Expression of obesity-related miR-1908 in human adipocytes is regulated by adipokines, free fatty acids and hormones

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Abstract. White adipose tissue mass is governed by competing processes that control lipid synthesis and storage, as well as the development of new adipocytes, and also trigger metabolic and inflammatory changes. microRNAs (miRNAs) have been suggested to act as negative regulators controlling varied biological processes at the level of post-transcriptional repression. The present study focused on investigating the expression of miR-1908 in mature human adipocytes and its responses to adipokines [tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), leptin and resistin), free fatty acids (FFAs), growth hormone (GH) and dexamethasone (DEX). miR-1908 was highly expressed in mature human adipocytes. The mature human adipocytes responded to proinflammatory cytokines (TNF-α and IL-6) by markedly increasing the expression of miR-1908 at 4 h of incubation. Adipokines (resistin and leptin) and FFAs were shown to downregulate the expression of miR-1908 in human adipocytes. Furthermore, the expression of miR-1908 was decreased 4 h after treatment with GH; however, DEX treatment of human adipocytes did not affect the expression of miR-1908 during the 24-h experimental period. In conclusion, the present study showed that the expression of miR-1908 is affected by a variety of factors that are associated with obesity and insulin sensitivity. miR-1908 may be an important mediator in the development of obesity-related complications.

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Introduction

The prevalence of obesity in children and adolescents is currently the major risk factor for the development of type 2 diabetes, heart disease, hypertension and stroke (1). Obesity occurs due to a positive energy balance in the body, which results in an increase in adipose tissue by an increase in either the number or the size of adipocytes (2). The expansion of adipose tissue that is associated with obesity eventually leads to adipose tissue dysfunction. The functions of adipose tissue are essential to energy metabolism as the tissue is not only an energy depot (3), but also a source of endocrine factors (4,5), secreting adipokines, free fatty acids (FFAs) and hormones. Increasing evidence has shown that adipokines, including tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), leptin and resistin, are associated with obesity, inflammation and insulin resistance (6,7). However, the molecular mechanisms underlying the effects of adipokines, FFAs and hormones on obesity and insulin sensitivity are elusive.

Over the past decade, microRNAs (miRNAs) have been shown to be involved in multiple biological processes, including glucose homeostasis and lipid metabolism (8,9). A number of miRNAs have been identified that appear to have a role in obesity and insulin sensitivity. For example, in vertebrates, miR-375 and miR-376, which are abundantly expressed in pancreatic β-cells, are involved in the control of insulin secretion (10). Furthermore, miR-34a overexpression was shown to decrease glucose-stimulated insulin secretion and mediate FFA-induced apoptosis in Min6 cells by targeting vesicle-associated membrane protein 2 and B-cell lymphoma 2, respectively (11). However, there is still little evidence regarding the expression of miRNAs in adipose tissue, particularly the association between their regulation and obesity and insulin sensitivity.

miR-1908 was first identified in human embryonic stem cells in 2008 (12). To the best of our knowledge, the present study is the first functional study of miR-1908. In this study, it was found that miR-1908 was highly expressed in mature human adipocytes. Thus, it was hypothesized in the present study that adipokines, FFAs and hormones may participate in regulating the miR-1908 expression involved in

the development of obesity. To evaluate this hypothesis, the expression of miR-1908 in mature human adipocytes was examined and its responses to adipokines, FFAs and hormones were investigated to clarify the role of miR-1908 in regulating the development of obesity and insulin resistance.

Materials and methods

Cell culture. Human visceral preadipocytes (ScienCell Research Laboratories, San Diego, CA, USA) were maintained in preadipocyte medium (PAM; cat. no. 7211; ScienCell Research Laboratories) containing 5% fetal bovine serum, 1% preadipocyte growth supplement and 1% penicillin/streptomycin solution at 37˚C in a humidified atmosphere under 5% CO_2. To induce differentiation, serum-free PAM [containing 50 nM insulin (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (DEX; Sigma-Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 100 μM rosiglitazone (Sigma-Aldrich)] was added to confluent human preadipocytes (day 0) and the medium was replaced every two days over four days. Thereafter, the medium was replaced with serum-free PAM containing 50 nM insulin, which was replaced every two days until lipid droplets had accumulated in the cells (day 15). Fat accumulation was assessed by staining formalin-fixed cells with Oil Red O (Sigma-Aldrich). The cells were collected at different time-points (days 0 and 15).

Treatment of adipocytes with adipokines, FFAs and hormones. Differentiated adipocytes were used for experiments 15 days after the induction of differentiation, at which point >80% of cells showed the morphological and biochemical properties of adipocytes. Following overnight incubation in serum-free PAM, human adipocytes were treated with different adipokines, including 10 ng/ml TNF-α (13), 30 ng/ml IL-6 (14), 30 ng/ml leptin or 60 ng/ml resistin, 1 mmol/l FFA cocktail (lauric, myristic, linoleic, oleic and arachidonic acids), 1 mmol/l DEX or 100 nmol/l growth hormone (GH) (all adipokines, Sigma-Aldrich) for different periods of time (4, 8 and 24 h). Adipocytes were collected at these time-points and prepared for further investigation.

RNA isolation and quantitative polymerase chain reaction (qPCR). Total RNA from human adipocytes was purified using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by DNase I treatment (Takara Bio Inc., Shiga, Japan). The quality and concentration of the RNA was assessed using a Nanodrop 2.0 instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA). To monitor levels of miRNA, cDNA was synthesized from 200 ng total RNA using the TaqMan® miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using a 7500 Sequence Detection system (Applied Biosystems), following the manufacturer's instructions. Briefly, samples were incubated at 95˚C for 10 min for an initial denaturation stage, followed by 40 PCR cycles consisting of incubation at 95˚C for 15 sec and then 60˚C for 1 min. miRNA expression was normalized to small nuclear RNA (snRNA) U6 and miR-103, respectively. The primer identification numbers were 121109 for miR-1908, 000439 for miR-103 and 001973 for snRU6 (Applied Biosystems).

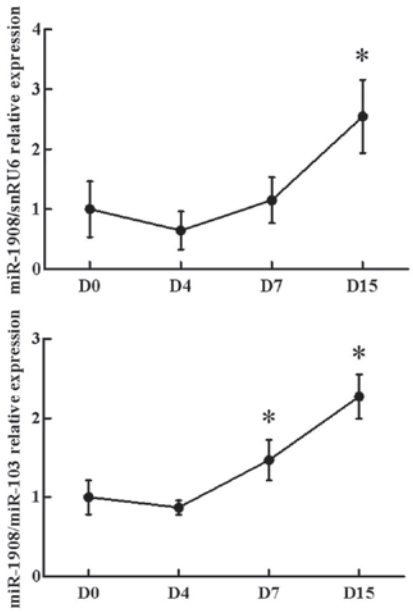

Figure 1. Expression of miR-1908 during the conversion of human preadipocytes (day 0) into adipocytes (day 15). Human preadipocytes were induced to differentiate and total RNA was harvested from the human preadipocytes on alternate days prior to (day 0) and after (day 15) replacement of growth medium with differentiation medium. miR-1908 and miR-103 levels were assessed using quantitative polymerase chain reaction and normalized to snRU6, and miR-1908 levels were also normalized to the levels of miR-103. The results are presented as the mean ± standard error of the mean of three experiments. *P<0.01 versus the basal levels (day 0). miR, micro RNA; snR, small nuclear RNA.

Statistical analysis. Representatives of replicate experiments are shown in the figures, and results are presented as the mean ± standard error of the mean. Statistical analysis was performed using the one-way analysis of variance. P≤0.05 was considered to indicate a statistically significant difference.

Results

miR-1908 expression is increased during differentiation of human preadipocytes. The present study firstly investigated the expression levels of miR-1908 during the maturation of human preadipocytes. As shown in Fig. 1, the miR-1908 expression levels were relatively low in human pre-adipocytes. Fifteen days after the induction of differentiation, >80% of preadipocytes exhibited typical adipocyte morphology. In addition to miR-1908 levels, the expression levels of miR-103 were analyzed. miR-103 expression levels were not altered during the differentiation of the human preadipocytes. Thus, miR-103 was used as a normalization control for the assessment of miR-1908 expression. Using snRU6 and miR-103 as positive controls, miR-1908 expression levels were observed to be significantly upregulated in the cells at days 7 and 15 relative to those at day 0. This observation demonstrated that miR-1908 expression was elevated during the differentiation of human preadipocytes into adipocytes.

miR-1908 is regulated by adipokines (IL-6, TNF-α, leptin and resistin) in human adipocytes. Without any treatment, expression levels of miR-1908 remained unchanged at different time-points (4, 8 and 24 h) (Fig. 2). Thus, the expression at

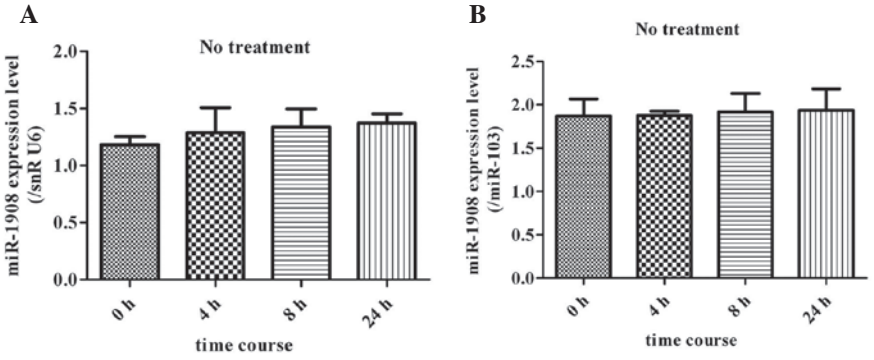

Figure 2. Alterations in miR-1908 expression levels at different time-points without any treatment. miR-1908 expression levels remained unchanged at different time-points (4, 8 and 24 h), as indicated by quantitative polymerase chain reaction. Levels were normalized to the (A) snRU6 and (B) miR-103 levels. Results are expressed as the mean ± standard error of the mean of three experiments. miR, microRNA; D, day; snR, small nuclear RNA.

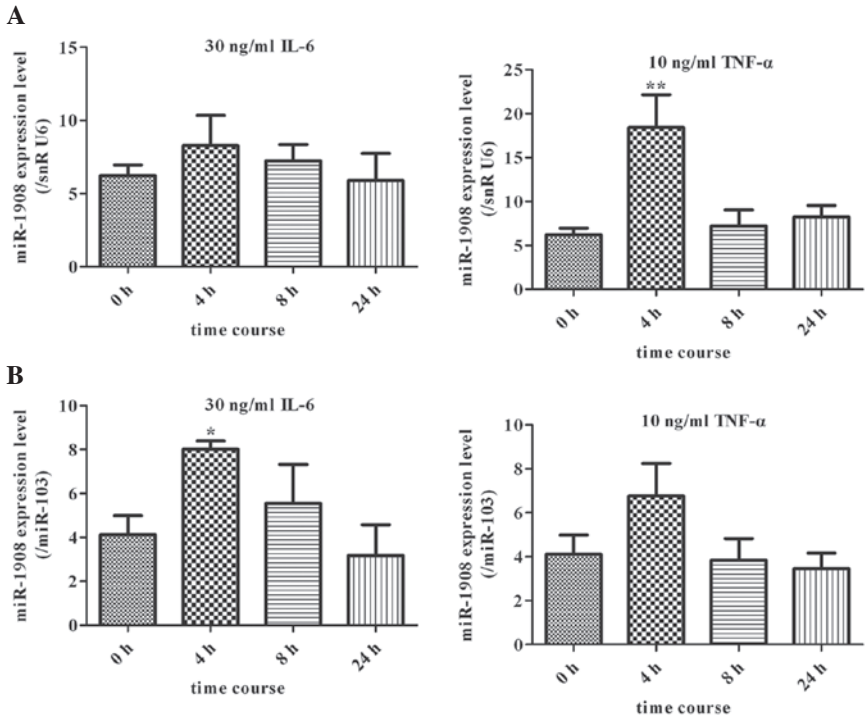

Figure 3. Inflammatory cytokines increase the expression of miR-1908 in human adipocytes. Cells were treated with IL-6 (30 ng/ml) and TNF-α (10 ng/ml), respectively, and miR-1908 expression was analyzed by quantitative polymerase chain reaction and normalized to (A) snRU6 and (B) miR-103 levels. *P<0.05 and **P<0.01 versus the cells at 0 h (untreated cells). Data shown are representative of three separate experiments. Results are expressed as the mean ± standard error of the mean. TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; miR, micro RNA; snR, small nuclear RNA.

0 h was used as a control during the assessment of miR-1908 expression. To assess the role of this miRNA in the association between obesity and insulin resistance, the effects of adipokines, including proinflammatory cytokines (TNF-α and IL-6), leptin and resistin, on the expression of miR-1908 in human adipocytes were assessed at different time-points (4, 8 and 24 h) (Figs. 3 and 4). When mature adipocytes were treated with 30 ng/ml IL-6, the expression of miR-1908, which was normalized to snRU6 expression, was not significantly altered at the different time-points (4, 8 and 24 h). By contrast, it was observed that miR-1908 expression levels in human adipocytes treated with 10 ng/ml TNF-α were significantly upregulated at 4 h as compared with levels in the controls (P<0.05) (Fig. 3A). In addition, human adipocytes were treated with the adipokines leptin (30 ng/ml) and resistin (60 ng/ml). Of note, this led to ~10-fold decreases (P<0.01) in the expression of miR-1908 at 4 h, with expression remaining low at 24 h of incubation (Fig. 4A). In summary, exposure of the cells to the adipokines leptin and resistin resulted in a decrease in the expression levels of miR-1908 (Fig. 4A). To further verify the effect of the aforementioned adipokines on miR-1908, miR-103 was also used for normalization, and the results were consistent with those obtained using snRU6 for normalization (Fig. 3B and 4B).

Effects of FFAs on miR-1908 expression in human adipocytes. The effects of 1 mmol/l FFAs on miR-1908 expression in cultured human adipocytes were analyzed using qPCR

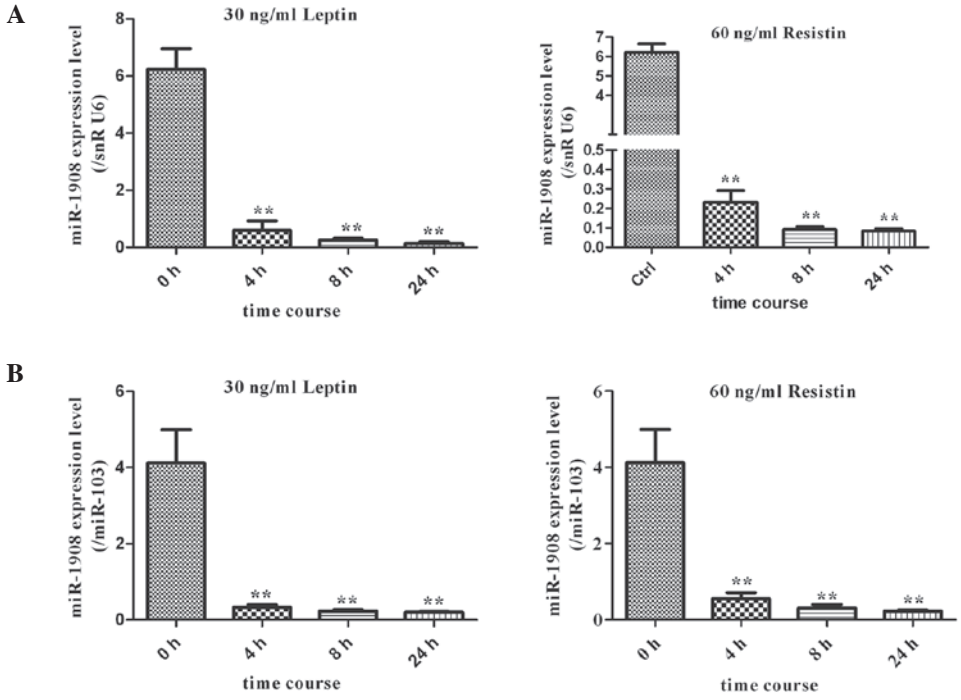

Figure 4. miR-1908 expression is regulated by adipokines (leptin and resistin) in human adipocytes. Differentiated human adipocytes were treated with 30 ng/ml leptin or 60 ng/ml resistin for the indicated periods (up to 24 h). miR-1908 levels were analyzed by quantitative polymerase chain reaction and normalized to the (A) snRU6 and (B) miR-103 levels. Results are presented as the mean ± standard error of the mean of three experiments. **P<0.01 versus the miR-1908 levels at 0 h (untreated cells). miR, micro RNA; Ctrl, control; snR, small nuclear RNA.

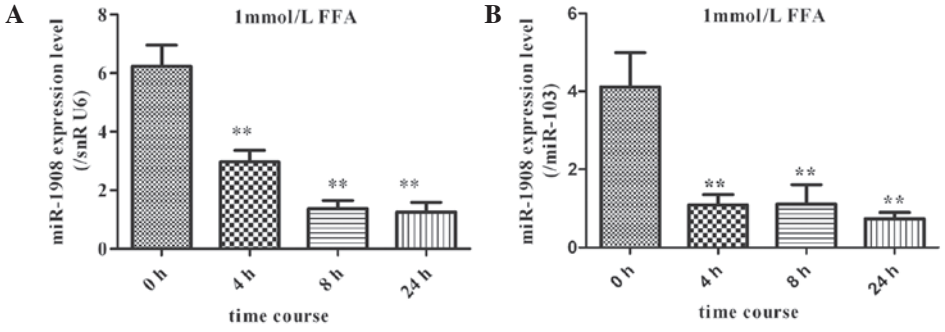

Figure 5. Effects of FFAs on miR-1908 expression in human adipocytes. Differentiated human adipocytes were treated with 1 mmol/l FFAs for the indicated periods (up to 24 h). miR-1908 expression was analyzed using quantitative polymerase chain reaction and normalized to the (A) snRU6 and (B) miR-103 levels. **P<0.01 versus the miR-1908 levels at 0 h (untreated cells). The data shown are representative of three similar experiments. Results are presented as the mean ± standard error of the mean. FFA, free fatty acid; miR, micro RNA; snR, small nuclear RNA.

(TaqMan probe method). Differentiation of human preadipocytes was induced and adipocyte cultures were prepared for use in experiments, as described in the materials and methods. Adipocytes were cultured in the presence of 1 mmol/l FFAs. The expression of miR-1908 was significantly downregulated in a time-dependent manner following initiation of FFA stimulation. This effect was maintained for up to 24 h (Fig. 5).

Response of miR-1908 expression levels to DEX and GH in human adipocytes. The effects of DEX and GH on the expression of miR-1908 in human adipocytes were investigated. Mature adipocytes were cultured in the presence of 1 mmol/l DEX and the effects of DEX on miR-1908 expression in cultured human adipocytes were analyzed using qPCR (TaqMan probe method). The expression of miR-1908 was slightly altered by stimulation with DEX; however, no statistically significant differences in expression were observed compared with expression at 0 h. In addition, miR-1908 expression in human adipocytes treated with 100 nmol/l GH for different periods of time (4, 8, and 24 h) was investigated. As shown in Fig. 6, miR-1908 was significantly downregulated 4 h after the initiation of GH stimulation. Thereafter, the expression levels of miR-1908 slightly increased to equal those of untreated cells.

Discussion

Research into the association between obesity and its related complications, including type 2 diabetes and cardiovascular diseases, has indicated that adipose tissue plays a key role in the regulation of glucose and lipid metabolism, acting through at least two different mechanisms: i) Storage of lipids (as triglycerides) and ii) adipokine secretion, for endocrine

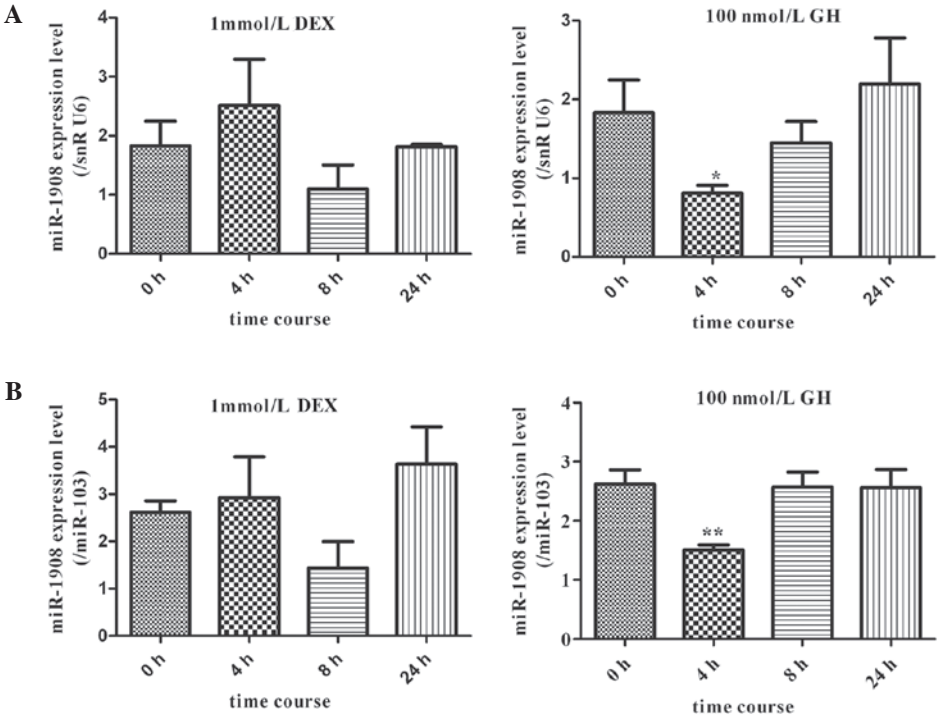

Figure 6. Response of miR-1908 expression levels to DEX and GH in human adipocytes. Differentiated human adipocytes were treated with 1 mmol/l DEX or 100 nmol/l GH for the indicated periods (up to 24 h). miR-1908 levels were assessed using quantitative polymerase chain reaction and normalized to the (A) snRU6 and (B) miR-103 levels. Results are expressed as the mean ± standard error of the mean of three experiments. *P<0.05 and **P<0.01 versus miR-1908 levels at 0 h (untreated cells). DEX, dexamethasone; GH, growth hormone; miR, micro RNA; snR, small nuclear RNA.

or paracrine signaling (15). The expansion of adipose tissue in obese individuals not only affects the storage of lipids as triglycerides in lipid droplets, but also results in qualitative and quantitative changes in a number of adipokines, including IL-6, TNF-α, leptin and resistin (16). miRNAs are currently of particular interest in research on obesity and metabolic syndrome, and it was found that the dysregulation of miRNA expression is closely associated with these diseases. However, there is still no evidence regarding the expression of miRNAs in adipose tissue, particularly concerning the association between their regulation and obesity. In the present study, the role of miRNAs in obesity and insulin resistance was investigated.

miR-1908 was first identified in human embryonic stem cells in 2008 (12), and has since been found to be closely associated with the processes of metastatic invasion, angiogenesis and the colonization of melanomas (17). miR-1908 may also be involved in the malignant progression of chordoma (18) and may participate in the formation of hepatoma cells (19). The function of miR-1908 in adipocytes has yet to be elucidated. The present study showed that miR-1908 is highly expressed in human adipocytes. The effects of adipokines, FFAs and hormones associated with obesity, as well as obesity-related insulin resistance, on miR-1908 expression were investigated in human adipocytes.

It is well known that IL-6 production by adipose tissue is enhanced in obese patients (20). A previous study reported that TNF-α inhibited 3T3-L1 adipocyte differentiation by upregulating miR-155 expression (21). In the present study, miR-1908 expression levels were significantly upregulated in human adipocytes following treatment with 10 ng/ml TNF-α at 4 h; however, IL-6 had no statistically significant effect on miR-1908 expression. Resistin, also known as adipocyte-secreted factor and 'found in inflammatory zone 3', is a protein whose expression is adipocyte-specific in mice (6,22,23). Leptin is an adipocyte-derived hormone and cytokine that is upregulated in patients with obesity-related type 2 diabetes mellitus, although leptin resistance may also occur (24). These two adipokines control food intake and energy expenditure. The functions of leptin and resistin have yet to be fully elucidated; however, there is evidence that these adipokines have a role in obesity-related insulin resistance as well as adipocyte differentiation (6,22). In the present study, it was of note that marked decreases in the expression of miR-1908 were observed with the administration of leptin and resistin. This indicates that miR-1908 is closely associated with the development of obesity.

Plasma FFA concentrations are usually elevated in obese individuals (25), which may lead to several components of the insulin resistance syndrome and a risk of diabetes (26). In the present study, the expression of miR-1908 was significantly downregulated in a time-dependent manner following the initiation of the stimulation with FFAs, which indicated that miR-1908 is likely to be involved in regulating the development of obesity and insulin resistance via increasing insulin sensitivity of human adipocytes.

Studies on DEX and GH have broadened the knowledge on lipid metabolism and insulin sensitivity. Studies have indicated that high levels of glucocorticoids (such as DEX) in the adipose tissue of obese individuals promote glucose uptake and storage of fatty acids by increasing lipoprotein lipase levels (27) and increasing lipogenesis and lipid storage (28,29).

Furthermore, GH has a pronounced lipolytic effect, particularly on abdominal fat (30). The present study showed that the expression of miR-1908 was slightly altered by stimulation with DEX, although these changes were not statistically significant. By contrast, miR-1908 was downregulated at 4 h following treatment with GH; however, the effects of the two hormones appeared to become weaker with increasing time. These findings suggest other underlying mechanisms regulating miR-1908 expression, involving multiple metabolic processes.

In conclusion, the present study identified a new role of obesity-associated cytokines, which are able to alter miR-1908 expression. It remains to be elucidated what accounts for the alteration in miR-1908 expression in response to different adipokines, FFAs and hormones. The mechanisms underlying the alteration in miR-1908 expression have not been clearly linked to specific obesity-related cytokines. However, this is likely to be an important focus of further studies.

Acknowledgements

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