

Autophagy Involves in Differentiation of Insulin-Secreting Cells from Adipose Derived Stem Cells

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Abstract

Objective: Destruction of pancreatic beta-cells induces an insulin deficiency and causes type 1 diabetes. The role of autophagy in inducing insulin-secreting cells (ISCs) from adipose-derived mesenchymal stem cells (AMSCs) was investigated in the current study.

Materials and Methods: In this experimental study, the isolated AMSCs were characterization and exposed to a cocktail differentiation medium (CDM) in the absence or presence of 3-methyladenine (3MA), an autophagy inhibitor. The differentiation of ISCs was confirmed by the evaluation of the expression of beta-cell-specific genes including pancreatic and duodenal homeobox 1 (*PDX1*), musculoaponeurotic fibrosarcoma oncogene homolog A (*MAF-A*), Nk class of homeodomain-encoding genes 6.1 and 2.2 (*NKX6-1* and *NKX2.2*), Glucose transporter 2 (*GLUT-2*) and *INSULIN*. Using Newport Green (NG), insulin-positive cells were identified. Insulin secretion in response to various glucose concentrations was measured. Autophagy was evaluated by Acridine orange (AO) staining. Also, expression of autophagy-associated genes, including autophagy-related gene 5 (*ATG-5*), autophagy-related gene 7 (*ATG-7*), *BCLIN-1*, and mammalian target of rapamycin (*mTOR*), was evaluated by Real-time polymerase chain reaction (PCR) method.

Results: We observed a significant increase of beta-cell specific genes expression in the CDM-treated cells ($P < 0.01$ or $P < 0.001$), whereas the expression of these genes was down-regulated in 3MA-exposed cells. Expression of *INSULIN* and *GLUT-2* genes ($P < 0.01$ and $P < 0.05$, respectively), insulin secretion in response to glucose ($P < 0.01$), and percentage of NG-positive cells ($P < 0.05$) in the 3MA-exposed cells were considerably lower than the cells treated with CDM. The percentage of AO-positive cells ($P < 0.01$) and the expression of autophagy-related genes ($P < 0.001$) was significantly enhanced in the CDM group. These events were significantly prevented by the 3MA.

Conclusion: Our data showed that autophagy is necessary for beta-cell differentiation, and preventing autophagy by 3MA causes the reduction of beta-cell differentiation and insulin secretion.

Keywords: Adipose Stem Cells, Autophagy, Differentiation, Insulin Secreting Cells

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Introduction

Diabetes, a widespread chronic disease, manifests in two types. Diabetes type I is caused by the insulin-producing cell death while diabetes type II is induced by beta-cell impairment and reduction of insulin generation (1). Islet transplantation can be used for type I diabetes treatment, however it accompanies with two major limitations, including low pancreas donor and immune rejection (2). In recent years, stem cell therapy is applied to the production of beta-cell (3, 4). Mesenchymal stem cells are appropriate choices for this aim (5, 6). Among stem cell sources, white adipose tissue is more abundant and accessible. Adipose mesenchymal stem cells (AMSCs) can successfully differentiate into insulin-secreting cells (ISCs) (7, 8).

Autophagy is a requirement of cell remodeling and plays critical role in differentiation. Size, shape, and polarity drives remodeling can develop new organelles into the cell (9). The autophagy is a catabolic process that causes intracellular homeostasis through the destruction of intra-cytoplasmic components, damaged organelles, and aggressive pathogens. Moreover, autophagy increases cell survival by deleting damaged organelles and aggregated proteins. In other hand, Mammalian target of rapamycin (mTOR) modulates autophagy by affecting autophagy-associated proteins, such as autophagy-related gene 7 (*ATG-7*), *BCLIN-1*, autophagy-related gene 1 (*ATG-1*), and autophagy-related gene 5 (*ATG-5*) (10-12). Previous studies suggested that autophagy is required for beta-cell function and number, and impairment of autophagy

causes beta-cell dysfunction and reduces insulin secretion (13-16). Until now, most studies have been focused on the role of autophagy on insulin secretion capacity of beta cells in diabetic subjects. But, less attention has been paid to explore the effects of autophagy on the differentiation of stem cells into beta cells. In this study, autophagy effect on the differentiation of AMSCs to ISCs has been investigated.

Materials and Methods

In this experimental study, 10 normal Wistar rats (6-8 weeks) were handled following the International Animal Ethics Committee guidelines and the experiments were permitted by Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.ABHC.REC.1398.001). Following euthanasia with sodium pentobarbital (800 mg/kg body mass) (17) epididymis fat pads were dissected under sterile conditions. The fat pieces were exposed to 1.0 mg/mL type I collagenase (CAS Number: 9001-12-1, Sigma, USA) for 30 minutes (37°C) and then, centrifuged at 1,200 rpm /10 minutes (160 × g/10 minutes) to obtain homogenous suspension. The cells were re-suspended in DMEM media and then cultured in 25 cm² flasks. Using flow cytometry, the cells of passage 3, were characterized to determine specific markers (Fig.S1, See Supplementary Online Information at www.celljournal.org). Osteogenic and adipogenic potentials were examined by alizarin-red and oil red O staining (Fig.S2, See Supplementary Online Information at www.celljournal.org).

Inducing insulin-secreting cells

In the first step, the AMSCs at passage 4 were incubated (5% CO₂ at 37°C) for 48 hours in serum-free DMEM (high-glucose) containing 0.5 mmol/L 2-mercaptoethanol (Cat number: 60-24-2, Sigma-Aldrich, Germany). In second step, the cells were exposed to a cocktail medium containing 30 ng/mL fibroblast growth factor (FGF, Cat Number: 62031-54-3, Sigma-Aldrich, Germany), L-glutamine (2 mmol/L; Cat Number: 11548876, Gibco, USA), 20 ng/mL epidermal growth factor (EGF, Cat Number: 62253-63-8, Sigma-Aldrich, Germany), 2% B27 (Cat Number: 17504001, Thermofisher, USA), 10 ng/mL Exendin-4 (Cat Number: 141758-74-9, Sigma-Aldrich, Germany), and 1% non-essential amino acids for 8 days. Finally, in terminal step, the cells were exposed to a different medium containing 10 mmol/L nicotinamide (Cat Number: 98-92-0, Sigma-Aldrich, USA), and 2% B27 for 8 days.

Experimental groups

The following groups were used in this study:

- I. Control: treated with only DMEM for 18 days.
- II. 3MA (3-methyladenine, an autophagy inhibitor): incubated in only DMEM for 2 days and was followed by 50 μmol/L 3MA (Cat Number: 5142-23-4, Sigma-Aldrich, USA) in DMEM for 16 days.
- III. CDM: received cocktail differentiation media (CDM).

IV. CDM+3MA: received differentiation media and 50 μmol/L 3MA in second and terminal steps.

MTT assessment

MTT (dimethylthiazol-yl)-diphenyl tetrazolium bromide) test was done to measure the viability of the cells. The cells with the density of 5,000 cells/well were cultured in a 96-well plate. At the end of the 18th day, the media were removed and the cells exposed to MTT solution (Cat Number: 298-93-1, Sigma-Aldrich, Germany) up to 50 μL/well for 4 hours. Subsequently, 100 μL dimethyl sulfoxide (Cat Number: 67-68-5, Sigma-Aldrich, Germany) was added into the wells and was incubated at room temperature, for 60 minutes in the dark. Finally, the OD value (absorbance) was read at 570 nm wavelength using an ELISA Reader (Bio-Rad, USA).

Newport green staining

Conforming insulin synthesis in the differentiated cells, Newport green (NG, Cat number: N7991, Invitrogen, USA) staining was used. NG is a fluorescent dye with a high affinity for zinc, and also zinc is an important factor for insulin granules formation. In brief, the cells were fixed in paraformaldehyde (4%, Cat number: 30525-89-4, Sigma-Aldrich, Germany) for 15 minutes and then exposed to 0.1 % Triton X-100 (Cat number: 9036-19-5, Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS) for 20 minutes. Subsequently, adding 25 μM NG, the cells were incubated at 37°C for 30 minutes. The percentage of NG-positive cells was estimated using a fluorescent microscope (Olympus TH4-200, Japan).

Radioimmunoassay (glucose challenge test)

The capability of cells for insulin secretion was measured using a rat RIA kit (Cat number: EZRMI-13K, Millipore, Germany). First, the cells of each group were exposed to glucose-free Krebs-Ringer Bicarbonate (KRB). Then, the cells were divided into three groups and exposed to KRB containing different concentrations of glucose 5.56, 16.7 and 25 mmol/L for one hour. Then, the media were removed and kept in a -80°C freezer till use. Finally, insulin secretion was measured by the rat RIA kit (Cat number: EZRMI-13K, Millipore, Germany).

Acridine orange staining

After trypsinization, the cells were fixed in 4% paraformaldehyde (15 minutes), then the cells were exposed to methanol (Cat number: 67-56-1, Sigma-Aldrich, USA) for 10 minutes and permeabilized with 0.1 % Triton X-100 (10 minutes). One μg/mL acridine orange (AO, Cat Number: 65-61-2, Sigma-Aldrich, USA) solution was added to dishes. Then, the cells were washed with PBS 2-3 times. The percentage of AO-stained cells was determined using a fluorescent microscope (Olympus TH4-200, Japan).

Real-time polymerase chain reaction

The expression of pancreatic and duodenal homeobox

1 (*PDX1*), musculoaponeurotic fibrosarcoma oncogene homolog A (*MAF-A*), Nk class of homeodomain-encoding genes 6.1 and 2.2 (*NKX6-1* and *NKX2.2*), Glucose transporter 2 (*GLUT-2*) and *INSULIN* genes were evaluated which provide further evidence of ISCs differentiation and maturation. The autophagic activity was also determined by evaluating the expression of *ATG-5*, *BECLIN-1*, *mTOR*, and *ATG-7* genes. The *GAPDH* gene expression was detected as a housekeeping gene. RNA extraction was performed by using the RNeasy Mini kit (Cat number: 74904, Qiagen, Germany). Then extracted RNA were converted to cDNA by cDNA synthesis kit (Cat number: 205311, Qiagen, Germany). For each reaction, a mixture of 10 μ L master mix Sybr Green (Cat number: DQ384-40h, Biofact, Korea), 7 μ L nuclease-free water, one μ L of each sense and antisense primers (Table 1) and one μ L cDNA, was used. Real-time RT-PCR were run with the following program 10 minutes at 95°C, over 45 cycles at 25 seconds at 95°C, 50 seconds

at 5°C, and 45 seconds at 60°C. Finally, the $2^{-\Delta\Delta CT}$ value was used for data analysis.

Statistical analysis

All data has been analyzed by SPSS 21.0 (Chicago, IL, USA). Using Bonferroni technique, we performed post-hoc pairwise comparison. $P < 0.05$ was regarded as statistically significant. Each experiment was done in quadruplicate.

Results

3MA decreases viability percentage of AMSCs and ISCs

The viability percentage of CDM group was similar to the control. In the 3MA group, the viability percentage was significantly lower compared with control group ($P=0.035$). In the 3MA+CDM group, the percentage of the viability was considerably less than the CDM group ($P=0.042$, Fig.1).

Table 1: Primer sequences of different genes are presented

Gene	Primer sequencing (5'-3')	Size (bp)	Annealing temperature (°C)
<i>PDX1</i>	F: AAA CGGCACACACAAGGAGAA R: AGACCTGGCGCTTCACATG	150	55
<i>GLUT-2</i>	F: CAGCTGTCTTGTGCTCTGCTTGT R: GCCGTCATGCTCACATAACTCA	150	58
<i>INSULIN</i>	F: TCTTCTACACACCCATGTCCC R: GGTGCAGCACTGATCCAG	148	59
<i>NKX6-1</i>	F: ACACACGAGCCCACTTTTT R: TTCTGGAACCAGACCTTGAC	147	53
<i>NKX2.2</i>	F: AGTACTCCCTGCACGGTC R: GTCTCCTTGTCATTGTCCG	103	54
<i>MAF-A</i>	F: CTGCTGTCCTACTATGCTCA R: TGTATTTCCCAGGAGTTACAG	137	57
<i>mTOR</i>	F: TGTGATGGCTGTGAAGATCC R: TTGCTTGCCCATCAGAGTCAG	117	52
<i>BECLIN-1</i>	F: CTCTGAAACTGGACACGAGC R: CCTGAGTTAGCCTCTTCCTCC	124	58
<i>ATG-7</i>	F: CCAAAGCAGCATTGATGACCA R: TTCTGGATGCTGCAAAACAG	154	56
<i>ATG-5</i>	R: GGACCTTCTACACTGTCCATCC R: TGTCATTCTGCAGTCCCATC	152	53
<i>GAPDH</i>	F: ACCCAGAAGACTGTGGATGG R: TTCTAGACGGCAGGTCAGGT	179	52

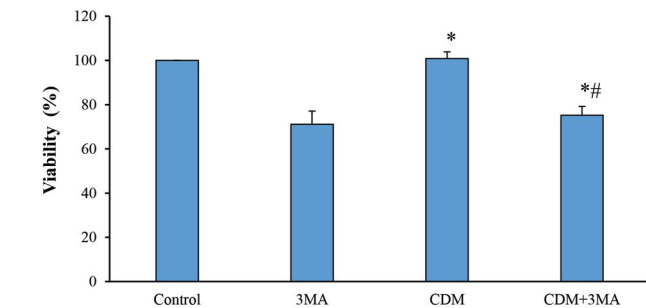
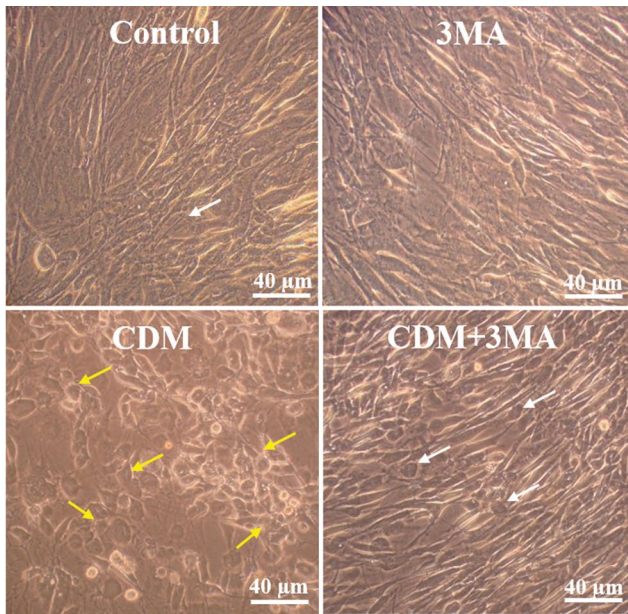


Fig.1: Morphological features and cell viability percentage in the different groups. Spindle fibroblast-like morphology is the most feature in the control and 3MA-treated cells. The majority of CDM-treated cells show round morphology. In 3MA+CDM group, round feature seems to be lower than spindle fibroblast-like morphology (white arrows indicate ISCs, and yellow arrows indicate clusters of the ISCs). Values are presented as mean \pm SD (n=6). *, P<0.05 (comparison with control), #, P<0.05 (comparison with CDM-treated cells), CDM; Cocktail differentiation media, and 3MA; 3-methyladenine.

The effects of CDM and 3MA on the morphology of cultured AMSCs

The cells of control group showed fusiform morphology at the first and at the end of the experiment, a few cells with spherical and neuron-like phenotype were observed. The majority of cells in the CDM group had a spherical shape. The number of spherical cells in the CDM+3MA group was lower than the CDM-treated cells (Fig.1).

3MA decreases percentage of NG-positive cells

The percentage of NG-stained cells (NG-positive cells) was significantly decreased in 3MA group, compared with the control (P=0.024). In the CDM group, the percentage of NG-stained cells was significantly increased compared with the control (P=0.004). The percentage of NG-stained cells in CDM+3MA group was significantly less than the CDM-treated cells (P=0.041, Fig.2).

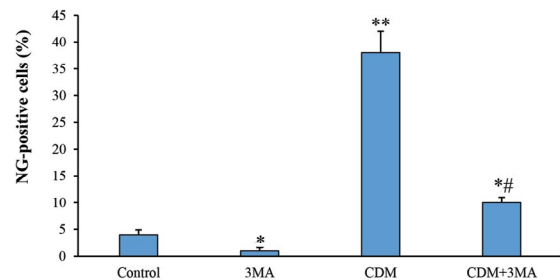
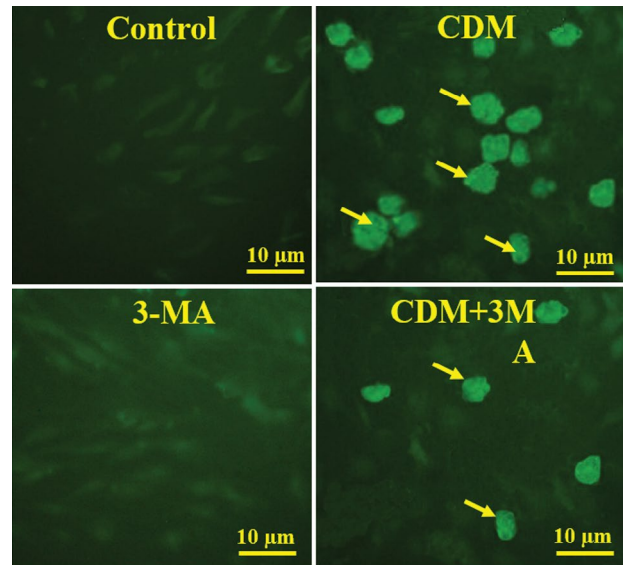


Fig.2: NG staining and percentage of NG-positive cells (mean \pm SD, n=4). Arrows indicate NG-positive cells. *, P<0.05, **, P<0.01 (comparison with control), #, P<0.01 (comparison with CDM-treated cells), CDM; Cocktail differentiation media, 3MA; 3-methyladenine, and NG; Newport green.

3MA reduces insulin secretion in response to different concentration of glucose

The amount of insulin secretion was elevated in response to the acceleration of glucose concentration in the all groups, although more insulin secretion was observed at 25 mmol/L in comparison with 5.56 mmol/L glucose. In the 3MA group, the amount of insulin secretion was significantly lower than the control (P=0.033). Insulin secretion in the CDM group was significantly more than the control group (P<0.001). The amount of insulin secretion in the CDM+3MA group was significantly lower than CDM group (P=0.006, Fig.3).

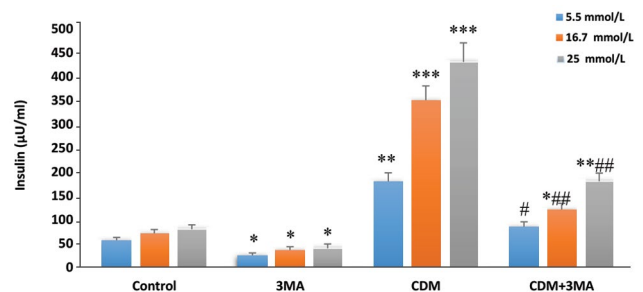


Fig.3: Secretion of insulin in response to different concentrations of glucose (mean \pm SD, n=5). *, P<0.05, **, P<0.01, ***, P<0.001 (comparison with control), #, P<0.05, ##, P<0.01 (comparison with CDM-treated cells), CDM; Cocktail differentiation media, and 3MA; 3-methyladenine.

The number of AO-positive cells increased in ISCs

The number of AO-staining cells in the 3MA group was lower than the control ($P < 0.001$). The number of AO-positive cells was significantly elevated in the CDM group, compared to the control ($P = 0.005$). These results are presented in Figure 4.

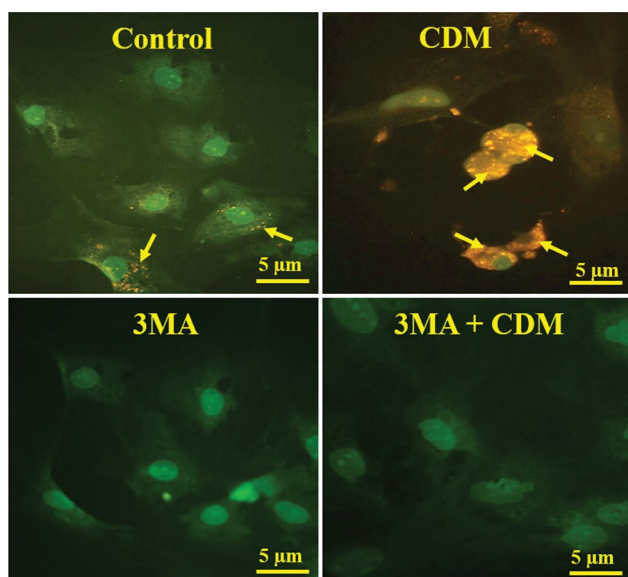


Fig.4: Acridine orange (AO) staining and percentage of AO-positive cells. Arrows indicate autophagosomes. Values are presented as mean \pm SD (n=6). *, $P < 0.01$.

3MA decreases expression of beta-cell-specific genes in differentiated AMSCs

The expression of *PDX1*, *NKX2.2*, *NKX6-1*, *GLUT2*, *MAF-A* and *INSULIN* genes in the CDM-treated cells were significantly more than the control group. The expression of these genes was significantly decreased in the 3MA-treated cells. In the CDM+3MA group, the expression of beta-cell-specific genes significantly decreased in comparison with the CDM group (Fig.5).

Autophagy-related genes up-regulates in ISCs

Gene expression value of *BECLIN-1*, *ATG-5*, and *ATG-7* in the 3MA group was significantly lower than the control group. The expression of these genes in the CDM group was significantly higher than the control group. Cells in the CDM+3MA groups had less expression than

the CDM group. The expression level of the *mTOR* gene in the DM group was significantly lower than the control group. In the 3MA group, the expression of this gene was significantly higher than the control group. The expression of *mTOR* in the CDM+3MA group was significantly more than the CDM-treated cells (Fig.5).

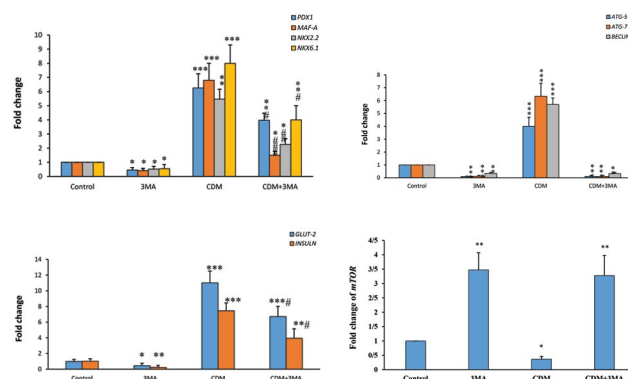


Fig.5: Gene expression in various groups. Values are presented as mean \pm SD (n=4). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ (comparison with control), #, $P < 0.05$, ##, $P < 0.01$, ###, $P < 0.01$ (comparison with CDM-treated cells), CDM; Cocktail differentiation media, and 3MA; 3-methyladenine.

Discussion

In the present study, adipose-derived mesenchymal stem cells (AMSCs) were successfully differentiated into the ISCs. The presence of ISCs was confirmed by evaluation of beta-cell specific genes *PDX1*, *NKX2.2*, *NKX6-1*, *GLUT2*, and *MAF-A*, and generation and secretion of insulin. *NKX2.2* is required for the beta-cells differentiation and expresses early in pancreatic progenitors (18). *NKX2.2* is also essential for the maintenance and function of the mature beta-cell in the postnatal islet (19). *NKX6-1* regulates differentiation and proliferation of beta cells, and insulin secretion in response to the glucose stimulation (20, 21). Taylor et al. (20) showed that inactivation of *NKX6-1* induces hypoinsulinemia and diabetes in mice. They concluded that *Nkx6.1* has a crucial role in maintaining the functional state of beta cells.

Also, *PDX1* is another requirement for development of the pancreatic beta-cells. In summary, *PDX1* binds to the regulatory regions and enhances *INSULIN* expression. In human Zhu et al. (22) reported that *PDX1* deletion causes pancreatic agenesis that reduces insulin secretion and attenuates beta-cell number. Also, *PDX1* binds to the enhancer region of the *MAF-A*, and regulates its expression. *MAF-A* binds to the promoter region of the *INSULIN* gene and stimulates insulin secretion in response to glucose (23). The increasing expression of beta-cell-specific genes was accompanied by increasing NG-positive cells, which indicating insulin synthesis in CDM-treated cells.

The ISCs not only generates insulin, but also secretes insulin in response to a glucose challenge. In the CDM-treated cells, expression of *INSULIN* and *GLUT-2* genes

were also significantly increased, which indicates that the ASC-derived ICSs have undergone differentiation and maturation. In the beta-cell, glucose uptake is regulated by *GLUT-2*, which is critical for insulin secretion.

We observed that 3MA significantly reduced the expression of *PDX1*, *NKX2.2* and *MAF-A* genes in the ASC-derived ICSs. Also, *PDX1* stimulates expression of several genes such as *Glut-2*, glucokinase (*GCK*) and *INSULIN*, which involve in the maturation of beta-cell. In 3MA-exposed cells, the percentage of NG-positive cells was reduced and expression of *GLUT-2* and *INSULIN* was down-regulated. In addition, 3MA significantly reduced the secretion of insulin in the glucose challenge conditions.

Knowing exact control of organelles degradation and turnover of proteins are essential to knowing cell differentiation. Autophagy is a highly conserved cellular degradation process. Cellular organelles that involve in beta-cell survival and insulin secretion, such as mitochondria and endoplasmic reticulum also, rely on autophagy for their proper function (24-29). It has been demonstrated that autophagy is essential for maintaining the normal function of pancreatic beta-cells. Autophagy also has a critical role in the insulin granules turnover of the beta-cell and insulin secretion. Autophagy dysfunction reduces beta-cell mass and functions and impairs islet architecture (29-31). Chen et al. (15) showed that autophagy had a crucial role in the organization and function of beta-cell. Watada and Fujitani (32) have confirmed that autophagy inhibition causes dysfunction of the beta-cells.

To confirm the importance of autophagy in the differentiation of AMSCs to ICSs, expression of *BECLIN-1*, *ATG-5*, *ATG-7*, and *mTOR* was evaluated in this experiment. As shown in result section, *ATG-7*, *ATG-5*, and *BECLIN-1* genes were up-regulated in the ICSs. In agreement with these results, Ren et al. (33) have shown that autophagy is involved in the differentiation of porcine pancreatic stem cells into ICSs.

In the study of *Atg-7* mutant mice by Ebato et al. (30), insulin serum level reduction, and impaired glucose tolerance was observed. In this study, a diminish in the beta-cell number, insulin content of pancreas, and beta-cell proliferation was observed, whereas, apoptosis was induced in the beta-cells. Inducing protective impact of autophagy against beta-cell apoptosis by high-glucose has been reported (15). Interestingly, the viability of ICSs in the presence of 3MA dramatically reduced. Hence, 3MA may increase beta-cell death and consequently decrease insulin secretion via suppression of autophagy.

As mentioned in "Result" section, AMSCs can partially differentiate into ICSs in the presence of CDM and 3MA. Thus, besides autophagy, other cellular pathways or molecules may also be involved in the ICSs inducing from AMSCs such as miRNAs. López-Beas et al. (34) reported that mir-7 and, enhances differentiation of embryonic

stem cells while Xu et al. (35) study showed that mir-690 induces pluripotent stem cells conversion into the ICSs.

Conclusion

In the present study, we observed successfully differentiation of AMSCs into the ICSs in the presence of cocktail differentiation medium (CDM). The ICSs effectively generated insulin and overexpressed the *INSULIN* gene. The current work has also demonstrated that 3MA, autophagy inhibitor, suppresses differentiation of AMSCs into ICSs. Our results collectively indicate that autophagy is required for differentiation and maturation of beta-cell.

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Authors' Contributions

F.R.-T., L.K.; Study design, all experimental work, data collection, and statistical analysis. M.H., L.K.; Data interpretation and responsible for overall supervision. S.A.M.; Study design and validation. All authors read and approved the final manuscript.

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