*Cytotherapy*, 2013; 15: 971–978

International Society for Cellular Therapy
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# Modulation of glucose and lipid metabolism by porcine adiponectin receptor 1–transgenic mesenchymal stromal cells in diet-induced obese mice

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**Abstract**

*Background aims.* Obesity and its associated diseases demand better therapeutic strategies. Regenerative medicine combined with gene therapy has emerged as a promising approach in various clinical applications. Adiponectin (ApN) and its receptors have been demonstrated to play beneficial roles in modulating glucose and lipid homeostasis. In the current study, we tested such an approach by transplanting mesenchymal stromal cells (MSCs) from porcine ApN receptor (pAdipoR) 1-transgenic mice into high-fat/sucrose diet (HFSD)-fed mice. *Methods.* Twenty 6-week-old Friend virus B/NJNarl male mice were randomly assigned into four groups with the control fed a chow diet (chow) and others HFSD for 10 months. The HFSD groups were then intraperitoneally injected once per week for 8 weeks with placebo (200 μL phosphate-buffered saline), wild-type MSC (WT-MSC, $2 \times 10^6$ cells/200 μL phosphate-buffered saline) or pAdipoR1-transgenic MSC (pR1-tMSC, $2 \times 10^6$ cells/200 μL phosphate-buffered saline), respectively. Body weights, blood samples, tissue histology, and gene expression and protein levels of metabolism-associated genes were analyzed. *Results.* Both WT-MSC and pR1-tMSC transplantations restored the messenger RNA expression of AdipoR1, with those of glucose transporter 4 and 5′-adenosine monophosphate-activated protein kinase catalytic subunit α-1 and protein levels of pyruvate kinase induced by pR1-tMSC in the muscles of HFSD-fed mice. In the liver, both WT-MSC and pR1-tMSC ameliorated HFSD-induced hepatosteatosis, with the gene expression of lipoprotein lipase and hormone-sensitive lipase upregulated by the latter. Lastly, pR1-tMSC transplantation reduced fatty acid synthase mRNA levels in the adipose tissues of HFSD-fed mice. *Conclusions.* This study demonstrates the modulatory actions of MSC and pR1-tMSC on genes associated with glucose and lipid metabolism and provides insights into its therapeutic application for obesity-associated metabolic complication.

**Key Words:** *adiponectin receptor 1, diet-induced obesity, gene therapy, mesenchymal stromal cells*

## Introduction

As the result of imbalanced diets and sedentary lifestyles, obesity-associated metabolic syndrome, including type 2 diabetes, insulin resistance, hypertension, atherosclerosis and nonalcoholic fatty liver disease, poses serious health problems for the modern society (1). Therefore, novel therapeutic approaches are needed. Mesenchymal stromal cells (MSCs) are defined as a heterogeneous cell population that is capable of self-renewing and differentiating into multiple cell types (2,3). These cells migrate to specific tissues during injury or secrete chemo-attractants (2,3) and have immuno-modulatory properties that allow for safe clinical applications (4). These unique properties make MSC the ideal candidates for regenerative medicine; they have been demonstrated to lead to improved prognosis of many diseases including type 1 diabetes, kidney injury and the infarcted heart (5–7). Moreover, their therapeutic potential can be further enhanced by integrated functional genes, such as CXCR4 (CXC chemokine receptor 4) or Akt1 (thymoma viral proto-oncogene 1) (8,9).

Adiponectin (ApN), a cytokine secreted from the adipose tissue, has previously been shown to have anti-atherogenic, anti-diabetic and insulin-sensitizing properties (10). Moreover, decreased circulating ApN

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*(Received 24 January 2013; accepted 13 March 2013)*


http://dx.doi.org/10.1016/j.jcyt.2013.03.008

concentrations were found to be associated with insulin resistance, obesity and type 2 diabetes (11,12), whereas administration of ApN to mice decreases plasma levels of glucose, free fatty acids and triglycerides, increases muscle fatty acid oxidation and induces weight loss (10). Originally cloned from human and mouse, the ApN receptors 1 (AdipoR1) and 2 are the receptors mediating the action of ApN through adenosine monophosphate-activated protein kinase (10). The expression of AdipoR1 has been shown to be regulated by peroxisome proliferator-activated receptor-γ (PPARγ) ligands or a combination of ligands for PPARα and PPARγ in obese patients and mice (13–15). By studying energy metabolism, adipogenesis and lipid metabolism in pigs, we initially cloned the genes encoding porcine ApN and its receptors (16) and subsequently generated the porcine AdipoR1-transgenic mice, which were shown to be resistant to high-fat/sucrose diet (HFSD)-induced obesity (Liu and Ding, unpublished data, 2009).

The aforementioned therapeutic potential of gene-modified MSC and beneficial actions of ApN and its receptors in metabolic syndrome led us, in the current study, to test the therapeutic potential of MSC isolated from our pAdipoR1-transgenic mice for obesity and its associated complications in HFSD-fed mice.

## Methods

### *Isolation, differentiation and characterization of murine MSC*

Bone marrow cells were flushed from the femurs and tibiae of 8-week-old Friend virus B/NJNar1 mice (purchased from the National Laboratory Animal Center, Taipei, Taiwan) and pAdipoR1-transgenic mice (Liu and Ding, unpublished data, 2009) killed by cervical dislocation. MSC were purified with the use of the transient lower-density plastic adherence strategy and used as the sources for cell therapy (17).

To characterize MSC, the cells were grown to confluence in 12-well culture plates containing minimum essential medium-α (Sigma-Aldrich, St Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) at 37°C in a humidified chamber in air containing 5% $CO_2$. For adipocyte differentiation, 24 h after confluence, the medium was changed to differentiation medium containing 10 μg/mL insulin (from bovine pancreas, Sigma-Aldrich), 1 μmol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 100 μmol/L indomethacin and 10% FBS. The differentiation medium was changed every 3 days. For osteoblast differentiation, the medium contained 0.1 μmol/L dexamethasone, 10 mmol/L glycerol-2-phosphate, 50 μmol/L ascorbate-2-phosphate and 10% FBS and was changed every 3 days. Oil red O staining and alizarin red staining were used to characterize lipid deposition during adipogenesis and calcium deposition during osteogenesis, respectively (18). For flow cytometry analysis, MSC were detached from the plate with the use of 0.25% trypsin/ethylenediamine tetra-acetic acid and stained with antibodies against cluster of differentiation (CD)11b, CD29, CD44, CD45, Sca-1, class I major histocompatibility complex (MHC) or class II MHC molecules (eBioscience, San Diego, CA, USA) for 30 min. After fixation, cells were stained by incubation with the appropriate antibody, and at least 10,000 cells were used for flow cytometry in a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA) with isotype used as control.

### *RNA extraction and real-time reverse transcription polymerase chain reaction*

Total RNA was extracted from animal tissues or cells through the use of the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Samples were digested with DNase I (Ambion, Austin, TX, USA) at 37°C for 30 min to remove genomic DNA contamination and then reverse transcribed (RT) with the use of a high-capacity complementary (c)DNA RT kit (Applied Biosystems, Foster City, CA, USA). The transcribed complementary cDNA was amplified by polymerase chain reaction (PCR), and the DNA products were reacted with SYBR green. Conditions for RT-PCR reactions were initial denaturation at 95°C for 7 min and 39 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds. Amplification of specific transcripts was confirmed by melting-curve profile analysis and agarose gel electrophoresis. The messenger (m)RNA levels of each gene were normalized through the use of β-actin levels in the same sample and calculated by the formula $(1/2)^{\text{Ct target genes} - \text{Ct } \beta\text{-actin}}$ (19). Sequences of specific PCR primers for target gene amplication are listed in the Supplemental Table.

### *Western blot analysis*

Tissues were homogenized in radio-immunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA); protein concentrations were determined with the use of Bradford reagent. Twenty to 40 μg of protein was used for 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis; the proteins were then transferred onto a polyvinylidene fluoride membrane (Perkin Elmer, Norwalk, CT,

USA) at 400 Ma for 70 min in 20% methanol, 192 mmol/L glycine and 25 mmol/L Tris/HCl. After transfer, the membrane was blocked with gelatin-NET (0.25% gelatin, 0.15 mol/L NaCl, 5 mmol/L ethylenediamine tetra-acetic acid 2Na, 0.05% Tween-20 and 50 mmol/L Tris) for 2 h at room temperature. After three washes with phosphate-buffered saline containing 0.1% Tween-20 (PBS/T), membranes were incubated with gentle agitation at 4°C overnight with the primary antibody at 1:1000 dilution for pyruvate kinase M1 and M2 isoform (Cell Signaling Technology) or 1:5000 dilution of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA sc-47778). After three washes with PBS/T, the membrane was incubated with the appropriate secondary antibody at room temperature for 1 h, briefly incubated with a chemiluminescence reagent (Immobilon Western, Millipore, Billerica, MA, USA) and exposed to the UVP BioSpectrum Imaging System (Upland, CA, USA). Target proteins were quantified by means of the UVP BioSpectrum Imaging System and referenced to the expression of β-actin in the same sample.

### *Histological analysis*

After 8 weeks of cell transplantation, paraffin-embedded liver sections were deparaffinized with xylene and then rehydrated with a graded series of ethanol dilutions and stained with hematoxylin and eosin. The accumulation of lipid droplets in the liver tissue was determined by ImageJ software. Five images were randomly selected for each liver section at ×400 magnification and quantified for the area percentage of the lipid droplets. The method for lipid droplet quantification is followed by Chen and Farese (20).

### *In vivo experiments*

Twenty 6-week-old Friend virus B/NJNarl male mice were randomly assigned into four groups with the control (C) fed a chow diet and the other three groups fed a HFSD (No. 58V8, TestDiet, Richmond, IN, USA) for 10 months. Mice were kept at 26°C with a 12 h/12 h light/dark cycle and fed *ad libitum* with water. After 10-month feeding, the HFSD diet groups were intraperitoneally injected once per week for the 8 weeks with placebo (200 μL PBS), wild-type MSC (WT-MSC, $2 \times 10^6$ cells/200 μL PBS) or porcine AdipoR1-transgenic MSC (pR1-tMSC, $2 \times 10^6$ cells/200 μL PBS), respectively. Body weights were measured each week. After 8 weeks of treatment, mice were killed by cervical dislocation, and the livers, epididymal fat pads, perirenal fat and thigh muscles were collected for gene expression analysis and tissue samples. All experimental procedures were approved by the Institutional Animal Care and Use Committee at National Taiwan University.

### *Glucose tolerance test*

After the 8-week treatment with MSC, mice were fasted for 12 h and injected intraperitoneally with glucose (2 mg/g body weight) in PBS. Blood samples were taken from the tail vein at 0, 15, 30, 60 and 120 min after glucose injection and measured for glucose levels with the use of a blood glucose meter (Roche, ACCU-CHEK Active, Roche Diagnostics, Mannheim, Germany). Total area under the time × glucose concentration curve was also calculated.

### *Statistical analysis*

All values are expressed as mean ± SEM. Differences between two groups were assessed by unpaired two-tailed *t* tests. Results involving more than two groups were assessed by one-way analysis of variance procedure. The Dunnett significant difference test was used to evaluate differences among means (GraphPad Prism 5, Version 5.01, La Jolla, CA, USA). A significant difference was considered at $P \leq 0.05$.

## Results

### *Isolation and characterization of MSC*

The multipotency of MSC was confirmed by the existence of surface markers CD44, Sca-1, CD29 and MHC-I, analyzed by flow cytometry and demonstrated by their capabilities to differentiate into osteoblasts or adipocytes under the appropriate differentiation conditions (Supplemental Figure). Figure 1 confirms the overexpression (~2-fold) of AdipoR1 gene in pR1-tMSC compared with WT-MSC.

### *Effects of MSC implantation on HFSD-induced body weight gain and blood glucose levels*

As expected, HFSD feeding induced greater body weight gain, and this was not significantly affected by MSC transplantation (Figure 2A). Fasting glucose concentrations were decreased in the pR1-tMSC group compared with the PBS group (Figure 2B); the total area under the glucose tolerance curve tended to be lowest ($P = 0.07$) in mice treated with pR1-tMSC (Figure 2C,D).

### *Effects of MSC transplantation on glucose metabolism—associated genes in the muscles of HFSD-fed mice*

Because the muscle is one of the major organs for glucose/energy metabolism, we analyzed the mRNA

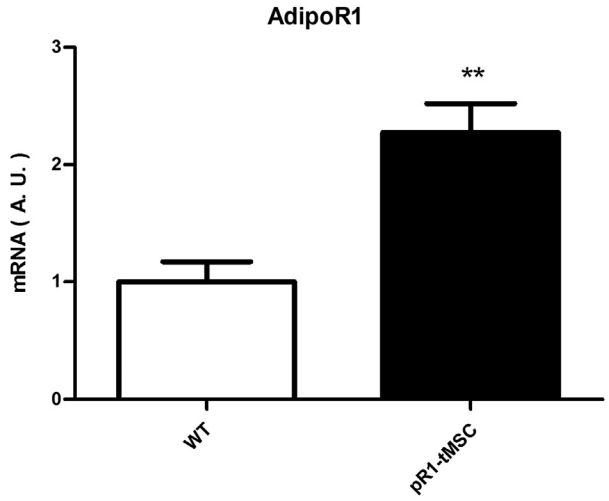


Figure 1. Overexpression of transgenic pAdipoR1 in pR1-tMSCs. Quantification of gene expression of ApNR1 in WT-MSC (WT) and pR1-tMSCs. Total RNA extracted from MSC was analyzed by RT-(quantitative)PCR with primers corresponding to AdipoR1 sequences of both mouse and pig. Data were normalized to $\beta$-actin and expressed as relative mean ± SEM. $^{**}P \leq 0.01$ ($n = 4$).

expression of several genes associated with this area and found that HFSD treatment lowered the expression of AdipoR1, which can be restored by MSC treatments, especially pR1-tMSC (Figure 3B). pR1-tMSC increased the mRNA expression of glucose transporter 4 compared with PBS (Figure 3A). Moreover, it also increased those of 5′-AMP-activated protein kinase catalytic subunit α-1 (AMPKα1) and 5′-AMP-activated protein kinase catalytic subunit α-2 (AMPKα2) compared with the control (or chow diet) and PBS, respectively (Figure 3C,D). Despite the lack of significant difference in the mRNA expression of glycolytic genes, such as pyruvate kinase II and hexokinase II, compared with the PBS group (data not shown), Western blot analysis of pyruvate kinase protein indicated a greater level in the pR1-tMSC treatment group compared with control, PBS and WT-MSC (Figure 3F).

### *MSC transplantation reduces hepatic lipid droplets and increases the expression of lipolytic genes in HFSD-fed mice*

Hepatosteatosis is one of the metabolic complications induced by HFSD and can be ameliorated by weight loss. Despite the lack of effects of MSC transplantation on HFSD-induced body weight gain (Figure 2A), histological analyses of the liver sections revealed that whereas the area of lipid droplets was significantly increased by HFSD feeding, it was significantly decreased by both WT-MSC and pR1-tMSC treatments compared with the PBS group (Figure 4A,B). Although the mRNA expressions of glucose metabolism—related genes such as

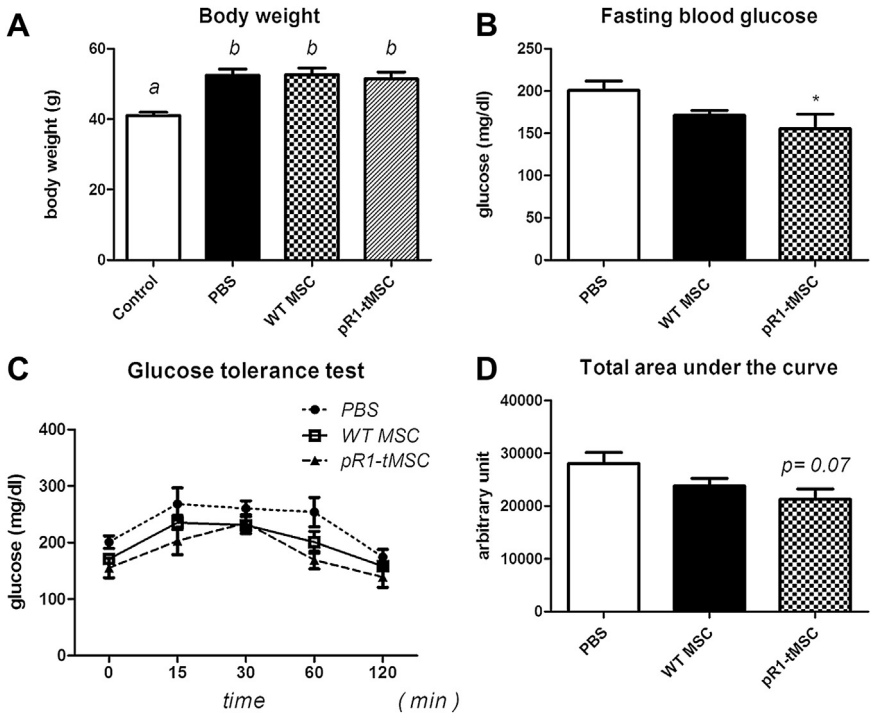


Figure 2. Effects of pR1-tMSC transplantation on body weight and blood glucose levels of HFSD-fed mice. (A) Body weight, (B) fasting blood glucose levels, (C) glucose tolerance test and (D) total area under the curve were measured and calculated as described in the text. Mice fed a HFSD were treated with PBS, WT-MSC or pR1-tMSC for 8 weeks, whereas the control was fed a chow diet. Data are expressed as mean ± SEM. Different letters indicate statistical significance, $P \leq 0.05$ (PBS versus MSC, $n = 6$). $^{*}P \leqq 0.05$.

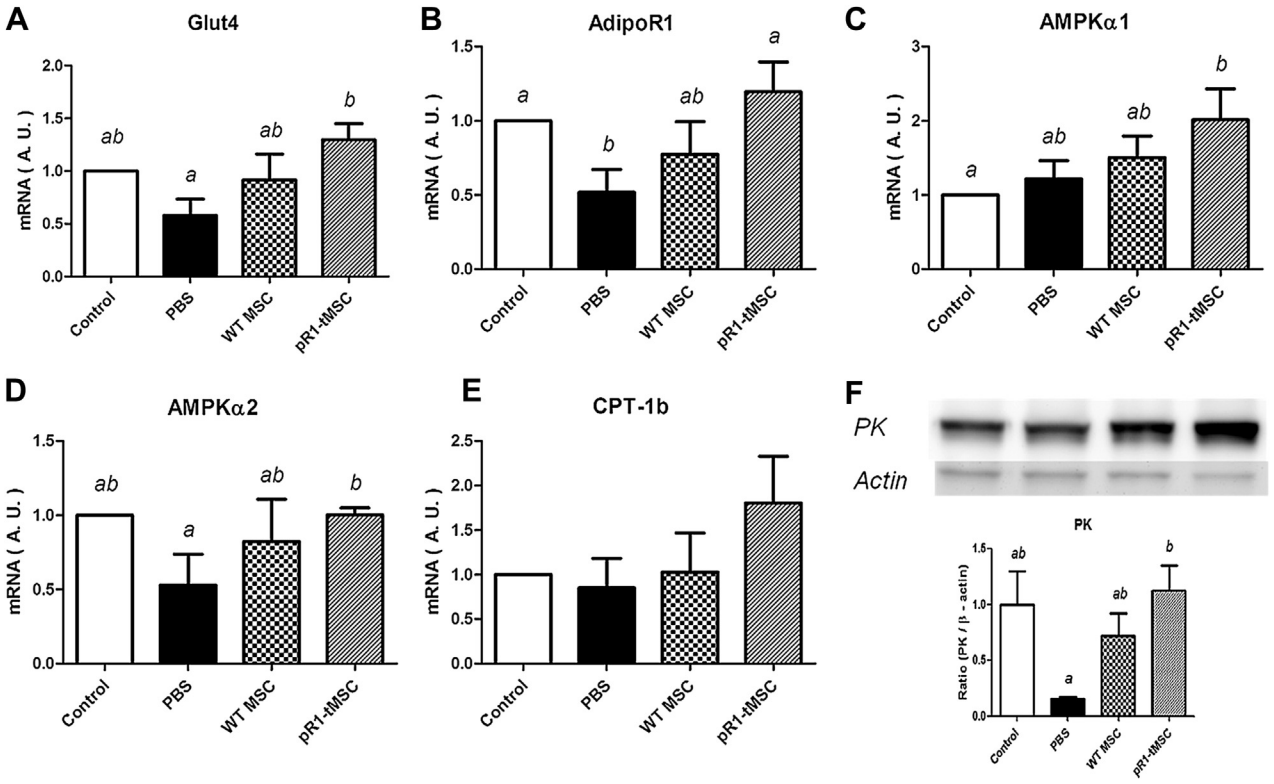


Figure 3. Effects of pR1-tMSC transplantation on gene expression of the muscles in HFSD-fed mice. Quantification of gene expression of (A) GLUT 4, (B) AdipoR1, (C) AMPKα1, (D) AMPKα2, (E) CPT-1b and (F) representative Western blot analysis of PK in the muscles of control (chow) and HFSD-fed mice treated as described in Figure 2. The mRNA levels were determined by RT-(quantitative)PCR and normalized to β-actin; protein levels were also used to generate the PK/β-actin ratio. Data are expressed as relative mean ± SEM. Different letters indicate statistical significance, $P \leq 0.05$ ($n = 6$). GLUT 4, glucose transporter 4; AdipoR1, ApN receptor 1; AMPKα1' 5′-AMP-activated protein kinase catalytic subunit α-1; AMPKα2' 5′-AMP-activated protein kinase catalytic subunit α2; CPT-1b, carnitine palmitoyltransferase 1b; PK, pyruvate kinase.

glucokinase and phosphoenolpyruvate carboxykinase were significantly suppressed by HFSD, neither WT-MSC nor pR1-tMSC had any effect on these genes (data not shown).

Whereas HFSD feeding lowered the mRNA expression of carnitine palmitoyltransferase (CPT)-1a, fatty acid synthase (FAS) and sterol regulatory element-binding protein 1c (SREBP1c) (Figure 5A–C), it raised that of lipoprotein lipase (LPL) and had no effect on that of hormone sensitive lipase (HSL) (Figure 5D,E). MSC transplantation had no effect on CPT-1a, FAS or SREBP1c mRNA expression (compared with PBS), whereas the mRNA expression of LPL and HSL was increased by pR1-tMSC.

### *Effects of pR1-tMSC transplantation on lipid metabolism–associated genes in the adipose tissues of HFSD-fed mice*

Despite the reduction of the mRNA abundance of adipogenic genes including CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, PPARγ or SREBP1c by a HFSD in the adipose tissue, they were not affected by the MSC treatments (Figure 6A–D). The fatty acid oxidation–associated and lipolysis-associated genes, such as ApN, AdipoR1, PPARα, HSL and LPL, were not affected by MSC treatment (compared with PBS) (data not shown). For FAS, HFSD decreased its mRNA expression, which was further lowered by pR1-tMSC transplantation (Figure 6E).

## Discussion

Our previous study observed that when fed a HFSD for 6 months, porcine AdipoR1-transgenic mice can better maintain their body weight and glucose homeostasis than the wild-type (Liu and Ding, unpublished data, 2009). This fact and the therapeutic potential of MSC and *AdipoR1* gene (4,10), prompted us to design the current approach for the improvement of obesity and glucose/lipid homeostasis in HFSD-challenged mice. Despite the lack of statistically significant effects on body weight and blood glucose levels, our MSC approach (both the wild-type and pR1-tMSC) lowered the lipid droplet accumulations in the livers and pR1-tMSC also altered the mRNA and protein levels of several genes

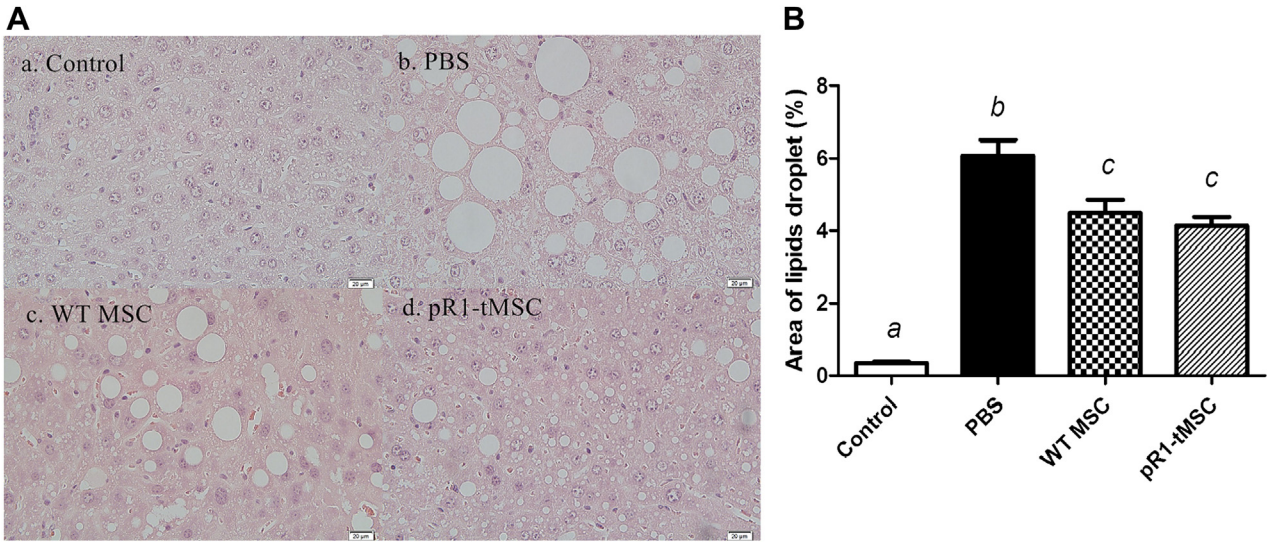


Figure 4. Effects of pR1-tMSC transplantation on hepatic lipid droplet accumulation in HFSD-fed mice. (A) Representative histological analyses of liver sections from control (a, chow diet) and HFSD-fed mice treated with (b) PBS, (c) WT-MSC and (d) pR1-tMSC as described in Figure 2. Sections were stained with hematoxylin and eosin and viewed at ×400 magnification with scale bars indicating 20 μm. (B) Quantification of the area of lipid droplets in histological sections of livers as determined by ImageJ software. Data are expressed as mean ± SEM ($n = 6$). Different letters indicate statistical significance, $P \leq 0.05$.

associated with glucose and lipid metabolism. Thus, the current study represents the first attempt to apply gene-modified MSC to treat obesity-associated complications.

The lack of effect of MSC treatment on body weight and blood glucose (Figure 2) may be due to the inadequate treatment period or frequency. Despite the use of a higher number ($2 \times 10^6$) of cells

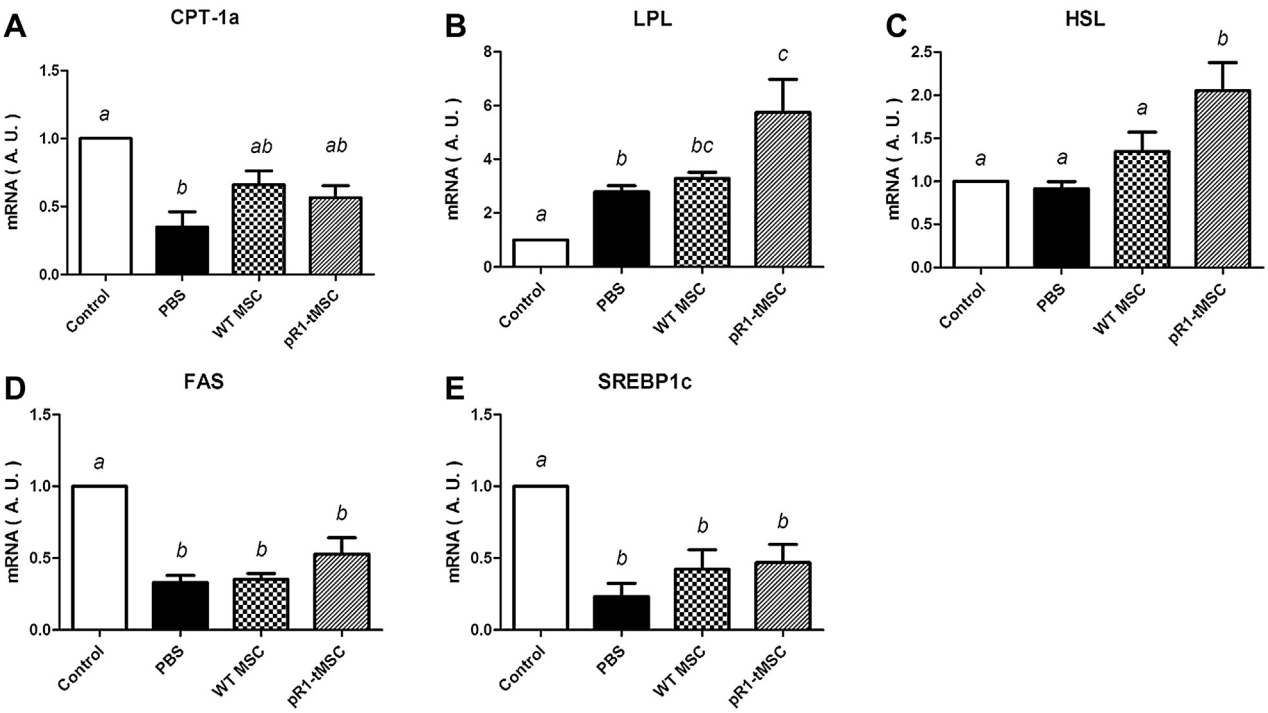


Figure 5. Effects of pR1-tMSC transplantation on the gene expression of the liver in HFSD-fed mice. Quantification of gene expression of (A) CPT1-a, (B) LPL, (C) HSL, (D) FAS and (E) SREBP1c in the livers of control (chow) and HFSD-fed mice treated as described in Figure 2. The mRNA levels were determined by RT-(quantitative)PCR and normalized to $\beta$-actin. Data are expressed as relative mean ± SEM. Different letters indicate statistical significance, $P \leq 0.05$ (n = 6). CPT1-a, carnitine palmitoyltransferase 1a; LPL, lipoprotein lipase; HSL, hormone sensitive lipase; FAS, fatty acid synthase; SREBP1c, sterol regulatory element-binding protein1c.

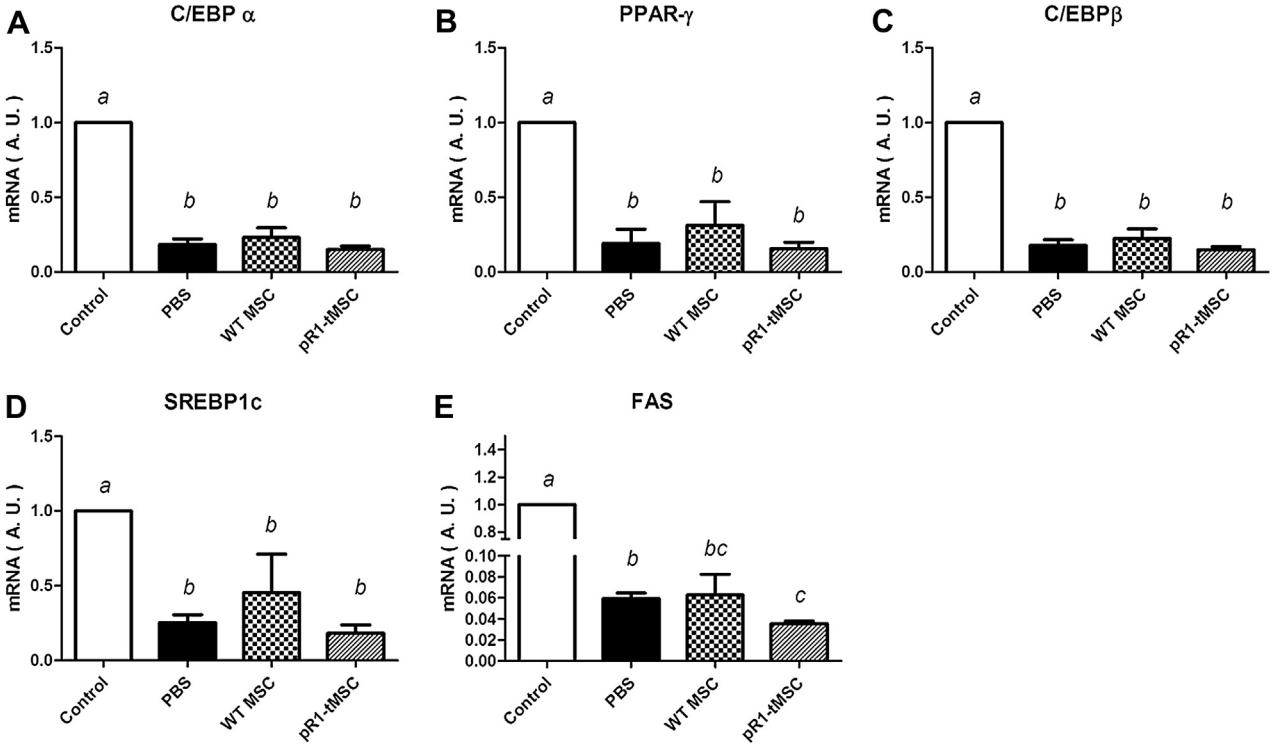


Figure 6. Effects of pR1-tMSC transplantation on gene expression of adipose tissue in HFSD-fed mice. Quantification of gene expression of (A) C/EBP$\alpha$, (B) PPAR-$\gamma$, (C) C/EBP$\beta$, (D) SREBP1c and (E) FAS in the adipose tissue of control (chow) and HFSD-fed mice treated as described in Figure 2. The mRNA levels were determined by RT-(quantitative)PCR and normalized to $\beta$-actin. Data are expressed as relative mean $\pm$ SEM. Different letters indicate statistical significance, $P \leq 0.05$ ($n = 6$). C/EBP$\alpha$, CCAAT/enhancer binding−protein-$\alpha$; PPAR-$\gamma$, peroxisomeproliferator-activated receptor-$\gamma$; C/EBP$\beta$, CCAAT/enhancer binding protein-$\beta$; SREBP1c, sterol regulatory element-binding protein 1c; FAS, fatty acid synthase.

than those used in others (5,7,8), in this study, we were unable to detect our pR1-tMSC, which over-expresses Flag-tagged pAdipoR1 (Figure 1) in the muscles, liver and adipose tissues by immunohisto-chemistry with the use of an anti-Flag tag antibody, suggesting that even higher numbers of cells may be needed to have beneficial effects seen in our pAdipoR1-transgenic mice (Liu and Ding, unpub-lished data, 2009). The other explanation may lie in the deficiency of ApN in obese condition (11,12) and thus the insufficient activation of AdipoR1 signaling events in our MSC system. Both can be tested in the future, especially the latter one by supplementing ApN or agonists or co-transplanting with ApN-expressing (-transgenic) MSC.

Our data suggest that the amelioration of HFSD-induced hepatosteatosis can be attributed to the upregulation of LPL and HSL gene expression by pR1-tMSC (Figure 5B,C). This is consistent with their lipolytic activities (21,22) and being the downstream targets of AdipoR1 and AMPK (10). Previous studies have demonstrated that MSCs exert their beneficial effects by migrating to the sites of injury and secreting a host of factors, including cytokines and chemokines (23), which may also contribute to pR1-tMSC action in the liver.

The increased mRNA expression of CPT-1 and HSL in the muscles and liver, respectively, by pR1-tMSC treatment (Figure 3E and Figure 5C), suggest that fatty acid oxidation was improved. This is consistent with previous studies showing that admin-istration of ApN to mice decreases plasma glucose levels and increases muscle fatty acid oxidation and treatment of C2C12 muscle cells with ApN stimulates fatty acid oxidation and glucose uptake through acti-vation of AMP-activated protein kinase and the increase in muscle fatty acid oxidation is accompanied by increased CPT-1 mRNA (10). Furthermore, pR1-tMSC treatment increased the expression of glucose transporter 4 and AdipoR1 (Figure 3A,B) as well as pyruvate kinase protein, an enzyme involved in glycolysis (Figure 3F), suggesting that the efficiency of glucose uptake by the muscles and glycolysis are increased by pR1-tMSC treatment.

In conclusion, our current study demonstrates the beneficial effects of both WT-MSC and pR1-tMSC on hepatosteatosis through potentially improved fatty acid oxidation and suggests that pR1-tMSC transplantation may enhance glucose

metabolism. Our data should provide insights into the therapeutic potential of gene-modified MSC for obesity and obesity-associated diseases.

## Acknowledgments

The authors would like to express our gratitude to the lab members for their help and input during the study. The study was supported by grants from the National Taiwan University. C.C. Chen was supported by a postdoctoral fellowship grant (NSC099-2811-B-029-003) from the National Science Counsel in Taiwan.

***Disclosure of interests:*** The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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## Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jcyt.2013.03.008