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ORIGINAL ARTICLE

Glucose regulates RMI1 expression through the E2F pathways in adipose cells

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Abstract RecQ-mediated genome instability 1 (RMI1) has been identified as a novel energy homeostasis-related molecule. While recent studies have suggested that change in RMI1 expression levels in adipose tissue may affect the body's energy balance, no reports have identified the mechanism behind this expression regulation. In the present study, we found that RMI1 expression increased on differentiation of 3T3-L1 fibroblasts to adipocytes. In addition, glucose stimulation induced RMI1 expression to approximately eight times the baseline level. Further, knockdown of either E2F5 or E2F8 mRNA using siRNA suppressed this glucose-induced up-regulation of RMI1 expression. These results suggest that RMI1 expression may be regulated by glucose, at least in part, via E2F expression.

Keywords RMI1 · Adipocyte hyperplasia · E2F

Introduction

We recently reported that recQ-mediated genome instability 1 (RMI1) plays an important role in regulation of energy homeostasis [1]. RMI1-deficient mice were found to be resistant to high-fat diet- and genetics-related obesity, exhibiting less abdominal fat and less food intake. In addition, RMI1 expression was found to be increased in the abdominal fat and liver of obese mice. Expression levels of various regulatory factors have been reported to be increased on development of metabolic disorders [2–4]. Taken together, these findings suggest that change in RMI1 expression level may be associated with the development of energy homeostasis disorders [1].

RMI1, an enzyme-binding protein, has previously been reported to mediate DNA recombination, chromosome organization, and biogenesis as well as to regulate cell-cycle checkpoint machinery [5–7]. In fact, we recently found that a deficiency in RMI1 could lead to decreased expression of E2F mRNA [1], a well-documented cell cycle-related molecule [8, 9]. E2F family members play a major role during the G1/S transition in the mammalian cell cycle. In addition, it has been reported that the E2F plays a central role in preadipocyte proliferation, leading to increased fat mass, and obesity [10, 11]. Given these previous findings, we therefore hypothesize that RMI1 modulates energy homeostasis via regulation of cell cycle progression in metabolic tissues.

Here, to determine the mechanism of RMI1 expression regulation, we examined inducers of RMI1 expression in adipose cells. In addition, we investigated the relationship between E2F molecules and RMI1 expression level.

Materials and methods

Differentiation induction

3T3-L1 fibroblasts, purchased from ATCC (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) until a state of density arrest at 2 days post-confluence was attained on 24-well plates. Growth medium was replaced at density arrest with the standard

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differentiation induction mixture containing insulin (10 mg/ml), dexamethasone (0.25 mM), and 3-isobutyl-1-methylxanthine (0.5 mM). At 2 days after initiation of differentiation induction, the medium was changed to DMEM supplemented only with 10% FBS. Cells were extracted with RLT reagent (Qiagen, Tokyo, Japan) at each time point after differentiation induction (0, 3, 6, 8, 10, 13, 15, and 17 days post-induction), and the total RNA was immediately purified using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) and following the manufacturer's instructions. Additionally, any contaminating genomic DNA in the RNA preparation was removed by DNase digestion of purified RNA using RNase-Free DNase Set (Qiagen, Tokyo, Japan) following the manufacturer's instructions. RNA purity was confirmed based on the ratio of the readings at 260 and 280 nm using a spectrometer Safire (TECAN, Switzerland). Total RNA (250 ng) was used in cDNA synthesis using SuperScript III (Invitrogen, Carlsbad, CA, USA).

Exploration of RMI1 expression inducers

At Day 10 post-induction, 3T3-L1 adipocytes, or HepG2 and SW872 cells (1×10^5 cells/well), purchased from ATCC (Manassas, VA, USA), were prepared on 24-well plates. After 24-h culture at 37°C, the cells were washed with phosphate-buffered saline and cultured in growth medium without FBS or glucose. After 24 h, several stimulants, including glucose, insulin, TNF-a, and palmitate, were added to the culture medium, and the cells were further cultured for 10 h. Cells were then extracted, and total RNA was purified as previously described.

Short interfering RNA (siRNA) knockdown

SW872 cells (4×10^4 cells/well) were prepared on 24-well plates. After 24 h in culture at 37°C, 15-nM siRNA strands were transfected into the cells using Lipofectamine RNAiMAX (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. At 5 h post-transfection, the medium was changed to DMEM supplemented with or without 4 mM glucose. After 20 h, cells were extracted, and total RNA was purified as previously described. All siRNA were purchased from Qiagen (Tokyo, Japan). E2F1: Hs_E2F1_6, SI02664410. E2F3: Hs_E2F3_6, SI03066966. E2F4: Hs_E2F4_5, SI02654694. E2F5: Hs_E2F5_1, SI00030436 and Hs_E2F5_7, SI03102141. E2F6: Hs_E2F6_1, SI00375445. E2F7: Hs_E2F7_3, SI00375487. E2F8: Hs_FLJ23311_1, SI00136885 and Hs_FLJ23311_4, SI00136864. Transcription factor DP1 (TFDP1): Hs_TFDP1_2, SI00094654. TFDP2: Hs_TFDP2_1, SI00086933. The negative control siRNA was SI03650325.

mRNA expression analysis

Real-time quantitative polymerase chain reaction (PCR) analysis was used to determine the relative mRNA expression levels of the mouse RMI1 (NM_028904), human RMI1 (NM_024945), human E2F1 (NM_005225), E2F3 (NM_001949), E2F4 (NM_001950), E2F5 (NM_001951), E2F6 (NM_198256), E2F7 (NM_203394), E2F8 (NM_024680), TFDP1 (NM_007111), and TFDP2 (NM_001178139). The SYBR green assay was carried out in a total volume of 20 µl containing each primer at 0.1 µM, 10 µl SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), and 2 µl of 10-fold diluted cDNA with a PRISM 7900 sequence detector based on the manufacturer's protocol (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The PCR conditions were: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles with denaturation for 15 s at 95°C and annealing, and extension for 1 min at 59°C. The analyses were performed in duplicate. No PCR signals were observed in the PCR condition without reverse transcription. The target mRNA expression levels were expressed as ratio to the reference gene: mouse acidic ribosomal phosphoprotein P0 (NM_007475) (mP0) (Fig. 1) or human β-actin (NM_001101) (Figs. 2, 3). Sequences of primers used for the respective genes are shown in Table 1.

Statistical analysis

The data represent the mean ± S.D. Statistical significance of between-groups was assessed using Dunnett's test for multiple comparison, with significance set at $P < 0.05$. All analyses were conducted using the SAS 8.2 software package (SAS Institute Japan, Ltd., Tokyo, Japan).

Results

Change in RMI1 expression in adipose cells

We first investigated the change in RMI1 expression levels during adipogenesis in mouse 3T3-L1 fibroblasts. Differentiation from fibroblast to adipocyte induced RMI expression in a time-dependent manner. At 17 days post-differentiation, the expression was 10 times greater compared to non-differentiated cells (Fig. 1a). We then explored induction of RMI1 expression in 3T3-L1 adipocytes. As shown in Fig. 1b, glucose stimulation strongly and concentration-dependently increased RMI1 expression, up to a 1.5-fold increase. This up-regulation of RMI1 by glucose was also observed in 3T3-L1 fibroblasts (data not shown). In contrast, treatment with tumor necrosis factor-α

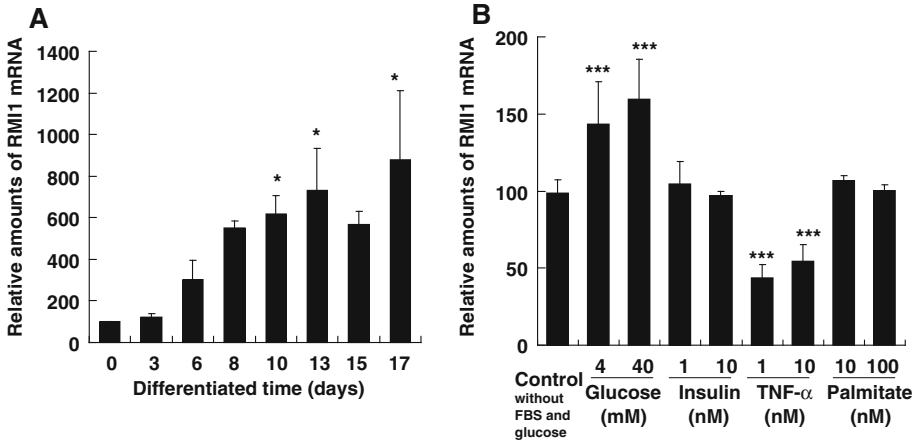

Fig. 1 RMI1 mRNA expression change in 3T3-L1 adipocytes. 3T3-L1 fibroblasts differentiated into adipocytes, and total RNA was extracted after the indicated duration (**a**). Differentiated 3T3-L1 adipocytes were stimulated using the indicated factors for 10 h, after which total RNA was extracted (**b**). Expression levels of RMI1 mRNA were analyzed using quantitative PCR. Values are the mean ± S.D. ($n = 3$), asterisks indicate significant differences: $^{*}P < 0.05$, $^{***}P < 0.001$ versus Day 0 (**a**) or control (**b** growth medium only without FBS and glucose), and the data shown is representative of three independent experiments

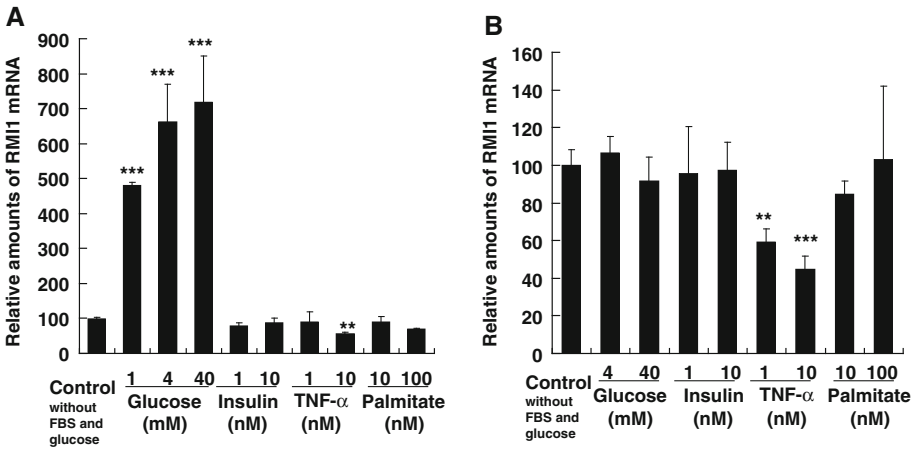

Fig. 2 RMI1 mRNA expression change in SW872 or HepG2 cells. SW872 (**a**) or HepG2 (**b**) cells were stimulated using the indicated factors for 10 h, after which total RNA was extracted. Expression levels of RMI1 mRNA were analyzed using quantitative PCR. Values are the mean ± S.D. ($n = 3$), asterisks indicate significant differences: $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (growth medium only without FBS and glucose), and the data shown is representative of three independent experiments

(TNF-α) reduced RMI1 expression, while insulin and palmitate exerted no effects at all.

On examination of specificity in expression regulation, we found that addition of glucose significantly and strongly induced RMI1 expression in a human adipocytic cell line (SW872), up to an approximately 8-fold increase (Fig. 2a). In contrast, TNF-α suppressed RMI1 expression levels in these cells (Fig. 2a). However, in contrast to these findings in adipocytic cells, glucose was found to have no ability to induce RMI1 expression in a human hepatocytic cell line (HepG2; Fig. 2b), whereas TNF-α retained its ability to reduce expression (Fig. 2b). In addition, treatment with glucose also failed to increase RMI expression levels in a human embryonic kidney cell line (HEK293; data not shown).

Relationship between E2F molecules and RMI1 expression

We investigated the potential association between E2F molecules and change in RMI1 expression by performing knockdown of E2F family molecules (E2F1, -3, -4, -5, -6, -7, -8, and TFDP1, -2) using siRNA in SW872 cells treated with glucose. E2F5 and E2F8 knockdown suppressed glucose-induced RMI1 expression in these cells by approximately 35% (Fig. 3a), and we confirmed that this knockdown phenotype is not due to the off-target effect using two different siRNA sequences for E2F5 and E2F8 (data not shown). We assessed the suppression of target molecules on treatment with siRNA and found that the

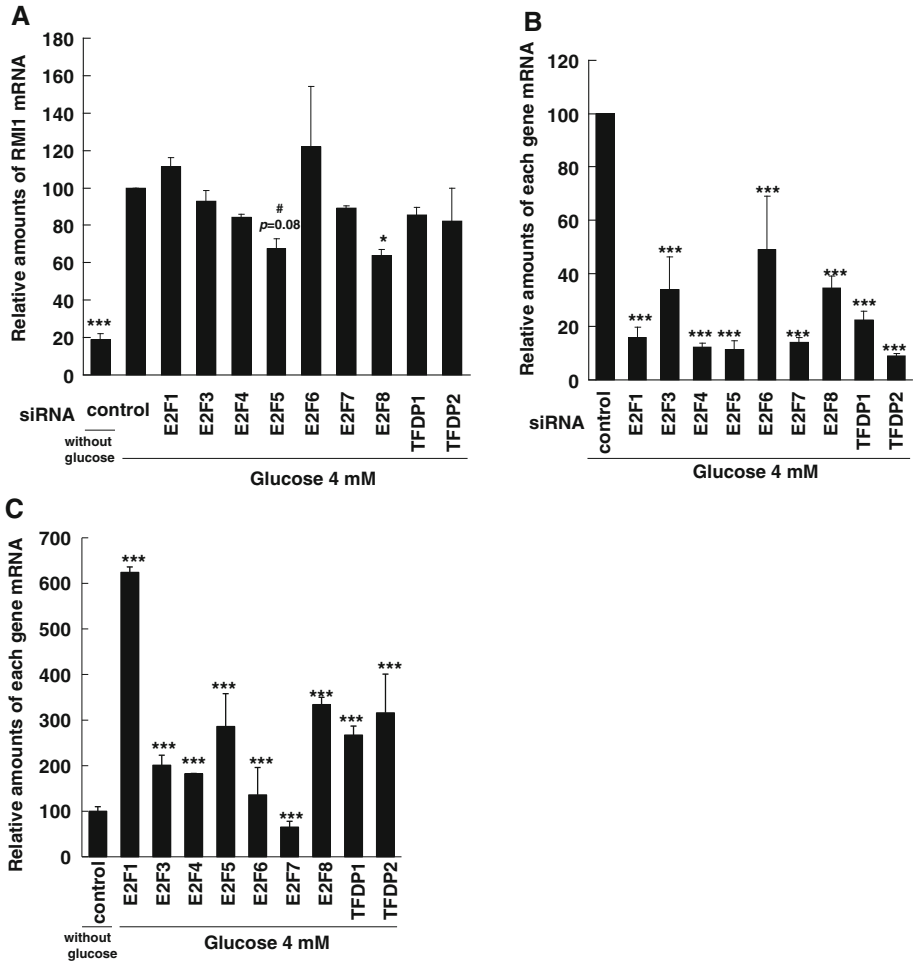

Fig. 3 Effect of E2F knockdown on RMI1 expression. SW872 cells were transfected with siRNA targeted against E2F genes. The transfected (**a**, **b**) or non-transfected (**c**) cells were treated with or without 4 mM glucose, after which total RNA was extracted. Expression levels of RMI1 (**a**) and E2F (**b**, **c**) mRNA were analyzed using quantitative PCR. Values are the mean ± S.D. ($n = 3$), asterisks indicate significant differences: $*P < 0.05$, $***P < 0.001$ versus control (**a**, **b** medium with glucose 4 mM, **c** growth medium only without FBS and glucose), and the data shown is representative of three independent experiments

target mRNA molecules were suppressed by more than 60% (Fig. 3b). In addition, expression of E2F family mRNA increased on addition of glucose, except for E2F7 (Fig. 3c).

Discussion

In the present study, we found that RMI1 expression increased during differentiation of fibroblasts to adipocytes. Treatment with glucose was found to increase RMI1 expression in 3T3-L1 and SW872 cells, but not in HepG2 or HEK293 cells. Further, this glucose-induced up-regulation of RMI1 was suppressed on knockdown of E2F5 or E2F8 genes. Taken together, these results suggest that RMI1 expression level is indeed regulated by glucose via E2F expression.

In a previous investigation, we suggested that RMI1 expression may play a role in controlling energy balance in adipocytes [1]. We demonstrated that RMI1 expression was up-regulated in abdominal fat tissue of obese and diabetic mice, suggesting that RMI1 may be associated with the development of lipid accumulation of metabolic tissues, leading to obesity [1]. However, despite these findings, the specific molecular mechanisms regulating RMI1 expression in adipose tissue remain unknown. Here, we found for the first time that the amount of RMI1 mRNA increased following adipogenesis. TNF-α has been found capable of regulating adipogenesis by inhibiting preadipocyte differentiation [12]; indeed, RMI1 expression was significantly suppressed on treatment with TNF-α. Taken together, these results suggest that RMI1 is associated with adipogenesis.

We explored inducers responsible for RMI expression under obese and diabetic conditions. Obesity and diabetes are known to have high correlation with hyperglycemia, insulinemia, and lipidemia. Our investigation into the effect of factors such as glucose, insulin, and fatty acid on RMI1 expression levels showed glucose to be a strong regulatory factor of RMI1 expression, a mechanism specific to adipose cells. We also suspected that E2F transcription factors might function as upstream molecules, and found that E2F5 and E2F8 mRNA expression increased on treatment with glucose, just as was seen with RMI1, and

Table 1 Primer sequences

Mouse RMI1	5′-primer; cctagcccacccagatacct
	3′-primer; agacgccattatcctctgcttct
Human RMI1	5′-primer; acttgagtcaggcccaaatg
	3′-primer; acattcgtgaaggctttgct
E2F1	5′-primer; catccagctcattgccaagaag
	3′-primer; gatcccacctacggtctcctca
E2F3	5′-primer; agggcccattgaggtttact
	3′-primer; gaggccagaggagaaaggtt
E2F4	5′-primer; cccacaggtgttttggaact
	3′-primer; caggcacatcaaagaggtca
F2F5	5′-primer; ctggaggtacccattccaga
	3′-primer; tgttgctcaggcagattttg
E2F6	5′-primer; caggccttccatgaacagat
	3′-primer; cttcttcctcagggccttct
E2F7	5′-primer; aaggccaagcagaaaacaga
	3′-primer; cagcgactccagcacattta
E2F8	5′-primer; ccaccacagcaaatatcgtg
	3′-primer; ctttggcctcaggtaatcca
TFDP1	5′-primer; tcgtcaacaccagcaagaag
	3′-primer; tggccattttaaggtcttcg
TFDP2	5′-primer; ggagtcaggcaaatgctctc
	3′-primer; gctaaggccacttctgcatc
mP0	5′-primer; cagatgcagcagatccgc
	3′-primer; tgttcttgcccatcagcac
β-actin	5′-primer; ctggcacccagcacaatg
	3′-primer; ccgatccacacggagtacttg

knockdown of these genes suppressed RMI1 expression. Computer analysis has shown evidence of E2F response element consensus sites in the RMI1 promoter (unpublished observation), suggesting that E2F may transactivate the RMI1 promoter. We previously reported that RMI1 deficiency suppressed E2F8 expression [1]. Taken together, these results indicate that RMI1 expression is indeed regulated positively by E2F feedback machinery in adipose tissue, influencing preadipocyte proliferation.

Recently, a number of adipogenesis-related genes have been found to be affected by glucose. Adiponutrin, an unsecreted protein specifically synthesized by adipocytes, is strongly induced during adipocyte differentiation, and its expression is strongly and rapidly regulated by insulin and glucose in humans [13, 14], a regulatory mechanism similar to that of RMI1. Carbohydrate response element-binding protein (ChREBP), which is a member of the basic helix–loop–helix leucine zipper transcription factor family, is activated in response to high-carbohydrate feeding and subsequently initiates gene transcription of glucose metabolism- and lipogenic-related molecules in the liver [15, 16]. ChREBP expression is also up-regulated by insulin and glucose in 3T3-L1 adipocytes [17]. Fibroblast growth factor 10 (FGF10), a member of the FGF family secreted by cultured 3T3-L1 preadipocytes, plays a crucial role in enhancing both adipogenesis and preadipocyte proliferation [18, 19]. Clarification of the molecular mechanisms underlying RMI1 regulation and the physiological significance of this phenomenon will require further investigation into the relationship between RMI1 and glucose-responsive and adipogenesis-related molecules.

In conclusion, we demonstrated that RMI1 expression is strongly regulated by glucose and E2F transcription factors. Although the physiological role of this protein has not yet been clarified, in vivo and in vitro results suggest that RMI1 may regulate whole body energy balance through cell cycle modulation via a glucose-responsive mechanism. As the next step, an additional future study would have to focus on the discovery of the physiological function and meaning of RMI1 induction in adipocyte. Findings from the present study may facilitate the development of a novel therapeutic strategy for treating disorders linked to energy homeostasis, such as obesity.

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Conflicts of interest The authors have no conflicts to disclose.

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