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Human embryonic stem cell differentiation into insulin secreting β-cells for diabetes

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Abstract

hESC (human embryonic stem cells), when differentiated into pancreatic β-ILC (islet-like clusters), have enormous potential for the cell transplantation therapy for Type 1 diabetes. We have developed a five-step protocol in which the EBs (embryoid bodies) were first differentiated into definitive endoderm and subsequently into pancreatic lineage followed by formation of functional endocrine β islets, which were finally matured efficiently under 3D conditions. The conventional cytokines activin A and RA (retinoic acid) were used initially to obtain definitive endoderm. In the last step, ILC were further matured under 3D conditions using amino acid rich media (CMRL media) supplemented with anti-hyperglycaemic hormone-Glp1 (glucagon-like peptide 1) analogue Liraglutide with prolonged t½, and Exendin 4. The differentiated islet-like 3D clusters expressed bonafide mature and functional β-cell markers-PDX1 (pancreatic and duodenal homeobox-1), C-peptide, insulin and MafA. Insulin synthesis de novo was confirmed by C-peptide ELISA of culture supernatant in response to varying concentrations of glucose as well as agonist and antagonist of functional 3D β islet cells in vitro. Our results indicate the presence of almost 65% of insulin producing cells in 3D clusters. The cells were also found to ameliorate hyperglycaemia in STZ (streptozotocin) induced diabetic NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mouse up to 96 days of transplantation. This protocol provides a basis for 3D in vitro generation of long-term in vivo functionally viable islets from hESC.

Keywords: agonists and antagonists of insulin pathway; C-peptide ELISA; hESC; pancreatic differentiation; β islets

1. Introduction

Diabetes is the most common metabolic disorder affecting 4–5% of the global population (Mandrop-Poulsen, 1998). It occurs in two forms: Type 1 (insulin-dependent) caused by an autoimmune destruction of insulin producing β-cells and Type 2 (on-insulin dependent), resulting from reduced insulin sensitivity as well as impaired insulin secretion from β islet cells of Langerhans in pancreas. The only effective way to cure Type 1 diabetes is pancreatic islet transplantation instead of insulin injections (Serup et al., 2001). However, due to limited supply of donor or cadaveric pancreas, researchers have been continuously exploring stem cells as cell therapy source that has the potential to differentiate into pancreatic β-cells (Bonner-Weir and Weir, 2005). hESC (human embryonic stem cells), which are immortal and capable of self-renewal and differentiation into any cell types of the body, can be a potential source for indefinite supply of insulin producing β-cells for transplantation into diabetic patients (Soria et al., 2001).

The adult pancreas is composed of exocrine and endocrine parts, the exocrine cells produce digestive enzymes and the endocrine cells produce insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells) and PP (pancreatic polypeptide) cells (Slack, 1995). During organogenesis within the mouse embryonic foregut, pancreatic fate is first specified by the expression of the homeobox gene PDX1 (pancreatic and duodenal homeobox-1) (Jonsson et al., 1994; Guz et al., 1995). Thus, activation of PDX1 is considered a prerequisite for pancreatic differentiation in vitro and should precede the progressive expression of more mature markers of the endocrine lineage including Ngn3, Nkx2.2, Nkx6.1, MafA and MafB (Jensen, 2004). Moreover, MafA has been identified as the master regulator of glucose stimulated insulin secretion in functional β islet cells (Hang et al., 2011; Matsuoka et al., 2004; Zhang et al., 2005; Wang et al., 2007). This transcription factor which is last expressed among all β islet transcription factors has been involved in regulating β-cell function by activating the insulin gene promoter via PDX1 and positive regulation of important β-cell genes such as GLUT2, PDX1, Nkx6.1 and Glp1 (glucagon-like peptide 1)-R. Also MafA and MafB have been implicated in functional maturation of pancreatic β islet cells from hESC (Wang et al., 2007).

More recently, the β islet differentiation in vitro strategies from ES cells were evolved which mimicked β-cells organogenesis. The approach taken is stepwise endodermal differentiation followed by the differentiation into pancreatic β islet cells through the use of various growth factors (Shi et al., 2005). D’Amour et al. (2006) described a five-step and a four-step two-dimensional (or monolayer) hESC differentiation protocol that leads to efficient PDX1 activation by day 12 and 15 respectively, and the impressive formation of up to 12% insulin-containing cells by day 18 (Madsen and Serup, 2006) and day 25 (Jiang et al., 2007a, 2007b). These cells responded to a variety of insulin secretagogues, but only

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Abbreviations: bFGF, basic fibroblast growth factor; DAPI, 4',6-diamidino-2-phenylindole; EB, embryoid body; E-Cadherin, epithelial Cadherin; Glp1, glucagon-like peptide 1; hESC, human embryonic stem cells; ILC, islet-like clusters; NOD, non-obese diabetic; PDX1, pancreatic and duodenal homeobox-1; qRT-PCR, quantitative real-time PCR; RA, retinoic acid; SCID, severe combined immunodeficiency; STZ, streptozotocin.
showed limited glucose responsiveness. Modified pancreatic differentiation by Kroon et al. (2008) reported the production of insulin-producing cells from hESC which were also in vivo responsive to glucose. Recently, Nostro et al. (2011) reported improved efficiency (25%) insulin-producing cells by stage specific in vivo regulation of TGFβ family members. Contemporarily, 3D β islets were also generated from mouse ES cells, iPSC and mouse fetal pancreatic cells (Wang and Ye, 2009; Saito et al., 2011). However, generation of pancreatic β islet cells in 3D in vitro culture systems from human cells has not yet been reported.

We present the differentiation of two hESC lines ReliCell hES1 (Mandal et al., 2006) and BGO1 (National Institutes of Health registered cell line) into ILC (islet-like clusters) using a five-step serum-free differentiation protocol that involves maturation of β islets under 3D in the last step. These 3D clusters when transplanted in STZ (streptozotocin) induced NOD (non-obese diabetic)/SCID (severe combined immunodeficiency) mice could ameliorate diabetes up to 96 days post transplantation, a novel finding of 3D differentiation of β islets from hESC.

2. Materials and Methods

For detailed protocols and reagents, please refer to the Supplementary Materials and methods section (available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm).

2.1. hESC Culture and Differentiation Conditions

hESC culture and differentiation to pancreatic lineage were performed as per the protocol schematically represented in Figure 1A.

2.2. Characterization of in vitro Differentiated Cells

Characterization was performed using qRT-PCR (quantitative real-time PCR), immunocytochemistry, FACS analysis and in vitro functional assays for insulin and C-peptide release by ELISA.

2.3. Animal Transplantation Studies

Animal studies were conducted after obtaining approval from the IAEC (Institutional Animal Ethics Committee), Reliance Life Sciences, India and CPCSEA (Centre for Prevention of Cruelty to Small Experimental Animals, Government of India). NOD/SCID mice (Jackson Laboratories) were maintained in Laboratory of Animal Sciences, Reliance Life Sciences Pvt Ltd, Mumbai, India.

Diabetes was induced in 10-week-old male mice 3–5 days before transplantation by two injections (intraperitoneal) of STZ (Sigma) with an interval of 72 h, and was used as the recipients of the implants. The dosage for the first shot of STZ was 100 mg/kg body weight and that of the second shot was 40 mg/kg body weight in citrate buffer (pH 4.5). Before transplantation, diabetes was confirmed by the presence hyperglycaemia (300 mg/dl), weight loss and polyurea. Random blood glucose levels was assured to be higher than 300 mg/dl at two instances on different days. The blood glucose was measured using a portable glucometer (Lifescan Inc.) from one drop of blood drawn by pricking the tail vein with a needle. For cell implantation, 200 insulin producing 3D islet clusters were resuspended in 20 μl step 5 pancreatic differentiation media. Cells were transplanted into the Group B animals whereas the animals in the controls (Group A) received only 20 μl step 5 pancreatic differentiation media under the left kidney capsule. Mice were anaesthetized using one intraperitoneal injection of 300 μl of 1:10 working solution of Avertin (1% solution of Tribromoethanol in tertiary amyl alcohol). Surgery was carried under a laminar flow hood. The left kidney was exposed through a ventral incision and the cells were transferred gently under the kidney capsule with the help of tuberculin-syringe needle 26,2 gauge (BD Biosciences). The kidney was put back into place and the incision closed. Animals were kept for post-operative recovery, with random sugar levels taken twice a week until they were killed 96 days post transplantation. Survival of the animals was followed in both the groups.

3. Results

3.1 Stepwise Pancreatic Differentiation of hESC Expressed Sequential Morphological Changes

Undifferentiated hESC colonies, as identified by their pluripotency marker expression analysis (Figure 1B) when subjected to a step wise pancreatic differentiation protocol (Fig. 1A), underwent the following morphological changes. The undifferentiated hESC successfully formed EBs (embryoid bodies) (Supplementary Figure S1A available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm) in suspension culture in step 1 of differentiation. On plating these EBs on matrigel-coated plates followed by the sequential addition of activin A and RA (retinoic acid) for 6 days, the cells acquired a morphology which resembled endoderm during step 2 of differentiation protocol (Supplementary Figure S1B). Furthermore, in the differentiation protocol (step 3), we obtained pancreatic progenitor cells with flattened morphology (Supplementary Figure S1C) after 12 days of treatment with bFGF (basic fibroblast growth factor) and Noggin. At the end of step 4 of differentiation, morphologically visible insulin secreting cell clusters were obtained. Due to the phenotypic resemblance of the in vitro generated/differentiated insulin secreting cell clusters to the naturally occurring β islet cell clusters present inside human pancreas, we termed these in vitro differentiated cell clusters as ILC (Supplementary Figure S1D) after treating the cells with the stipulated growth factors as mentioned in section 2 for a further 12 days. These typical ILC were interconnected with long tubular structures (Supplementary Figure S1E). Finally, in the last step, step 5 of differentiation, the 3D islet-like clusters occurred with two morphological phenotypes – hollow clusters and compact clusters (Figure 2A and Supplementary Figure S1F). These two types of clusters eventually fused together to form compact clusters.
Figure 1 Stage-specific morphologies and marker expression during stepwise pancreatic differentiation of hESC into 3D islet-like clusters

(A) Schematic representation of stepwise pancreatic differentiation protocol for ReliCellhES1 and B6O1. (B) (a) Phase contrast picture, (b) Oct4 staining, (c) DAPI (4',6-diamidino-2-phenylindole)-nuclear staining and (d) Oct4/DAPI merged staining of single colony of hESC indicating pluripotency. Immunofluorescence staining of hESC-derived cells after: (C) step 2 (DE) induction for (a) Sox17, (b) Oct4, (c) nuclear-DAPI and (d) merged for Sox17/Oct4 and DAPI. (D) Step 3 – pancreatic endoderm induction for (a) E-Cadherin, (b) PDX1, (c) nuclear-DAPI, (d) merged for E-Cadherin/PDX1 and DAPI. (E) Step 4 – pancreatic differentiation, 2D islets for (a) insulin, (b) C-peptide, (c) nuclear-DAPI and (d) merged for insulin/C-peptide and DAPI. (F) Step 5 – pancreatic 3D islets formation for (a) MafA, (b) PDX1, (c) nuclear-DAPI and (d) merged for MafA/PDX1 and DAPI. Scale bar: 100 μm.
3.2. Insulin-secreting 3D ILC expressed sequential markers specific to various stages of differentiation

The 3D ILC differentiated from two hESC lines were characterized by qRT-PCR, FACS and immunofluorescence analysis at different stages of differentiation. RNA samples from human fetal pancreas were used as a positive control and undifferentiated hESC and human embryonic fibroblasts were used as negative control in all the qRT-PCR experiments. Treatment with activin A and RA (step 2) induced the cells to express definitive endoderm specific markers, i.e. Sox17 and FoxA2 as evident from qRT-PCR (Supplementary Figure S2 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm). Approximately 89% of the cells at this stage expressed Sox17 as evident from FACS analysis (Supplementary Figure S3 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm) and immunostaining (Figure 1C, a), while 58.3% cells expressed CXCR4 (CXC chemokine receptor 4) (Supplementary Figure S3). Moreover, as expected the cells at the end of step 2 of differentiation showed very low Oct4 gene expression by qRT-PCR analysis (Supplementary Figure S2) to complete absence of Oct4 protein, evident from immunostaining (Figure 1C, b). Interestingly, further during step 3 differentiation, the induced epithelial-like cells started expressing PDX1, the gene expression in differentiated cells at step 3 being comparable to PDX1 expression in human fetal pancreas (Supplementary Figure S2). Furthermore, 73.8% of the cells at step 3 of differentiation expressed PDX1 protein, as also evident from FACS analysis (Supplementary Figure S4 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm).

Finally, step 4 of differentiation yielded cells with islet-like morphology connected by inter-islet tubular structures (Supplementary Figure S1E). Such ILC-expressed mature pancreatic genes, i.e. Ngn3, GLUT2, insulin and MafA (qPCR Supplementary Figure S2). Such mature ILC co-expressed insulin and its precursor C-peptide as evident from immunofluorescence analysis (Figure 1E). Although ~65% of the cells in hESC-derived ILC-stained positive for GLUT2 (data not shown), only 24.5% of the cells in these clusters expressed C-peptide protein, shown by FACS analysis (Supplementary Figure S5 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm). This essentially indicates a differentiation protocol yielding 24.5% insulin-producing cells.
Furthermore, when the 2D islets were cultured under 3D culture conditions with β-cell enrichment media, increased expression of insulin positive cells to 68.4% occurred as analysed by FACS and immunofluorescence (Figure 2B, Supplementary Figure S6 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm) and mature pancreatic markers such as MafA (Figure 1F). Interestingly, the 3D clusters had low expression of other genes such as glucagon and somatostatin as compared with the 2D clusters (Supplementary Figure S2). Mature insulin-secreting islet-like 3D clusters obtained by this method also stained positive with dithizone (Supplementary Figure S7C available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm), a zinc containing dye staining insulin-producing cells. The maximum intensity of staining was observed in the actively insulin producing regions of the islet clusters (Supplementary Figure S7C). However, other regions (non-insulin producing) of the islet cluster did not stain positive with dithizone.

3.3 3D ILC derived from hESC responded well to glucose stimulation and activators and inhibitors of insulin pathway in a dose-dependent manner in vitro

To assess the in vitro functionality of these 3D ILC derived from the two hESC lines, insulin and C-peptide secretion were estimated in the cell culture media in response to low (3.3 mM) and high (16.7 mM) concentrations of glucose alone and in addition to activators (Tolbutamide and Diazoxide) and inhibitors (Nifedipine) in the culture media. Plasma from human blood was taken as the control for basal levels of circulating insulin.

For insulin ELISA, the basal levels of insulin in the normal cell culture supernatant and the control human plasma were comparable (Figure 2C). However, overnight glucose starvation of the cells led to zero levels of secreted insulin in cell culture media. A 3-fold increase in insulin release was observed in the cells treated with 16.7 mM glucose as compared with the insulin levels in the normal cell culture supernatant. However, stimulation of the starved cells with only 3.3 mM glucose failed to show an increase in insulin release over the basal level. The activator 10 mM Tolbutamide along with 16.7 mM glucose and 250 μM Diazoxide along with 16.7 mM glucose had, however, stimulated the cells to secrete increased levels of insulin to the extent of 3.8- and 4-fold respectively over the normal basal level. Also when the starved ILC were stimulated with the inhibitor 50 μM Nifedipine along with 16.7 mM glucose, a remarkable drop in the levels of circulating insulin was observed which was almost comparable to the basal levels of circulating insulin (Figure 2C). Furthermore, a similar pattern was also observed in terms of C-peptide secretion in response to various treatments (Figure 2D). These results essentially indicated in vitro functionally active 3D ILC.

3.4 Glucose levels were maintained in NOD/SCID diabetic mice in response to hESC-derived 3D ILC

The last part of characterization of differentiated cells focused on in vivo experiments. To assess the capacity of differentiated cells to maintain normal glucose homeostasis (Grossman et al., 2010), 200 intact 3D ILC differentiated from hESC lines were transplanted under the left kidney capsule of each of STZ-induced diabetic mice (Group B). Group A (the control group) received only the media under the left kidney capsule. Survival of both the groups of animals was studied for 96 days. The majority of the animals (n=4) of Group A survived up to 38 days (Figure 3A) and the others beyond 64 days. In the transplanted group survival was better. The majority of the animals of this group survived the 96 days (n=5), whereas only one animal (Animal ID #B5) died at 60 day (Figure 3B).

Random non-fasted glucose levels for both the groups of animals were assessed on the control group of animals, and all the animals showed a continuous increase in the random sugar levels from a starting value of 300 mg/dl (before injection) to a value of >600 mg/dl with time post-injection (Figure 3A). One animal in this group, however, continued to survive beyond 40 days post injection despite a very high random glucose level. Random sugar levels of Group B (cell transplanted group) of animals showed a decreasing trend in their respective random sugar levels from a starting value of 300 mg/dl (prior to transplantation) from 16 days post transplantation onwards and then maintained a low value of ~200 mg/dl thereafter till 96 days post transplantation (Figure 3B). However, one animal in this group died earlier before the end of the experiment.

Finally, we studied the presence of insulin positive cells in the kidney grafts removed from the animals of both the groups (control and islets transplanted) (Supplementary Figure S8 available at http://www.cellbiolint.org/cbi/036/cbi0361013add.htm, showing the kidney grafts) by immunohistological studies. Interestingly, no teratomas were detected in the kidney grafts receiving hESC-derived 3D islets indicating complete differentiation. Furthermore, when scored for insulin and C-peptide double positive cells (estimated by counting ten random fields), in the transplanted portion of the grafts which received 3D islets, ~80% double positive cells were detected in the Group B animals (Figure 3D). However, no insulin or C-peptide positive cells were detected in the graft sections of the control group of animals (Figure 3C).

4. Discussion

Due to the need for β-cell replacement therapy, much work has been done to generate β-cells from a variety of cell sources. hESC can be directed to become fully mature β-cells (D’Amour et al., 2006; Jiang et al., 2007a, 2007b; Nostro et al., 2011; D’Amour et al., 2005; Van Hoor et al., 2009; Lumelsky et al., 2009). With the knowledge about embryonic development and progress made with mouse ESCs, a stepwise approach was used to direct hESC towards islet cells, first to definitive endoderm next to gut-tube pancreatic endoderm and then to islet cells. It was possible to generate cells in vitro that had characteristics of islet cells, but they were not fully mature. However, after immature precursor cells were transplanted into immunodeficient mice, maturation progressed to produce β-cells that were convincingly normal with
regard to multiple characteristics. Importantly, these cells could make and store fully formed insulin, release insulin in response to a glucose stimulation and could cure diabetes in mice. However, much further research is needed before this advance can be brought to clinical application. For example, there is concern that these populations of precursor cells might contain cells that will form teratomas. Moreover, the percentage of insulin producing cells that could be accomplished in vitro was merely 25% (Kroon et al., 2008). A 3D approach can be taken to the culture systems to differentiate beta islets from mouse ES cells (Wang et al., 2009) and have shown that up to 60% of the beta islets could be generated in vitro. Also, another group has reported 3D differentiation of beta islets from mouse iPS cells (Saito et al., 2011).

In this investigation, we present preferment in the method of pancreatic differentiation from hESC under 3D culture conditions. This protocol (Figure 1A) involves stepwise differentiation of hESC through definitive endoderm, pancreatic endoderm and finally the generation of endocrine cells and enrichment of beta-cell mass that secretes insulin and C-peptide. The sequential morphology transition of the hESC during the entire process of in vitro differentiation to obtain finally 3D beta ILC is represented in Supplementary Figure S1. Moreover, expression of important marker proteins right from the undifferentiated hESC until the formation of 3D ILC have been represented as immunostaining photomicrographs in Figures 1(B)–1(F). In this protocol the very early EBs (48 h; Supplementary Figure S1A) were first subjected to conventional definitive endoderm induction by activin A and then with RA for first 6 days (Supplementary Figure S1B). This step also confirms the down-regulation of pluripotency marker Oct4 and also simultaneous up-regulation of definitive endoderm marker Sox17 (Figure 1C and Supplementary Figure S2). The step 3 involves differentiation of the endodermal cells into

Figure 3 Outcome of in vivo transplantation of hESC-derived 3D ILC underneath the kidney capsule of STZ-induced diabetic NOD/SCID mice
(A) Random blood glucose levels (mg/dl) represented in the y-axis for control group of diabetic mice which did not receive any ILC underneath the kidney capsule at various days post-transplantation/injection (x-axis) and instead received 20 µl of step 5 differentiation media. Six mice were used in the control group and were named as A1, A2, A3, A4, A5 and A6. (B) Random blood glucose levels (mg/dl) represented in the y-axis for transplanted group of diabetic mice which received ILC underneath the kidney capsule at various days post transplantation/injection (x-axis). Six mice were used in the transplanted group and were named as B1, B2, B3, B4, B5 and B6. (C) The kidney grafts where step 5 differentiation media was injected in control group were excised/sectioned and stained for (a) insulin, (b) C-peptide, (c) nuclear-DAPI and (d) merged for insulin/C-peptide/DAPI. (D) Portion of kidney grafts which received ILC in transplanted group were excised/sectioned and stained for (a) insulin, (b) C-peptide, (c) nuclear-DAPI and (d) merged for insulin/C-peptide/DAPI.
islet cell maintain and enriching components, such as Exendin 4 and Liraglutide. The morphology of 3D ILC is shown in Supplementary Figure S1(F). Interestingly, MafA, the mature pancreatic β-cell marker was expressed at the end of step 3 of differentiation and showed a very high expression at the end of step 5 after formation of 3D ILC (Supplementary Figure S2). Gip1, an anti-hyperglycaemic hormone, enhances functional β-cell mass also enhances glucose-dependent insulin secretion and glucagon suppression has a very short $t_{1/2}$. In order to overcome this, we have used Liraglutide which has a relatively longer $t_{1/2}$ (Bregenholt et al., 2005; Michael et al., 2009; D’Alessio and Vahl, 2004; Farilla et al., 2003).

The 2D islets as well as 3D islets express high amounts of mature β islet marker gene MafA (Supplementary Figure S2). Immunostaining of the 3D ILC for MafA protein is shown in Figure 1(F). Furthermore, the 2D islets continued to co-express non-β-cell endocrine pancreatic markers, e.g. glucagon and somatostatin (Supplementary Figure S2). However, 3D islet cultures upon analysis for the presence of other exocrine or endocrine hormone producing cells of pancreas other than β islet cells, had a much lower expression of glucagon and somatostatin (Supplementary Figures S2, S7A and S7B). 3D ILC also did not express amylase, a marker of exocrine pancreas (data not shown). This observation regarding negligible expression of non-β-cell endocrine pancreatic markers by 3D ILC proves a purer population of β ILC obtained by this protocol. This five-step protocol generated 68% 3D ILC with secretory granules containing insulin (Figures 2B and 2C and Supplementary Figure S6). Furthermore, the β-cell mass generated by this method produced C-peptide in response to glucose stimulation in vitro (Figure 2D).

Here the in vitro differentiation is shown to be completed in 42 days and the cells were found to ameliorate diabetes in STZ-induced diabetic mice (Figure 3). First, the mice were induced into diabetic state by injecting STZ intraperitoneally. The mice that showed random (non-fasted) sugar levels of $>300$ mg/dl upon diabetes induction were selected for 3D ILC transplantation experiments as well as their non-transplanted control counterparts. Interestingly, the correction of diabetic state occurred after 16 days post 3D ILC transplantation and was indicated by an attainment of $\leq 200$ mg/dl (non-fasted) random sugar levels (Figure 3B) and was maintained until 96 days post transplantation. As in studies of other investigators, as well in euglycaemia mice (random non-fasted sugar levels), a level is indicated as $\leq 200$ mg/dl. However, normoglycaemia in mice under non-fasting conditions has been given as 100 mg/dl (Yin et al., 2006). In our case, although complete reversal of diabetes was not achieved in Group B animals (3D ILC transplanted), we successfully achieved significant amelioration of diabetes that was sufficient to keep the mice alive until day 96 post 3D ILC transplantation. In contrast, in the non-transplanted control group (Group A), majority of animals ($n=4$) died by day 38, primarily due to hyperglycaemia.

In conclusion, using the specific growth factors and signalling molecules, we have developed a reproducible well-defined culture system for generating purer population of 3D ILC from hESC which are similar to pancreatic β-cells. The cells generated by this method may represent future candidates for cell-based therapy in diabetes type 1.

**Author contribution**

Bipasha Bose was instrumental in experimental design, execution, data analysis and manuscript writing. Sudheer Shenoy P participated in experimental execution, data analysis and manuscript writing. Sudhakar Konda and Pralhad Wangikar have participated in animal experimental design, execution and data analysis.

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