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Evaluating KIND1 human embryonic stem cell-derived pancreatic progenitors to ameliorate streptozotocin-induced diabetes in mice

Varsha Pursani[1], Sona Kapoor[1], S.M. Metkari[2], Prabha Nair[3] & Deepa Bhartiya[1]

Departments of [1]Stem Cell Biology & [2]Experimental Animal Facility, ICMR-National Institute for Research in Reproductive Health, Mumbai & [3]Division of Tissue Engineering and Regeneration Technologies, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Thiruvananthapuram, India

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***Background & objectives*: Diabetes is a global disease burden. Various stem cell types are being explored to serve as an alternative source of islets. This study was conducted to evaluate the ability of in-house developed human embryonic stem (hES) cells-derived pancreatic progenitors to ameliorate diabetic symptoms in mice.**

***Methods*: Pancreatic progenitors were packed in macro-capsules and transplanted into six male Swiss mice and four mice were taken as controls. Thirty days post-transplantation, diabetes was induced by streptozotocin treatment. Mice were then followed up for >100 days and body weight and blood glucose levels were regularly monitored.**

***Results*: Control mice lost weight, maintained high glucose levels and did not survive beyond 40 days, whereas transplanted group maintained body weight and four of the six mice had lowered blood glucose levels. About five-fold increase was observed in human C-peptide levels in the recipients of progenitor transplants as compared to diabetic control.**

***Interpretation & conclusions*: The beneficial effect of transplanted cells was not long-lasting. Further studies are required to critically evaluate and compare the potential of endogenous pluripotent stem cells and hES cells-derived progenitors before moving from bench to the bedside.**

Key words Diabetes - differentiation - hESC - pancreas - progenitors - stem cells - transplantation

Currently, 415 million people suffer from diabetes worldwide compared to 151 million in 2000, with an expected rise to 642 million by 2040. Every sixth second, a person dies from diabetes[1]. The first report of reversal of diabetes as a part of Edmonton protocol using cadaveric islets in 2000 raised hopes for the treatment of this debilitating disease. However, the scarcity of pancreas and cadaveric islet donors has made stem cells a promising alternative cell source to differentiate into islets for treating diabetes[2].

Because of their ability to self-renew indefinitely, human embryonic stem cells (hESC) could be an ideal source of islets *in vitro* and may allow treatment of a large number of patients. D'Armor and colleagues[3] first reported the differentiation of hES cells into pancreatic

progenitors, later placed the differentiated cells overlaid on a scaffold followed by transplantation in SCID mice and detected human insulin and C-peptide release[4], developed scalable system for producing functional progenitors and documented the efficiency of their product PEC-01[5]. Similarly, in another study 30 per cent of transplanted mice showed reduction in hyperglycaemia on transplanting insulin positive cells obtained by differentiating ES cells, for over a period of six months[6]. Bruin *et al*[7] improved the differentiation protocol further which resulted in grafts containing >80 per cent endocrine cells and resulted in single hormonal cells expressing either insulin or glucagon or somatostatin in contrast to earlier polyhormonal cells. Kirk *et al*[8] have demonstrated that human insulin is secreted by seven weeks after transplantation of encapsulated pancreatic progenitors, and by 20 wk, enough human insulin is produced to ameliorate alloxan-induced diabetic symptoms. The differentiated endocrine cells were monohormonal and insulin was produced in response to a glucose challenge.

The current research efforts are focussed on optimizing the encapsulation procedures of transplanted hES-derived progenitors. Vegas *et al*[9] achieved normoglycaemia upto 174 days on using triazole-thiomorpholine dioxide (TMTD)-coated alginate microspheres where TMTD resists fibrosis on the surface. Modifications of existing protocols by including scale-up, enrichment and maturation of islet-like cells before transplantation, have also been reported[5].

Cell lines developed by our group (KIND1 and KIND2) were derived on human feeders[10] and were evaluated for their propensity to develop into various germ layers[11]. These were later adapted to feeder-free conditions and differentiated into pancreatic progenitors[12] using published protocols. The present study was undertaken to evaluate the feasibility, efficacy and safety of pancreatic progenitors developed from KIND1 hES cells which had earlier shown a propensity to form endoderm. The cells were encapsulated in biocompatible macro-capsules for intra-peritoneal transplantation in mice as reported earlier[13]. Mice were later treated with streptozotocin (STZ) and followed up long-term to evaluate functional maturation of islets and their ability to lower blood glucose levels.

Material & Methods

The study was undertaken in Stem Cell Biology department of the ICMR-National Institute for Research in Reproductive Health, Mumbai, India. All chemicals for cell culture were obtained from Life Technologies (Carlsbad, CA, USA) unless otherwise indicated. The study was approved by the Institute Committee for Stem Cells Research and Animal Ethics Committee.

Human embryonic stem (hES) cells culture: hES cells (KIND1) were adapted to feeder-free conditions[10,12]. Cells were cultured and expanded in 60 mm Petri dish coated with Geltrex using StemPro hESC serum-free medium supplemented with 8 ng/ml of basic fibroblast growth factor (FGF) (R&D Systems, MN, USA) at 37°C in a humidified atmosphere with 5 per cent CO_2. Passaging was done mechanically using cell lifter (Sigma-Aldrich, MO, USA) in 1:3 ratio.

In vitro differentiation of hESC: hES cells were differentiated into endoderm lineage as described earlier[12]. Briefly, feeder-free hES cells showing 80 per cent confluency were cultured in RPMI 1640 medium containing 100 ng/ml activin A (R&D Systems), 1mM sodium butyrate (Sigma-Aldrich), and 25 ng/ml Wnt-3a (R&D Systems). After 24 h, 0.2 per cent foetal bovine serum (FBS) was added to RPMI media along with 100 ng/ml activin A, 0.5 mM sodium butyrate. On days 3 and 4, the RPMI medium was supplemented with 2 per cent FBS and 100 ng/ml activin A. From day 4 onwards, the basal medium used was DMEM-F12 supplemented with 1×B27, 2 µM retinoic acid (Sigma-Aldrich), 50 ng/ml noggin (R&D Systems), 0.25 µM cyclopamine (Sigma-Aldrich) for four days. During the last stage of differentiation protocol, the cells were cultured in DMEM along with 1×B27 supplement, 2 µM retinoic acid, 50 ng/ml noggin, 1mM nicotinamide (Sigma-Aldrich), 1× non-essential amino acids and 25 µg/ml FGF-10 (R&D Systems).

Characterization of hESC and differentiated pancreatic progenitors by specific markers: The KIND1 hES cells grown in feeder-free conditions maintained pluripotent characteristics (expressing *OCT4, NANOG, SOX2, REX1, TERT, SSEA4*) and differentiated into pancreatic progenitors which were further characterized at both protein and mRNA levels by immunofluorescence (*SOX9, SOX17, PDX1*) and quantitative reverse transcription polymerase chain reaction (qRT-PCR, *SOX9, SOX17, NKX6.1, PDX1*) studies using specific markers. qRT-PCR studies were also undertaken to detect the presence of mesoderm (*MESP1* and *NKX2.5*) and ectoderm (*SOX1* and *MAP2*)-specific cell types among the pancreatic progenitors obtained by directed differentiation.

Immunofluorescence: The KIND1 cells were fixed with freshly prepared 4 per cent paraformaldehyde and washed thrice with phosphate buffered saline (PBS) and 0.02 per cent Tween 20 (Sigma-Aldrich). For intracellular antigens, permeabilization was done with 0.3 per cent Triton X-100 (Sigma-Aldrich). Blocking of non-specific sites was carried out with blocking buffer composed of 5 per cent bovine serum albumin (Sigma-Aldrich) and one per cent normal goat serum (Bangalore Genei, Bangalore) in PBS for 60 min at room temperature. The cells were incubated overnight at 4°C with primary antibodies against rabbit anti- SOX9 (1:200, Millipore), rabbit anti-SOX17 (1:200, Millipore) and rabbit anti-PDX1 (1:100, Epitomics CA, USA). After three washes, the cells were incubated with goat Alexa Fluor 488 (Molecular Probes, Invitrogen, USA) secondary antibody diluted (1:1000) in blocking buffer for two hours at room temperature. The cells were washed, counterstained with 300nM DAPI (Molecular Probes, Invitrogen) and photographed under confocal microscope (Carl Zeiss, Germany).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR): Total RNA was extracted using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. Spectrophotometric quantification of the extracted RNA was done using Ultrospec 3100 Pro (GE Healthcare, PA, USA). The cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) in a 20µl reaction volume according to the manufacturer's instructions using G-STORM thermal cycler (Gene Technologies, Braintree, UK). qRT-PCR was performed using CFX96 Real-Time Machine (Bio-Rad, CA, USA) and iQ SYBR Green SuperMix (Bio-Rad). The threshold values (Ct) were obtained from CFX 96 manager software (Bio-Rad) and normalized using housekeeping gene GAPDH. The primer sequences[12,14,15] are given in Table I. The amplification conditions comprised initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 62°C for 20 sec and elongation at 72°C for 30 sec. The fold change in expression was calculated by $2^{-\Delta\Delta Ct}$ method. Each reaction was carried out in duplicate. The expression of gene transcripts specific for definitive endoderm (day 4), pancreatic gut tube (day 8), and pancreatic progenitor (days 12-16) is expressed relative to undifferentiated hES cells.

Table I. List of primers used for reverse transcription polymerase chain reaction

Gene	Primer sequence 5'-3'	PCR product size (bp)
GAPDH	F-GTCAGTGGTGGACCTGACCT R-CACCACCCTGTTGCTGTAGC	255
OCT4A	F-AGCCCTCATTTCACCAGGCC R-TGGGACTCCTCCGGGTTTTG	448
SOX2	F-GGGGGAAAGTAGTTTGCTGCCTCT R-TGCCGCCGCCGATGATTGTT	135
SOX9	F-GACGCTGGGCAAGCTCTGGAGACTT R-TTCTTCACCGACTTCCTCCGCCG	141
SOX17	F-AAGGGCGAGTCCCGTATC R-TTGTAGTTGGGGTGGTCCTG	221
NKX6.1	F-CAGAGAGTCAGGTCAAGGTCTGGT R-CTCGGACGCGTGCAGTAGGA	260
PDX1	F-GAAAGGCCAGTGGGCAGGCGG R-GGCGCGGCCGTGAGATGTAC	137
MESP1	F-CTCTGTTGGAGACCTGGATG R-CCTGCTTGCCTCAAAGTG	278
NKX2.5	F-ACCTCAACAGCTCCCTGACTCT R-ATAATCGCCGCCACAAACTCTCC	155
SOX1	F-TGTAATCACTTTAACGAATGAGTGG R-AGTTTAATGAGAACCGAATTCAGC	133
MAP2	F-GCATGAGCTCTTGGCAGG R-CCAATTGAACCCATGTAAAGCC	192

Source: Refs 12,14,15

Transplantation studies in mice: Initially, establishment of model, its characterization and developing methods to transplant capsules were undertaken using several batches of mice. This study was undertaken using 10-12 wk old male mice, control (n=4) and transplanted (n=6). Macro-encapsulated day 16 derived pancreatic progenitors were transplanted intraperitoneally into six male Swiss mice. After 30 days of transplantation, all mice were treated with STZ and 15 days later were maintained on human insulin (1 IU/kg) given subcutaneously. Human insulin was discontinued after two weeks and mice were regularly monitored for their weight and blood glucose levels for a period of 110 days. They were carefully monitored for their body weight and blood glucose levels. Glucose was measured in blood collected from tail vein using contour glucometer.

Encapsulation and transplantation of hES-derived pancreatic progenitors: For transplantation of pancreatic progenitors, biocompatible macro-capsules made up of polyurethane-polyvinyl pyrrolidone semi-interpenetrating network, were employed[16]. The encapsulation procedure was carried out in strictly aseptic conditions as described earlier[13]. Confluent hES cells differentiated into pancreatic progenitors were detached mechanically from culture plates using a cell lifter, centrifuged and re-suspended into 300 µl basal medium (DMEM-F12). This cell suspension was transferred into the capsules and the open ends were sealed using a heated forceps. Transplanted group animals were anaesthetized using xylazine (20 mg/kg) and ketamine (80 mg/kg) and approximately $0.8\text{-}1.0\times10^6$ pancreatic progenitor cells encapsulated in the biocompatible device were transplanted in the peritoneal cavity of each of the six mice. Post-transplantation animals were maintained on a healthy diet.

Streptozotocin (STZ) treatment and follow up studies: After 30 days of transplantation, all mice (n=10) were rendered diabetic with multiple low-dose STZ (50 mg/kg) injections given for four consecutive days. Body weight and blood glucose were monitored daily post-STZ treatment. Once the blood glucose levels reached >300 mg/dl, all the mice received exogenous insulin support (1 IU/kg, subcutaneously) for 15 consecutive days. Animal survival, overall health, body weight and blood glucose levels were monitored at regular intervals for the next 120 days.

ELISA for human C-peptide: C-peptide levels were measured to assess further differentiation and maturation of the transplanted pancreatic progenitor cells. Blood (30 µl each) was collected from one control and two transplanted animals by retro-orbital bleeding, centrifuged and serum was collected for ELISA for human C-peptide as per the manufacturer's instruction (EZHCP-20K, Merck Millipore, Germany). Secreted C-peptide levels were quantified using BioTek plate reader (BioTek Instruments, VT, USA).

Results

Human embryonic stem cells culture: Undifferentiated hES cell line KIND1 was grown and maintained in feeder-free and monolayer condition (Fig. 1A and B). Feeder-free KIND1 cells were regularly characterized at transcript and protein level by qRT-PCR and immunofluorescence for the maintenance of their pluripotent state using markers such as OCT4, NANOG, SOX2, REX1, TERT and SSEA4 (Fig.1C and D).

In vitro differentiation of hESC: Our optimized directed differentiation protocol for endoderm differentiation from hES cells led to the formation of pancreatic progenitors by day 16 (Fig. 2A). During differentiation, sequential addition of growth factors led to the appearance of transcripts specific to the developing endoderm lineage.

Characterization of differentiated pancreatic progenitors: Formation of endoderm lineage from hES cells was evident by detection of genes such as *SOX17* and *SOX9* marking the formation of definitive endoderm and primitive gut tube, respectively, as well as *NKX6.1* and *PDX1* representing the formation of pancreatic progenitors. This was supported by significant downregulation of pluripotent markers *OCT4A* and *SOX2* along with low levels of ectoderm- and mesoderm-specific genes such as *SOX1, MAP2* and *MESP1, NKX2.5*, respectively, as compared to undifferentiated feeder-free hES cells (Fig. 2B). Immunofluorescence studies of hES cell-derived pancreatic progenitors also revealed the formation of pancreatic endoderm lineage as evident by the localization of the specific markers such as SOX9, SOX17 and PDX1 by confocal microscopy (Fig. 2C).

Transplantation studies in mice: Approximately 7-8 weeks post-STZ treatment, blood glucose level in four out of six experimental animals started decreasing and achieved normal levels (Table II). Normalization of

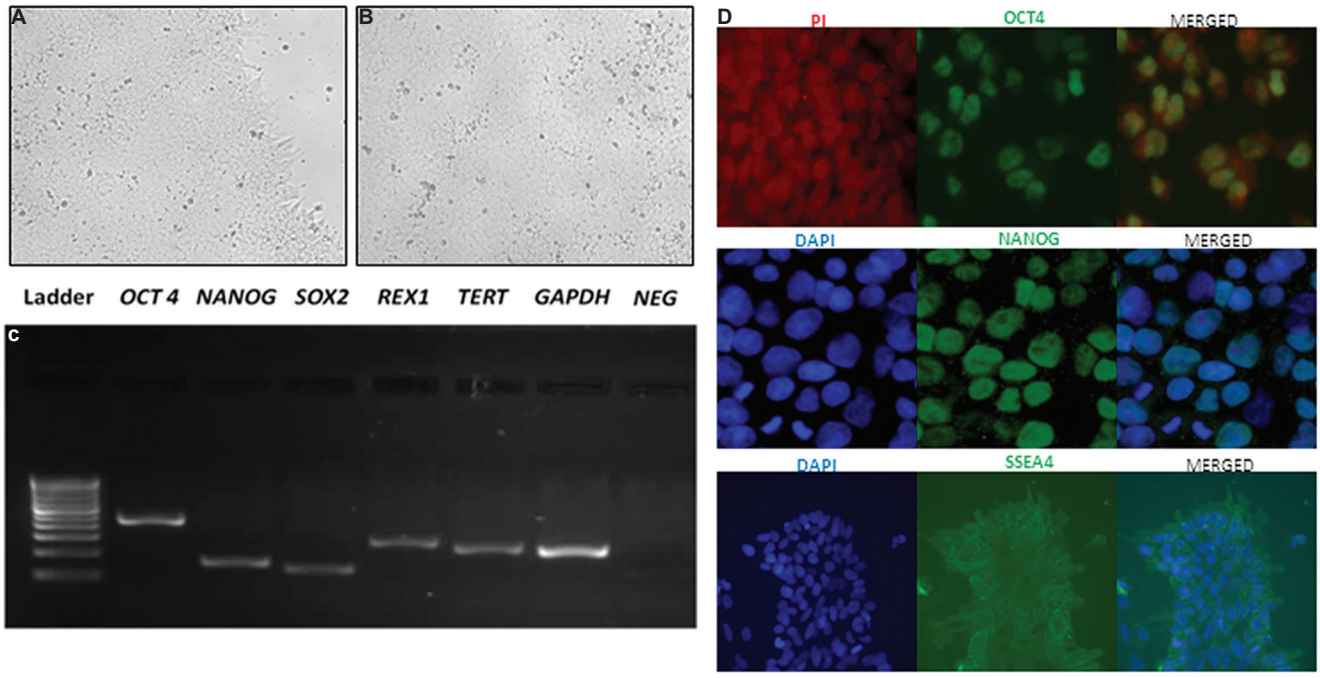

Fig. 1. Human embryonic stem cells (KIND1) used for the study. (**A** & **B**) KIND1 human embryonic stem cells colony grown as monolayer under feeder-free conditions on a Geltrex-coated dish (×10). (**C**) Pluripotent transcripts amplified by reverse transcription polymerase chain reaction using KIND1 human embryonic stem cells OCT4 (octamer-binding transcription factor 4), NANOG, SOX2 (SRY-Box 2), REX1 (Reduced Expression Protein 1) and TERT (Telomerase Reverse Transcriptase). GAPDH was used as housekeeping gene. (**D**) Immunofluorescence staining revealed the localization of markers such as OCT4, NANOG and SSEA4 (stage-specific embryonic antigen-4) (×40) (top & middle panel) and ×20 (bottom panel). Counterstaining was done using PI for OCT4 while DAPI was used for NANOG and SSEA4.

hyperglycaemia was accompanied with an increase in body weight (Table II). Two mice in the experimental group died on days 20 and 32, respectively, post-STZ. On the other hand, diabetic control animals (n=4) maintained high blood glucose levels and gradual weight loss throughout the study and were dead by day 45 of STZ treatment (Table II). The remaining transplanted mice continued with lowered blood glucose level and normal body weight for 4-5 weeks, after which the experiment was terminated due to ill health of the mice.

ELISA for human C-peptide: As a measure of development and functionality of transplanted progenitors, human C-peptide levels were analyzed on day 100 of experiment in two of the experimental animals (1.99 and 1.45 ng/ml) compared to that of diabetic control animal (0.37 ng/ml). More than five folds increased release in human C-peptide levels were noticed in the recipients of progenitor transplants as compared to diabetic control. Similar results were obtained in a smaller experiment done earlier transplanting pancreatic progenitors in four mice.

Discussion

The present study attempted to assess the effect of hES cells derived pancreatic progenitors in diabetic mouse model. Pancreatic progenitors obtained by *in vitro* differentiation of KIND1 hES cells[10] were packed in an immunoisolatory device and transplanted in mice. One month later the mice were made diabetic and the transplanted progenitors evidently became functional after another two months (3 months post-transplantation) and helped to maintain low blood glucose levels and body weight for a period of 4-5 weeks. The time taken by the progenitor cells to become functional was in agreement to the *in vivo* maturity period[17], and also in agreement with another published report[4]. C-peptide estimation is an indirect measurement of human insulin in circulation. The progenitors had the ability to further mature into beta islets as shown by secretion of human C-peptide in mouse circulation. It was feasible to transplant the progenitors, achieve full maturation into islets in mice and the approach was found to be safe since no teratoma was observed in any of the

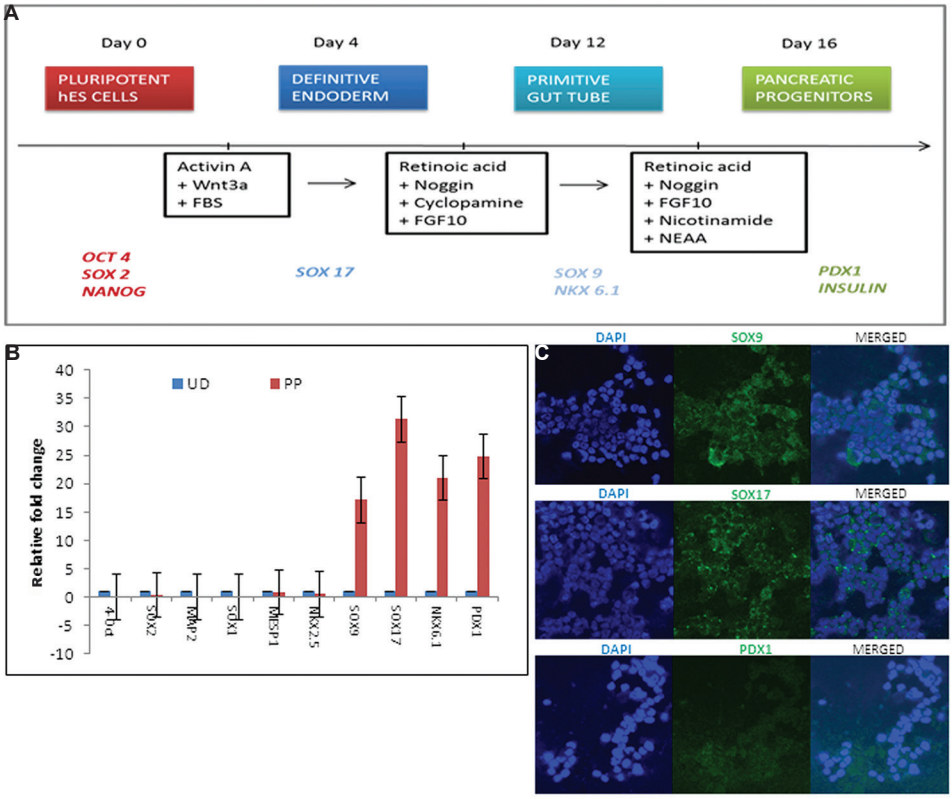

Fig. 2. Differentiation of KIND1 cells into pancreatic progenitors. (**A**) Schematic representation of protocol. (**B**) Expression of pluripotency (OCT4, SOX2), pancreatic progenitor-specific (SOX17 (SRY-Box 17), SOX9 (SRY-Box 9), NKX6.1 (NK6 homeobox 1), PDX1 (Pancreas/duodenum homeobox protein 1), ectodermal SOX1 (SRY-Box 1), MAP2 (Microtubule-associated protein 2) and mesodermal MESP1 (mesoderm posterior bHLH transcription factor 1), NKX2.5 (NK2 homeobox 5) transcripts in undifferentiated (UD, blue) and pancreatic progenitors on day 16 (PP, red). Data represent mean±standard error of the mean. (**C**) Immunofluorescence for SOX9, SOX17 and PDX1 (×10).

Table II. Changes in body weight and blood glucose of the four control and six mice after transplantation in the study

Days post-STZ treatment	Animals																			
	Body weight (g)†										Blood glucose (mg/dl)††									
	Control				Transplanted						Control				Transplanted					
	1	2	3	4	1	2	3	4	5	6	1	2	3	4	1	2	3	4	5	6
0	24	25	24	26	26	24	24	26	24	25	108	113	139	124	146	120	110	102	178	97
12	22	21	21	24	22	22	21	23	22	24	433	494	542	561	489	600	413	421	593	460
32	21	20	20	23	22	-	22	26	-	26	600	600	600	600	347	-	431	356	-	316
48	-	-	-	-	21	-	22	28	-	27	-	-	-	-	416	-	301	313	-	180#
56	-	-	-	-	22	-	23	26	-	26	-	-	-	-	385	-	283#	315	-	134#
68	-	-	-	-	24.9	-	23.2	28.2	-	28.8	-	-	-	-	222#	-	279#	294#	-	192#
88	-	-	-	-	25.1	-	24.6	27.8	-	-	-	-	-	-	153#	-	186#	161#	-	139#
96	-	-	-	-	24	-	-	26	-	-	-	-	-	-	154#	-	-	139#		-

†As evident body weight in control mice gradually declined whereas body weight was maintained in transplanted mice; ††Mice with blood glucose levels >300 mg/dl are considered diabetic. #Values show normalization of blood glucose post-transplantation. Boxes with dash show where glucose levels could not be estimated because of death of mice. Two of the transplanted mice survived for 110 days, but glucose levels were not estimated any further. STZ, streptozotocin

transplanted mice. However, the study was terminated by day 110 because the mice were sick and would not have survived any longer. This could be a limitation of the model or might be due to poor efficiency of

Table III. A critical review of various pre-clinical studies done using pancreatic progenitors

References	Study design	Number of mice	Site of implantation	Follow up	Results	Conclusion
Kroon *et al*, 2008[4]	5-20 million pancreatic progenitors were transplanted per animal 90 days post-transplantation in group 2 and 98-120 days post-transplantation in group 3, mice received 70 mg STZ per kg body weight, through intraperitoneal injection on 5 consecutive days for a total dose of 350 mg/kg.Function was assessed by IPGTT.	105	Into the epididymal fat pads and subcutaneously into kidney capsule	100 days post-STZ	C-peptide levels were detected in serum on day 30-60. Blood glucose levels in transplanted mice started decreasing before the diabetic controls after 2-3 wk; 100 days post-STZ, grafts were removed that led to increased blood glucose.	Hyperglycaemia after graft removal confirmed that hES cell-derived progenitors prevented the STZ-induced diabetes.
Matveyenko *et al*, 2010[20]	Athymic nude male rats were implanted with pancreatic endodermal cells-derived from hES cells	16	Into epididymal fat pads and subcutaneous implantation into TheraCyte.	140 days	Blood glucose, C-peptide and human insulin remained undetectable in the transplanted animal.	-
Bruin *et al*, 2013[7]	Mice were treated with STZ (either 190 mg/kg or low daily injections of 50 mg kg for five days). High-dose STZ mice were implanted slow-release insulin pellet while low-dose STZ mice were not treated with exogenous insulin. Mice received 0.5 million cells in TheraCytemacro-encapsulation device.	25 mice	Either under the left kidney capsule or subcutaneously.	200 days	Blood glucose decreased by eight weeks post-transplantation along with human C-peptide secretion noted between 8 and 10wk. H and E staining revealed that the majority of cells within the encapsulation devices resembled pancreatic endocrine cells and a minority resembled ductal cells.	Low dose STZ and macroencapsulated cells led to maturation of progenitor stage cells.
Sui *et al*, 2013[22]	2.5 million of pancreatic progenitors were transplanted. Mice were sacrificed at two, six, nine, and 12 wk post-transplantation.	21	Epididymal fat pad or the dorsal subcutaneous space.	15-85 days	Teratoma formation was observed in 3 of the 24 grafts. Grafts harvested at weeks six, nine and 12 showed that further differentiation of endoderm progenitors occurred in the grafts transplanted into subcutaneous space and not in those in the epididymal fat pads.	Subcutaneous transplantation is better than epididymal fat pads.
Rezania *et al*, 2014[23]	1.27 million cells were transplanted in non-diabetic and diabetic mice following which mice were checked for blood glucose and C-peptide.	45	Under the kidney capsule	Apprx 120 days	Transplantation resulted in normalization of blood glucose and significant levels of C-peptide.	The study favors the transplantation of mature beta cells expressing insulin and MAFA over pancreatic endodermal or pancreatic progenitor stage cells.
Rezania *et al*, 2013[21]	STZ given for five days (50 mg/kg/day).	9 mice in each group.	5 million cells transplanted in TheraCyte device.	135 days	NKX6.1 high cells receiving mice reversed hyperglycemia by 90 days post-transplantation while NKX6.1 low cells receiving mice maintained hyperglycemia	

Contd...

References	Study design	Number of mice	Site of implantation	Follow up	Results	Conclusion
					throughout the study. Insulin secretion was observed following meal or glucose or arginine intake in NKX6.1 high injected mice by day 85.	
Kirk *et al,* 2014[8]	1.5-3×105 cells/µl naive or pancreatic epithelium stage cells were encapsulated into TheraCyte 90 mg/kg alloxan given.	23	Subcutaneous	154 days	Circulating human C-peptide was first observed at seven weeks post-transplantation. Bioluminescent imaging confirmed that the cell biomass remained constant during maturation process *in vivo*. Blood glucose was lowered down post-alloxan treatment for 23 days.	Study demonstrated maintenance of cell biomass within the capsule and absence of cell loss from the capsule.
Pagliuca *et al,* 2014[24]	8-10 wk old immunodeficient SCID-beige mice and NRG-Akita mice were employed in the study. Mice were transplanted human islets 5 million human pluripotent cells (hES cells or iPS cells)-derived beta-cells or pancreatic progenitor cells or polyhormonal cells.	6 mice in each group	Below the kidney capsule	Two weeks	Human insulin was secreted into the bloodstream of the mice transplanted with hES- or iPS-derived beta cells and human islet cells; however, no human insulin was detected in mice transplanted with pancreatic progenitors or polyhormonal cells within two weeks of transplantation detected by ELISA. In addition, IHC performed post-two weeks in the kidneys grafted with stem cells derived beta cells and human islet cells revealed C-peptide positive clusters. Glucose clearance was found in the mice transplanted with stem cells derived beta cells comparable to the ones transplanted with human islet cells even after 18 wk of transplantation.	The study supports the transplantation of pluripotent stem cells derived insulin secreting beta cells rather than pancreatic progenitors
Shim *et al,* 2015[25]	Directed differentiation of embryoid bodies derived from hESC lines (HSF6, H1, Miz-hES6) to functional islet-like 3D cellular spheroids. Single STZ injection given 200 mg/kg. Cells transplanted 3-4 days after STZ.	Controls=4 Sham controls=4 Transplanted=6	3 million of the differentiated cells were injected into the kidney capsule.	110 days	Controls and sham controls sustained hyperglycemia and diabetic weight loss until they died 30-40 days after STZ injection. Transplanted profoundly decreased glucose levels within 50 days and maintained normal glucose levels. IPGTT revealed normalization	Long-term study required to assess safety of 3D aggregates.

STZ, streptozotocin; hES, human embryonic stem; iPS, induced pluripotent stem; IHC, immunohistochemistry; hESC, human embryonic stem cell; 3D, three-dimensional; IPGTT, intra-peritoneal glucose tolerance test; SCID, severe combined immunodeficient; NRG, NOD.Cg-Rag1^{tm1Mom} IL2rg^{tm1Wjl}

differentiation of hES cells into pancreatic progenitors or maturation post-transplantation.

Based on the work published from our laboratory[3,18,19], we were keen to compare the potential of endogenous pluripotent stem cells to regenerate a diabetic pancreas with hES cells-derived pancreatic progenitors (grown in a Petri dish). The analyses of various published pre-clinical studies describing the outcome of transplanted pancreatic progenitors (Table III) suggest that ES cells have the potential to differentiate into islets and human C-peptide and insulin are detected in circulation. Majority of studies were for 120-175 days including the present study and only one study[21] followed up mice for 238 days. This group reported that the pancreatic progenitors *in vivo* exhibited gene and protein expression profiles remarkably similar to the developing human foetal (not adult) pancreas. Jiang and Morahan[26] have concluded that although ES/induced pluripotent stem (iPS) theoretically has the ability to differentiate into functional beta cells, the field has not advanced as expected.

While studying the epigenetic changes involved during differentiation of ES cells into pancreatic progenitors, we have earlier reported that polycomb group proteins including both PRC1 (RING1, BMI1, CBX) and PRC2 (SUZ12, EED, EZH2) specific transcript levels are different in D16 progenitors compared to adult pancreas[2,12]. These differences may get ameliorated when the progenitors differentiate post-transplantation into mature islets or this may be the basic underlying cause which results in foetal-like state of ES-derived progenitors in STZ-treated mice and prevent their further differentiation into the adult state. We postulate that these epigenetic differences between ES-derived progenitors compared to adult human pancreatic cells are of significance and further careful studies need to be undertaken to address this in details.

A careful review of the literature reveals that even though several groups have attempted to differentiate both mouse and hES/iPS cells into gametes, research has not progressed as expected. The main reason is the inefficient conversion of ES cells into primordial germ cells (PGCs) which is the first and most crucial step to convert ES cells into gametes[27]. ES cells are derived by *in vitro* expansion of the inner cell mass of blastocyst stage embryo, whereas PGCs are developmentally more mature cells with a distinct epigenetic profile which appear in the yolk sac of developing epiblast stage embryo and migrate towards the gonadal ridge where they differentiate into gametes. Thus, by closely monitoring hES cells differentiation into pancreatic progenitors and gametes, it is postulated that various differentiation protocols transition hES cells into desired cell types at genetic level but fall short at epigenetic level and thus remain in foetal state and may not mature to an adult state (which is crucial for effective and long-term regeneration).This may be a major limitation for use of hES/iPS cells for regenerative medicine. Various adult cell types are also being used to regenerate a diabetic pancreas with varying results including autologous bone marrow cells and mesenchymal cells[2]. Autologous bone marrow cells failed to benefit ST elevation in cases of acute myocardial infarction in a multi-centric clinical trial concluded in India[28]. Bhansali *et al*[29] reported beneficial effect of transplanting mesenchymal cells through the tail vein in diabetic mice, but whether the beneficial effect was due to mesenchymal stem cells or very small embryonic-like stem cells (VSELs) needs further clarification[30].

The best strategy to alleviate the disease symptoms will possibly be to manipulate endogenous stem cells in the diabetic pancreas[31]. Various mechanisms have been proposed to explain regeneration of pancreas. It has earlier been demonstrated that VSELs are present in mouse pancreas and get mobilized in large numbers after STZ treatment in mice and also during pancreatic cancers in humans[32]. Two groups have reported distinct OCT-4 positive, small-sized cells in the human pancreas[33,34]. The small size and being present in very few numbers have led to the non-detection of VSELs[35] during otherwise a very good lineage tracing study[36]. We have recently observed VSELs involvement in regenerating a diabetic mouse (STZ treated) pancreas after partial pancreatectomy[18]. Preliminary data from our lab shows that when STZ treated mice were subjected to partial pancreatectomy, they remained healthy and maintained normal body weight and normoglycaemia for more than six months (unpublished observations).

To conclude, the potential of endogenous pluripotent stem cells (VSELs) to regenerate a diabetic pancreas needs to be compared with hES cells-derived pancreatic progenitors (grown in a Petri dish) to develop strategies to increase the effective span of cell therapy.

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***Conflicts of Interest*:** None.

References

1. International Diabetes Federation (IDF). *IDF diabetes atlas*. 7th ed. Brussels, Belgium: IDF; 2014.
2. Bhartiya D. Stem cells to replace or regenerate the diabetic pancreas: Huge potential & existing hurdles. *Indian J Med Res* 2016; *143* : 267-74.
3. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, *et al.* Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat Biotechnol* 2006; *24* : 1392-401.
4. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, *et al.* Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells *in vivo*. *Nat Biotechnol* 2008; *26* : 443-52.
5. Agulnick AD, Ambruzs DM, Moorman MA, Bhoumik A, Cesario RM, Payne JK, *et al.* Insulin-producing endocrine cells differentiated *in vitro* from human embryonic stem cells function in macroencapsulation devices *in vivo*. *Stem Cells Transl Med* 2015; *4* : 1214-22.
6. Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, *et al. In vitro* derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Res* 2007; *17* : 333-44.
7. Bruin JE, Rezania A, Xu J, Narayan K, Fox JK, O'Neil JJ, *et al.* Maturation and function of human embryonic stem cell-derived pancreatic progenitors in macroencapsulation devices following transplant into mice. *Diabetologia* 2013; *56* : 1987-98.
8. Kirk K, Hao E, Lahmy R, Itkin-Ansari P. Human embryonic stem cell derived islet progenitors mature inside an encapsulation device without evidence of increased biomass or cell escape. *Stem Cell Res* 2014; *12* : 807-14.
9. Vegas AJ, Veiseh O, Gürtler M, Millman JR, Pagliuca FW, Bader AR, *et al.* Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. *Nat Med* 2016; *22* : 306-11.
10. Kumar N, Hinduja I, Nagvenkar P, Pillai L, Zaveri K, Mukadam L, *et al.* Derivation and characterization of two genetically unique human embryonic stem cell lines on in-house-derived human feeders. *Stem Cells Dev* 2009; *18* : 435-45.
11. Nagvenkar P, Pethe P, Pawani H, Telang J, Kumar N, Hinduja I, *et al.* Evaluating differentiation propensity of in-house derived human embryonic stem cell lines KIND-1 and KIND-2. *In Vitro Cell Dev Biol Anim* 2011; *47* : 406-19.
12. Pethe P, Nagvenkar P, Bhartiya D. Polycomb group protein expression during differentiation of human embryonic stem cells into pancreatic lineage *in vitro*. *BMC Cell Biol* 2014; *15* : 18.
13. Kadam S, Muthyala S, Nair P, Bhonde R. Human placenta-derived mesenchymal stem cells and islet-like cell clusters generated from these cells as a novel source for stem cell therapy in diabetes. *Rev Diabet Stud* 2010; *7* : 168-82.
14. Pethe P, Pursani V, Bhartiya D. Lineage specific expression of Polycomb Group Proteins in human embryonic stem cells *in vitro*. *Cell Biol Int* 2015; *39* : 600-10.
15. Pawani H, Nagvenkar P, Pethe P, Bhartiya D. Differentiation of human ES cell line KIND-2 to yield tripotent cardiovascular progenitors. *In Vitro Cell Dev Biol Anim* 2013; *49* : 82-93.
16. Nair PD. A Process for the Preparation of a Biocompatible, Polymeric Composition of an Interpenetrating Polymeric Network (IPN). Indian Patent No. 230740. Filed by Sree ChitraTirunal Institute for Medical Sciences and Technology, Kerala; 2009.
17. Anderson S, Seeberger K, Ellis C, Eshpeter A, Korbutt G. Immunohistochemical characterization of insulin, glucagon, PDX1, SOX17 and NGN3 expression in human fetal pancreatic development. *J Stem Cell Res Ther* 2013; *3* : 148.
18. Bhartiya D, Mundekar A, Mahale V, Patel H. Very small embryonic-like stem cells are involved in regeneration of mouse pancreas post-pancreatectomy. *Stem Cell Res Ther* 2014; *5* : 106.
19. Bhartiya D, Patel H. Very small embryonic-like stem cells are involved in pancreatic regeneration and their dysfunction with age may lead to diabetes and cancer. *Stem Cell ResTher* 2015; *6* : 96.
20. Matveyenko AV, Georgia S, Bhushan A, Butler PC. Inconsistent formation and non-function of insulin-positive cells from pancreatic endoderm derived from human embryonic stem cells in athymic nude rats. *Am J Physiol Endocrinol Metab* 2010; *299* : E713-20.
21. Rezania A, Bruin JE, Xu J, Narayan K, Fox JK, O'Neil JJ, *et al.* Enrichment of human embryonic stem cell-derived NKX6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells *in vivo*. *Stem Cells* 2013; *31* : 2432-42.
22. Sui L, Mfopou JK, Chen B, Sermon K, Bouwens L. Transplantation of human embryonic stem cell-derived pancreatic endoderm reveals a site-specific survival, growth, and differentiation. *Cell Transplant* 2013; *22* : 821-30.
23. Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, *et al.* Reversal of diabetes with insulin-producing cells derived *in vitro* from human pluripotent stem cells. *Nat Biotechnol* 2014; *32* : 1121-33.
24. Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, *et al.* Generation of functional human pancreatic β cells *in vitro*. *Cell* 2014; *159* : 428-39.
25. Shim JH, Kim J, Han J, An SY, Jang YJ, Son J, *et al.* Pancreatic islet-like three-dimensional aggregates derived from human embryonic stem cells ameliorate hyperglycemia in streptozotocin-induced diabetic mice. *Cell Transplant* 2015; *24* : 2155-68.
26. Jiang FX, Morahan G. Pancreatic stem cells remain unresolved. *Stem Cells Dev* 2014; *23* : 2803-12.
27. Bhartiya D, Hinduja I, Patel H, Bhilawadikar R. Making gametes from pluripotent stem cells - A promising role for very small embryonic-like stem cells. *Reprod Biol Endocrinol* 2014; *12* : 114.
28. Nair V, Madan H, Sofat S, Ganguli P, Jacob MJ, Datta R, *et al.* Efficacy of stem cell in improvement of left ventricular function in acute myocardial infarction – MI3 Trial. *Indian J Med Res* 2015; *142* : 165-74.

29. Bhansali S, Kumar V, Saikia UN, Medhi B, Jha V, Bhansali A, *et al.* Effect of mesenchymal stem cells transplantation on glycaemic profile & their localization in streptozotocin induced diabetic Wistar rats. *Indian J Med Res* 2015; *142* : 63-71.

30. Bhartiya D. Is the improved function of streptozotocin treated pancreas truly due to transdifferentiation/fusion of transplanted MSCs? *Indian J Med Res* 2016; *143* : 111-2.

31. German MS. Anonymous sources: Where do adult ß cells come from? *J Clin Invest* 2013; *123* : 1936-8.

32. Starzynska T, Dabkowski K, Blogowski W, Zuba-Surma E, Budkowska M, Salata D, *et al.* An intensified systemic trafficking of bone marrow-derived stem/progenitor cells in patients with pancreatic cancer. *J Cell Mol Med* 2013; *17* : 792-9.

33. White MG, Al-Turaifi HR, Holliman GN, Aldibbiat A, Mahmoud A, Shaw JA. Pluripotency-associated stem cell marker expression in proliferative cell cultures derived from adult human pancreas. *J Endocrinol* 2011; *211* : 169-76.

34. Zhao M, Amiel SA, Christie MR, Muiesan P, Srinivasan P, Littlejohn W, *et al.* Evidence for the presence of stem cell-like progenitor cells in human adult pancreas. *J Endocrinol* 2007; *195* : 407-14.

35. Bhartiya D, Shaikh A, Anand S, Patel H, Kapoor S, Sriraman K, *et al.* Endogenous, very small embryonic-like stem cells: Critical review, therapeutic potential and a look ahead. *Hum Reprod Update* 2016; *23* : 41-76.

36. Xiao X, Chen Z, Shiota C, Prasadan K, Guo P, El-Gohary Y, *et al.* No evidence for β cell neogenesis in murine adult pancreas. *J Clin Invest* 2013; *123* : 2207-17.

Reprint requests: Dr Deepa Bhartiya, Department of Stem Cell Biology, ICMR-National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai 400 012, Maharashtra, India
e-mail: bhartiyad@nirrh.res.in