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Modulator recognition factor-2 regulates triglyceride metabolism in adipocytes

Takahiro Yamakawa [1], Kaori Sugimoto [2], Robert H. Whitson, Keiichi Itakura *

Department of Molecular Biology, City of Hope Beckman Research Institute, 1500 E. Duarte Road, Duarte, CA 91010, USA

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ABSTRACT

Mice lacking modulator recognition factor-2 (Mrf-2; ARID5B) have less fat in brown and white adipose tissues, partly because of a defect in adipocyte differentiation. We have also shown that knockdown of Mrf-2 decreases the expression of the adipogenic transcription factors C/EBPα and PPARγ, and inhibits adipogenesis in 3T3-L1 preadipocytes. Since these transcription factors may also contribute to the maintenance of adipocyte function, we examined the effects of siRNA targeted to Mrf-2 on triglyceride metabolism in mature 3T3-L1-derived adipocytes. As it did in differentiating adipocytes, knockdown of Mrf-2 decreased the expression of both C/EBPα and PPARγ. Knockdown of Mrf-2 also activated both lipolysis and triglyceride synthesis, and caused a significant increase in the ratio of glycerol release to free fatty acid release. This suggests that knockdown of Mrf-2 increases the rate of fatty acid recycling in 3T3-L1-derived adipocytes. Continual cycling of fatty acids through lipolysis and triglyceride synthesis could lead to dissipation of energy. Therefore, the activation of such a futile cycle via the suppression of Mrf-2 could be an effective treatment for obesity and diabetes.

Introduction

We have reported that mice lacking modulator recognition factor-2 (Mrf-2; also known as ARID5B) exhibit a lean phenotype with significant decreases in brown and white adipose tissues [1]. We have also shown that mouse embryo fibroblasts lacking Mrf-2 have a defect in *in vitro* adipogenesis, and that small interference RNA targeted to Mrf-2 (siMrf-2) inhibits adipogenesis in 3T3-L1 preadipocytes [2]. These results demonstrate that Mrf-2 is required for adipogenesis, and in this paper, we present evidence that Mrf-2 is also required to maintain normal functions in mature adipocytes.

siMRF-2 decreases the expression of CCAAT/enhancer-binding protein α (C/EBPα), and peroxisome proliferator-activated receptor-γ (PPARγ) when 3T3-L1 preadipocytes are treated with adipogenic factors [2]. Normally, these transcription factors are induced at the late stages of adipogenesis [3], and stimulate the expression of enzymes that catalyze fatty acid and triglyceride synthesis, as well as lipolysis [4,5]. C/EBPα and PPARγ are also highly expressed in mature adipocytes, and their continual high expression is required for maintenance of adipocyte function [6]. In mature adipocytes, PPARγ regulates the expression of perilipin and fat-specific protein of 27 kDa (FSP27) [7,8], both of which are required for lipid storage [9–12]. Over-expression of dominant-negative PPARγ mutants in 3T3-L1-derived adipocytes suppresses the expression of perilipin and FSP27, increases lipolysis and decreases triglyceride content [10]. Given its effects on C/EBPα and PPARγ expression in developing adipocytes, we hypothesized that knocking down Mrf-2 would also affect metabolism in mature adipocytes.

In order to investigate this, we stimulated 3T3-L1 cells to differentiate normally, then suppressed Mrf-2 expression in the mature adipocytes using siMrf-2. We found that knockdown of Mrf-2 decreased expression of C/EBPα, and PPARγ and its target genes. Knockdown of Mrf-2 also increased both lipolysis and triglyceride synthesis, but did not alter triglyceride content, implying that an energy-dissipating cycle is activated in Mrf-2-deficient adipocytes.

Materials and methods

Materials

The 3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA). Dulbecco's modified Eagle's (DME) high glucose medium was purchased from Irvine Scientific (Santa Ana, CA), and bovine serum was from Omega Scientific (Tarzana, CA). Lipofectamine 2000 and TRIzol reagent were from Invitrogen (Carlsbad, CA), RNasin was

Abbreviations: Mrf-2, modulator recognition factor-2; C/EBPα, CCAAT/enhancer-binding protein-α; PPARγ, peroxisome proliferator-activated receptor-γ; ARID, AT-rich interaction domain; siRNA, small interference RNA; NEFA, nonesterified fatty acid; PEPCK, phosphoenolpyruvate carboxykinase.

* Corresponding author. Fax: +1 626 301 8271.
E-mail address: KItakura@coh.org (K. Itakura).

[1] Present address: Phytomedicines & Nutraceuticals Research Laboratory, Healthcare Research Institute, Wakunaga Pharmaceutical Co. Ltd., Akitakata-shi, Hiroshima 739–1195, Japan.

[2] Present address: New Lead Pharmacology Laboratory, Institute for Medical Research, Wakunaga Pharmaceutical Co. Ltd., Akitakata-shi, Hiroshima 739–1195, Japan.

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from Promega (Madison, WI), and AMV reverse transcriptase was from Life Sciences. (St. Petersburg, FL). The Real-time PCR Detection System, iQ SYBR Green reagent, Zeta probe, and Trans-blot membranes were from Bio-Rad (Hercules, CA). Protease inhibitor cocktail was from Roche (Indianapolis, IN), and MicroBCA and NEFA-C) assay kits were from Pierce Biotechnology (Rockford, IL) and Wako Chemicals (Richmond, VA), respectively. Enhanced chemiluminescence (ECL) system and [U-^{14}C]glucose were from Amersham (Piscataway, NJ), and ULTRAhyb was purchased from Ambion (Austin, TX). [α-^{32}P]dCTP and [^{3}H]oleic acid were from Perkin-Elmer (Boston, MA). Other reagents were from Sigma (St. Louis, MO).

Methods

Cell culture and small interference RNA treatment. 3T3-L1 preadipocytes were grown and stimulated to differentiate as described previously [2]. Throughout this paper "3T3-L1-derived adipocytes" refers to the cells obtained after 10 days of treatment. Insulin-containing medium was withdrawn from 3T3-L1 derived adipocytes 48 h prior to transfection with small interference RNA against Mrf-2 (siMrf-2) or scrambled siRNA. (siRNA sequences are given in Ref. [2]). After transfection (12–24 h), the cells were processed for RNA and protein analyses, lipolysis assays, or measurement of triglyceride content and synthesis.

RNA and protein analysis. Extraction of total RNA, cDNA synthesis, and gene expression analyses using Northern blotting or real-time RT-PCR were performed as described previously [2]. Primer sets for FSP27 are 5′-GATGGACTACGCCATGAAGTC-3′ (forward), and 5′-GTGCTCACTGCCACATGC-3′ (reverse).

For analyses of proteins, cell monolayers were washed with ice-cold PBS, and harvested in ice-cold lysis buffer as described previously [2]. Protein concentration was determined using MicroBCA assay kits. Whole cell extracts were analyzed by Western blotting, with ECL as the detection reagent.

Lipolysis assay. 3T3-L1-derived adipocytes in monolayer culture were washed with DME high glucose medium without FBS, then incubated for 2 h in medium containing 2% fatty acid-free bovine serum albumin instead of FBS. Conditioned medium was removed and stored at −80 °C until analyzed. Glycerol and NEFA were assayed in aliquots of the same samples using Free Glycerol Reagent and NEFA-C kits, respectively. After removal of conditioned medium cell monolayers were lysed, and protein was assayed as described above.

Triglyceride determination. 3T3-L1-derived adipocytes were treated with scrambled siRNA or siMrf2, washed with ice-cold PBS, and lysed. Whole cell extracts were mixed with chloroform:methanol (2:1) and centrifuged to remove insoluble materials. The organic phase was collected, and solvents were evaporated. Triglyceride was measured in these residues using Triglyceride Reagent and Free Glycerol Reagent. Protein was assayed in replicate cell extracts.

Measurement of triglyceride synthesis. Triglyceride synthesis was measured as the rate of incorporation of ^{14}C from [U-^{14}C]glucose into triglyceride glycerol [13–15]. 3T3-L1 cells were plated in triplicate in 6-well plates, differentiated, and treated with siRNA's. Cells in one of the wells were used for protein assays. Cells in the other two wells were washed with Krebs–Ringer phosphate Hepes buffer (130 mM NaCl, 4.7 mM KCl, 1.24 mM $MgSO_4$, 2.5 mM $CaCl_2$, 100 mM Hepes, 2.5 mM NaH_2PO_4, pH 7.4; KRPH), and incubated with 5 mM [U-^{14}C]glucose (0.1 mCi/mmol) in 1 ml of KRPH at 37 °C. After 2 h, the medium was acidified with 0.25 ml of 0.5 M H_2SO_4, and cells were harvested. Lipophilic components were extracted with 10 ml of Dole's reagent [16], 6 ml of water, and 6 ml of heptane. The organic phase was collected and washed with 6 ml of water, then evaporated. The residue was dissolved in 1 ml of heptane, and an aliquot was subjected to liquid scintillation counting (LSC) to measure incorporation of ^{14}C into total lipid. The remainder of the sample was re-dried, and saponified with 0.5 ml of 20% KOH:ethanol, 1:1 (v/v) at 95 °C for 30 min. Samples were neutralized with 0.4 ml of 6 M HCl, and extracted with 2 ml of heptane, and the organic phase was dried and counted. Incorporation of ^{14}C into triglyceride glycerol was determined by subtracting ^{14}C that remained in the organic phase after saponification from total lipid ^{14}C. CPM were converted to nmol of glycerol using the specific activity of [U-^{14}C]-glucose [13].

Measurement of uptake and incorporation of exogenous fatty acids into cellular lipids. Uptake of exogenous [^{3}H]oleic acid and its incorporation into cellular lipids were measured using slight modifications of the method of Ruan and Pownall [17]. Briefly, adipocytes in six-well plates were incubated in 1 ml of KRPH, plus 0.7% fatty acid-free bovine serum albumin and 5 mM glucose for 1 h at 37 °C. Insulin was added (1 μg/ml) and the incubation was continued for 1 h. The assay was initiated by addition of 100 μM [^{3}H]oleic acid (50 mCi/mmol). At 0 and 90 min after addition of radioactive oleic acid, aliquots of the medium were removed and ^{3}H was quantitated by LSC. After the 90 min sample was collected, the medium was removed, and cell monolayers were washed with KRPH and lysed. Aliquots of the extracts were used to determine cell-associated radioactivity by LSC. The uptake of exogenous [^{3}H]oleic acid was calculated as: [(cell lysate ^{3}H)/(medium ^{3}H + cell lysate ^{3}H)] × 100%. Lipids were extracted from the remainder of the extracts using modifications of the method of Folch et al. [18] and the organic phase was dried. The residue was re-dissolved in $CHCl_3$:MeOH (2:1), and samples were loaded onto thin-layer chromatography (TLC) plates. The plates were developed in hexane:diethyl ether:acetic acid (80:20:2), which separates neutral lipids. For some plates, non-radioactive diglyceride (DG), triglyceride (TG), phosphatidic acid (PA) and lysophosphatidic acid (LPA) standards were visualized using iodine vapor. Spots corresponding to these lipids were scraped from the plates and dissolved in scintillation fluid, and ^{3}H was measured by LSC. For other plates, radioactive neutral lipids were visualized and quantitated directly, using a phosphor screen (GE Healthcare Life Sciences, Piscataway, NJ). The relative incorporation of oleic acid into triglycerides and diglycerides is expressed as: (^{3}H-TG + ^{3}H-DG)/(^{3}H-TG + ^{3}H-DG + ^{3}H-PA + ^{3}H-LPA).

Statistical analysis. Results were expressed as means ± SE, and compared using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Knockdown of Mrf-2 decreases expression 0f C/EBPα and PPARγ and their downstream targets in 3T3-L1-derived adipocytes

Knockdown of Mrf-2 in 3T3-L1 preadipocytes decreased C/EBPα and PPARγ expression and inhibited adipogenesis [2]. Here, we examined whether knockdown of Mrf-2 would have similar effects in mature adipocytes. First, we tested whether the siRNA that was used in the previous study (Mrf-2 site 3 as noted in Ref. [2]) could knock down Mrf-2 in 3T3-L1-derived adipocytes. Mrf-2 expression was analyzed 12–24 h after 3T3-L1-derived adipocytes were transfected with either scrambled siRNA or siMrf-2. Both Northern blots (Fig. 1A) and RT-PCT (Fig. 1B) showed that Mrf-2 expression was reduced strongly 12 h after transfection, but only slightly 24 h after transfection. siRNA's that were targeted to different sites in Mrf-2 [See Ref. 2] also knocked down Mrf-2 expression, but siMrf-2 site 3 had the strongest effects, and this siRNA was used in all of our subsequent experiments.

We next examined whether this transient knockdown of Mrf-2 affected the expression of adipogenic transcription factors and their target genes. Knockdown of Mrf-2 decreased expression of

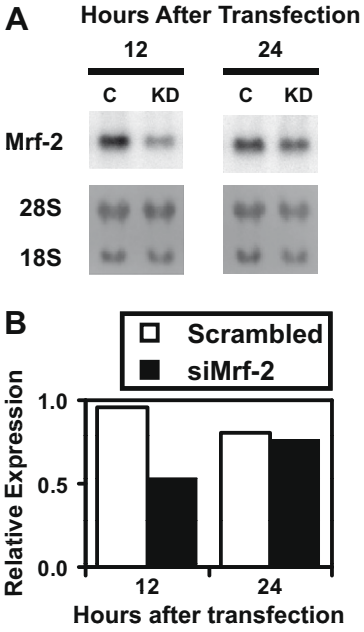

Fig. 1. Mrf-2 mRNA expression in scrambled siRNA- or siMrf-2-treated 3T3-L1 adipocytes. 3T3-L1-derived adipocytes were treated with scrambled siRNA or siMrf-2, and total RNA was extracted at the indicated times. (A) Northern blots. Total RNA was separated on 1% denatured agarose gels, and transferred to Zeta Probe membranes. Membranes were hybridized with ^{32}P-labeled Mrf-2 probes. "C" indicates scrambled siRNA treated controls; "KD" indicates siMrf-2 treatment. (B) Real-time RT-PCR. Expression data are relative to 18S levels. The results shown are representative of at least two independent experiments.

C/EBPα mRNA after 12 h, and the decrease was sustained for at least 24 h (Fig. 2A). C/EBPα has two active translational isoforms arising from the same transcript: a full-length 42 kDa isoform (p42) and a truncated 30 kDa form (p30) [19]. The expression of both of these protein isoforms was analyzed by Western blots. As shown in Fig. 2B, the levels of both C/EBPα p42 and p30 were unaffected 12 h after transfection, but there was a substantial decrease of both isoforms by 24 h. Expression of PPARγ mRNA was also decreased by knockdown of Mrf-2 at 12 and 24 h after transfection (Fig. 2A). PPARγ has two transcriptional variants (PPARγ1 and PPARγ2), with PPARγ2 being the adipocyte specific form [20]. Western blots revealed that PPARγ1 did not change as a result of siRNA treatment, but PPARγ2 expression was decreased at 12 and 24 h after transfection with siMrf-2 (Fig. 2B). Thus, the knockdown of Mrf-2 decreased the expression of both PPARγ and C/EBPα in mature 3T3-L1-derived adipocytes, just as it did in differentiating 3T3-L1 cells [2].

Lipid droplets in adipocytes are coated with specific stabilizing proteins such as perilipin and FSP27, and decreased expression of these proteins increases basal lipolysis [9–12]. High levels of perilipin and FSP27 expression are dependent on PPARγ activity [7,8], and as expected, the decrease in PPARγ expression resulting from knockdown of Mrf-2 led to the suppression of these target genes (Fig. 2A and B).

Knockdown of Mrf-2 activates lipolytic activity in 3T3-L1-derived adipocytes

We next examined the effects of siMrf-2 on lipolytic activity in 3T3-L1-derived adipocytes. Cells were treated with siRNA's for 24 h, then incubated with serum-free growth medium supplemented with 2% BSA for 2 h. Glycerol and NEFA content in the conditioned media were then analyzed as measures of lipolytic activity. As shown in Table 1, both glycerol and NEFA release were significantly higher in siMrf-2-treated adipocytes than in controls,

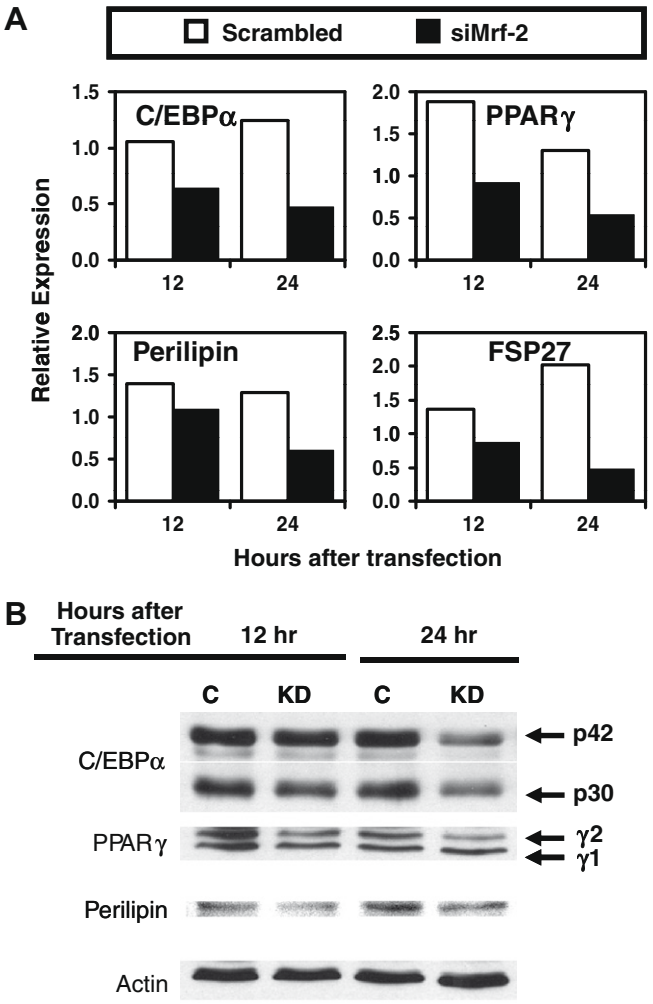

Fig. 2. Expression of adipogenic transcription factors and lipid droplet-associated proteins in 3T3-L1-derived adipocytes treated with scrambled siRNA and siMrf-2. Knockdown of Mrf-2 was performed as described in Fig. 1, and total RNA and whole cell extracts were prepared after 12 and 24 h. (A) Real-time PCR. Expression data are relative to 18S levels. (B) Western blots. Equal amounts of cellular protein (25 μg) were resolved by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blotted successively with antibodies to C/EBPα, PPARγ, perilipin and actin, as indicated. The results shown are representative of at least two independent experiments.

indicating that knockdown of Mrf-2 activated lipolysis. Interestingly, siMrf-2 also increased the ratio of glycerol released to NEFA released by about twofold (Table 1). Although there may be alternative explanations for this, our hypothesis is that suppression of Mrf-2 increases the rate of NEFA recycling into triglycerides.

Knockdown of Mrf-2 activates triglyceride synthesis and recycling of NEFA in 3T3-L1-derived adipocytes

We next measured triglyceride content and triglyceride synthesis in 3T3-L1-derived adipocytes treated with either scrambled siRNA or siMrf-2. Twenty-four hours after transfection, triglyceride content was not significantly different in scrambled siRNA- versus siMrf-2-treated cells (Table 1). Maintaining cellular triglyceride levels in the face of increased lipolysis would require a countervailing increase in triglyceride synthesis. To test this, we measured the incorporation of ^{14}C from glucose into triglyceride glycerol. By this measure, triglyceride synthesis was significantly higher in siMrf-2-treated 3T3-L1-derived adipocytes than in the controls (Table 1). As an independent test, we also measured incorporation of exogenous fatty acids into cellular triglycerides. As shown in Table 1,

Table 1
Triglyceride metabolism in 3T3-L1-derived adipocytes treated with siMrf-2.

Parameter	siRNAs used	
	Scrambled	Mrf-2 site 3
Glycerol release (nmol/2 h/mg protein)	29.8 ± 1.3	100.5 ± 10.5*,†
NEFA release (nmol/2 h/mg protein)	20.7 ± 2.1	32.6 ± 1.5*
Ratio of glycerol to NEFA released	1.48 ± 0.22	3.09 ± 0.31*
Triglyceride content (μmol/mg protein)	1.00 ± 0.047	0.94 ± 0.022NS
Triglyceride synthesis (nmol/2 h/mg protein)	45.6 ± 4.1	67.8 ± 1.4**
Fatty acid uptake (%)	53.6 ± 1.9	47.1 ± 1.6**
Fatty acid incorporation into cellular triglyceride (Ratio of DG + TG to total Radioactivity in neutral lipids)	0.50 ± 0.12	0.75 ± 0.14*

NS, not significant; compared with scrambled siRNA group.
† Results are expressed as means ± SE ($n = 3$).
* $p < 0.05$.
** $p < 0.01$.

exogenous oleic acid was incorporated into cellular diacylglycerides and triglycerides at a higher rate in siMrf-2-treated cells than in controls. Incorporation was increased despite the fact that fatty acid uptake into siMrf-2-treated cells was slightly decreased (Table 1). Taken together, these data indicate that triglyceride synthesis in 3T3-L1-derived adipocytes is stimulated by knockdown of Mrf-2. The simultaneous increase of lipolysis and triglyceride synthesis, coupled with the maintenance of total triglyceride content and an increased ratio of glycerol:NEFA release, all support the hypothesis that knockdown of Mrf-2 stimulates recycling of NEFA in 3T3-L1-derived adipocytes.

Discussion

We reported previously that induction of the adipogenic transcription factors C/EBPα and PPARγ is prevented and adipogenesis is inhibited when 3T3-L1 preadipocytes are treated with siMrf-2 before the addition of adipogenic hormones [2]. Here, we presented evidence that Mrf-2 is also required for maintenance of C/EBPα and PPARγ expression in mature 3T3-L1-derived adipocytes. We also found that knockdown of Mrf-2 in the mature cells increases lipolysis and triglyceride synthesis, resulting in an increased rate of NEFA recycling.

Complete hydrolysis of triglyceride yields glycerol and NEFA in a molar ratio of 1:3. NEFA that are released in this way can be secreted from the cell, consumed by β-oxidation or re-cycled into triglycerides [21]. Since the rate of β-oxidation is usually quite low in white adipocytes [22], the great majority of NEFA are secreted or re-cycled. In fact, up to 60% of NEFA are re-cycled into triglycerides in both mouse and human adipose tissues [21]. By contrast, the glycerol that is released by lipolysis cannot be re-cycled into triglycerides, since white adipocytes express vanishingly low levels of glycerokinase [Ref. 21, and our unpublished data]. Thus, the ratio of secreted glycerol to secreted NEFA provides an estimate of the rate of NEFA recycling. We found that in control 3T3-L1-derived adipocytes 29.8 nmol of glycerol per mg of cellular protein was released during the 2 h assay. In the absence of recycling, 89.4-nmol of NEFA would have been released. Since only 20.7 nmol were actually released (Table 1), it can be estimated that approximately 70 nmol of NEFA was re-cycled into triglyceride. Using the same calculation, it can be estimated that 270 nmol of NEFA was re-cycled in siMrf-2-treated adipocytes. Although these calculations imply that recycling of NEFA is higher in 3T3-L1-derived adipocytes treated with siMrf-2 than in the controls, we cannot exclude the possibility that NEFA are oxidized following knockdown of Mrf-2.

By analyzing both the incorporation of glucose-derived glycerol into triglycerides, and the incorporation of exogenous oleic acid into triglycerides and diglycerides, we showed that knockdown of Mrf-2 increases triglyceride synthesis by about 50% (Table 1). Since triglyceride synthesis is an energy-requiring process, the simultaneous increase of lipolysis and triglyceride synthesis could lead to energy dissipation (Fig. 3A). The activation of this "futile cycle" by knockdown of Mrf-2 resembles the effects of leptin and thiazolidinediones, which also accelerate both lipolysis and triglyceride synthesis [23–25]. *In vivo* studies in humans have shown that lean subjects have a higher rate of NEFA recycling [26,27]. These observations suggest that enhanced NEFA recycling may have a

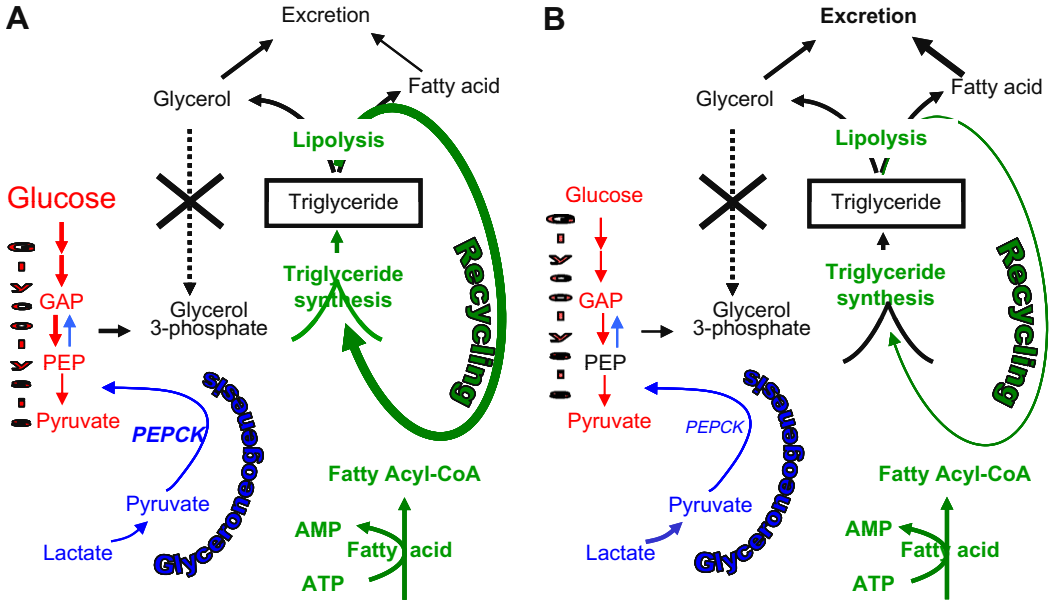

Fig. 3. Putative mechanisms for the alteration of triglyceride metabolism in adipocytes caused by knockdown of Mrf-2. The effects of Mrf-2 ablation on triglyceride metabolism are shown for high glucose (A) and low glucose conditions (B). (A) Under high glucose conditions, both lipolysis and triglyceride synthesis were activated by knockdown of Mrf-2 as indicated by green arrows, suggesting that an energy-dissipating futile cycle (green pathway) is activated. Glycerol from lipolysis cannot be re-cycled due to the absence of glycerokinase (X), but glyceraldehyde 3-phosphate (GAP) from glycolysis (red pathway) provides glycerol 3-phosphate. (B) When glucose supplies are limiting, lactose and pyruvate can supply glycerol 3-phosphate through glyceroneogenesis (blue pathway). In Mrf-2-deficient cells, however, lower levels of PEPCK could reduce the supply of glycerol 3-phosphate, inhibit recycling and lead to the excretion of free fatty acids.

protective effect against the development of obesity and type 2 diabetes.

We did not observe a significant reduction of triglyceride content in adipocytes treated with siMrf-2. This implies that the increase in lipolysis is balanced by the increase in triglyceride synthesis in siMrf-2-treated adipocytes. In the data presented in Table 1, the rate of lipolysis (100 nmol/2 h/mg protein) exceeds the rate of triglyceride synthesis (67.4 nmol/2 h/mg protein) in siMrf-2-treated cells. It should be noted, however, that lipolysis was measured in the presence of 25 mM glucose, and triglyceride incorporation was measured in the presence of 5 mM glucose. It is reasonable to assume that the rate of triglyceride synthesis would be higher in DME high glucose, but whether this would exactly match the rate of lipolysis cannot be determined from our data. It is possible that more prolonged knockdown of Mrf-2 expression would reveal subtle differences in the rates of lipolysis and triglyceride synthesis, and result in changes in total triglyceride content. Because the cells in these experiments were cultured in DME high glucose, there was a constant supply of glycerol-3-phosphate from glycolysis. In the adipocytes of fasted mice, however, the main supply of glycerol-3-phosphate is glyceroneogenesis [21]. PEPCK is the rate-limiting enzyme in this pathway, and adipose-specific over-expression of PEPCK leads to obesity in mice [28]. This underscores the potential importance of adipocyte fatty acid recycling in the regulation of total body energy balance. PEPCK expression is significantly reduced in siMrf-2-treated 3T3-L1 cells, in *Mrf-2*$^{-/-}$ mouse embryo fibroblasts [2], and in adipose tissue from *Mrf-2*$^{-/-}$ mice [Whitson, unpublished data]. This could decrease the amount of glycerol-3-phosphate available for NEFA recycling and accelerate the depletion of triglycerides during fasting in *Mrf-2*$^{-/-}$ mice (Fig. 3B).

Mice lacking Mrf-2 have a lean phenotype with reductions in both brown and white adipose tissues [1]. We have postulated that one cause of the lean phenotype is a defect in adipocyte differentiation [2], but other factors may contribute. In this study, we found that knockdown of Mrf-2 activated an energy-dissipating cycle involving hydrolysis and synthesis of triglyceride in adipocytes. This may contribute to the higher basal metabolic rate we have observed in Mrf-2 knockout mice [Whitson, unpublished data]. Further investigations into the molecular mechanisms by which loss of Mrf-2 expression leads to these metabolic perturbations may result in novel strategies for the treatment of obesity and diabetes.

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