Mol. Cells *31*, 33-38, January 31, 2011
DOI/10.1007/s10059-011-0009-7

Molecules and Cells

Murine Hepatic miRNAs Expression and Regulation of Gene Expression in Diet-Induced Obese Mice

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MicroRNAs are short, non-coding RNA molecules that regulate gene expression primarily by translational repression or by messenger RNA degradation. MicroRNAs play crucial roles in various biological processes. However, little is known regarding their role in obesity. We investigated differences of microRNA (miRNA) expression in liver tissue from diet-induced obese mice and potential effects of them on gene and protein expression. We used a miRNA microarray and quantitative RT-PCR to determine miRNA expression in murine liver tissue. Gene and protein expression were determined by qRT-PCR and Western blot analysis. Effects of miRNA by knock-down using RNAi or overexpression on putative target genes and/or proteins in a murine hepatic cell line were also investigated. MicroRNA array and qRT-PCR analsysis revealed that > 50 miRNAs were down- or upregulated more than 2-fold in the liver of diet-induced obese mice. While changes in expression of many genes were observed at the mRNA level, some were only altered at the protein level. Overexpression or knock-down of miR-107 in murine hepatic cells revealed that the expression of its putative target, fatty acid synthase, was dramatically decreased or increased, respectively. In conclusion, more than 50 hepatic miRNAs were dysregulated in diet-induced obese mice. Some of them regulate protein expression at translation level and others regulate mRNA expression at transcriptional level. MiR-107 is downregulated while FASN, a putative target of miR-107, was increased in diet-induced obese mice. These findings provide the evidence of the correlation of miRNAs and their targets in diet-induced obese mice.

INTRODUCTION

Obesity is a metabolic disease that is increasingly assuming epidemic proportions throughout the world, with more than one billion individuals predicted to suffer from obesity by 2030 (Kelly et al., 2008). Obesity is associated with an increased risk of various life-threatening diseases such as cardiovascular disease, type 2 diabetes, hypertension, and dyslipidemia, leading to premature mortality (Miranda et al., 2005). As suggested by Mayer in 1967, not only genetic but also environmental factors such as the diet may be etiological factors for development of obesity (Mayer et al., 1967). This proposition is supported by an epidemiological study in which Japanese-Brazilians residing in Brazil were shown to have a greater prevalence of obesity as compared to those residing in Japan (Schwingel et al., 2007). Moreover, the prevalence of the metabolic syndrome is significantly higher in Japanese-Americans than in the native Japanese population (Yoneda et al., 2008). Therefore, investigating the effect of diet is critical to the understanding of the pathophysiology of obesity.

Many previous reports have clearly shown that diet or dietary components affect the expression of genes and proteins (Kumar et al., 2009), activities of enzymes (Nguyen et al., 2008), and changes in the epigenetic status (Dolinoy et al., 2006). These findings suggest that diet exerts various biological influences through different regulatory pathways and subsequently affects phenotypes or disease processes. Importantly, recent findings have demonstrated that dietary components such as folate, retinoids, and curcumin modulate miRNA expression and play various biological roles (Kutay et al., 2006).

MicroRNAs (miRNA) are small non-coding evolutionarily conserved RNAs (Shyu et al., 2008). A single miRNA regulates hundreds of its targets primarily by translational inhibition or by mRNA degradation (Baek et al., 2008). Consequently, miRNAs can regulate various biological processes, including development, cell proliferation, apoptosis, metabolism, and oncogenesis (Stefani et al., 2008). Since the first identification of miR-14 as a regulator of fat metabolism in fruit flies in 2003, more emerging data suggest a critical role of miRNAs in lipid metabolism, adipogenesis, and obesity-related diseases (Esau et al., 2006; Lovis et al., 2008). However, little is known regarding the miRNA profile of diet-induced obese animals.

Therefore, in the present study, we examined the miRNA profiles in diet-induced obese mice. We also determined the expression of the putative targets, including mRNA and protein, by quantitative real-time PCR (qRT-PCR) and Western blot analysis. In addition, we investigated whether changes in miR-107 expression affect its potential target expression in murine hepatic cells.

MATERIAL AND METHODS

Mice and diets

Six-week-old male C57BL/6J mice were purchased from Cen-

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Received June 15, 2010; accepted October 20, 2010; published online November 25, 2010

Keywords: gene expression, high-fat diet, microRNA, miRNA array, obesity

Table 1. Diet composition

	AIN76	Obese
Casein	200	200
Corn oil	50	50
Beef tallow	0	70
Lard	0	70
Cocoa butter	0	50
Coconut oil	0	30
Cholesterol	0	2.5
Corn starch	150	97.5
Sucrose	500	330
Cellulose	50	50
Mineral mix	35	35
Vitamin mix	10	10
Methionine	3	3
Choline bitartrate	2	2
Total	1,000 g	1,000 g

tral Lab. Animal Inc. (Korea) and were housed in a facility under a 12-h light-dark cycle. All animals were cared for according to the institutional guidelines, and all experiments were approved by the Institutional Ethics Committee. The mice were divided into the control and obese groups (n = 6 per group). The mice were provided the experimental diets (see Table 1) and water *ad libitum*. The body weight of the mice was measured weekly for 6 weeks. Subsequently, the mice were fasted for 12 h and sacrificed.

Cell culture and transient transfection

Murine hepatic cells (CRL-1830) were obtained from American Type Culture Collection (ATCC) and maintained at 37°C in DMEM containing 10% fetal bovine serum (FBS). MicroRNA inhibitors for knock-down and mimic microRNA for overexpression were obtained from Dharmacon (USA). Negative control for knock-down and overexpression were also obtained from Dharmacon. The inhibitors or mimic microRNA for RNAi or overexpression, respectively, were preincubated with Lipofectamine 2000 (Invitrogen, USA) transfection reagent according to the manufacturer's instructions. The DNA-Lipofectamine 2000 complexes were added to murine hepatic cells and then incubated in DMEM (10% FBS) for 48 h. The cells were washed, and the cell lysates were used for subsequent RNA or protein extraction.

Microarray

Hepatic tissues from 4 normal and 4 obese mice were pooled and then microRNA was extracted using a miRNeasy Mini kit (Qiagen, USA) according to manufacturer's instructions. MicroRNA was labeled and hybridized by using the GenoExplorer microRNA array labeling kit and GenoExplorer microRNA chips (GenoSensor Corporation, USA). The hybridized microRNA chips were scanned and analyzed using an Axon GenePix 4000B scanner and GenePix Pro software (Molecular Devices, USA). The prediction of miRNA target genes was carried out using miRanda algorithm at microcosm (http://www.ebi.ac.uk/enright-srv/microcosm) (Griffiths-Jones, 2006; Griffiths-Jones et al., 2008). Then, candidate obesity genes were selected using database of obesity genes (www.obesitygene.pbrc.edu) and pathways (www.pantherdb.org).

Quantitative real-time PCR

Total RNA was isolated from 3 mice in each group by using the RNeasy Plus Mini kit (Qiagen) as described previously (Kim et al., 2005). MicroRNA was prepared as described above. Total RNA or microRNA was reverse-transcribed into cDNA using CycleScript Reverse Transcriptase (BIONEER, Korea) or the GenoExplorer miRNA First-Strand cDNA Core Kit (GenoSensor), respectively.

The level of mRNA of each gene was determined by using the QuantiTect SYBR Green PCR kit (Qiagen). After initial preincubation (50°C for 2 min and 95°C for 15 min), amplification was carried out for 40 cycles (95°C for 15 s, 60°C for 30 s, and 72°C for 30 s) using the LightCycler 480 Real-Time PCR System (Roche Applied Science, USA). The primer sequences were as follows: *Dlk1* (5′-CCCAGGTGAGCTTCGAGTG-3′ and 5′-GGAGAGGGGTACTCTTGTTGAG-3′), *Esrra* (5′-GAC GGC AGAAGTACAAACGG-3′ and 5′-CAACCAGCAGATGCGACA C-3′), *Fasn* (5′-GGCTCTATGGATTACCCAAGC-3′ and 5′-CC AGTGTTCGTTCCTCGGA-3′), *Ihh* (5′-TCTTGCCTACAAGCA GTTCA-3′ and 5′-CCGTGTTCTCCTCGTCCTT-3′), *Pip5k3* (5′-TCCCCGACACTGGACTCTG-3′ and 5′-GGCTGGCCCAACT TGAACT-3′), *Myc* (5′-ATGCCCCTCAACGTGAACT TC-3´ and 5′-CGCAACATAGGATGGAGAGCA-3′), *Crhr1* (5′-GGAACCT CATCTCGGCTTTCA-3′ and 5′-GTTACGTGGAAGTAGTTGT AGGC-3′), and *β-Actin* (5′-CCAGGCACCAGGGCGTGAT-3′ and 5′-TGACGATGCCGTGCTCGATG-3′). The level of mRNA of each gene was normalized to *β-Actin*.

The expression of microRNA was examined by using the GenoExplorer miRNA First-Strand cDNA Core Kit (GenoSensor Corporation). Amplification was performed for 40 to 60 cycles (95°C for 20 s, 60°C for 15 s, 72°C for 30 s). The microRNA-specific primers were purchased from GenoSensor Corp. U6 snRNA was the reference gene used to normalize microRNA expression.

All qRT-PCR were carried out more than 3 times and final numbers in figure legend are the result of multiplying mice number by repeats. If positive and negative controls were abnormal (not detected, saturated at earlier cycles, or strong signals), the sets of the experiments were excluded.

The ratio of gene or microRNA expression was compared to the internal control and was calculated based on the formula 2 ^ ($Cp_{control}$ - Cp_{sample}).

Western blot analysis

A total of 40 μg of protein from 3 mice was separated in a 10% polyacrylamide gel under reducing conditions and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, USA). The membranes were incubated overnight at 4°C with several primary antibodies. After incubation with horseradish peroxidase-conjugated antibodies for 1 h at room temperature, detection was performed with an enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech) using a JP33 X-ray film processor (Jungwon Precision Ind. Co. Ltd., Korea).

Statistics

All statistics were carried out using two-tailed Student *t*-test.

RESULTS

A high-fat and high-cholesterol diet induces obesity

Male C57BL/6J mice were fed with a high-fat and high-cholesterol diet (HFCD) or normal chow (NC) for 6 weeks. At 3 weeks during the experimental period, significant increases in body weights began to show in the diet-induced obese (DIO) mice (Fig. 1). After 6 weeks on the experimental diets, the obese mice gained an average weight of 12.9 g, while the normal mice gained an average weight of 6.7 g.

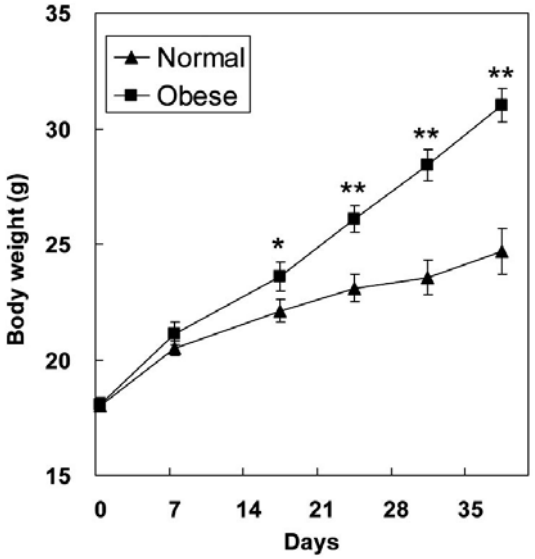

Fig. 1. Weight of the normal and obese mice (n = 6 per group). C57BL/6J mice were fed with a high-fat and high-cholesterol diet or with normal chow for 6 weeks. The data are expressed as the mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

MicroRNA profiling of the diet-induced obese mice

To identify the miRNAs that are up- or downregulated in the DIO mice, miRNA array profiling was carried out without duplication. The miRNA array revealed that 5 miRNAs were upregulated and 52 were downregulated in the obese mice, i.e., more than 2-folds as compared to the normal mice (Table 2).

To confirm the results of the miRNA array analysis, quantitative RT-PCR (qRT-PCR) of 6 miRNAs was carried out. When the miRNA expression profiles by miRNA array analysis and qRT-PCR were compared, most of the patterns were in the same direction and of the same degree (Fig. 2A). MiR-16, miR-103, miR-107, and miR-451 were downregulated up to 6-fold, while miR-351-pre was upregulated 3.5-fold in the DIO mice. However, miR-669c was upregulated up to 3-fold as shown by qRT-PCR analysis.

MicroRNAs and the expression of obesity-related genes

To identify the putative obesity-related genes regulated by the 5 miRNAs, the miRNA target genes predicted using the miRanda algorithm (Griffiths-Jones et al., 2006; 2008) were compared with candidate obesity genes (www.obe-sitygene.pbrc.edu, www.pantherdb.org), and it was found that there were 7 candidate obesity genes: *Dlk1*, *Esrra*, *Fasn*, *Ihh*, *Pip5k3*, *Myc*, and *Crhr1* (Table 3). To confirm the regulation of expression of these genes by miRNAs, qRT-PCR was performed on murine hepatic tissues. The transcripts of *Esrra* and *Ihh* increased significantly, while the *Crhr1* transcript was downregulated in the DIO mice (Fig. 2B). The other transcripts remained unchanged.

To further investigate the role of the miRNAs in regulating the expression of the candidate genes, Western blot analysis was carried out. The level of FASN increased, while MYC expression decreased in the DIO mice (Fig. 2C). However, the CRHR1 level remained unchanged.

miR-107 and FASN expression

To extend the observation that miR-107 expression was inversely correlated with FASN expression as illustrated in Fig.

Table 2. Differentially regulated miRNAs in the liver of diet-induced obese mice

	qRT-PCR			
	Normal	Obese	Obese/normal	Obese/normal
Mmu-mir-010-a	260	103	0.40	-
Mmu-mir-016	1163	249	0.21	0.14
Mmu-mir-019-b	492	94	0.19	-
Mmu-mir-023-a	640	175	0.27	-
Mmu-mir-023-b	654	198	0.30	-
Mmu-mir-024-1	189	45	0.24	-
Mmu-mir-026-a	2299	972	0.42	-
Mmu-mir-026-b	1946	538	0.28	-
Mmu-mir-027-a	680	240	0.20	-
Mmu-mir-027-b	497	195	0.39	-
Mmu-mir-027-b-pre	22	256	11.64	-
Mmu-mir-029-a	1946	966	0.50	-
Mmu-mir-029c	3085	1147	0.37	-
Mmu-mir-030-b	4209	1247	0.30	-
Mmu-mir-030-c	1601	712	0.44	-
Mmu-mir-030-d	1686	463	0.27	-
Mmu-mir-093	477	124	0.26	-
Mmu-mir-099-b-pre	94	1	0.01	-
Mmu-mir-101	2868	561	0.20	-
Mmu-mir-101-a	862	424	0.49	-
Mmu-mir-103	236	37	0.16	0.63
Mmu-mir-106-a	282	73	0.26	-
Mmu-mir-107	195	44	0.22	0.62
Mmu-mir-126-pre	181	13	0.07	-
Mmu-mir-126-3p	814	247	0.30	-
Mmu-mir-130-a	1229	403	0.32	-
Mmu-mir-143	199	86	0.433	-
Mmu-mir-148-a	481	176	0.366	-
Mmu-mir-184-pre	26	71	2.73	-
Mmu-mir-192	882	403	0.46	-
Mmu-mir-194	865	363	0.42	-
Mmu-mir-195	486	128	0.26	-
Mmu-mir-215	515	171	0.33	-
Mmu-mir-292-3p	327	98	0.30	-
Mmu-mir-297-1	676	120	0.18	-
Mmu-mir-297-2-pre	201	56	0.28	-
Mmu-mir-346-pre	277	94	0.34	-
Mmu-mir-350	122	5	0.04	-
Mmu-mir-351-pre	29	208	7.18	4.07
Mmu-mir-365-1-pre	6	470	83.00	-
Mmu-mir-370	88	238	2.71	-
Mmu-mir-378	189	10	0.05	-
Mmu-mir-381-pre	137	1	0.01	-
Mmu-mir-451	544	158	0.29	0.15
Mmu-mir-467-pre	291	76	0.26	-
Mmu-mir-468	825	209	0.30	-
Mmu-mir-469-pre	281	105	0.37	-
Mmu-mir-470	190	54	0.29	-
Mmu-mir-669-a	190	32	0.17	-
Mmu-mir-669-b	338	113	0.33	-
Mmu-mir-669-c	411	74	0.18	1.98
Mmu-mir-669-c-pre	235	25	0.11	-
Mmu-mir-673-pre	4459	9	0.01	-
Mmu-mir-683	185	43	0.23	-
Mmu-mir-711	219	107	0.49	-
Mmu-mir-719-pre	199	23	0.11	-
Mmu-mir-720-pre	1158	10	0.01	-

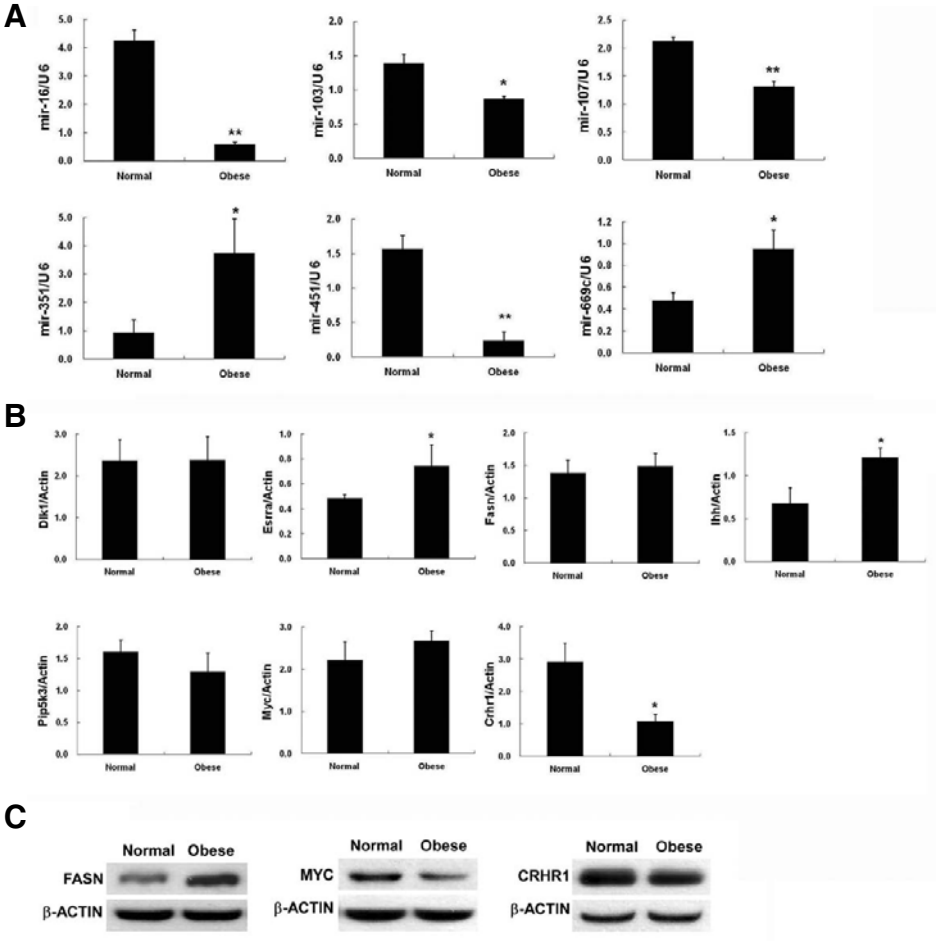

Fig. 2. Validation of expression of the 6 miRNAs and their targets, i.e., mRNAs and proteins. (A) Expression of the 6 miRNAs in the liver of obese and normal mice was normalized to the internal control, U6 RNA, by qRT-PCR (n = 6~9). The data are expressed as an arbitrary unit and as the mean ± SEM. $*p < 0.05$ and $**p < 0.01$. (B) The mRNA levels of the putative target genes were quantified by qRT-PCR, and their expression levels were normalized to *β-Actin* (n = 4~6). The data are expressed as an arbitrary unit and as the mean ± SEM. $*p < 0.05$ and $**p < 0.01$. (C) The protein levels of the putative target genes were determined by Western blot analysis. A total of 40 μg of hepatic protein from 4 mice was mixed and then subject to this analysis.

2B, miR-107 was overexpressed or knocked down by RNAi in murine hepatic cells. As shown in Fig. 3A, FASN expression was dramatically increased up to 3 times in cells expressing miR-107 RNAi compared to control group. Conversely, in the hepatic cells overexpressing miR-107, up to 2 times decreased FASN expression was observed (Fig. 3B).

DISCUSSION

In this study, we demonstrated that feeding mice with an HFCD for 6 weeks resulted in a 25% increase in their body weight, which is consistent with the findings of previous reports (Matsuzawa et al., 2007). An HFCD has been used to investigate the metabolic syndrome, including atherosclerosis, hypertension, and diabetes, in various mammals (Miriyala et al., 2006; Osto et al., 2008; Wendt et al., 2009; Wu et al., 2006). In wild-type or genetically modified animals, an HFCD induces a dramatic increase in the body weight, hepatomegaly, splenomegaly, high blood pressure, and an increase in the plasma lipid content. Thus, a murine model fed with an HFCD can be suggested as a good model to investigate the pathophysiology of the metabolic syndrome in mammals.

In the DIO mice, we, for the first time, demonstrated that the expression of more than 50 miRNAs changed more than 2-fold (Table 2). Some of them were already known to be involved in obesity. However, other miRNAs have been newly identified by our study. Consistent with a previous report showing that miR-103, miR-143, and miR-148a are downregulated in genetically modified obese (GMO) mice (Xie et al., 2009), our study also revealed decreased expression of these miRNAs in the DIO mice. However, many other miRNAs that were dysregulated in the DIO mice had not been recognized in the GMO mice. These findings suggest the existence of a distinct pathophysiology in the DIO and GMO mice. Moreover, it is possible that the expression of a set of miRNAs may be conserved in both mice.

In addition, the expression of miR-29a, one of the miRNAs downregulated in this study, has been shown to be inversely correlated with the body mass index (BMI) of 43 individuals (Iliopoulos et al., 2008). Thus, underexpression of miR-29a was observed in high BMI individuals. Conversely, miR-103, another miRNA shown to be underexpressed in our study, is positively correlated with BMI. These phenomena should be carefully interpreted because three quarters of the individuals were osteoarthritic patients. Since several hundred miRNAs may regulate 30% of human genes (Lewis et al., 2005), it is possible that an miRNA has many different targets and thus may be involved, at the same time, in several different pathogenetic mechanisms such as obesity, inflammation, and osteoarthritis. Moreover, it is important to note that the 6 miRNAs dysregulated in the present study were also differentially expressed in the osteoarthritic patients. This finding supports a proposition of Iliopoulos et al. that obesity is related to osteoarthritis through miRNA deregulation. Another miRNA, i.e., miR-451, that was downregulated in this study was also shown to have been underexpressed in

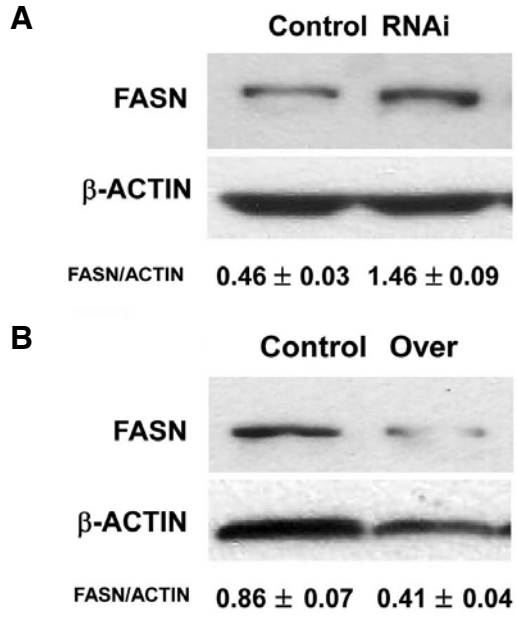

Fig. 3. The effect of miR-107 on FASN expression in murine hepatic cells. (A) Knock-down of miR-107 using RNAi increased FASN expression in murine hepatic cells. (B) Overexpression of miR-107 decreased FASN expression in the cells.

human nonalcoholic steatohepatitis (NASH) (Cheung et al., 2008), which has a major prevalence among obese individuals. Together with our results, these findings suggest that several miRNAs play an important role in obesity and may potentially be involved in a conserved pathogenic pathway in mammals.

A computational analysis of the microcosm algorithm, the pantherDB and the obesitygene databases has predicted 7 candidate obesity genes potentially regulated by 6 miRNAs (miR-16, miR-103, miR-107, miR-351-pre, miR-451, and miR-669c). Transcripts of *Essra* and *Ihh*, the putative targets of miR-16 and miR-103/miR-107, respectively, were significantly increased, whereas *Crhr1* mRNA, a potential target of miR-669c, was downregulated in the DIO mice. These findings illustrate that downregulated miRNAs no longer destabilize the target mRNAs, and the expression of their putative targets thus increases. Conversely, upregulated miRNAs stimulate the degradation of their potential target mRNAs. However, the other transcripts remain unchanged. Since miRNAs regulate their potential targets in 2 different ways, i.e., by translational inhibition or mRNA destabilization (Nushati et al., 2007), it is possible that miRNAs inhibit the translational processes of their targets without stimulating mRNA degradation. To confirm these findings, Western blot analysis was carried out. As mentioned above, FASN was observed to have increased, while MYC was shown to have decreased in the DIO mice, which suggest that miR-107 and miR-669c inhibit translation of their putative targets, FASN and MYC, rather than stimulate mRNA degradation.

Wilfred et al. (2007) used bioinformatics and predicted that the miRNA paralogs, miR-103 and miR-107, may regulate FASN; hence, we extended our study and used RNAi or overexpression *in vitro* to determine whether changes in miR-107 affect FASN expression. As predicted by the previous report, overexpression of mature miR-107 in murine hepatic cells decreased FASN expression, whereas almost complete knockdown of miR-107 in the cells increased polypeptide expression. FASN is a central enzyme in the *de novo* synthesis of fatty acids and plays a crucial role in determining body fat (Berndt et al., 2007). Moreover, an inhibitor of FASN has been shown to efficiently decrease body weight in a murine model (Kumar et al., 2002). Together with our data showing that changes in miR-107 affected FASN expression and caused miR-107 dysregulated in the DIO mice, it can be suggested that FASN is one of the important enzymes in lipid metabolism, and its expression is regulated at least partially by miR-107 at the translational level. Further, the potential interaction between miR-107 and FASN may be associated with the etiology of obesity.

In conclusion, the present study demonstrated that more than 50 miRNAs were dysregulated in the DIO mice. Some of them are already known to be involved in obesity, whereas some others were newly identified. In particular, miR-107 at least partially inhibits the translation of its target, FASN. Thus, miR-107 may potentially play a role in diet-induced obesity or in obesity-related diseases. However, each miRNA appears to regulate many target genes (Baek et al., 2008), and multiple miRNAs can act synergistically at different sites on a single mRNA (Krek et al., 2005). Hence, our findings must be extended further to investigate the role of miRNAs in obesity and the biological relevance of their targets in obesity.

ACKNOWLEDGMENT

This work was supported by grant from the Korea Food Research Institute. The authors declare no conflict of interest.

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