Role of miR-383 and miR-146b in different propensities to obesity in male mice

Shu-Fang Xia1,2,*, Xiao-Mei Duan2,3,*, Xiang-Rong Cheng2, Li-Mei Chen1, Yan-Jun Kang1, Peng Wang4, Xue Tang2, Yong-Hui Shi2 and Guo-Wei Le2

1Wuxi School of Medicine, Jiangnan University, Wuxi, China
2State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, China
3Shandong Sport Training Center, Jinan, China
4COFCO Corporation Oilseeds Processing Division, Beijing, China
*(S-F Xia and X-M Duan contributed equally to this work)

Abstract

The study was designed to investigate the possible mechanisms of hepatic microRNAs (miRs) in regulating local thyroid hormone (TH) action and ultimately different propensities to high-fat diet (HFD)-induced obesity. When obesity-prone (OP) and obesity-resistant (OR) mice were fed HFD for 7 weeks, OP mice showed apparent hepatic steatosis, with significantly higher body weight and lower hepatic TH receptor b (TRb) expression and type 1 deiodinase (DIO1) activity than OR mice. Next-generation sequencing technology revealed that 13 miRs in liver were dysregulated between the two phenotypes, of which 8 miRs were predicted to target on Dio1 or TRb. When mice were fed for 17 weeks, OR mice had mild hepatic steatosis and increased Dio1 and TRb expression than OP mice, with downregulation of T3 target genes (including Srebp1c, Acc1, Scd1 and Fasn) and upregulation of Cpt1a, Atp5c1, Cox7c and Cyp7a1. A stem-loop qRT-PCR analysis confirmed that the levels of miR-383, miR-34a and miR-146b were inversely correlated with those of DIO1 or TRb. Down-regulated expression of miR-383 or miR-146b by miR-383 inhibitor (anti-miR-383) or miR-146b inhibitor (anti-miR-146b) in free fatty acid-treated primary mouse hepatocytes led to increased DIO1 and TRb expression than OP mice, with downregulation of T3 target genes (including Srebp1c, Acc1, Scd1 and Fasn) and upregulation of Cpt1a, Atp5c1, Cox7c and Cyp7a1. A stem-loop qRT-PCR analysis confirmed that the levels of miR-383, miR-34a and miR-146b were inversely correlated with those of DIO1 or TRb. Down-regulated expression of miR-383 or miR-146b by miR-383 inhibitor (anti-miR-383) or miR-146b inhibitor (anti-miR-146b) in free fatty acid-treated primary mouse hepatocytes led to increased DIO1 and TRb expressions, respectively, and subsequently decreased cellular lipid accumulation, while miR-34a inhibitor (anti-miR-34a) transfection had no effects on TRb expression. Luciferase reporter assay illustrated that miR-146b could directly target TRb 3' untranslated region (3'UTR). These findings suggested that miR-383 and miR-146b might play critical roles in different propensities to diet-induced obesity via targeting on Dio1 and TRb, respectively.

Introduction

Obesity, resulting from imbalance between energy intake and expenditure, is a major metabolic and nutritional disorder worldwide, and represents one of the most prevalent risk factors for the occurrence of chronic metabolic diseases (Haslam & James 2005, Puhl & Heuer 2009), such as atherosclerosis, type 2 diabetes, hypertension and liver dysfunction (Dixon et al. 2001). High-fat diet (HFD), especially the saturated fat and monounsaturated
fat, could be responsible for the epidemic. In the past few decades, researchers have been intrigued by the fact that some people maintain a normal weight during their whole lifetime despite living in an obesogenic environment that promotes weight gain. Epidemiological investigation has confirmed that weight regulatory system is biased toward obesity, which means that not everyone gains weight when consuming the high-fat rich diet (Schwartz et al. 2003). Some individuals tend to increase body weight on a HFD (illustrated as obesity-prone, OP), while others resist the development of obesity (obesity-resistant, OR) (Cornier et al. 2009). Animal studies also verified that animals on the same genetic background had different susceptibilities to HFD-induced obesity (Harrold et al. 2000, Huang et al. 2003). Generally, OR animals either may eat less or elevate energy expenditure compared to OP animals (Jackman et al. 2010a). On account of the multi-etiologic nature of obesity and the diverse metabolic perturbations in obese individuals, identifying the causative factors is very difficult.

Thyroid hormones (THs) play critical roles in the regulation of anabolic function, energy expenditure and lipid homeostasis in mammals via direct and indirect regulation in its target genes (Sinha et al. 2014). The liver might serve as an alternative depot for lipid storage when adipose capacity is exceeded as in obesity (Agarwal & Garg 2006), as well as an important target organ of T3 (3,5,3-triiodothyronine) (Pihlajamaki et al. 2009). It has been estimated that nearly 8% of the hepatic genes are regulated by TH in vivo (Oppenheimer et al. 1987). These TH responsive genes can be both positively and negatively regulated by TH through thyroid hormone receptor b (TRb) in liver. It has been suggested that the top-ranking gene set related to obesity was a set of genes positively regulated by T3, such as Atp5c1 and Cox7c (Pihlajamaki et al. 2009). Although there is no consensus with regard to the serum profiles of TH in obesity, it is widely accepted that iodothyronine deiodinases in central and peripheral tissues determine tissue-specific TH levels and TH primarily exerts its actions through interaction with TRs, and finally affecting TH-dependent physiological activities. Our laboratory has found that different propensities to obesity might be related to HPT axis function and deiodinases activities (Xia et al. 2015). Hence, the regulation of steps involved in type 1 iodothyronine deiodinase (DIO1) and TRb action in liver are expected to play major roles in adjusting metabolic rate and substrate partitioning under conditions of surplus energy supply. However, the mechanism is not well understood.

MicroRNAs (miRs) are small non-coding RNAs of 19–24 nucleotides that can cause degradation of the target mRNA or translation block to regulate crucial biological processes, such as metabolism, cell growth and apoptosis (Bartel 2004). Emerging results revealed that various miRs were involved in the function of the TH (Nishi et al. 2011, Boguslawska et al. 2014) and have come in the spotlight of research in obesity and metabolic syndrome (Chartoumpekis et al. 2012). It has been proved that miR-21, miR-146a, miR-181a and miR-221 could react with the TRb directly (Jazdzewski et al. 2011). miR-224 and miR-383 have also been demonstrated to target on Dio1 3′UTR (Boguslawska et al. 2011). Considering the important roles of TH in regulating hepatic energy homeostasis, and the potential importance of miRs in regulating genes coding for proteins critical in these function, we sought to test the hypothesis that miRs might play a critical role in TH action by targeting Dio1 or TRb, and consequently induced different propensities to obesity.

Materials and methods

Animals

Male C57BL/6J mice (80, four-week-old) were purchased from Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China) and housed in plastic cages under a controlled environment (a 12/12-h light/darkness cycle, 08:00–20:00 h, temperature: 23 ± 2°C, humidity: 60 ± 5%). After acclimatization for one week on standard laboratory chow, the mice were randomly divided into a control group (Con, n = 16; fed a standard diet with energy density 3.85 kcal/g, 10, 20 and 70% energy from fat, protein, and carbohydrate, respectively) and a HFD group (n = 64; fed a HFD with energy density 4.73 kcal/g, 45, 20 and 35% energy from fat, protein and carbohydrate, respectively). Diets were provided by Research Diets (New Brunswick, NJ, USA). All mice had free access to the test diets and purified water during the whole experiment. In the 7th week, HFD mice were classified by body weight into OP and OR mice, based on the method described previously (Entiorti et al. 2007). Mice, whose body weights were ranging between the average ± 3 S.D. of the body weight of Con group, were designated as OR mice (n = 16, 27.34 ± 0.42 g) and kept on HFD feeding. Whereas the mice that body weight was 1.3-fold higher than that of the Con mice were selected, of which 16 mice were randomly chosen and defined as OP (n = 16, 34.98 ± 0.39 g). Interestingly, the frequency of OR and OP mice per cage was normally distributed. Furthermore, initial body weight of mice significantly
correlated to final weight and was the only factor that predicted OR vs OP, which was in accordance with previous research (Enriori et al. 2007). In the 7th week, half of mice were randomly selected from each group and sacrificed to assess the effects of short-term HFD consumption on the differentiation between OP and OR phenotypes. The remaining mice were kept on feeding and sacrificed in the 17th week. Body weight and food intake were measured weekly. The study was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Jiangnan University (JN no. 16 2015).

Indirect calorimetry analysis

One week before the end of their respective dietary manipulation, all mice were placed in the Comprehensive Laboratory Animal Monitoring System (C.L.A.M.S; Columbus Instruments, Columbus, OH, USA) to evaluate oxygen consumption (VO₂), carbon dioxide production (VCO₂), respiratory exchange ratio (RER), energy expenditure (EE = (3.815 + 1.232 × RER) × VO₂) and ambulatory activity. Animals were allowed to acclimatize in each individual metabolic cage for one day, and then the data of the second day were used for further analysis. Ambulatory activity was assessed in both horizontal and vertical directions using infrared beams to count the beam breaks during the study.

Tissue samples preparation and serum parameters measurement

At each period, the mice were fasted overnight and slightly anesthetized by intraperitoneal injection of pentobarbital (50mg/kg). Blood was collected, and then liver and white adipose tissues (including perirenal, epididymal, and mesenteric fat) were thoroughly removed, weighed, frozen with nitrogen liquid and stored at −80°C. The experiments were proceeded between 08:00 and 10:00 to minimize possible circadian mRNA expression variation.

Serum total T4 (TT4) and total T3 (TT3) concentrations were measured by radioimmuno assay kits (Diagnostic Products, Los Angeles, CA, USA) with the modifications described elsewhere to optimize the assay for mouse serum (Weiss et al. 1998). The detection limits were 1 μg/dL and 0.02 μg/dL for total T4 and total T3, respectively. Serum triglycerides (TG) and total cholesterol (TC) were also determined using commercial kits (Wako Pure Chemical Industries).

Next-generation sequencing and bioinformatics analysis

Total RNA was isolated from liver samples (n = 3) that were randomly selected from OP and OR mice using Trizol (Invitrogen). Then, each RNA sample was processed to generate a cDNA library that was used for small RNA sequencing. Briefly, a small RNA library was generated from the samples using Illumina Truseq Small RNA Preparation kit (Illumina, San Diego, CA, USA). The quality and size of the library were evaluated by Agilent 2100 Bioanalyzer. The purified cDNA library was used for cluster generation with Illumina TruSeq Rapid SR Cluster Kit-HS using Illumina HiSeq 2500. The small RNA sequencing data were first cleaned by executing small RNA sequencing data cleaning pipeline. Next, the clean sequence data were aligned to miRBase version 20 (v20) to detect and estimate the expression of microRNAs. Because variances did not vary systematically by group, the t-test (unpaired, two-tailed with equal variance) was used to identify the altered expressions of miRNAs between groups, miRNAs satisfying P < 0.05 were recorded.

Analysis of miRs predicted targets was performed using the following three algorithms: TargetScan (Lewis et al. 2003) (http://www.targetscan.org/), PicTar (Krek et al. 2005) (http://pictar.mdc-berlin.de/) and miRanda (John et al. 2004) (http://www.microrna.org/microrna/getMirnaForm.do).

Primary hepatocyte isolation and transient transfection

Primary hepatocytes were isolated from male C57BL/6j mice and cultured as previously described (Sharma et al. 2011). The miR-34a, miR-383 and miR-146b mimics and inhibitors were synthesized by GenePharma Co. Ltd (Shanghai, China) with the following sequences: miR-383 mimic (5′-AGAUCAGAACGUCUGGGCU-3′), miR-34a mimic (5′-UGGCAUGUCUUAGCUGGUUG-3′), miR-146b mimic (5′-UGAGAACUGAAUCCAUAGGCU-3′), anti-miR-383 (5′-AGCCACACUCACCUUCUGAUCU-3′, 2′Ome modification), anti-miR-34a (5′-A C A A C C A G C U A A G A C A C C A C G C C A - 3′, 2′Ome modification) and anti-miR-146b (5′-AGCCUAUGGAUUCCUGUCA-3′, 2′Ome modification). Hepatocytes were transfected with 50 nM mimic or 100 nM inhibitor using X-tremeGene siRNA transfection reagent (Roche Diagnostics). To induce lipid accumulation, primary mouse hepatocytes were incubated with or without 0.5 mM or 1 mM FFAs.
(2:1 oleate/palmitate, Sigma), 6 h after miRs transfections. Hepatocytes were harvested at 24-h post transfection for RNA extraction and 48-h post transfection for protein extraction.

Quantitative RT-PCR analysis

Total RNA was isolated from liver and primary mouse hepatocytes using Trizol reagent (Invitrogen). Quantitative RT-PCR was performed with an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using SYBR Green Real-time PCR Master Mix (Bioneer, Daegu, Korea). The relative amount of each transcript was normalized to cyclophilin A. microRNA PCRs were performed using a microRNA-specific qPCR kit (Qiagen) and U6 was used for normalization. The relative expression was calculated with the comparative ΔΔCt method, the data were expressed as 2−ΔΔCt. The primers used in the current study were illustrated in Table 1. All reactions were conducted in triplicate.

Table 1 Primers used for real-time PCR.

<table>
<thead>
<tr>
<th>Direction</th>
<th>Sequence (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dio1 Forward</td>
<td>GGT GCA ACA TTT GGG AGT TTA TG</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAA TAA GCC TCT TGA ACT GGT CA</td>
</tr>
<tr>
<td>TRb Forward</td>
<td>AAA TCT CCA TCC ATC ATA CTA C</td>
</tr>
<tr>
<td>Reverse</td>
<td>CT TGA AGT CAA CTC TCC CA</td>
</tr>
<tr>
<td>Srebp1c Forward</td>
<td>ATC GGC GCG GAA GCT GTC GGG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTA GTC GCC GTG TC</td>
</tr>
<tr>
<td>Acc1 Forward</td>
<td>ATT GGG CAC CCC AGA GCT A</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCC GCT CCT TCA ACT TGC T</td>
</tr>
<tr>
<td>Scd1 Forward</td>
<td>TGG GTT GGC GTC TGG TG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCG TGG GCA GGA TGA AG</td>
</tr>
<tr>
<td>Fasn Forward</td>
<td>TTC TAC GGC TCC AGG CTC TCC C</td>
</tr>
<tr>
<td>Reverse</td>
<td>GAA GAG TCT TCG TCA GGCAGG A</td>
</tr>
<tr>
<td>Cpt1a Forward</td>
<td>GGC AAG TG TGC CAG ACC TA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGT TCC GAT TCG TGC AAC GT</td>
</tr>
<tr>
<td>Lpl Forward</td>
<td>GGA GAG CAT CCG TGT G</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCC ACC TGC ACC TGG</td>
</tr>
<tr>
<td>Fabp Forward</td>
<td>GTC GTC CGC AAT GAG TCC AC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTA TTG GTG ATG TCC CT</td>
</tr>
<tr>
<td>Atp5c1 Forward</td>
<td>GCC AAC ATC ATC TAC TAC TCT C</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCC TTT ACC TCT TGT CTG</td>
</tr>
<tr>
<td>Cox7c Forward</td>
<td>CCA CCT ATC ATG TTT GCC ACA C</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCG TAA AGC ATT TGG TCA GG</td>
</tr>
<tr>
<td>Cyp7a1 Forward</td>
<td>CATCTGAAGCAGACACCATTTCC</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCACTTTCTAGAGGCTGTTTTC</td>
</tr>
<tr>
<td>Ldlr Forward</td>
<td>GGA AAA GCA TGG CTA GCA AGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>ATT GGA CTG ACA GGT GAC AGA CA</td>
</tr>
<tr>
<td>Cyclophilin A Forward</td>
<td>AGA CAA GGT CCC AAA GACAGC AGA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TGT GAA GTC ACC ACC CTG ACA CAT</td>
</tr>
</tbody>
</table>

Western blot analysis

Western blot analysis was performed as previously described. Protein extracted from mouse liver and hepatocytes was separated with 12% SDS-PAGE and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore). After being blocked with 5% nonfat milk for 1 h, the membranes were incubated using the primary antibodies (anti-DIO1, Proteintech, Wuhan, China; anti-TRb, Santa Cruz Biotechnology; anti-GAPDH, Abcam) at 4°C overnight. After washing with TBST, bolts were incubated with HRP-labeled secondary antibodies. Detection was accomplished using enhanced chemiluminescence reagents (Immobilon Western Detection Reagents, Millipore) and exposure to film.

DIO1 activity determination

DIO1 activity in liver and primary hepatocytes was measured based on previously published literature (Araujo et al. 2010). A specific DIO1 inhibitor (PTU) and excess T4 were used to confirm that type 2 deiodinase could not be detectable. Briefly, 25 mg of liver samples (n=8) was used for homogenization. Triplicates of homogenates (5 μg protein) were incubated with rT3 (1 μM) and [125I] rT3 (PerkinElmer Life Sciences) that were previously purified using Sephadex LH-20 (Sigma Chemical) and potassium phosphate buffer (containing DTT and EDTA). DIO1 activity was expressed as picomoles of rT3 deiodinated per minute per milligram of protein.

Histological analysis

After the liver was weighed, small part of the liver from the same position (n=4) was embedded in OCT compound (Sakura Finetech, Tokyo, Japan) and then stained with Oil Red O (Baso, Taiwan, China) for lipid deposition. Histological evaluation of tissue morphology was conducted under an Olympus light microscope (Olympus).

Triglyceride determination

Hepatic and intracellular lipids were extracted with the mixed solvents of methanol: chloroform (vol/vol=1:2) (Folch 1957) from livers and cell lysates. The resulting extract was re-suspended in water for TG determination using commercial kit (Wako Pure Chemical Industries).
Luciferase reporter assay
To generate a 3' untranslated region (3'UTR) luciferase reporter construct, the full length of the 3'UTR from TRb containing putative miR-146b-binding sites was subcloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) located 3' to the firefly luciferase translational stop codon. Site-directed mutagenesis in the seed regions of the putative miR-146b-binding sites in the TRb 3'UTR was generated using overlap-extension PCR with mutagen primer pairs (TRb-3'UTR-MUT1-PF and TRb-3'UTR-MUT1-PR, TRb-3'UTR-MUT2-PF and TRb-3'UTR-MUT2-PR). For the luciferase reporter assay, HEK293T cells were placed in 24-well plates and co-transfected with pmirGLO-3'UTR-WT1 (TRb-WT1) or pmirGLO-3'UTR-MUT1 (TRb-MUT1), and pmirGLO-3'UTR-WT2 (TRb-WT2) or pmirGLO-3'UTR-MUT2 (TRb-MUT2), respectively, and miR-146b mimic or negative control with Lipofectamine 2000 (Invitrogen). Assays were conducted 24 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Transfections were performed three times in independent experiments.

Statistical analysis
Data were presented as mean±S.E.M. Statistical analysis was performed using SPSS 20.0. Significant differences were determined either by two-tailed Student t-test or one-way ANOVA followed by post hoc Duncan's test for group comparisons. P<0.05 was considered statistically significant.

Results
High-fat diet-induced OP and OR phenotypes
When the experiment started, average body weight of mice that fed a standard diet or a HFD was not significantly different. Gradually, HFD-fed mice gained body weight at a faster rate than Con group. However, part of mice from HFD group had similar and even lower body weight gain compared to the Con mice. In the 7th week, OP and OR mice were selected. Over a period of 17 weeks, the OP group became 67.24% heavier than the Con group. In the first 13 weeks, OR mice had similar body weight compared to Con group. In the subsequent 4 weeks, OR mice increased body weight at a higher rate and became 21.68% heavier than Con mice (Fig. 1A). Finally, OP mice consumed significantly higher calorie than Con mice, which was also remarkably higher than those of OR mice.

No significance on energy intake was observed between OR and Con mice during the first 9 weeks, and in the subsequent 8 weeks, OR mice consumed significantly increased calorie compared to Con mice (Fig. 1B and C), indicating that increased body weight of OR mice might be partly attributed to increased energy intake.

Analysis of tissue indexes revealed that the difference on liver and visceral fat (perirenal, epididymal and mesenteric fat) pad mass indexes between OR and Con mice was not significant in the 7th week and reached statistical significance in the 17th week (Fig. 1D, E, F and G). OP mice demonstrated significantly higher liver and visceral fat pad mass indexes, as well as increased TG levels than Con mice in both periods (Fig. 1H). OR mice showed no significant difference on TG and TC levels relative to Con mice in the 7th week, but exhibited remarkably higher TC levels in the 17th week (Fig. 1I).

Differences in RER, energy expenditure and ambulatory activities between OR and OP mice
Determination of RER over a 24-h period displayed that OP mice showed remarkably lower RER values than Con group both in the 7th and 17th week (Supplementary Fig. 1A, B, C and D, see section on supplementary data given at the end of this article), indicating a shift in metabolism toward an increase in the utilization of lipids as substrate. Although OR mice had significantly lower energy expenditure than Con mice in the daytime in both periods, the significance disappeared in the nighttime (Supplementary Fig. 1E, F, G and H). In the whole day of both periods, OR mice had significantly higher energy expenditure than OP mice, suggesting that lean phenotype of OR mice might be partially attributed to elevated energy expenditure.

In the 7th week, OR mice had more ambulatory activities than Con and OP mice both in the light and darkness cycles (P<0.05), which might be one factor for differentiation of lean and obese phenotypes (Supplementary Fig. 1J). In the nighttime of the 17th week, OP mice were 214% and 34% more active than Con and OR mice, respectively. OR mice were 134% more active than Con mice (Supplementary Fig. 1L).

Differences in serum thyroid hormones and hepatic DIO1 activity between OR and OP mice
As illustrated in Fig. 2, although no significance on serum TT4 levels was observed among the three groups in the
7th week (Fig. 2A), OP mice showed remarkably lower T4 levels compared to Con mice in the 17th week, as well as significantly decreased T3 levels in both periods (Fig. 2B). However, during the whole experiments, OR mice demonstrated similar TT4 and TT3 levels compared to Con and OP mice. Considering the importance of peripheral deiodinases activities in deciding circulating TH levels, hepatic DIO1 activity was further measured. The results showed that compared to Con and OP mice, OP mice showed significantly decreased DIO1 activity in liver in both periods, suggesting the possibility of a decreased cellular T3 level in liver. No significant difference was observed in DIO1 activity between OR mice and Con mice in the 7th week and reached statistical significance in the 17th week (P<0.05).

Differential miRs expression in livers of OR mice vs OP mice and putative gene targets

To investigate the potential role of miRs in different propensities to HFD-induced obesity, next-generation sequencing was used in livers of OP and OR mice in
the 7th week, respectively. As displayed in Table 2, the expression levels of 13 miRNAs in livers of OR mice were significantly different from those of OP mice, including 8 downregulated miRNAs (miR-144, miR-200b, miR-34a, miR-142-3p, miR-383, miR-146b, miR-541 and miR-592) and 5 upregulated miRNAs (miR-223, miR-1247, miR-720, miR-139-5p and miR-100).

The computer-based algorithms (PicTar, TargetScan and miRanda) were used to get a list of the miRNAs whose predicted target genes included Dio1 or TRb or both. As illustrated in Table 2, Dio1 was one of the putative targets of miR-383 predicted by TargetScan, PicTar and miRanda. Additionally, miR-1247 and miR-100 were also revealed to target on Dio1 only predicted by TargetScan. With the same algorithm, miR-34a, miR-146b, miR-541 and miR-592 were illustrated to target on TRb. Furthermore, miR-139-5p was also shown to possibly target on TRb by miRanda.

### Differential expressions of Dio1 and its possible upstream miRs in livers between OR and OP mice

As shown in Fig. 3, Dio1 mRNA and protein expression in livers of OP mice was significantly downregulated than that of Con mice in both periods. OR mice showed significantly increased Dio1 levels than the other two groups in the 7th week and significantly lower level than Con mice in the 17th week (Fig. 3A and B). Based on predicting results by TargetScan, PicTar and miRanda, in order to investigate the possible miRs that might be involved in deciding Dio1 expression, a stem-loop qRT-PCR assay was used to preliminarily reveal the putative miRs expression. Compared to Con mice, the relative expression of hepatic miR-383 was significantly increased in OP mice in both periods. OR mice showed significantly increased Dio1 levels than the other two groups in the 7th week and remarkably higher miR-383 expression than Con mice in the end, which was lower than OP mice (Fig. 3C). The levels of miR-383 were inversely correlated with Dio1 mRNA and protein expression, suggesting the...
possible role of miR-383 in controlling DIO1 expression. Additionally, in the 7th week, OP mice demonstrated significantly lower expression of miR-100 and miR-1247 than Con mice, while OR mice showed remarkably higher miR-100 expression than the other two groups and higher miR-1247 expression than OP mice. In the 17th week, OP mice displayed significantly higher expression of miR-100 and miR-1247 than Con mice, while OR mice showed remarkably lower expression of miR-100 and miR-1247 than OP mice (Fig. 3D and E).

**Differential expressions of TRb and its possible upstream miRs in livers between OR and OP mice**

In the 7th week, OP mice showed significantly increased TRb mRNA and protein expression compared to Con mice, while OR mice displayed remarkably higher TRb expression than the other two groups. In the 17th week, OP mice showed significantly lower TRb expression than Con mice, while OR mice demonstrated significantly lower TRb expression than Con mice, which was significantly higher than that of OP mice (Fig. 4A and B). Results from qRT-PCR showed that OR mice demonstrated a significant downregulation of miR-34a and miR-146b compared to Con and OP mice in the 7th week, but showed significantly higher miR-34a and miR-146b expression than Con mice in the 17th week, which was remarkably lower than that of OP mice (Fig. 4C and D). No significance on miR-541 relative expression was observed in the 7th week, and OP mice displayed a significant upregulation of miR-541 in relative to Con mice in the 17th week (Fig. 4E). OR mice demonstrated a significant decrease in miR-592 relative expression compared to Con mice in the 7th week (Fig. 4F). No significance on miR-139-5p expression was observed among the three groups both in the 7th week and 17th week (Fig. 4G).
Different hepatic steatosis possibly regulated by TH between OR and OP mice

In the 7th week, hepatic TG levels of OP mice were increased significantly compared to those of Con and OR mice (Fig. 5A). When the mice were fed for a longer time, OP mice showed significantly higher hepatic TG levels than the other two groups, while OR mice also showed a remarkably increased TG levels compared to Con mice. Additionally, Oil Red O staining of liver sections of mice confirmed that there was a progressively severe steatosis seen in OP mice compared with Con mice (Fig. 5B). Though, no significance was found in OR mice in the 7th week. Only in the 17th week, OR mice showed observable hepatic steatosis relative to Con mice.

Here, we further investigated part of TH responsive genes that involved in lipogenesis (Srebp1c, Acc1, Scd1 and Fasn), lipid mobilization and fatty acid oxidation genes (Cpt1a, Lpl and Fabp), energy metabolism (Atp5c1 and Cox7c) and cholesterol metabolism (Cyp7a1 and Ldlr) (Fig. 5C and D). In the 7th week, OP mice had significantly increased expressions of genes involved in lipogenesis (about 3.47 fold of Srebp1c, 1.79 fold of Acc1, 6.87 fold of
Scd1 and 13.47 fold of Fasn), lipid mobilization and fatty acid oxidation (2.58 fold of Cpt1α, 3.89 fold of Lpl and 6.20 fold of Fabp) and cholesterol metabolism (1.98 fold of Ldlr), as well as decreased expressions of genes involved in energy metabolism (57% of Atp5c1 and 52% of Cox7c) when compared to control mice, while OR mice showed significantly higher expressions of some genes (2.38 fold of Scd1, 3.54 fold of Cpt1α, 2.29 fold of Lpl and 2.48 fold of Fabp, 4.35 fold of Cyp7a1) than Con mice (Fig. 5C). In the 17th week, OP mice demonstrated significantly higher expression of genes involved in lipogenesis (about 4.69 fold of Srebp1c, 5.81 fold of Acc1, 12.21 fold of Scd1 and 17.38 fold of Fasn) and remarkably lower expression of genes involved in lipid mobilization and fatty acid oxidation (46% of Cpt1α, 68% of Lpl and 79% of Fabp), energy metabolism (38% of Atp5c1 and 31% of Cox7c), and cholesterol metabolism (40% of Cyp7a1 and 57% Ldlr) when compared to control mice, whereas OR mice showed increased expressions of Scd1, Fasn and Cyp7a1, as well as decreased expressions of Lpl, Atp5c1 and Cox7c compared to Con mice (Fig. 5D).

MicroRNAs are robustly induced in free fatty acid treated mouse hepatocytes

Hepatocytes are the major cells that control lipid metabolism and the primary location of lipid accumulation in NAFLD (Ngetal. 2014). We used primary mouse hepatocytes and found that free fatty acids (0.5 mM and 1 mM) increased intracellular TG concentrations (Supplementary Fig. 2A), upregulated the expression of miR-383, miR-34a and miR-146b expression, and downregulated TRb and Dio1 relative expression (Supplementary Fig. 2B), as well as protein levels (Supplementary Fig. 2C). Additionally, lipogenesis genes (including Srebp1c, Acc1, Scd1, Fasn) were all upregulated, while Cpt1α, Lpl, Cyp7a1 and Ldlr expressions were downregulated in 1.0 mM FFA-treated hepatocytes (Supplementary Fig. 2D).
microRNAs participate in the regulation of DIO1 and TRb expression

The introduction of miR-383 mimic into hepatocytes decreased the amount of Dio1 mRNA (downregulation of 51.3%, \( P < 0.05 \)) (Fig. 6A) and protein (downregulation of 41.0%, \( P < 0.05 \)) (Fig. 6B), while anti-miR-383 increased Dio1 mRNA and protein levels. Next, we incubated hepatocytes with anti-miR-383 and further used FFA treatment to induce hepatocytes lipid accumulation. The results showed that anti-miR-383 upregulated Dio1 expression (Fig. 6C), increased DIO1 level (Fig. 6D) and activity (Fig. 6E), as well as upregulating of expression of TH responsive genes involved in lipid metabolism, including Srebp1c, Acc1, Scd1, Fasn, Cpt1a, Lpl, Fabp, Atp5c1, Cox7c, Cyp7a1 and Ldlr (Fig. 6F), and ultimately decreased intracellular TG contents (Fig. 6G).

We further explored the roles of miR-34a and miR-146b targeting TRb in lipid accumulation of hepatocytes. Enforced expression of miR-34a (Supplementary Fig. 3A and B) and miR-146b (Fig. 7A and B) both resulted in an obvious reduction of TRb mRNA and protein levels. In contrast, knockdown of miR-34a and miR-146b resulted in an increase in TRb mRNA and protein levels. However, knockdown of miR-34a failed to significantly increase TRb mRNA and protein levels (Supplementary Fig. 3C and D) and decrease intracellular lipid content (Supplementary Fig. 3E) upon FFA treatment. On the contrary, knockdown of miR-146b attenuated the decrease in TRb mRNA and protein levels (Fig. 7C and D) and reduced the TG content (Fig. 7E) upon FFA treatment. Expressions of lipid metabolism genes were also altered accordingly, including upregulation of Srebp1c, Acc1, Scd1, Fasn, Cpt1a, Lpl, Fabp, Atp5c1, Cox7c, Cyp7a1 and Ldlr (Fig. 7F). Thus, it seemed...
that miR-146b could specifically regulate TRb expression and T3-TR-dependent genes expressions, and thereof regulate hepatic lipid metabolism.

**The 3′ UTR of TRβ is a direct target for miR-146b**

To understand whether miR-146b directly target TRβ 3′ UTR, TargetScan7.1 online prediction was used. The prediction showed that 2 putative miR-146b-binding sites (3111–3117 bp and 4050–4056 bp) were present in the TRβ 3′ UTR (Fig. 8A). To confirm the relationship between miR-146b and TRβ, two mutated TRβ 3′ UTR dual-luciferase reporter vectors were constructed (pmiGLO-TRβ-3′UTR-MUT1 contained 7-base substitutions within the first putative miR-TRβ seed-matched sequence in 3111–3117 bp, and pmiGLO-TRβ-3′UTR-MUT2 contained 7-base substitutions within the second putative miR-146b seed-matched sequence in 4050–4056 bp) and transfected into HEK293 cells. miR-146b mimics had no effects on the activity of pmiGLO-TRβ-3′UTR-WT1 and pmiGLO-TRβ-3′UTR-MUT1 (Fig. 8B), but significantly inhibited the activity of pmiGLO-TRβ-3′UTR-WT2, and had no apparent inhibitory effect on pmiGLO-TRβ-3′UTR-MUT2 (Fig. 8C). These results demonstrated that the second binding site of miR-146b (4050–4056 bp) in the TRβ 3′ UTR existed.

**Discussion**

The main findings of the present study were that different propensities to high-fat diet-induced obesity might be related to hepatic TH action, which might be regulated...
by miR-383 and miR-146b via directly targeting Dio1 and TRβ, respectively. The results of this study provided insight into the roles of miRs in mediating the actions of TH on liver function. Additionally, the ability of OR mice to resist obesity was impaired, possibly due to the impairment of hepatic DIO1 and TRβ action caused by upregulation of miR-383 and miR-146b, and subsequent alteration of TH target gene expressions.

Obesity is a major health concern worldwide which is related to elevated risk of chronic diseases, including metabolic syndrome, cardiovascular disease and cancer (Wright & Aronne 2012). Previous research studies found that despite living in an obesogenic environment, some individuals maintain a thin phenotype compared to the majority who are at risk for weight gain and obesity.

However, the involved possible mechanism is largely unknown. Most genetic studies in the field of human obesity have been focused on genes and polymorphisms associated with an obese phenotype (Soulage et al. 2008). Considerably, less attention has been paid to the understanding of the reason why certain people remain thin, and do not develop obesity even in excess nutritional environment. It is still not clear whether the OR phenotype individuals can resist HFD-induced obesity forever. In our previous study, mice were fed 7 and 27 weeks to explore short- and long-term HFD consumption on the different susceptibilities to obesity, and the results showed that the ability of OR mice to resist obesity was impaired in the 27th week (Xia et al. 2015). We speculated that this impairment might happen earlier and in the present study, we ended the experiment in the 17th week. Normally, the increase in adiposity is the outcome of a positive energy balance as a result of less energy expenditure as compared with energy intake (Huang et al. 2004). In the present study, compared with the OP phenotype, OR mice showed higher energy expenditure both in the 7th week and 17th week, and also had significantly lower calorie intake, which might contribute to the obesity-resistant phenotype. Additionally, OR mice were more active than Con mice during the nighttime of the 17th week possibly because of the imbalance between calorie intake and energy expenditure. However, OP mice was less active than OR mice in the 7th week but more active than OR mice in the 17th week. We speculated that the difference on energy expenditure between the two phenotypes in the 7th week was relatively small, which became larger in the 17th week, so OP mice tried to consume more calories by means of elevating ambulatory activities. It is well known that locomotion is typically regulated by the dopamine system, particularly the nucleus accumbens (Ikemoto & Panksepp 1999). In the current study, it is hard to elucidate the reason for these changes, and thorough research studies should be conducted in the future.

THs play a central role in lipid and glucose metabolism and possibly regulation of body weight through direct and indirect regulation of expression in target genes (Boelaert & Franklyn 2005). The liver plays a critical role in energy metabolism and lipid regulation and is a major target organ of TH. Previous studies have demonstrated that TH regulated the expressions of genes involved in these physiological processes (Pihlajamäki et al. 2009). Although no consensus has been found as to the circulating TH levels in obesity, it is clear that TH perform its function through deiodination pathways (DIO1 in liver) that are...
inside the target cells and could not be deduced in the view of plasma concentrations (Bianco 2011). Besides, TH primarily exerts their actions through interaction with TR, which initiates or blocks target gene expression by binding to TH response elements (TREs) in the gene promoter regions. Therefore, hepatic DIO1 and TRb might be involved in different propensities to obesity, and the factors that affecting these steps need to be clarified.

MicroRNAs are emerging as critical, although poorly characterized, gene-regulatory elements that have been implicated in disease processes including obesity. The function of miRs is highly dependent on their target genes via suppressing translation and enhancing degradation of target gene transcripts by binding to comprehensive regions within the target transcripts (Hobert 2004). However, very little is known about the role of miRs in mediating the action of TH. Although previous research has explored the role of miRs in governing TH response (Dong et al. 2010), so far no research has focused on miRs when exploring the different propensities to obesity. We speculated that miRs might also play important roles in TH action through regulating expressions of some genes, such as Dio1 and TRb and thereafter to influence hepatic DIO1 and TRb levels and ultimately decide the different propensities to obesity. Results from next-generation sequencing and bioinformatics algorithms indicated that expressions of miR-34a, miR-383, miR-100, miR-1247, miR-146b, miR-541 and miR-592 were significantly different between OP and OR mice and might target on Dio1 or TRb. Among these miRs, miR-383, miR-34a and miR-146b were inversely correlated with Dio1 or TRb expression, in attempt to normalize the TH target genes expression, while OR mice resisted obesity possibly through suppressing these corresponding miRs expression.

When the mice were fed for a longer time, both OP and OR mice had elevated miR-383 and miR-146b expression, as well as decreased miR-146b expression, which possibly be directly regulated by TRb action, which might lead to decrease of Dio1 and increase of TRb expression, in attempt to normalize the TH target genes expression, while OR mice resisted obesity possibly through suppressing these corresponding miRs expression to enhance hepatic deiodination and TRb expression. When the mice were fed for a longer time, both OP and
OR mice had upregulation of miR-383 and miR-146b, possibly resulting in impaired deiodination and reduction of TRβ expression, and subsequent dysregulation of TH responsive genes. Hence, therapeutic strategies for obesity should take into consideration the role of miR-383 and miR-146b in hepatic deiodination and TRβ action.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-17-0044.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
The study was supported by the Fundamental Research Funds for the Central Universities (JUSRP115A33), Wuxi Science and Technology Development Fund (CSE31N1625) and Natural Science Foundation of Jiangsu Province, China (BK2014147).

Author contribution statement
S F X, X M D, X R C, L M C, Y J K, P W, Y H S and G W L conceived, designed and performed the experiments. S F X, X M D and Y J K analyzed the data. Y H S and X T contributed reagents/materials/analysis tools. S F X drafted the article. All authors read and approved the final manuscript.

References
Cornier M-A, Salzberg AK, Endly DC, Bessesen DH, Rojas DC & Tregellas JR 2009 The effects of overfeeding on the neuronal response to visual food cues in thin and reduced-obese individuals. PLoS ONE 4 e6310–e6310. (doi:10.1371/journal.pone.0006310)
Dong H, Paquette M, Williams A, Zoeller RT, Wade M & Yauk C 2010 Thyroid hormone may regulate mRNA abundance in liver by acting on microRNAs. PLoS ONE 5 e12136. (doi:10.1371/journal.pone.0012136)


Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW & Freake HC 1987 Advances in our understanding of thyroid hormone action at the cellular level. *Endocrine Reviews* **8** 288–308. (doi:10.1210/edrv-8-3-288)


Weiss RE, Murata Y, Cua K, Hayashi Y, Seo H & Refetoff S 1998 Thyroid hormone action on liver, heart, and energy expenditure in thyroid hormone receptor beta-deficient mice. *Endocrinology* **139** 4945–4952. (doi:10.1210/endo.139.12.6412)


Received in final form 17 May 2017
Accepted 31 May 2017
Accepted Preprint published online 1 June 2017