Isolation, Characterization, and *In Vitro* Differentiation of Mesenchymal Stem Cells from Human Umbilical Cord into Islet-Like Clusters

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Introduction: Mesenchymal stem cells (MSCs) are easy to isolate, culture, and manipulate in ex-vivo culture. MSCs were identified in several other organs such as umbilical cord which is focused in present project. Type 1 Diabetes mellitus is caused due to e damage of the pancreatic beta cells resulting in absolute insulin insufficiency. Transplantation of insulin-producing islet cells could be considered as an effective therapy for type 1 diabetes. **Materials and Methods***:* In this project we aimed to enzymatically isolate stem cells from human umbilical cord. The mesenchymal properties of these isolated cells were checked by flow cytometry technique. Their differentiation potentials were further studied, particularly with respect to islet like clusters (ILCs) which were generated through a three steps induction protocol. The amount of secreted insulin has been measured using insulin ELISA kit. **Results***:* Stem cells were successfully isolated from human umbilical cord. Cells expressed high level of mesenchymal markers and they had both osteogenenic and adipogenic differentiation potential. Through a 10 days of islet induction, most of the obtained islet like aggregates were positive for DTZ, a zinc-chelating agent known to selectively stain pancreatic β-cells. They were also able to secrete insulin. **Conclusion***: MSCs isolated from the human umbilical could be differentiated into ILCs that* possess the ability to produce insulin. Therefore human umbilical cord blood-derived MSCs (hUCMSCs) seem to be an excellent source of stem cells that can be used in the therapeutic procedures for type 1 diabetes.

Keywords: Umbilical cord blood-derived stem cells; Islet; Diabetes; Insulin; Stem cells

Introduction

Stem cells are cells that exist in all multi-cellular organisms. They can proliferate and differentiate into other specialized cell types and have self-renewal potential for creation of more number of stem cells. In mammals, there are two broad categories of stem cells; embryonic stem cells (ESCs) and adult stem cells (ASCs). Mesenchymal stem cells (MSCs) as a type of adult stem cells are easy to isolate, culture, and manipulate in ex-vivo culture. These cells have great plasticity and potential for therapeutic applications. According to a well-defined standards, MSCs are plastic adherent cells with specific surface phenotype that have the potential to self-renew and to differentiate into several lineages including bone, cartilage and adipose (1). MSCs were identified in several other organs such as adipose tissues, dental pulp and umbilical cord (UC) which is focused in present project.

The UC is the connecting cord from the developing embryo or fetus to placenta. Through prenatal development, the UC is physiologically and genetically part of the fetus and routinely contains two blood vessels and one blood vessel hidden inside Wharton's jelly (2). The umbilical vein possesses two major layers an external layer and an internal layer (3) at least two types of stem cells have been found in the human UC so far; the hematopoietic stem cells (HSCs) and the MSCs and throughout recent years many published papers on cord stromal cells explained their mesenchymal characters and applications in a variety of treatments (4,5).

Type 1 diabetes mellitus is caused due to autoimmune damage of the pancreatic beta cells ensuing in absolute insulin deficiency. Transplantation of insulin-producing islet cells isolated from a donor pancreas could be considered as an effective therapy for type 1 diabetes (6). However, deficiency of contributor organs and the side-effects of immunosuppressive therapy limit its therapeutic application, prompting an investigation for other sources of islet cells. Recently vast improvements have been happened in the understanding of endocrine development. These afford a significant guide to further efforts to produce islet cells in vitro (7). ESCs have been favorites in this concept because of their

remarkable differentiation capacity. However, the current ethical and legal concerns involved in ES cell research limit its usage in translational medicine (8).

Hence, MSCs are now being widely evaluated for their differentiation potential for cell replacement therapy. Recently, many studies have revealed that hepatic stem cells (9), and bone-marrow (BM)-derived MSCs (10) have the capacity to differentiate into insulin-producing cells. However, shortage of the source and the invasive procedures required to isolate and culture these cells, have restricted their practice.

By this explained situations, the main goal of this project was to isolate MSCs from discarded postnatal tissues like human UC. These isolated cells were used for characterization. Their differentiation potentials were further studied, particularly with respect to ILCs capable of producing insulin in vitro.

Materials and Methods

Isolation of MSCs from human UC

hUCMSCs were obtained from human UC of 30- to 35-year-old pregnant ladies who were admitted at the Manipal Hospital, Bangalore, India for delivery purpose. Informed consent was obtained from donors and the protocol was approved by Institutional Ethical Committee (Manipal Hospital, Bangalore). Under laminar air flow obtained human UC samples were separated from placenta and blood and blood clots were removed by washing with dulbecco's phosphate buffered saline (DPBS) containing antibiotics. Then Human UC samples were cut down into small pieces and about 5-10 ml collagenase and dispase (1mg/ml, 7:1) was added for 30-40 min. The digests were filtered through cell strainer and subsequently centrifuged at 1800 rpm for 10 min. Then 5-10 ml 0.05% trypsin was added to the pellet and incubated for 15-20 min. Again digests were filtered through cell strainer and centrifuged at 1800 rpm for 10 min. The supernatant was discarded and all the pellets were re-suspended in 2 ml of growth medium with 10% FBS. The medium were changed every two days and the attached cells were observed under microscope.

Characterization of hUCMSCs using flow cytometry

The media from (80-85%) confluent hUCMSCs plates was aspirated and washed thrice with 1ml PBS for 5 minutes. Then the PBS completely removed and 1ml of warmed 0.25% trypsin-EDTA was added to the T25 flask. Cells were observed under the microscope and allowed all the cells to detach. Then we neutralize the action of trypsin-EDTA with 3 ml of complete culture medium in a 1:3 proportion and subsequently the cell suspension was transferred to a 15 ml tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and a PBS wash given to the pelts. Then the pellet was re-suspended in 600 µl of PBS. Then 6 flow cytometry tubes were labeled as follow: CD166, CD90, CD73, CD34, HLA DR (all conjugated with PE) and ISO-PE. Subsequently the 600µl of cell suspension was distributed equally among the 6 tubes and 4µl of each antibody added to the respective tubes and 2 µl to the ISO-PE tube. The cells were incubated for an hour in the darkroom. The cells were read using flow cytometry machine.

Differentiation to adipocytes

Cells were plated in 24-well plate using normal MSCs media and incubated at 37°C in a 5% CO2 humidified incubator overnight till cells attached and reached 100% confluent. Then the medium was aspirated from each well and sufficient adipogenic induction medium was added. Adipogenic media changed every 2–3 days for 21 days. Lipid droplets were detected by microscopic examination as early as 5 days into the differentiation period. After 21 days of differentiation, adipocytes were fixed and the lipid droplets stained with Oil Red O solution. Contents of adipogenic induction medium were 200 mM glutamine, 1 mM dexamethasone, 1 mg/mL insulin, 100 mM indomethacin and 0.5 mM 3-isobutyl-1-1-methylxanthine (IBMX).

Differentiation to osteoblast

Cells were plated in 24-well plate using normal MSCs media and incubated at 37°C in a 5% CO2 humidified incubator overnight till cells attached and reached 100% confluent. Then the medium was aspirated from each well and sufficient osteogenic induction medium was added. Induction media replaced with fresh osteogenic medium every 2–3 days for 21 days. After this period of time mineralization (osteoblast) were detected by Von Kossa staining.

Contents of osteogenic induction medium were 5 mM glutamine, 10^{-8} M dexamethasone, 50 mg/mL ascorbic acid and10 mM β-glycerophosphate.

In vitro differentiation of hUCMSCs into ILCs

hUCMSCs were cultured in glass petridishes and induced for differentiation into islet cells in a three steps protocol. In first step the cells were cultured for two days in KO-DMEM, supplemented with 1% BSA Cohn fraction V Fatty acid free, 1 X Insulin-transferin-sodium selenite, 4 nM Activin A, 1 mM Sodium butyrate, 50 µM Beta-mercaptoethanol. In second step, on 3rd day after induction, the cells were cultured in Ko-DMEM supplemented with 1% BSA, ITS and 0.3 mM Taurine. And on 5th day (final step) the cells were cultured in KO-DMEM basal media supplemented with 1.5% BSA, ITS and 3mM Taurine, 100 nM Glucogon like peptide 1 (GLP-1), 1mM Nicotinamide and 1X Non-essential amino acids.

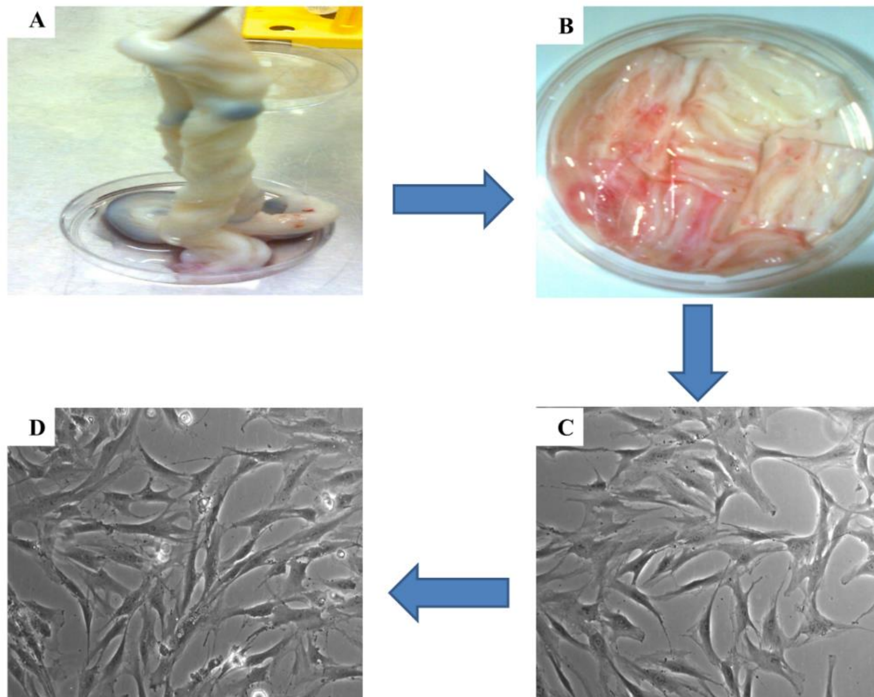

Figure1. Shows processing of human UC for isolation**.** Blood clot in the cord (A), Opened pieces of UC (B), hUCSCs at passage No.1(C) & No.5 (D).

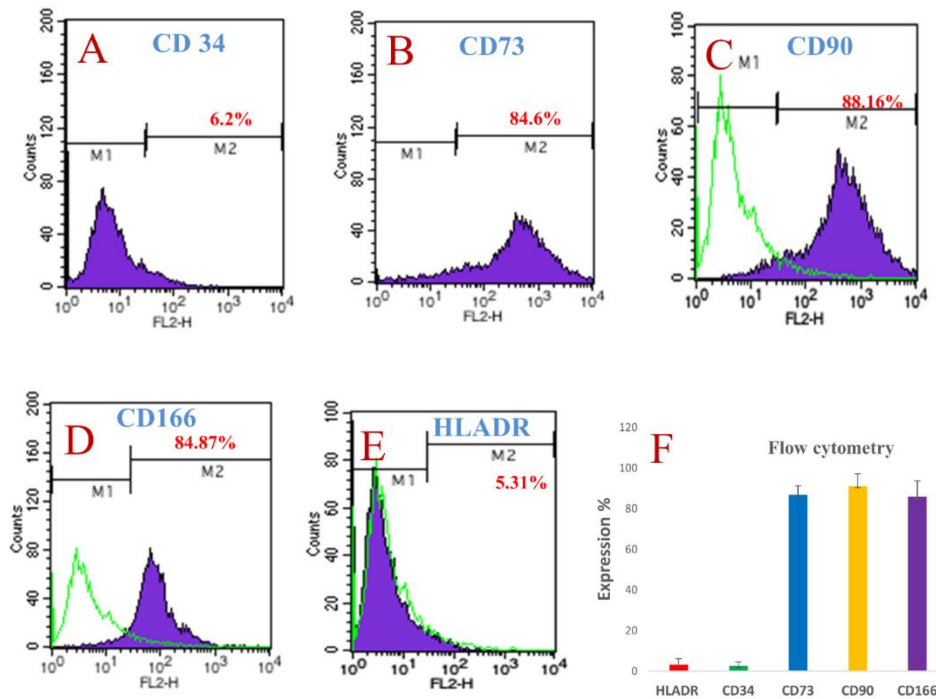

Figure2. Flow cytometric analysis revealed that the cells did not express haemopoietic markers CD34 and HLADR (A & E). On the contrary, cells expressed high levels of mesenchymal markers CD73, CD90, and CD166 (B, C & D). Graph presents the average of expression of each markers obtained from five samples (F)

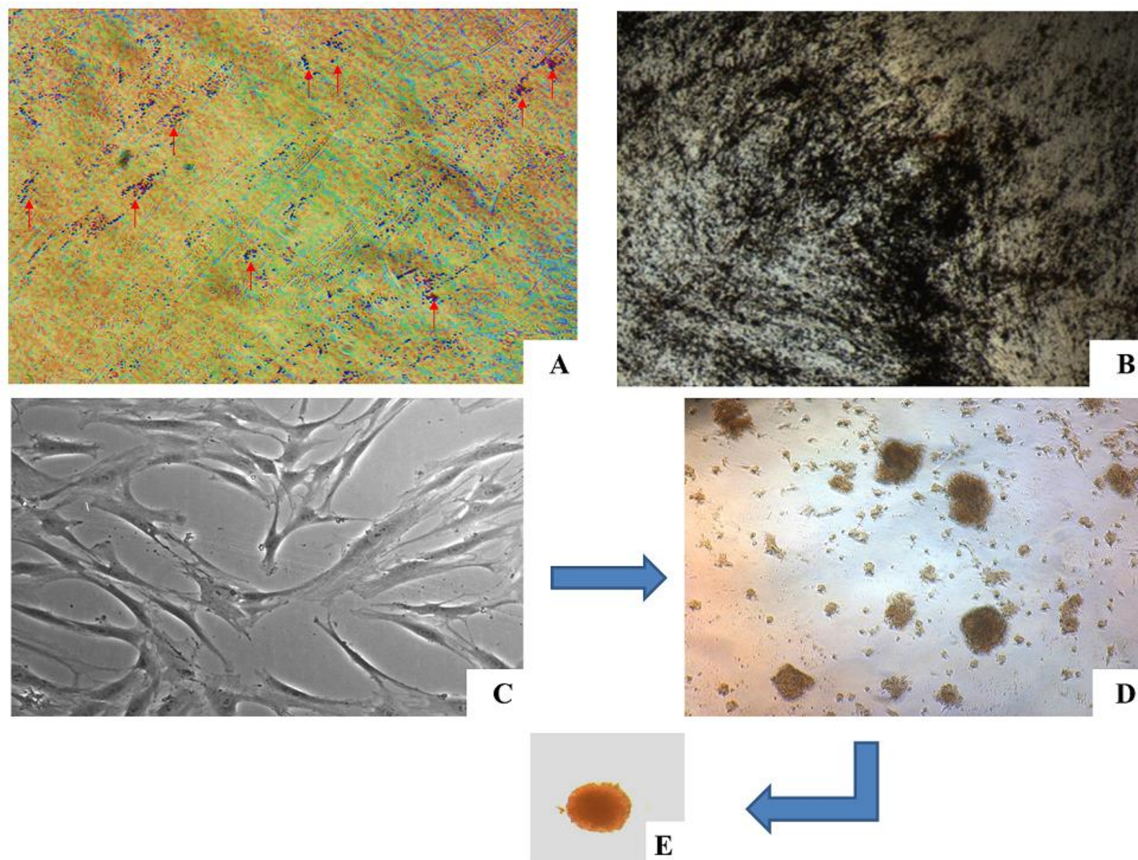

Figure3. Red arrows indicating Adipogenic potential of hUCSCs and accumulation of lipid vacuoles which were stained with oil red O (A). Black particles demonstrate calcium depositions in extracellular matrix of isolated hUCSCs which were stained by Von Kossa staining (B). Generation of islets like clusters from isolated hUCSCs through a three-step induction protocol demonstrating morphologic modifications in shape and size from monolayer (C) to islets like clusters formation (D). STZ positive stained day 10 islets like clusters (E).

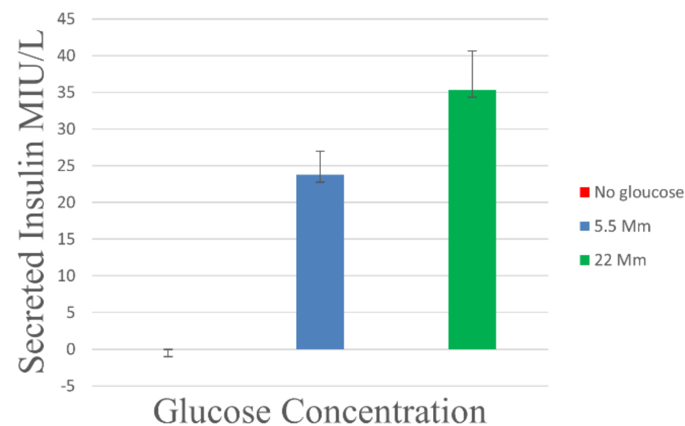

Figure 4. Graph displays the amount of secreted insulin from hUCSC-derived islet like clusters in response to stimulation with different concentrations of glucose.

Characterization of islet-like clusters using DTZ staining

Specificity of hUCMSC-derived ILCs were determined by Diphenylthiocarbazone (DTZ) staining. To prepare DTZ dye 10 mg of DTZ powder was mixed with 1ml of DMSO in an appropriate tube and mixed with PBS in 1:100 proportion. Then about 20 ILC were handpicked and kept in a 35 mm dish and incubated with 10 µL of prepared DTZ stain for 1 hour at 37°C. Subsequently the ILCs viewed under inverted phase contrast microscope (bright light).

Insulin release assay

About 200–300 ILCs (day-14) were selected and kept in eppendorf tube. ILCs were then washed several times with PBS and incubated with prepared KRBH buffer without glucose for 6 hours. ILCs were incubated with KRBH buffer containing 5.5 mM glucose for 1 h at 37°C. Then the supernatant were collected and the same clusters were further incubated in 22 mM glucose for additional 1 h at 37°C and again its supernatant was collected. In all three obtained supernatant human insulin concentration was measured with the use of an insulin ELISA kit.

Statistical analysis

Results were presented as mean ± standard of the mean (SEM). Statistical comparisons were performed using the Student's t-test. $P \leq 0.01$ were considered statistically significant.

Results

Isolation: Stem cells were successfully isolated from human UC and expanded for five passages. The cells maintained their normal fibroblastic morphology in all five passages (Figure 1, A-D).

Flow cytometry: Flow cytometric analysis revealed that the cells did not express haemopoietic markers CD34 and HLADR. On the contrary, cells expressed high levels of mesenchymal markers CD73, CD90, and CD166 (Figure 2A-F).

Adipogenic and Osteogenic differentiation: Adipogenesis potential of hUCMSCs was demonstrated by the accumulation of lipid vacuoles stained with oil red O (Figure 3A).

Their osteogenenic differentiation was also confirmed by calcium depositions in extracellular matrix stained by Von Kossa staining (Figure 3B).

In vitro differentiation hUCMSCs into ILCs: hUCMSCs that expanded as an adherent monolayer aggregated into spherical cells when their normal media replaced by islet induction media in a 3 steps days protocol (Figure 3C-D). After 10 days of incubation most of the islet like aggregates was positive for DTZ, a zinc-chelating agent known to selectively stain pancreatic β-cells (Figure 3E).

Insulin Release Assay: The total insulin contents of day 10 islet like aggregates when exposed to 22 mM glucose were higher than those ones which were stimulated with 5.5 mM glucose (Figure 4).

Discussion

The main aim of our study was to assess the potential of culture-expanded undifferentiated hUCMSCs as a source of insulin-producing cells. MSCs were successfully isolated from human UC using simple isolation protocols. These hUCMSCs were characterized for their morphology, and other phenotypic surface markers using confocal and flow cytometric studies. These cells similar to the other types of MSCs demonstrated typical fibroblast-like appearance (11) and expressed MSCs associated markers such as CD166, CD90, CD73, CD34, and SSEA 4 (1). Their stemness was confirmed by their differentiation into adipogenic and osteogenic lineages after exposure to lineage-specific differentiation cocktails of differentiating agents, as reported earlier by several groups (12). Following a stepwise protocol and using a taurine enriched medium, hUCMSCs were induced to differentiate into ILCs in vitro. The total time needed for differentiation into insulin-producing spheroid bodies was 4 weeks, in presence of serum-free medium containing ITS. But in present study we followed a modified pancreatic lineage differentiation protocol that has been reported earlier by other groups (13,14). In this procedure by adding of nicotinamide and GLP1 along with ITS into serum-free differentiation medium, we reduced the time needed for islet differentiation, the same result has been reported by other group when they used same protocol for induction of human placenta-derived MSCs (13).

These ILCs were identified as islets using phase contrast microscopy, DTZ staining (islet specific), and insulin secretion in response to glucose challenge. These properties of possessing of islet-like morphology and insulin-producing ability *were similar to the other types of MSCs which were induced with the same three steps protocol* (13,14).

Conclusion

It is evident from the data that MSCs isolated from the human UC are pluripotent and could be differentiated into ILCs *in addition to* adipogenic, and osteogenic lineages. These islet-like *aggregates* possess the ability to produce insulin in vitro. Therefore hUCMSCs seem to be an excellent source of stem cells to differentiate into islet-like clusters, because of the large potential donor pool, its rapid availability, less ethical concerns, no discomfort for the donor, and low risk of rejection.

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Conflict of Interest: 'None declared'.

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