BD Biosciences, USA), left at room temperature for 1 h and centrifuged at 10 000g, 4 °C for 2 min to obtain serum. Epididymal, perirenal, and retroperitoneal adipose tissues were dissected and weighed; epididymal adipose tissues were fixed in 10% neutral-buffered formalin. Serum and adipose tissues were stored at  $-80$  °C until use. All experimental procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-200819-3-2).

### 2.2. Serum biochemical analysis

The serum glucose, triglyceride, and total cholesterol (TC) levels were determined using commercial colorimetric kits (Asan Pharmaceutical Co., Korea). The monocyte chemoattractant protein 1 (MCP-1), leptin, and adiponectin levels were determined using commercial enzyme-linked immunosorbent assay kits (R&D Systems, USA) according to the manufacturer's protocol.

### 2.3. Adipose tissue histology analysis

Epididymal adipose tissue fixed using formalin was cut into 4  $\mu$ m thick paraffin sections and stained with hematoxylin and eosin (H&E) for histological evaluation. The morphology was observed under an ECLIPSE Ni-U microscope with a DS-Ri2 camera (Nikon, Japan). The diameter of adipocytes was measured in each 200 $\times$  image and the number of crown-like structures (CLS) was counted in each 100 $\times$  image. All stained analysis was blindly determined by an experienced pathologist.

### 2.4. Microarray hybridization analysis and microRNA target gene prediction

Serum miRNA was extracted using a miRNeasy serum/plasma kit (Qiagen, USA), and the miRNA expression profiles were analyzed using GeneChip miRNA 4.0 (Affymetrix, USA) (Fig. S2†). The comparative analysis between the test and control samples was carried out using one-way analysis of variance (ANOVA) with *post-hoc* analysis ( $|\text{fold change}| \geq 1.5$  with  $p < 0.05$ ). Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measurement of similarity and visualized as a heatmap using Morpheus software (<https://software.broadinstitute.org/morpheus>). Serum miRNA extraction and microarray hybridization analysis was

conducted blindly by Macrogen (Korea). To predict the target genes of each differentially expressed miRNA (DEM), miRWalk 3.0,<sup>20</sup> TargetScanMouse 7.2,<sup>21</sup> and miRDB 6.0<sup>22</sup> were used, and the target genes that are predicted by all the three algorithms were selected. Also, miRTarbase 9.0<sup>23</sup> was used to obtain the experimentally validated target genes. Only when the genes with 'strong evidence' existed, they were additionally added to the list. All predictions were conducted under default criteria. The target gene list was used for the Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis conducted by the DAVID program (<https://david.ncifcrf.gov>).

### 2.5. Statistical analyses

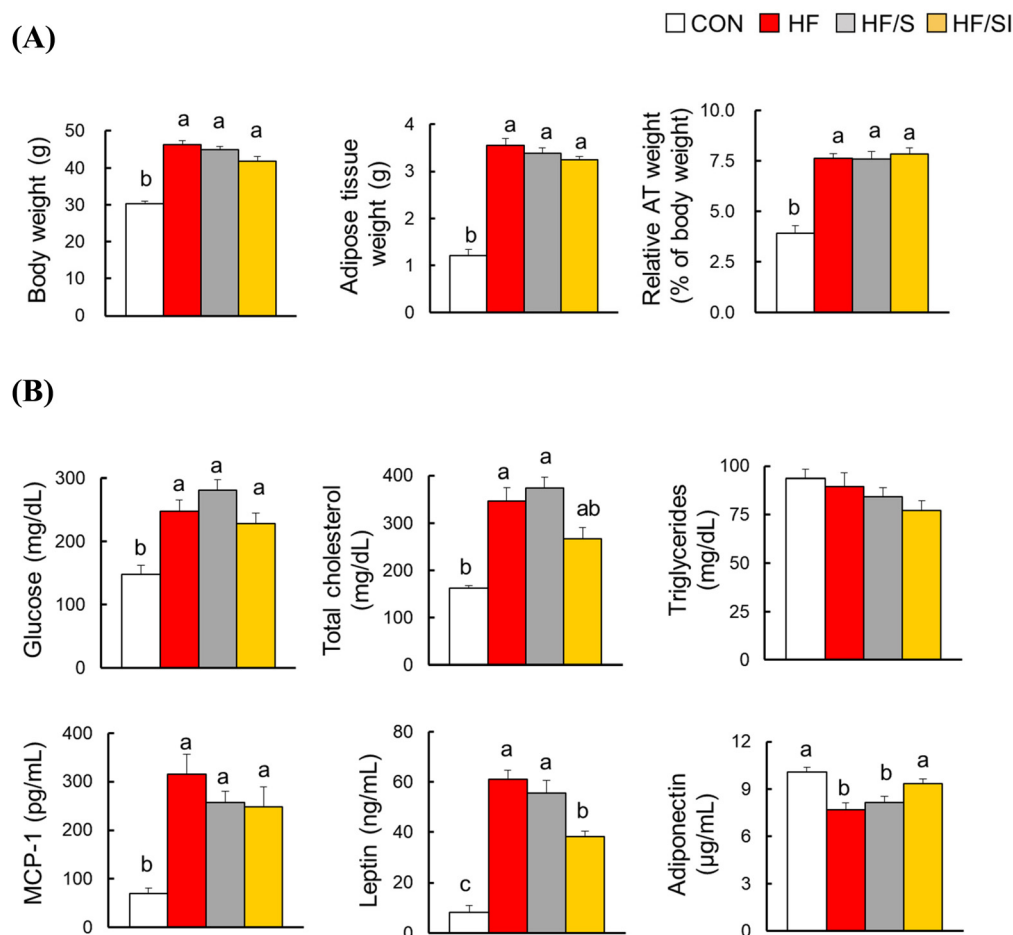
Data were analyzed by one-way ANOVA using SPSS software (ver. 26.0; SPSS Inc., USA). Where appropriate, *post hoc* comparisons were made using Duncan's multiple-range test. Prior to conducting the ANOVA, the assumption of normality was

evaluated by the Shapiro–Wilk test. When the normality was not satisfied (body weight, adipose tissue weight, and serum TC), a Kruskal–Wallis test and Dunn's multiple comparison test were conducted. The data are reported as the mean  $\pm$  SEM and the differences were considered significant at  $p < 0.05$ . The association between factors was examined by the calculation of Pearson's correlation coefficient.

## 3. Results

### 3.1. The effects of soy isoflavone on obesity, serum adipokine levels, and adipose tissue inflammation in mice fed a high-fat diet

The body and adipose tissue weights were significantly increased in the HF group compared to those of the CON group (Fig. 1A). Among the HF, HF/S, and HF/SI groups, no



**Fig. 1** The effects of soy isoflavone on obesity and serum adipokine levels in mice fed a high-fat diet for 16 weeks. (A) Body weight and adipose tissue weight ( $n = 11$ – $12$  per group). (B) Serum biochemical parameters ( $n = 11$ – $12$  per group for glucose, total cholesterol (TC) and triglycerides;  $n = 4$ – $6$  per group for MCP-1, leptin and adiponectin). Each bar represents the mean  $\pm$  SEM. Differences were considered statistically significant by one-way ANOVA followed by Duncan's multiple comparison test or the Kruskal–Wallis test with Dunn's multiple comparison test (body weight, adipose tissue weight, and serum TC) ( $p < 0.05$ ). Bars with different superscripts are significantly different. CON, the group fed with a control diet; HF, the group fed a high-fat diet; HF/S, the group fed with a high-fat/low-isoflavone soy protein diet; and HF/SI, the group fed with a high-fat/high-isoflavone soy protein diet.

significant differences in the body and adipose tissue weights were observed. In the HF group, serum glucose, TC, MCP-1, and leptin levels were significantly increased, and serum adiponectin levels were significantly decreased compared to those of the CON group (Fig. 1B). The changes in the serum leptin and adiponectin levels driven by a high-fat diet were significantly restored by the consumption of high-isoflavone soy protein. The serum TC and MCP-1 levels were similar among the three high-fat groups. Meanwhile, there was no significant difference in serum triglyceride levels among the groups.

H&E analysis of epididymal adipose tissue revealed significant increases in adipocyte size and the number of CLS, a well-known marker of adipocyte inflammation, in the HF group. These parameters were significantly reduced in response to isoflavone supplementation (Fig. 2A & B). In the HF/S group, only the number of CLS was significantly lower compared to that of the HF group. These biochemical and histological data implicate that lipid accumulation and inflammation in adipose tissue were alleviated in the HF/SI group compared to the HF group.

### 3.2. The effects of soy isoflavone on the levels of circulating microRNAs in mice fed a high-fat diet

The levels of circulating miRNAs of each experimental group were investigated using microarray hybridization analysis. Through one-way ANOVA, we identified 23 DEMs (18 mature and 5 immature) among the groups. Hierarchical clustering analysis of DEMs showed that the expression profile of the CON group was most similar to that of the SPI/SI group (Fig. 3A).

Compared to the CON group, the circulating levels of 12, 19, and 2 miRNAs were significantly different in the HF, HF/S, and HF/SI groups, respectively ( $|\text{fold change}| \geq 1.5$ ,  $p < 0.05$ ) (Fig. 3B). Results showed significant increases in circulating

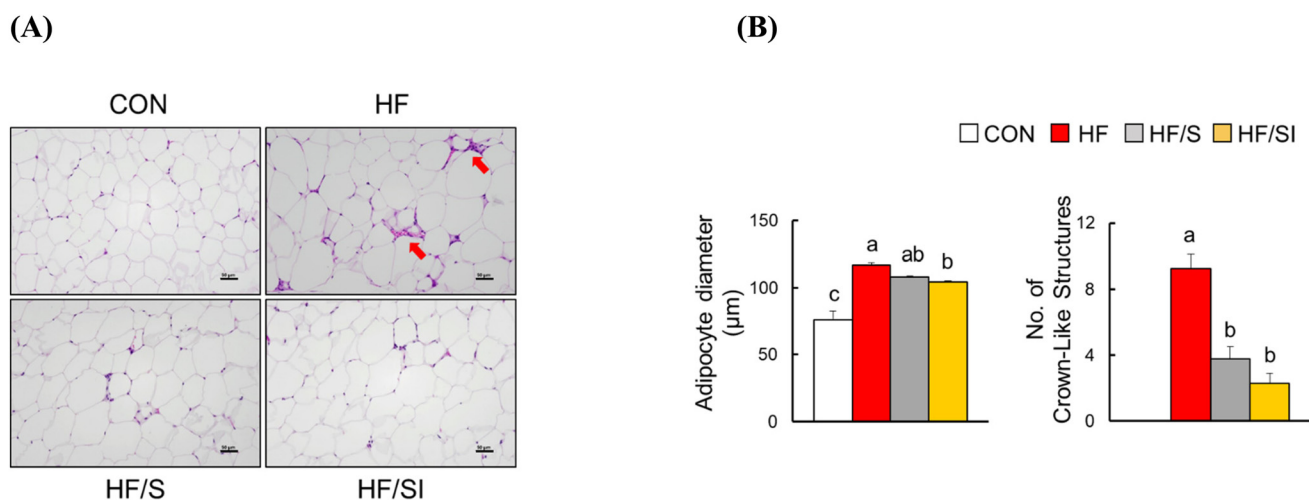
miR-193a-5p and miR-122-5p levels in all 3 comparisons. Between the HF/SI and the HF/S groups, the circulating levels of miR-193a-5p, miR-486a-5p, and miR-7682-3p were significantly different. While the circulating miR-486a-5p levels were upregulated, those of miR-193a-5p and miR-7682-3p were downregulated in the HF/SI group compared to those of the HF/S group.

To investigate the possible role of adiposity and adipose tissue inflammation in the expression profile of miRNA, correlation analyses between anthropometric, biochemical and histological parameters, and the levels of circulating DEMs were conducted (Fig. 4). All parameters were closely correlated with the circulating levels of most DEMs. In the case of CLS incidence, only miR-193a-5p, miR-7002-5p, miR-7085-5p, and miR-467h were significantly associated. Interestingly, 2 DEMs in the HF/SI vs. HF/S comparison, miR-486a-5p and miR-7682-3p, showed different correlation aspects. The circulating miR-486a-5p levels showed an inverse correlation with the parameters; meanwhile, none of the parameters were significantly associated with the circulating miR-7682-3p levels.

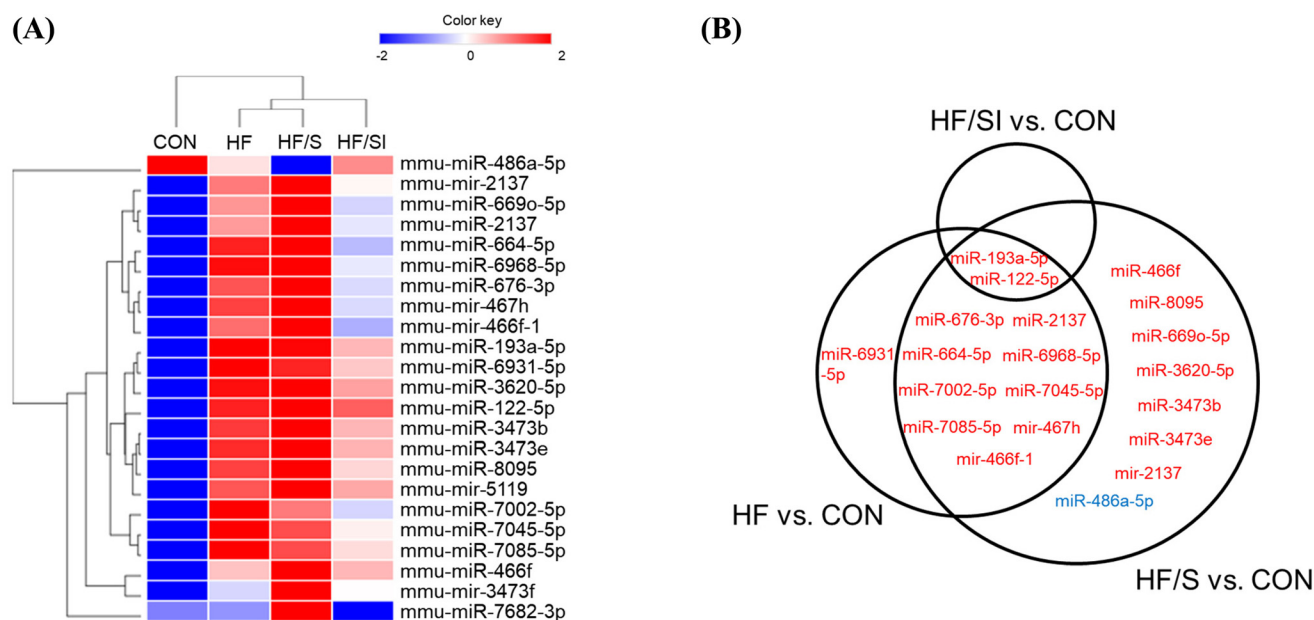
### 3.3. The effects of soy isoflavone on functional enrichment analysis of predicted target genes of circulating miRNAs

We conducted the KEGG pathway analysis of predicted target genes of 10 mature DEMs derived from the HF vs. CON comparison (Fig. 5A). The analysis results showed that the predicted target genes were significantly enriched in a total of 51 pathways. Among the top 20 significant pathways, we observed several cancer-related pathways, including 'Pathways in cancer' ( $\text{FDR} = 3.49 \times 10^{-5}$ ), 'Proteoglycans in cancer' ( $\text{FDR} = 1.95 \times 10^{-4}$ ), and 'Focal adhesion' ( $\text{FDR} = 5.21 \times 10^{-4}$ ).

To find out the potential effect of low-isoflavone soy protein on the replacement of casein, KEGG pathway analysis of the



**Fig. 2** The effects of soy isoflavone on adiposity and adipose tissue inflammation in mice fed a high-fat diet for 16 weeks. (A) Representative images of H&E staining ( $n = 3-4$  per group). The bar in the images represents  $50 \mu\text{m}$ . Crown-like structures (CLS) are annotated with red arrows. (B) The diameter of adipocytes and the number of CLS were determined in  $200\times$  and  $100\times$  image, respectively. Each bar represents the mean  $\pm$  SEM. Differences were considered statistically significant by one-way ANOVA followed by Duncan's multiple comparison test ( $p < 0.05$ ). Bars with different superscripts are significantly different. CON, the group fed with a control diet; HF, the group fed a high-fat diet; HF/S, the group fed with a high-fat/low-isoflavone soy protein diet; and HF/SI, the group fed with a high-fat/high-isoflavone soy protein diet.



**Fig. 3** Effects of soy isoflavone on the circulating microRNA expression profile measured by microarray analysis. (A) A heatmap overview of the hierarchical clustering of differentially expressed miRNAs (DEMs). Each cell represents the mean value of the Z-score normalized miRNA expression of each group ( $n = 3$ ). (B) Venn diagram of 23 DEMs in 3 different comparisons (HF vs. CON, HF/S vs. CON and HF/SI vs. CON). Twenty-two upregulated DEMs are presented in red and 1 downregulated DEM is presented in blue by the criteria of  $|\text{fold change}| \geq 1.5$  with  $p < 0.05$ . CON, the group fed with a control diet; HF, the group fed a high-fat diet; HF/S, the group fed with a high-fat/low-isoflavone soy protein diet; and HF/SI, the group fed with a high-fat/high-isoflavone soy protein diet.

predicted target genes of 7 mature DEMs that were unique to the HF/S vs. CON comparison was conducted (Fig. 5A). As a result, the predicted target genes were significantly enriched in 11 KEGG pathways, including 'Pathways in cancer' ( $\text{FDR} = 1.27 \times 10^{-3}$ ). Four KEGG pathways, the 'Thyroid hormone signaling pathway' ( $\text{FDR} = 1.27 \times 10^{-3}$ ), 'Rap1 signaling pathway' ( $\text{FDR} = 6.05 \times 10^{-3}$ ), 'Fc epsilon RI signaling pathway' ( $\text{FDR} = 2.26 \times 10^{-2}$ ), and 'Platelet activation' ( $\text{FDR} = 2.60 \times 10^{-2}$ ), were identified in the HF/S vs. CON comparison, but not in the HF vs. CON comparison.

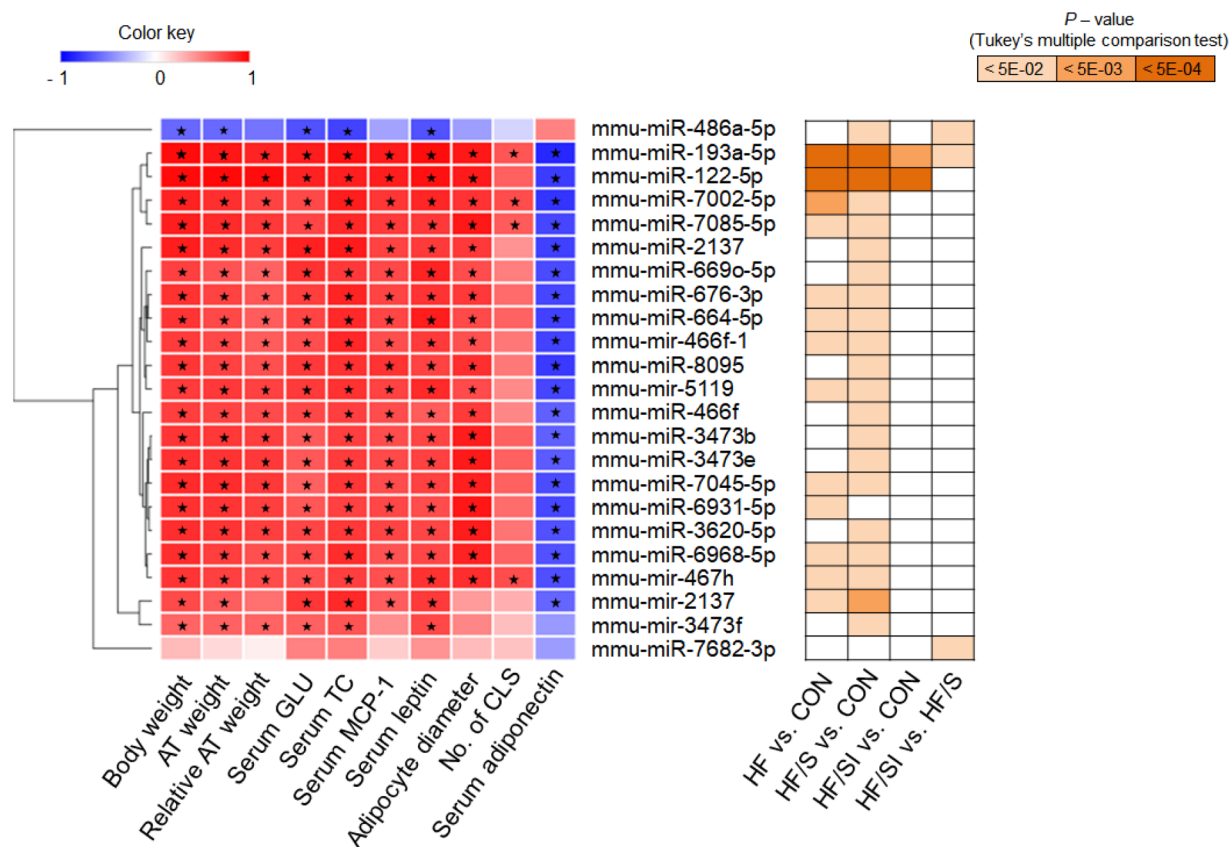
Functional enrichment analysis of the predicted target genes of 3 mature DEMs (miR-193a-5p, miR-486a-5p, and miR-7682-3p) derived from the HF/SI vs. HF/S comparison revealed that target genes of miR-486a-5p and miR-7682-3p were significantly enriched in the 'Sphingolipid signaling pathway' ( $\text{FDR} = 2.48 \times 10^{-2}$ ) and the 'mTOR signaling pathway' ( $\text{FDR} = 2.48 \times 10^{-2}$ ) (Fig. 5B). There were no DEMs in the HF/S vs. HF comparison; meanwhile, 2 down-regulated DEMs, miR-193a-5p and miR-7002-5p, were derived from the HF/SI vs. HF comparison. However, the functional enrichment analysis of the predicted target genes of these 2 mature DEMs did not reveal any enriched KEGG pathways.

## 4. Discussion

This study aimed to investigate the effects of low- and high-isoflavone soy protein on obesity and miRNA profiles in diet-induced obese mice. A few studies reported the alleviating

effects of a high-isoflavone soy protein diet on adiposity and circulating adipokine concentrations in rodents fed a high-fat diet.<sup>24,25</sup> Since these studies compared soy protein isolate *per se* with casein, the specific bioactive component(s) were not identified. In the present study, high-isoflavone soy protein reduced the enlargement and inflammation of adipocytes and ameliorated the dysregulated secretion of adipokines. Meanwhile, decreases in adipose tissue inflammation were observed in mice fed with a low-isoflavone soy protein diet. Furthermore, the circulating miRNA profiles were apparently different in the HF/SI group compared to those of the HF group. In particular, the levels of circulating miRNAs showed significant associations with adiposity and serum levels of adipokines, including leptin and adiponectin.

Various physiological and pathological states, including obesity-related metabolic disorders, are shown to regulate circulating miRNA levels.<sup>18</sup> In the present study, we observed that circulating levels of 12 miRNAs (miR-193a-5p, miR-122-5p, miR-664-5p, miR-676-3p, miR-2137, miR-6931-5p, miR-6968-5p, miR-7002-5p, miR-7045-5p, miR-7085-5p, mir-466f-1, and mir-467h) were significantly changed in mice fed a high-fat diet compared to mice fed with a control diet. Among these DEMs, miR-122-5p and miR-193a-5p were previously identified in serum from subjects with overweight or obesity, and their levels were reduced in response to diet-induced weight loss.<sup>26</sup> The levels of these two circulating miRNAs were also reduced in morbidly obese subjects after surgery-induced weight loss.<sup>27</sup> Moreover, inhibition of miR-122 by miR-122 antisense oligonucleotide injection reduced hepatic steatosis and plasma



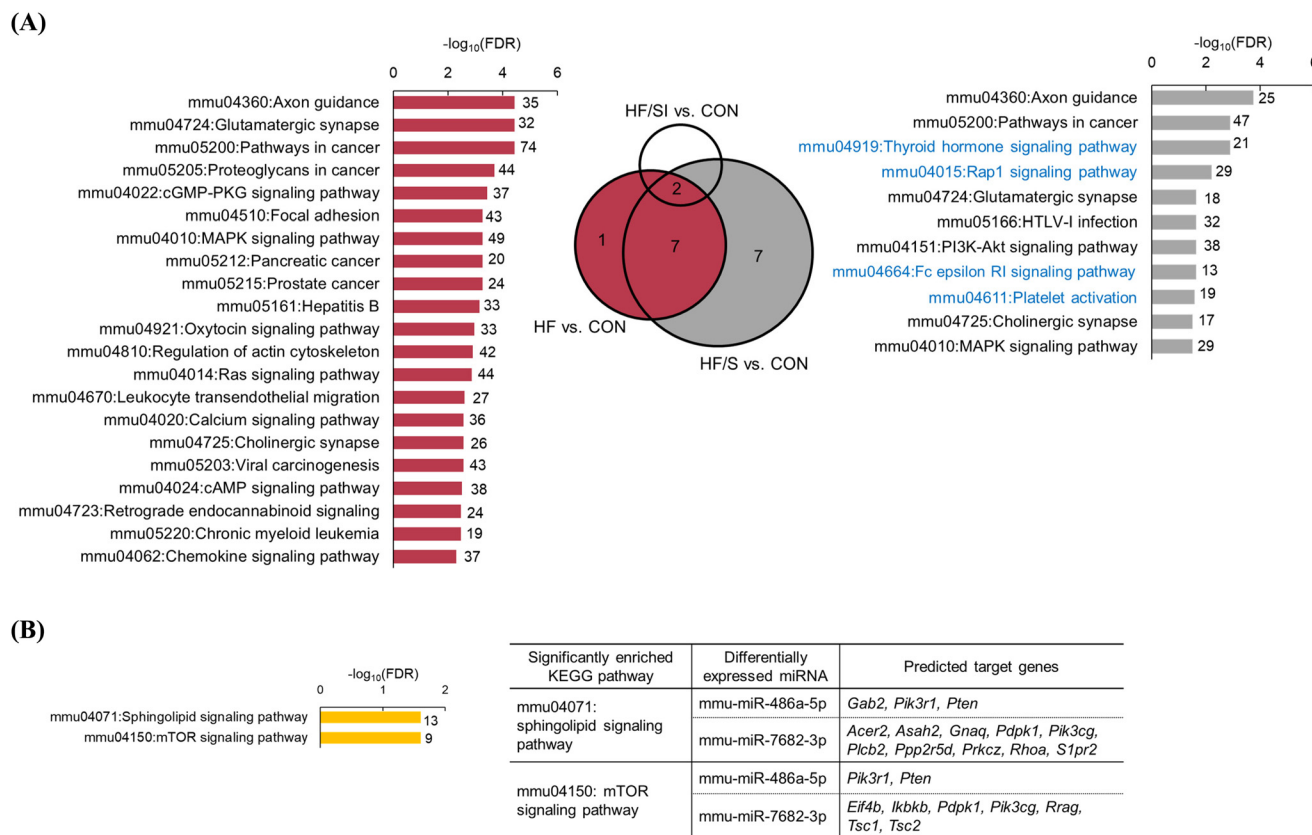
**Fig. 4** Effects of soy isoflavone on the correlations between obesity-associated parameters and the expression levels of differentially expressed miRNAs (DEMs). Left: a heatmap of Pearson's correlation coefficients between two factors. Red color represents positive correlation and blue color represents negative correlation. Asterisk represents statistical significance at  $p < 0.05$ . Right: a heatmap of  $p$ -values for pairwise differences in the expression levels of each DEM. CON, the group fed with a control diet; HF, the group fed a high-fat diet; HF/S, the group fed with a high-fat/low-isoflavone soy protein diet; and HF/SI, the group fed with a high-fat/high-isoflavone soy protein diet.

cholesterol levels in a diet-induced obese mouse model.<sup>28</sup> In accordance with our KEGG analysis data, previous studies have reported several DEMs in the HF vs. CON comparison to be associated with cancer development. The levels of miR-122-5p and miR-193a-5p, 2 DEMs identified in the intersection of three comparison sets, were upregulated in the plasma of hepatocellular carcinoma patients compared to normal individuals.<sup>29</sup> In the case of miR-664-5p, its higher expression levels in tumor tissues were associated with poor prognosis and survival of liver cancer patients.<sup>30</sup> Although reports on other DEMs are lacking, we observed that the circulating levels of these novel miRNAs were significantly correlated with the extent of obesity.

DEMs that are unique to the HF/S vs. CON comparison are miR-486a-5p, miR-466f, miR-3473b, miR-3473e, miR-6690-5p, miR-3620-5p, and mir-2137. Previous studies have reported that miR-486a-5p suppresses hepatocellular carcinoma<sup>31</sup> and non-small cell lung cancer<sup>32</sup> by targeting insulin-like growth factor 1 receptor and its downstream pathways. Except for miR-486a-5p, other DEMs were not studied extensively. Functional analysis of predicted target genes revealed that several KEGG pathways, including the 'thyroid hormone sig-

nal pathway', are exclusively identified in the HF/S vs. CON comparison, but not in the HF vs. CON comparison. From this result, it could be inferred that a low-isoflavone soy protein diet exerted distinct effects on high-fat diet induced obesity, through regulating rather novel miRNAs. Among the 3 DEMs from the HF/SI vs. HF/S comparison, the circulating levels of miR-486a-5p and miR-7682-3p have distinct correlation profiles with obesity-associated parameters. In addition, the putative target genes of these DEMs are associated with the 'sphingolipid signaling pathway' and the 'mTOR signaling pathway' that are known to be involved in the regulation of homeostasis of cell cycle and metabolism.<sup>33,34</sup> Previous studies reported that isoflavones regulate the expression of several genes associated with the biosynthesis and degradation of sphingolipids in human fibroblasts<sup>35</sup> and reduce ceramide levels by inhibiting its biosynthesis in the heart of rats fed a high-fat diet.<sup>36</sup>

Here, we observed that consumption of isoflavone at a dose contained in commercial soy protein isolate products reduced obesity-associated complications in high-fat-fed mice. The total isoflavone content of a commercial soy protein isolate ranges from 0.26 to 2.28 mg per g of soy protein.<sup>37</sup> The data



**Fig. 5** The effects of soy isoflavone on the functional enrichment analysis of predicted target genes of differentially expressed microRNAs (DEMs). (A) Significant KEGG pathways of predicted target genes of 10 mature DEMs (left) in the red section (HF vs. CON) and 7 mature DEMs (right) in the gray section (HF/S vs. CON only). In the case of the HF vs. CON comparison, top 20 significant pathways are depicted. The pathways colored in blue represent the pathways identified only in the gray section of the Venn diagram. (B) Significant KEGG pathways of predicted target genes of DEMs from the 'HF/SI vs. HF/S' comparison. DEMs and the predicted target genes contributing to each significant pathway are indicated in the table. KEGG pathways are indicated in ascending order of FDR. The number of target genes included in each pathway is depicted on the right side of each bar. CON, the group fed with a control diet; HF, the group fed a high-fat diet; HF/S, the group fed with a high-fat/low-isoflavone soy protein diet; and HF/SI, the group fed with a high-fat/high-isoflavone soy protein diet.

could be converted to about 50 to 450 mg of isoflavone per kg diet under the assumption that soy protein isolate accounts for 19.5% of the total diet as in the present study. Considering the content of isoflavones in a high-isoflavone diet (405 mg as aglycone equivalents per kg), the daily consumption of isoflavones is  $\sim 1.1 \mu\text{g day}^{-1}$  in mice, which is converted to  $\sim 130 \text{ mg day}^{-1}$  in humans based on the body surface area. In a human intervention study,  $\sim 100 \text{ mg}$  of isoflavone as aglycone equivalents per day was used to determine the effects of soy protein isolate on circulating hormone profiles in men having a higher risk of advanced prostate cancer.<sup>38</sup> Furthermore, the bioavailability of isoflavone was investigated in American<sup>39</sup> or Japanese women<sup>40</sup> after the consumption of  $250 \text{ mg day}^{-1}$  or  $130 \text{ mg day}^{-1}$ , respectively. According to a population-based prospective study, the daily estimated mean intake of total isoflavone by US adults was reported to be  $2.35 \text{ mg}$ .<sup>41</sup> Meanwhile, daily consumption of isoflavone (median value of Q1 to Q5) ranged from  $14.0$  to  $73.1 \text{ mg day}^{-1}$  and from  $14.5$  to  $72.8 \text{ mg day}^{-1}$  in Japanese men and women, respectively.<sup>42</sup> The isoflavone contents in soy-based foods that are typically consumed

by Eastern Asian counties were in the range of  $\sim 20$  to  $80 \text{ mg}$  per serving.<sup>43</sup>

In previous studies, consuming similar ( $\sim 200$  to  $500 \text{ mg kg}^{-1}$ ) or higher amounts ( $1$  to  $4 \text{ g kg}^{-1}$ ) of pure isoflavone, either genistein or daidzein, during various periods (12 to 24 weeks) reduced body weight in diet-induced obese rodent models.<sup>8,9,44,45</sup> However, we did not observe any significant decreases in body and adipose tissue weights in the HF/SI group. A variety of factors may contribute to the differential results, including the use of soy isoflavone concentrates instead of pure isoflavones. The proportion of aglycone form, genistein and daidzein, accounts for only 4.6% of total aglycone equivalents in the HF/SI diet. Although still controversial, the bioavailability of isoflavones in aglycone form was shown to be higher than those in glucoside form.<sup>40</sup> In addition, the weight-lowering effects of the high-isoflavone soy protein diet were observed in rodents fed a high-fat diet for either too short (5 weeks to 40 days)<sup>46,47</sup> or too long periods (160 to 180 days)<sup>24,25</sup> compared to ours (16 weeks). The relatively low impacts of low-isoflavone soy protein on diet-induced obesity

may be caused by the low amounts of bioactive peptides from soy protein since we did not conduct additional food processing like enzymatic treatments or microbial fermentation to derive more bioactive peptides from soy protein.

One of the limitations of the present study is the lack of a quantitative PCR assay to validate the levels of circulating DEMs. To replicate and extend the current observations, future research with an appropriate model to get larger blood volumes appears warranted. In addition, the secretory and target tissue(s) of circulating miRNAs were not defined. Further studies are needed to clarify whether the observed alterations in the miRNA profiles are due to the dysregulated adipose tissue or the obesity-associated changes in other tissues. Profiling of miRNAs isolated from the explant culture medium of candidate tissues as well as the candidate tissue *per se* would provide important information regarding the biogenesis and secretion of circulating miRNAs. Moreover, the proposed functions of DEMs in their target tissues need to be identified to provide the causal relationships between circulating miRNAs and obesity-related diseases.

In this study, a high-isoflavone soy protein diet suppressed obesity-mediated changes in circulating adipokine levels as well as adipocyte size, suggesting that soy isoflavone could possibly be the main contributor to the health-beneficial effects of soy protein. To the best of our knowledge, this is the first study that investigated the effects of a soy protein diet on circulating miRNA alterations. Overall, these findings provide additional insights into the effects of soy protein on diet-induced obesity and circulating miRNA levels. Since increasing evidence has shown the role of circulating miRNAs as the important mediator between obesity and cancer, the regulatory effects of putative miRNAs on the progression of cancer development are interesting and worthy of further study.

## Author contributions

Hyo Bin Lee: methodology, validation, investigation, writing – original draft, review & editing, and visualization. Ah Young Lee: investigation and writing –review & editing. Yumi Jang: writing – original draft and review & editing. Young Hye Kwon: conceptualization, methodology, validation, writing – original draft, review & editing, supervision, and funding acquisition.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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