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## **MafA-positive insulin-producing cells derived from rat adipose stem cells are efficient in glucose hemostasis in vitro**

**Dian Dayer**

PhD of Molecular Medicine, Cellular and Molecular Research Center, Medical Basic Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

**Elham Ghalami Negad**

MSC of Cellular and Molecular Sciences, Group of Biotechnology, Institute of Persian Gulf, Persian Gulf University, Bushehr 75169, Iran.

**Seyed Javad Hosseni\***

PhD in Cellular and Molecular Biology, Group of Biotechnology, Institute of Persian Gulf, Persian Gulf University, Bushehr 75169, Iran and Department of Biological Science and Technology, Faculty of Nano and Bio Science and Technology, Persian Gulf University, Bushehr 75169, Iran.

\*Coresspondance email: [sjh1348@gmail.com](mailto:sjh1348@gmail.com)

**Abstract**--Objective: MafA has been recognized as a specific pancreatic  $\beta$ -cells activator for Insulin expression. This study investigated the changes in glucose transport and consumption by MafA-positive Insulin-producing cells resulting from the differentiation of rat adipose tissue-derived mesenchymal stem cells. Methods: Adipose tissue-derived mesenchymal stem cells were differentiated into Insulin-producing cells in a 14-day protocol using nicotinamide and ITS. The ability to produce Insulin was confirmed by adipogenesis differentiation capacity and expression of Oct4, Nanog, and Sox2. Insulin and Glucagon concentrations in Insulin-producing cells were determined by ELISA. The ability of Insulin-producing cells for the presence of MafA, Glut2, ENO1, and IGF-I receptor genes was determined by Western blotting analysis. The amounts of Glucokinase and Pyruvate Carboxylase activities were measured by Fluorometric and Colorimetric methods, respectively. Results: The un-differentiated cells showed a promising capacity to differentiate toward adipocytes. The adipose tissue mesenchymal stem cells revealed significantly elevated levels of Oct4, Nanog, and Sox2 compared to differentiated adipocytes. The cells that differentiated into Insulin-producing cells secreted considerably more Insulin and Glucagon than undifferentiated cells. The differentiated cells showed significantly

higher amounts of MafA, Glut2 and IGF-I receptor protein expression compared with undifferentiated cells. While At the same time, the expression of ENO1 protein was not significantly different between the two groups. Conclusion: The obtained Insulin-producing cells seem to exhibit the ability to absorb glucose naturally, perform glycolysis, metabolism-secretion coupling and glucose-dependent Insulin secretion in vitro.

**Keywords**--*Insulin-producing cells, Adipose tissue-derived mesenchymal stem cells, Glut2, ENO1, IGF-I receptor, Glucagon.*

## Introduction

Diabetes type 1 is characterized by diminished  $\beta$  cell's proliferative capacity, associated with an obvious reduction of pancreatic  $\beta$  cell mass (1). In this view, maintaining a healthy population of beta cells can be regarded as an important goal in treating type 1 diabetes (2-5). For this purpose, identifying the signals that govern the survival and regeneration of beta cells seems inevitable (6). In recent years, laboratory-made Insulin-producing cells (IPCs) have been used to regenerate the beta cell population. Several studies have been performed to produce efficient IPCs (7,8). Researchers seek access to efficient IPCs in gene expression, protein translation, and post-translational modifications of insulin in addition to Insulin secretion in response to elevated glucose concentration. The efficient IPCs must be able to maintain glucose homeostasis (9). Considering the adipose-derived stem cells' easy access, high proliferative capacity, and high differentiation capacity, they have been considered one of the best sources for cell therapy (10). Experience has shown that the IPCs resulting from some differentiation protocols cannot secrete Insulin in vitro. In most other cases, the obtained IPCs with the ability to secrete Insulin in vitro show no capacity for insulin secretion in vivo. Many different variables may cause IPC's inefficacy (11). The increased level of intracellular calcium concentration is regarded as the main stimulus for Insulin secretion. The  $\beta$  cells release Insulin in two phases. In the first phase, Insulin is released rapidly in response to increased blood glucose levels. The second phase is independent of blood glucose concentration and consists of the stable and slow release of newly formed Insulin vesicles (12). Glucose enters the beta cells via glucose transferases (GLUTs). Then the anaerobic oxidation of glucose in the glycolysis cycle results in ATP production. The obtained ATP induces potassium channel blocking, cell membranes depolarizing, and elevated intracellular calcium levels, leading to the breakdown of cell membrane phospholipids and insulin release. The cAMP-dependent signaling pathways must be regarded as the other main regulator of insulin secretion (13). Free amino acids such as alanine and glutamine stimulate Glucagon production and Insulin secretion. Food digestion results in elevated blood levels of amino acids and glucose, stimulating intestinal L cells and stimulating GLP1 secretion. GLP1, in turn, directly affects beta cells and enhances the Glucose-dependent Insulin secretion process. Insulin secretion from beta cells depends on the proper signaling of Glucagon (14). Therefore, despite the contrasting activities of Glucagon and Insulin in regulating blood sugar and GLP1, normal Glucagon concentration is an essential factor in insulin secretion in

vivo (15). Some studies reported simultaneous secretion of Insulin and Glucagon by differentiated IPCs. (16). Type 1 diabetes is characterized by drastically reduced Insulin secretion from  $\beta$  cells, accompanied by the increased level of Glucagon to insulin ratio in the blood. Therefore, inhibiting Glucagon-dependent signaling pathways can be regarded as one of the diabetes treatment methods (17). The ability of IPCs to glucose uptake can be regarded as another significant factor in regulating Insulin secretion. Because elevated blood glucose concentrations play a major role in stimulating insulin secretion, any defect in the structure or function of GLUT proteins may lead to the inability of Insulin secretion. Glut2 plays the main role in glucose transport into  $\beta$  cells in rodents. When glucose binds to Glut2 on the surface of  $\beta$  cells, several signaling pathways are activated and induce glucose concentration-sensitive gene expression (18). Previous research showed that inhibition of Glut2 in liver cells prevents Insulin secretion in response to increased glucose concentrations (19). The glucose absorbed by the cell is consumed in the glycolysis cycle. Prior research revealed several major metabolic abnormalities in pancreatic  $\beta$  cells of diabetic rats. Some metabolic abnormalities contain the significantly increased expression of the genes involved in glycolysis and the greatly reduced expression of oxidative phosphorylation-responsible genes. This situation results to  $\beta$  cells' inability in NADH2 and ATP production and insufficient Insulin secretion, accompanied by elevated levels of peripheral glucose concentration. In  $\beta$  cells, a close relationship between glycolysis and Insulin secretion is noted. Insufficient Insulin secretion leads to abnormalities in the glycolysis process that ultimately lead to hyperglycemia (20). Glucokinase is a key glucose sensor and rate-limiting enzyme in glucose metabolism in  $\beta$ -cells. The Km value of Glucokinase is in the physiological range of blood glucose concentration (21). In patients with diabetes, the oxidative phosphorylation pathway in beta cells is disrupted (22). Therefore, glycolysis pathways mediators cannot be used for subsequent steps in oxidative phosphorylation. The mediator's accumulation in  $\beta$  cells disrupts the sensitivity of  $\beta$  cells to glucose concentration fluctuations (22). Alfa enolase (ENO1) is considered one of the important mediators of glycolysis (23). The diabetic rats have been shown significantly reduce symptoms of diabetes when treated with ENO-block (24). The normal  $\beta$  cells express a basal balanced concentration of ENO1 (24). The pyruvate as glycolysis end-product introduces three carboxylic acid cycles through enzymatic reaction with pyruvate carboxylase. Pyruvate carboxylase is regarded as another crucial regulator of the insulin secretion process (25). pyruvate carboxylase contributes to metabolism-secretion coupling in  $\beta$  cells (25). Previous studies revealed MafA-enhancing effects on Insulin promoter activity. The cooperation between MafA, Pdx1 and Nkx6.1 results in converting pancreatic acinar cells to mature Insulin-secreting cells (26). MafA also influences insulin secretion by enhancing Glut2, Glucokinase and Pyruvate Carboxylase expression (27,28). Insulin-Like Growth Factor-1 (IGF-I) is another effective factor in blood glucose homeostasis and maintenance of efficient  $\beta$  cells (29). The IGF-I is a peptide hormone with a similar structure to Insulin. IGF-I affects insulin receptors and induces glucose absorption. Similarly, Insulin binds to the IGF-I receptor (IGF-IR) and mimics its tissue effects. Type 1 diabetes is associated with some GH-IGF-I axis abnormalities. IGF-I is an essential factor for the growth and maturation of  $\beta$  cells. IGF-IR expression by IPCs reflects the cell's efficiency in maintaining glucose homeostasis (30). Both insulin and IGF-1 receptor deficiency in  $\beta$  cells results in reduced expression of MafA, reduced Glut2

expression, reduced  $\beta$ -cell mass and diabetes (31). Our previous study revealed that the IPCs produced from 14 days of differentiation of ATDMSCs (with or without MafA overexpression) were effective in glucose-dependent Insulin secretion and specific beta cell gene expression. However, transplantation of the IPCs to diabetic rats caused no significant modulation of blood glucose concentration until the sixth week after transplantation (31). In this regard, we investigated the factors that might affect glucose hemostasis in differentiated IPCs. We evaluated the effectiveness of in vitro produced IPCs in IGF-I response, glucose uptake, normal glycolysis performance, and metabolism-secretion coupling.

## **Materials and methods**

### **Preparation of adipose tissues**

Five normal Sprague Dawley rats (3 months old, 180-200 g) were chosen for the experiment. Animal work was carried out by the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health Publication No. 86-23). The Animal Experiments Committee of the Ahvaz Jundishapur University of Medical Sciences approved all experiments (AJUMS.REC.1393.100). Rats were euthanized with a mixture of 100 mg/kg ketamine (Sigma, USA) and 10 mg/kg xylazine (Sigma, USA). The adipose tissue was harvested from the splanchnic region under sterile conditions. The tissues were washed three times with sterile PBS containing 3% Pen /Strep (Gibco, UK).

### **Cell culture**

Some small pieces ( $\approx$ 5 mg) of adipose tissues were separated. After the isolation of blood vessels, the explants were washed with PBS (Sigma, USA) three times. The explants were cultured into a culture flask. The flasks were incubated at 37°C for 15 minutes to adhere the explants to the bottom of the culture flask. Then 500  $\mu$ l of pre-warmed FBS (Sigma, USA) was added to each 25 ml flask. The explants were incubated at 37°C and 5% CO<sub>2</sub> for 24 h. Then 2 ml of DMEM-HG contained 1% Pen/strep, and 20% FBS was added to each 25 ml flask. The culture medium was changed every 2 two days. After the cells reached confluent, the explants were discarded, and the cells were passaged. The cells in the third passage were utilized for the study.

### **Characterization of adipose-derived mesenchymal stem cells**

#### **Adipogenic differentiation**

To evaluate the ATDMSCs differentiation capacity, the cells were differentiated into adipose tissue. ATDMSCs at the third passage were cultured in 6 well plates at  $3 \times 10^5$  cells/well density. After 24 h incubation in 37° C and 5% CO<sub>2</sub>. The supernatant medium was discarded, and the differentiation medium (I8280, USA) was added. The differentiation medium was exchanged every 3 three days. After 21 days of differentiation, cells were incubated with 4% Formaldehyde (Sigma, USA) for 60 min and then washed with 70% Ethanol. After 20 min incubation

with O-red oil (Sigma, USA), the cells were washed with 70% Ethanol and visualized using a phase-contrast microscope (Olympus IX71, Japan).

### **Real Real-time PCR**

#### **Precipitation of the cells**

The expression of the genes related to the stemness of ATDMSCs was evaluated using real-time PCR. The ATDMSCs in passage 3 three were trypsinized. After 8 minutes of centrifugation at 1200 rpm, the supernatant was discarded, and the precipitated cell plate was used for RNA extraction.

#### **RNA extraction**

According to manufacturer instructions, the total RNA of ATDMSCs was extracted using the SinaPure™ RNA extraction kit (Sinaclon, Iran). The density absorption ratio at 260/280 nm was determined using NanoDrop spectrophotometry (Thermo Scientific NanoDrop). The samples with OD 260/280 nm between 1.8 to 2 were considered optimal pure RNAs for cDNA synthesis.

#### **cDNA synthesis**

Reverse transcription was carried out with a Sinaclon cDNA synthesis kit (SinaClon, Iran) using one µg RNA and random hexamer as recommended by the manufacturer.

### **Real Real-time PCR**

The gene expression pattern was determined by real-time PCR analysis. The analysis was performed using the Takara Master kit for SYBR Green (Takara, Japan) on a Step One Plus Detection System (ABI, USA). The designed primers were as follows: Oct4 forward (5'-CGAACCTGGCTAAGCTTCCA-3') and reverse (5'-GCCATCCCTCCACAGAACTC-3') primers, Nanog forward (5'-TACCTCAGCCTCCAGCAGAT-3') and reverse (5'-CATTGGTTTTTCTGCCACCT-3') primers; Sox2 forward (5'-CTCGCAGACCTACATGAAC-3') and reverse (5'-TCGGACTTGACCACAGAG-3') primers, Rex-1 forward (5'-GCTCCGCGGAATCGAGTGG-3') and reverse (5'-GCACGTGTTGCTTGCGACC-3'), and Tert forward (5'-CCCGAGTATGGCTGCATGAT-3') and reverse (5'-AAAGTCCGAGTGTCAGCAG-3') primers. β-actin forward (5'-GCAGGAGTACGATGAGTCCG-3') and reverse (5'-ACGCAGCTCAGTAACAGTCC-3'). Reactions were prepared in a total volume of 25µl mixture containing 12.5 µl of master mix kit and 1 µl of each primer (200 nmol/l), 100 ng cDNA, and an appropriate volume of nuclease-free water. The polymerization consisted of a 10 min denaturation at 95°C followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. β-actin was used as the reference gene. Two reactions without cDNA or with RNA were used as negative controls. Relative quantification was performed according to the comparative  $2^{-\Delta\Delta Ct}$  method and using Step One Plus software. All reactions were performed in triplicate.

### **Differentiation of ATDMSCs to IPCs**

The adipose-derived mesenchymal stem cells in the third passage were counted and randomly divided into two groups. When confluency reached 70%, the experimental group of cells was differentiated to IPCs through a three-step differentiation protocol described previously (33). On day 14 of differentiation, the supernatant of the cells was collected and stored at -70 ° C. The cells of the control and experimental groups were trypsinized. The plates of the cells were collected and kept at -70 ° C.

### **Glucose-dependent- Insulin secretion assay**

On day 14 of differentiations, the differentiated and control groups were treated with KRB buffer and high glucose concentration glucose as described previously (32). The supernatant was used for the Insulin assay. The manufacturer recommended rat-specific Insulin ELISA kit (Monobind, Inc, Lake Forest, CA, USA) was used for Insulin concentration determination. The Insulin concentration was reported in ng/ml. All experiments were performed in triplicate.

### **Glucagon secretion assay**

Glucagon concentration in IPCs supernatant was determined by Sandwich ELISA using DuoSet® ELISA kit (DuoSet® ELISA Development System, USA) as recommended by the manufacturer. The Glucagon concentration was reported in ng/ml. All experiments were performed in triplicate.

### **Western blotting**

The expression of Glut2, ENO1 and IGF-IR proteins by the IPCS compared to ATDMSCs was evaluated using western blotting analysis. The protocol was performed as a previously described method (33). The primarily used antibodies consisted of anti-Glut2, anti ENO1, anti-IGF-IR, anti-MafA and anti- $\beta$ -actin antibodies (sc-518022 (SANTA CRUZ BIOTECHNOLOGY, INC, USA), sc-271384 (SANTA CRUZ BIOTECHNOLOGY, INC, USA), sc-390491(SANTA CRUZ BIOTECHNOLOGY, INC, USA), and E-AB-63684 (Elabscience, USA). The primary antibody detection was performed using mouse anti-rabbit IgG-HRP: sc-2357 (SANTA CRUZ BIOTECHNOLOGY, INC, USA) and m-IgG $\kappa$ BP-HRP: sc-516102 (SANTA CRUZ BIOTECHNOLOGY, INC, USA). The densitometric quantification of proteins concerning  $\beta$ -actin as the calibrator was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The western blot analysis was performed in three independent experiments for each sample.

### **Determination of Glucokinase Activity**

Glucokinase Activity was determined by Fluorometric assay using PicoProbe™ Glucokinase Activity Assay Kit (Bio Vision, USA, Cat No: K969-100) as recommended by the manufacturer.

### **Determination of Pyruvate Carboxylase activity**

Pyruvate Carboxylase Activity was determined by colourimetric method using Pyruvate Carboxylase Assay Kit (Bio Vision, USA, Cat No: K2075-100 ) as recommended by the manufacturer.

### **Statistical analyses**

Data analyses were done using the SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). All analyses were done in triplicate. One-way ANOVA followed by Tukey post-hoc analysis was used to test differences between various means, including the expression level of different genes and Insulin concentration. All experimental data were presented as the mean  $\pm$  SEM. The level of significance for all tests was set at  $P < 0.05$ .

## **Results**

### **Morphological changes in isolated ATDMSCs**

The newly isolated cells showed a large number and small round morphology (Fig1.A). On day 4 of isolation, the morphology of cells was changed to a spindle-like shape. However, some non-specific cells were distinguished (Fig1.B). The number of non-specific cells was decreased in passage 2 (Fig1.C). The cells in passage 3 exhibited uniform fibroblast-like morphology (Fig 1.D).

### **Characterization of ATDMSCs**

#### **Adipogenic differentiation capacity**

The ATDMSCs were successfully differentiated to adipocytes using the adipogenic differentiation medium. Oil-red O Staining of the differentiated cells confirmed fat droplet formation (Fig 2).

#### **Expression of proliferation and differentiation markers by ATDMSCs**

The un-differentiated ATDMSCs showed a significantly higher capacity for Oct4, Nanog, and Sox2 expression when compared with the differentiated adipocytes ( $p < 0.05$ ) (Fig 3).

#### **Morphological changes during differentiation of ATDMSCs to IPCs**

The Undifferentiated ATMSCs with spindle-like shape (Fig4.A). During differentiation toward IPCs, the spindle-like ATDMSCs were gradually shortened (Fig4.B) (day 3 of differentiation), (Fig 4.C) (day 7 of differentiation). The fully differentiated ATDMSCs revealed an epithelial-like shape (Fig 4.D) (day 14 of differentiation).

### **Characterization of obtained IPCs**

#### **IPCS's ability to glucose-dependent- Insulin secretion**

Measurement of the amount of secreted Insulin by ELISA demonstrated the IPC's significantly higher capacity for Insulin secretion in response to high glucose concentration than the un-differentiated ATDMSCs ( $p < 0.05$ ) (Fig 5.A).

#### **IPCS's ability to Glucagon secretion**

The resulting IPCs could produce significantly higher Glucagon amounts than ATDMSCs ( $p < 0.05$ ) (Fig 5.B).

#### **The ability of IPCs to glucose sensing**

The IPCs showed significantly elevated expression of the Glut2 gene as a glucose transporter compared to ATDMSCs ( $p < 0.05$ ) (Fig 6.A).

#### **The balance condition in the glycolysis process in IPCs**

The IPCs resulting from ATDMSCs differentiation showed regular expression of ENO1 as a glycolysis mediator gene. The expression of ENO1 was not significantly different among the ATDMSCs and IPCs ( $p > 0.05$ ) (Fig 6.B).

#### **The ability of IPCs to IGF-IR expression**

The final resulting IPCs showed significantly higher expression of IGF-IR than un-differentiated ATDMSCs ( $p < 0.05$ ) (Fig 6.C).

#### **The ability of IPCs to Glucokinase secretion**

The obtained IPCs were able to have Glucokinase secretion. The IPCs showed significantly higher amounts of Glucokinase activity when compared with the control group ( $p < 0.05$ ) (Fig 7.A)

#### **The ability of IPCs to Pyruvate Carboxylase secretion**

The obtained IPCs were able to Pyruvate Carboxylase secretion. The IPCs showed significantly higher amounts of Glucokinase activity when compared with the control group ( $p < 0.05$ ) (Fig 7.B).

#### **The ability of IPCs to MafA expression**

The IPCs showed significantly higher amounts of MafA expression when compared with the control group ( $p < 0.05$ ) (Fig 7.C).

### **Discussion**

The definition of an efficient protocol for healthy  $\beta$  cell-like cell production requires proper identification of the signals governing the survival and regeneration of  $\beta$  cells (34). In the present study, adipose-derived stem cells were differentiated into IPCs with the ability to secrete Insulin in response to increased glucose concentrations. Several studies have produced efficient IPCs (7-10). Amer et al. differentiated adipose-derived stem cells into IPCs. The differentiated cells

were injected into the pancreas of type 1 diabetic rats. The proliferative and regenerative capacity of beta cells and the ability of C-peptide secretion showed significantly increased levels following transplantation of IPCs to diabetic rats. The researchers concluded that IPCs transplantation improves the function and morphology of islet cells in diabetic rats (35). Liao et al. injected adipose-derived stem cells into diabetic rats with type 2 diabetes and hepatic fibrosis and concluded that ADMSCs transplantation results in significantly reduced insulin resistance and hyperglycemia. However, the blood sugar levels did not return to normal (36). Xie et al. differentiated rat bone marrow-derived mesenchymal stem cells into IPCs and transplanted the resulting cells into diabetic rats. In this study, the Insulin secretion rate by transplanted IPCs was equivalent to one-tenth of the Insulin secreted by normal islet beta cells. However, the transplanted IPCs could modulate glucose concentration after transplantation to the kidney capsule of diabetic rats (37). In Hu et al. study, Umbilical cord mesenchymal cells differentiated into IPCs and transplanted into diabetic rats. Immunofluorescence results showed that many Insulin-positive cells were present beneath the kidney capsule. On day 14 after transplantation, the human serum Insulin level in the treatment group showed a significantly higher amount than in the control group. Although after transplantation of IPCs, diabetic rats remained hyperglycemic and continued to lose weight. The results of this study indicate the in-efficiency of transplanted IPCs in controlling glucose concentration (35). In prior studies, the transplanted pancreases revealed a higher capacity to regulate blood glucose concentration than transplanted IPCs. However, the host's immune-rejection of the transplanted pancreas must be considered a significant limiting factor in this way. The inability of IPCs to secrete Insulin may result from several different factors (8). Svendsen et al. showed that insulin secretion depends on the proper signaling of Glucagon inside pancreatic  $\beta$  cells. The Glucagon secretion stimulant factors contribute to blood glucose level elevation. In turn, high glucose concentration stimulates Insulin secretion. Therefore, in the normal state, the increased level of Glucagon production leads to Insulin secretion stimulation *in vivo*. The complementary effects of Insulin and Glucagon eventually lead to glucose homeostasis (39). In the present study, the IPCs were able to produce Glucagon. The Glucagon expression by these cells showed significantly increased levels compared to the control group (16). The ability of IPCs to glucose uptake can be regarded as another significant factor in regulating Insulin secretion. Considering the elevated glucose as the main stimulator of Insulin secretion, the deficiencies in GLUTs expression may result in insufficient Insulin secretion. In rodents, GLUT2 is the main glucose transporter protein on the pancreatic  $\beta$ -cell surface (15, 16). Previous studies show that GLUT2 inhibition in liver cells is associated with glucose-dependent Insulin secretion suppression (18). The extent of GLUT2 expression determines glucose uptake, the main identifier of pancreatic  $\beta$  cells efficacy. In humans, GLUT2 inhibition results in Fanconi syndrome, accompanied by the impaired function of the liver and kidney (40). The deficiency of Glut2 expression causes transient neonatal diabetes in infants (41). Some abnormalities in the major metabolic pathways of  $\beta$  cells have been detected in diabetic rats (42). The greatly reduced activity of oxidative phosphorylation-responsible genes results in the accumulation of glycolytic metabolites. As a result of these abnormalities,  $\beta$  cells can not produce NADH<sub>2</sub> and ATP in proportion to the increase in peripheral glucose concentration (42). Burkhardt et al. inserted the recombinant adenovirus containing the Glut2 promoter gene into

HepG2 hepatocytes. The resulting cells were injected through a port vein into diabetic mice. All diabetic rats showed normal blood glucose concentrations 36 days after injection, and 50% maintained normal glucose concentrations 77 days after injection. The study results confirmed the vital role of Glut2 in regulating Insulin secretion and blood glucose concentration (43). Moshtagh et al. differentiated adipose-derived mesenchymal cells into IPCs and reported that the obtained cells could express GLUT2, Pdx1, Pax4, and Ngn3 (44). Rattananinsruang et al. differentiated embryonic stem cells into IPCs and showed that the resulting cells expressed Insulin, Pdx1, Nkx6.1, Ngn3, and Glut2 genes. In vivo study revealed that both encapsulated and free forms of transplanted IPCs induced hyperglycemia modulation and significantly reduced IL-1 $\beta$  expression in mice. However, the blood glucose concentration did not receive the normal range (8). In line with the results of previous studies, the present study showed that the expression of the Glut2 gene in differentiated IPCs was significantly higher than in the cells of the control group. This finding indicates the effectiveness of the existing differentiation protocol and the ability of the obtained cells to absorb peripheral glucose to stimulate Insulin secretion from secretory vesicles. The absorbed glucose should be consumed during the glycolysis phase in  $\beta$  cells. Glycolysis is a vital regulator of Insulin secretion from IPCs. Various nutritional and hormonal signals regulate the activity of glycolysis enzymes in the transcription, translation, and post-translational stages. In  $\beta$  cells, the glycolysis process and Insulin secretion are closely dependent. The lack of Insulin secretion by pancreatic  $\beta$  cells disrupts the glycolysis cycle and causes hyperglycemia. Given the important role of glycolysis stages in glucose uptake and ATP production, any enzyme production defect may impair Insulin secretion from IPCs. Glucokinase is the common glucose sensor in the  $\beta$  cells (45). Hence, any alteration in Glucokinase activity results in to change in glucose hemostasis (45). Grimsby et al. reported that allosteric glucokinase activators contribute to glucose sensing elevation and Diabetes treatment (46). Kiyoshi et al. experience demonstrated the usefulness of glucokinase activators in treating type 2 Diabetic patients (47). Prior studies showed that Pyruvate Carboxylase is essential in coupling mitochondrial metabolism to insulin secretion in normal  $\beta$  cells. Pancreatic  $\beta$  cells of Diabetic patients show reduced levels of Pyruvate Carboxylase (48). The study by Xu et al. revealed that inhibition of Pyruvate Carboxylase activity inhibits Insulin secretion (49). Our survey showed a significant elevation in Glucokinase and Pyruvate Carboxylase activity in differentiated IPCs. This finding indicates elevated levels of glucose sensing and metabolism-Insulin secretion capacity following differentiation of ATDMSCS to IPCs. It has been postulated that MafA is the key modulator of Insulin secretion in response to glucose concentration. In the study of Zhang et al., MafA deficient mice exhibited impaired glucose-stimulated Insulin secretion accompanied by diminished levels of Pdx1, Beta2, and Glut-2 expression (50). In our study, the differentiated IPCs expressed significantly higher MafA protein levels than the undifferentiated stem cells. This result is consistent with the increased glucose-stimulated Insulin secretion and Glut2, Glucokinase and Pyruvate carboxylase overexpression. Ueki et al. reported reduced MafA and Glut2 expression and reduced  $\beta$ -cell mass following Insulin and IGF-I receptors depletion in mice  $\beta$  cells (51). The  $\beta$  cells of the islets of Langerhans lack the enzyme phosphoenol carboxypyruvate kinase. Therefore, the glycolysis in  $\beta$  cells is unilateral, and gluconeogenesis does not occur in these cells (52). ENO1 is one of the important

mediators of the glycolysis pathway (53). Previous research has shown that in patients with diabetes, the oxidative phosphorylation pathway in  $\beta$  cells is disrupted. Therefore, glycolysis mediators cannot be used for subsequent steps in oxidative phosphorylation. These mediators accumulate in the  $\beta$  cells. The abnormal accumulation of glycolysis mediators in  $\beta$  cells must be considered an inhibiting factor in IPCs sensitivity to glucose concentration (54). The Study by Cho et al. has shown that ENO-block modulates diabetes symptoms and tissue inflammation in diabetic mice. In this study, the antidiabetic properties of ENO-block were significantly higher than rosiglitazone. Therefore Cho et al. Suggested an ENO-block application to treat diabetes (24). The research by Qian et al. showed that glycolysis acceleration plays an important role in cisplatin resistance of gastric cancer cells. The researchers reported that inhibition of ENO1 by siRNA accelerates the glycolysis inhibition and leads to loss of cisplatin resistance (54). The studies by Principe et al., Showed greatly increased activity of ENO1 in patients with metastatic pancreatic adenocarcinoma. Principe et al. proposed the administration of anti-ENO1 monoclonal antibody as a new treatment for pancreatic adenocarcinoma (55). In the present study, the IPCs that resulted from the differentiation of ATDMSCs did not show a significant difference from the control group in ENO1 expression. It can be concluded that the rate of glycolysis is balanced with the rate of glycolysis mediators production. Diabetic people reveal abnormalities in glucose metabolism associated with decreased IGF-I levels in the blood (56). Carroll et al. evaluated the therapeutic effects of IGF-I on protein and glucose metabolism in patients with type 1 diabetes. In Carroll et al.'s study, the therapeutic use of IGF-I reduced the need for Insulin injection. Therefore, the researchers concluded that IGF-I could effectively treat diabetes (57). The different effects of Insulin or IGF-I on growth or metabolism depend on the extent of access of each hormone to its specific receptor. The effects of GH and IGF-I on  $\beta$ -cell growth depend on the effect of these hormones on glucose metabolism (58). Glucose is the best nutrient in  $\beta$  cell growth promotion. The normal glucose concentration induces proper growth of  $\beta$  cells. In contrast, glucose levels above baseline cause  $\beta$ -cell apoptosis (59). The IGF-1-mediated  $\beta$ -cell mitogenesis requires the induction of phosphatidylinositol-3-kinase (PI3K) activity downstream of the Insulin receptor or IRS-2. Further, IGF-1 activates the protein kinase B or Akt, essential for maintaining  $\beta$  cells. It can be concluded that IGF-I is an essential factor for the growth and maturation of  $\beta$  cells. The cooperation between GH and IGF-I leads to blood glucose homeostasis adjustment and maintaining efficient  $\beta$  cells (60). The study by Dimitriadis et al. showed that the effect of IGF-I on monocytes of hyperthyroid patients induced over-expression of Glut3 and Glut4 transporters. This study showed that IGF-IR over-expression leads to glucose transporters production and glucose uptake by monocytes. Therefore, IGF-I can be considered an effective factor in glucose homeostasis (61). Casellas et al. found that topical expression of IGF-I in the islets of Langerhans prevented lymphocytic inflammation and beta-cell death in diabetic mice (62). In line with the results of previous studies, the present study showed a significantly elevated level of IGF-IR expression by in vitro-produced IPCs. This finding confirms the efficiency and maturity of the obtained IPCs. The results of the present study indicate that the IPCs obtained in vitro were sensitive to glucose concentration and showed increased expression of MafA and IGF-IR, efficient glucose hemostasis and a promise glucose-dependent Insulin secretion ability.

## Conclusion

The IPCs produced *in vitro* were efficient in MafA expression, IGF-I responsiveness, glucose uptake, glycolysis performance and metabolism-Insulin secretion coupling. Besides that, the cells secreted Insulin correctly in response to glucose. Therefore, the insufficient ability of the IPCs to control blood glucose concentration *in vivo* must be due to some metabolic abnormalities other than the inability of IPCs to maintain glucose homeostasis.

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## Disclosure of interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article. The authors declare no duality of interest associated with this manuscript.

## Author contribution

DD, and SJH designed the study. DD and ELG performed the analysis, researched the data, analysed the results, and revised the article critically for important intellectual

content. DD and SJH wrote the manuscript, analysed the data, and revised the manuscript. All authors gave final approval of the version to be published.

SJH is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## Figure legends

**Figure 1.** Changes in cell morphology during the expansion of ATMSCs in culture medium. (A) Newly isolated cells from rat adipose tissue with round morphology. (B, C) Spindle-like specific cells with some nonspecific cells during the first and second passages. (D) Specific ATMSCs with a fibroblast-like shape at passage 3.

**Figure 2.** Characteristics of ATDMSCs. (a) Fibroblast-like morphology of undifferentiated ATDMSCs (b) Adipogenic differentiation potential of ATDMSCs was determined by oil red O–stained oil droplets.

**Figure 3.** Characterization of ATDMSCs. The undifferentiated ATDMSCs showed significantly higher expression of Oct4, Nanog, and Sox2 than adipocytes. Demonstrating the proliferation and differentiation capacity of ATDMSCs.

**Figure 4.** Morphological characteristics of ATDMSCs during differentiation into IPCs. The number of cells with an epithelial-like shape increased, consistent with differentiation progress. (A) Undifferentiated ATDMSCs. (B) Day 3 of differentiation. (C) Day 7 of differentiation. (D) Day 14 of differentiation.

**Figure 5.** Comparison of Insulin and Glucagon secretion capacity between control ATDMSCs and IPCs. The differentiated cells showed significantly elevated levels of glucose-dependent- Insulin secretion and Glucagon. Bars with different letters differ significantly ( $P < 0.05$ ).

**Figure 6.** Comparison of protein expression of pancreas-related genes: Glut-2, ENO1, and IGF-IR between control ATDMSCs and IPCs. Data are means  $\pm$  SD. Bars with different letters differ significantly ( $P < 0.05$ ).

**Figure 7.** Glucokinase, Pyruvate Carboxylase secretion capacity and MafA expression. The differentiated cells showed significantly elevated levels of Glucokinase and Pyruvate Carboxylase compared to control ATDMSCs (A, B). The differentiated cells showed significantly elevated levels of MafA protein expression compared to the control group (C). The concentrations of Glucokinase and Pyruvate Carboxylase were reported as mIU/mg and IU/mg, respectively. Bars with different letters differ significantly ( $P < 0.05$ ).

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