Research Article **Cell Biology International**
10.1002/cbin.10567

Induction of pancreatic β cell gene expression in mesenchymal stem cells†

Running Head: β cell induction of mesenchymal stem cells

Zahra Mehrfarjam[1], Fariba Esmaeili[2,3*], Leila Shabani[3], Esmaeil Ebrahimie[4,5,6,7]

[1] Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, P.O. Box 681499468, Khorramabad, Iran.
[2] Department of Biology, Faculty of Basic Sciences, University of Isfahan, P.O. Box 8174673441, Isfahan, Iran.
[3] Research Institute of Biotechnology, Shahrekord University, P.O. Box 115, Shahrekord, Iran.
[4] Institute of Biotechnology, Shiraz University, Shiraz, Iran.
[5] School of Information Technology and Mathematical Sciences, Division of Information Technology Engineering & Environment, University of South Australia, Aselaide, Australia.
[6] Department of Genetics and Evolution, School of Biological Sciences, The University of Adelaide, Adelaide, Australia.
[7] School of Biological Sciences, Faculty of Science and Engineering, Flinders University, Adelaide, Australia

***Corresponding author:** Fariba Esmaeili, PhD.,
Department of Biology, Faculty of Sciences, University of Isfahan, P.O. Box 8174673441, Isfahan, Iran. Phone: +98 31 37934151, Fax: +98 31 37932170,
E-mail: f.esmaeili@sci.ui.ac.ir
esmaeili-f@sci.sku.ac.ir

Authors' E-mail:
Zahra Mehrfarjam: bahar.mehrfarjam@gmail.com
Fariba Esmaeili: f.esmaeili@sci.ui.ac.ir & esmaeili-f@sci.sku.ac.ir
Leila Shabani: shabani-l@sci.sku.ac.ir
Esmaeil Ebrahimie: esmaeil.ebrahimie@adelaide.edu.au

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/cbin.10567]

Received July 19, 2015; Revised November 10, 2015; Accepted November 23, 2015

List of abbreviations

MSCs: mesenchymal stem cells

IPCs: insulin-producing cells

MPE: mouse pancreas extract

PCM: pancreas conditioned medium

RPE: Rat regenerating pancreas extract

Sox-2: SRY (Sex Determining Region Y)-Box 2

Oct3/4: POU Class 5 Homeobox 1

Nanog: Nanog Homeobox

INS1: insulin1

INS2: insulin2

PDX-1: pancreatic and duodenal homeobox 1

EP300: E1A binding protein p300

CREB1: cAMP responsive element binding protein 1

β-2M: β-2 microglobulin

Abstract

Transdifferentiattion potential of mesenchymal stem cells (MSCs) into insulin-producing cells (IPCs) has been suggested recently. In our recent works, we demonstrated the high performance of mouse neonate pancreas extract (MPE) in production of functional IPCs from carcinoma stem cells. In this study, MPE was used to generate IPCs from MSCs without any genetic manipulation. To this end, bone marrow MSCs were isolated and characterized. In order to differentiate, MSCs were induced by selection of nestin-expressing cells and treatment with 100 μg/ml MPE. Morphological features of the differentiated cells were confirmed by dithizone staining. Immunoreactivity to insulin receptor beta, proinsulin, insulin and C-peptide was observed by immunoflourescence. We also quantified glucose-dependent insulin production and secretion by ELISA. Real-time PCR indicated the expressions of β cell related genes, *PDX-1*, *INS1*, *INS2*, *EP300* and *CREB1* in IPC cells. Possible pathways governed by CREB1, EP300, and PDX-1 transcription factors in differentiation of MSCs to IPCs were determined based on Gene Set Enrichment (GSE) approach at p=0.05. Pathway discovery highlighted the negative regulatory effects of *MIR124-2*, HDAC5 protein, REST and NR0B2 transcription factors on expression of *CREB1*, *EP300*, and *PDX-1* and inhabitation of IPC differentiations. In contrast, a crosstalk between FOXA2 and TCF7L2 transcription factors, DNA-PK complex, KAT2B protein positively interacting with PDX-1, CREB1, EP300 resulted in the induction of IPC and following insulin production. In conclusion, we report an efficient, simple, and easy method for production of functional IPCs from MSCs by MPE treatment.

Key words: Mesenchymal stem cells; Pancreas extract; stem cell differentiation; Insulin-producing cells; Nestin-positive cells

1. Introduction

Ex vivo differentiation systems using stem cells provide valuable experimental tools to explore underlying regulatory mechanisms of pancreatic β cell mass development, expansion and maintenance. To reach this goal, developing appropriate and effective methods for differentiation and maturation of stem cells into insulin-producing cells (IPCs) are highly required. Different elicitors such as fibroblast growth factor (FGF), betacellulin, activin, and exendin-4 can induce stem cell differentiation into IPCs (Ackermann and Gannon, 2007; Chao et al., 2008; Jafary et al., 2008; Yang et al., 2002). Interestingly, signals from mesenchymal stem cells (MSCs) play a significant role in the development of normal pancreas. Reddy et al. (2000) showed that administration of whole pancreatic extract to non-obese diabetic (NOD) mice inhibited autoimmune diabetes (Reddy et al., 2000). Subcutaneous administration of pancreas extract to human decreased blood glucose and increased utilization of carbohydrates (Banting et al., 1991). Cotransplantation of differentiated human embryonic stem cells (hESCs) with mouse embryonic pancreas under the kidney capsule resulted in differentiation of β cell (Brolén et al., 2005). Coculture of MSCs with regenerating pancreas conditioned medium (Choi et al., 2005) or rabbit pancreas tissue (Chang et al., 2007) remarkably stimulated the differentiation of the stem cells to generate IPCs. It has been reported that the required factors for pancreas regeneration are either secreted or exist in pancreatic milieu (Kanitkar and Bhonde, 2004). Rat regenerating pancreas extract (RPE) contains factors that induce islet neogenesis in diabetic animals as well as transdifferentiation of MSCs into IPCs (Lee et al., 2008; Xie et al., 2013; Zhang et al., 2012). Self-renewable MSCs can be isolated from a range of adult tissues such as adipose, pancreas, and bone marrow. They can be easily separated, grown in culture and transdifferentiated into a wide variety of cell types. MSCs from various tissues were induced to differentiate into functional IPCs under the defined conditions (Chao et al., 2008; Liu and Han, 2008; Oh et al., 2004; Xie et al., 2013). However, the resulting IPCs appeared to be not fully matured and functional (Xie et al., 2013).

We recently introduced two successful induction systems using neonate mouse pancreas extract (MPE) (Ebrahimie et al., 2014) and conditioned medium (PCM) (Mansouri et al., 2014) as natural native inducers to produce IPCs from P19 embryonal carcinoma (EC) cells. Based on our observations both MPE and PCM are able to efficiently induce IPC differentiation. It should be emphasized that islets of mouse pancreas are not fully formed before birth and undergo further remodeling and development for 2-3 weeks after birth (Habener et al., 2005). Numbers and mean sizes of mouse pancreatic islets and β cells increase significantly during postnatal life (Herbach et al., 2011). Therefore, we hypothesized that neonatal MPE may also contains factors similar to embryonic and regenerating pancreas able to induce in vitro transdifferentiation of MSCs into pancreatic lineages**.** Based on the mentioned hypothesis, in this study, we used whole pancreas extract to generate functional IPCs from MSCs without any genetic manipulation. We showed MPE is highly efficient for transdifferentiation of bone marrow derived MSCs into mature IPCs. The differentiated cells expressed specific β cell markers. In addition, they were able to make and secret insulin in response to glucose.

2. Materials & Methods

2.1. Preparation of MPE

BALB/c pregnant mice were provided from Azad University of Shahrekord (Shahrekord, Iran). Whole pancreas of 1-2 weeks old neonate mice were carefully removed and homogenized in PBS containing protease inhibitors (PMSF, Roche, 10837 091 001). The homogenates were then centrifuged and protein contents of the samples were rapidly analyzed by Bradford method.

2.2. Isolation, culture and expansion of MSCs

Isolation of MSCs from bone marrow was performed according to previously described method (Peister et al., 2004) with slight modifications. Briefly, the femurs and tibiae of two-months-old male mice were dissected away from the attached tissues. Bone marrow cells were perfused and

cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 31600), 10% fetal bovine serum (FBS, Gibco, 10270-106), penicillin (50 μg/ml, Sigma, P3032), and streptomycin (50 μg/ml, Sigma, S1277). After 24 h, the nonadherent hematopoietic cells were removed. At 80% confluency, the cells were treated with 0.25% trypsin/0.04% EDTA and seeded into fresh flasks.

2.3. Multipotency characterization of MSCs

The cells were cultured in a six-well plate and then treated with osteogenic or adipogenic induction medium for 21 days. Alizarin red and oil red staining were used to confirm the osteocytes and adipocytes differentiation, respectively, as described previously (Peister et al., 2004). Expressions of undifferentiated stem cell markers including *Oct3/4*, *Sox-2* and *Nanog* in MSCs were detected using real-time PCR (see below).

2.4. Phenotypic characterization of MSCs by fluorescence activated cell sorting (FACS)

FACS analysis of MSCs was performed as previously described (Kobune et al., 2003). Briefly, cell suspension was washed twice with 0.1% bovine serum albumin (BSA) in PBS. 1×10^6 cells were incubated with anti-CD44 (Abcam, ab119348) or anti-CD45 (Abcam, ab10558) primary antibody. FITC-conjugated anti-mouse IgG (Sigma, St. Louis, MO, F9137) was used as secondary antibody and re-suspended for analysis.

.2.5 Transdifferentiation of MSCs into IPCs

Outline of differentiation protocol is summarized in Figure 1. Bone marrow MSCs were isolated and grown in DMEM supplemented with 10% FBS (stage 1). Multipotency characterization of the cells was then carried out (see above). To direct differentiation, cells were cultured in DMEM supplemented with 3% FBS (stage 2) and induced based on selection of nestin-positive cells (Lumelsky et al., 2001) (stage 3). To select nestin-positive cells, the media were changed with serum-free medium including DMEM/F12 medium (DMEM nutrient mixture F-12 HAM, Sigma, D8900) and 1 mg/ml ITSFn (Insulin-transferrin-sodium selenite media supplement, Sigma, I1884).

Four to seven days later, the cultures were grown in N2 medium (Stem Cell Technologies INC, 07152) containing B27 (Invitrogen, 17504-044), 5 μg/ml insulin, 10 ng/ml bFGF (FGF-Basic human, Sigma, F0291) and 10 ng/ml EGF (Epidermal Growth Factor human, Sigma, E9644) (stage 4). EGF and bFGF were then withdrawn and 10 mmol/l nicotinamide (Sigma, N0636) was added for the next 7-14 days (stage 5). For differentiation induction of the cells into IPCs, MPE (100 μg/ml) was added in this stage. The cultures with no MPE were considered as control.

2.6. Dithizone staining

To confirm the existence of MSCs-derived IPCs, we used dithizone (DTZ) staining (Shiroi et al., 2005). Stock solution of DTZ (diphenylthiocarbozone, Merck, DX2370-3) (50 mg) in dimethylsulfoxide (DMSO, Sigma, D2650) was prepared. Staining was performed by working solution. After rinsing with HBSS (hank's balanced salt solution), clusters of crimson red cells were tested by stereomicroscope. A cell suspension was prepared after trypsinization following DTZ staining. The cells were counted using a hemocytometer at 40X magnification. The assay was repeated at least three times.

2.7. Immunoflourescence staining

The primary antibodies used in this study were: anti-nestin (Sigma, N5413), mouse monoclonal proinsulin+insulin, (Abcam, ab8304-50), rabbit polyclonal anti-C peptide (Abcam, ab14181) and mouse monoclonal insulin receptor beta (Abcam, ab8304-100) antibody. Cy5.29-conjugated anti-rabbit IgG (Abcam, Cambridge, USA, ab6564) and FITC-conjugated anti-mouse IgG (Sigma, St. Louis, MO, F9137) were used as secondary antibodies. For immunoflourescence, the cells were cultured in six-well plates, fixed and permeabilized. They were then treated by normal goat serum (NGS, Sigma, G9023) to prevent nonspecific binding. Then, the cells were incubated with the primary antibody, and relevant secondary antibody. All of the antibodies were used at 1:1000

dilutions. Glycerol (70%) was used to mount the cover slips. Primary antibody was removed as negative controls for immunostaining.

2.8. Enzyme-linked immunosorbent assay (ELISA) for evaluating insulin content and secretion

IPCs and undifferentiated MSCs were grown in six-well plates to assay the secreted and intracellular insulin**.** Before the measurement, the cells were washed and incubated in a fresh serum-free medium containing 0.5% bovine serum albumin (BSA) to allow the detection of insulin secretion without fetal serum interference. Sequential adding of serum-free media containing low (5.5 mmol/l) - or high (25 mmol/l)-glucose was used for glucose challenge of the cells. The conditioned media was frozen at -70°C until assay for insulin content. Sonicating in acid-ethanol was used for assessing intracellular insulin content of cell pellets. The values were normalized regarding the total protein content. ELISA was used on the conditioned media and cell extract by insulin mouse ultrasensitive ELISA kit (Alpco, 80-insmsu-E01). The assay was repeated at least three times.

2.9. Real-time RT-PCR

Total RNA was extracted from IPCs and undifferentiated MSCs using Qiazol lyses reagent (Qiagene, 79306). cDNAs were made (Qiagene-QuantiTectRev_Transcription Kit, 205311) and RT-PCR assays were carried out (Qiagen, Rotor-Gene, California, USA). Real-time of undifferentiated cell markers, *Sox-2* [SRY (Sex Determining Region Y)-Box 2], *Oct3/4* (POU Class 5 Homeobox 1), and *Nanog* (Nanog Homeobox) as well as pancreatic β cell genes, *INS1* (insulin1), *INS2* (insulin2), *PDX-1* (pancreatic and duodenal homeobox 1), *EP300* (E1A binding protein p300), and *CREB1* (cAMP responsive element binding protein 1) and a housekeeping gene, *β-2M* (β2 microglobulin) were performed using specific primers (Supplementary 1) and SYBR Premix Ex Taq. RT samples and negative controls (with no template) were run together with the test samples. Standard curves were designed for each tested gene to analyze the efficiency of the reaction. Values

of the expressions were normalized in relation to individual *β-2M* as internal control. The profile was obtained by plotting the relative gene expression levels compared to the undifferentiated cells.

2.10. Statistical analysis

All the experiments were performed in triplicate. Statistical differences were measured using Student's t-test, in two groups' comparison. For more than two groups (treatments), one-way analysis of variance (ANOVA) and Duncan's Test were applied. The results were expressed as the mean ± standard deviation (SD) and $p<0.05$ was pointed out as statistically significant.

.2.11 Subnetwork discovery based on Gene Set Enrichment (GSE) approach

After the confirmation of the up regulation of *CREB1*, *EP300*, and *PDX-1* transcription factors as the major regulators in differentiation of MSCs to IPCs by real-time RT-PCR, these transcription factors as well as insulin protein were subjected to subnetwork (pathway) discovery based on Gene Set Enrichment (GSE) approach at $p=0.05$ (Subramanian et al., 2005). Statistically significant subnetworks determined the possible biological functions and ultimate goals by which *CREB1*, *EP300*, and *PDX-1*are upregulated.

For running GSE algorithm, enriched mammalian database of Pathway Studio 10 package (Elsevier) including gene/protein/small RNA interaction was employed (Novichkova et al., 2003). Then, the contribution of *CREB1*, *EP300*, and *PDX-1* transcription factors and insulin to different deposited pathways in Pathway Studio database was measured by Fisher's exact test. The deposited pathways in Pathway Studio database (Mammal) is presented in Supplementary 2. Pathway Studio is highly enriched in terms of pathways, gene ontologies, and relationships which are constructed using literature (text) mining of scientific publications of major domains (pubmed, science direct, etc.) by Medscan language text mining (Hosseinpour et al., 2012; Nikitin et al., 2003; Novichkova et al., 2003). Medscan is able to efficiently mine the text and convert that to biological interaction formula such as promoter binding, expression/regulation, inhibitory microRNA effect, etc.

2.12. Combining the subnetworks based on union selected network algorithm

Union selected network algorithm was used to combine the statistically significant subnetworks and form an integrated network. Union selected subnetwork is an effective algorithm in unraveling the key regulatory elements (ligands, receptors, transcription factors and microRNAs) (Alanazi et al., 2013; Ebrahimie et al., 2014; Nikitin et al., 2003). Transcription factors, ligands, receptors, and microRNAs are the main coordinators in integrative networks which build crosstalk between different pathways and synchronize them (Alisoltani et al., 2014).

3. Results

3.1. Isolation and characterization of MSCs

To obtain the adherent MSCs, cells from mouse bone marrow were plated and cultured (Figure 2 A). The unattached cells were removed and the adherent MSCs were cultured. After subculture, the majority of adherent cells had uniformly appearance. Figure 2 B-D shows morphological changes of undifferentiated MSCs. The attached undifferentiated MSCs show fibroblast-like cell morphology (Figure 2 B, C). After 10 days of culturing, the cells started to form cell clusters (Figure 2 D). To confirm the multipotency characters of MSCs, the cells were incubated in osteogenic or adipogenic differentiation medium for 21 days. Calcium depositions were detected by Alizarin red in osteogenic differentiated MSCs (Figure 2 E). The result of oil red staining showed the presence of lipid droplets in adipogenic differentiated MSCs (Figure 2 F). Furthermore, MSCs markers were analyzed by FACS (Figure 2 G). The data indicated that the majority of the cells were positive for CD44 (99.6%) and negative for CD45 (98.7%).

3.2. Selection of nestin-positive cells

In the first step of differentiation protocol, MSCs were cultured in a serum-free medium to select nestin-positive cells. Immunoflourescence analysis showed that the differentiating cells express

nestin at the stage 3. Figure 3 shows individual nestin-positive cells after staining with antibody against nestin (Figure 3 A). The nuclei were counterstained by Hoechst **(**Figure 3 B**)**. Figure 3 C shows combined picture of nestin and Hoechst.

3.3. Morphological studies

DTZ was used to assess the presence of MSC-derived IPCs after differentiation induction. The outcomes of DTZ-staining are shown in Figure 4 A-E. During differentiation, spindle fibroblast-shape cells became round or oval-type cells. Then, they organized into small clusters, and in final, formation of islet-like aggregates happened (Figure 4 A-C). Distinct DTZ^+ cells were observed in MPE-treated MSCs while they could not be seen at the intact undifferentiated cells (Figure 4 D). To estimate the frequency of DTZ^+ cells, the number of crimson red cells in the cultures was counted. Figure 4 E shows the percentage of DTZ^+ cells which is significantly higher than that of the untreated group (74.00±5.09%, 15.20±2.64%, respectively, $p<0.05$).

3.4. Protein analysis of MSC-derived IPCs

Nestin-positive cells were expanded by culturing in the presence of bFGF and EGF and differentiated into IPCs by supplementing the culture medium with nicotinamide and 100 µg/ml concentration of MPE**.** Using this procedure, cells that displayed expression of pancreatic β cell specific proteins were obtained. Immunoflourescence evaluation of the differentiated MSCs showed that these cells were immunoreactive to insulin receptor beta (Figure 5 B), proinsulin+insulin (Figure 5 E), and C-peptide (Figure 5 H) after MPE treatment. The nuclei were counterstained by Hoechst (Figure 5 A, D, G, J). The primary antibody was omitted in several groups as controls for immunostaining (Figure 5 K).

3.5. Insulin alterations responding to glucose challenge

To evaluate the functional differentiation of developed IPCs in our culture system, we analyzed glucose-dependent insulin release by ELISA. Glucose inducible insulin secretion was examined in undifferentiated MSCs and differentiated IPCs. The results are presented in Figure 6. The statistical differences between the groups revealed that compared to MSCs, differentiated IPCs demonstrated

an obvious enhancement in secreted (Figure 6 A) and intracellular (Figure 6 B) insulin, when normalized to the total protein content. No significant insulin content or secretion was observed in undifferentiated cells. The amount of secreted and intracellular insulin in the treated cells was 4.6 and 3.9 times respectively over that in untreated cells ($P<0.05$).

3.6. QPCR

To determine the effects of MPE on IPCs transdifferentiation of MSCs, the expression of pancreas specific genes was examined by qPCR. Untreated cells (with no MPE treatment) were also considered as control. The results of representative experiments are presented in Figure 7. To categorize MSC cells, a qPCR analysis was initially performed on these undifferentiated cells and IPCs for comparing undifferentiated marker gene expression, including *Oct3/4*, *Sox-2* and *Nanog* (Figure 7 A). Expression of genes was significantly down regulated in IPC differentiated cells by 84, 15.38 and 1.44 times, respectively.

The data showed that, in our culture system mRNA expression of pancreatic specific markers were significantly increased in the differentiated cells (Figure 7 B). The expression levels of *PDX-1*, *INS1* and *INS2* in IPCs were significantly higher than those of undifferentiated MSCs. Differentiated cell markers*, EP300* and *CREB1* were also selected and quantified in MSCs and IPCs. The results indicated that during IPCs differentiation, the expressions of EP300 and CREB1 transcription factors were increased by 5 and 9.7 times, respectively (Figure 7 B).

3.7. Subnetwork discovery and network construction

Based on GSE approach (Subramanian et al., 2005), different pathways (subnetworks) governed by upregulation of *CREB1*, *EP300*, and *PDX-1* transcription factors were identified at $p<0.05$ and are presented in Table 1 and Supplementary 3. Some of these pathways are visualized in Figure 8 and underpinning references are mentioned in Supplementary 4. As presented in Figure 8, some subnetworks have negative and some have positive regulatory effect on CREB1, EP300, PDX-1, and insulin production.

The first significant pathway belongs to MIR124-2. This microRNA has negative regulatory effect on expression of CREB1, and PDX-1 as well as insulin production (Figure 8). Another regulatory subnetwork belongs to HDAC5. HDAC5 is a nuclear protein which down regulated CREB1, EP300, PDX-1, and insulin production mechanism (Figure 8). REST and NR0B2 transcription factors are the other negative regulators of expression of CREB1, EP300, and PDX-1 and inhibitors of IPC differentiations.

In contrast, FOXA2 and TCF7L2 transcription factors, DNA-PK complex, KAT2B protein positively interact with CREB1, EP300, PDX-1 and lead to differentiation of IPCs and insulin production. Due to the ability of transcription factors in activation of promoter regions of different genes (Deihimi et al., 2012), transcription factors are the major element establishing the link between different subnetworks.

Union selected subnetwork constructed an integrative network based on the significant subnetworks (presented in Supplementary 5). Underlying relationships and types of relationships can be retrieved from Supplementary 6. Interestingly, SIRT1 is placed on mitochondria contributes in IPCs differentiation.

4. Discussion

Adult bone marrow-derived MSCs are pluripotent stem cells with the potential to differentiate into a wide variety of cell types (Abdi et al., 2008). Some studies have reported that MSCs isolated from various tissues are able to differentiate into functional IPCs (Chang et al., 2007; Chao et al., 2008; Liu and Han, 2008; Oh et al., 2004; Sun et al., 2007; Xie et al., 2013; Zhang et al., 2009). We recently introduced two successful induction systems using neonate MPE (Ebrahimie et al., 2014) or PCM (Mansouri et al., 2014) as natural native inducers to produce IPCs from P19 EC cells. Our observations documented that both MPE and PCM efficiently induced IPC differentiation in this cell line. To the best of our knowledge, this work is the first attempt demonstrating that pancreatic extract from neonatal mouse has the capacity to induce pancreatic differentiation of MSCs. Here,

we report that pluripotent MSCs similar to P19 cells efficiently respond to differentiation cues presented in MPE through transdifferentiation into functional IPCs for the first time.

MSCs can be easily separated, grown and multiply in vitro. These cells are recognized by various criteria including adherence to culture plastic, differentiation ability, and presence/absence of specific markers (Le Blanc and Pittenger, 2005). Therefore, at the first part of the present study, undifferentiated state of MSCs was assessed by 1) adherence to plastic culture flasks: they were plastic-adherent when maintained in expansion conditions, 2) morphology: they indicated elongated spindle or fibroblast-like shape as well as a vortex-like growth (Xie et al., 2013), 3) osteogenic and adipogenic differentiation potential of MSCs, 4) positive and negative reaction for MSCs markers CD44 and CD45, respectively, and finally 5) expression of stem cell markers, including *Oct3/4*, *Sox-2* and *Nanog*. In line with the findings of this study, other investigators have reported the expression of these three genes in MSCs (Gade et al., 2013; Lee et al., 2008). Pierantozzi et al. (2011) showed that *Nanog*, but not *Oct-4* and *Sox-2* is expressed in cultured human adult MSCs (Pierantozzi et al., 2011). Our results demonstrated that the expressions of these undifferentiated stem cell markers were down regulated during differentiation. Furthermore, results of FACS analysis showed that isolated MSCs were positive for CD44, but negative for CD45.

The extract utilized in our study derived from whole pancreas of 1-2 weeks mouse neonate. Our hypothesis was that MPE is able to provide a tissue-specific microenvironment for pancreatic differentiation. It is well known that tissue extract mimics tissue specific microenvironment and is able to stimulate the differentiation of bone marrow MSCs to the target lineage (Liu et al., 2009). Human diabetic MSCs (dBM-MSCs) exposed to fetal pancreatic extract (Phadnis et al., 2009) or paracrine factors secreted from regenerating pancreas (Phadnis et al., 2011) were differentiated into a pancreatic lineage. Pancreatic tissue extract can effectively improve transdifferentiation efficiency and maturity of IPCs (Xie et al., 2013). Both insoluble and soluble factors from the surrounding microenvironment influenced the fate of stem cell differentiation (Phadnis et al., 2009). Differentiation induction of ES cells into IPCs was observed by fetal soluble factors, released

during pancreas embryogenesis (Vaca et al., 2006). Mouse pancreas undergoes further development for 2-3 weeks after birth (Habener et al., 2005). Therefore, mouse neonate MPE probably contains some soluble factors that are necessary for pancreas development. Soluble factors act in up-regulation of some of the pancreatic endocrine and exocrine markers (Metrakos et al., 1993; Narayanan et al., 2014; Talavera-Adame et al., 2011). Altogether, these findings indicate that the developing pancreatic tissues contained some important soluble factors that are able to promote stem cells differentiate to β cells. This approach provides new strategies for developing an efficient induction method for differentiation of stem cells into IPCs.

To direct differentiation, MSCs were induced based on nestin-expressing cell selection and treatment with 100 μg/ml MPE**.** Differentiation of nestin-positive cells into IPCs after treatment, suggested that MPE could provide the necessary cues for promoting pancreatic induction of MSCs. In our earlier investigation using MPE, this native medium could efficiently induce β cell features in P19 EC cells. The peak response for pancreatic induction was at 100 μg/ml (Ebrahimie et al., 2014), which was used for further investigation at the present study. According to some reports, nestin as a neural stem cell marker may not be specific for precursors of β cell (Yang et al., 2011). In contrast, some studies have shown the expression of nestin in islet progenitor cells (Kim et al., 2004; Kim et al., 2010). Our results indicated that it is possible to generate IPCs from undifferentiated mouse bone marrow MSCs with the functional and molecular characteristics of pancreatic β cells via nestin selection protocol plus MPE as an inducer. These findings are consistent with the earlier demonstrations that nestin-expressing cells can be differentiated into IPCs (Chao et al., 2008; Fujikawa et al., 2005; Kim et al., 2004; Kim et al., 2010; Wei et al., 2013; Zulewski, 2006).

Pancreatic characterizations of the differentiated cells, produced in our culture system, were analyzed by means of morphology, insulin production and secretion assay and specific protein and gene expression analysis. DTZ staining indicated that MSCs are able to form crimson red islet-like clusters showing morphological features of pancreatic β cells. DTZ stains β cells in the islet due to

the presence of zinc in insulin secretory granules (Latif et al., 1988). Secreted and intracellular insulin was quantified in the differentiated cells via glucose-dependent insulin release by ELISA. An important feature of mature β cells is their ability to sense blood glucose level and respond by synthesizing and secretion of insulin (Ackermann and Gannon, 2007). Furthermore, immunoreactivity for proinsulin along with C-peptide in the current study exhibited the presence of endogenously produced insulin. De novo synthesis and processing of insulin as a feature of normal β cells, was reported previously (Brolén et al., 2005; Ebrahimie et al., 2014; Mansouri et al., 2014; Zulewski, 2006). In the current study, insulin production by IPCs was confirmed by immunoflourescence, ELISA and real-time PCR analysis. We found that two mature forms of insulin transcripts, INS1 and INS2, were expressed in IPCs. Brolénet al. (2005) claim that "It is expected that cells, which synthesize and secrete insulin, should express key β cell transcription factors which participate in the transcriptional regulation of β cell differentiation and function "(Brolén et al., 2005). Quantitative PCR highlighted that IPCs produced in our culture system could express pancreatic β cell-related genes, such as *INS1*, *INS2*, *PDX-1*, *EP300* and *CREB1*. *PDX-1* is necessary for maintaining function of mature β cell and plays a role in the final differentiation of β cells by inducing expression of insulin (Ackermann and Gannon, 2007; Edlund, 2001). It has been reported that PDX-1, NeuroD1 and MafA promote transcription of insulin gene via binding to its promoter (Qing-Song et al., 2012). Expression of *PDX-1* increase the regeneration and proliferation of β cells (Park et al., 2010). Based on our recent reports INS2 and PDX-1 are the central proteins for induction of IPCs. Furthermore, several transcription factors including EP300 and CREB1 have positive regulatory effects on crosstalk and activation of *PDX*-1 and *INS2* (Ebrahimie et al., 2014; Mansouri et al., 2014).

The data presented here demonstrated that differentiated MSCs could express insulin and insulin receptor beta after treatment with MPE. As stated earlier in various reports, MSCs treated with tissue extract, secrete a variety of cytokines and growth factors such as IGF-1, VEGF, and bFGF that improve the glycometabolism of diabetic rats (Park et al., 2010; Xu et al., 2009). These factors

have both paracrine and autocrine activities and stimulate mitosis and differentiation of stem cells (Caplan and Dennis, 2006). The transmembrane insulin receptor is activated by IGF-I, IGF-II and insulin (Ward and Lawrence, 2009). These factors stimulate β cell hypertrophy and replication (Rabinovitch et al., 1982; Swenne et al., 1987). Therefore, IPC induction in our culture system may be operated via a paracrine or an autocrine induction loop between insulin receptor and its ligans, expressed by MSCs. The detail mechanism of action of MPE on IPCs differentiation needs further investigation in the future.

Possible pathways governed by CREB1, EP300, and PDX-1 transcription factors in differentiation of MSCs to IPCs were determined by subnetwork discovery based on Gene Set Enrichment (GSE) approach at p=0.05. Pathway discovery highlighted the negative regulatory effects of MIR124-2, HDAC5 protein, REST and NR0B2 transcription factors on expression of *CREB1*, *EP300*, and *PDX-1* and inhabitation of IPC differentiations. Contrasting, FOXA2 and TCF7L2 transcription factors, DNA-PK complex, KAT2B protein positively interact with CREB1, EP300, PDX-1 and lead to differentiation of IPC and insulin production. This study documents the practicability of Gene Set Enrichment (GSE) and union selected subnetwork in unraveling the regulatory mechanism of insulin production and discovery on new candidate genes.

The imported genes in constructed network were *CREB1*, *EP300*, and *PDX-1* which are the major regulators in differentiation of MSCs to IPCs. Interestingly, we observed the upregulation of these transcription factors at the following two experiments: (1) Production of insulin-producing cells from P19 EC stem cells under the treatment of pancreas-conditioned medium (Mansouri et al., 2014), and (2) Production of insulin-producing cells from P19 EC stem cells using mouse neonate pancreas extract (Ebrahimie et al., 2014). Upregulation of *CREB1, EP300,* and *PDX-1* transcription factors in production of IPCs from different types of stem cells such as P19 EC stem cells and MSCs and under different inducers such as pancreas conditioned medium and mouse neonate pancreas extract confirms the key role of *CREB1, EP300,* and *PDX-1* in generation of IPCs. Consequently, it can be expected that the networks governed by *CREB1, EP300,* and *PDX-1* to be

present at successful generation of IPCs and secretion of functional insulin irrespective of the input stem cell and applied inducer.

.5 Conclusion

In the present report, we aimed to verify the effects of MPE on differentiation of MSCs from EC cells via nestin positive selection. Our results demonstrated that MSCs have a high potential to transdifferentiate into IPCs in response to MPE. Furthermore, mouse neonate pancreas extract provides an excellent native tissue specific microenvironment to induce pancreas cell lineages.

Acknowledgments

We would like to thank Dr. Behnaz Saffar (head of Research Institute of Biotechnology, Shahrekord University) and Dr. Manijeh Mohammadi-Dehcheshmeh (School of Agriculture, Food, and Wine, The University of Adelaide) for their excellent assistance. The work carried out in Research Institute of Biotechnology was supported by grant from Shahrekord University.

Author Contributions

Conceived and designed the experiments: Fariba Esmaeili. Performed the experiments: Zahra Mehrfarjam. Analyzed the data: Leila Shabani, Esmaeil Ebrahimie, Fariba Esmaeili. Wrote the paper: Fariba Esmaeili. Edit the manuscript: Esmaeil Ebrahimie, Manijeh Mohammadi-Dehcheshmeh.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

This work carried out in Research Institute of Biotechnology was supported by grant from Shahrekord University. The funders had no role in design of the study, data collection and analysis, preparation of the manuscript and decision to publish.

References

Abdi R, Fiorina P, Adra CN, Atkinson M, Sayegh MH. Immunomodulation by mesenchymal stem cells a potential therapeutic strategy for type 1 diabetes. Diabetes. 2008; 57:1759-67.

Ackermann AM, Gannon M. Molecular regulation of pancreatic β-cell mass development, maintenance, and expansion. J Mol Endocrinol. 2007; 38:193-206.

Alanazi I, Ebrahimie E, Hoffmann P, Adelson DL. Combined gene expression and proteomic analysis of EGF induced apoptosis in A431 cells suggests multiple pathways trigger apoptosis. Apoptosis. 2013; 18:1291-305.

Alisoltani A, Fallahi H, Ebrahimi M, Ebrahimi M, Ebrahimie E. Prediction of Potential Cancer-Risk Regions Based on Transcriptome Data: Towards a Comprehensive View. PloS one. 2014; 9:e96320, Doi:10.1371/journal.pone.0096320.

Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. Pancreatic extracts in the treatment of diabetes mellitus: preliminary report. 1922. Can Med Assoc J. 1991; 145:1281-6.

Brolén GKC, Heins N, Edsbagge J, Semb H. Signals from the embryonic mouse pancreas induce differentiation of human embryonic stem cells into insulin-producing-cell–like cells. Diabetes. 2005; 54:2867-74.

Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006; 98:1076-84.

Chang C, Niu D, Zhou H, Li F, Gong F. Mesenchymal stem cells contribute to insulin-producing cells upon microenvironmental manipulation in vitro. Transplant Proc: Elsevier; 2007. p. 3363-8, Doi:10.1016/j.transproceed.

Chao KC, Chao KF, Fu YS, Liu SH. Islet-like clusters derived from mesenchymal stem cells in Wharton's Jelly of the human umbilical cord for transplantation to control type 1 diabetes. PLoS One. 2008; 3:e1451, Doi:10.371/journal.pone.0001451.

Choi KS, Shin JS, Lee JJ, Kim YS, Kim SB, Kim CW. In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. Biochem Biophys Res Commun 2005; 330:1299-305.

Deihimi T, Niazi A, Ebrahimi M, Kajbaf K, Fanaee S, Bakhtiarizadeh MRet al. Finding the undiscovered roles of genes: an approach using mutual ranking of coexpressed genes and promoter architecture-case study: dual roles of thaumatin like proteins in biotic and abiotic stresses. SpringerPlus. 2012; 1:1-10.

Ebrahimie M, Esmaeili F, Cheraghi S, Houshmand F, Shabani L, Ebrahimie E. Efficient and Simple Production of Insulin-Producing Cells from Embryonal Carcinoma Stem Cells Using Mouse Neonate Pancreas Extract, As a Natural Inducer. PloS one. 2014; 9:

Edlund H. Developmental biology of the pancreas. Diabetes. 2001; 50:S5-S9.

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol. 2005; 166:1781-91.

Gade NE, Pratheesh MD, Nath A, Dubey PK, Sharma B, Saikumar Get al. Molecular and Cellular Characterization of Buffalo Bone Marrowâ€ Derived Mesenchymal Stem Cells. Reprod Domest Anim. 2013; 48:358-67.

Habener JF, Kemp DM, Thomas MK. Minireview: transcriptional regulation in pancreatic development. Endocrinology. 2005; 146:1025-34.

Herbach N, Bergmayr M, Goke B, Wolf E, Wanke R. Postnatal development of numbers and mean sizes of pancreatic islets and beta-cells in healthy mice and GIPR dn transgenic diabetic mice. PLoS ONE 2011; 6(7): e22814:doi:10.1371/journal.pone.0022814.

Hosseinpour B, HajiHoseini V, Kashfi R, Ebrahimie E, Hemmatzadeh F. Protein Interaction Network of Arabidopsis thaliana Female Gametophyte Development Identifies Novel Proteins and Relations. PloS One. 2012; 7:e49931.

Jafary H, Larijani B, Farrokhi A, Pirouz M, Mollamohammadi S, Baharvand H. Differential effect of activin on mouse embryonic stem cell differentiation in insulin-secreting cells under nestin-positive selection and spontaneous differentiation protocols. Cell BioL Int. 2008; 32:278-86.

Kanitkar M, Bhonde R. Existence of islet regenerating factors within the pancreas. Rev Diabet Stud. 2004; 1:185-92.

Kim S-Y, Lee S-H, Kim B-M, Kim E-H, Min B-H, Bendayan Met al. Activation of nestin-positive duct stem (NPDS) cells in pancreas upon neogenic motivation and possible cytodifferentiation into insulin-secreting cells from NPDS cells. Dev Dyn. 2004; 230:1-11.

Kim S-Y, Lee S, Hong S-W, Min B-H, Lee K-U, Bendayan Met al. Nestin action during insulin-secreting cell differentiation. J Histochem Cytochem. 2010; 58:567-76.

Kobune M, Kawano Y, Ito Y, Chiba H, Nakamura K, Tsuda Het al. Telomerized human multipotent mesenchymal cells can differentiate into hematopoietic and cobblestone areaâ€“supporting cells. Experimental hematology. 2003; 31:715-22.

Latif Z, Noel J, Alejandro R. A simple method of staining fresh and cultured islets. Transplantation. 1988; 45:827-30.

Le Blanc K, Pittenger MF. Mesenchymal stem cells: progress toward promise. Cytotherapy. 2005; 7:36-45.

Lee J, Han DJ, Kim SC. In vitro differentiation of human adipose tissue-derived stem cells into cells with pancreatic phenotype by regenerating pancreas extract. Biochem Biophys Res Commun. 2008; 375:547-51.

Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med 2008; 12:1155-68.

Liu Y-X, Ji L, Yue W, Yan Z-F, Wang J, Xi J-Fet al. Cells extract from fetal liver promotes the hematopoietic differentiation of human embryonic stem cells. Cloning and stem cells. 2009; 11:51-60.

Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science. 2001; 292:1389-94.

Mansouri A, Esmaeili F, Nejatpour A, Houshmand F, Shabani L, Ebrahimie E. Differentiation of P19 embryonal carcinoma stem cells into insulin-producing cells promoted by pancreas-conditioned medium. J Tissue Eng Regen Med. 2014; 8, DOI: 10.1002/term.1927.

Metrakos P, Yuan S, Agapitos D, Rosenberg L. Intercellular communication and maintenance of islet cell mass--implications for islet transplantation. Surgery. 1993; 114:423-7.

Narayanan K, Lim VY, Shen J, Tan ZW, Rajendran D, Luo S-Cet al. Extracellular Matrix-Mediated Differentiation of Human Embryonic Stem Cells: Differentiation to Insulin-Secreting Beta Cells. Tissue Eng Part A. 2014; 20:424-33.

Nikitin A, Egorov S, Daraselia N, Mazo I. Pathway studio-the analysis and navigation of molecular networks. Bioinformatics. 2003; 19:2155-7.

Novichkova S, Egorov S, Daraselia N. MedScan, a natural language processing engine for MEDLINE abstracts. Bioinformatics. 2003; 19:1699-706.

Oh SH, Muzzonigro TM, Bae SH, LaPlante JM, Hatch HM, Petersen BE. Adult bone marrow-derived cells trans-differentiating into insulin-producing cells for the treatment of type I diabetes. Lab Invest. 2004; 84:607-17.

Park K-S, Kim Y-S, Kim J-H, Choi B, Kim S-H, Tan AH-Ket al. Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. Transplantation. 2010; 89:509-17.

Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood. 2004; 103:1662-8.

Phadnis SM, Ghaskadbi SM, Hardikar AA, Bhonde RR. Mesenchymal stem cells derived from bone marrow of diabetic patients portrait unique markers influenced by the diabetic microenvironment. The review of diabetic studies: RDS. 2009; 6:260-70.

Phadnis SM, Joglekar MV, Dalvi MP, Muthyala S, Nair PD, Ghaskadbi SMet al. Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage in vivo. Cytotherapy. 2011; 13:279-93.

Pierantozzi E, Gava B, Manini I, Roviello F, Marotta G, Chiavarelli Met al. Pluripotency regulators in human mesenchymal stem cells: expression of NANOG but not of OCT-4 and SOX-2. Stem Cells Dev. 2011; 20:915-23.

Qing-Song G, Ming-Yan Z, Lei W, Xiang-Jun F, Yu-Hua L, Zhi-Wei Wet al. Combined transfection of the three transcriptional factors, PDX-1, NeuroD1, and MafA, causes differentiation of bone marrow mesenchymal stem cells into insulin-producing cells. Exp Diabet Res. 2012; 2012:Doi:10.1155/2012/672013.

Rabinovitch A, Quigley C, Russell T, Patel Y, Mintz D. Insulin and multiplication stimulating activity (an insulin-like growth factor) stimulate islet (beta-cell replication in neonatal rat pancreatic monolayer cultures. Diabetes. 1982; 31:160-4.

Reddy S, Stefanovic N, Karanam M. Prevention of autoimmune diabetes by oral administration of syngeneic pancreatic extract to young NOD mice. Pancreas. 2000; 20:55-60.

Shiroi A, Ueda S, Ouji Y, Saito K, Moriya K, Sugie Yet al. Differentiation of embryonic stem cells into insulin-producing cells promoted by Nkx2. 2 gene transfer. World J Gastroenterol 2005; 11:4161-6.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MAet al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005; 102:15545-50.

Sun Y, Chen L, Hou X-g, Hou W-k, Dong J-j, Sun Let al. Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells in vitro. Chinese Med J. 2007; 120:771-6.

Swenne I, Hill D, Strain A, Milner R. Growth hormone regulation of somatomedin C/insulin-like growth factor I production and DNA replication in fetal rat islets in tissue culture. Diabetes. 1987; 36:288-94.

Talavera-Adame D, Wu G, He Y, Ng TT, Gupta A, Kurtovic Set al. Endothelial cells in co-culture enhance embryonic stem cell differentiation to pancreatic progenitors and insulin-producing cells through BMP signaling. Stem Cell Rev Rep. 2011; 7:532-43.

Vaca P, Martín F, Vegara-Meseguer JM, Rovira JM, Berna G, Soria B. Induction of Differentiation of Embryonic Stem Cells into Insulin-Secreting Cells by Fetal Soluble Factors. Stem Cells. 2006; 24:258-65.

Ward CW, Lawrence MC. Ligand-induced activation of the insulin receptor: a multi-step process involving structural changes in both the ligand and the receptor. Bioessays. 2009; 31:422-34.

Wei R, Yang J, Hou W, Liu G, Gao M, Zhang Let al. Insulin-producing cells derived from human embryonic stem cells: comparison of definitive endoderm-and nestin-positive progenitor-based differentiation strategies. PloS one. 2013; 8:e72513.

Xie H, Wang Y, Zhang H, Qi H, Zhou H, Li F-R. Role of injured pancreatic extract promotes bone marrow-derived mesenchymal stem cells efficiently differentiate into insulin-producing cells. PloS one. 2013; 8:e76056, Doi:10.1371/journal.pone.0076056.g002.

Xu YX, Chen L, Hou WK, Lin P, Sun L, Sun Yet al. Mesenchymal stem cells treated with rat pancreatic extract secrete cytokines that improve the glycometabolism of diabetic rats. Transplant Proc; 2009. p. 1878-84.

Yang K-M, Yong W, Li A-D, Yang H-J. Insulin-producing cells are bi-potential and differentiatorsprior to proliferation in early human development. World J Diabetes. 2011; 2:54-8.

Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BEet al. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci USA. 2002; 99:8078-83.

Zhang Y-H, Wang H-F, Liu W, Wei B, Bing L-J, Gao Y-M. Insulin-Producing Cells Derived from Rat Bone Marrow and Their Autologous Transplantation in the Duodenal Wall for Treating Diabetes. Anat Rec 2009; 292:728-35.

Zhang Y, Wang D, Zeng H, Wang L, Sun J, Zhang Zet al. Differentiation of human amniotic mesenchymal stem cells into insulin-secreting cells induced by regenerating pancreatic extract. Sheng wu gong cheng xue bao, Chinese J Biotechnol. 2012; 28:214-21.

Zulewski H. Stem cells with potential to generate insulin-producing cells in man. Swiss Med Wkly. 2006; 136:647-54.

Table 1. Statistically significant subnetworks which CREB1, EP300, and PDX-1 transcription factors and insulin can contribute (generate) in order to differentiate mesenchymal stem cells (MSCs) into functional insulin-producing cells (IPCs) under defined conditions based on Gene Set Enrichment (GSE) approach. Pathways deposited in database of Pathway Studio 10 (Elsevier) were used as the source of pathway discovery.

Subnetwork (pathway)	Total number of neighbors in subnetwork	Number of overlap	Percent of Overlap %	Overlapping Entities	p-value
Neighbors of MIR124-2	12	3	23	CREB1,INS,PDX-1	1.08E-10
Neighbors of HDAC5	211	4	1	CREB1,EP300,INS,PDX-1	7.70E-10
Neighbors of HIPK2	230	4	1	CREB1,EP300,INS,PDX-1	1.09E-09
Neighbors of PDX-1	336	4	1	CREB1,EP300,INS,PDX-1	4.97E-09
Neighbors of KAT2B	344	4	1	CREB1,EP300,INS,PDX-1	5.46E-09
Neighbors of HCFC1	48	3	6	EP300,INS,PDX-1	6.93E-09
Neighbors of DNA-PK	365	4	1	CREB1,EP300,INS,PDX-1	6.93E-09
Neighbors of REST	392	4	1	CREB1,EP300,INS,PDX-1	9.22E-09
Neighbors of NR0B2	400	4	0	CREB1,EP300,INS,PDX-1	1.00E-08
Neighbors of FOXA2	440	4	0	CREB1,EP300,INS,PDX-1	1.46E-08
Neighbors of TCF7L2	441	4	0	CREB1,EP300,INS,PDX-1	1.48E-08
Neighbors of ATF3	485	4	0	CREB1,EP300,INS,PDX-1	2.16E-08
Neighbors of GPR39	76	3	3	CREB1,INS,PDX-1	2.75E-08
Neighbors of MAFA	79	3	3	EP300,INS,PDX-1	3.09E-08

Neighbors of OGT	546	4	0	CREB1,EP300,INS,PDX-1	3.47E-08
Neighbors of SUMO1	546	4	0	CREB1,EP300,INS,PDX-1	3.47E-08
Neighbors of SIK2	87	3	3	CREB1,EP300,INS	4.12E-08
Neighbors of MIR296	89	3	3	CREB1,INS,PDX-1	4.41E-08
Neighbors of MAPK9	605	4	0	CREB1,EP300,INS,PDX-1	5.24E-08
Neighbors of PLAGL1	98	3	3	CREB1,EP300,INS	5.89E-08

Figure legends

Figure 1. Schematic representation of 6-stages protocol for differentiation of insulin-producing cells (IPCs) from mesenchymal stem cells (MSCs) based on the selection of nestin-positive precursors. The right panel shows cell morphologies at different stages. DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, ITSFn: insulin-transferrin-sodium selenite, bFGF: basic fibroblast growth factor, EGF: epidermal growth factor, MPE: mouse pancreas extract.

Figure 2. Isolation, culture and characterization of bone marrow mesenchymal stem cell (MSCs). (A) Mouse MSCs were plated and cultured to obtain the adherent mature cells. (B) About 10 days after culture, the cells show fibroblast-like cell morphology. (C) Subculture of undifferentiated MSCs. (D) a cell aggregate of MSCs. Representative micrographs of (E) osteogenic and (F) adipogenic differentiation, after incubation for 21 days in specific medium. (G) Flow cytometry analysis of MSCs indicated that majority of the cells were positive for CD44 (99.6%) and negative for CD45 (98.7%).

Figure 3. Immunofluorescence for nestin. (A) Immunofluorescence staining with antibody against nestin reveals individual nestin-positive cells. (B) The nuclei are counterstained by Hoechst. (C) Merged image of nestin and Hoechst.

Figure 4. Dithizone staining of transdifferentiated mesenchymal stem cells (MSCs). (A) During differentiation, spindle fibroblast-shape cells became round or oval-type cells (arrows). Then, they started to organize into small aggregates (arrowheads), and finally formed islet-like clusters (asterisks). (B) Higher magnification of an islet-like cluster, shown in panel A. (C) DTZ^{+} single cells distinctly

stained crimson red by dithizone. (D) Untreated MSCs are not stained by DTZ. (E) Percentage of DTZ^+ cells among total cells. MPE induced the differentiation of MSC cells at a higher mean than that of the untreated group. The experiments were performed at least in triplicate. Each value represents mean±SD. The lowercase letters represent statistical significance at $p < 0.05$. The values with different letters are statistically different at $p<0.05$.

Figure 5. Fluorescence micrographs indicating the expression of pancreas β cell markers in insulin-producing cells (IPCs) derived from MSCs. Staining of IPCs with antibodies against insulin receptor beta (B), proinsulin+insulin (E), and C-peptide (H) shows that most of the cells treated with MPE express pancreatic β cell markers. The nuclei are counterstained by Hoechst. (C) Merged image of insulin receptor beta and Hoechst. (F) Merged image of proinsulin+insulin and Hoechst. (I) Merged image of C-peptide and Hoechst. (J, K) The primary antibody was omitted as control for immunostaining.

Figure 6. Detection of secreted (A) and intracellular versus secreted (B) insulin in undifferentiated mesenchymal stem cells (MSCs) and differentiated cells (IPCs). Significant insulin concentration was observed in MPE–treated IPCs. To normalize the amount of insulin secretion, total protein of the cells was measured by the Bradford method. The experiments were performed at least in triplicate. Each value represents mean±SD. The lowercase letters represent statistical significance at $p < 0.05$. The values with different letters are statistically different at $p<0.05$.

Figure 7. Real-time PCR of the genes involved in differentiation of mesenchymal stem cells (MSCs) into pancreatic cells. (A) Comparison of expression of undifferentiated stem cell markers, *Oct3/4*, *Sox-2*, and *Nanog* between MSCs and IPCs. Expression of these genes was significantly down regulated in differentiated cells. (B) Relative gene expression of pancreas specific genes, *PDX-1*, *INS1*, *INS2*,

EP300 and *CREB1* in MSCs-derived IPCs. During differentiation of IPCs from MSCs, the expression of these genes was significantly increased in comparison to undifferentiated cells. The data are expressed as relative gene expression to *β-2M* and are presented as mean±SD. The experiments were performed at least in triplicate. The lowercase letters represent statistical significance at $p<0.05$. The values with different letters are statistically different at $p<0.05$.

Figure 8. Statistically significant pathways (subnetworks) generated by up regulation of CREB1, EP300, and PDX1 transcription factors ($p = 0.05$) during differentiation of mesenchymal stem cells to insulin producing cells.

Supplementaries

Supplementary 1. Real time PCR Primer Sequences

Supplementary 2. Database of pathways deposited in Pathway Studio package for Gene Set Enrichment analysis

Supplementary 3. Statistically significant subnetworks which CREB1, EP300, and PDX-1 transcription factors and insulin can contribute (generate) in order to differentiate mesenchymal stem cells (MSCs) into functional insulin-producing cells (IPCs) under defined conditions based on Gene Set Enrichment (GSE) approach (Subramanian et al., 2005). Pathways deposited in database of Pathway Studio 10 (Elsevier) were used as the source of pathway discovery.

Supplementary 4. Relationships underlying the predicted statistically significant sunetworks in differentiation of insulin producing cells from mesenchymal stem cells.

Supplementary 5. Predicted integrative network based on "union selected algorithm" in differentiation of insulin producing cells from mesenchymal stem cells.

Supplementary 6. Relationships underlying the predicted integrative network in differentiation of insulin producing cells from mesenchymal stem cells.

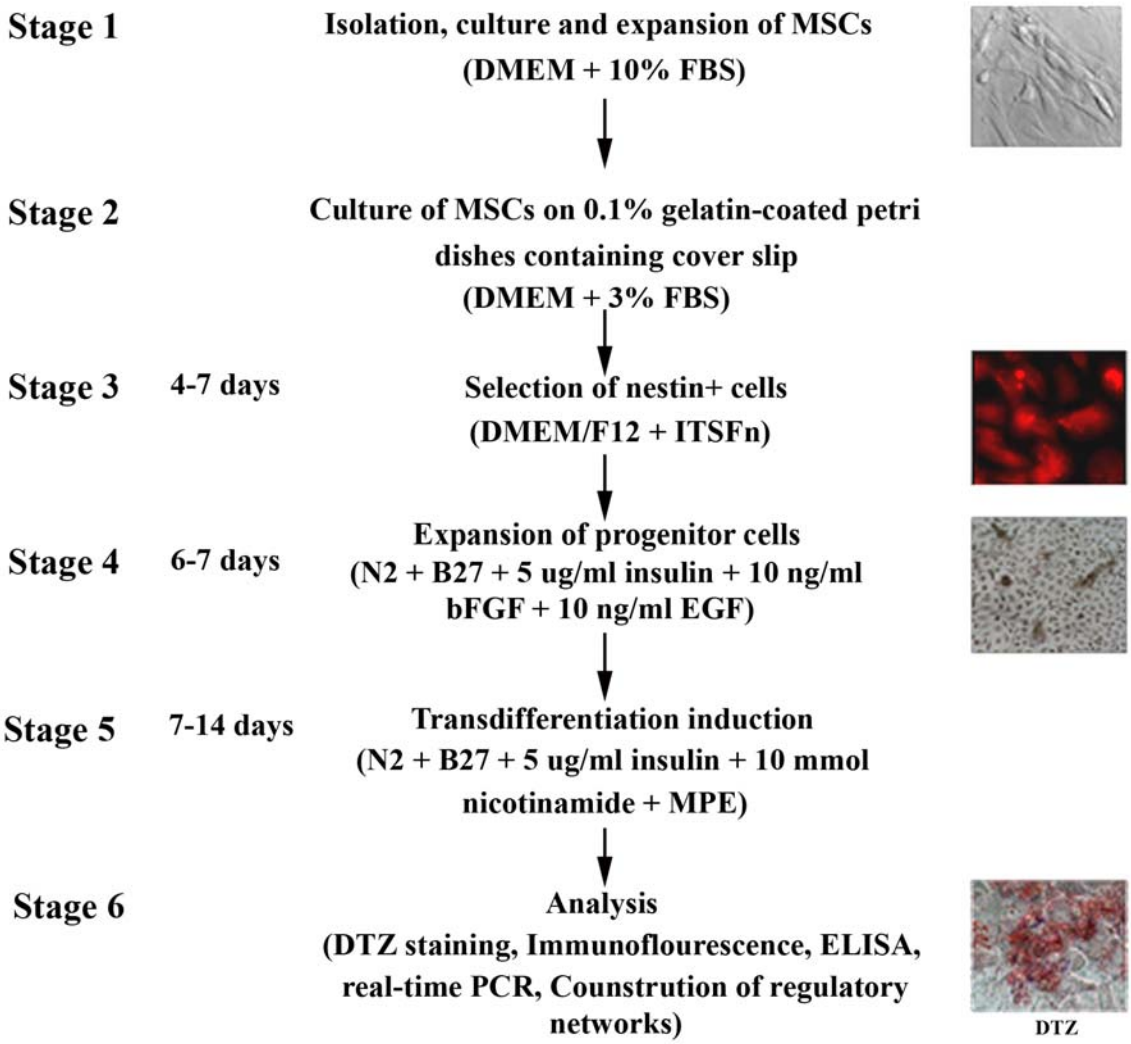


Figure 1

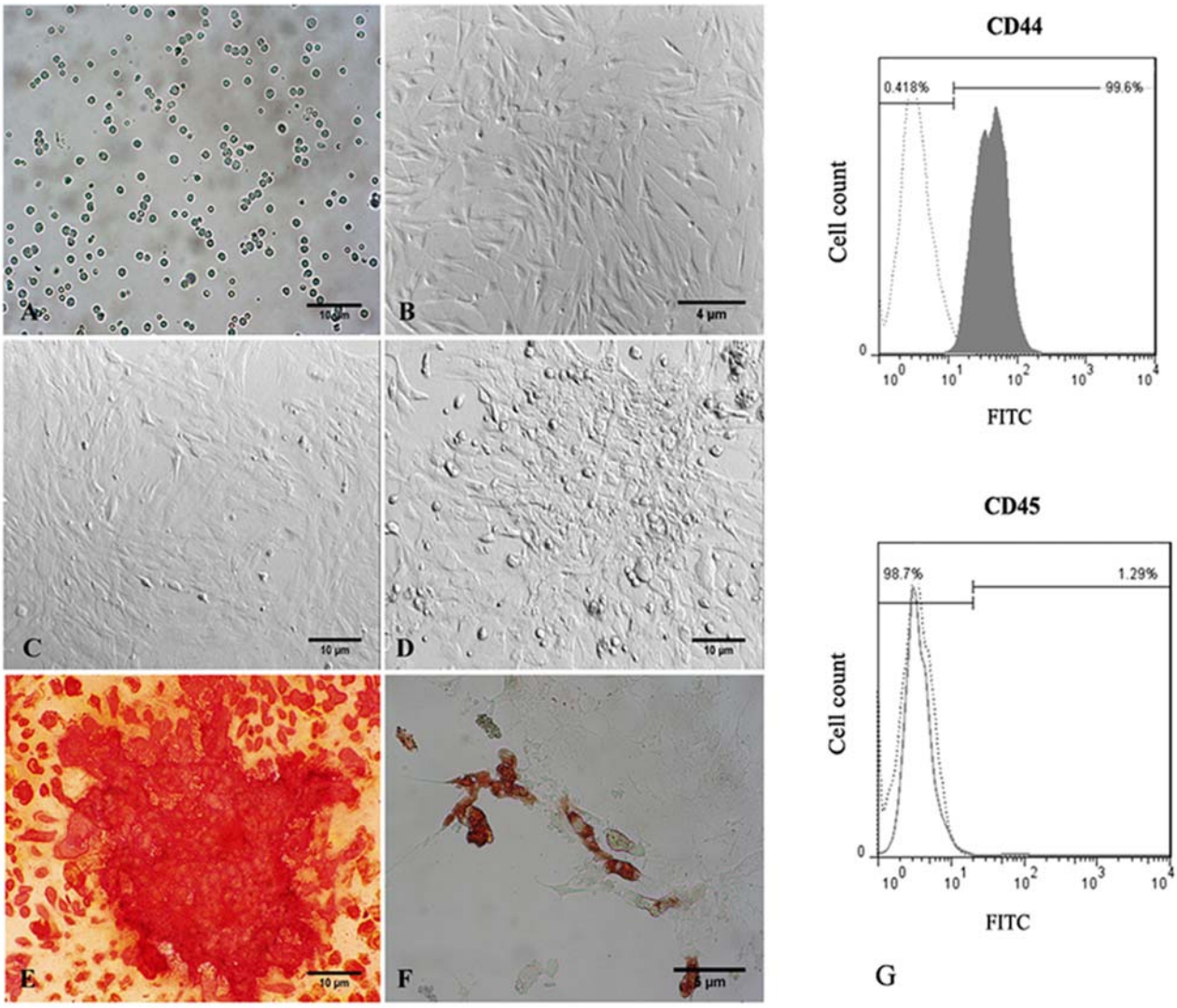


Figure 2

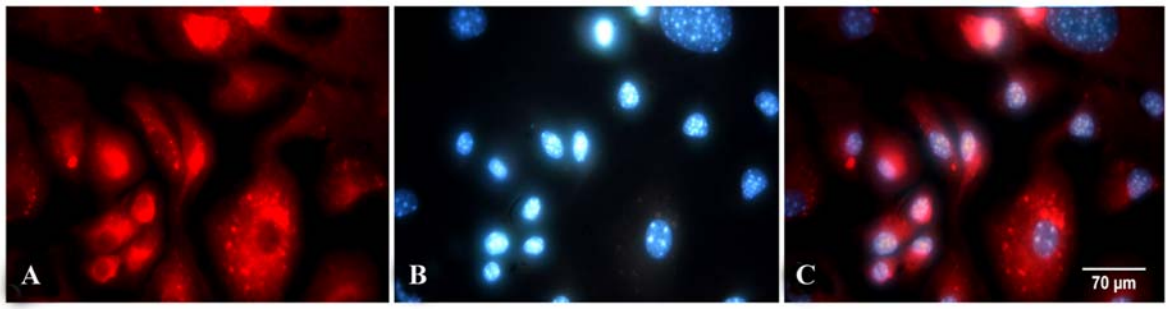


Figure 3

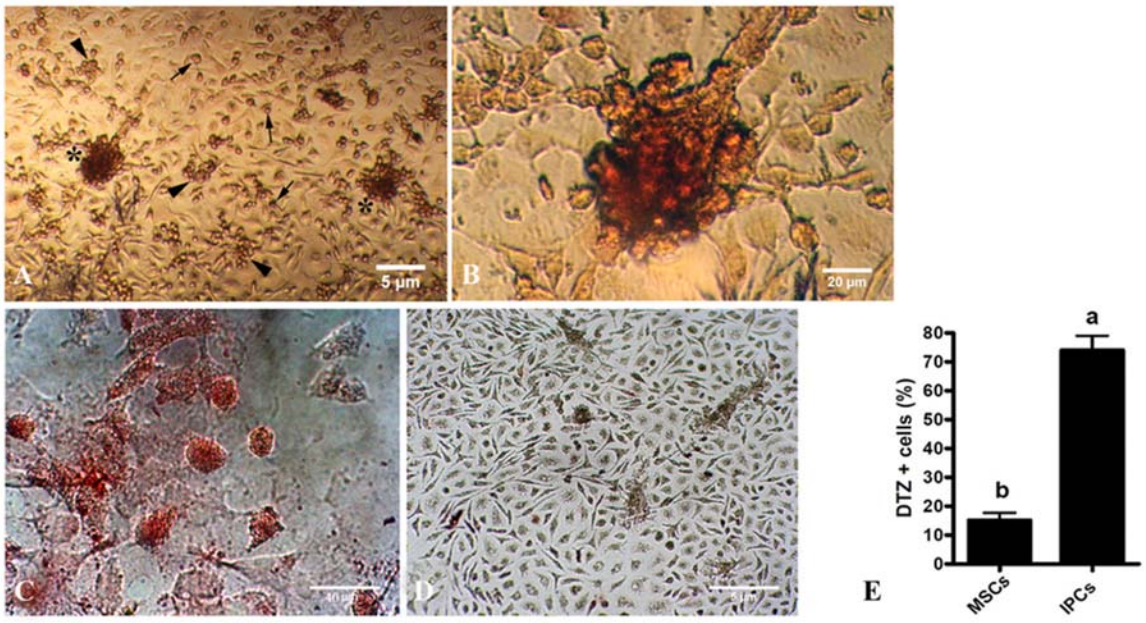


Figure 4

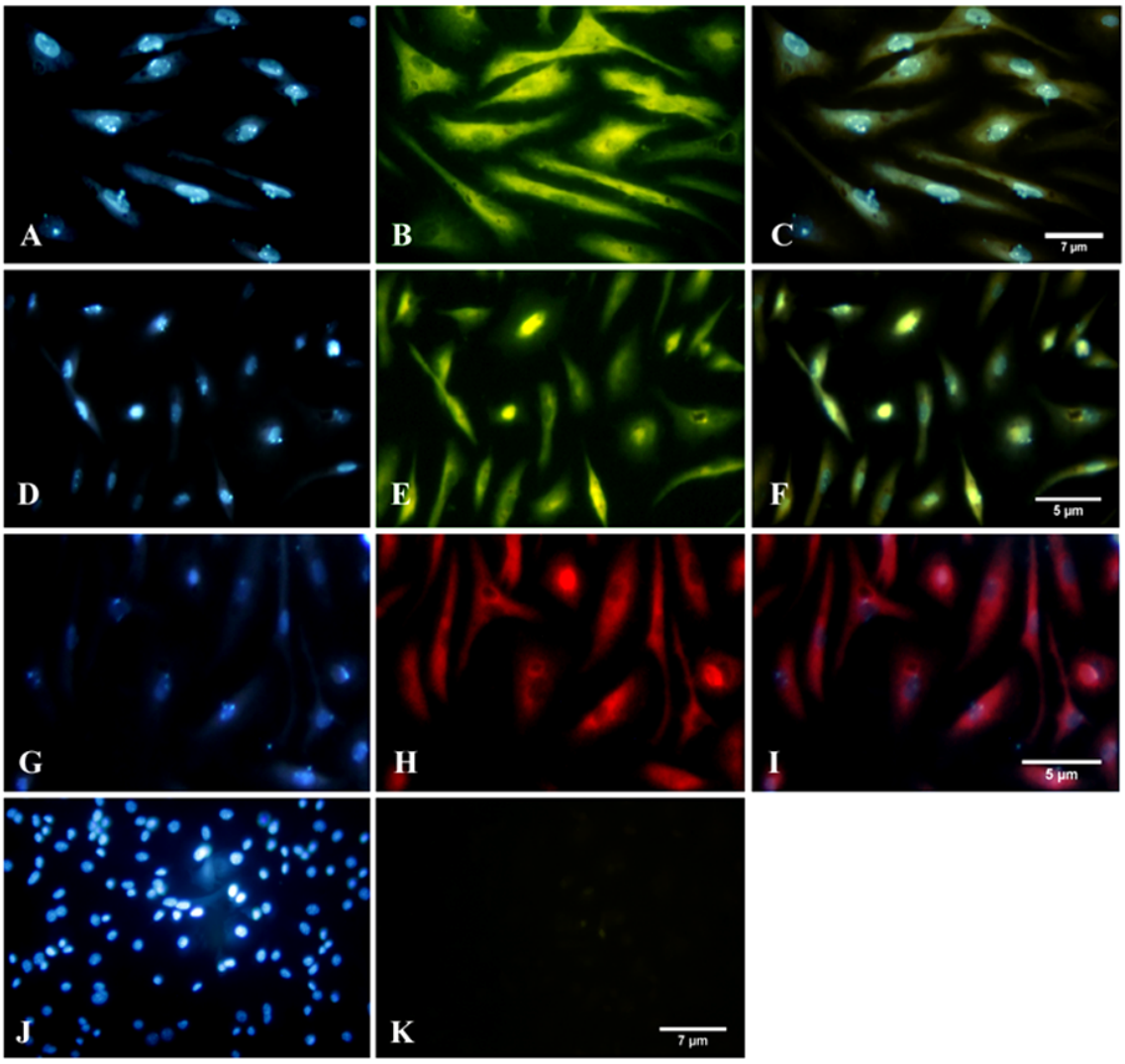
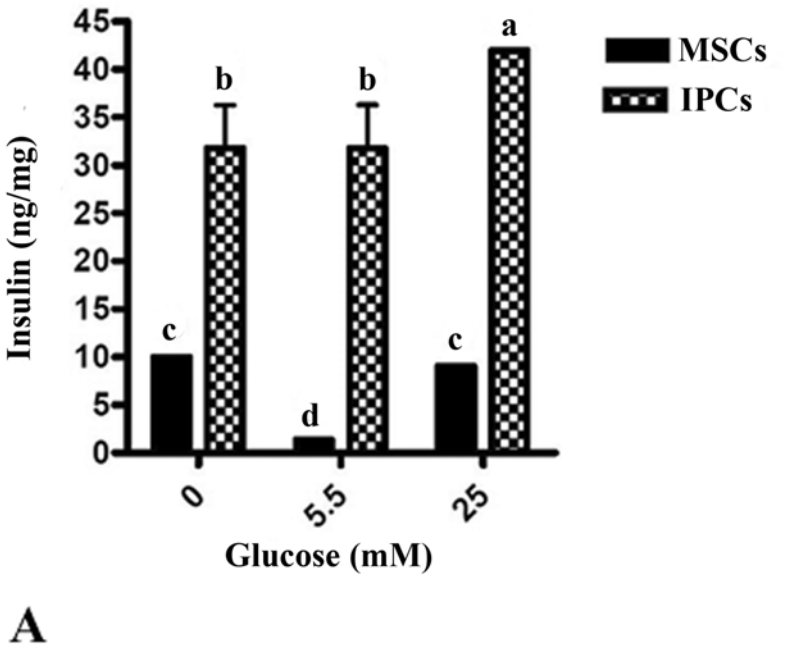
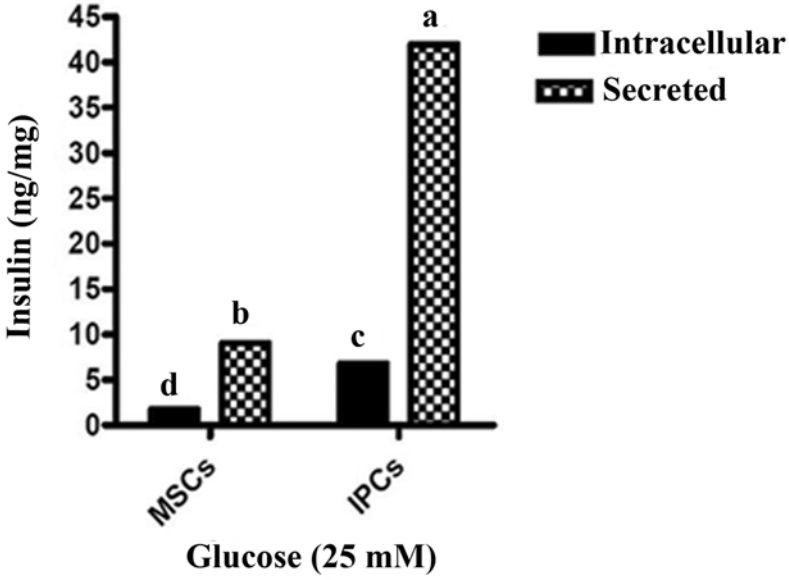


Figure 5



A



B

Figure 6

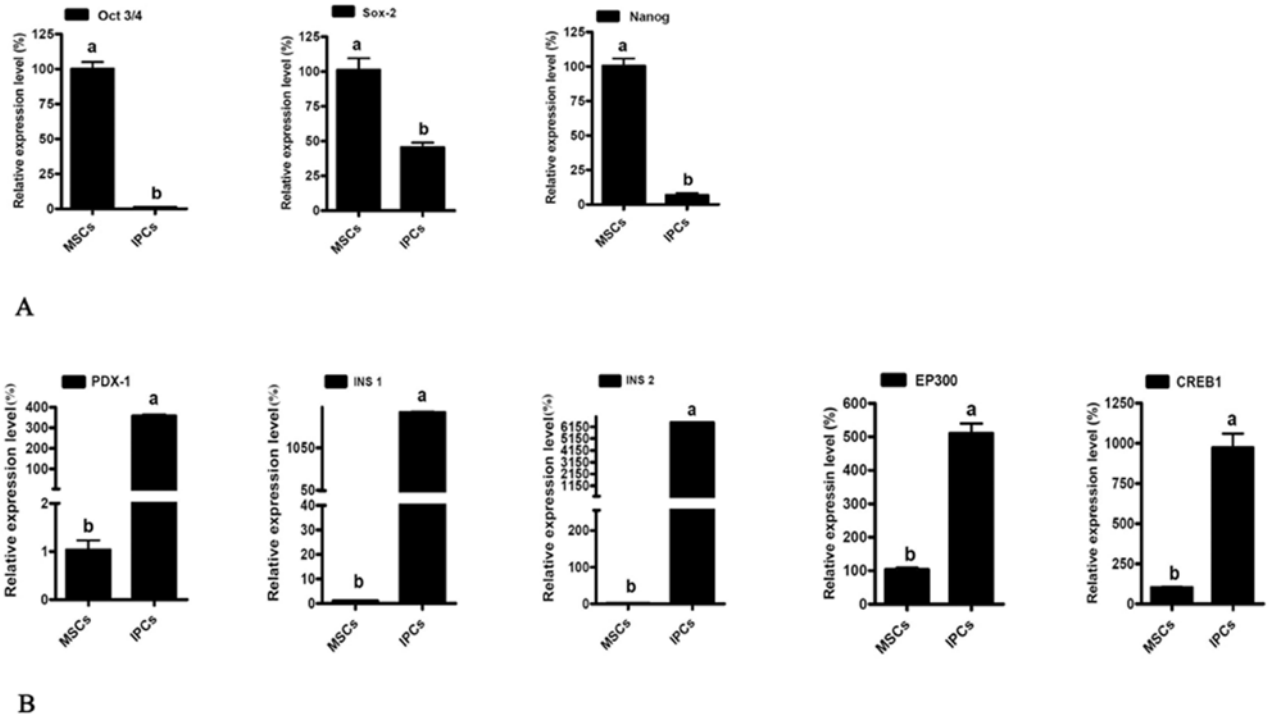


Figure 7

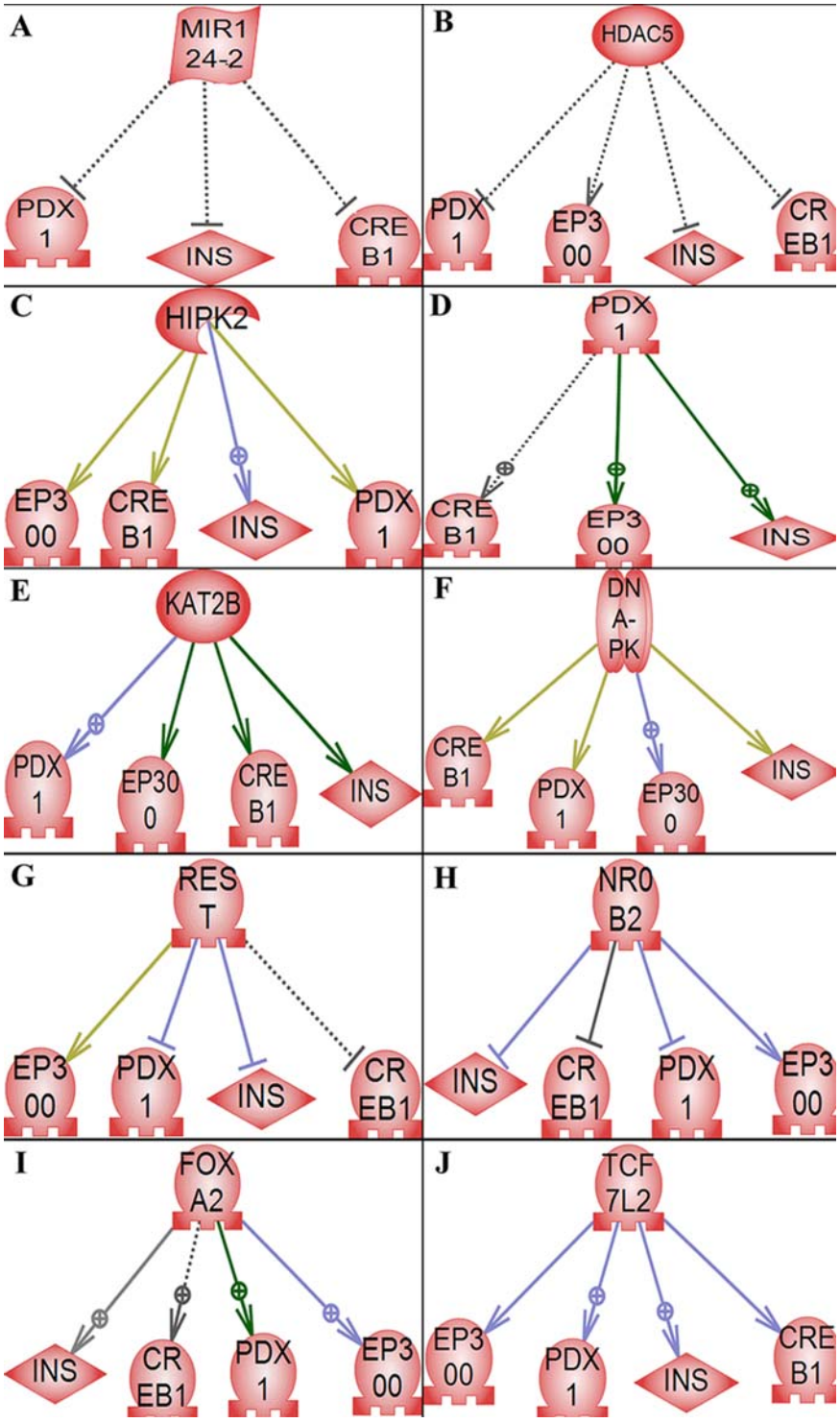


Figure 8