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Islet Generation from Intra Islet Precursor Cells of Diabetic Pancreas: *In Vitro* Studies Depicting *In Vivo* Differentiation

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ABSTRACT

Context Beta-cells have a limited replicative capacity; hence, there is always a quest for sources of islet regeneration to compensate for the loss of functional beta-cells in diabetes.

Objective To test the hypothesis of whether intra islet precursor cells of islets isolated from a diabetic pancreas and *in vitro* streptozotocin treated islets are capable of giving rise to neoislets.

Interventions Streptozotocin treatment was given to mice and to islets isolated from normal mice. Islets were isolated from diabetic mice, cultured on matrigel coated plates with a well-defined serum free medium containing mitotic (nicotinamide) and differentiating (keratinocyte growth factor) agents. Initially, islets gave rise to an epithelial-like cell monolayer and, later on, differentiated into islet-like clusters. These were characterized for the ductal epithelial cell specific markers cytokeratin-19 and cytokeratin-7 and for the islet specific markers-insulin and PDX1. Insulin secretion in response to glucose and L-arginine was estimated by ELISA.

Results A cytokeratin-19 and cytokeratin-7 positive precursor cell population was found scattered throughout the epithelial monolayer. Upon addition of the keratinocyte growth factor, these precursor cells gave rise to islet-like clusters which were confirmed to be islets by marker studies. Though streptozotocin treatment on islets of normal mice allowed proliferation of the epithelial monolayer, it did not give rise to neoislets under similar growth conditions.

Conclusion The present study reveals that streptozotocin treatment of normal islets *in vitro* leads to the loss of the potential of intra islet precursor cells to form neoislets; however, in streptozotocin-induced experimental diabetes, they retain their potential to generate new islets opening a novel putative way of treating diabetes.

INTRODUCTION

Unraveling the mechanisms of islet cell development and regeneration represents an important medical issue because it could lead to strategies aimed at restoring the functional beta-cell mass which is known to be deficient or even absent in patients suffering from diabetes. For instance, the transplantation of islets or islet cells in diabetics would greatly benefit from the availability of methods to increase the number of beta-cells after their isolation from donor organs since they are always in short supply. The problem is that beta-cells have a limited, if any, proliferative potential and trials to significantly expand beta-cells in culture have remained unsuccessful. Evidence of the existence of a progenitor cell in the pancreas rests primarily on the phenomenon of islet neogenesis, which can be experimentally induced by the

cellophane wrapping of the pancreas [1], partial pancreatectomy [2], streptozotocin induced diabetes [3] and has also been observed during pregnancy [4]. Although a protein that induces islet neogenesis (islet neogenesis associated protein, INGAP) has been purified and characterized, and it has had c-DNA cloned [5, 6], a potential target progenitor cell remains unknown.

At present, most of the studies favor the pancreatic duct as a potential source of progenitor cells in the adult pancreas [4, 5]. This is based on established information that development of the islets during embryogenesis is closely associated with the ductal epithelium [7]. The budding of new islets from the small ducts has been described in humans [8]. In embryos and postnatal mice, precursors present in the pancreatic duct [9] migrate into the tissue parenchyma where they differentiate into mature islets. The ability of ductal cells to expand *in vitro* and to form insulin-producing islet-like structures has been demonstrated [10, 11]. A few recent reports indicate that another potential source of progenitor cells is the islet itself. For example, the analysis of islet regeneration in the mouse pancreas after the administration of streptozotocin suggests the presence of beta progenitor cells in the islets which differentiate into insulin-producing cells following diabetogenic insult [12]. Another report demonstrates that rat and human islets contain a distinct population of nestin positive and hormone negative immature cells, which proliferate extensively *in vitro* and appear to be multipotential [13].

In our present study, we were interested in testing the hypothesis of whether intra islet precursor cells within an experimental diabetic pancreas could be stimulated to give rise to new islets opening the possibility of utilizing them for islet regeneration in diabetes. In this effort, we found a distinct population of intra islet precursor cells which exhibited differential behavior to a streptozotocin challenge *in vitro* and *in vivo*.

MATERIALS AND METHODS

Islet Isolation

Thirty Balb/C mice of both sexes (6 to 8 weeks old) were obtained from an inbred colony maintained at the animal house of the National Centre for Cell Science, Pune, India. Pancreatic islets were isolated from the Balb/C mice following the previously reported protocol from our lab [14]. Islets were purified from a collagenase (Sigma, St Louis, MO, USA) digested pancreas on a Ficoll gradient and cultured in RPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% FCS (Trace Biosciences PTY Ltd., New South Wales, Australia) and antibiotics (penicillin 200 U/mL and streptomycin 0.2 mg/mL) in culture grade Nunclon flasks at 37 °C, 5% CO_2 for 48 hours.

Induction of Diabetes in Balb/C Mice by Streptozotocin

After fasting overnight, adult Balb/C mice were injected with freshly prepared streptozotocin (200 mg/kg body weight, prepared in chilled citrate buffer, pH 4.5). Pancreases were excised from mice showing severe hyperglycemia (blood glucose level greater than 350 mg/dL).

In Vitro Streptozotocin Treatment of Isolated Normal Balb/C Mouse Islets

Islets cultured for 48 hours as described above were washed twice with PBS and then counted under an inverted microscope (Olympus, Tokyo, Japan). Islets were then transferred into 35 mm plastic Petri dishes with PBS and given streptozotocin (5 mM) treatment for one hour at 37 °C. A streptozotocin solution was prepared in PBS for *in vitro* experimentation.

In Vitro Proliferation of Intra Islet Precursor Cells

Cultured islets (for 48 hours) were overlaid on matrigel (BD Biosciences, Bedford, MA, USA) (50 µL of reconstituted matrigel used to coat each well of a six well plate) coated plates and fed with DMEM/F-12 (1:1) media with 5% FCS (Trace Biosciences PTY Ltd., New South Wales, Australia) for another 24 hours and left for attachment at 37°C in a CO_2 incubator. Then, the medium was totally removed along with the floating cell population, the wells were washed once with same media and then replenished by well-defined serum free medium containing DMEM/F-12, 1:1 (8 mM glucose) basal medium supplemented with 1 g/L supplement (5 mg/L insulin, 5 mg/L transferrin, 5 mg/L selenium), 50 µM 2-mercaptoethanol, 2 g/L bovine serum albumin, 10 mM nicotinamide (Sigma, St Louis, MO, USA), and antibiotics (penicillin 200 U/mL and streptomycin 0.2 mg/mL). The media was changed on the 4th day and the plates were replenished with same media containing keratinocyte growth factor (KGF) (Sigma, St Louis, MO, USA) (10 ng/mL). The intra islet precursor cells gave rise to patches of well-organized polygonal epithelial-like cells within 4 days of the addition of serum-free media. In time, these cell patches started differentiating and gave rise to islet-like budding structures which eventually detached and started floating in the medium.

Dithiazone Staining

To evaluate the total number and assess the specificity of the newly generated islets from diabetic mouse pancreases, islets were collected along with the media in 1.5 mL Eppendorf tubes, centrifuged, washed once with PBS and then collected in 35 mm Petri dishes for dithiazone (DTZ) staining (Sigma, St Louis, MO, USA). A 39 mM DTZ stock solution was prepared by dissolving 100 mg of DTZ in 10 mL DMSO, filtered, aliquoted and stored at -15°C). Routine staining was carried out by adding 10 µL DTZ stock to islets suspended in 1 mL Krebs Ringer bicarbonate buffer (pH 7.4) with: N-(2-hydroxyethyl)piperazine-N'-(4-butanesulfonic acid) (HEPES) (10 mM) and incubated at 37°C for 10 to 15 minutes. The stained islets were counted under an inverted microscope (Olympus, Tokyo, Japan).

Immunostaining of Insulin

The islets cultured in the six well plates were collected and washed 3 times with PBS. Cytospin preparation was performed at 500 *g* for 5 minutes. The islets were fixed with 4% paraformaldehyde (w/v) in PBS (pH 7.2) with 0.5% bovine serum albumin and 0.1% sodium azide for 10 minutes at room temperature. They were washed with 0.5% bovine serum albumin and 0.1% sodium azide. Permeabilization was carried out with 0.2% Triton X-100 for 5 minutes at 4˚C and the remaining procedure was carried out at room temperature. Draining was used to remove reagents between each step, but drying of the specimens was avoided. Sufficient reagent was used to cover each specimen (approximately 20 µL was used) which was then blocked with 2% bovine serum albumin for 30 min to block non-specific binding of IgG. The specific primary polyclonal antisera (goat polyclonal anti-insulin; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100) was used. The antibody was diluted in PBS containing 1% bovine serum albumin. The specimen was incubated overnight with primary antibody at 4°C. After overnight incubation the slides were washed 3 times in PBS with bovine serum albumin and sodium azide; each was washed for 5 min, then incubated with fluorescein isothiocyanate (FITC) labeled secondary antibody (rabbit antigoat IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500) for 1 hr at room temperature and then washed 3 times again with PBS and mounted on mowiol (antifade). Quantification was performed using LSM 510 software on a Zeiss workstation (Carl Zeiss Meditec AG, Jena,

Germany) using an argon air cooled laser of wavelength 488 nm (LASOS Lasertechnik GmbH, Jena, Germany).

Immunostaining for Epithelial Cell Marker Cytokeratins (CK-7 and CK-19)

For CK staining of epithelial cell patches, islets (derived from diabetic mouse pancreases) were seeded on 13 mm round thermanox coverslips (Nunc Inc., Naperville, IL, USA) kept in 35 mm plastic petri dishes. They were cultured for 4 days to obtain patches of epithelial cells under similar conditions to those described earlier. Coverslips were then removed and washed twice with PBS. Fixation, permeabilization and blocking steps are similar to those described for insulin immunostaining. Staining was carried out using mouse monoclonal IgG_1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:40) as a primary antibody followed by a secondary antibody (FITC-goat antimouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:250). Analysis was done with set-up similar to those described earlier for insulin immunostaining.

Insulin Secretion Assay

The islets were washed twice for 10 minutes each in fresh modified Krebs Ringer bicarbonate buffer (pH 7.4) supplemented with 1 mg/mL bovine serum albumin (Sigma, St Louis, MO, USA), 1 mM glucose and 10 mM HEPES. The Krebs buffer was kept at 37°C and was gassed with a mixture of O_2 (95%) and CO_2 (5%, pH 7.4). The islets were then transferred into 6 new well plates and incubated at 37°C with the same fresh modified Krebs buffer with 0.5% bovine serum albumin, and with 5.5 mM and then 16.6 mM glucose for 1 hr as a stationary set-up. At the end of each incubation period, the buffer was removed from the 6 well plates. Each condition was assayed in three ELISA tests (each test used triplicate samples). The supernatant was collected and stored at -20˚C and assayed later on using insulin ELISA (BioSource Europe S.A., Nivelles, Belgium).

Western Blot Analysis

The cells were lysed and separated on 12% SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia, Bukhimhamshire, United Kingdom) using a transfer cell (HoeferT22, Amersham, Tokyo Japan). After blocking the membranes for 1 hr in 5% BSA in PBST (phosphate buffer saline plus 0.1% Tween 20), the membrane was washed with PBST and incubated overnight in primary insulin antibody (goat polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1,000 dilution) and washed three times with PBST. The membrane was then incubated for 2 hours at room temperature in PBST containing HRP-rabbit antigoat IgG (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by several washings with PBST. Immunoreactive bands were visualized using an ECL system (Amersham, Tokyo, Japan) and exposed to film (Kodak, Kalina Santacruz, Mumbai, India).

RT-PCR for PDX-1 Gene

Total RNA was extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). A reverse transcription reaction was carried out using total RNA (500 ng) at 50°C for 30 minutes. The PCR conditions were 35 cycles of 94°C (1 minute), annealing temperature (1 minute), 72°C (1 minute) (Thermoscript® RT-PCR system; Invitrogen Life Technologies, Carlsbad, CA, USA). The final extraction was at 72°C for 10 minutes. The respective sequences of PCR primer PDX1 (Gemini Biotech, Alachua, Florida) (50°C, 569 bp) designed on software available in our lab were:
(Sense) 5’CAGTGAGGAGCAGTACTAC3’
(Antisense) 5’GATGTGTCTCTCGGTAAGTT3

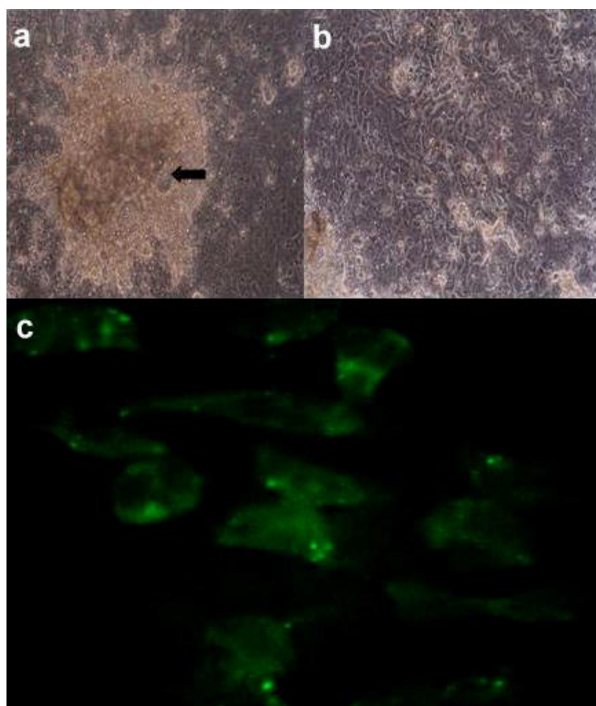

Figure 1. Mother explant culture of islets (arrow) from STZ-treated experimental diabetic mouse pancreas giving rise to epithelial-like cells on matrigel coated plates within 24 hours after islet attachment (Figure 1a). Epithelial-like cell monolayer containing intra islet precursor cells formed in the culture with well-defined serum-free medium (Figure 1b). Intra islet precursor cells of epithelial-like cell sheet stained with epithelial cell specific marker CK-19; CK immunostaining was examined by confocal microscopy (green fluorescence, Figure 1c).

ETHICS

The local animal research committee approved the experimental protocol. The animals used were cared for in accordance with the principles of the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 86-23 revised 1985).

STATISTICS

Insulin secretion is reported as mean±SD. Two-tailed P values of less than 0.05 were considered statistically significant. Statistical evaluations were performed by means of the Student t-test by using the SPSS/PC+® software version 5.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In Vitro Proliferation and Expansion of Intra Islet Precursor Cells and Their Characterization

The islets seeded on the matrigel coated plates attached within 24 hours when cultured in serum-free medium without KGF. The proliferation of epithelial cells was observed from the periphery of the islet mother explant (Figure 1a) and formed a confluent monolayer in the subsequent four days (Figure 1b).These epithelial-like cell sheets stained positive for cytokeratins CK-19 and CK-7 (data are shown only for CK-19) as revealed by immunocytochemistry (Figure 1c). However, these epithelial cells remained unstained when subjected to DTZ staining.

In Vitro Differentiation of Intra Islet Precursor Cells

These epithelial cell monolayers successively started showing intense zones of activity throughout the cell monolayer in response to the addition of KGF in the serum free culture medium. Subsequently, these intense activity zones converted themselves into islet-like clusters (Figure 2a) which, in turn, developed

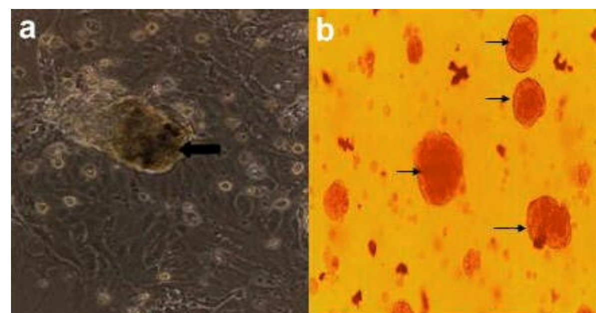

Figure 2. In response to the growth factor KGF, the intra islet precursor from the epithelial cell sheet starts differentiating into islet like clusters which primarily form an outgrowth-like structure within the cell sheet as shown by the arrow (Figure 2a). The islet bud turns into a well-defined islet in another 4 days under the influence of KGF. Specificity of the neogenerated islets of varying size were examined by islet specific stain DTZ; neogenerated islets appeared crimson red in colour (islets shown by arrow marks) after DTZ staining (Figure 2b).

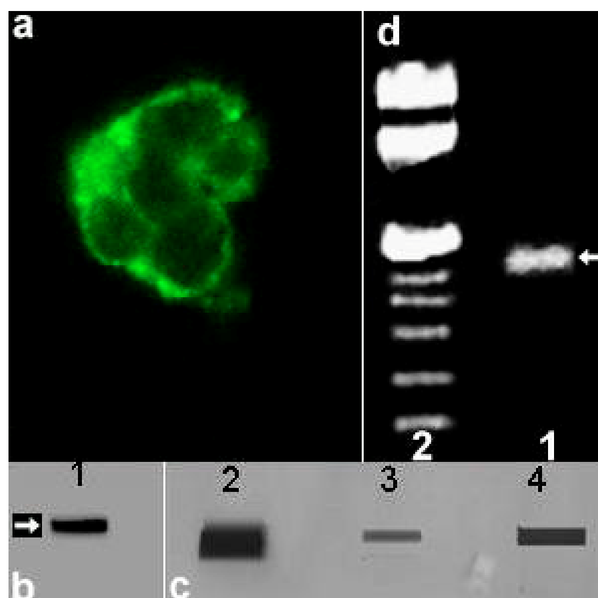

Figure 3. Newly generated islets stained with insulin antibody. Immunostained islets were analyzed by confocal microscopy (green fluorescence showing the insulin immunopositive islets, Figure 3a). Western blot analysis of neogenerated islets; arrow mark representing the 5.8 KDa insulin band (Figure 3b, 3c) in recombinant human insulin 25 µg (1), islet extract of 20 islets from adult non-diabetic mice (2), islet extract of 10 neoislets (3), and islet extract of 20 neoislets (4). RT-PCR analysis of islet specific molecular marker PDX-1 (Figure 3d); arrow showing the amplification of 569 bp expected size of product (1) and DNA molecular weight marker (2).

into well-defined islets that stained positive for DTZ and appeared crimson red in colour (Figure 2b).

Structural and Functional Characterization of Newly Generated Islets

The presence of insulin in these newly generated islets was confirmed by immunofluoresence (Figure 3a) as well as by western blotting (Figure 3c) with a characteristic band of insulin at 5.8 KDa as a positive control (Figure 3b). Characteristic features of the newly generated islets were further analyzed by gene expression of the endocrine pancreatic marker using the RT-PCR method. mRNA expression of PDX1 (pancreatic duodenal homeobox transcription factor) was demonstrated in these islets (Figure 3d). Insulin secretary response of these newly generated islets was recorded against the secretagogues glucose and L-arginine, and compared with that of freshly isolated islets (Table 1). It was observed that neo-islets were highly responsive to the L-arginine challenge as compared to the glucose challenge whereas neo-islets were less responsive to glucose as compared to freshly isolated islets. The insulin release by the neo-islets in response to L-arginine stimulation was comparable (P=0.104) to that of freshly isolated islets in response to 16.5 mM glucose stimulation indicating differential secretagogue response.

DISCUSSION

In the present investigation, we showed that the intra islet precursor cells from the pancreases of experimental diabetic mice had the potential to differentiate *in vitro* into neo-islets when stimulated appropriately. The number of islets obtained from the intra islet precursor cells from the pancreases of diabetic mice is comparable to that obtained from non-diabetic mice (data not shown) suggesting the preservation of precursor cells under the influence of hyperglycemia. Our study supports the report of Guz *et al.* [12] wherein the presence of intra islet precursor cells has been shown in diabetic mice.

Table 1. Comparison of insulin secretion levels of control islets (islets from non-diabetic mice) with neoislets against glucose and L-arginine secretagogue response. Quantitative analysis of insulin secretion was done by ELISA. Insulin levels were measured in µIU/mL against a standard curve. Inter-assay variability is less than 5.2%. (Three observations were made for each cell).

Secretagogue response	Islets (control)	Neo-islets	P value
Glucose			
- Basal (5.5 mM)	136.7±2.7	4.32±2.2	<0.001
- High (16.5 mM)	263.0±3.2[a]	5.68±2.6	<0.001
L-arginine (10 mM)	4.08±3.4	257.6±3.1[a]	<0.001

[a] L-arginine is acting as a secretagogue for the newly generated islets as the level of insulin secreted by the neoislets in response to L-arginine is comparable (P=0.104) to the level of insulin secreted by control islets in response to 16.5 mM glucose.

However, our study differs from their study [12] regarding normalization of the euglycemia by insulin treatment as we did not make any attempt to achieve normoglycemia in our mouse model. Our finding is of therapeutic importance in the treatment of diabetes as there is hope of stimulating the intra islet precursor cell population within a diabetic pancreas which retains its integrity despite the diabetes. Another feature of our study is the demonstration of a distinct precursor cell population within islets exhibiting $CK\text{-}19^{+}$ and $CK\text{-}7^{+}$ and IN^{-} markers similar to that of ductal epithelial cells [10] which was not reported earlier.

Our study also indicates the differential action of streptozotocin *in vitro* and *in vivo*. The intra islet precursor cells remain intact in streptozotocin-induced experimental diabetic mice [12]. However, *in vitro* streptozotocin treatment leads to the destruction of these precursor cells due to the cytotoxic effect of streptozotocin (data not shown). We found that streptozotocin-induced diabetic mice retain their pool of intra islet precursor cells intact as depicted in Figure 1 even in a severe hyperglycemic state and give rise to mature functional islets upon appropriate induction (Figure 2). However, when islets isolated from normal mice are treated with streptozotocin, they lose their pool of intra islet precursor cells, eventually losing their capacity to differentiate into mature islets.

It is interesting to note here the role of extra cellular matrix protein and the growth factor in the induction of proliferation and differentiation of cells into desired lineage. The islets seeded onto matrigel coated plates in a serum free medium proliferated into an epithelial cell monolayer that stained positive for CK-19 and CK-7 and, at the same time, stained negative for IN. In the absence of matrigel, we did not get any proliferation of epithelial cells from islets (data not shown), proving the importance of extra cellular matrix. The addition of KGF to serum-free medium made the cell monolayer differentiate into islet-like clusters which, in time, were converted into functional islets emphasizing the potential of KGF not only as pancreatic duct mitogen [15] but also as a differentiating agent for the intra islet precursor cell population. KGF is a member of the fibroblast growth factor (FGF) family it specifically binds to KGFR/KGFR2 and is mainly synthesized in stromal cells, stimulating epithelial cells via the KGFR receptors. KGF is known as an inducer of pancreatic ductal epithelial cell proliferation [16] and has also been shown to cause changes in epithelial growth, differentiation and insulin expression [17]. However, the role of KGF in inducing proliferation and differentiation of intra islet precursor cells is shown here for the first time. Such an *in vitro* system which developed during the course of this investigation allows tailormade regulation of the differentiation of intra islet precursor cells depending upon the presence or absence of KGF. Our study brings *ex vivo* regeneration therapy in diabetes closer as suggested by Yamaoka [15], promoting beta-cell proliferation and neogenesis.

The present work thus provides direct evidence for the differentiation of intra-islet precursor cells from the pancreas of a diabetic mouse into islets as confirmed by the presence of insulin and the expression of the PDX1 gene (Figure 3). PDX1 is a homeodomain containing transcription factor which binds and transactivates the insulin promoter and is crucial for pancreatic organogenesis. In postnatal animals, PDX1 is expressed in pancreatic beta cells and recent studies suggest that PDX1 is required for functions in mature beta cells [18, 19, 20]. Thus, PDX1 serves distinct roles at different developmental stages [21]. The expression of PDX1 in newly generated islets not only supports these observations but also indicates their ontogeny. In this respect, the secretagogue response of these islets to L-arginine (Table 1) further depicts their foetal ontogeny. In other words, the *in vitro*-generated islets show an embryonic pattern of development supporting Hackle’s theory that “Ontogeny recapitulates phylogeny”.

The present data is of significance to a diabetic subject whose islet beta-cell mass could be increased by stimulation of their

intra islet precursor cells thus compensating for beta-cell loss in diabetes. Furthermore, this strategy could also be applied to expand the beta-cell mass of the transplanted islets [22], thus eliminating the need of transplanting high number of islets.

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Abbreviations CK: cytokeratin; DTZ: diphenyle dithio carbazone; FITC: fluorescein isothiocyanate; HEPES: N-(2-hydroxyethyl)piperazine-N'-(4-butanesulfonic acid); IN: insulin; INGAP: islet neogenesis associated protein; KGF: keratinocyte growth factor; PBS: phosphate buffered saline; PBST: phosphate buffer saline plus Tween 20

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