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Pioglitazone up-regulates MALAT1 and promotes the proliferation of endothelial progenitor cells by increasing c-Myc expression in type 2 diabetes mellitus

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Abstract

Objective: Evidence suggests that pioglitazone improves the function of endothelial progenitor cells (EPCs); however, knowledge of its molecular mechanism remains limited. In the present study, the role of long non-coding RNA MATAL1 in modulating the number and function of EPCS in type 2 diabetes mellitus (T2DM) was characterized.
Methods: Circulating EPCs were obtained from the peripheral blood of healthy subjects and T2DM patients with or without pioglitazone treatment. The in vitro experiments were conducted in bone marrow-EPCs to evaluate the effect of pioglitazone and its possible mechanism.
Results: Pioglitazone increased the number of circulating EPCs and improved their function in T2DM patients. By transfecting the EPCs with siRNA-MALAT1, the increase in c-Myc protein expression induced by pioglitazone treatment was canceled. In db/db diabetic mice, a glucose tolerance test showed that pioglitazone increased glucose tolerance, which was reversed by siRNA-MALAT1.
Conclusion: Pioglitazone improves EPC function, possibly through regulating MALAT1 and c-Myc expression in T2DM.

Keywords: Pioglitazone; diabetes mellitus; MALAT1; endothelial function; glucose tolerant

Introduction

Pioglitazone, a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist, is an insulin-sensitizing agent acting as a prescription drug for the treatment of type 2 diabetes mellitus (T2DM) [1, 2]. It has been demonstrated that pioglitazone provides benefits in a diverse population of patients with diabetes, lowering the risk of death, myocardial infarction, or stroke [3]. A subset of stem cells called endothelial progenitor cells (EPCs) exist with the vascular circulation and play important roles in the maintenance of endothelial homeostasis and vascular integrity, contributing to vessel repair following endothelial damage [4]. Reduced EPC number and function are associated with the presence of cardiovascular events, including T2DM [5-7]. Pioglitazone involves controlling blood glucose through insulin-sensitizing effects [8]. In addition, emerging evidence indicates that improvements in endothelium-dependent vascular function also contributes to the protective effect of pioglitazone on T2DM and its associated complications [9]. In the last decade, the role of pioglitazone in EPCs has been highlighted. Spigoni et al. showed that pioglitazone treatment improves the in vitro viability and function of EPCs and reduces cardiovascular risk in impaired glucose tolerant individuals independent of the insulin-sensitizing action [10], and the PI3K/Akt signal pathway has been proposed in this process [11]. However, knowledge remains limited on the molecular

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mechanisms of pioglitazone in EPC regulation.

Non-coding RNAs have been widely considered to take part in various pathophysiologic processes of animal and human diseases [12, 13]. Long non-coding RNA (lncRNA) has more than 200 nucleotides, is a type of novel non-coding RNA, and participates in the modulation of gene transcription, post-transcriptional regulation, and epigenetic regulation [14, 15]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT 1), also known as noncoding nuclear-enriched abundant transcript 2 (NEAT2), was first found in lung cancer and regulates the expression of metastasis-associated genes [16, 17]. A newly published paper showed that MALAT1 controls a phenotypic switch in endothelial cell and regulates its function and vessel growth [18]. It has also been demonstrated that MALAT1 plays a pathogenic role in endothelial cell dysfunction in diabetes mellitus [19]. However, whether MALAT1 is involved in regulating EPC function in T2DM remains unknown. The objective of this study was to examine the effect of pioglitazone on EPC function and evaluate whether MALAT1 participates in this process.

Materials and methods

Subjects

The human study was designed as a prospective clinical trial of healthy subjects and patients with T2DM. All participants were recruited from Tianjin Medical University Chu Hisen-I Memorial Hospital. The trial was performed with the approval of the Ethics Committee of Tianjin Medical University Chu Hisen-I Memorial Hospital. Informed consent was obtained from all participants.

Thirty-two healthy subjects (control) and 65 T2DM patients were recruited. Thirty-two patients with T2DM received a treatment of pioglitazone (30 mg/day) for four weeks, and the other 33 patients received no treatment. In this study, T2DM was defined according to the American Diabetes Association criteria [20]. Patient with one or more of the following criteria were excluded: type 1 diabetes, treatment with insulin, treatment with diet and/or exercise, treatment with any thiazolidinedione, and treatment with medications known to affect EPC biology. Four weeks after treatment, fasting venous blood was collected. The fresh whole blood was used to assess the number and function of the circulating EPCs. The serum was obtained to perform the routine examination.

Flow cytometry analysis of EPC number

Hematopoietic stem cells (CD34 and CD133) and endothelial cells (KDR) are the surface markers of EPCs, and the determination of these three markers represents the number of EPCs. Flow cytometry was used to determine the expression of CD34, CD133, and KDR in mononuclear cells (MNCs). A volume of 10 mL fresh peripheral venous blood treated with heparin (20 u/mL) was used to obtain MNCs via a Ficoll-Hypaque Solution (Tianjin Hanyang Biologicals Technology Co., Ltd, China). The MNCs were then re-suspended in a M199 medium (Hyclone, USA) and diluted in a FACS buffer for the measurement of CD34, CD133, and KDR via incubation with anti-CD34-PC-5 (Becton Dickinson, USA), anti-CD133-FITC (Beijing Bo Orson Biological Technology Co., Ltd., China), and anti-KDR-PE (R&D Systems, USA) in the dark. Cells were washed twice with the FACS buffer and fixed with 2% paraformaldehyde. Analysis was performed via the assessment of 100,000 events for each separate examination. The expression of CD34, CD133, and KDR was reported as a percentage of the total events.

EPC adhesion

Dissociated adherent cells with 0.25% trypsin and re-suspended them in the medium. The cell suspension was washed with PBS for 5 min. 1×10^5 cells were seeded in a fibronectin-coated culture plate for 30 min. The adhesion cells were counted under a light microscope.

Migration assay

Boyden chambers were used to assess the migration of the EPCs, which were treated in a Boyden chamber (Haimen Qi LinBeiEr instrument manufacturing co., LTD., China) with ultraviolet irradiation overnight. Cells were dissociated with trypsin (200 µL/well) and re-suspended in a M199 medium with 5% fetal bovine serum. The concentration of cells was adjusted to 2.0×10^5 cells/well. The lower chamber was added to a M199 medium containing 20 µL vascular endothelial growth factor (VEGF, 50ng/mL). Placed 200 µL cell suspension in the upper chamber with polycarbonate-free membrane (8.0 µm proes) to culture for 90 min. The cells were then removed from the upper chamber, and the migrated cells were stained on the lower surface of the membrane with haematoxylin and eoxsin. The cells were then counted under the microscope.

In vitro angiogenesis assays

The angiogenic capacity of the EPCs was measured by the in vitro Angiogenesis Assay Kit (Chemicon, USA) according to the manufacturer's instructions. Cells were harvested with trypsin and cultured in a 96-well plate pre-coated with ECMatrix gel for 24h. The cells were then counted under a microscope, and tube formation was calculated.

Bone marrow-EPC culture

In vitro experiments were performed using a bone marrow-EPC culture. Healthy male C57BL/6 mice aged 8–10 weeks were sacrificed after anesthetization with ketamine (25 mg/kg). Femurs and tibias were dissected, and the adhering tissues were completely removed. Both ends of the bones were excised. Bone marrow cells (BMCs) were harvested via flushing with Endothelial Cell Growth Medium 2 (EGM-2, Lonza, USA) and the density gradient centrifugation method. The cells were then re-suspended with EGM-2 containing 20% fetal calf serum (FCS), and the cell suspension (1×10^6 cells/mL) was seeded in a culture

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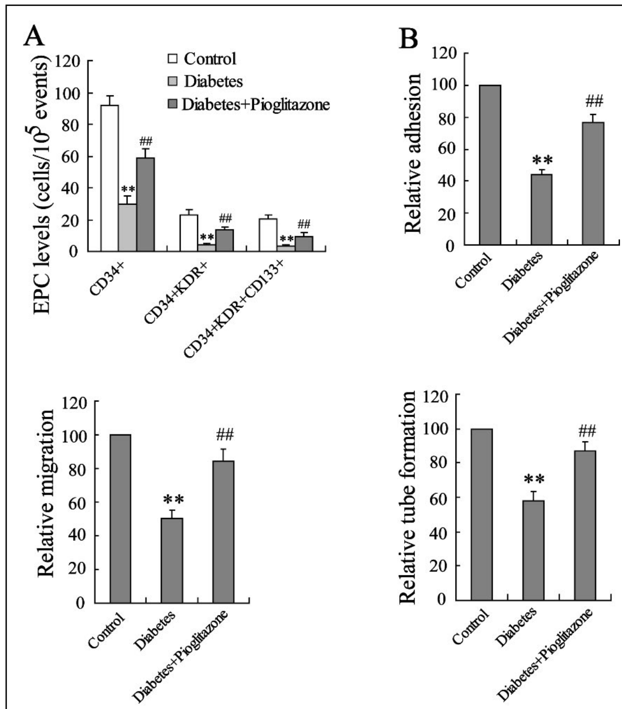

Figure 1. Effects of pioglitazone on the number and function of circulating EPCs in T2DM patients. EPC number (A), adhesion (B), migration (C) and tube formation (D) were detected in healthy subjects or T2DM patients with or without the treatment of pioglitazone (30 mg pd for four weeks). ** P <. 0.01, vs. control; ## P < 0.01, vs. diabetes.

plate at 37°C with 5% CO_2. Adherent cells were incubated with fresh EGM-2 three days after removing the unattached cells. The medium was replaced every two days, and the morphology of the cells was monitored. When the cells had grown to 80% confluence (days 7 to 10), 0.125% trypsin was used for cell dissociation.

RNA extraction and quantitative real-time PCR

The circulating EPCs or bone marrow-EPCs were lysed with a TRIzol reagent (Invitrogen, USA), and total RNA was extracted according to the manufacturer's instruction. The PrimeScriptTM RT-PCR Kit (Takara, China) was used to synthesize the cDNA with RNA. For the quantitative analysis of MALAT1 and c-Myc, the BIO-RAD CFX96 touch q-PCR system was applied with a IQ SYBR Green supermix (Bio-Rad Laboratories, USA). All samples were read in triplicate, and the relative expression values were normalized to GAPDH and β-actin expression via the $2^{-\Delta\Delta CT}$ method, respectively.

Western blot

For western blot analysis, the cells were harvested and washed twice in cold PBS. Then, they were lysed in a RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with a protease inhibitor for 1 h. The lysates were then centrifuged at 10, 000 × g for 10 min at 4 °C, the supernatants were collected. Protein concentrations were measured by the Bradford Protein Assay Kit (Beyotime, China). Equal amounts of protein extract for each sample were loaded on 10% SDS-PAGE electrophoresis for separation, and then the proteins were transferred to PVDF membranes (Millipore, USA) and blocked in 5% nonfat milk prior to incubation with the primary antibodies against c-Myc and β-actin (1:1000, Cell Signaling Technology, USA) overnight at 4 °C. A HRP-conjugated secondary antibody (1:5000, Cell Signaling Technology, USA) was then incubated in a membrane for 90 min.

Animal experiments

C57BL/6J db/db diabetic mice at ten weeks of age were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animals were fed with a standard chow diet in a temperature-controlled room. Isolated autologous EPCs from mice were used to treat diabetic mice. Before injection, EPCs were pre-incubated with 10 mM Pioglitazone for 2 h and transfected with pLVX-IRES-ZsGreenl-si-MALAT1 (pLVX-IRES-ZsGreenl as control) for 24 h. The EPCs were intravenously injected into the mice once a week for 16 weeks. A glucose tolerance test was performed to determine glucose tolerance. After fasting for 15 hours, the mice received 0.5g/kg glucose via intraperitoneal injection. The blood was then collected from the tail vein, and the blood glucose concentration was measured. The animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the animal experimental ethics committee of Tianjin Medical University Chu Hisen-I Memorial Hospital.

Statistical analysis

All statistical analyses were performed using SPSS 13.0

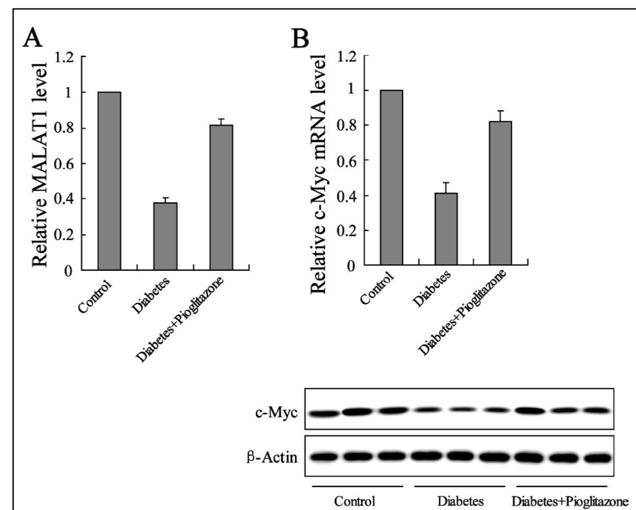

Figure 2. Effects of pioglitazone on MALAT1 and the c-Myc expression of circulating EPCs in T2DM patients. The expression levels of MALAT1 RNA (A) and c-Myc mRNA and protein (B) were determined in healthy subjects or T2DM patients with or without the treatment of pioglitazone (30 mg pd for four weeks). ** P < 0.01, vs. control; ## P < 0.01, vs. diabetes.

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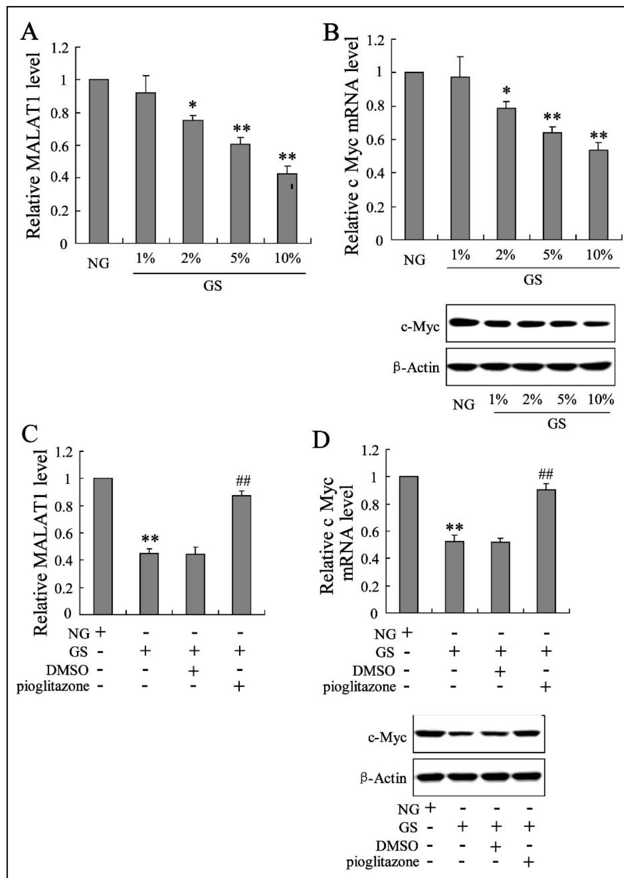

Figure 3. Effects of pioglitazone on MALAT1 and c-Myc expression in bone marrow-EPCs exposed to high glucose in vitro. The expression levels of MALAT1 RNA (A) and c-Myc mRNA and protein (B) were determined in bone marrow EPCs with different concentrations of glucose (1%, 1%, 2%, 5%, and 10%). These expressions (C, D) was also determined in high glucose (10%)-induced bone marrow-EPCs with the treatment of pioglitazone. ** P < 0.01, vs. control; ## P < 0.01, vs. diabetes.

statistic software, and the data was represented as mean ± standard error (SEM). The difference in the means between the two groups was analyzed by Student's t test. A P value less than 0.05 was regarded as statistically significant.

Results

Pioglitazone improves the function of circulating EPCs in T2DM patients.

The number and function of the circulating EPCs in T2DM patients were first evaluated. As expected, T2DM patients exhibited decreased EPC markers CD34+, CD34+KDR+, and CD34+KDR+CD133+ numbers compared with healthy subjects (Figure 1A). The function of the circulating EPCs was then measured. The results showed that the migration, adhesion, and formation of the EPCs were reduced to 43.4%, 50.9% and 58.1% in the T2DM patients, respectively (Figure 1B-1D). Administrated with 30 mg pioglitazone for 4 weeks, the number and function of circulating EPCs in T2DM were significantly elevated.

MALAT1 and c-Myc expression of circulating EPCs in T2DM patients

The MALAT1 level of the circulating EPCs was then investigated in the T2DM patients, and the results showed that it had significantly down-regulated to 38.3%, whereas the pioglitazone treatment resulted in an increase of the MALAT1 expression level to 81.3% (Figure 2A). c-Myc has been demonstrated to play a vital role in adequate angiogenesis and regulating endothelial dysfunction [21, 22]. The c-Myc mRNA and protein expression levels of the circulating EPCs in the T2DM patients presented a similar trend with respect to MALAT1, while the pioglitazone treatment up-regulated these expressions (Figure 2B).

Pioglitazone affected the MALAT1 and c-Myc expression in bone marrow-EPCs exposed to high glucose

The effects of pioglitazone in the in vitro model of T2DM were demonstrated. Co-incubated bone marrow-EPCs with different concentrations of glucose (1%, 2%, 5% and 10%) as well as a decreasing MALAT1 and c-Myc mRNA and protein expression were shown in a dose-dependent manner (Figure 3A and 3B). However, the decrease of MALAT1 and c-Myc expression in EPCs exposed to high glucose content (10%) could also be reversed by the treat-

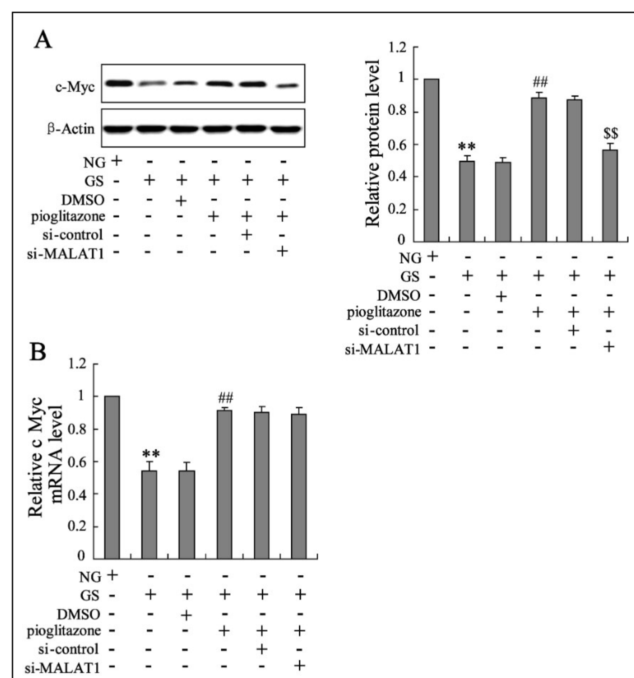

Figure 4. MALAT1 is involved in the effect of pioglitazone on c-Myc expression in bone marrow-EPCs exposed to high glucose content in vitro. The expression of c-Myc in the protein levels (A) and mRNA levels (B) were determined in bone marrow EPCs with different treatments. ** P < 0.01, vs. NG; ## P < 0.01, vs. NG+DMSO; $$ P < 0.01, vs. NG+DMSO+si-control.

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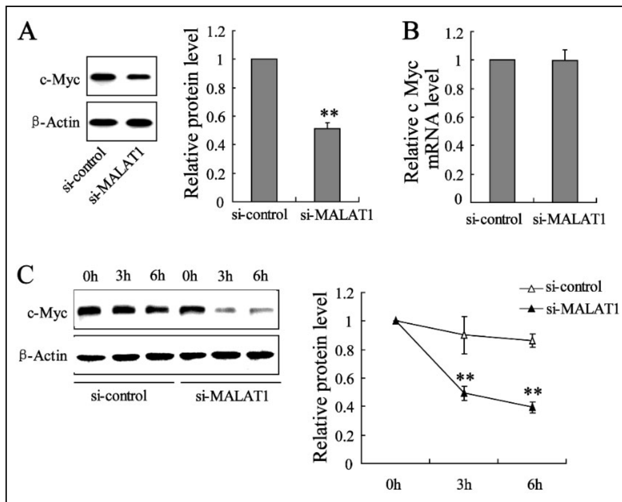

Figure 5. MALAT1 down-regulation decreased c-Myc expression in protein levels. The expression of c-Myc in the protein levels (A, C) and in mRNA levels (B) were determined in bone marrow EPCs with the treatment of si-MALAT1 (or si-control). ** $P < 0.01$, vs. si-control.

ment of pioglitazone in vitro (Figure 3C and 3D).

MALAT1 down-regulation affected the effect of pioglitazone on c-Myc expression

Pioglitazone has previously been confirmed to play a role in reversing the effect of high glucose on MALAT1 and c-Myc expression in bone marrow-EPCs. Then, bone marrow-EPCs were administered si-MALAT1 for down-regulating its expression, and the role of pioglitazone in increasing c-Myc protein expression was largely canceled (Figure 4A). However, the mRNA expression of c-Myc was not changed by si-MALAT1 (Figure 4B).

MALAT1 down-regulation affected c-Myc expression and stability

In order to confirm the role of MALAT1 in regulating c-Myc expression, co-incubated bone marrow EPCs with si-MALAT1 alone to observe the c-Myc expression. As shown in Figure 5, c-Myc protein expression was decreased to 51.0% (Figure 5A), but its mRNA expression was not altered (Figure 5B) by si-MALAT1. In addition, c-Myc protein expression decreased at the time of si-MALAT1 treatment (Figure 5C).

The glucose tolerance of db/db mice with the treatment of EPCs

The therapeutic effect EPCs with the treatment of pioglitazone and si-MALAT1 on the glucose tolerance of db/db mice was investigated. The results showed that EPCs with the treatment of pioglitazone resulted in the improvement of glucose tolerance; however, administrated EPCs with pioglitazone and si-MALAT1, the benefit of pioglitazone for blood glucose control was largely reversed (Figure 6).

Discussion

In the present study, we found that pioglitazone significantly increased the number and function of circulating EPCs. Pre-incubated EPCs with pioglitazone and then treated db/db diabetic mice, a significant decrease of the blood glucose levels and improvements in glucose tolerance were observed. Moreover, the up-regulation of MALAT1 expression induced by pioglitazone was proposed to take part in the increasing number and function of EPCs and as providing a benefit for the treatment of T2DM.

The EPCs co-expressed the surface markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (KDR) [23]. The measurement of these three cell markers is commonly used to determine EPC counts. In agreement with previous observations, we demonstrated that T2DM patients had a reduced number and function of circulating EPCs [5, 24]. In the last two decades, emerging studies have illustrated that circulating EPCs act as a biomarker of endothelial function, and their alteration is characterized by a high risk of cardiovascular disease [25-27]. The impairment and reduction of EPCs are also hallmark features of type 1 and type 2 diabetes [28-30]. Autologous EPCs are considered to be a novel cellular therapy for diabetic vascular complications [31]. However, this application is limited in diabetic individuals due to their dysfunctional circulating EPCs. Previous studies have confirmed that pioglitazone increases the number and function of EPCs in individuals with diabetes mellitus [32], coronary artery disease, normal glucose tolerance [33], and impaired glucose tolerance [10], contributing to the better use of EPCs in cardiovascular disease treatment. However, few studies have been shown to investigate the molecular mechanism of pioglitazone in EPC regulation.

LncRNAs are implicated in diverse human diseases, especially in cancer, cardiovascular disorders, and immunological diseases [34]. MALAT1 was first reported in lung cancer and is associated with metastasis, migration, tumor growth, and cell proliferation in various cancers [16, 35,

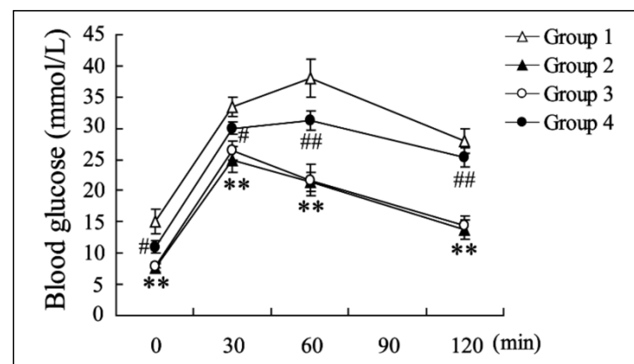

Figure 6. Effects of EPC treatment on glucose tolerance in db/db mice. The db/db mice were administrated using autologous EPCs with different treatments. Group 1: EPCs without any treatment; Group 2: EPCs with the treatment of pioglitazone; Group 3: EPCs with the treatment of pioglitazone and pLVX-IRES-ZsGreenl (control); Group 4: EPCs with the treatment of pioglitazone and pLVX-IRES-ZsGreenl-si-MALAT1. A glucose tolerance test was then performed in the db/bd mice. ** $P < 0.01$, vs. Group 1; # $P < 0.05$, ## $P < 0.01$, vs. Group 3.

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36]. Recently, Michalik et al. reported that MALTA1 indirectly triggers cell cycle blockade via the up-regulation of cell-cycle-related factors; enhances the migration capacity via thus far unknown mechanisms; and that the deficiency of MALAT1 leads to lower endothelial cell numbers and angiogenic defects [37]. It has also been reported that circulating MALAT1 expression was higher in acute myocardial infarction patients than that in healthy volunteers [38]. Poller et al. showed that MALAT1 participates in the process of cardiac innate immunity [39]. In the current study, a decreased MALAT1 expression was observed in the circulating EPCs of T2DM patients. Its expression was also reduced in bone marrow EPCs exposed to high glucose content in a dose-dependent manner. Considering the reduced number and function of circulating EPCs, we proposed that MALAT1 was correlated with EPC function in T2DM patients. As expected, pioglitazone treatment contributed to the elevation of MALAT1 levels in EPCs both in vivo and in vitro.

c-Myc is a regulator gene that codes for a transcription factor and plays a critical role in cell growth, apoptosis, and metabolism in mammals [40]. It has been commonly considered to be a proto-oncogene and is vital for adequate vascular development and angiogenesis [22]. The dysregulation of c-Myc expression is closely related to cancers and cardiovascular disorders [41]. Florea et al. demonstrated that the loss of c-Myc expression in endothelial cells results in a pro-inflammatory senescent phenotype, suggesting a central regulator of endothelial dysfunction [21]. In the present study, T2DM patients exhibited the decreased c-Myc expression of circulating EPCs in mRNA and protein levels. Pioglitazone treatment up-regulated the expression of EPCs exposed to high glucose conditions both in vivo and in vitro. Previous studies have shown that c-Myc expression is essential for the transcriptional repression of p21 and p15 [42, 43]. Therefore, decreased c-Myc levels in circulating endothelial cells may lift the repression of these cell cycle inhibitors, triggering cell cycle arrest. Transfected bone marrow-EPCs with si-MALAT1 for down-regulating its expression, the c-Myc expression was reduced only in protein level. In addition, si-MALAT1 largely canceled the role of pioglitazone in increasing the c-Myc protein expression of bone marrow-EPCs exposed to high glucose content. This data indicated that MALAT1 is involved in the effects of pioglitazone on EPC function via the regulation of c-Myc expression.

The main finding of this study was that MALAT1 is down-regulated in the circulating EPCs of T2DM patients and that decreased MALAT1 disrupts glucose tolerance. We also observed that pioglitazone benefits from increasing the number and function of EPCs and controlling blood glucose as well as that MALAT1 down-regulation greatly affects the effects of pioglitazone by modulating c-Myc expression. These findings suggest that autologous EPCs with pioglitazone administration hold great promise in the treatment of diabetes mellitus and diabetic vascular complications. Thus, MALAT1 may act as a new molecular target for modulating EPC function.

Declaration

Conflict of Interest: The authors declare that they have no conflict of interest.

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