Differentation of Umbilical Cord Mesenchymal Stem Cell toward Insulin Producing Cells

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ABSTRACT
Type1 diabetes is characterized by autoimmune destruction of pancreatic β cells, leading to reduced insulin secretion. Differentiation of mesenchymal stem cells (MSCs) into β-like cells offers new ways of diabetes treatment. MSCs can be isolated from the human umbilical cord tissue and differentiate into insulin-producing cells. Human umbilical cord-derived stem cells (hUCSCs) were attained after birth, selected by plastic adhesion, and considered by flow cytometric analysis. For differentiation induction, miR-375 was lentivirally overexpressed in hUCSCs. Meaningful that microRNAs (miRNAs) are key players in several stages of pancreatic Differentiation, we present a novel and cost-effective strategy in which over-expression of miR-375 promotes pancreatic differentiation in hUCSCs in the absence of any other stimulator. Major expressions of PDX1 and insulin were identified by quantitative RT-PCR (P < 0.05). In conclusion, Morphological assessment and expression analysis of islet marker genes demonstrated that hUCSCs are able to differentiate into insulin-producing cells by transduction with lentiviral vector miR-375.

INTRODUCTION
Type1 diabetes occurs by autoimmune damage of islet cells, leading to reduced insulin production and growing blood glucose level. Treatment of Type1 diabetes has been usually performed by injection of exogenous insulin or by pancreas replacement. Pancreatic transplantation has some difficulties such as induction of immunosuppression and trouble in surgery, which limit the transplantation of pancreas tissue. Currently, stem cell therapy is under investigation as another candidate for islet cell replacement. Embryonic stem cells (ESCs) derived from blastocyst [1] and adult stem cells derived from different adult tissues [2] have been used for obtaining enough number of β-cells [3, 4]. ESCs are pluripotent cells but are limited by immune rejection, tumorigenicity, and ethical problems [5]. Adult stem cells can be obtained from different sources like skin [6], salivary gland [7], liver [8], spleen [9], bone marrow [10], and umbilical cord tissue [11]. Among them, umbilical cord-derived stem cells (UDSCs) are good applicants for possible clinical applications. UDSCs are easily available without ethical concerns. UDSs may become a valuable target for islet cell replacement in diabetic patients because these cells have the potential to adopt a pancreatic islet-like phenotype [11]. Different strategies like transfer vectors, and differentiating medium has been investigated to generate insulin producing cells [12]. MicroRNAs (miRNAs) are small, 22-nucleotide non-coding RNAs with posttranscriptional regulatory roles with cleavage or translational repression of mRNAs. They have important roles in different cellular processes like development, differentiation, and apoptosis [13].

miRNA-375 has been involved in pancreas development in Zebrafish, mice and human. In zebrafish embryos, miR-375 knockdown led to aberrant formation of the endocrine pancreas [14]. In addition, this miRNA is essential for normal glucose homeostasis, and adaptive β-cell expansion in insulin resistance in mice [15].

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human, miR-375 is also expressed in adult islets with major role in generation of hormone-producing cells [16–18]. Therefore, miR-375 is considered as a useful tool for genesis of pancreatic islet cells. In the present study, we developed a growth factor-free strategy to differentiate hUDSCs toward insulin producing cells by lentiviral overexpression of miR-375.

MATERIALS AND METHODS

Collection and culture of human UDSCs:

Human UDSCs (hUDSCs) were isolated from fresh umbilical cord tissue after birth. Informed consent was obtained from all pregnant mothers, and experiments were performed according to the guidelines of Ethics Committee at Shiraz University of Medical Sciences. The sample was washed by phosphate buffered saline (PBS), and was cut into small pieces (1–2 mm²) and cultured in plates (10 cm) with DMEM/F12 supplemented with 10% fetal calf serum (Gibco, Germany) and antibiotics. After 7–10 days, adherent spindle shape cells were proliferated from explanted tissue. After 70-80% confluence, the cells were harvested using 0.25% Trypsin 0.02% ethylenediaminetetraacetic acid (EDTA) and frozen in liquid nitrogen for later use.

Flow cytometry analysis:

hUDSCs at the third passage were isolated and stained with fluorescence-labeled antibodies against human CD90-FITC, CD45-FITC, CD133-PE, CD44- FITC, CD-34- FITC, CD133-PE and CD105-FITC (BioLegend, San Diego, Calif., USA). Then the labeled cells were analyzed by FACS Calibur flow cytometer (Becton Dickinson, N.J., USA).

UDSC transduction with LV- miR-375:

hUDSCs in third passage (1/5 × 10⁶ cells/mL) were cultured in a 25 cm² plastic culture flask in glucose DMEM supplemented with 10% FBS for 18 H. The cells were incubated with 150 μL concentrated LV-miR-375 for 16H. The hUDSCs miR-375⁺ were exposed to 2.5 μg/mL puromycin for 3days to obtain stable transduction.

Analysis of expressions islet specific markers by real-time RT-PCR:

Total RNA was extracted from hUDSCs and hUDSCsat the third passage using the High Pure RNA isolation kit (CinnaGen Co, Iran) following the manufacturer’s instructions. RNA was then reverse-transcribed into cDNA according to the manufacturer’s instructions by cDNA synthesis kit (Fermentas). Quantitative real-time RT-PCR was performed with Rotor GeneTM 6000 Real-time rotary analyzer (Corbett, Australia) using MaximaTM SYBR Green, qPCR Master Mix (Takara, Japan), forward and reverse primers and the template cDNA. The expression level of each gene at every checkpoint was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The relative gene expression data were analyzed, using the 2ΔΔCt method.

RESULTS AND DISCUSSION

Isolation and characterization of hUDSCs:

hUDSCs were successfully isolated from fresh umbilical cord tissues and expanded in DMEM culture medium at a density of 2 × 10⁴ cells/mL after plastic adherence selection. Hematopoietic cells were removed during subsequent changes of medium and passaging. hUDSCs exhibited their fibroblast-like morphology and made colonies after 7–10 days of culture. The hUDSCs were harvested and ThehUDSCs were harvested and labeled with antibodies against CD45, CD34, CD90, CD105, CD133, and CD44. The hUDSCs were negative for CD45,CD34, CD133 and expressed high levels of CD44, CD105, and CD90 (Fig. 1).

Morphological Changes:

To investigate the effects of introduction of miR-375 into hUDSCs the changes in cell morphology of the control and experimental groups were studied on days 0, 10 and 14. Phenotypic changes in the control group were not observed from day 0 to 14 while, the phenotypic changes in the experimental group could observe from day 3 to 14 (Fig.2). After the third day of differentiation, the induced cells showed a remarkable transition from bipolar fibroblast- like morphology to a round epithelial- like shape (Fig.2B). During further culturing, the rate of cell proliferation become slower and spindle- like cells become short and changed into round epithelial- like cells. Meanwhile, some new islet- like clusters started to appear. After the 5th day of differentiation, cellular aggregation occurred as a gradual process (Fig.2C). After the 10th day of differentiation, complete aggregates were formed (Fig.2D). The cells formed tight clusters that resembled pancreatic islets. Moreover, the control group of hUDSCs did not produce clusters.
Fig. 1: Phenotype analysis of hUDSCs. hUDSCs in third passage were tested against antibodies listed below and analyzed by FACS Caliber. The hUDSCs were negative for CD45, CD34, CD133 and expressed high levels of CD44, CD105.

Fig. 2: Morphologic changes of hUDSCs during differentiation. hUDSCs in day 0 (Fig. A). Morphologic changes in hUDSCs after the 5 days (Fig. C). Appear clusters after 10 days (Fig. D).

The Expression of Pancreatic-Specific Transcription Factors During Differentiation of hUDSCs:

To confirm the production of β-like cell (β-LCs) after transfection, RT-qPCR was performed on the genes involved in pancreas differentiation, such as insulin and PDX1 on the 0, 7 and 21 days after transfection. After formation of the induced definitive β-LCs, the specific marker gene expression patterns in the pancreatic endocrine cells were monitored, and PDX1 expression peaks were observed on the 7 day its expressions remained high until the end of the experiment (Diagram 1A). Meanwhile insulin expression level exclusively up at the end of training (Diagram 1B). The increased expression of these factors in the differentiated cells suggests that miR-375 induce hUDSCs cells toward β-LCs.
Diagram 1: Real-time qPCR analysis of gene expression. PDX1 expression peaks were observed on the 7 day its expressions remained high until the end of the experiment (Diagram 1A). meanwhile insulin expression level exclusively up at the end of training (Diagram 1B).

Discussion:
Diabetes mellitus is one of the most attractive candidate diseases for medical cellular therapeutic intervention via the replacement of defective β-cells. hUDSCs are one of excellent candidate cell types for providing a bank of cells to produce new β-cells. LVs can deliver transgenes to a wide variety of dividing and non-dividing cells and maintain stable long-term transgene expression [20]. Expression profile analysis has demonstrated that several microRNAs exhibit increased expression and target genes controlling cellular growth and proliferation during development of human pancreatic cells [21,22]. MicroRNAs, with their small size, fast synthesis, resistance to nuclease action and long half-life/bioactivity, might be ideal substitutes for growth factors and inhibitors to guide differentiation towards any particular cell type [23]. With the known significance of miR-375 in islet development, we established a new protocol to differentiate hUDSCs into insulin-producing β-like cells by lentivirally-mediated miR-375 overexpression in the lack of any modulators. Phenotypic changes observed in miR-375 overexpressing group led to more compact aggregation, followed by the formation of islet-like clusters at the end of experiment. Similar morphologies were not detected in the normal hUDSCs. Quantitative RT–PCR analysis revealed that the expression pattern of selected β cell-marker genes in LV-miR-375 transduced cells is similar to that detected during pancreatic differentiation in humans [24, 25]. pdx1 was significantly expressed from day 7 until the end of induction. Insulin arisen and reached a peak on day 21, which indicated the appearance of mature β-like cells. No important change was seen in the mRNA level of this marker in the normal hUDSCs. In conclusion, we present a new approach to produce β-like cells in vitro by miR-375 overexpression in the absence of any extrinsic factors. Our findings provide evidence that constitutive miR-375 expression in hUDSCs led to the expression of β cell markers as well as the production of insulin in response to glucose in islet-like clusters.
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