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ORIGINAL ARTICLE

In vitro quantitative and relative gene expression analysis of pancreatic transcription factors Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 in trans-differentiated human hepatic progenitors

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ABSTRACT

Aims/Introduction: Diabetes is a major health concern throughout the world because of its increasing prevalence in epidemic proportions. β-Cell deterioration in the pancreas is a crucial factor for the progression of diabetes mellitus. Therefore, the restoration of β-cell mass and its function is of vital importance for the development of effective therapeutic strategies and most accessible cell sources for the treatment of diabetes mellitus.

Materials and Methods: Human fetuses (12–20 weeks gestation age) were used to isolate human hepatic progenitor cells (hHPCs) from fetal liver using a two-step collagenase digestion method. Epithelial cell adhesion molecule-positive (EpCAM+ve)-enriched hHPCs were cultured *in vitro* and induced with 5–30 mmol/L concentration of glucose for 0–32 h. Pdx-1 expression and insulin secretion was analyzed using immunophenotypic and chemifluorescence assays, respectively. Relative gene expression was quantified in induced hHPCs, and compared with uninduced and pancreatic cells to identify the activated transcription factors (Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1) involved in β-cell production.

Results: EpCAM+ve cells derived from human fetal liver showed high *in vitro* trans-differentiation potential towards the β-cell phenotype with 23 mmol/L glucose induction after 24 h. The transcription factors showed eminent expression in induced cells. The expression level of transcription factors was found significantly high in 23 mmol/L-induced hHPCs as compared with the uninduced cells.

Conclusions: The present study has shown an exciting new insight into β-cell development from hHPCs trans-differentiation. Relative quantification of gene expression in trans-differentiated cells offers vast possibility for the production of a maximum number of functionally active pancreatic β-cells for a future cure of diabetes.

INTRODUCTION

In the past few years, islet transplantation has been used for the treatment of diabetes mellitus, but the limited yield of quality donor pancreata makes this strategy inadequate[2]. Therefore, patients affected by diabetes mellitus are in great need of the new source of cells to enable a better transition into clinical programs of cell therapy and regenerative medicine. β-Cells reside within the pancreatic islets, and are the only cell type in

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mammalians that synthesize and secrete the endocrine hormone, insulin. Insulin has a significant role in the regulation of carbohydrate, fat and protein metabolisms. Loss or dysfunction of β-cells results in an insufficient insulin production that leads to a high glucose level in the blood, ensuing a metabolic disorder called diabetes mellitus. This metabolic disorder is increasing in prevalence rate day-by-day throughout the world. It has been estimated that between 2010 and 2030, there will be a 69% increase in the number of patients with diabetes in developing countries, and a 20% increase in developed countries[1,21].

The pancreas is a dual-function organ composed of various types of endocrine and exocrine cells. Among different types of pancreatic cells, the insulin-producing β-cell is of the most interest because of its close physiological connection to diabetes. Although both type I and type II diabetes can be effectively treated by insulin administration, the best way to cure diabetes is to restore the β-cell population. Unlike hepatocytes that have stem cell-like regenerative capability, the adult β-cells have very limited regenerative ability, which is insufficient to compensate the cell loss in diabetes. Pancreatic exocrine cells and endocrine β-cells have closely related lineages in development, and are derived from common progenitors. Conversion between them might require fewer epigenetic changes, thus representing an appealing source of β-cell regeneration. In this setting, the liver is becoming the most promising and available source of such cells because of its common origin with the ventral pancreas during embryonic development[3]. The knowledge of adult stem cells has added a new dimension to the study of the early events of liver development. Hepatic progenitors can be defined as the bipotential cells residing in human and animal livers, which have the ability to differentiate into a lineage of hepatocytes and cholangiocytes. Hepatic progenitors serve as the major component of the hepatic parenchyma in the early stages of liver organogenesis[4]. Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein mediating Ca^{2+} independent homotypic cell–cell adhesion in epithelia, and is involved in cell signaling, migration, proliferation, and differentiation[5,6]. EpCAM has also been identified as a surface marker on human hepatic stem/progenitor cells that is absent on mature hepatocytes[7–9], and can be isolated using EpCAM as a phenotypic marker.

Trans-differentiation is defined as the conversion of one differentiated cell type to another. The process of trans-differentiation is essential to study, because it helps to intensify our understanding of the developmental biology of tissues that interconvert. Tissues that undergo trans-differentiation generally arise from adjacent regions in the developing embryo[10]. In normal development, the expression of one or more transcription factors encoded by the 'master-switch' gene is responsible for distinguishing the adjacent tissue type. Although different sets of transcription factors and specialized gene cascades are expressed in the liver and pancreas that generate specialized cell type after induction. Pdx-1 has been a major switch in order to control the activation of other transcription factors of different cell lineages[4,12]. Therefore, identifying gene expression profiles and their expression levels is the fundamental element for the biological inquiry at the cellular level to further investigate its potential and application in regenerative medicine. Techniques such as northern blot have been in existence for decades to carry out this task, but advances in molecular biology and bioinstrumentation have led to the development of a variety of new techniques with a wide range of sensitivities, throughputs and quantitative capabilities. The present study focused on the latter issue. For several commonly used gene expression techniques, the extent and range of quantitative applicability were used, and approaches for maximizing the accuracy and precision of these measurements were done.

The identification of a particular cell type involves an important aspect of the configuration of active and regulatory genes for the specific differentiation product in a stable manner. There are several transcription factors involved in both neogenesis and the replication of pancreatic β-cells (Table 1). However, it is not clear whether they work alone or in combination with other transcription factors. Therefore, it becomes crucial to have a better understanding of the various molecular pathways involved in pancreatic islet cell rejuvenation.

MATERIALS AND METHODS

Hepatocytes Isolation

After receiving the informed consent from the parents, 12–20 weeks' gestation aged human fetuses were obtained from the local maternity hospitals as a result of spontaneous abortion. Liver cells were isolated by a two-step collagenase digestion method described by Habibullah *et al.*[3] Briefly, liver tissue was dissociated by two-step collagenase (0.3%) digestion at 37°C for 1 h, and filtered through a 40-μm nylon mesh to obtain single cell suspension. The study protocol was approved by the Institutional Ethics Committee, Deccan College of Medical Sciences, Hyderabad, India.

Table 1 | Major transcription factors involved in pancreatic neogenesis

Gene	Function	Reference
Pdx-1	Initiate endocrine neogenesis	15
Ngn-3	Initiate endocrine differentiation and activates NeuroD1	1
Isl-1	Essential for promoting pancreatic islets proliferation and maintaining endocrine cells survival in embryonic and postnatal pancreatic islets	2
Pax-4	Expressed in endocrine progenitor cells, and directs formation of β- and δ-cells	16
Pax-6	Pax6 acts upstream of MafB, which in turn might trigger the expression of insulin and regulate the PDX-1 and MafA expression required for β-cell maturation	17
Nkx-6.1	Maintenance and expanding population of β-cell precursors as these progress from precursors to differentiated β-cells	18

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Cell Sorting

Enrichment of hepatic stem/progenitor cells was carried out by magnetic activated cell sorting (MACS) using human direct anti-EpCAM magnetic beads according to the manufacturer's instructions (MiltenyiBiotec, Bergisch Gladbach, Germany). Briefly, single cell suspension of enzymatically dissociated cells was first incubated with 60 μL EpCAM antibody for 30 min at 4°C. Cells were washed twice with 1X phosphate buffer saline (PBS; Invitrogen, Carlsbad, CA, USA) and then treated with magnetic microbeads (MiltenyiBiotec) for 15 min at 4°C. The magnetically labeled cells were applied onto a MS column (MiltenyiBiotec) followed by washing. After the suspension had gone through the column, the cells were washed three times using DMEM-F12 (Invitrogen), and the total effluent was collected as an unlabeled negative fraction (EpCAM-ve). The MS column was removed from the magnet and medium was applied onto the column. The retained magnetically labeled cells were gently flushed out by a plunger and collected as EpCAM+ve cells fraction.

Cell Viability Assays

Cell viability of EpCAM+ve cells was assessed by 0.2% Trypan-blue exclusion and fluorescien di-acetate (FDA) assays. Cell viability counting and enumeration was carried out using a hemocytometer.

In Vitro Proliferation of EpCAM+ve Cells

Enriched EpCAM+ve hHPCs cells (5×10^4) were cultured on collagen coated six-well plates in serum-free medium containing 20 ng/mL epidermal growth factor (EGF; PrepoTech, Londo, UK), 10 ng/mL basic fibroblast growth factor (b-FGF; PrepoTech), 20 ng/mL hepatocyte growth factor (HGF; Sigma, St. Louis, MO, USA), and 0.61 g/L nicotinamide (Sigma) with antibiotics and antimycotics. The 50% medium was replaced every after third day and cultured for 7 days.

Maturation of hHPCs

After proliferation for 7 days in serum-free medium, hepatic progenitors were induced to differentiate into mature hepatocyte lineage cells using DMEM-F12 (Sigma) supplemented with 20 ng/mL oncostatin M (Sigma), 1 μmol/L dexamethasone (Sigma) and 50 ng/mL insulin–transferrin–selenium premix (Sigma) for 15 days. The medium was changed twice a week, and differentiation was assessed by reverse transcriptase polymerase chain reaction (RT–PCR) for pancreatic β-cell transcription factors. All the experiments were repeated at least three times to eliminate any technical error.

Trans-Differentiation of Cultured EpCAM+ve hHPCs

Cultured EpCAM+ve hHPCs were harvested after 7 days of initial proliferation in serum-free medium and subcultured in six-well plates in conditioned medium containing antibiotics and antimycotics. The cells were induced with 5–30 mmol/L glucose concentration and maintained for 30 h at 37°C in a humidified atmosphere of 5%CO_2. After 2 h of post-induction, culture supernatants were collected every 4 h interval for 30 h, and the total insulin content secreted by the cells was estimated by chemiluminescence assay according to the manufacturer's instructions (Auto Bio Labtech, Zhengzhou, China). The insulin production was highest (1 mU/L) in 23 mmol/L-induced glucose concentration and was lowest in 5 mmol/L glucose concentration after 24 h of incubation. These two cell sources were considered to correlate with the changes in relative gene expression profile of pancreatic transcription factors Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 involved in β-cell production.

Immunocytochemical Staining

Cultured hHPCs and trans-differentiated cells were harvested by trypsinization and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Cells were incubated at 4°C for 2 h with mouse anti-human Pdx-1-PE (1:100 in PBS; R&D System, Delhi, India) and anti-human insulin-FITC (1:100 in PBS; R&D System, India) separately. After incubation cells were washed twice with cold 1X PBS and analyzed by inverted fluorescent microscopy (Axiovert; CarlZeiss, Gottingen, Germany). 4′,6-Diamidino-2-phenylindole, dihydrochloride (Sigma) was used as a counter dye to stain the cell nuclei.

Flow Cytometry

The 5 mmol/L and 23 mmol/L-induced hHPCs were permeabilized with 0.1% Triton X-100 (v/v) at room temperature for 15 min and stained with mouse anti-human Pdx-1 monoclonal antibody (1:100; R&D Systems, Minneapolis, MN, USA) at 4°C in the dark. PE-conjugated mouse immunoglobulin IgG1 was used as an isotype control. The expression was analyzed on FACS Caliber flow-cytometry using CellQuest software (BD Biosciences, San Jose, CA, USA).

Ribonucleic Acid Isolation and RT–PCR

Total ribonucleic acid (RNA) was extracted from uninduced, 5 mmol/L and 23 mmol/L-induced and pancreatic cells using the Trizol (Invitrogen) method. Complementary deoxyribonucleic acid (cDNA) was prepared using Oligo dT (Invitrogen) and reverse transcriptase II (Fermentas, Burlington, ON, Canada). Then, 5 ng of cDNA was used for reverse transcription quantitative polymerase chain reaction (RT–qPCR) analysis using SYBR chemistry in StepOne Real-Time PCR (Applied Biosystems, Carlsbad, CA, USA). Pancreatic transcription factors were amplified for 40 cycles using primers specific for Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 gene transcripts. Primer pairs used in the study are listed in Table 2.

Relative Quantification

Fold changes in gene expression for pancreatic transcription factors in induced hHPCs were evaluated using relative quantification in reference to pancreatic cells. Glyceraldehyde

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Table 2 | Primer details used for the relative quantification of pancreatic transcription factors

Target gene	Primer sequences (5′ to >3′)	Product size	GenBank #
Pdx-1	FP- CCCATGGATGAAGTCTACC RP- GTCCTCCTCCTTTTTCCAC	262 bp	NM_000209
Ngn-3	FP- AAGAGCGAGTTGGCACTGAGC RP- CGTACAAGCTGTGGTCCGC	229 bp	NM_020999
Isl-1	FP- AGAGAGTCAGGTCAAGGTCTGGTT RP- ACTTGTGCTTCTTCAACAGCTGCG	233 bp	NM_006168
Pax-4	FP- ATACCCGGCAGCAGATTGTG RP- AAGACACCTGTGCGGTAGTAA	127 bp	NM_006193
Pax-6	FP- TCAGCACCAGTGTCTACCAACCAA RP- ATCATAACTCCGCCCATTCACCGA	239 bp	NM_000280
Nkx6.1	FP- AGAGAGTCAGGTCAAGGTCTGGTT RP- ACTTGTGCTTCTTCAACAGCTGCG	215 bp	NM_006168
GAPDH	FP- TGTGGGCATCAATGGATTTGG RP-ACACCATGTATTCCGGGTCAAT	116 bp	NM_014364

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3-phosphate dehydrogenase (GAPDH) was used as endogenous control for normalization of the test samples. Before quantifying the test samples, cDNA from each sample was validated by GAPDH qPCR using 100, 10, 5, 2 and 0.1 ng as templates. Each transcript was amplified in triplicates. PCR efficiency was calculated using Y = mx + c. The mean was taken for each sample and the fold difference was calculated using the $2^{-\Delta\Delta CT}$ method by StepOne (version 2.2) software in StepOne Real-Time PCR (Applied Biosystems).

Statistical Analysis

All data were expressed as mean ± standard error of the mean. An independent-sample *t*-test (SPSS 10.0; SPSS Inc., Chicago, IL, USA) was used to compare the purity and viability of EpCAM+ve cells. Microsoft Excel was used to generate the graphical representation of EpCAM+ve cells quantitative yield and cell viability. $P < 0.05\%$ was considered to be significant. RT–qPCR efficiency was calculated with the help of StepOne (version 2.2) software. A regression value (R^2) ≥0.99 was considered to be the significant with 100% PCR efficiency.

RESULTS

Isolation of Primary Hepatocytes From Human Fetal Liver

Isolation of fetal hepatocytes by a two-step collagenase digestion method resulted in the utmost cell viability (95.2 ± 6.5%). The cells were morphologically normal, as assessed by confocal microscopy, with maintenance of gap junctions between cells and limited vacuolation. After MACS, EpCAM+ve cells were found to be homogeneous in nature with highest viability (>95%), whereas EpCAM-ve cells were heterogeneous in nature with less viability (90–92%). The average percentage of EpCAM+ve cells after MACS was calculated to be ~60–80%.

In Vitro Proliferation and Characterization of EpCAM+ve Cells

Magnetically sorted (EpCAM+ve) cells were maintained in culture for 7 days and did not show any morphological changes in proliferation medium. After induction with different concentrations of glucose, slight morphological changes were observed after 10 h. After 24 h of induction, cells completely changed in their morphology and started to secrete insulin that was further observed by immunocytochemical (ICC) staining.

Immunofluorescence Staining

Intracellular staining of uninduced and induced hHPCs cells was carried out with anti-human Pdx-1, which resulted in high expression in 23 mmol/L-induced cells. The morphology of Pdx-1 stained cells was different from that of normal hepatocytes. More than 80% of cells showed a strong and steady expression pattern in 23 mmol/L-induced cells (Figure 1).

Flow Cytometry Analysis

Flow cytometry data showed high expression for Pdx-1 in 23 mmol/L-induced cells after 24 h. More than 80% of 23 mmol/L-induced cells showed positive expression for Pdx-1, whereas a much smaller percentage of 5 mmol/L-induced cells were found to be positive, and no significant expression was observed in uninduced EpCAM+ve cells (Figure 2).

Glucose-Induced Insulin Secretion by Cultured hHPCs

Insulin estimation was carried out in 5–23 mmol/L-induced cells at 0–32 h. Insulin content measured the highest in 23 mmol/L-induced cells at 24 h of induction by chemifluorescence assay (Figure 3a,b). Further identification of insulin-producing cells was carried out by staining with anti-human insulin-FITC antibody. A high expression level was observed in trans-differentiated cells after 24 h of glucose induction. The expression pattern of insulin production was found to be similar to the pancreatic β-cells (Figure 4).

Relative Gene Expression in Induced Hepatic Progenitors

For gene expression analysis, samples were categorized in four groups: uninduced hHPCs (negative control), 5 mmol/L-induced cells, 23 mmol/L-induced cells and pancreatic cells (positive/reference control). Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 transcription factors primarily involved in insulin secretion were examined *in vitro* by RT–qPCR. None of these transcription factors were expressed in uninduced hHPCs, except a minimal expression of Pdx-1. Although, their expression was slightly enhanced in 5 mmol/L-induced cells, and 23 mmol/L-induced cells have shown significantly increased expression (similar to the pancreatic cells).

The results obtained from the validation of cDNA for test and control samples showed constant expression levels for all target gene transcripts. The 5 ng cDNA resulted in better PCR efficiency and even curve for all the transcripts. Relative

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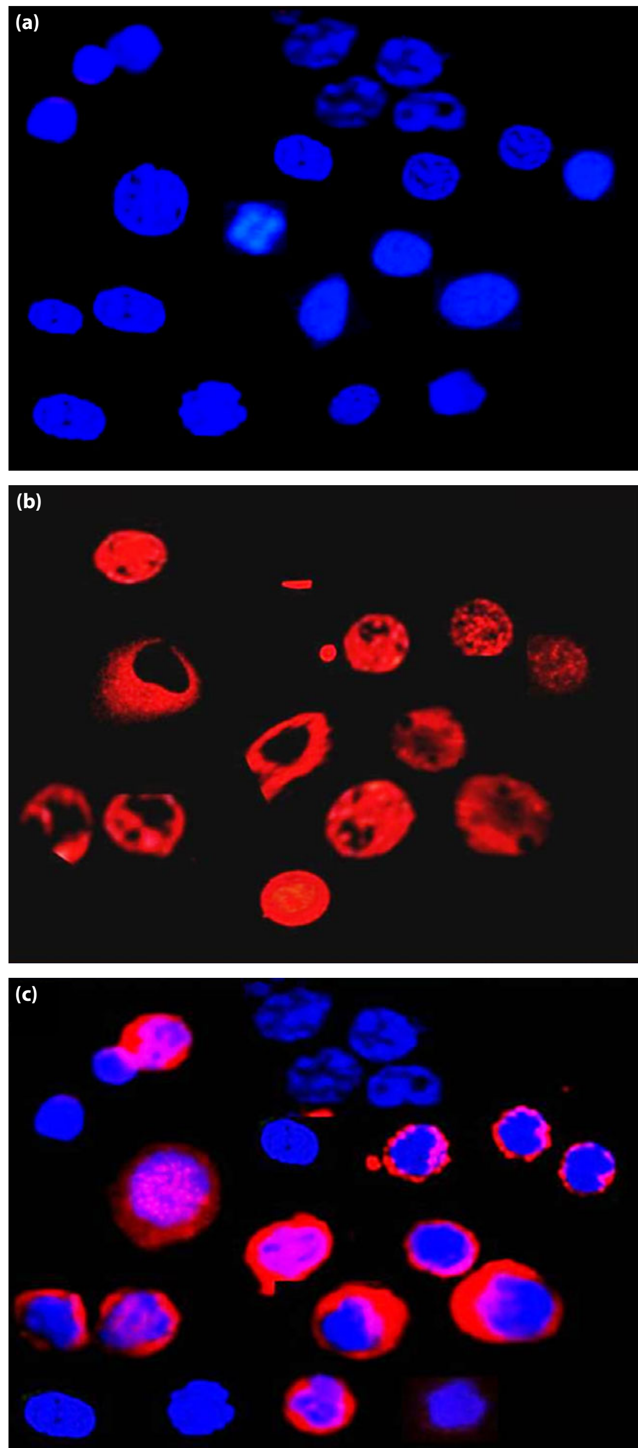

Figure 1 | Immunocytochemical (ICC) staining with Pdx-1-PE antibody for 23 mmol/L-induced human hepatic progenitor cells after 24 h glucose induction (magnification, ×100). (a) High nuclear content was stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; blue). (b) Intracellular staining was observed for Pdx-1 (red). (c) Merged ICC image showing the nuclear staining for DAPI (blue) and cytoplasmic content for Pdx-1 (red).

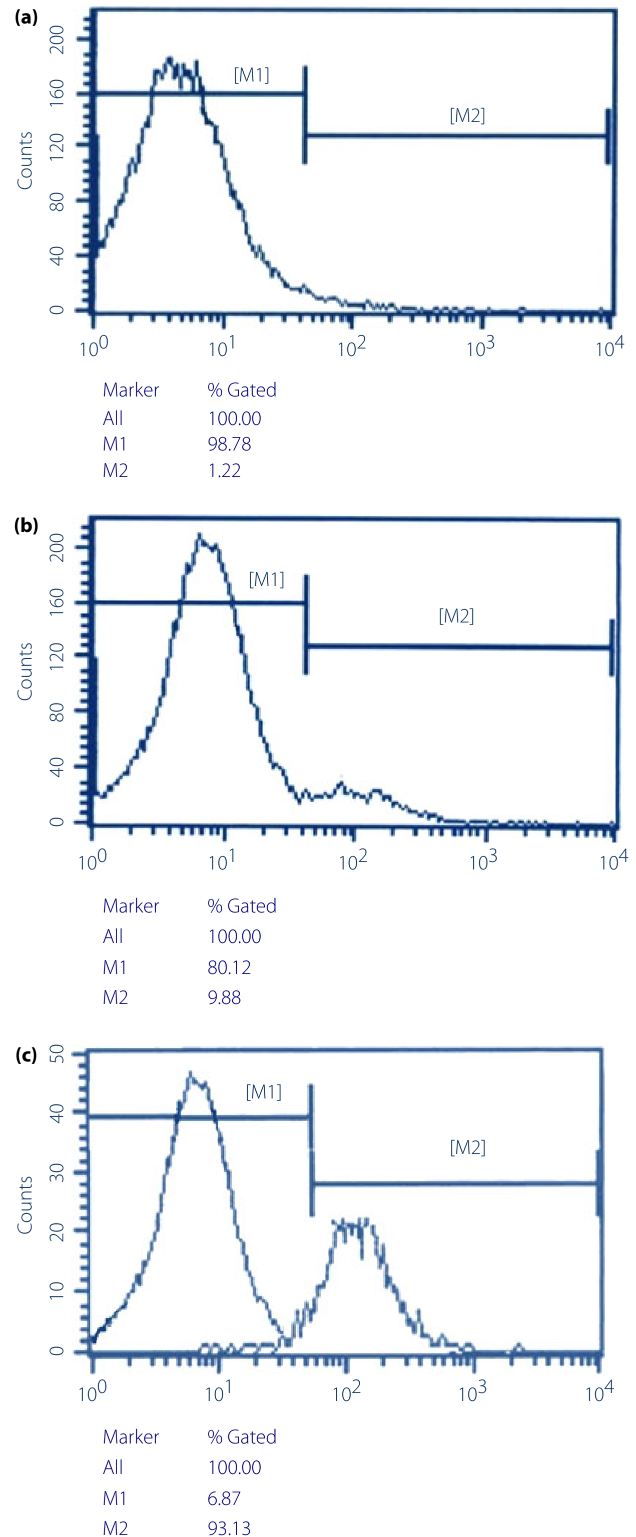

Figure 2 | (a) Flow cytometry analysis of Pdx-1 expression in cultured uninduced hHPCs. (b) Only 9.88% cells showed positive expression for Pdx-1 after 5mmol/L glucose induction. (c) More than 93% cells showed positive expression for Pdx-1 after 23 mmol/L glucose induction.

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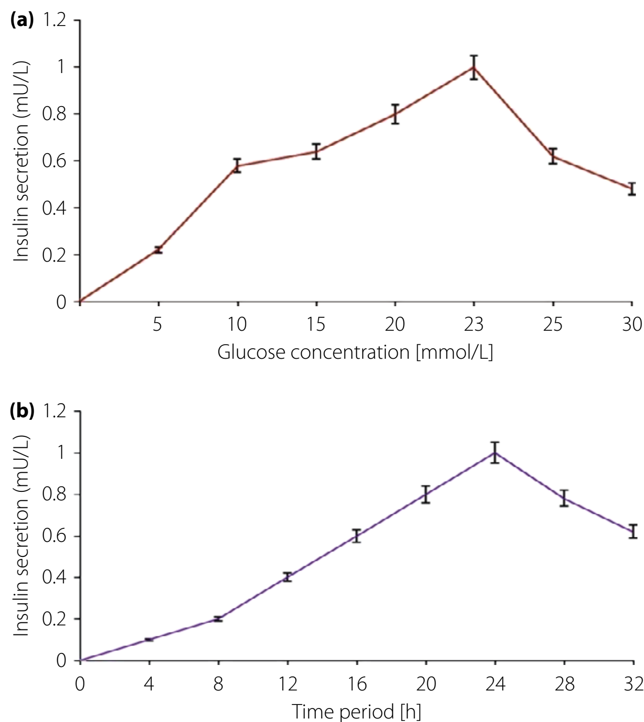

Figure 3 | Insulin estimation in cultured epithelial cell adhesion molecule-positive human hepatic progenitor cells. (a) Maximum insulin secretion was observed at 23 mmol/L glucose concentration after 24 h of the induction period. (b) Insulin production was found to be a maximum after 24 h of induction, which later gradually decreased (error bars are represented as 5% value).

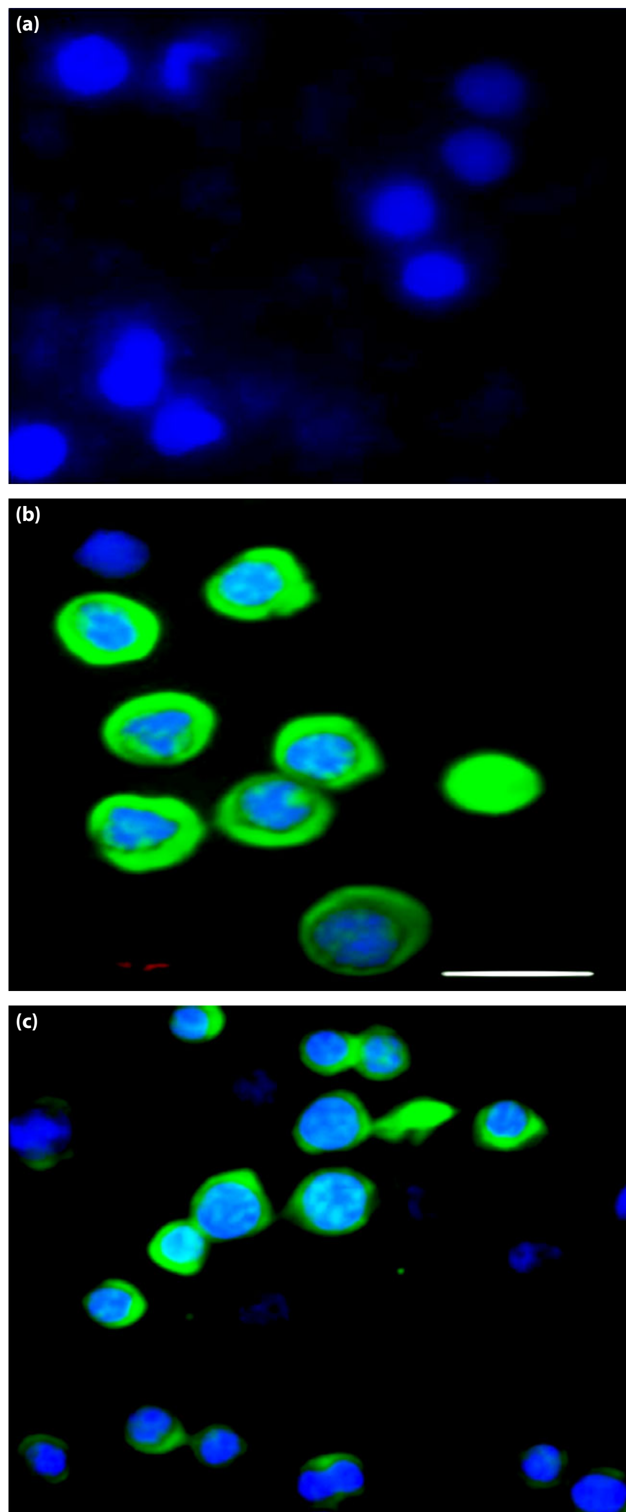

Figure 4 | Immunocytochemical staining of cells using anti-human insulin-FITC. (a) Uninduced epithelial cell adhesion molecule-positive sorted human hepatic progenitor cells did not show expression for insulin. (b) High expression was observed in 23 mmol/L-induced cells after 24 h. (c) Pancreatic cells were used as a positive control to examine the expression pattern for insulin secretion from β-cells.

quantification based on the $2^{-\Delta\Delta Ct}$ method showed that the human EpCAM+ve HPCs, when induced with 23 mmol/L glucose after 24 h, significantly increased the gene expression levels for all the pancreatic transcription factors in comparison with uninduced and 5 mmol/L-induced cells. While comparing the fold difference between the 23 mmol/L-induced hHPCs after 24 h and pancreatic cells, very less fold difference was observed between them (two-tailed $P = 0.0206$; Figure 5a,b).

DISCUSSION

Patients affected by diabetes mellitus are in great need of better therapeutic strategies and new source of cells to enable a better transition into clinical programs of cell therapy and regenerative medicine. In this setting, liver is becoming the most promising source of cells as it has the same embryonic origin as the pancreas. Stem cell biology is making waves around the globe with great anticipation for eventual diabetes therapy. Current work on hHPCs has offered new hope for the development of cell replacement therapy for diabetes. It has been proved that hHPCs constitute an average of 2% of the parenchyma of fetal liver, and can be isolated and enriched by EpCAM as potential phenotypic markers[11].

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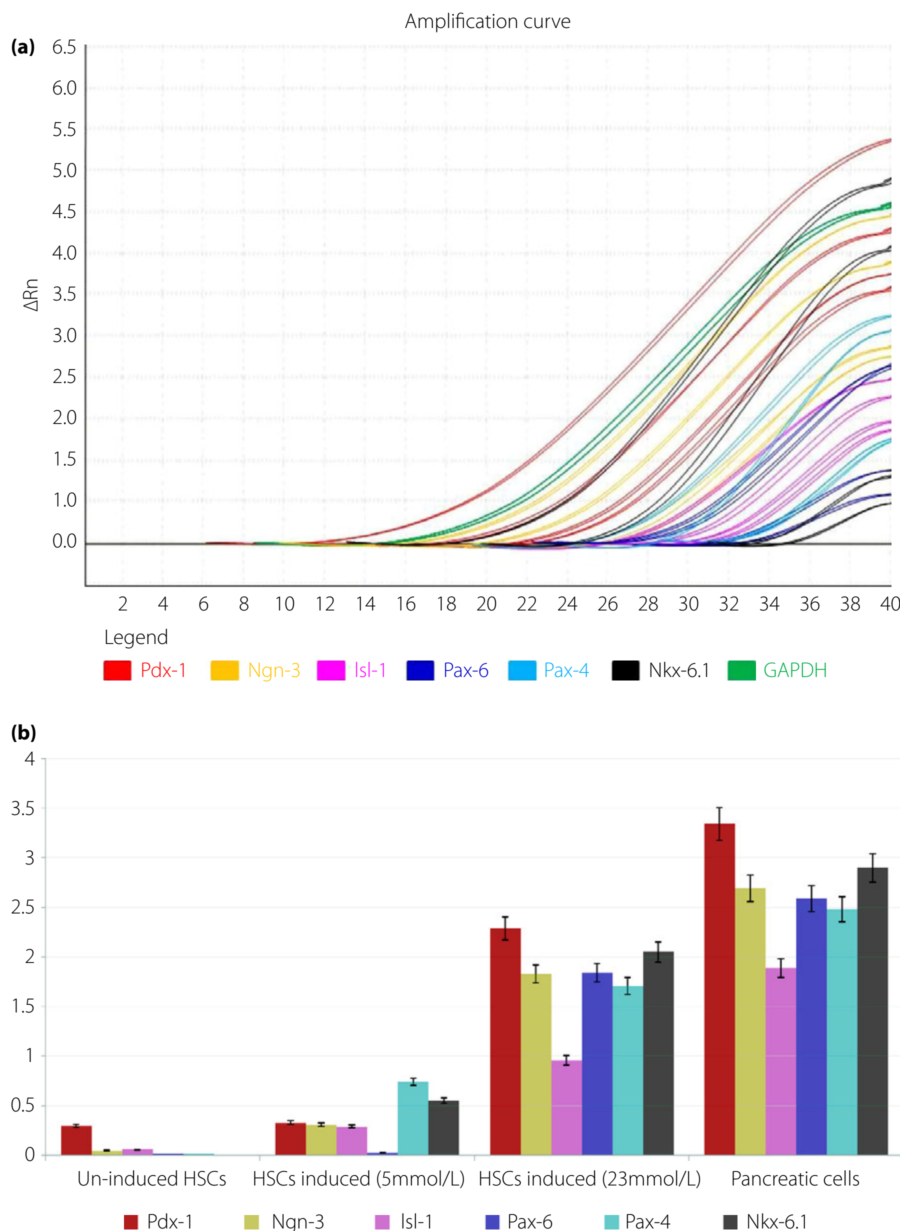

Figure 5 | (a) Real-time amplification plot for major transcription factors showing uniform amplification curve during SYBR Green assay. Different colors of curves represents respective gene transcript. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. (b) Relative gene expression profile in terms of fold difference of major pancreatic transcription factors involved in β-cell production. *P*-value <0.05 was considered to be significant. HSCs, hepatic stem cells.

As the liver and pancreas arise from the same bipotential precursors in the anterior endoderm, it is reasonable to speculate that two closely related tissues might be interconvertible. Several animal studies have shown that Pdx-1 expression could lead to the conversion of hepatocytes into pancreatic β-like cells that secret insulin[12,13]. Likewise, it has also been found that the combination of Pdx-1 and Ngn-3 could synergistically induce expression of β-cell factors and insulin biosynthesis in the liver, and drastically ameliorate glucose tolerance[14]. Taken together, these studies showed that liver and pancreatic cells might possess the plasticity of converting into each other under certain circumstances. Though many of these studies might not make an instant breakthrough in the clinic, they contribute enormously to our understanding of cell lineage determination and therefore could eventually lead to the success of cell therapies. In this scenario, the present study strongly suggests that culture

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of EpCAM+ve-enriched hHPCs possesses the capacity to trans-differentiate into functional endocrine pancreatic β-cells after 24 h of 23 mmol/L glucose induction. RT–qPCR analysis of major pancreatic transcription factors in induced cells has shown the increased expression of their messenger RNA transcripts. Interestingly, a significant increase in expression level of Nkx-6.1 almost equal to Pdx-1 shows a more committed β-cell phenotype for insulin production. An endocrine pancreatic β-cell phenotype was further supported by ICC staining of Pdx-1 and chemifluorescence assay, which showed the presence of insulin-producing cells and insulin content, respectively, in trans-differentiated cells.

Characterization of Pdx-1 in induced hHPCs is of utmost importance, as it activates expression of other islet-enriched genes. Several studies have shown Pdx-1 as a 'master regulator' of directing cell fate towards the pancreatic endocrine cells[19,20]. In the present study, after 23 mmol/L glucose induction, >80% of cells were found to express Pdx-1, which later activates other transcription factors and shows the potential to generate pancreatic β-cells. Relative quantification of Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 genes showed a significantly high expression level in trans-differentiated cells and defined the parameters to achieve the desired β-cell population.

Furthermore, with the increasing rate of diabetes patients throughout the world, it is necessary to have a new approach for the development of suitable therapies[21] and diagnosis. Confirmation of chronic hyperglycemia is essential for the diagnosis of diabetes mellitus. In 1999, the Japan Diabetes Society (JDS) introduced a new parameter (glycated hemoglobin [HbA1c]) for better diagnosis of the diabetic condition[22]. Later, the value of HbA1c was defined in different diabetic conditions, and it was shown that ≥6.5% HbA1c can be considered to indicate the diabetic type. The value of HbA1c is equivalent to the internationally used HbA1c (%) (HbA1c [NGSP]) defined by the National Glycohemoglobin Standardization Program (NGSP), and is expressed by adding 0.4% to the HbA1c (JDS) (%) defined by the JDS[23].

In this scenario, the present study with the molecular and cellular biology aspect provides the continual promise of exciting new insights into β-cell development and survival. In summary, the present study has shown a potential approach in cell therapy-based treatment of insulin-dependent diabetes by generating insulin-producing cells from induced hHPCs. While foreseeing immense potentials of these discoveries, certain issues remain to be addressed within the coming years, such as the efficiency of the cell trans-differentiation, the maturation of the hepatocytes or β-cells, and the elimination of any possible tumor-initiating cells resulting from incomplete epigenetic conversion. Once we achieve a comprehensive understanding of the mystery behind hHPCs trans-differentiation, we might be able to use the knowledge of refined and controlled production of β-cells from alternative sources to move further from bench to bedside for the treatment of diabetes mellitus.

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