• Lipids (Saturated/Unsaturated Fatty Acids and Cholesterol)

It is essential to reduce the intake of saturated fatty acids and cholesterol, which are contained in large amounts in animal fat; however, there are significant individual differences in the absorption rate of cholesterol. Increased intake of saturated fatty acids has been reported to exacerbate insulin resistance and increase the LDL-C levels in Japan as well as in Western countries[43, 44]. In contrast, it has been reported in Japan that an extremely low intake of saturated fatty acids is associated with an increased incidence of cerebral hemorrhage[45, 46]; thus, the percentage of energy derived from saturated fatty acids should be at least 4.5% but less than 7%[47]. Meanwhile, excessive intake of trans unsaturated fatty acids, produced by the hydrogenation of polyunsaturated fatty acids which are contained in hard margarine and shortening, increases oxidized LDL, decreases HDL-C, and thereby increases the risk of CAD[48]. In order to reduce the intake of saturated fatty acids and cholesterol, meat with less fat should be selected and excessive intake of meat, dairy products and eggs should be avoided.

While the intake of saturated fatty acids should be reduced, the intake of unsaturated fatty acids should be increased. Patients should be instructed to consume more fish, especially bluefish, which is rich in n-3 polyunsaturated fatty acids. Epidemiological studies conducted in Japan have revealed a negative correlation between the intake of fish and n-3 polyunsaturated fatty acids and mortality from coronary events and myocardial infarction[49, 50]. These effects are considered to be mediated by TG-lowering effects, hypotensive effects, platelet aggregation inhibitory effects and improvements in the endothelial function achieved by n-3 polyunsaturated fatty acids, which are contained in large amounts in fish oil[51-54]. On the other hand, polyunsaturated fatty acids are easily oxidized; therefore, it should be noted that excessive intake of these fatty acids results in increased levels of oxidized LDL and decreased levels of HDL-C.

• Selection of Carbohydrates

Carbohydrates include sugar, which is digestible and absorbable, and dietary fiber, which is indigestible. The type and intake of carbohydrates affect glucose metabolism and the levels of TG and HDL-C. The glycemic index (GI) and glycemic load (GL) are indexes used to evaluate postprandial blood glucose following the intake of carbohydrates. Many studies have reported that these indexes exhibit positive correlations with the obesity index and the levels of TG and fasting blood glucose and a negative correlation with the level of HDL-C[55, 56].

An increased intake of dietary fiber inhibits fat absorption in the intestines and decreases the GI and GL. The intake of dietary fiber, especially soluble dietary fiber, has a LDL-C-lowering effect[56-59]. A relationship between the consumption of greater amounts of dietary fiber and decreased mortality from CAD and CVD has been reported[60, 61]. To ensure sufficient dietary fiber intake, consuming adequate amounts of plant foods, such as unrefined grains (e.g., brown rice, barley), soy (e.g., tofu, bean curd: natto, fermented soybeans), vegetables, seaweed, fruits and potatoes is useful. This leads to a low GI/GL diet.

• Soy, Soy Products, Vegetables and Fruits

It has been reported that the intake of plant foods from soy and soy products and their major components, isoflavones, is associated with inhibition of the development of CAD and cerebral infarction in women[62]. This is thought to be due to the mild decreases in the level of LDL-C, antioxidant effects, hypotensive effects and estrogen effects induced by the isoflavones[63-68], protein[69, 70] and polyunsaturated fatty acids contained in soy. The consumption of plant foods other than soy, such as fruits, vegetables[29, 71], pulses[71] and grains[72], as well as green tea, coffee and oolong tea, is also associated with inhibition of the development of CAD. In particular, a relationship between an increased intake of green tea and decreased mortality from CAD has been reported in Japanese women[73]. Plant sterols, rich in soy and germ, are expected to inhibit the absorption of exogenous cholesterol in the gastrointestinal tract[74-77]. A meta-analysis showed that an intake of plant sterols of ≥2 g/day results in decreases in the LDL-C level of up to 9%[78].

Frequent consumption of fruit and vegetables is recommended because these foods are low in calories and rich in dietary fiber, vitamins and minerals. It has also been reported that the intake of potassium[79], vitamin C[80] and vitamin B_6[81, 82] is associated with inhibition of the development of CAD.

• Salt and Alcohol

An excessive intake of salt increases blood pressure and promotes atherosclerosis. The intake of salt should be reduced to <6 g/day. Light to moderate alcohol consumption has been shown to be associated with the prevention of CAD[83], while excessive consumption of alcohol increases blood pressure and enhances TG synthesis in the liver.

3) Diet to Improve Risk Factors

Diet modification is essential for preventing CVD

Table 2. Nutrient Recommendations for the Prevention of Cardiovascular Disease

1. Maintain an ideal body weight (height $[m]^2 \times 22$) in consideration of energy intake and the amount of physical activity.
2. Limit the energy percent derived from fat to 20%-25%, saturated fatty acids to ≥4.5% but <7%, and cholesterol intake to <200 mg/day.
3. Increase the intake of n-3 polyunsaturated fatty acids.
4. Limit the energy percent derived from carbohydrates to 50%-60%, and increase the dietary fiber intake.
5. Aim to reduce the salt intake to <6 g/day.
6. Limit alcohol consumption to ≤25 g/day.

because it is effective for managing the risk factors of CVD, as has been demonstrated in many studies. Patients should be given individualized dietary instructions in consideration of prior assessments of their lifestyles including their nutrient intakes (**Table 2**).

• Hyper-LDL Cholesterolemia and Diet

The intake of saturated fatty acids, cholesterol and trans unsaturated fatty acids, which increase the level of LDL-C, should be reduced. The percent energy from saturated fatty acids should be less than 7%, while cholesterol intake should be less than 200 mg/day. Specifically, the intake of meat, milk and eggs, which contain high amount of fat, should be limited. Furthermore, the intake of foods with LDL-C-lowering effects, particularly soluble dietary fiber and plant sterols, should be increased[84-86].

• Hypertriglyceridemia and Diet

The percentage of energy derived from carbohydrates should be slightly reduced, and excessive consumption of alcohol should be limited. The intake of n-3 polyunsaturated fatty acids should be increased. In patients with hyperchylomicronemia, fats should be limited more strictly. The percentage of energy derived from fat should be limited to less than 15%, comprised primarily of medium-chain fatty acids[87] or n-3 polyunsaturated fatty acids.

• Hypo-HDL Cholesterolemia and Diet

If the patient consumes alcohol moderately and exhibits no abnormalities in TGs, alcohol consumption does not need to be limited. Excessive intake of trans unsaturated fatty acids and n-6 polyunsaturated fatty acids should be limited.

• Metabolic Syndrome and Diet

In general, for patients with visceral fat accumulation and high insulin resistance, the total energy intake should be limited and a diet with a low percentage of energy derived from carbohydrates should be consumed. When selecting carbohydrates, low-GI/GL diets are desirable. Total caloric reduction with a moderate amount of fat in combination with exercise can improve insulin resistance and the components of metabolic syndrome, even if weight loss is modest.

• Hypertension and Diet

Efforts should be made to reduce the salt intake while increasing the fruit and vegetable intake. This leads to sodium restriction and sufficient intake of potassium, resulting in the promotion of urinary excretion of sodium. Excessive consumption of alcohol should be avoided because it increases blood pressure.

• Diabetes Mellitus and Diet

In patients with type 2 diabetes, amelioration of obesity is the most important component of disease management. Overeating should be avoided and the energy intake should be tailored for the level of daily physical activity. Hyperglycemia should be corrected by dividing the energy intake equally into the three meals, i.e. breakfast, lunch and dinner, whenever possible. Regarding the levels of energy intakes of nutrients, the ratio of sugar to other nutrients should not be increased. In particular, the intake of sugar and saturated fatty acids should be limited. In patients with type 1 diabetes, appropriate quantities of dietary energy should be consumed to maintain an ideal body weight, with consumption of a nutrient-balanced diet.

Glossary

Glycemic Index (GI) and Glycemic Load (GL)

The GI is a ranking of carbohydrates based on how much they raise blood glucose levels after the consumption of foods containing 50 g of carbohydrate. It is a relative index with 50 g of glucose serving as the reference value of 100. The GL is the value calculated from GI and is the amount of carbohydrate by which postprandial blood glucose change is predicted.

5. Exercise Therapy

Physical inactivity is associated with increased body fat (obesity), dyslipidemia, metabolic syndrome, hypertension, diabetes mellitus/impaired glucose toler-

Table 3. Guidelines for Exercise Therapy

Exercise intensity*	About 50% of maximum oxygen uptake
Intensity and frequency	At least 30 min per day (daily if possible) and at least 180 min per week
Type	Brisk walking, slow jogging, social dancing, swimming, cycling, bench step exercise, etc.

*Exercise intensity
(1) Estimated from the pulse rate during exercise (when exercise intensity is 50%) Heart rate (pulse rate/min) = 138 − (age/2)
(2) Estimated from perceived exertion:
11-13 on the Borg's scale (perceived exercise intensity)
(Fairly light to somewhat hard)
Maximum oxygen uptake: Index of whole-body endurance (cardio-respiratory endurance)

Borg's scale

Scale	Perceived
20	
19	Very, very hard
18	
17	Very hard
16	
15	Hard
14	
13	Somewhat hard
12	
11	Fairly light
10	
9	Very light
8	
7	Very, very light
6	

(Borg GA: Med Sci Sports Exerc. 1973; 5: 90-93.)

ance, vascular endothelial dysfunction, decreased exercise capacity and an increased risk of atherosclerotic CVD, such as CAD and cerebrovascular disease[88-98].

Increased physical activity maintains and increases exercise capacity, improves the serum lipid profile, decreases blood pressure, increases insulin sensitivity and glucose tolerance, improves the vascular endothelial function and prevents thrombosis[99-101]. In addition, it decreases mental stress and preserves the cognitive function[102, 103]. The amount of daily physical activity and leisure time physical activity and the level of physical fitness have been shown to be negatively correlated with mortality resulting from CVD and cancer as well as all-cause mortality[104-114]. Similar results have been observed in cohort studies conducted in Japan[88].

Exercise therapy includes aerobic endurance exercise and muscle resistance exercise. Aerobic exercise is effective in improving lipid metabolism[115-118]. The most commonly observed change in the serum lipid levels induced by exercise is an increase in the level of HDL-C. A meta-analysis of 25 randomized controlled trials (RCTs) that compared the effects of exercise therapy comprising ≥ 15 minutes of aerobic therapy for eight weeks with those of non-exercise therapy showed that the exercise therapy significantly increased the levels of HDL-C (Δ2.53 mg/dL, 95% CI: 1.36 to 3.70)[119]. The increased HDL-C levels exhibited a positive correlation with the length of exercise, and exercise of ≥ 121 minutes/week significantly increased the HDL-C levels. A meta-analysis of four RCTs conducted in Japan comparing the effects of aerobic exercise of mild to moderate intensity for 10 weeks to 24 months with those of non-exercise therapy showed that the exercise significantly increased the levels of HDL-C (Δ10.0 mg/dL, 95%CI: 5.39 to 14.65)[88].

Table 3 shows the basic guidelines for exercise therapy. Efforts should be made to increase physical activity in daily living and undertake exercise suited to the lifestyle of the individual. Aerobic exercise should be primarily performed, and brisk walking and slow jogging are recommended. Regarding the intensity of exercise, approximately 50% of the maximum oxygen uptake is suitable in terms of efficacy and safety. At 50% intensity, the increase in blood pressure observed during exercise is mild, blood lactate is not accumulated and exercise can be performed for an extended period of time. Exercise of at least 30 minutes duration per day at least three times per week (daily if possible) or of at least 180 minutes per week is desirable. For the elderly with a reduced muscle mass, aerobic exercise in combination with mild resistance (muscle) exercise is useful, and bench-stepping training, which can be performed in a room, is recommended[88]. The Ministry of Health, Labour and Welfare established the "Exercise and Physical Activity Guide for Health Promotion 2006" to prevent lifestyle-related diseases (**Supplemental Tables 3A to 3C**)[120]. A unit to express the quantity of exercise, the "Ekusasaizu (Ex) (= METs · hour)," was established, and the target quantity of physical activity to prevent lifestyle-related diseases was set at 23 Ex or more per week. For example,

walking or bicycling for 15 minutes, jogging or aerobics for 10 minutes and swimming for seven to eight minutes are equivalent to 1 Ex.

On the other hand, unaccustomed exercise carries a risk of musculoskeletal injury. For patients with CVD, strenuous exercise may cause sudden death or myocardial infarction[121, 122]. This requires careful consideration, and when exercise therapy is performed, complications of potential CVD and bone and joint disease should be assessed.

Footnotes

This is an English version of the guidelines of the Japan Atherosclerosis Society (Chapter 7-A) published in Japanese in June 2012.

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Supplemental Table 1. 5A Approach

Step
Step 1: Ask (Identify all smokers systematically at each examination.)
Step 2: Advise (Clearly, strongly, and individually advise all smokers to stop smoking.)
Step 3: Assess (Assess the desire to smoking cessation.)
Step 4: Assist (Assist patients in smoking cessation.)
Step 5: Arrange (Arrange a schedule of follow-up examinations.)

Strategies for implementation

Step 1

- Prepare a system within the medical organization to ensure that all patients are asked about smoking at each examination and the answers are recorded.
- Add a space for smoking (to distinguish current, former, and non-smokers) to the section of vital signs such as the blood pressure, heart rate, body temperature, and body weight. Alternatively, attach a sticker indicating the smoking status to all charts.

Step 2

- Clearly: "It is important for you to stop smoking now. I am ready to help you." or "It is not enough to cut back on smoking only when you are sick."
- Strongly: "As your attending physician, I must let you know that smoking cessation is the most important step you can take to protect your health. I and the hospital staff are ready to help you."
- Individually: Relate smoking with the current state of health/disease, social and economic cost, motivation/desire to quit smoking, and impact on children and family.

Step 3

- Ask all smokers if they are willing to stop smoking now (within 30 days).
  If they are, support them in cessation.
  If they are not, motivate them to cessation.

Step 4

Assist patients to make a plan of smoking cessation.

- Set a date to start smoking cessation (preferably within 2 weeks)
- Tell family, friends, and colleagues about smoking cessation and ask for their understanding and support.
- Mentally prepare for problems that will arise in smoking cessation (particularly during the first few weeks) in advance. They include nicotine-withdrawal symptoms.
- Eliminate tobacco from the environment on smoking cessation. Before smoking cessation, avoid smoking in places where you spend prolonged periods of time such as the office, home, and car.

Counsel the patients (training in problem-solving skills)

- It is important not to smoke even a single cigarette: Not even a puff is permitted after the day you start to quit.
- History of smoking cessation: Look back on what helped and interfered with smoking cessation during past attempts.
- Alcohol: Since alcohol consumption may lead to a resumption of smoking, patients should reduce or give up drinking during smoking cessation.
- Smokers in the family: Smokers in the family make smoking cessation difficult. Persuade these family members to quit smoking at the same time or not to smoke in the patient's presence.

Provide social support in medical activities

- Say, "I and my staff are always ready to help you."

Help the patients to receive social support from people other than medical professionals.

- Say, "Ask your spouse/partner, friends, and colleagues for social support."

Recommend undergoing drug therapy

- Recommend the use of drugs with established efficacy. Explain how these drugs increase the success rate of smoking cessation and alleviate withdrawal symptoms.
- Use a nicotine-replacing agent and bupropion hydrochloride SR (not approved in Japan) as the first choices.

Provide supplementary study materials

- Select study materials appropriate for the characteristics of the patient from those published by the government or NPOs.

Step 5

- Timing: The first follow-up examination should be scheduled immediately after the beginning of smoking cessation, within 1 week if possible. The second should be scheduled within 1 month. Make a schedule for subsequent follow-ups.
- What should be done in follow-up examinations: Congratulate the patient on smoking cessation. If the patient has started smoking again, study the situation, and advise them to try again. Advise the patient to regard the failure as a chance to learn for future success. Anticipate problems that have actually arisen and those expected to arise.
- Assess the use of drug therapy and its problems. Evaluate the possibility of the use of, or suggestion to use, stronger treatments.

Supplemental Table 2. Classification of Overweight and Diagnostic Criteria for Obesity

Definition of overweight

A state in which fatty tissue is excessive and BMI is ≥ 25 kg/m^2.

Classification of overweight

Overweight should be classified according to the table below based on the body mass index: BMI = body weight (kg)/[height (m)]^2.

Overweight index

BMI (kg/m^2)	Category	WHO criteria
< 18.5	Low weight	Under weight
18.5 ≤ - < 25	Normal weight	Normal range
25 ≤ - < 30	Overweight (grade I)	Pre-obese
30 ≤ - < 35	Overweight (grade II)	Obese class I
35 ≤ - < 40	Overweight (grade III)	Obese class I
40 ≤	Overweight (grade IV)	Obese class III

Note 1: It should be noted that overweight (BMI ≥ 25) is not always a state that medically requires weight loss. The standard body weight (ideal body weight) should be calculated with the following formula: standard body weight (kg) = height (m)^2 × 22. This is based on a BMI of 22, which is most unlikely to be associated with disease.

Note 2: BMI ≥ 35 should be defined as severe overweight.

Definition of obesity

Obesity is a state in which health problems have been caused by or are related to overweight, or a state in which weight loss is indicated medically because problems are anticipated, and should be treated as a disease entity.

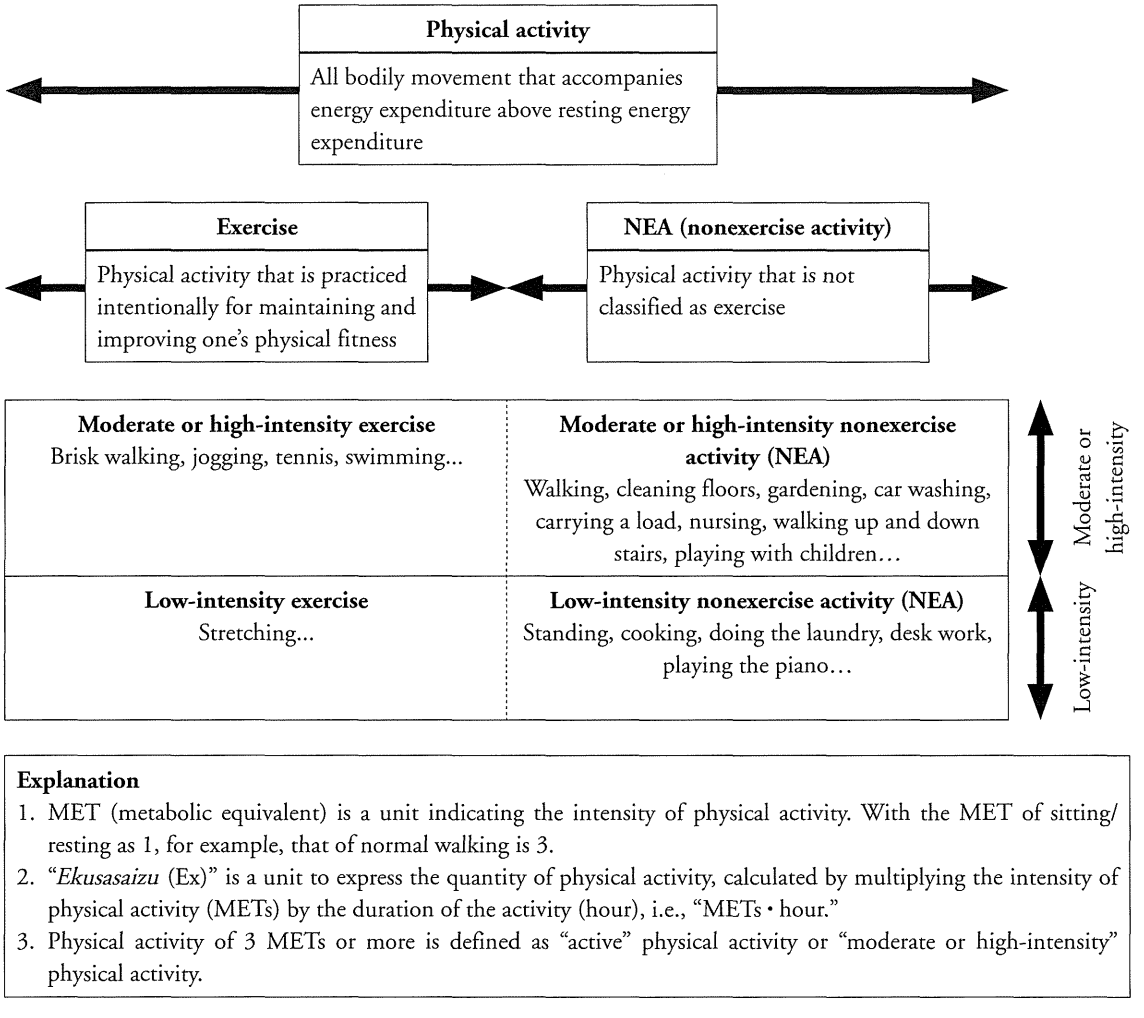
Diagnosis of obesity

The patient is overweight (BMI ≥ 25) and meets any of the following criteria:

1. The patient has health problems caused by or related to overweight and weight loss is required (the problems can be improved or progression can be prevented by weight loss).
2. There is a high risk of health problems if the patient does not lose weight.
   Visceral fat accumulation is suspected by screening of the waist circumference and visceral obesity is definitively diagnosed by abdominal CT scanning.

Japan Society for the Study of Obesity, ed.: 2011 Diagnostic Guidelines for Obesity. Study of Obesity, 2011:17 (extra edition) opening chart P1, Table A

Supplemental Table 3A.



Explanation

1. MET (metabolic equivalent) is a unit indicating the intensity of physical activity. With the MET of sitting/resting as 1, for example, that of normal walking is 3.
2. "*Ekusasaizu* (Ex)" is a unit to express the quantity of physical activity, calculated by multiplying the intensity of physical activity (METs) by the duration of the activity (hour), i.e., "METs • hour."
3. Physical activity of 3 METs or more is defined as "active" physical activity or "moderate or high-intensity" physical activity.

Adapted from the Ministry of Health, Labour and Welfare's "Exercise and Physical Activity Guide for Health"

Supplemental Table 3B. Goals for the Quantity of Physical Activity to Prevent Lifestyle-Related Diseases

Basic goal	23 Ex (METs • hour) per week by physical activity, of which 4 Ex is active exercise.
Goal to reduce visceral fat	About 10 Ex/week or more of exercise is required to ensure a reduction in visceral fat.
Specific examples of physical activity	Example 1) Physical activity corresponding to 23 Ex per week: walking 8,000 to 10,000 steps per day 7 days per week Example 2) Exercise corresponding to 4 Ex: brisk walking for 60 mins or tennis for 40 min Example 3) Exercise corresponding to 10 Ex: 30 min brisk walking 5 days per week

(Examples of exercise and physical activity corresponding to 1 Ex are shown in Supplemental Table 4)

Adapted from the Ministry of Health, Labour and Welfare's "Exercise and Physical Activity Guide for Health Promotion 2006."[123]

Supplemental Table 3C. Examples of Physical Activity Corresponding to 1 Ex

	Activities	Time(min)
Examples of exercise corresponding to 1 Ex	Bowling, volleyball, frisbee, weight lifting (light or moderate effort)	20
	Brisk walking, radio calisthenics, golf (using a power cart), table tennis, badminton, aquabics, Tai Chi	15
	Light jogging, weight lifting (vigorous effort), jazzercise, aerobics, basketball, swimming (leisurely), soccer, tennis, skiing, skating	10
	Running, swimming, judo, karate	7～8
	Walking, sweeping the floor, loading/unloading a car, childcare, car washing	20
Examples of NEA corresponding to 1 Ex	Brisk walking, cycling, nursing, gardening, walking/running - playing with child(ren), moderate intensity	15
	Mowing the lawn, walking, using a power mower; moving furniture; climbing stairs; shoveling snow by hand	10
	Carrying heavy loads	7～8

Cited from the Ministry of Health, Labour and Welfare's "Exercise and Physical Activity Guide for Health Promotion 2006."[123]

Explanation

In 2006, the preparation committee proposed the creation of an exercise reference and exercise guide for health promotion[124]. In this guide, "physical activity" is defined as "all bodily movement that accompanies energy expenditure above resting energy expenditure," and is classified into "exercise" that is practiced intentionally for maintaining and improving one's physical fitness and "nonexercise activity (NEA)" (Supplemental Table 3A). To prevent lifestyle-related diseases, walking about 8,000 to 10,000 steps per day or corresponding physical activity and moderate exercise suitable for individuals (e.g., brisk walking for 60 minutes per week, tennis for 40 minutes) is recommended (Supplemental Tables 3B and 3C). Trying to use the stairs instead of an escalator or lift is considered to be an effective way to increase muscle strength in daily living. Furthermore, exercise involving 30 minutes brisk walking 5 times a week is required to ensure a reduction in visceral fat.

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MicroRNA-33 regulates sterol regulatory element-binding protein 1 expression in mice

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MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recent reports have indicated that miR-33, which is located within the intron of sterol regulatory element-binding protein (SREBP) 2, controls cholesterol homoeostasis and may be a potential therapeutic target for the treatment of atherosclerosis. Here we show that deletion of miR-33 results in marked worsening of high-fat diet-induced obesity and liver steatosis. Using miR-33$^{-/-}$ *Srebf1*$^{+/-}$ mice, we demonstrate that SREBP-1 is a target of miR-33 and that the mechanisms leading to obesity and liver steatosis in miR-33$^{-/-}$ mice involve enhanced expression of SREBP-1. These results elucidate a novel interaction between SREBP-1 and SREBP-2 mediated by miR-33 *in vivo*.

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Sterol regulatory element-binding proteins (SREBPs) are the predominant transcription factors controlling the synthesis of cholesterol and fatty acids in the liver[1]. The family of SREBPs essentially encompasses two isoforms, SREBP-1 and SREBP-2, encoded by the corresponding genes *SREBF1* and *SREBF2* (refs 2,3). In contrast to SREBP-2, SREBP-1 is transcribed into two major splicing variants, SREBP-1a and SREBP-1c, which differ only in their first exon through the use of alternative promoters[2,3]. Although there is some functional overlap among the three SREBP isoforms[4], these proteins regulate different metabolic pathways. SREBP-2 is the master regulator of cholesterol synthesis and metabolism, whereas SREBP-1c controls fatty acid synthesis in the liver and adipose tissue[5]. In replicating tumour cell lines, SREBP-1a mostly transactivates both lipogenic and cholesterogenic genes. Although SREBP-1a and SREBP-1c share the same bHLH and regulatory domains, SREBP-1a is a stronger activator than SREBP-1c owing to a longer amino-terminal transactivation domain[6]. Therefore, SREBP-1a, -1c and -2 have specific roles in the regulation of cholesterol and fatty acids. In order to fine-tune cellular metabolism efficiently, it may be important to regulate their functions in an interdependent manner. However, limited evidence has been obtained about the potential interactions between SREBP-1 and SREBP-2 until date.

MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. miR-33 is encoded in an intron of *SREBF2*. The sequence of miR-33 is identical, and the stem-loop of the pre-miRNA is highly conserved in mammals[7–10]. Recent reports, including ours, have indicated that miR-33 controls ABCA1 expression and reduces HDL-C levels, and that miR-33 is a potential target for the treatment of atherosclerosis[11,12]. To determine the organ/cell type-specific function of miRs in the long term *in vivo*, studies on miRNA-deficient mice and analysis of specific organ/cell types from these mice are needed. Therefore, we generated miR-33-deficient mice and studied their phenotypes. We noted that miR-33-deficient mice gradually gained more weight than control mice, and the obese phenotype was evident after 26 weeks of age when receiving normal chow (NC). When we fed them a high-fat diet (HFD), miR-33-deficient mice became severely obese and suffered from liver steatosis. Microarray analysis showed that genes involved in fatty acid metabolism were upregulated in miR-33$^{-/-}$ mice fed NC before becoming obese. We searched for potential target genes of miR-33 in a public database (TargetScan, http://www.targetscan.org), and found that one of the targets of miR-33 is SREBP-1. *In vitro* experiments indicated that SREBP-1 is a likely target of miR-33. We further intercrossed miR-33$^{-/-}$ mice with *Srebf1*$^{+/-}$ mice and fed them HFD. The difference in body weight (BW) between miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice and miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice decreased and hepatic steatosis was reversed in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice under pair-feeding conditions. These data demonstrate that miR-33 targets SREBP-1 *in vivo*.

In the present study, we demonstrated that miR-33 deficiency increases SREBP-1 levels, fatty acid synthesis, and fatty acid accumulation in the liver and adipose tissue. These results indicate a novel relationship between SREBP-1 and SREBP-2 through miR-33.

Results

miR-33-KO mice become obese and develop hepatic steatosis. Twenty-six-week-old male miR-33-knockout mice weighed more than wild-type (WT) littermates after being fed NC (Fig. 1a). Up to 24 weeks of age, the BWs of the miR-33$^{-/-}$ mice and those of age- and sex-matched miR-33$^{+/+}$ control mice did not differ, but 26-week-old male miR-33$^{-/-}$ mice were 20% heavier than controls. After feeding with HFD from 8 to 20 weeks of age, miR-33$^{-/-}$ mice become markedly obese compared with controls of both genders (Fig. 1b,c). Computed tomography (CT) of 20-week-old miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed HFD showed a severe increase in body fat of miR-33$^{-/-}$ mice compared with miR-33$^{+/+}$ mice (Fig. 1d). We estimated fat weight from CT values because there is a good correlation of visceral fat weight and calculated weight from CT values (Supplementary Fig. S1a). Both visceral and subcutaneous fat weights were higher in miR-33$^{-/-}$ mice fed HFD (Supplementary Fig. S1b). Figure 2a indicates that the increased BW was caused by an increase in liver and adipose tissue weight. The livers of miR-33$^{-/-}$ mice fed HFD were severely enlarged and pale in colour (Fig. 1c). Histological examination revealed that miR-33$^{-/-}$ mice fed HFD developed severe fatty liver with the accumulation of lipid droplets (Fig. 2b). We measured total cholesterol and triglyceride levels in the liver and found that triglyceride levels were significantly increased in the liver of miR-33$^{-/-}$ mice fed HFD compared with miR-33$^{+/+}$ mice fed HFD and mice fed NC (Fig. 2c right). On the other hand, cholesterol levels in the liver were increased in mice fed HFD compared with mice fed NC and there was no difference between miR-33$^{+/+}$ and

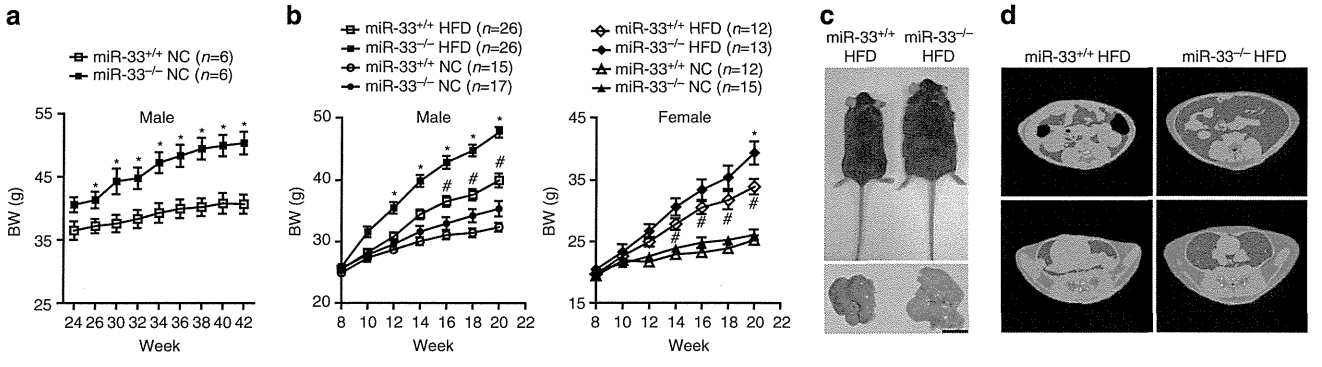

Figure 1 | miR-33$^{-/-}$ mice become obese and develop hepatic steatosis. (**a**) Development of BW of miR-33$^{+/+}$ and miR-33$^{-/-}$ male mice fed NC. *P<0.05 versus NC-fed miR-33$^{+/+}$ mice. Statistical comparisons were made by Student's *t*-test. (**b**) Development of BW of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD. *P<0.05 versus HFD-fed miR-33$^{+/+}$ mice, #P<0.05 versus NC-fed miR-33$^{+/+}$ mice. Statistical comparisons were made by one-way analysis of valiance test. (**c**) Representative image of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed with a HFD. Lower images show the livers of these mice. Scale bars, 1.0 cm. (**d**) Representative CT images of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed HFD. Values are the means ± s.e.m.

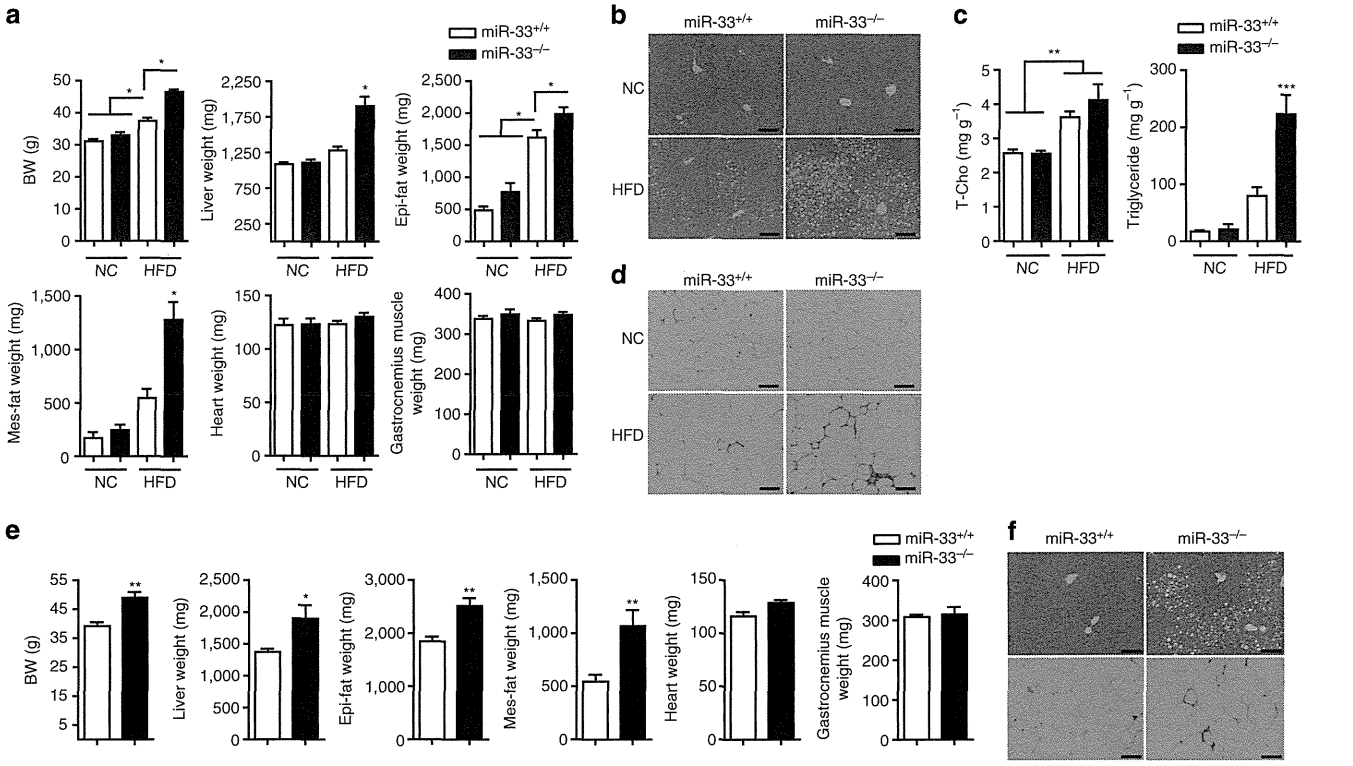

Figure 2 | Pathophysiological features of miR-33$^{-/-}$ mice. (**a**) Body, liver, adipose tissue (epi-fat; epididymal fat, mes-fat; mesenteric fat), heart and muscle weights of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD ($n=12$–13 for NC, $n=21$ for HFD each. *$P<0.05$ in one-way analysis of valiance test). (**b**) Representative microscopic images of the livers of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD. Scale bars, 200 μm. (**c**) Cholesterol and triglyceride levels in the livers of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD ($n=5$ each. **$P<0.01$, ***$P<0.001$ in one-way analysis of valiance test). (**d**) Representative microscopic images of the adipose tissue (epididymal fat) of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD. Scale bars, 200 μm. (**e**) Body, liver, adipose tissue, heart and muscle weights of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at the age of 50 weeks ($n=6$ each, *$P<0.05$, **$P<0.01$ in Student's *t*-test). (**f**) Representative microscopic images of the liver and the adipose tissue of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at the age of 50 weeks. Scale bars, 200 μm. Values are the means ± s.e.m.

miR-33$^{-/-}$ mice (Fig. 2c left). Figure 2d shows the increase in adipocyte size with the accumulation of infiltrated cells in white adipose tissue in miR-33$^{-/-}$ mice fed HFD. It is of note that the same phenotypes as those of miR-33$^{-/-}$ mice fed HFD were also observed in miR-33$^{-/-}$ mice fed NC at 50 weeks of age (Fig. 2e,f). Thus, genetic ablation of miR-33 induces obesity and hepatic steatosis.

miR-33-KO mice have abnormal glucose and insulin tolerance. miR-33$^{-/-}$ mice fed HFD from 8 weeks to 20 weeks of age showed higher fasting glucose levels and severely impaired glucose tolerance at 20 weeks (Fig. 3a,b).

However, miR-33$^{-/-}$ mice fed NC showed the same glucose levels as miR-33$^{+/+}$ mice at this age. Baseline glucose levels of NC-fed miR-33$^{+/+}$ mice, NC-fed miR-33$^{-/-}$ mice, HFD-fed miR-33$^{+/+}$ mice and HFD-fed miR-33$^{-/-}$ mice were 110.5 ± 8.3, 122 ± 2.5, 120.5 ± 5.6 and 155.6 ± 6.7 mg dl^{-1}, respectively (All values represent mean ± s.e.m.). Impaired insulin tolerance was observed only in miR-33$^{-/-}$ mice fed HFD (Fig. 3c–f). Insulin levels in intraperitoneal glucose tolerance test (IPGTT) were significantly elevated in miR-33$^{-/-}$ mice fed HFD (Fig. 3g,h). Plasma leptin levels were also elevated in miR-33$^{-/-}$ mice fed HFD (Fig. 3i). Impaired glucose tolerance and insulin tolerance were also evident at the age of 50 weeks even in mice fed NC (Fig. 3j–m).

Serum levels of ALP, T-cho and HDL-C were elevated in miR-33$^{-/-}$ mice compared with that in WT mice at the age of 20 weeks, as indicated in our previous report (Table 1)[10]. When these mice were fed HFD from 8 to 20 weeks of age, increases in serum levels of AST, ALT, NEFA and LDL-C became evident (Table 1). Similar elevation of T-cho was observed in miR-33$^{-/-}$ mice fed NC at the age of 50 weeks compared with controls (Supplementary Table S2).

miR-33-KO mice find HFD more palatable. Food intake, as analysed by housing in metabolic cages, was higher in miR-33$^{-/-}$ mice fed HFD than that in their control counterparts (Fig. 4a). The difference in food intake was only observed when they were fed with HFD (Supplementary Fig. S2a, b), which suggests that miR-33$^{-/-}$ mice find HFD more palatable. These mice showed similar body temperatures (37.38 °C versus 37.27 °C) and O_2 consumption rate or activity did not differ between these strains during the day or night at the age of 16 weeks when fed NC (Fig. 4c–f). Moreover, urinary excretion of adrenaline, noradrenaline and dopamine were also the same between these strains at the same age (Fig. 4g).

miR-33 regulates SREBP-1 expression *in vivo*. In order to determine the cause of the phenotypic changes observed in miR-33$^{-/-}$ mice fed HFD or in older miR-33$^{-/-}$ mice, we analysed the gene expression profiles by microarray analysis using the livers of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at the age of 16 weeks when their weights were the same. The pathways altered in the livers of miR-33$^{-/-}$ mice were determined by

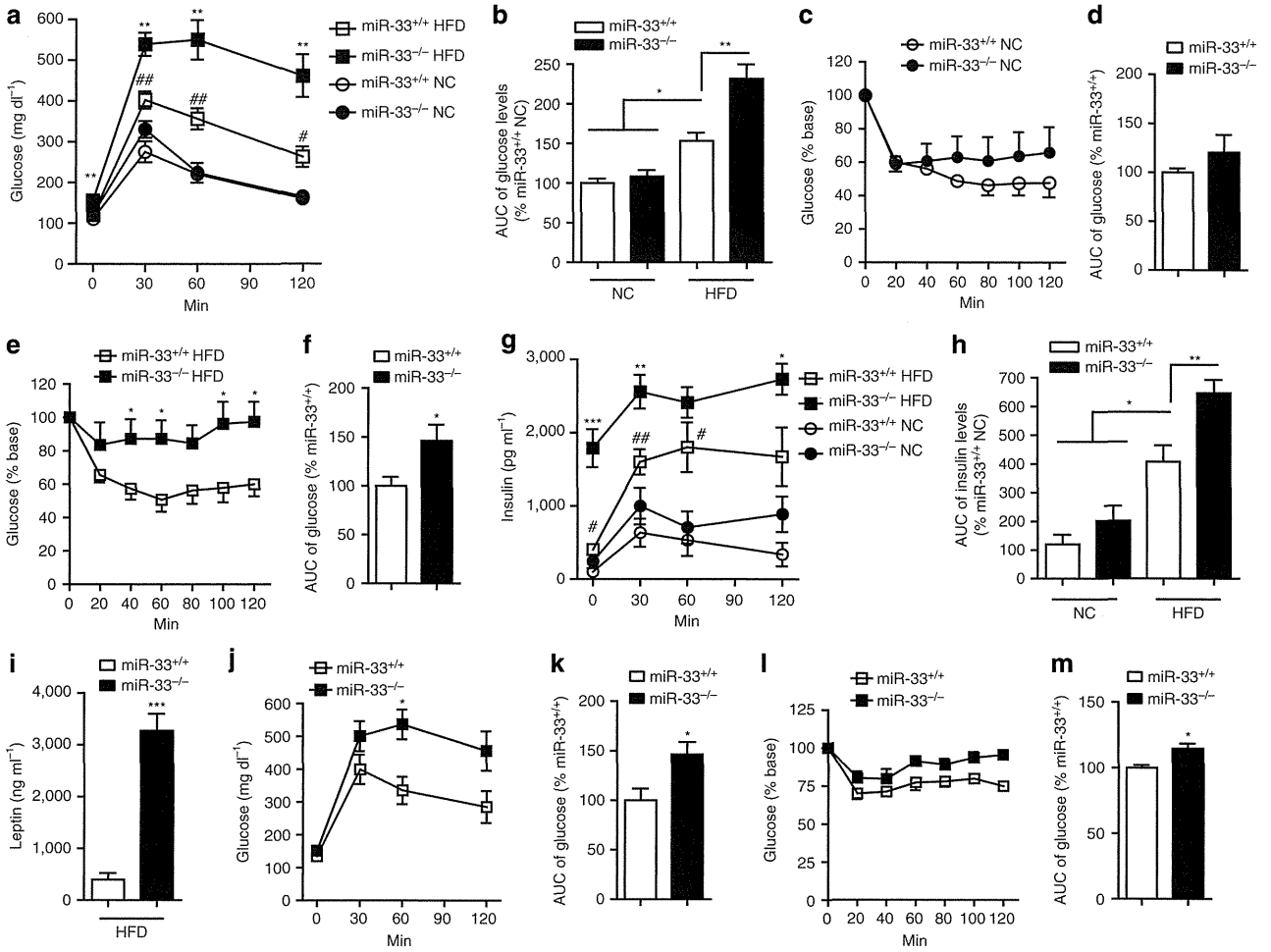

Figure 3 | Analysis of glucose and insulin tolerance. (**a**,**b**) Serial changes in glucose levels (**a**) and area under curve (AUC) of glucose levels (**b**) after intraperitoneal injection of glucose in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD ($n=6$ for NC, $n=11$–12 for HFD each. $^{*}P<0.05$ versus mice fed NC. $^{**}P<0.01$ versus miR-33$^{+/+}$ mice fed HFD. $^{\#}P<0.05$ versus miR-33$^{+/+}$ mice fed NC, $^{\#\#}P<0.01$ versus miR-33$^{+/+}$ mice fed NC in one-way analysis of valiance test). (**c**,**d**) Serial changes in glucose levels (**c**) and AUC of glucose levels (**d**) after intraperitoneal injection of insulin in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC ($n=5$ each). (**e**,**f**) Serial changes in glucose levels (**e**) and AUC of glucose levels (**f**) after intraperitoneal injection of insulin in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed HFD ($n=9$ each, $^{*}P<0.05$ in Student's *t*-test). (**g**) Serial changes in insulin levels after intraperitoneal injection of glucose in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD ($n=6$ for NC, $n=11$–12 for HFD each. $^{*}P<0.05$ versus miR-33$^{+/+}$ mice fed HFD, $^{**}P<0.01$ versus miR-33$^{+/+}$ mice fed HFD, $^{***}P<0.001$ versus miR-33$^{+/+}$ mice fed HFD, $^{\#}P<0.05$ versus miR-33$^{+/+}$ mice fed NC, $^{\#\#}P<0.01$ versus miR-33$^{+/+}$ mice fed NC in one-way analysis of valiance test). (**h**) AUC of insulin levels after intraperitoneal injection of glucose in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed or not fed HFD ($n=6$ for NC, $n=11$–12 for HFD each, $^{*}P<0.05$, $^{**}P<0.01$ in one-way analysis of valiance test). (**i**) Serum leptin levels in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed HFD ($n=10$ for each, $^{***}P<0.001$ in Student's *t*-test). (**j**,**k**) Serial changes in glucose levels (**j**) and AUC of glucose levels (**k**) after intraperitoneal injection of glucose in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at the age of 50 weeks ($n=6$ each, $^{*}P<0.05$ in Student's *t*-test). (**l**,**m**) Serial changes in glucose levels (**l**) and AUC of glucose levels (**m**) after intraperitoneal injection of insulin in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at the age of 50 weeks ($n=6$ each, $^{*}P<0.05$ in Student's *t*-test). Values are the means ± s.e.m.

Table 1 | Serum profile of miR-33$^{+/+}$ and miR-33$^{-/-}$ on NC or HFD.

	miR-33$^{+/+}$ NC ($n=8$)	miR-33$^{-/-}$ NC ($n=8$)	miR-33$^{+/+}$ HFD ($n=5$)	miR-33$^{-/-}$ HFD ($n=5$)
AST (IU l^{-1})	67.63 ± 5.43	56.50 ± 11.89	53.80 ± 2.89	134.40 ± 18.48**
ALT (IU l^{-1})	39.38 ± 6.02	41.00 ± 11.95	31.40 ± 3.92	167.40 ± 29.15**
ALP (IU l^{-1})	186.88 ± 15.29	238.13 ± 14.57*	138.80 ± 9.83	210.60 ± 20.79*
T-CHO (mg dl^{-1})	85.75 ± 3.83	110.25 ± 6.16**	157.40 ± 13.25	244.8 ± 21.42**
TG (mg dl^{-1})	35.63 ± 4.39	34.50 ± 4.82	21.80 ± 2.33	17.60 ± 3.72
NEFA (μEq l^{-1})	766.8 ± 30.89	867.1 ± 53.68	778 ± 61.21	979 ± 55.40*
LDL-C (mg dl^{-1})	5.50 ± 0.63	6.88 ± 0.72	11.40 ± 0.51	26.00 ± 5.30*
HDL-C (mg dl^{-1})	52.38 ± 2.08	66.13 ± 2.72**	79.00 ± 4.71	83.60 ± 1.25

Values are the means ± s.e.m. Statistical comparisons were made by Student's *t*-test ($^{*}P<0.05$, $^{**}P<0.01$).

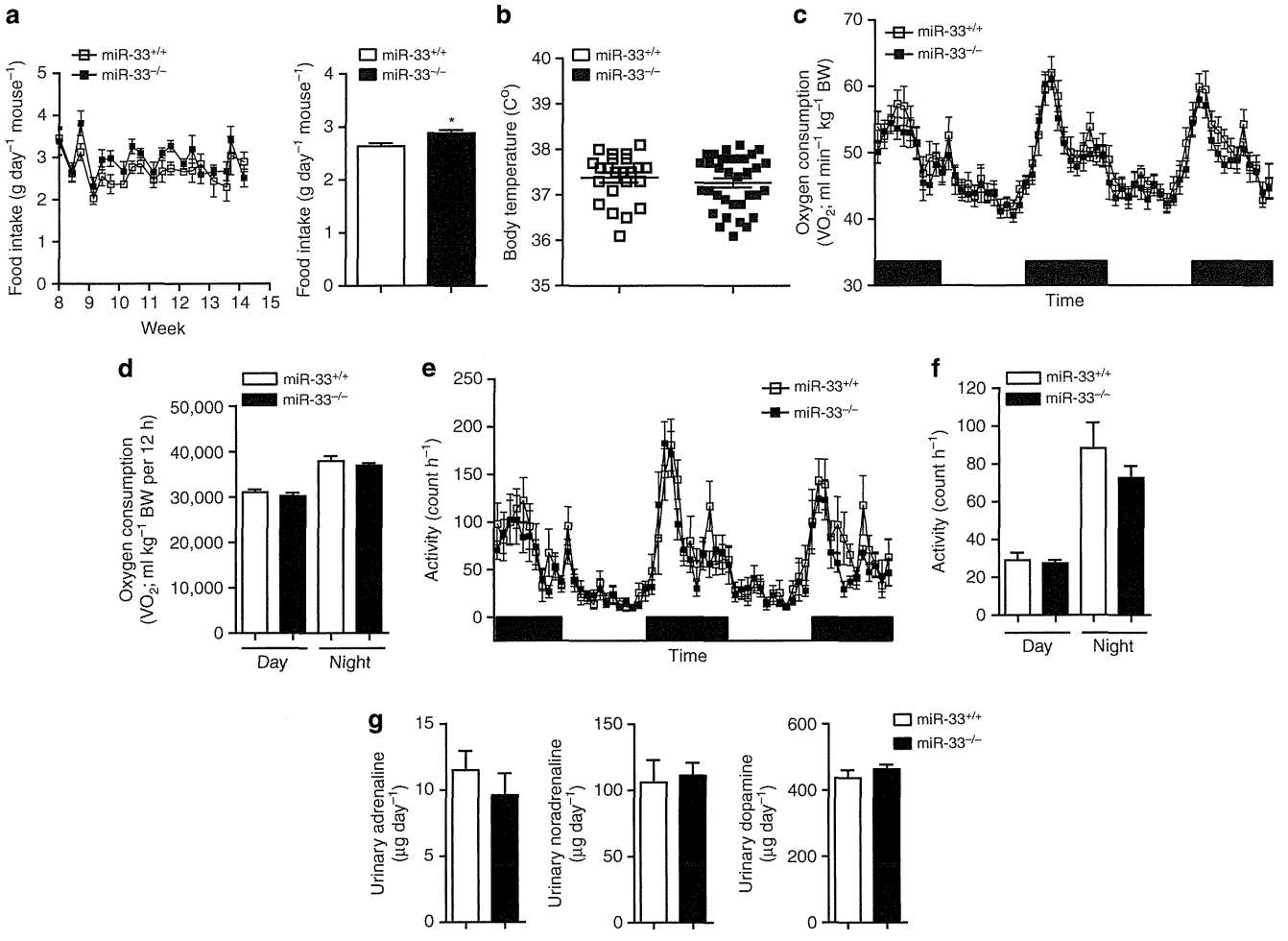

Figure 4 | Analysis of energy balance. (**a**) Serial changes in food intake of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed HFD in metabolic cages ($n=5$–6 each, *$P<0.05$ in Student's *t*-test). (**b**) Body temperature of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice at 16 weeks of age ($n=23$, 34 each). (**c**) Oxygen consumption rate of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at 16 weeks of age ($n=8$ each). (**d**) Oxygen consumption during 12 h by miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at 16 weeks of age ($n=8$ each). (**e**) Serial changes in activity of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at 16 weeks of age ($n=8$ each). (**f**) Day and night activity of miR-33$^{+/+}$ and miR-33$^{-/-}$ mice fed NC at 16 weeks of age ($n=8$ each). (**g**) Urinary secretion of adrenaline, noradrenaline and dopamine ($n=3$–4 each). Values are the means ± s.e.m.

GenMAPP analysis (http://www.genmapp.org/about.html). Most strikingly, the fatty acid metabolism pathway showed the highest Z score (Supplementary Table S3). We picked up genes related to fatty acid metabolism and validated their expression levels in the liver by quantitative RT–PCR (PCR with reverse transcription). Interestingly, significant differences were observed in the expression levels of several lipogenic genes including *Srebf1, Pparg* and its downstream genes (Fig. 5a). We also measured *de novo* hepatic fatty acid synthesis rate, as previously described[13,14]. It was increased significantly in the miR-33$^{-/-}$ mice compared with that of the miR-33$^{+/+}$ mice (Supplementary Fig. S3a). The *Srebf1* 3′UTR has a potential binding site for miR-33 in many species (TargetScan; http://www.targetscan.org; Fig. 5b). Overexpression of miR-33 reduced the luciferase activity of a reporter gene fused with *Srebf1* 3′UTR sequences from humans and mice (Fig. 5c). Moreover, miR-33 decreased luciferase activity dose-dependently, whereas miR-146a, which has no binding site in the *Srebf1* 3′UTR, could not (Fig. 5d). Mutation in this binding site abolished the reduction of luciferase activity in 293T cells (Fig. 5e). The same results were also obtained in COS-7 cells (Supplementary Fig. S3b, c). We also measured the activity of SREBP-1 by sterol regulatory element (*SRE*) and fatty acid synthase (*FAS*) promoter reporter analysis by the use of *Srebf1* with or without the 3′UTR. Luciferase activity of the *SRE* and *FAS* reporter genes was significantly reduced by miR-33 expression when *Srebf1* with the 3′UTR was present. This reduction was not observed in the experiments conducted with *Srebf1* without the 3′UTR (Fig. 5f,g). Overexpression of miR-33 reduced protein levels of SREBP-1 and ABCA1 but not of IRS-2 in HepG2 cells (Fig. 5h and Supplementary Fig. S4a). The decrease in *SREBF1* expression was mainly caused by reduction in *SREBF1c* (Supplementary Fig. S4b). Overexpression of miR-33 also reduced the protein levels of SREBP-1 and ABCA1 but not of IRS-2 in miR-33$^{+/+}$ primary hepatocytes (Fig. 5h and Supplementary Fig. S4c). It was confirmed that miR-33$^{-/-}$ mice had higher protein expression levels of SREBP-1 and ABCA1 but not of IRS-2 (Fig. 5h and Supplementary Fig. S4d). We measured the expression levels of lipogenic genes in the primary hepatocyte transduced with miR-33 or the control. As shown in Supplementary Fig. S4e, expression levels of *Srebf1*, *Abca1* and several lipogenic genes were downregulated. Moreover, *Srebf1*, *Abca1* and several lipogenic genes were upregulated in miR-33$^{-/-}$ primary hepatocytes compared with miR-33$^{+/+}$ primary hepatocytes (Supplementary Fig. S4f). SREBP-1 levels were further enhanced in miR-33$^{-/-}$ mice fed HFD (Supplementary Fig. S5a). We also measured the levels of

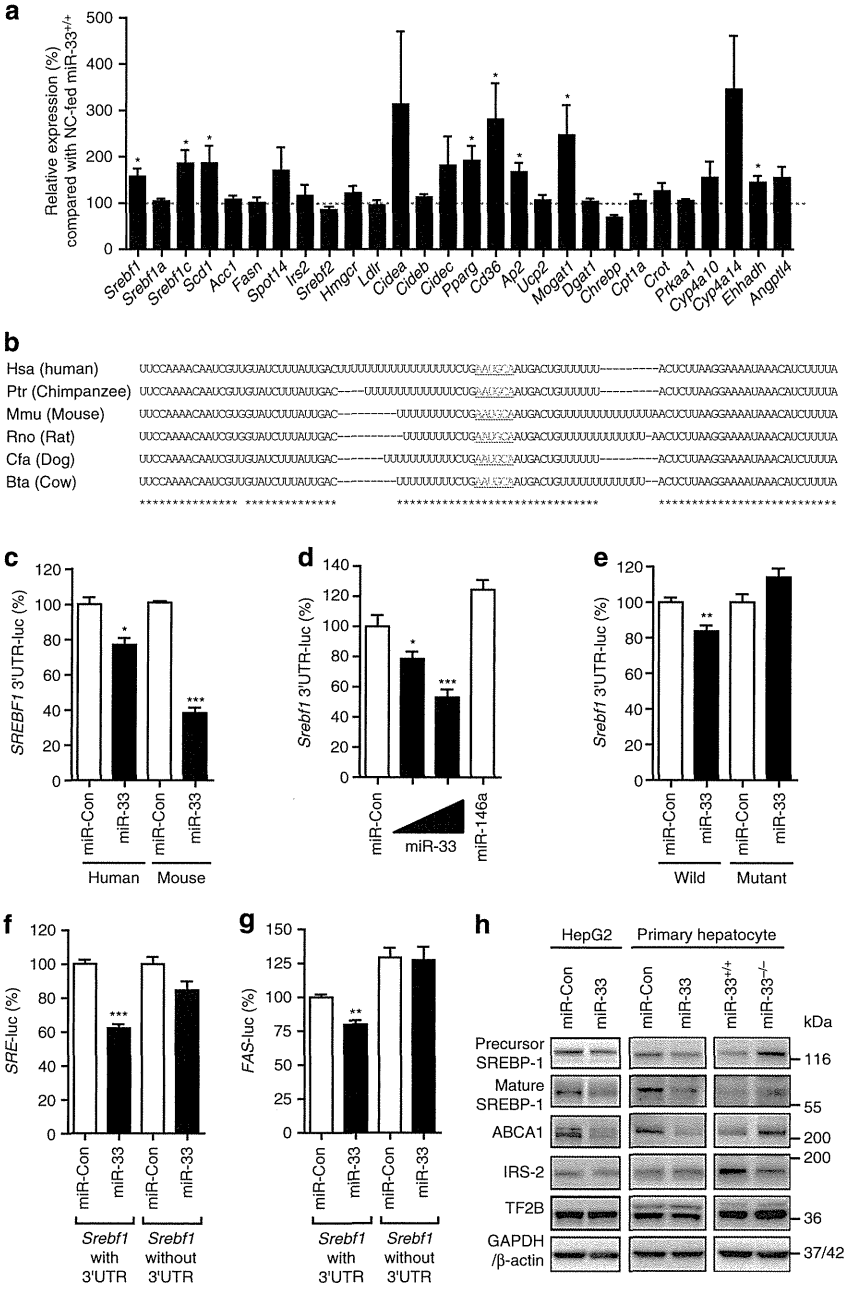

Figure 5 | *Srebf1* is a miR-33 target gene. (**a**) Relative changes in lipid metabolism-related genes in the livers of miR-33$^{-/-}$ mice compared with miR-33$^{+/+}$ mice fed NC at 16 weeks of age. ($n=5$–8 each,*$P<0.05$ in Student's *t*-test). (**b**) Conservation of miR-33 target regions in the 3′UTR of *Srebf1*. Underlined sequences are the potential binding site of miR-33 seed sequences. * indicates the conservation among spieces. (**c**) 3′UTR reporter assay used to verify the target. Luciferase reporter activity of human and mouse SREBP-1 gene 3′UTR constructs in 293T cells overexpressing miR-control (miR-Con) and miR-33 ($n=4$ each, *$P<0.05$ and ***$P<0.001$ in Student's *t*-test). (**d**) miR-33 dose-dependent changes in luciferase reporter activity of mouse *Srebf1* 3′UTR construct in 293T cells. miR-Con and miR-146a is used as a negative control ($n=4$ each, *$P<0.05$ and ***$P<0.001$ in one-way analysis of valiance test). (**e**) Luciferase reporter activity of the WT or mutant *Srebf1* 3′UTR at the potential miR-33 binding site in 293T cells ($n=4$ each, **$P<0.01$ in Student's *t*-test). (**f**,**g**) Luciferase reporter activity of *SRE*-promoter (**f**) or *FAS*-promoter (**g**) in 293T cells. 293T cells were co-transfected with mouse *Srebf1* with the full-length 3′UTR or without the 3′UTR, along with expression plasmids for miR-negative control, or miR-33. Values are the mean ± s.e. ($n=4$ each, **$P<0.01$ versus miR-Con. ***$P<0.001$ versus miR-Con in one-way analysis of valiance test). (**h**) Western blotting analysis of SREBP-1, ABCA1, and IRS-2 in miR-33 transduced HepG2 cells and primary hepatocytes and hepatocytes prepared from miR-33$^{+/+}$ and miR-33$^{-/-}$ mice. Representative western blot images are shown ($n=4$). Values are the means ± s.e.m.

AMPKα, previously described as a potential miR-33 target, but we could not detect any difference between miR-33$^{+/+}$ and miR-33$^{-/-}$ mice (Supplementary Fig. S5b). We further checked whether PPAR-γ is regulated by miR-33 in primary hepatocytes. Overexpression of miR-33 did not change the protein expression level of PPAR-γ after transduction in HepG2 cells (Supplementary Fig. S5c) and primary hepatocytes (Supplementary Fig. S5d), and found non-enhancement in miR-33$^{-/-}$ mice (Supplementary Fig. S5d). Moreover, we conducted peroxisome proliferator-activated receptor response

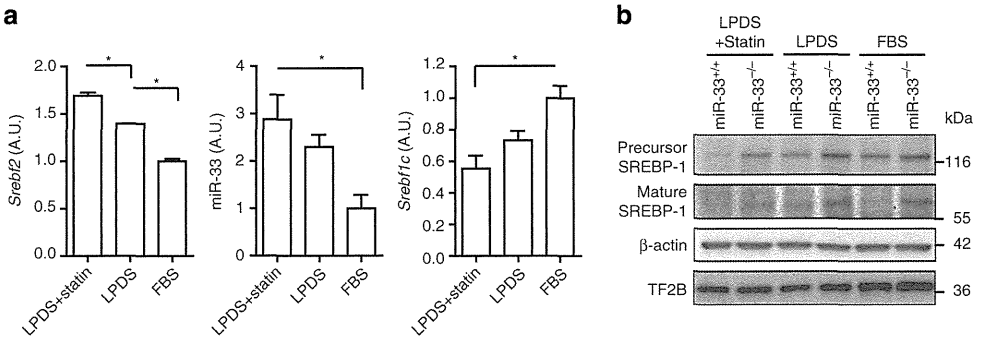

Figure 6 | SREBP-1 is regulated by endogenous changes in miR-33 *in vitro*. (**a**) RNA expression levels in *Srebf2*, miR-33 and *Srebf1c* in primary hepatocytes cultured in DMEM supplemented with 5% FBS or 5% LPDS with or without statin treatment. Values are the mean ± s.e.m. ($n=3$ each for *Srebf2* and *Srebf1c*, $n=4$–6 each for miR-33, $^*P<0.05$ in one-way analysis of valiance test). (**b**) Protein levels of SREBP-1 in primary hepatocytes cultured in DMEM supplemented with 5% FBS or 5% LPDS with or without statin treatment. Representative western blot images are shown ($n=4$).

elements (*PPRE*)-driven luciferase assay in HepG2 cells. No difference was observed in *PPRE* activity in control and miR-33 transduced cells with or without PPAR ligand pioglitazone (Supplementary Fig. S5e). Therefore, these results indicate that *Pparg* is not a direct target of miR-33.

SREBP-1 is regulated by endogenous changes in miR-33 *in vitro*. We further attempted to confirm whether the expression of SREBP-1 was affected by endogenous changes in miR-33 expression via modulating the cellular cholesterol level in primary hepatocytes. When the cells were depleted of sterols by prior incubation in medium containing lipoprotein-deficient serum (LPDS) with or without pitavastatin, mRNA levels of *Srebf2* and miR-33 were significantly increased in parallel (Fig. 6a). In this situation, *Srebf1c* and protein levels of SREBP-1 were decreased in miR-33$^{+/+}$ primary hepatocytes, whereas they were still elevated in miR-33$^{-/-}$ hepatocytes (Fig. 6b). There is a potential binding site of miR-33 in the 3′UTR of human *SCAP*. However, it is not conserved in mice and mRNA and protein levels of SCAP are the same in miR-33$^{+/+}$ and miR-33$^{-/-}$ mice (Supplementary Fig. S6a–c). Thus, the levels of precursor and mature forms of SREBP-1 are regulated in parallel.

Reduction of SREBP-1 reverses fatty liver in miR-33-KO mice. To elucidate the role of SREBP-1 in the phenotypic changes in miR-33$^{-/-}$ mice fed HFD, we generated miR-33$^{-/-}$ mice that have SREBP-1 expression levels similar to WT mice. As shown in Fig. 7a, protein levels of SREBP-1 are the same in miR-33$^{-/-}$ *Srebf1*$^{+/-}$ and miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice. Aberrant bands of SREBP-1 were observed in *Srebf1*-deficient mice (Fig. 7a and Supplementary Fig. S7a)[15]. mRNA levels of *Srebf1* in these mice are shown in Fig. 8a. Because miR-33$^{-/-}$ mice showed positive palatability for HFD compared with miR-33$^{+/+}$ mice (Fig. 4a), we analysed these four groups of mice under pair-feeding conditions. miR-33$^{+/+}$*Srebf1*$^{+/-}$, miR-33$^{-/-}$*Srebf1*$^{+/+}$ and miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice received HFD in amounts that matched the rate of food intake of miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice. As shown in Fig. 7b's left panel, miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice gained significantly more weight than miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice under these conditions. Therefore, the BW gain in miR-33$^{-/-}$ mice compared with miR-33$^{+/+}$ mice is not caused by a change in food intake. The BW increase caused by miR-33 deficiency was abolished in a *Srebf1*$^{+/-}$ background (Fig. 7b right and Supplementary Fig. S7b). On the other hand, glucose tolerance was ameliorated in miR-33$^{-/-}$*Srebf1*$^{+/-}$ compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice (Fig. 7c,d). There was no difference in BW or glucose tolerance among miR-33$^{+/+}$*Srebf1*$^{+/+}$, miR-33$^{+/+}$*Srebf1*$^{+/-}$ and miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice (Fig. 7b–d). Serum insulin levels were reduced in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice (Supplementary Fig. S7c, d). A striking difference was observed in the liver. Hepatic steatosis was reversed in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with that in miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice in both macro- and microscopic images and the liver triglyceride content of these mice was almost the same as that of miR-33$^{+/+}$*Srebf1*$^{+/+}$ and miR-33$^{+/+}$*Srebf1*$^{+/-}$ mice (Supplementary Fig. S7b and Fig. 7e,f). As shown in Fig. 7g, adipocyte size was partially reduced in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice and there were still many infiltrating cells in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice. Serum leptin levels also reduced to baseline levels in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice (Fig. 7h). These results indicate that obesity and hepatic steatosis were ameliorated in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice. There was no difference in the mRNA and protein levels of AMPKα and SIRT6, which are negative regulators of SREBP-1 and potential targets of miR-33, as shown in previous reports (Fig. 8c and Supplementary Fig. S7e). Finally, we examined the lipogenic gene profiles in the liver of these mice. As shown in Fig. 8a, the expression levels of *Srebf1* were significantly increased in miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice compared with miR-33$^{+/+}$*Srebf1*$^{+/+}$ mice, and this was reversed in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice. The same pattern was observed in *Scd1*. Although they were not statistically significant, the expression levels of *Fasn*, *Acc1* and *Pparg* were reduced in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice compared with miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice (Fig. 8a,b). Serum data for these mice are summarized in Supplementary Table S4. Serum ALP levels were significantly elevated in miR-33$^{-/-}$*Srebf1*$^{+/+}$ mice, which was reversed in miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice. *Srebf1* and other lipogenic genes were also increased in adipose tissue of miR-33$^{-/-}$ mice compared with that of miR-33$^{+/+}$ mice (Supplementary Fig. S8).

Discussion

In the current study, we showed that obesity and hepatic steatosis are observed in miR-33-deficient mice at the age of 50 weeks or when fed HFD for 12 weeks. We demonstrated that miR-33 targets SREBP-1, and miR-33$^{-/-}$ mice had an enhanced expression of SREBP-1 in the liver. Study of miR-33$^{-/-}$*Srebf1*$^{+/-}$ mice clearly showed that enhanced expression of SREBP-1 caused obesity and fatty liver in miR-33$^{-/-}$ mice