

Differentiation of Human MSCs Into Nsulin Producing Cells by Using Lentiviral Vector Carrying PDX-1

Running title: **Differentiation of MSC Cells Into IPCs**

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Received: 17/Mar/2014, Accepted: 15/May/2014

Abstract

Introduction: Type I diabetes is an immunologically-mediated devastation of the insulin producing cells (IPCs) in pancreatic islet. Stem cells for producing β cells are new promising tool. Adult stem cells such as mesenchymal stem cells (MSCs) are multipotent cells and have self renewal capacities that capable them differentiating to ectodermal, mesodermal, and endodermal tissues. Pancreatic and duodenal homeobox factor 1 (PDX1), is a master regulator gene for the embryonic development of the pancreas and is crucial for normal pancreatic islet in adult.

Materials and methods: In this experimental study, for generating IPCs, we induced the over expression PDX1 gene in human BM-MSCs. After being infected with Lenti-PDX1, MSCs were induced to differentiate into IPCs.

Results: After the transduction, MSCs changed their morphology at day 5 and gradually differentiated into IPCs. Insulin and PDX-1 expression were confirmed by RT-PCR and immunostaining. IPCs secreted insulin and c-peptide in the mediums containing different glucose concentration.

Conclusion: In this experimental research we could differentiate MSCs to IPCs by genetic manipulating. Our result showed that lentiviral vectors can deliver PDX-1 gene to MSCs and induce pancreatic differentiation.

Keyword: PDX-1, Diabete type I, MSCs (MSCs), Insulin Producing Cells (IPCs)

Introduction

Diabetes involved more than 300 million people in the world and consumes about 12% of the world's total health cost. The most cases of diabetes include in two broad categories type

1 diabetes (5–10%) and type 2 diabetes (90–95%)(1, 2). Type I diabetes is an immunologically-mediated devastation of the insulin-producing-cells in pancreatic islet (3). Insulin therapy is an ordinary treatment of type 1 diabetes, but several daily injections regimens may be difficult to achieve optimal glycaemic control (4). Other treatments contain pancreas transplantation, but these processes have disadvantages; the lack of donors islet transplantation and immune rejection (5). Stem cells for producing beta cells are new promising tool. Embryonic stem cells (ESCs) are pluripotent cells can differentiate into three germ layers. These cells enable to differentiate into insulin positive cells but the cells showed to raise the risk for tumor formation in host recipient(6). Adult stem cell such as MSCs are multipotent cells and have self renewal capacities that capable them to differentiating into ectodermal, mesodermal, and endodermal tissues(7). Human MSCs isolated from somatic tissues, such as bone marrow (BM), adipose tissue and from fetal origin, such as amniotic fluid (AF), Wharton's jelly and umbilical cord blood(8). Pancreatic and duodenal homeobox factor 1 (PDX-1) is a transcriptional factor that requires for the embryonic development of the pancreas. In other words, PDX-1 plays a distinct role in proliferation and differentiating. Heterozygote missense and frame shift mutations caused not enough secretion of insulin from beta cells and creates a type of diabetes, known as juvenile diabetes, early puberty (MODY4)(9). Lentiviral vectors caused the high transmission efficiency and stable gene expression in target cells and used as a useful tool for genetic manipulation(10). In this study we used lentiviral vector to deliver PDX-1 gene into MSCs to make differentiate. After being transducing cells we examined the ability of the cells by measuring extracellular and intracellular insulin/c-peptide production and then evaluate insulin and PDX-1 gene expressions by Real-time PCR and immunocytochemical analysis.

Materials and Methods

Cell isolation and culture analysis of human MSC

Three healthy sample for bone marrow transplantation were got from healthy donor (age 20-40 years old). These samples were got pre-informed consent and permission from the local Ethics Committee at Taleghani hospital (Tehran, Iran). 5 ml of BM samples were got from posterior superior iliac crest and then transfer to clean heparinized collection tube . Diluted sample with PBS with ratio 1:1 and then added to 3-4 ml Ficoll(ficoll-paque; 1.073g/ml, Pharmacia Uppsala, Sweden) and centrifuged for 20 minutes at 400 g. The mononuclear cells were at the plasma: density gradient medium interface collected and transfer the cells to a new tube and added PBS to the cells and centrifuge for 5 minutes at 400 g. Then, discarded the supernatant and cultured in 5 ml of DMEM-LG (Gibco) supplemented with 10 % fetal bovine serum (FBS; Gibco), and 100u/ml penicillin/streptomycin (Gibco), then cells were incubated at 37 °c in 5 % humidified CO₂. After 3-4 days half of medium was replaced by fresh medium to remove non adherent cells. When the cells reached 80–90% confluency, they were collected by treatment with 0.25 % trypsin and 1 mM EDTA for 2–5 min at 37 °c. After centrifugation, MSCs were cultured in 75 cm² flasks to reach confluency.

Flow cytometry analysis of human MSCs

After the fourth passages, MSCs were detached by trypsinization . The cells were washed with PBS, and incubate with labeled phycoerythrin (PE)-conjugated monoclonal antibodies at the dilution recommended by manufacturer at 4 °c for 25 minutes in the dark: The antibodies used were CD 90, CD34, CD105, and CD31-PE (BD Biosciences, USA) and PE-labeled isotype-matched immunoglobulin used as a negative control. The labeled cells were analyzed on a FACS Caliber (Becton-Dickinson, FACScan, San Jose, CA, USA).

Adipocytes and osteoblastic differentiation of BM MSCs

MSCs at passages 4 were used for induction of osteoblastic and adipocyte differentiation.

Adipocyte differentiation were performed by medium containing L-DMEM(bioidea.iran), 10%

FBS ,10⁻⁶ M dexamethasone(sigma , USA), 0.05mg/dl ascorbic acid (Merck,Germany).

Osteoblastic differentiation were performed by medium containing L-DMEM(bioidea.iran),

10% FBS ,10⁻⁸ M dexamethasone(sigma,USA),10 mM β-glycerophosphate (Merck,

Germany), 0.05mg/dl ascorbic acid(Merck,Germany). After 2 weeks, differentiated adipocyte

cells were stained with Oil Red staining (Sigma, USA) and after 3 weeks differentiated

osteoblasts cells were stained with Alizarin Red staining (Sigma, USA).

Oil-Red O staining

The cells were washed with PBS and were fixed with 4% para-formaldehyde for 20 min

.Then added 60% isopropanol and after 5 min isopropanol were removed. Oil Red O was

dissolved in 99% isopropanol and added to cells and Incubated 15 min at room temperature.

After that, dye removed and cells washed with PBS until the water rinsed off clear.

Alizarin-Red staining

The cells were washed with PBS and fixated by 4% para-formaldehyde, immersed in a 1%

alizarin red solution for 10 min and rinsed with DW.

Plasmids

EX-M0942-Lv105 plasmid carried the PDX-1 gene, was purchased from (genecopoeia,

USA). PsPAX2 and pMD2.G vectors for viral packaging were purchased from (Invitrogen,

USA). These vectors were transformed in DH5α. Plasmids were purified using the real-

biotech, plasmid mini kit (Taiwan).one single copy of EX-M0942-Lv105 containing 7730 bp.

The copy number of the plasmid PDX-1 was calculated by software that exists at:

<http://cels.uri.edu/gsc/cndna.html>. So, 7.23×10¹⁰ copies were per micro liter were obtained. This

calculation was needed for viral particles titration. Serial dilutions of the plasmid (7.23×10^9 - 7.23×10^7) were prepared and then PDX-1 plasmid standard curve was created for determining the copy number of integrated lentiviruses.

Production lentiviral vector in HEK 293 cells

HEK-293T cells with density of 4×10^6 /ml were seeded at 100 mm dishes (**JET BIOFIL, china**) containing L-DMEM supplemented with 10% FBS. When the cells reached 80% confluency, transfection with PDX-1 plasmid and the two packaging viral vector (pMD2.G and psPAX2) was performed through the calcium-phosphate protocol. The cell supernatant were collected every 24 hr and fresh medium containing serum was added to the cells. After 72 hours, the total viral medium was centrifuged (10 min 2100g) and filtrated with 0.45 micron syringe filter. The concentration of virus was performed using poly ethylene glycol (PEG) 50% (sigma, USA) and NaCl 5M (Merck, Germany) were added to the supernatant and incubated for 16-20 hours on Shaker at 4°C . Then samples were centrifuged (10 min 4100g at 4°C). Sediment dissolved at 1 ml of DMEM-F12 medium and aliqueted in sterile 1.5 μl microtubes that stabled for one year at -70°C or at 4°C for a week. To confirm virus production, cells collected and PDX-1 gene expression were examined by RT-PCR and western blot.

Titration lenti-vector by Real-time PCR

HEK 293T cells were seeded at the density of 6×10^4 /mL per well in 12-well tissue culture plates (**JET BIOFIL , china**). 50 and 100 μl of concentrated virus were added to the cells. After 16 hr, the culture medium replaced with fresh DMEM media supplemented with 10% FBS. After 96 hr, DNA extracted from traduced cells by Spin Blood Mini Kit (Invisorb®),

Germany) and stored at -20°C until use. In qPCR a standard curve was generated by using 10 log including serial dilution (7.23×10^7 - 7.23×10^9 particle/ μl) of plasmid EX-M0942-Lv105 containing puromycin resistance gene sequence. The standard curve was calculated by Applied Biosystems Step One software v2.2. Recombinant DNA (unknown sample) that extracted from HEK-293 and standard samples run on Applied Biosystems Step One (Applied Biosystems, USA) with a SYBR-Green kit (Takara, Japan) using the in a total reaction volume of $10 \mu\text{l}$. Specificity of products were evaluated by melting curve analysis. For quantifying of copy numbers of viral particles Step One software v2.2. Primers specific for puromycin resistance gene were shown in Table 1. The qPCR values are mean \pm SEM.

Transduction of MSCs

5×10^5 /well human MSCs at passages 4 with 70-80% confluences used were seeded in a plate 6 wells and were transduced with the medium containing viral particles at 37°C for 6-8 hours by the multiplicity of infection (MOI) of 50 incubated and then replaced with fresh L-DMEM containing 10% FBS. Medium replacement performed 2 days a weeks.

RT-PCR and real-time PCR

Total RNA was extracted from transfected HEK-293 cells by Trizol (Invitrogen, USA) according to the manufacturer's instructions. The first strand cDNA was synthesized from RNA template. 50 ng of total RNA was reverse transcribed by random hexamer priming using high capacity cDNA synthesis kit (Intron, Korea) at 42°C for 60 min and at 70°C for 5 min. PCR performed in $25 \mu\text{l}$ final volume that contained $20.75 \mu\text{l}$ PCR master mix (Vivantis, Malaysia), $0.25 \mu\text{l}$ Taq polymerase (VIVANTIS, Malaysia), $2 \mu\text{l}$ PDX-1 forward and reverse primer (listed in table 1) and $2 \mu\text{l}$ cDNA. Amplification conditions was initial

denaturation at 95 °c for 3 min and followed by 35 cycles in 60°c and extension at 72°c for 1 min .Also Total RNA was extracted at days 7,14 and 21 post infection from MSCs and cDNA was synthesized from RNA template according the instructions above. The real-time PCR was performed using the Applied Biosystems Step One (Applied Biosystems,USA) with a SybrGreen master mix kit (Takara, Japan) and performed in 10 µl volume containing 1 µl cDNA and 1 µl primers that listed in table 1. GAPDH was used as a reference gene. Device Program was primary denaturation at 95 °C and annealing 60 °C for 40 cycles. The qRT-PCR values are mean ± SEM of 3 independent Real-time PCR experiments.

Western blotting

Western blot analysis was performed to discriminate of PDX1 in produced from transfected HEK293. Total protein was extracted from differentiated and undifferentiated MSCs by radioimmunoprecipitation assay (RIPA) buffer containing 10mM Tris-HCl (PH=8.0), 1% NP-40, 10% Glycerol, 0.1% SDS,1mM EDTA and 100mM Nacl with protease inhibitor cocktail (Roche Diagnostic, GmbH, Germany). 50µg of lysates were loaded to each well of 15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and electrophoresis was performed. After transfer of separated proteins on nitrocellulose membrane (Amersham Biosciences) the membrane was blocked with 3% nonfat skim milk in Tris-buffered solution with 0.1% Tween 20 (TBST) for 2h and then incubated overnight at 4 °C with mouse anti-human PDX1 primary antibody (#ab84987, Abcam, Cambridge, MA, UK). After three times washing with TBST, the blots were incubated with polyclonal anti-mouse horseradish peroxidase-conjugated (HRP) secondary antibody (1:500) (#AP8036, Razi Biotech, Iran) for 1h at room temperature, after 3 times washing with TBST, bands were visualized using

enhanced chemiluminescence (ECL) reagent (Ariyatous Biotech, Iran) according to the manufacturer's instructions.

MTT assay

To assess the viability of MSCs during differentiation to IPCs in this study, MTT assay was performed. Transduced cells on days 0, 7, 14 and 21 were collected and cells were seeded into 96-well plates at a density of 105 cells per well. The cells incubated 24 hr at 37 ° C. The supernatant was removed and added freshly prepare MTT (5mg/ml) solution to the cells and incubated 4 hr at 37 ° C. After incubation, 100 µl DMSO was added to the wells and were incubated for 20 min at room temperature. The optical density(OD) was read at 570 nm wavelengths. Viability compared to control cells was calculated.

Immunostaining

For immunocytochemical analysis, differentiated cells were scraped at day 21 and 105 cells were washed in cold 2% FBS-PBS twice and dilute in 100 µl of cold 1% BSA-PBS. Slides were cytopspined (Shandon Cytospin) and 100 µl cell suspension was spun for 1-2 minutes. Cells were coated on slide and were fixed by with 4% Paraformaldehyde (PFA) for 20 min at RT. Then, the cells were permeablized with 0.2% Triton 100-X for 5 min. Slides were washed twice and incubated in 5% goat serum for 45 minutes. The primary antibody were used, mouse anti-human insulin (1:100) (# ab7760, Abcam, Cambridge, MA, UK), mouse anti-human Neurogenin3 (1:100) (# ab87108) and mouse anti-human PDX1 (#ab84987). Slides were washed with PBS 3 times and incubated with Texas Red-labeled goat anti-mouse IgG secondary antibody (1:32) (ab5884). The cells were kept in the dark for 45 min. Nuclear DNA was stained with 0.1µg/ml of blue-fluorescent 4',6-Diamidino-2-

phenylindole (DAPI) (Sigma) at 30°C for 5 min. The slides were visualized under a fluorescence microscope (Nikon.US).

Insulin and C-peptide secretion and content

14 days after induction, the cells were washed with PBS. Then, incubated in Krebs–Ringer bicarbonate (KRB) buffer without glucose for 2 hr. Then the cells were incubated in KRB containing 5.5,15 and 25 mM glucose at 37°C. After incubation, cells were centrifuged and the supernatant was collected and stored at -70°C until assayed. Also for measuring of intracellular Insulin and c-peptide, total protein was extracted from cells by lysis buffer and protease inhibitor and then sonicated 3 times for 15 second each at 40w,and centrifuged(12000rpm,15min,4°C) and supernatant collected and kept at -70°C until used. Insulin and c-peptide determined by enzyme immunoassay ELISA kit((#10-1132-01,#10-1141-01, Mercodia, Uppsala, Sweden). Conversion factor for insulin 1µg/ml= 23mu/l; 1mu/l= 6.0 pmol/l and for C-peptide 1µg/l corresponded to 331 pmol/l.

Statistical analysis

The data were analyzed by Graph Pad Prism 5(GraphPad, La Jolla, CA, USA). The method One-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for comparison of experimental groups with control was used to compare results. P values <0.05 were considered as statistically significant.

Results

Cell culture and characterization of hBM.DSCs

Bone marrow samples were collected from healthy donors. Mononuclear cells were got through density centrifugation by using ficoll-paque solution. The nonadherent cells were removed at 48 hours after primary culture and adherent cells were cultured over 28 days. Then, the cells were seeded into plastic flask. Primary MSCs showed their characteristic spindle-shaped morphology (Fig. 1a). Flow cytometry analysis showed that sub cultured MSCs expressed high level of CD105 and CD90 low level of CD31, CD34. (Fig.1b). MSC were successfully differentiate to osteogenic and adipogenic lineage. We observed osteogenic differentiation and mineral deposit in MSCs after 21 days of culture in osteogenic media as confirmed by alizarin-red staining and oil-red O-positive and adipogenic cells after 14 days of exposure to adipogenic media.(Fig.2)

Production of LV-PDX1 in HEK 293 cells

The LV-PDX1 vector was amplified and packaged in HEK -293 cells. Virus production was confirmed by detection of PDX1 in HEK 293. RNA extracted from transfected cell and cDNA synthesized. RT-PCR analysis was performed to examine expression PDX-1 in transfected cells (Fig.3A). We also examined expression of PDX1 protein by Western blot in transfected cell. (Fig.3B).

Titration of lentivector by qPCR

To perform an titration by quantitative real-time PCR, plasmid EX-M0942-Lv105 containing puromycin resistance gene and puromycin primers were used to derive the standard curves. The standard curve was created automatically with the Applied Biosystems (Step One V2.0) software in each run by plotting the Ct number against the copy numbers of each standard and quantification of

viral DNA for unknown samples was inferred from the regression line viral titer were (Fig.4a,b). Melting Curve for standard and unknown samples occurs at 93.85°C. Also we can see that no contaminating products are present in this reaction.(Fig.4c)

Morphological changes of transduced MSCs

MSCs at passage 4 were induced into islet-like cells In 21 days. The MSCs exhibited a spindle-shape morphology before differentiation.(Fig.5a), initial morphological changes observed in transduced cells at day 5 and cuboidal endodermal cells was determined(Fig.5b). At day 7, cells from spindle-shaped changed to round form and the cells were gradually accumulated together (Fig.5c), at day 14, cells became aggregates and new islet-like clusters began to appear (Fig.5d). In addition, cell proliferation became slower and islet-like cluster were formed at day 21 (Fig.5e).

Pancreas-specific genes expression

In this study, we analyzed the PDX-1 gene expression as a pancreatic development key transcription factor and insulin gene expression as endocrine related marker by RT-PCR analysis in triplicate on cDNA samples of transduced cells at day 0,7,14 and 21. Our result showed that PDX-1 gene expressions were increased at day 7th and gradual rising at day14th and 21th.(Fig.6,left part). As illustrated in (Fig.6,right part) the differentiated PDX-1 hMSCs 14 days after induction expressed gene characteristic insulin gene expression and gene expression level strongly increased at day 21. Insulin gene expression was detectable at day 14

of differentiation and at day 21. Our results showed that PDX-1 gene expression was determined earlier than insulin gene expression.

Viability of MSCs during differentiation to IPCs

The MTT assay was performed on transduced cell at day 0, 7, 14, 21 post inductions to determine Percentage of cell death during differentiation. Data were from 3 independent experiments. The viability of IPCs was slightly reduced at days 7 and 21 of differentiation compared with undifferentiated MSCs (day 0) which was statistically significant ($p < 0.05$). (Fig.7)

Immunofluorescence analysis

Expressions of PDX1 and insulin proteins were revealed in transduced hADSCs+PDX1 using anti-PDX1 and anti-insulin antibodies, which were represented as green dots in the immunofluorescence assay at days 21. Immunofluorescence analysis detected nuclei localization of PDX1 and cytoplasmic localization of Insulin. Quinter-staining of nucleus (blue) was performed by DAPI.(Fig.8)

Glucose-induced insulin and C-peptide secretion and content

Insulin producing cells in response to different glucose concentrations were examined. The insulin level was 3.75 ± 0.70 ng/ 10^6 Cells prior induction. At Concentration of 5.5 ,15 and 25 mM insulin levels respectively was 14.32 ± 1.25 ng/ 10^6 , 22.35 ± 2.3 ng/ 10^6 , 16.1 ± 1.4 ng/ 10^6 Cells(Fig.9a). The c-peptide secreted was 0.24 ± 0.01 μ g/ 10^6 cells before induction. At Concentration of 5.5, 15 and 25 mM c-peptide level respectively was 0.89 ± 0.06

$\mu\text{g}/10^6$, $1.53 \pm 0.11 \mu\text{g}/10^6$ and $1.30 \pm 0.15 \mu\text{g}/10^6$ cells (Fig.9b). the value of intracellular insulin and c-peptide were noted on (Fig.9c) and (Fig.9d). Secreted insulin and c-peptide were proportional to glucose concentrations between 5.5 - 15 mM glucose and at 25 mM glucose decreased insulin and c-peptide level.

Discussion

Generation of insulin-producing cells from MSCs is new approach to treat type 1 diabetes (11, 12). Different cell sources such as cord blood, adipose tissue, bone marrow were used to generate IPC (13-15). BM.MSCs generate more insulin producing cells than Ad.MSC because BMSCs had more islet-like clusters and predominant pancreatic gene expression than ADSC that differentiate to IPC (16). Our result showed that Culture of MSCs produced adherent spindle-like cells and expression of CD90 and CD105 MSC surface markers confirmed characterization of these cells. We also examined MSCs differentiate into adipogenic and osteogenic lineages. In this study we evaluated the potential of BM-MSCs to generate morphologically and functionally pancreatic islet-like structures by genetic manipulation. Our findings revealed that PDX1 makes a strong change in gene MSCs and can induce its differentiation to pancreatic lineage. Lentiviral vectors transport transgenes better than adenoviral vectors and therefore higher transgene expression levels were found. Lentiviral vectors can introduce PDX-1 gene to MSCs to produce IPCs (17, 18). Karneili et al in same study performed differentiation human BM-MSC into insulin-producing cells by over expression of rat Pdx-1 gene and they could show expression of islet genes in manipulated BM-MSC that indicate a predisposition for differentiation toward islet-cell phenotype(19). The homeodomain protein PDX-1 is a transcription factors possessing the hallmark 60 amino acid homeodomain that is highly conserved from yeast to human(20), so rat Pdx1 gene can

differentiate hMSC to IPC. Transfection by lipofectamine is common in other same studies, but we did transfection by calcium-phosphate method that generated sufficient transduced cells. Cell-toxicity of the procedure determined by MTT assay, mortality at day 7 was not considerable but at days 14 and 21 was Significant. Our result exhibited that IPCs released insulin in response to physiological concentrations of glucose. Boroujeni *et al* In order to promote insulin secretion, added B27, nicotinamide and β FGF into the culture medium (21), but we didn't used any chemical materials or growth factor in differentiation protocol. In this study IPCs secreted insulin in response to various concentrations of glucose (0,5.5,15,25 mM) and peak insulin was 23ng/l at 15 mM glucose concentration. Peak insulin secretion in same researches was 6.85 ng/l (Rahmati *et al*) (22), 40 ng/l (Boroujeni *et al*) (21) and 27 ng/l (karneili *et al*) (19). C-peptide in the cell clusters confirmed that insulin release resulted from endogenous synthesis. Our findings showed that Insulin was released from IPCs in response to rising glucose up to 15 mM glucose because the PDX-1 activated a number of genes involved in maintaining beta cell identity and function, such as insulin, Glut2 and GK, which suggested that these cells might have glucose response(23), but poor response to high concentration glucose result lack of expression KIR6.2 and SUR1 in transduced MSC PDX-1+ cells (19). In this study, the expression of the genes and protein related IPCs was detected by immunocytochemistry, RT-PCR. PDX-1 gene was expressed earlier at 7th day of post induction and insulin gene expressed at day 14 due to insulin gene activity-dependent PDX-1 gene activity(24). Morphological transformation initiated at day5 and cells gradually accumulated together at day 14 and islet –like cluster formed at day 21. The morphological changes MSCs began from spindle-shaped to round form after 5 day post induction. We showed morphological changes of insulin producing cells obviously at different days of differentiation and these were similar to pancreatic islet cells.

Conclusion

Our findings indicate that BM.MSCs can differentiate into insulin producing cells efficiently by PDX-1 over expression. Lentiviral vectors can deliver PDX-1 gene to MSCs and induce pancreatic differentiation. We used simple and effective methods to generate effective IPCs. Furthermore additional experiment including in vivo needed to confirm our findings.

Author's contributions

AA performed the experiments, wrote the manuscript, interpreted the results and analyzed the data. AJ, SA, MS and MT designed the study provided laboratory space and equipment and revised the manuscript. FS helped to do the laboratory tests.

Acknowledgments

This research is financially supported by a grant from Tarbiat Modares University, Tehran, Iran. We would like to thank parviz fallah his technical help. There is no conflict of interest in this article.

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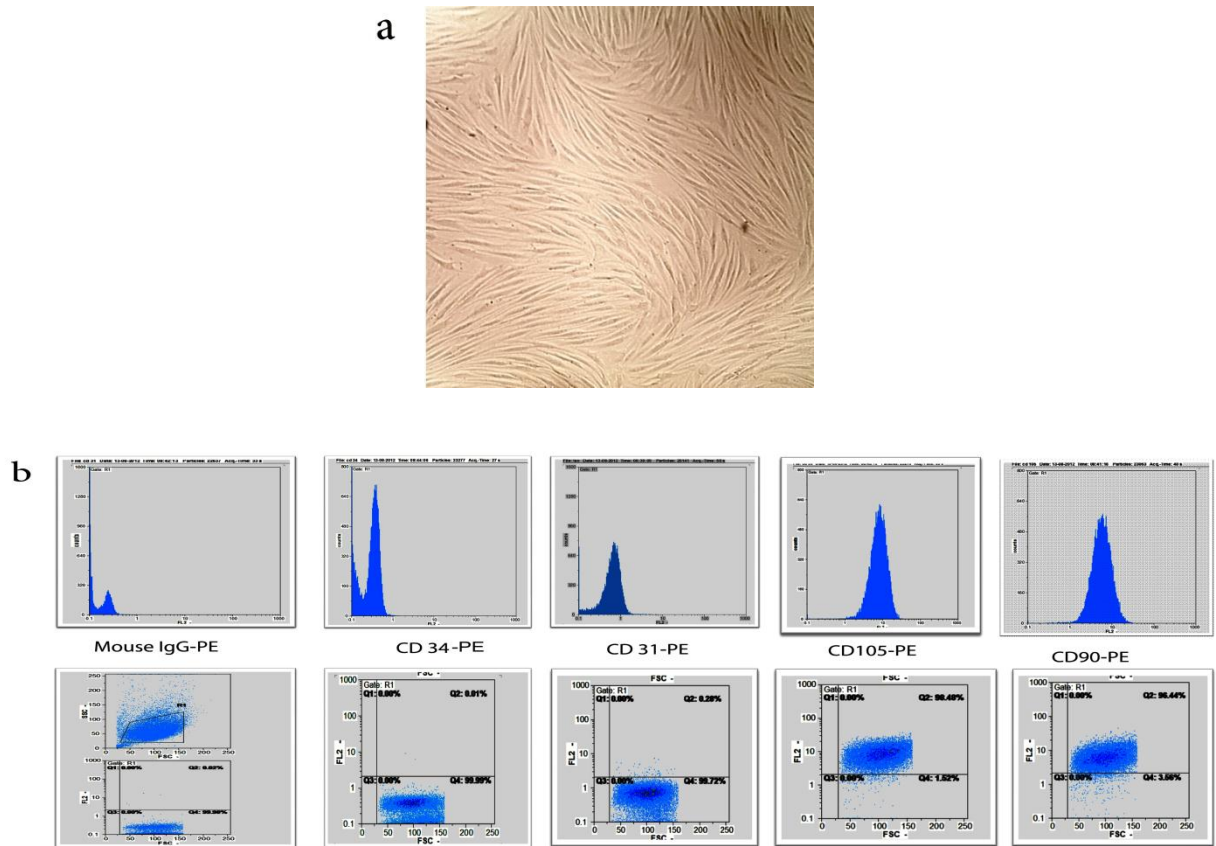


Fig.1 Morphological appearance of cultured cells and Flow cytometric analysis. A. MSCs showed a spindle-shaped fibroblastic b: Flow cytometry analysis of the cell surfacemarkers of MSC. These cells were positive for CD 105 and CD90 and were negative for CD31 and CD34.

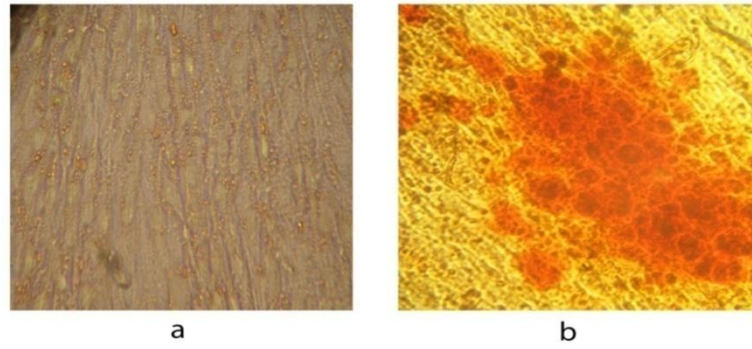


Fig.2 Multilineage differentiating of MSCs. MSC were differentiated toward adipogenic and osteogenic lineage using induction media. After 2 weeks adipogenic differentiation was assessed by Oil Red O staining(a). Osteogenic differentiation was analyzed after 3 weeks by Alizarin Red staining(b).

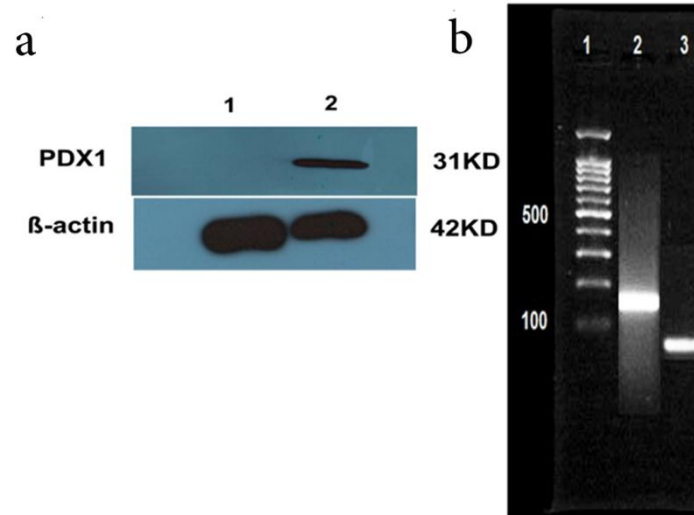


Fig.3 Confirmation of lentiviral transfection. (A)Western blotting was used to detect PDX-1 protein(31KD) and β -actin (42 kD). (B) Reverse transcription polymerase chain reaction (RT-PCR) analysis of PDX-1 gene from HEK 293 cells , Lane 1,DNA ladder 100bp. Lane 2,transfected HEK 293 cells. Lane 3. untransfected HEK 293.

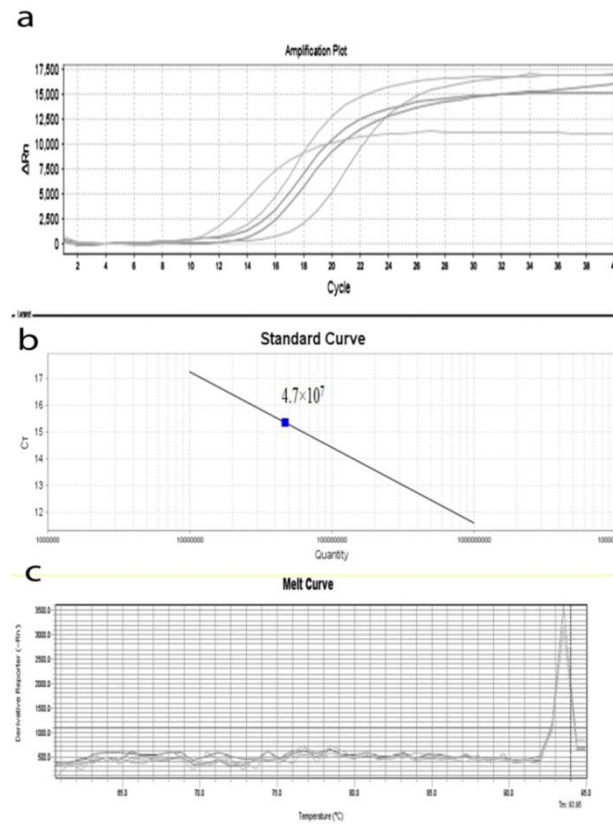


Fig. 4 Quantitation of lentivirus vector samples by real-time PCR. (a) Amplification plot of samples with each dilution represented in order from left to right on the graph. The x-axis represents the cycle of PCR amplification curve. (b) A representative of standard curve for quantification of viral samples. Three 10-fold-dilutions (7.23×10^7 - 7.23×10^9 copies of the plasmid/ μ l) were used and viral titer was 4.7×10^7 particle/ μ l. (c) Melting curve analysis of the samples. The products from vector and viral samples have the same melting point peak at $93.85^{\circ}C$.

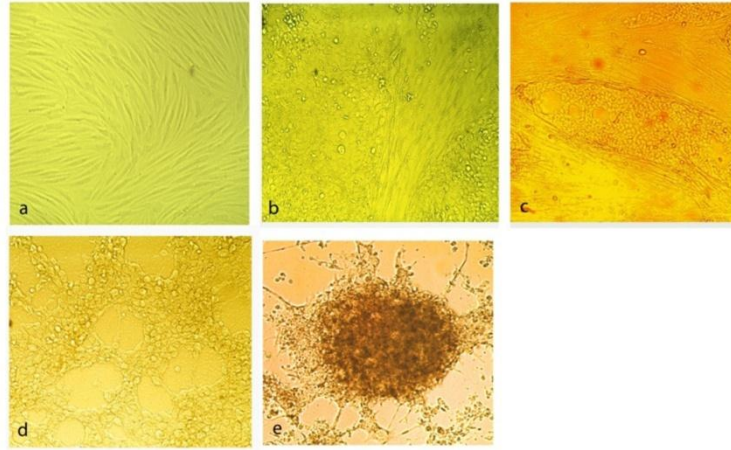


Fig. 5 Morphological changes transduced MSCs. (a) The morphological change of transduct cells at day1 ($\times 200$). (b) Transduct cells at day5, cells lost their spindle shape and got endodermal cuboidal shape ($\times 200$). (c) Transduct cells at day 7, cells changed to round shape ($\times 200$). (d) After 14 days cells finished their spherical shape accumulate together ($\times 200$). (e) The cells after 21 days differentiated to islet-like cluster ($\times 200$).

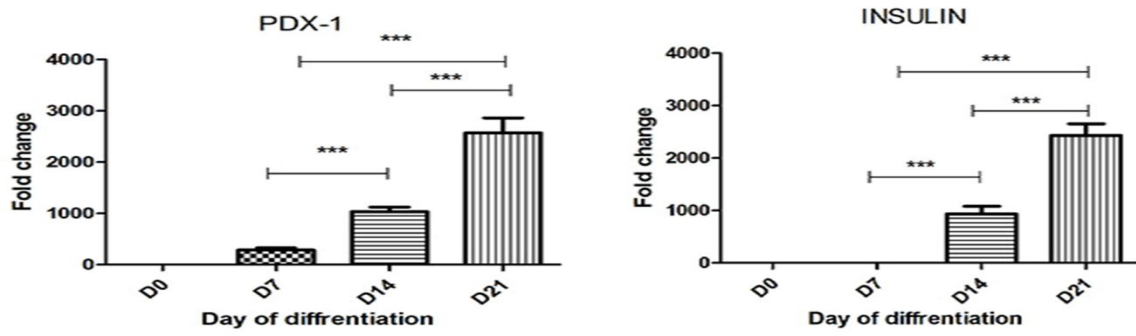


Fig.6 insulin and PDX-1 gene expression at days 7,14 and 21. qRT-PCR analysis of insulin and PDX-1 gene. At 3 stage of differentiation (day 7, day 14 and day 21) to islet-like clusters in comparison with undifferentiated MSCs (D0), The expression levels were normalized with GAPDH, The data are presented as mean \pm SEM .Data were got from 3 separate experiments and analysis after one-way ANOVA analysis showed no significant changes. (**p<0.01, ***P <0.0001).

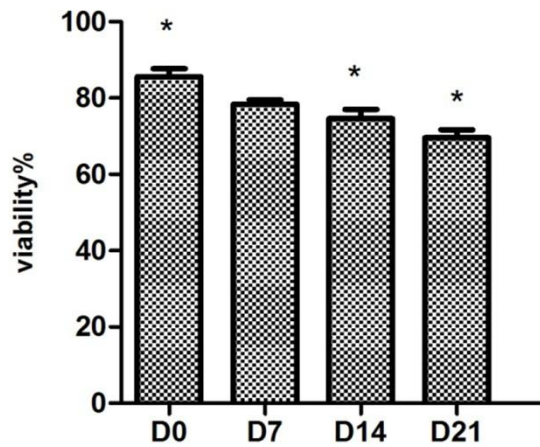


Fig.7 Relative cell viability of transduced MSC on days 0,7,14 and 21. The cells viability was determined by MTT test. There was no difference between viability cells on day 0 and 7, but viability of the cells at day 14 and 21 were reduced and was statically significant (* $p < 0.05$). The data are presented as mean \pm SEM.

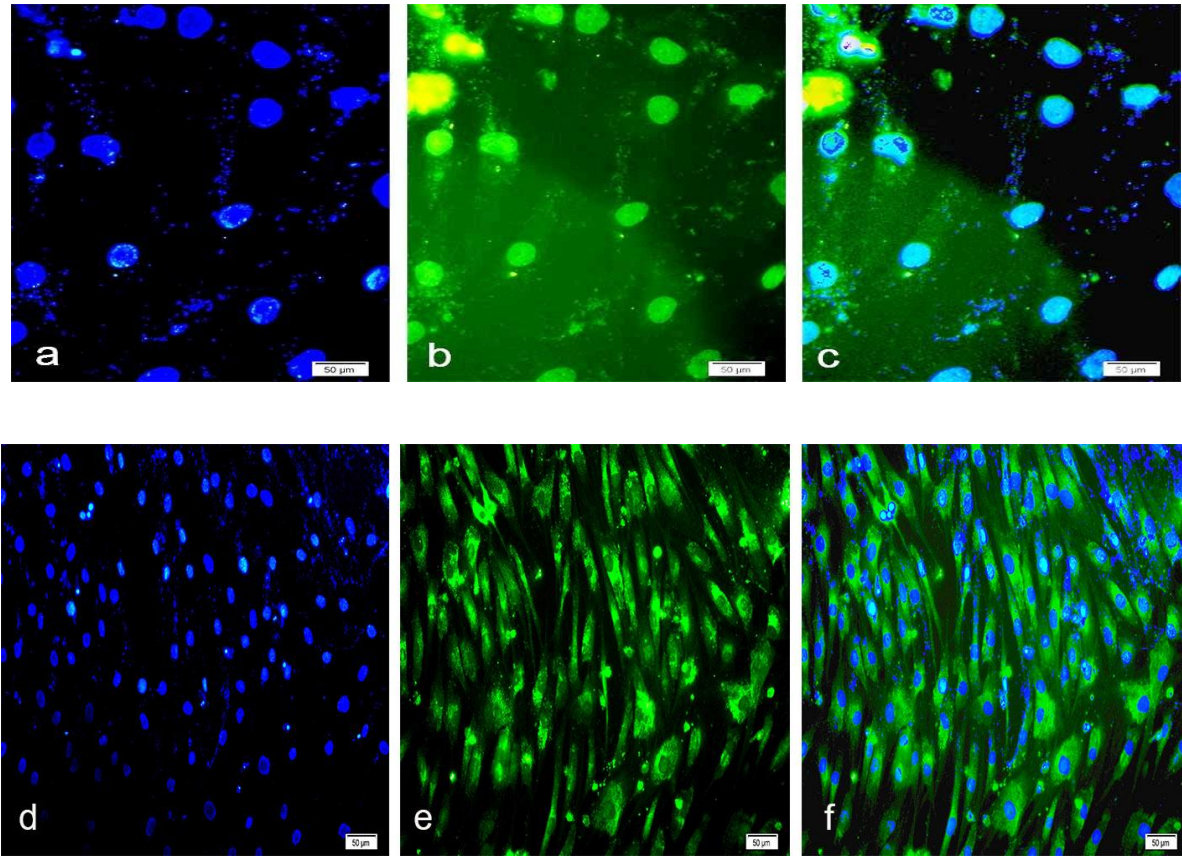


Fig.8 Expression of PDX-1 and insulin in IPCs. FITC conjugated PDX-1 antibody detected nuclei localization of PDX-1 (b). The merged image of nuclei immunostaining was shown in Figure (c). FITC conjugated insulin antibody detected cytoplasmic localization of Insulin(e) and merged(f). Quinter-staining of nucleus (blue) was performed byDAPI(a,d). Images were obtained by a fluorescence microscope (magnification X200).

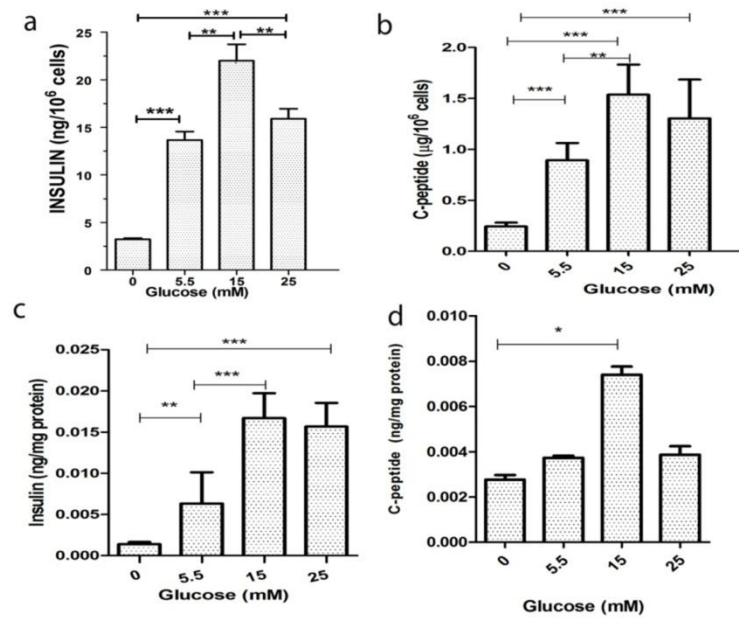


Fig.9 Insulin and c-peptide assay . (a)Insulin secretion, and (b) c-peptide secretion in response of 0, 5, 15 and 25 mM glucose.(c)Intracellular insulin content in each concentration of glucose versus of cellular total protein.(d) Intracellular c-peptide content in each concentration of glucose versus of cellular total protein. Maximum amount of insulin and c-peptide were obtained in 15 mM glucose concentration. **Results are shown** as mean \pm SEM. (** p < 0.01, *** p < 0.001)

Table1.Primers sequences

primer	Forward	reverse	T m	Prod uct size
PDX1	ATGGATGAAGTCTACCAAAGC	CGTGAGATGTACTTGTTGAATA G	60	159
Insulin	GAACGAGGCTTCTTCTACAC	ACAATGCCACGCTTCTG	59	143
GAPDH	GACAAGCTTCCCGTTCTCAG	GAGTCAACGGATTTGGTCGT	58	166
Prumycin	TAAATATAGTCAATGTCCCTCAG C	TGTGGTTCTGTGTTGGTAGC	58	174