

Ex vivo generation of glucose sensitive insulin secreting mesenchymal stem cells derived from human adipose tissue

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ABSTRACT

Background: Diabetics are incapable of producing insulin/have autoimmune mechanisms making it ineffective to control glucose secretion. We present a prospective study of glucose-sensitive insulin-secreting mesenchymal stem cells (IS-MSC) generated from human adipose tissue (h-AD) sans xenogenic material. **Materials and Methods:** Ten grams h-AD from donor anterior abdominal wall was collected in proliferation medium composed of α -Minimum Essential Media (α -MEM), albumin, fibroblast-growth factor and antibiotics, minced, incubated in collagenase-I at 37°C with shaker and centrifuged. Supernatant and pellets were separately cultured in proliferation medium on cell+ plates at 37°C with 5% CO₂ for 10 days. Cells were harvested by trypsinization, checked for viability, sterility, counts, flow-cytometry (CD45/90⁺/73⁺), and differentiated into insulin-expressing cells using medium composed of DMEM, gene expressing up-regulators and antibiotics for 3 days. They were studied for transcriptional factors Pax-6, Isl-1, pdx-1 (immunofluorescence). C-peptide and insulin were measured by chemiluminescence. *In vitro* glucose sensitivity assay was carried out by measuring levels of insulin and C-peptide secretion in absence of glucose followed by 2 hours incubation after glucose addition. **Results:** Mean IS-AD-MSC quantum was 3.21 ml, cell count, 1.5 x10⁹ cells/ μ l, CD45/90⁺/73⁺ cells were 44.37% /25.52%. All of them showed presence of pax-6, pdx-1, and Isl-1. Mean C-Peptide and insulin levels were 0.36 ng/ml and 234 μ U/ml, respectively, pre-glucose and 0.87 ng/ml and 618.3 μ U/ml post-glucose additions. The mean rise in secretion levels was 2.42 and 2.65 fold, respectively. **Conclusion:** Insulin-secreting h-AD-MSC can be generated safely and effectively showing *in vitro* glucose responsive alteration in insulin and C-peptide secretion levels.

Key words: Adipose tissue, C-peptide, insulin, insulin-secreting mesenchymal stem cells

INTRODUCTION

Insulin is a naturally-occurring protein hormone, responsible for allowing glucose to enter from the blood into cells. Hence, the phrase “curing diabetes type 1” means

“causing a maintenance or restoration of the endogenous ability of the body to produce insulin in response to the level of blood glucose” and cooperative operation with counter-regulatory hormones. In connection with the discovery of insulin biosynthesis, pro-insulin C-peptide was first described in 1967, which serves as an important link between the α and β chains of insulin. C-peptide is a marker of insulin secretion and helps in furthering the understanding of pathophysiology of type 1/ 2 diabetes.^[1]

Stem cell (SC) research in the last few decades has shown promising field for neogenesis of beta cells using embryonic SC, adult SC residing in the pancreas, or other non-

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pancreatic cell types. Fully functional islets have not yet been derived from any of these sources.^[2] Both embryonic and adult SC have been used to generate surrogate beta cells or restore beta cell functioning demonstrating generation of insulin-secreting cells that normalized blood glucose values when transplanted into diabetic animal models.^[3]

Mesenchymal stem cells (MSCs) were first identified in the bone marrow (BM) and characterized by Friedenstein and colleagues in 1974. They are multipotent cells capable of differentiating into several lineages including cartilage, bone, muscle, tendon, ligament, and adipose tissue.^[4-6] In their undifferentiated state MSC are spindle-shaped and resemble fibroblasts.^[5,6] The diversity of characteristics associated with MSC can be explained by differences in tissue origin, isolation methods and culture conditions between laboratories, in addition there appear to be strain-to-strain differences in murine-derived MSC.^[4,7-9] According to Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, MSC have to be plastic-adherent when maintained under standard culture conditions, they must have the ability of osteogenic, adipogenic, and chondrogenic differentiation; must express CD73, CD90, and CD105; and must lack expression of hematopoietic lineage markers c-kit, CD14, CD34, CD 45, CD 11b, CD 29, CD79 α , and HLA-DR.^[4,5,10]

In the present prospective study we present experience of generation of glucose sensitive insulin secreting MSCs derived from adipose tissue (IS-AD-MSC) of voluntary human donors who underwent abdominal surgeries after taking their written informed consent. No xenogenic material was used.

MATERIALS AND METHODS

Isolation of mesenchymal stem cells from human adipose tissue

After Institutional Review Board approval of the methodology and informed consent forms from donors, 10 gram adipose tissue was resected from anterior abdominal wall under local anesthesia after making a small incision on left lateral side below umbilicus. Sutures were taken after hemostasis was secured. This adipose tissue was collected in self-designed proliferation medium with Minimum Essential Media with alpha modification (α -MEM) (Sigma, USA), 20 % human albumin (Reliance Life Sciences, India), basic-human fibroblast growth factor (B-hFGF), 1% sodium pyruvate and standard antibiotics which included penicillin, streptomycin, cefotaxime, and anti-fungal fluconazol.

The adipose tissue was minced with knife into fine pieces

with addition of an enzyme Collagenase type I (10 mg/10 ml) in 75 cm² tissue culture dishes. After mincing the tissue, culture dish was placed in incubator at 37°C with shaker arranged at 35 RPM (self-designed) for 1 hour for removal of extra cellular matrix from the adipose tissue. Subsequently the contents were transferred to 15 ml centrifuge tubes and centrifuged at 780 RPM X 8 minutes. Subsequently the supernatant and pellets were separately cultured in proliferation medium on 100 sq. cm and 25 sq. cm. cell+ plates (Sarstedt, USA), respectively, at 37°C with 5% CO₂ for 10 days. Medium was changed every other day.

Differentiation of human adipose tissue-mesenchymal stem cells into insulin secreting cells

On 10th day of culture in proliferation medium, the cells were washed in Phosphate Buffered Saline (PBS) (1 N) and were harvested by means of trypsinization (0.25% Trypsin EDTA solution, Hi Media, India). Cells were checked for morphology, viability using trypan blue, sterility (Bactec, USA), and counts in modified Neubauer chamber. MSC characteristics were confirmed by flow cytometric analysis with CD 45 (Per CP) negativity and CD90 (FITC)/ CD73 (PE) (Beckton Dickinson, USA) positivity. A small aliquot was also stained by hematoxylin and eosin for morphologic analysis. Remaining cells were further subjected to differentiation to insulin-secreting cells using differentiation medium comprising of Dulbecco's Modified Eagle's Medium (DMEM) (4500 mg glucose/L), DMEM: F 12, growth factors and serum supplements like Nicotinamide, Activin A, Exendin 4, Pentagastrin, Hepatocyte Growth Factor, B-27, N-2 and antibiotics. No xenogenic material was used.

The cells were kept in this medium for 3 days and then subjected to isolation on Ficoll Hypaque by density gradient centrifugation. Cell pellet was then diluted with phosphate buffered saline (PBS) (1N) and subsequently subjected after testing for sterility, viability, and cell counts to immunofluorescence for islet transcriptional factor 1 (isl-1), the gene up-regulating expression of insulin, paired box genes-6 (Pax-6) and key regulator for normal islet cell development, pancreatic and duodenal homobox 6 (pdx-6), regulator of β -cell specific gene expression, function, and for self-renewal of β progenitor cells. C-peptide and insulin secretion from cells were measured by chemiluminescence assay (Lumax, USA).

Glucose sensitivity assay

Cells were further incubated in 6 well-Tissue culture test plate (SPL Life sciences, Korea) at the concentration of 5 cells/cm² area in absence of glucose and insulin and c-peptide levels were measured and test was repeated after addition of glucose (Dextrose, 25% w/v) for 2 hours. This

was treated as test batch. Insulin and c-peptide levels were measured by chemiluminescence assay subsequently.

Negative controls

For negative controls, after MSC were isolated from proliferation media on 10th day of culture, followed by sterility, quantification tests and characterization by IF and flow cytometry, half of the inoculum was subjected to culture without differentiation media. The cells were isolated at the end of 3 days and subsequently aliquots were incubated in 6 well-Tissue culture test plate (SPL Life sciences, Korea) at the concentration of 5 cells/cm² area in parallel to the test batch. Insulin and c-peptide levels were measured and test was repeated after addition of glucose (Dextrose, 25% w/v) for 2 hours.

Statistical analysis

Statistical Analysis was performed using SPSS version 12. Data are expressed as mean ± SD (min-max) for continuous variables. Continuous variables were compared using Wilcoxon signed rank test. *P* < 0.05 was considered to be statistically significant.

RESULTS

Totally 33 cell lines were generated from AD-MSc derived insulin-secreting cells from 33 volunteers. Wet preparation showed large round to polygonal cells with large nuclei surrounded by cytoplasm [Figure 1a] and hematoxylin and eosin stained cells showed large basophilic nuclei with distinct margins surrounded by eosinophilic cytoplasm [Figure 1b]. The mean cell quantum was 3.21 ± 0.59 ml (range: 2-4 ml), mean cell count, 1.5 ± 0.95 x10³ /µl (0.78-2.5 x10³ /µl), mean CD45-/90+ cells were 44.37 ± 16.69 % (range: 16.62–81.38%) and mean CD45-/73+ cells were 25.52 ± 15.36 % (range: 2.68–65.72%) [Figure 2]. All the test samples showed presence of transcriptional factors Paired box genes-6 (Pax-6), Islet 1 transcriptional factor (Isl-1) and Pancreatic and duodenal homobox 6(Pdx-6) [Figure 3 a-c]. Mean insulin level secreted by the cells themselves was 234 ± 815.09 µU/ml (0–3800 µU/ml) in absence of glucose and 2 hours after addition of glucose (following incubation at 37°C) the rise in insulin secretion level was observed with the mean of 618.3 ± 1845.08 µU/ml (range: 0.5–9500 µU/ml).

Mean C-peptide level secreted by the cells themselves was 0.36 ± 0.48 ng/ml (range: 0–2.26 ng/ml) in absence of glucose and after 2 hours after addition of glucose the rise in C-peptide secretion level was observed with the mean of 0.87 ± 1.91 ng/ml (range: 0.4– 9.35 ng/ml).

All the negative controls showed absence of insulin

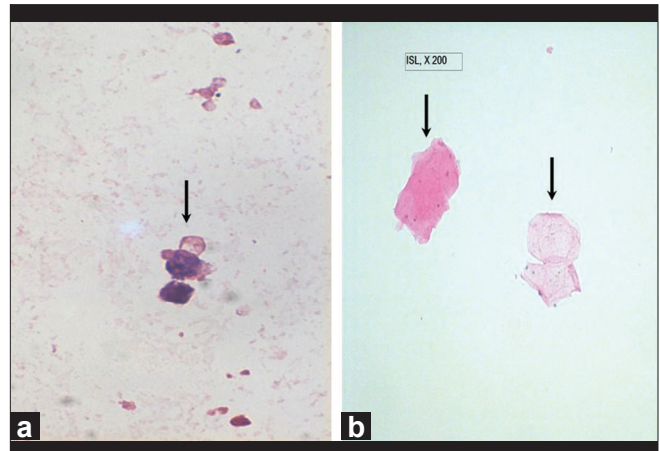


Figure 1: Hematoxylin and eosin stain: (a) on left showing mesenchymal stem cells with centrally placed round nucleus with clear nuclear margin and surrounding fine granular eosinophilic cytoplasm, x200 and (b) on right showing insulin secreting cells, x200

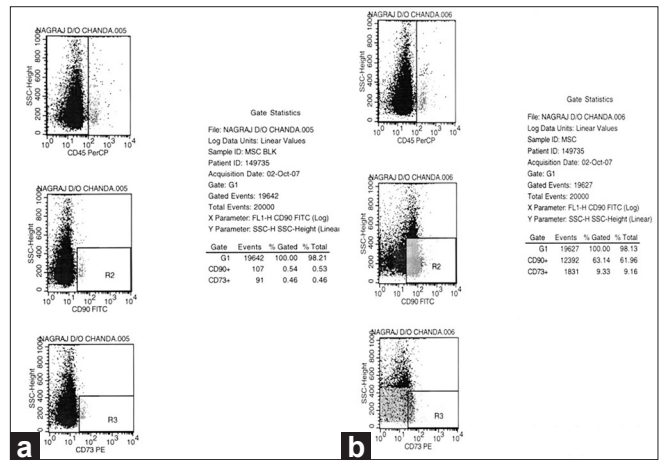


Figure 2: Flow cytometric analysis using FAC scan demonstrating characteristics of mesenchymal stem cells (CD45-, CD90+, CD 73+) with blank (a) and test (b) values

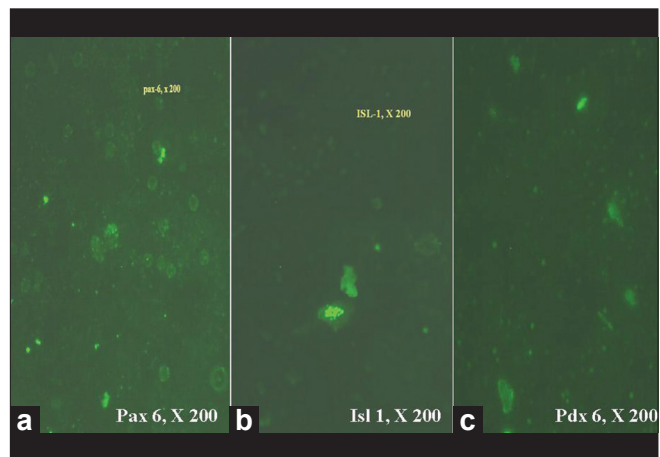


Figure 3: Indirect immunofluorescence demonstrating expression of pax-6 (a), isl-1 (b), pdx-6 (c), from left to right, x200

secretion and C-peptide in absence of glucose as well as 2 hours after addition of glucose.

The mean rise in insulin secretion level observed was 2.65 folds ($P = 0.000001$) and C-peptide level secreted by the cells themselves was 2.42 folds ($P = 0.007$) after addition of glucose to the cells after 2 hours incubation.

DISCUSSION

Diabetes is a group of autoimmune diseases characterized by abnormally high levels of glucose in the blood stream. Type 1 diabetes (juvenile-onset diabetes), typically affects children and young adults. Diabetes develops when the body's immune system sees its own cells as foreign and attacks and destroys them. As a result, islet cells of pancreas, which normally produce insulin, are destroyed. In the absence of insulin, glucose cannot enter the cell and hence accumulates in the blood. Type 2 diabetes (adult-onset diabetes) tends to affect older, sedentary, and overweight individuals with a family history of diabetes. Type 2 diabetes occurs when the body cannot use insulin effectively, which is called insulin resistance and the result is the same as with type 1 diabetes- a buildup of glucose in the blood.

Diabetes is predicted to be the major killer of population all across the world with likelihood of 366 million people suffering from diabetes by the year 2030.^[11] Strategies to curb this problem are being established in the form of pancreatic transplantation or islet cell replacement from adult and embryonic SC producing insulin. However, steroid immunosuppression becomes mandatory to prevent them from being rejected by the host immune system, which in itself increases metabolic demands on insulin-producing cells and eventually they may exhaust their capacity to produce insulin. The deleterious effect of steroids is greater for islet cell transplants than for whole-organ transplants.^[3,12] In developing a potential therapy for patients with diabetes, researchers hope to develop a system that meets several criteria, i.e. cells should be self-renewing and should also be able to differentiate *in vivo* to produce the desired kinds of cells. Fetal tissue, embryonic SC and adult tissues have been used for culturing islet cells as a potential source of islet progenitor cells. Although differentiated beta cells are difficult to proliferate and culture, some researchers have succeeded in engineering such cells to some extent. The major problem in dealing with these cells is maintaining the delicate balance between growth and differentiation. Cells that proliferate well do not produce insulin efficiently, and those that do produce insulin do not proliferate well. The major issue is developing the technology to grow large numbers of these cells that will reproducibly generate adequate amounts of insulin.^[12-15]

MSCs represent a population of non-hematopoietic precursor cells that have generated marked interest and attention for their capacity to elicit tissue regeneration.^[16] These cells can be obtained from BM, synovium, fetal liver, adipose tissue, cord blood, and even peripheral blood.^[17] Adipose tissue derived MSCs are similar to BM and umbilical cord blood derived MSC with respect to their morphology, immune phenotype, success rate of isolating MSC, colony frequency, and differentiation capacity. Ideal conditions for culture include cultures of low density, use of anti-oxidants like N-acetyl-L-cysteine, L-ascorbic acid-2 phosphate, low Ca concentration can increase growth rate and lifespan of ADSC. AD-MSCs have better proliferating potential than BMSC.^[18] These cells are reported to provide critical growth factors for tissue regeneration and also possess potent immunoregulatory properties that could be exploited to suppress allograft rejection following transplantation.^[19] MSCs also have immunomodulatory, immunosuppressant and regenerating activities in addition to preventing graft versus host diseases in transplantation.^[20-23] Jaeseok Han and *et al.*, studied insulin secretion in response to glucose and reverse hyperglycemia from engineered entero-endocrine cells in diabetic mice in which they genetically engineered an entero-endocrine cell line (STC-1) to express insulin under control of the glucose-dependent insulinotropic polypeptide promoter, screened clones and chose one, Gi-INS-7, based on its high production of insulin.^[24] By detecting expression of glucose transporter 2 (GLUT2) and glucokinase (GK) they studied insulin secretion in response to elevated glucose levels *in vitro*. They also measured insulin secretion in the culture supernatant and insulin content in MIN6 and Gi-INS-7 cells after incubation in low (5.5 mM) and high (27.5 mM) glucose for 2 hours and calculated insulin secretion as a percentage of total cellular insulin content. They also studied glucose tolerance tests *in vivo*, in mice after transplantation with Gi-INS-7 cells showing that exogenous glucose was cleared appropriately.

We have generated *in vitro* MSC from human adipose tissue which qualify the definition standardized by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy.^[19,25] We further differentiated them in to insulin secreting cells under defined culture conditions phenotypically identical to pancreatic β -cells without using any xenogenic material.^[25] These cells expressed transcription factors pdx-1, pax-6, and isl-1, all three are central controlling genes capable of reprogramming non-pancreatic cells to surrogate β -cell functions.^[25] Addition of this differentiation medium to the cells upregulate gene expression, nourishes the cells and prevents their further proliferation. In this study, we have also established that absence of glucose switches off their insulin secreting response. Reconstitution of a

functional beta-cell mass by cell therapy using organ donor islets of Langerhans and pluripotent embryonic SC has been demonstrated to restore euglycemia in the absence of insulin treatment.^[26]

To our knowledge this is the first study using AD-MSK which is an easily available source, demonstrating effective and practically simple reprogramming of non-pancreatic cells as compared to vector based gene transfer or any other genetically engineered techniques and shows change in insulin and C-peptide secretion levels in response to addition of glucose in *in vitro* condition.

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REFERENCES

- Steiner DF, Cunningham D, Spigelman L, Aten B. Insulin biosynthesis: Evidence for a precursor. *Science* 1967;157:697-700.
- Seissler J, Schott M. Generation of insulin-producing beta cells from stem cells-perspectives for cell therapy in type 1 diabetes. *Horm Metab Res* 2008;40:155-61.
- Soria B, Bedoya FJ, Tejado JR, Hmadcha A, Ruiz-Salmerón R, Lim S, et al. Cell therapy for diabetes mellitus: an opportunity for stem cells? *Cells Tissues Organs* 2008;188:70-7.
- Barry FP, Murphy JM. Mesenchymal stem cells: Clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;36:568-84.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multi-lineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143-7.
- Friedenstein AJ, Petrokova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;16:381-90.
- Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41-9.
- Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells: A subpopulation of mesenchymal stem cells. *Ann N Y Acad Sci* 2001;938:231-3.
- Jorgensen C, Gordeladze J, Noel D. Tissue engineering through autologous mesenchymal stem cells. *Curr Opin Biotechnol* 2004;15:406-10.
- Jorgensen C, Djouad F, Apparailly F, Noel D. Engineering mesenchymal stem cells for immunotherapy. *Gene Ther* 2003;10:928-31.
- Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* 2004;27:1047-53.
- Madsen OD. Stem cells and diabetes treatment. *APMIS* 2005;113:858-75.
- Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Mortiz W, Müller B, et al. Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate *ex vivo* into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 2001;50:521-33.
- Timper K, Sebok D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, et al. Human adipose-tissue derived mesenchymal stem cells differentiate in to insulin, somatostatin and glucagon expressing cells. *Biochem Biophys Res Commun* 2006;341:1135-40.
- Marion NW, Mao JJ. Mesenchymal stem cells and tissue engineering. *Met Enzymol* 2006;420:339-61.
- 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.
- Phinney DG, Prockop DJ. Concise review: Mesenchymal stem/multipotent stromal cells: The state of trans differentiation and modes of tissue repair--current views. *Stem Cells* 2007;25:2896-902.
- Schaffler A, Buchler C. Concise Review: Adipose tissue derived stroma cells- Basic and Clinical Implications for novel cell- based therapies. *Stem Cells* 2007;25:818-27.
- Brusko TM. Mesenchymal stem cells: A potential border patrol for transplanted islets? *Diabetes* 2009;58:1728-9.
- Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* 2003;3:199-210.
- Koc ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, et al. Rapid hematopoietic recovery after co infusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000;18:307-16.
- Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;105:1815-22.
- Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;102:3837-44.
- Han J, Lee HH, Kwon H, Shin S, Yoon JW, Jun HS. Engineered enteroendocrine cells secrete insulin in response to glucose and reverse hyperglycemia in diabetic mice. *Mol Ther* 2007;15:1195-202.
- Trivedi HL, Vanikar AV, Thakker U, Firoze A, Dave SD, Patel CN, et al. Human adipose tissue-derived mesenchymal stem cells combined with hematopoietic stem cell transplantation synthesize insulin. *Transplant Proc* 2008;40:1135-9.
- Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. *In vivo* reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* 2008;455:627-32.

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