Circulating miR-130b mediates metabolic crosstalk between fat and muscle in overweight/obesity

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Abstract

Aims/hypothesis Adipose tissue is a dynamic endocrine organ that regulates whole-body energy homeostasis through the secretion of signalling molecules. Recent reports suggest that secreted microRNAs (miRNAs) may function as biologically active molecules for intercellular communication. This study aims to identify obesity-related circulating miRNA that could be secreted from adipocytes and to explore its possible role in the pathogenesis of metabolic diseases.

Methods Real-time RT-PCR was used to evaluate the circulating level of miR-130b in mouse models of obesity as well as in humans. Luciferase assay and immunoblotting were used to verify the miRNA target. The effect of miR-130b on mouse peroxisome proliferator-activated receptor γ coactivator-1 α was also investigated by electroporation.

Results The circulating level of miR-130b was elevated in mouse models of obesity as well as in obese Chinese individuals.

More interestingly, the circulating level of miR-130b was positively correlated with BMI. Moreover, circulating miR-130b was a better predictor of the metabolic syndrome than was triacylglycerol level. Mechanistically, adipocytes secreted miR-130b during adipogenesis. TGF- β , which is proportionately increased with obesity, stimulated miR-130b secretion from adipocytes. Furthermore, miR-130b was able to target muscle cells and reduce the expression of its direct target gene, *PGC-1 α* (also known as *PPARGC1A*), which plays a key role in lipid oxidation in muscle.

Conclusions/interpretation Circulating miR-130b reflects the degree of obesity and could serve as a potential biomarker for hypertriacylglycerolaemia and metabolic syndrome. Circulating miR-130b could function as a metabolic mediator for adipose–muscle crosstalk and might be involved in the pathogenesis of obesity-associated metabolic diseases.

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Keywords Circulating · MiR-130b · Obesity · PGC-1 α · TGF- β

Abbreviations

BF%-BIA	Body fat percentage estimated by bioelectrical impedance analysis
CPT1B	Carnitine palmitoyltransferase 1b
DIO	Diet-induced obese
EWAT	Epididymal WAT
GAS	Gastrocnemius
HFD	High-fat diet
miRNA	microRNA
MRE	miRNA regulatory element
nSMase	Neutral sphingomyelinase 2
PGC-1 α	Peroxisome proliferator-activated receptor γ coactivator-1 α
ROC	Receiver operating characteristic
siRNA	Small interfering RNA
SOL	Soleus
WAT	White adipose tissue

Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation due to augmented number and size of adipocytes in fat depots. Adipose tissue dysfunction is known to be an important factor in the pathogenesis of type 2 diabetes and other metabolic diseases. In obesity, adipocytes undergo hypertrophy, which leads to adipokine secretion. An increasing body of evidence indicates that dysregulated production of the adipokines participates in the pathogenesis of obesity-associated metabolic disorders [1]. Nowadays, adipose tissue is considered to be a dynamic endocrine organ that regulates nutritional balance and energy homeostasis through crosstalk with other metabolic organs. For example, adipose tissue releases endocrine and metabolic mediators, including TNF- α , IL-6, adiponectin and leptin, to affect insulin signalling and energy metabolism in skeletal muscle.

TGF- β , a key cytokine, regulates adipose tissue metabolism and acts as an adipokine in the pathogenesis of many diseases [2]. Systemic blockade of TGF- β signalling protects mice from obesity, diabetes and hepatic steatosis [3], suggesting that the TGF- β signalling pathway plays an important role in regulating whole-body homeostasis. In humans, circulating TGF- β levels are proportionately increased with obesity in overweight and obese individuals compared with normal-weight individuals [4, 5]. In addition, circulating TGF- β levels, as well as protein levels of TGF- β , in adipocytes are elevated in leptin-deficient (*ob/ob*) mice and diet-induced obese (DIO) mice [6]. However, the mechanisms underlying the action of TGF- β in the pathogenesis

of obesity and obesity-associated metabolic diseases remain largely unknown.

Growing evidence indicates that microRNAs (miRNAs) are key regulators of numerous physiological and pathological processes [7]. During the past several years, studies have demonstrated that miRNAs also exist in various body fluids such as serum, plasma, saliva, urine and milk [8–10]. Altered profiles of circulating miRNAs have been shown to be associated with various diseases, including tissue injury, cancers and diabetes. Consequently, circulating miRNAs have become promising biomarkers for assessing pathophysiological status and monitoring the effectiveness of clinical treatment. More interestingly, it has recently been reported that secreted miRNA could target neighbouring cells or organs and perform a regulatory function in a new location [11, 12]. Although miRNA has been discovered in adipocyte-derived microvesicles [13], and the miRNA expression has been profiled in adipose tissue [14, 15] and the circulation [16, 17] in obese animal models as well as in humans, it remains unclear whether adipocyte-secreted miRNAs are involved in the pathogenesis of obesity-related diseases. Therefore, further investigation is required to determine whether the abnormal circulating miRNA levels could have pathogenic effects.

The objective of this study was to identify a potential miRNA biomarker for obesity and investigate its relationship with obesity-related metabolic diseases and the molecular mechanism involved. Based on our results, we propose that circulating miR-130b could be an early obesity biomarker for clinical investigation and might mediate a metabolic crosstalk between fat and muscle in obesity. Our findings presented here help us to further understand the molecular mechanisms that link obesity and obesity-related metabolic diseases and provide us with new therapeutic targets for defeating obesity-related diseases.

Methods

Reagents, cell culture and differentiation Adipogenesis of 3T3-L1 pre-adipocytes was induced as described previously with modification [18]. Briefly, on the third day after reaching confluence, 3T3-L1 cells were incubated with 10% FBS, methylisobutylxanthine (115 μ g/ml), insulin (1 μ g/ml) and dexamethasone (390 ng/ml) for 2 days. Afterwards, cells were maintained in medium containing 10% FBS and insulin (1 μ g/ml) for up to 6 days. The culture medium of adipocytes was collected for RNA extraction. Adipocytes were starved of serum for 4 h and then stimulated with 200 nmol/l insulin for 12 h. Myogenesis of C2C12 myoblasts was induced as described previously [19]. Cells were incubated with 5 ng/ml TGF- β (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or as indicated, 50 ng/ml TNF- α (gift from Yingying Le, Shanghai Institutes for Biological Sciences, Chinese Academy of

Sciences, China) and 1 $\mu\text{mol/l}$ rosiglitazone (Cayman Chemical, Ann Arbor, MI, USA) for 12 h. Small interfering RNA (siRNA) oligos and miR-130b mimics were purchased from GenePharma (Shanghai, China).

Mouse and human studies Mice were maintained and experiments were performed according to the protocol approved by the Animal Care and Use Committees of Institute for Nutritional Sciences (permit No.: 2011-AN-14). ‘Principles of laboratory animal care’ were followed, as well as specific national laws where applicable. Male *ob/ob* mice, 6–8 weeks old, provided by the Model Animal Research Center of Nanjing University (Nanjing, China), on a C57BL/6 genetic background, were used. To obtain DIO mice, C57BL/6 mice were fed a high-fat diet (HFD; 45% calories from fat) for 4 weeks. Ten- to thirteen-week-old male mice were used in this study. Fat pads were harvested, frozen in liquid nitrogen and stored at -80°C . Blood samples were centrifuged to prepare serum and stored at -80°C .

For the human study, only men were recruited and the study was reviewed and approved by the Ethics Committee of Shanghai Xuhui Central Hospital (Shanghai, China). All participants gave written informed consent. The fasting serum samples were collected at Shanghai Xuhui Central Hospital. The percentage of body fat was estimated by using TANITA-TA-410 body composition analyser (Tanita, Tokyo, Japan). Lipid and glucose measurements were automated by using Siemens Advia 2400 Clinical Chemistry System (Siemens, Erlangen, Germany). Blood pressure was measured by using an Omron blood pressure monitor (Omron, Tokyo, Japan). Human insulin level was measured by using a human INS ELISA Kit (R&D Systems, Minneapolis, MN, USA). The WHO recommends using BMI cut points to define obesity and overweight in adults. In China, BMIs of 24 kg/m^2 and 28 kg/m^2 are considered to be the best cut points to classify normal weight, overweight and obesity [20].

RNA isolation, quantitative RT-PCR analysis and western blot analysis The miRNAs were extracted from serum and cell culture medium using the miRcute miRNA isolation kit (Tiangen, Shanghai, China). Total RNA was reverse-transcribed by using the PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan). Small RNA was reverse-transcribed by using stem-loop RT, which was performed according to the protocols [21]. Real-time PCR was performed on an ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The expression levels of miRNAs in the cells, tissues, or culture medium were normalised to U6 level. The level of circulating miRNA was normalised to miR-223 level. Western blot analysis was performed as described previously, with modification [18]. Briefly, cultured cells were homogenised in RIPA lysis buffer (50 mmol/l Tris HCl, pH 7.5, 150 mmol/l NaCl, 1.0 mmol/l EDTA, 0.1% wt/vol.

SDS, 1% wt/vol. sodium deoxycholate and 1% wt/vol. Triton X-100), which was supplemented with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Sigma, St Louis, MO, USA) immediately before use. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher, Waltham, MA, USA). Protein lysates were resolved on 10% SDS-PAGE gels using standard procedures. Anti-PGC-1 α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (Kang Chen, Shanghai, China) were used for western blot analysis.

Plasmid and RNA oligonucleotide Primers used in the current study are listed in electronic supplementary material (ESM) Table 1. For construction of the luciferase reporter plasmid, the 3'UTR of *PGC-1 α* (also known as *PPARGC1A*) was amplified from human cDNA by PCR, and inserted into the pRL-TK vector. A mutant derivative (*PGC-1 α* 3'UTR mutation) was obtained by using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). siRNA information is provided in ESM Table 1.

Transfection and luciferase assay Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase assays were performed by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as described previously [22]. Luciferase activity was measured on a luminometer (Berthold Technologies, Bad Wildbad, Germany). Electroporation was performed as described previously [23]. Briefly, mice soleus (SOL) muscles were injected with 100 μmol miR-130b mimics. Five 50 V/cm pulses, with a 200 ms interval, were applied. Muscles were collected 6 days after gene delivery.

Prediction of miRNA targets and bioinformatic analysis miR-130b target genes were predicted by TargetScan (<http://www.targetscan.org/>). The association between potential miR-130b target genes and human diseases was analysed using the web tool FunDO (<http://django.nubic.northwestern.edu/fundo/>, accessed July 2012). Only miR-130b target genes associated with metabolic diseases with a *p* value <0.01 were selected for plotting and listed in a table. The *p* value was calculated using Fisher's exact test. The web-based computational tool from DIANA Lab (www.microrna.gr/miRPathv2, accessed July 2012) was used to identify signalling pathways potentially altered by miR-130b targets.

Statistical analysis Data are presented as means $\pm$ SE. For two-group comparisons, Student's *t* test was performed; *p* <0.05 was considered to represent a significant difference. Receiver operating characteristic (ROC) curves were plotted to evaluate the diagnostic effects of the profiles. The AUC for the ROC curve was calculated for the measurement of discrimination accuracy. GraphPad Prism 5.0 (GraphPad

Software, La Jolla, CA, USA) was used for all the statistical analysis.

Results

Circulating levels of miR-130b are significantly elevated in obese mice We hypothesised that adipose tissue can secrete functional miRNAs to crosstalk with other metabolic organs, thereby regulating whole-body metabolism. To test our hypothesis, we carried out experiments to identify miRNAs that are expressed in adipose tissue, dysregulated in obesity and detectable in circulation. We first profiled miRNAs expressed differentially among mouse inguinal white adipose tissue (IWAT), epididymal white adipose tissue (EWAT), brown adipose tissue (BAT), gastrocnemius (GAS) muscle and SOL muscle by microarray, and found that *Mir130b* expression was relatively higher in white adipose tissue (WAT; ESM Fig. 1). To discover whether *Mir130b* expression was altered in obesity, we determined its expression in various tissues from *ob/ob* mice. As shown in Fig. 1a, *Mir130b* expression was increased dramatically in the EWAT of *ob/ob* mice compared with that of the wild-type group, which is consistent with an early report [14]. We also observed a slight but significant increase of *Mir130b* expression in GAS muscle of *ob/ob* mice compared with wild-type mice.

Fig. 1 miR-130b levels are elevated in the WAT and serum of obese mice. **(a)** Relative expression of *Mir130b* in the brain (BRA), heart (HEA), kidney (KID), pancreas (PAN), stomach (STO), EWAT, GAS muscle and liver (LIV) of *ob/ob* mice (black bars) and wild-type mice (white bars). $n=3-6$, $*p<0.05$ and $**p<0.01$ compared with wild-type mice. **(b)** Relative level of miR-130b in the serum of *ob/ob* mice as compared with wild-type mice (WT). $n=3-6$, $***p<0.001$ compared with WT. **(c)** Relative expression of *Mir130b* in the EWAT of DIO mice that were on an HFD and control mice fed a chow diet. $n=4-6$, $*p<0.05$ compared with control. **(d)** Relative level of miR-130b in the serum of DIO mice as compared with their controls. $n=3-6$, $*p<0.05$ compared with control

To determine whether miR-130b also exists in serum and whether the serum levels of miR-130b are altered in obesity, we measured the circulating level of miR-130b in *ob/ob* mice. Interestingly, we found that the level of miR-130b was significantly elevated in the serum of *ob/ob* mice (Fig. 1b). A similar elevation of miR-130b level was observed in both EWAT and serum of DIO mice that were on an HFD (Fig. 1c, d). These exciting observations prompted us to investigate the serum level of miR-130b in human samples.

The serum level of miR-130b is positively correlated with obesity in humans Anthropometric and metabolic characteristics of study participants are summarised in ESM Table 2. As we expected, miR-130b levels were elevated in both overweight and obese participants. Interestingly, a positive correlation between miR-130b level and BMI was evident (Fig. 2a; $n=44$, $r^2=0.6022$, $p<0.0001$), suggesting a link between the degree of obesity and miR-130b level. Moreover, serum levels of miR-130b could detect individuals who were overweight/obese ($\text{BMI} \geq 24 \text{ kg/m}^2$) with 70% sensitivity at 95% specificity (Fig. 2b). The ROC curves of miR-130b serum level reflected strong separation between normal and overweight/obese groups, with an AUC of 0.905 ($p<0.0001$) (Fig. 2c). Taken together, these results demonstrated that the level of circulating miR-130b is a marker of obesity.

Circulating miR-130b is a risk factor for metabolic syndrome To determine whether the circulating level of miR-130b is correlated with other clinical characteristics, correlation analysis was performed. As listed in Table 1, serum levels of miR-130b were also correlated well with body fat percentage estimated by bioelectrical impedance analysis (BF%-BIA), arm circumference, triceps skinfold, calf circumference, waist circumference and triacylglycerol level (TG). These results suggested that the elevated miR-130b levels might associate with poor metabolic profile in humans. In addition, ROC analysis was performed to discover whether miR-130b could be used as a potential biomarker for total cholesterol, TG, hypertension, hyperglycaemia and metabolic syndrome [24]. As shown in Table 2, the ROC analysis of serum level of miR-130b reflected strong separation between normal and metabolic syndrome groups ($p=0.0002$). Moreover, the ROC analysis of serum level of miR-130b reflected separation between participants with and without hypertriacylglycerolaemia ($p=0.0043$). The separation between participants with and without hypertension also tended to be significant ($p=0.07$).

Next, we evaluated the differentiating power of circulating miR-130b levels by comparison with that of BF%-BIA or TG. The ROC curve analysis indicated that BF%-BIA was only useful in discriminating the metabolic syndrome group from the control group with an AUC of 0.727, as shown in

Fig. 2 The circulating level of miR-130b is positively correlated with obesity in humans. **(a)** Circulating level of miR-130b is positively correlated with BMI in humans; $n=44$, $r^2=0.6022$, $p<0.0001$. **(b)** Sensitivity and specificity of serum miR-130b level in the diagnosis of overweight/obesity ($\text{BMI}\geq 24 \text{ kg/m}^2$). The dashed line indicates a 95% specificity threshold. Serum levels of miR-130b could detect overweight/obese individuals with 70% sensitivity. **(c)** A ROC curve was drawn with the data on serum miR-130b from 44 participants; AUC 0.905, $p<0.0001$. **(d, e)** Circulating miR-130b displays higher sensitivity and specificity for diagnosis of the metabolic syndrome than BF%-BIA. Sensitivity and specificity of serum miR-130b level **(d)** and BF%-BIA **(e)** in the diagnosis of metabolic syndrome (MetS). The dashed line indicates a 96% specificity threshold. Serum level of miR-130b could detect individuals with metabolic syndrome with 55% sensitivity **(d)**, while BF%-BIA was less sensitive (30%) **(e)**. The relative level of circulating miR-130b was normalised to miR-223 level **(a, b, d)**

Table 2 ($p=0.011$). Similarly, the ROC curve analysis indicated that TG was useful in discriminating the metabolic syndrome group from the control group with an AUC of 0.739 as shown in Table 2 ($p=0.0074$). These data suggested that miR-130b is a better predictor of individuals with metabolic syndrome than TG, while TG is slightly better than BF%-BIA. Furthermore, serum level of miR-130b could detect individuals with metabolic syndrome with 55% sensitivity at 96% specificity (Fig. 2d), while BF%-BIA was less sensitive (30% and 96%, respectively) for metabolic syndrome diagnosis (Fig. 2e). These results suggested that circulating level of miR-130b displays higher sensitivity and specificity for diagnosis of metabolic syndrome than BF%-BIA.

miR-130b is secreted into culture medium during adipogenesis Since the levels of miR-130b in serum and

Table 1 Correlation between serum levels of miR-130b with clinical characteristics

Variable	r^2	p value
BMI	0.6022	$<0.0001^a$
BF%-BIA	0.1858	0.0035^b
AC	0.4597	$<0.0001^a$
TSF	0.2043	0.0021^b
CC	0.3059	$<0.0001^a$
WC	0.4742	$<0.0001^a$
SBP	0.0678	0.0879^c
DBP	0.0509	0.1410^c
GLU	0.0220	0.3421^c
TC	0.0002	0.9305^c
TG	0.1773	0.0049^b
LDL-cholesterol	0.0006	0.8774^c
HDL-cholesterol	0.0784	0.0690^c
Insulin	0.0137	0.4490^c

^a $p<0.001$

^b $p<0.01$

^cNS

The p value is calculated using a two-tailed test

AC, arm circumference; CC, calf circumference; DBP, diastolic blood pressure; GLU, glucose; SBP, systolic blood pressure; TC, total cholesterol; TG, triacylglycerol; TSF, triceps skinfold; WC, waist circumference

WAT were elevated in obese mice (Fig. 1), we speculated that the adipose-derived miR-130b might contribute to the high circulating level of miR-130b. To test this possibility, we first determined whether miR-130b was secreted from adipocytes during adipogenesis. 3T3-L1 pre-adipocytes were differentiated into matured adipocytes by using a standard protocol. As we expected, mRNA levels of *Pparg* and *Adipsin* (also known as *Cfd*), two markers of adipocyte differentiation, as well as *Pgc-1 α* , were significantly increased after differentiation (ESM Fig. 2). Consistent with the findings of a previous report [25], the expression level of *Mir130b* in adipocytes was slightly decreased during adipogenesis (Fig. 3a). In contrast, we found the level of miR-130b in culture medium was significantly increased (Fig. 3b). Our data indicated that miR-130b is released from differentiating adipocytes during adipogenesis.

TGF- β stimulates the secretion of miR-130b from matured adipocytes In addition to investigating the secretion of miR-130b during adipogenesis, we also examined some other signalling factors that might have impact on the release of miR-130b from matured adipocytes. PPAR γ ligands and cytokines, such as TNF- α and TGF- β , as well as insulin, are believed to exert complex regulatory actions on adipose tissue under various physiological and pathological

Table 2 ROC analysis of miR-130b, BF%-BIA and triacylglycerol

Disorder	miR-130b		BF%-BIA		Triacylglycerol	
	AUC	<i>p</i> value	AUC	<i>p</i> value	AUC	<i>p</i> value
Hypercholesterolaemia (TC $\geq$ 5.18 mmol/l)	0.580	0.4124 ^d	0.665	0.0881 ^d	0.678	0.0661 ^d
Hypertriacylglycerolaemia (TG $\geq$ 1.7 mmol/l)	0.758	0.0043 ^b	0.600	0.2680 ^d		
Hypertension (blood pressure $\geq$ 140/90 mmHg)	0.661	0.0700 ^d	0.511	0.9057 ^d	0.614	0.2049 ^d
Hyperglycaemia (fasting plasma glucose $\geq$ 7.0 mmol/l)	0.750	0.1527 ^d	0.567	0.7029 ^d	0.575	0.6679 ^d
Metabolic syndrome ^c	0.833	0.0002 ^a	0.727	0.0110 ^c	0.739	0.0074 ^b

^a*p*<0.001^b*p*<0.01^c*p*<0.05^dNS

^cThe metabolic syndrome was defined based on the updated National Cholesterol Education Program Adult Treatment Panel III criteria for Asian-Americans as presenting at least three of the following components: (1) waist circumferences 90 cm or greater in men or 80 cm or greater in women; (2) triacylglycerols 1.7 mmol/l or greater; (3) HDL-cholesterol less than 1.03 mmol/l in men or less than 1.30 mmol/l in women; (4) blood pressure 130/85 mmHg or greater or current use of antihypertensive medications; (5) fasting plasma glucose 5.6 mmol/l or greater or previously diagnosed type 2 diabetes or on oral glucose-lowering agents or insulin

The *p* value determines if the AUC is significantly different from 0.5

TC, total cholesterol

conditions. Here we found that treatment with rosiglitazone, TNF- α or insulin did not change the level of miR-130b in the culture medium of matured 3T3-L1 adipocytes (Fig. 3c and ESM Fig. 3a). In contrast, addition of TGF- β dramatically elevated the level of miR-130b in the culture medium (Fig. 3c). Concomitant with TGF- β -induced secretion of miR-130b, the intracellular level of miR-130b was slightly decreased (Fig. 3d). Consistent with previous reports, we also found that the mRNA expression levels of *Tgf- β* (also known as *Tgfb1*) were increased in WAT of *ob/ob* mice compared with wild-type mice (Fig. 3e). These data implicated that high levels of TGF- β in obese individuals might contribute to the increased secretion of miR-130b from the WATs. It is worth noting that the mRNA expression of *nSMase2* (also known as *Smpd3*), encoding neutral sphingomyelinase 2, a regulator of miRNA secretion [26], was also increased in WAT of these obese mice, suggesting the miRNA secretion process is activated in obesity (Fig. 3f). Moreover, TGF- β -induced secretion of miR-130b from adipocytes could be attenuated by knock-down of *nSMase2* by siRNA (Fig. 3g). This result indicated that the secretion of miR-130b stimulated by TGF- β in adipocytes is dependent on *nSMase2*.

Interestingly, TGF- β had no effect on the level of miR-130b in the culture medium of the myotubes or on the intracellular miR-130b level (ESM Fig. 3b, c). Moreover, TGF- β did not increase the level of other miRNAs, such as miR-96 and miR-182, in the culture medium of matured adipocytes (ESM Fig. 3d, e). These data suggested that TGF- β might regulate the secretion of miR-130b in a tissue-specific and miRNA-specific manner.

Potential miR-130b target genes are involved in metabolic pathways and diseases Since miRNA could have multiple targets and have a differential effect on various signalling pathways, to identify molecular pathways potentially altered by miR-130b, we performed analysis by using a web-based computational tool from DIANA Lab. As shown in ESM Table 3, through its target genes miR-130b might be able to regulate the mTOR and adipocytokine signalling pathways, both of which are important for metabolic regulation. In addition, the correlation between potential miR-130b target genes and diseases was analysed by the FunDO web tool. As shown in Fig. 4 and Table 3, predicted miR-130b target genes were associated with diabetes mellitus, obesity, heart failure, hypertension and atherosclerosis. All these results based on computational analysis implied that miR-130b might regulate metabolism under both physiological and pathological conditions.

Circulating miR-130b might affect muscle metabolism by targeting peroxisome proliferator-activated receptor γ coactivator-1 α It has been shown that adipose tissue could function as an endocrine organ and crosstalk with other tissues through various mechanisms. Since the expression of *Mir130b* was elevated in the skeletal muscle of *ob/ob* mice (Fig. 1a), we speculated that circulating miR-130b could be taken up by muscle cells and influence the metabolism in its new location. To rule out the possibility that the increased level of miR-130b observed in the skeletal muscle of *ob/ob* mice was due to the activation of transcription of *Mir130b*, the level of miR-130b precursor was measured. As shown in

Fig. 3 The secretion of miR-130b from adipocytes is regulated in physiological and pathological processes. (a, b) miR-130b is released from adipocytes during adipogenesis. The level of miR-130b in adipocytes declines gradually during adipogenesis (a) while the relative level of miR-130b in culture medium increases gradually (b). The relative level of miR-130b was analysed by real-time PCR individually at different time points after induction as indicated (D0, day 0; D2, day 2; D6, day 6). * $p < 0.05$ and ** $p < 0.01$ compared with D0. (c, d) TGF-β, but not rosiglitazone or TNF-α, is able to increase the secretion of miR-130b from matured adipocytes. The effect of TGF-β, rosiglitazone and TNF-α on the level of miR-130b in the culture medium (c) and on the expression of *Mir130b* in matured adipocytes (d) was examined by using real-time PCR. * $p < 0.05$ compared with vehicle (Veh). (e, f) The expression of TGF-β (e) and *nSMase2* (f) is increased significantly in the EWAT of *ob/ob* mice. $n = 3$, * $p < 0.05$ and ** $p < 0.01$ compared with wild-type (WT) mice. (g) Knockdown of *nSMase2* by siRNA reduces the amount of miR-130b in the culture medium of TGF-β-treated adipocytes. *** $p < 0.001$ compared with control siRNA

Fig. 5a, an increased level of miR-130b precursor was found only in adipose tissues but not in the other tissues examined, including muscle tissues of *ob/ob* mice, indicating that the

Fig. 4 Predicted miR-130b target genes are associated with human metabolic diseases. The association between potential miR-130b target genes predicted by TargetScan and human diseases was analysed by the FunDO web tool. Predicted miR-130b target genes associated with metabolic diseases, including diabetes mellitus, obesity, heart failure, hypertension and atherosclerosis, are shown

high circulating miR-130b level might contribute to the increase of miR-130b level in the muscle of *ob/ob* mice. In addition, we compared the level of matured miR-130b and miR-130b precursor in three metabolic organs (EWAT, GAS muscle and liver) of wild-type mice. We found that the levels of miR-130b, as well as its precursor, were higher in EWAT than in GAS muscle or liver (Fig. 5b). These results further supported the idea that adipose tissue expression of *Mir130b* might be the main driver of the changes in circulating miR-130b.

To provide direct evidence that miR-130b secreted by adipocytes could be taken up by muscle cells, we treated the matured 3T3-L1 adipocytes with different concentrations of TGF-β; afterwards, the culture medium of adipocytes was collected and added to the culture medium of C2C12 myotubes to mimic a co-culture system. As we expected,

Table 3 Predicted miR-130b target genes are associated with human metabolic diseases

DO term	Number of target genes	<i>p</i> value
Heart failure	12	6.589×10^{-7}
Diabetes mellitus	23	7.887×10^{-6}
Obesity	14	2.910×10^{-5}
Hypertension	11	9.915×10^{-4}
Atherosclerosis	11	6.405×10^{-3}

The *p* value is calculated using Fisher's exact test

Fig. 5 miR-130b regulates muscle metabolism by targeting PGC-1 α . **(a)** The level of miR-130b precursor (pre-miR-130b) is increased significantly only in EWAT but not in brain (BRA), heart (HEA), kidney (KID), pancreas (PAN), stomach (STO), GAS muscle or liver (LIV) of *ob/ob* mice (black bars) compared with wild-type mice (white bars). $n=3-6$, $*p<0.05$. **(b)** The relative level of miR-130b and pre-miR-130b in EWAT, GAS muscle and liver (LIV) of wild-type mice. **(c)** The effect of culture medium from TGF- β -treated adipocytes on the expression of *Mir130b* in myotubes. Matured 3T3-L1 adipocytes were treated with different concentrations of TGF- β as indicated; afterwards, the culture medium of adipocytes was collected and added to the culture medium of C2C12 myotubes to mimic a co-culture system. The muscle cells were harvested 12 h after addition of the adipocyte-derived culture medium and miR-130b levels were determined by quantitative RT-PCR. $*p<0.05$ compared with no TGF- β treatment. **(d)** miR-130b regulatory element in the 3'UTR of human *PGC-1 α* was identified by Targetscan. MRE-wt, wild-type MRE; MRE-mut, mutated MRE. **(e)** The effect of miR-130b mimics on a reporter containing *PGC-1 α* -3'UTR with MRE-wt or MRE-mut was determined in HEK-293T cells. Relative luciferase units (RLU) are shown. White bars, negative control; black bars, miR-130b mimics. Error bars represent the SE of three independent

experiments. $*p<0.05$ compared with control. **(f)** *Mir130b* expression in C2C12 myotubes was determined by quantitative RT-PCR 24 h post-transfection of miR-130b mimics. White bars, negative control; black bars, miR-130b mimics. $n=3$, $**p<0.01$ compared with control. **(g)** The effect of miR-130b mimics on PGC-1 α protein expression was evaluated by western blot analysis in C2C12 myotubes. Total protein was harvested 24 h or 18 h post-transfection, respectively. NC, negative control. **(h)** *Cpt1b*, a PGC-1 α target gene, is repressed by miR-130b mimics in C2C12 myotubes. Total RNA was harvested 24 h post-transfection and real-time PCR analysis was performed. White bars, negative control; black bars, miR-130b mimics. Error bars represent the SE of three independent experiments. $*p<0.05$ compared with control. **(i)** The in vivo transfection efficiency was evaluated by quantitative RT-PCR for miR-130b. SOL, soleus muscle. White bars, negative control; black bars, miR-130b mimics. $n=3$, $***p<0.001$ compared with control. **(j)** The effect of miR-130b on PGC-1 α protein expression was evaluated by western blot analysis in the soleus muscle of mice. NC, negative control. **(k)** The effect of miR-130b on the mRNA expression of *PGC-1 α* and *Cpt1b* was evaluated by real-time PCR analysis in the soleus muscle of mice. White bars, negative control; black bars, miR-130b mimics. $n=3$, $*p<0.05$ compared with control

the culture medium from the adipocytes treated with TGF- β elevated the miR-130b levels in myotubes in a dose-dependent manner (Fig. 5c), suggesting that miR-130b could be taken up by muscle cells and act as a mediator for adipose–muscle crosstalk.

Among the predicted miR-130b targets, *PGC-1 α* is known to be a crucial regulator for multiple metabolic processes, including lipid oxidation, mitochondrial biogenesis and respiration and fibre-type switching in muscle [27, 28]. To confirm that *PGC-1 α* is a real target of miR-130b, we first

cloned 3'UTR of *PGC-1 α* containing the putative miRNA regulatory element (MRE) for miR-130b (Fig. 5d) into a luciferase reporter plasmid to determine whether miR-130b could have inhibitory effect. As shown in Fig. 5e, miR-130b significantly suppressed the luciferase activity suggesting that miR-130b could function through the 3'UTR of *PGC-1 α* to inhibit the reporter gene expression. In addition, we made a luciferase reporter with a mutation in the MRE for miR-130b (Fig. 5d). This mutation abrogated the suppressive effect of miR-130b on the 3'UTR of *PGC-1 α* (Fig. 5e). Moreover, we investigated whether miR-130b could affect the expression of peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) in cells. Since PGC-1 α expression is abundant in muscles, we examined the effect of miR-130b on PGC-1 α expression in myotubes. First, we evaluated the transfection efficiency of miR-130b in C2C12 myotubes. As shown in Fig. 5f, *Mir130b* expression in myotubes was elevated about 300-fold post-transfection. As we expected, the PGC-1 α protein expression, as well as the mRNA expression of its downstream target gene *Cpt1b* (encoding carnitine palmitoyltransferase 1b [CPT1B]), was decreased by *Mir130b* overexpression (Fig. 5g, h). These findings suggested that PGC-1 α is a direct target gene of miR-130b in muscles.

To determine whether miR-130b could affect PGC-1 α and CPT1B in vivo, we delivered miR-130b into the SOL muscle of mice as described in **Methods**. First, we evaluated the transfection efficiency of miR-130b in SOL muscle. As shown in Fig. 5i, the miR-130b level was elevated by more than 30-fold in vivo. In support of our hypothesis, overexpression of *Mir130b* in SOL muscle led to a decrease in PGC-1 α protein level and mRNA level (Fig. 5j, k). Concomitant with the reduced expression of PGC-1 α , the mRNA levels of *Cpt1b* and those genes involved in mitochondrial electron transport and fatty acid β -oxidation, were decreased (Fig. 5k and ESM Fig. 4), suggesting that the oxidative capacity was impaired in the muscle. Based on these findings, we proposed that the repression of *PGC-1 α* and *Cpt1b* by miR-130b in the skeletal muscle of obese individuals might contribute to the reduction of oxidative capacity and mitochondrial dysfunction, and lead to metabolic disorder.

Discussion

Since secreted miRNAs can be detected in biological fluids such as serum and reflect the physiological status of the cells and organs they originate from, they could potentially serve as predictive and prognostic biomarkers for human diseases. Also, measurement of circulating miRNA levels is believed to be an alternative or complementary diagnostic strategy to biopsy profiling, especially for asymptomatic patients. In this study, our results revealed a novel obesity biomarker,

circulating miR-130b, the level of which was positively correlated with the degree of obesity. In addition, the elevated miR-130b levels in overweight participants before the onset of obesity seemed to be associated with a poor metabolic profile, suggesting that miR-130b level could be used for early detection of metabolic syndrome. Indeed, according to our analysis, the level of circulating miR-130b displayed diagnostic power in detecting hypertriacylglycerolaemia and metabolic syndrome. Moreover, the serum miR-130b level was more closely related to the metabolic syndrome than was BF%-BIA. A recent paper by Ortega et al reported that circulating miR-130b levels are reduced in morbidly obese individuals (BMI ≥ 40 kg/m²) [17]. This discrepancy is probably due to the different BMI ranges and patient populations used in these two studies. It also suggests that in morbidly obese individuals, the crosstalk between tissues might be dysregulated. Actually, Ortega et al also detected a slight increase in miR-130b level in obese (40 kg/m² > BMI ≥ 30 kg/m²) as compared with non-obese individuals (30 kg/m² > BMI), although the difference was not statistically significant.

Recently, the TGF- β signalling pathway has been shown to play an important role in regulating whole-body energy homeostasis [3]. Our studies suggested that the miR-130b secreted from adipose tissues might mediate the metabolic regulatory action of TGF- β . In addition, we discovered that TGF- β but not the other factors that we examined could stimulate the secretion of miR-130b from adipocytes. TGF- β promoted the secretion of miR-130b from adipocytes but not from myocytes. TGF- β only induced the secretion of miR-130b of the examined miRNAs from adipocytes in this study. These findings suggested that the secretion of a particular miRNA from certain tissues or organs is tightly controlled under both physiological and pathological conditions to coordinate whole-body energy homeostasis.

Fig. 6 Schematic representation of the role of secreted miR-130b in obesity

WAT dysfunction is believed to be an important factor in the pathogenesis of obesity-related metabolic diseases including type 2 diabetes. Dysregulation of miRNA in adipose tissues and miRNA function in adipocytes has been extensively studied and alteration of circulating miRNA profiles in obese patients has been reported recently. Nonetheless, it is still unclear whether miRNAs could be considered to be a novel class of adipokine and provide a new way for adipose tissues to communicate with other organs. Although the serum pool of miR-130b may arise from other tissues not examined in this study, we believe that we revealed for the first time that miR-130b could function as an ‘adipokine’ and mediate the crosstalk between adipose tissues and skeletal muscle.

Reduced oxidative capacity in skeletal muscle is known to increase the risk of developing metabolic diseases, including type 2 diabetes. Genes involved in oxidative phosphorylation exhibit reduced expression levels in the skeletal muscle of individuals with type 2 diabetes and those in an impaired glucose tolerance stage. Such alteration might present before the onset of impaired glucose tolerance. PGC-1 α plays a key role in the control of oxidative capacity and is suppressed in the skeletal muscle in animal models of type 2 diabetes [29]. We demonstrated that circulating miR-130b was able to decrease *PGC-1 α* mRNA levels in recipient myocytes, which might lead to reduced muscle oxidative capacity and accelerate the pathogenesis of metabolic syndrome or type 2 diabetes.

Taken together, we identified miR-130b as a potential biomarker for overweight, hypertriglyceridaemia and metabolic syndrome, and discovered a novel mechanism linking obesity and obesity-related metabolic diseases, which involves circulating miRNA-based adipose–muscle crosstalk (Fig. 6). This work also broadened our awareness of the crosstalk between adipose tissue and skeletal muscle through secreted miRNA.

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Contribution statement This study was designed by YW, YL and HY. Cell culture and animal studies were carried out by YL, YW, DZ and HZ. YW, XW, QW, YH, JW, LZ and HX performed the clinical part of the work. YL, YW and HY analysed the data and wrote the paper. JY, XL and HY contributed to discussion and supervised the project. All authors participated in data interpretation and revising the paper and approved the final version of the manuscript.

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