Islet-Like Cell Aggregates Generated from Human Adipose Tissue Derived Stem Cells Ameliorate Experimental Diabetes in Mice

Vikash Chandra¹, Swetha G¹, Sudhakar Muthyala², Amit K. Jaiswal³, Jayesh R. Bellare³, Prabha D. Nair², Ramesh R. Bhonde¹*

¹ Tissue Engineering and Banking Laboratory, National Centre for Cell Science, Ganeshkhind, Pune, Maharashtra, India, ² Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India, ³ Department of Chemical Engineering, Indian Institute of Technology Bombay, Mumbai, Maharashtra, India

Abstract

Background: Type 1 Diabetes Mellitus is caused by autoimmune destruction of insulin producing beta cells in the pancreas. Currently available treatments include transplantation of isolated islets from donor pancreas to the patient. However, this method is limited by inadequate means of immuno-suppression to prevent islet rejection and importantly, limited supply of islets for transplantation. Autologous adult stem cells are now considered for cell replacement therapy in diabetes as it has the potential to generate neo-islets which are genetically part of the treated individual. Adopting methods of islet encapsulation in immuno-isolatory devices would eliminate the need for immuno-suppressants.

Methodology/Principal Findings: In the present study we explore the potential of human adipose tissue derived adult stem cells (h-ASCs) to differentiate into functional islet like cell aggregates (ICAs). Our stage specific differentiation protocol permit the conversion of mesodermic h-ASCs to definitive endoderm (Hnf3b, TCF2 and Sox17) and to PDX1, Ngn3, NeuroD, Pax4 positive pancreatic endoderm which further matures in vitro to secrete insulin. These ICAs are shown to produce human C-peptide in a glucose dependent manner exhibiting in-vitro functionality. Transplantation of mature ICAs, packed in immuno-isolatory biocompatible capsules to STZ induced diabetic mice restored near normoglycemia within 3–4 weeks. The detection of human C-peptide, 1155±165 pM in blood serum of experimental mice demonstrate the efficacy of our differentiation approach.

Conclusions: h-ASC is an ideal population of personal stem cells for cell replacement therapy, given that they are abundant, easily available and autologous in origin. Our findings present evidence that h-ASCs could be induced to differentiate into physiologically competent functional islet like cell aggregates, which may provide as a source of alternative islets for cell replacement therapy in type 1 diabetes.

Introduction

Type 1 diabetes is characterized by the autoimmune destruction of β cells, resulting in life-long dependency on insulin injection that often results in complications of hypo- or hyperglycemia. Although Edmonton protocol for islet transplantation is the most preferred therapy available for type 1 diabetes, a major obstacle with this therapy is the limited supply of cadaveric donor islets in retention to the high demand of eligible patients and the need for lifetime immunosuppression [1–4]. Recent studies have demonstrated that Embryonic stem cells (ESCs) [5–7], Induced pluripotent stem cells (iPSCs) [8,9], and adult stem cells like bone marrow (BM) [10], pancreas [11,12], liver [13], umbilical cord blood [14], Wharton’s jelly [15], placenta [16], could be differentiated into insulin producing cells. ESCs have tremendous pluripotency, however, ethical/legal issues and risks of teratoma formation limit its use in translational medicine. In this scenario, Adipose-derived adult stem cells (ASCs) appear to be an ideal population of stem cells for practical cell replacement therapy, given that they are abundant, autologous and easy availability [17]. Even lean adult men and women have at least 3.0–4.5 kg of adipose tissue, and in individuals with severe obesity, adipose tissue can constitute up to 45 kg or more of body weight [18], sufficient quantities of ASCs can be harvested and cultured from a minimum of 1.0 g of fat. Moreover adipose tissue is a remarkable organ that play important role in glucose homeostasis and hormone production (adipokine) [19]. Some of the adipokines like leptin and adiponectin have direct impact on glucose homeostasis and fatty acid oxidation [20].

Findings by Timper et al [21] and Lee et al [22] with human ASCs and our earlier experience with murine ASCs [23] indicate...
that h-ASCs are the ideal candidates for cell replacement therapy in diabetes.

In the present study we explore the potential of h-ASCs to differentiate into functional islet like cellular aggregates (ICAs) with stage specific differentiation conditions. The differentiated ICAs are packed in immunosoluation capsules for transplantation studies. These ICAs when transplanted into STZ induced diabetic mice can restore near normoglycemia within 3–4 weeks. The detection of human specific C-peptide in transplanted mice serum further strength our differentiation approach. Our studies thus raise the possibility that patient own adipose tissue can serve as a source of ASCs to differentiate into functional autologous ICAs for cell replacement therapy in type 1 diabetes.

Results

Human adipose tissue derived adult stem cells (h-ASCs) were isolated from the resected fat tissue samples (n = 6, female donor of age group 25–50 years) as earlier reported [17,21]. The initial culture of stromal vascular fraction resulted in the growth of plastic adherent cell population with typical mesenchymal morphology. Although freshly isolated h-ASCs showed heterogeneous phenotype in culture, single fibroblastoid cell populations were clonally expanded which exhibited homogenous morphology. Four clones of h-ASCs were evaluated for all the differentiation studies and all experiments were carried out using h-ASCs of passages 4–8. The homogeneity of the cloned population was confirmed by the triple stained FACS analysis of h-ASCs at passage-4 which showed uniform co-expression of CD90, CD44 and CD29 surface markers (Figure 1).

Surface phenotype characterization of h-ASCs with flow cytometry showed that, unlike murine ASCs, h-ASCs showed high expression of CD13, CD59, CD105 (Figure S1). h-ASCs showed expression of cytoskeletal proteins vimentin, alpha-smooth muscle actin, nestin and extracellular matrix protein fibronectin (Figure 2A–D). Cells were highly mitotic in culture and expressed proliferation marker Ki-67 (Figure 2E). h-ASCs exhibited in-vitro competence to differentiate into adipogenic, chondrogenic and osteogenic lineages upon specific induction as confirmed by Oil red O (Figure 2G), Saffranin-O (Figure 2H) and Alizarin-red staining respectively (Figure 2I).

Differentiation of h-ASCs to definitive endoderm ICAs

h-ASCs were grown in low adherence culture dishes and exposed to serum free media (SFM-A) for 2 days. By 24–48 hrs, cells migrated and clustered to become islet like cells aggregates (ICAs) (Figure 3). By day 2, the cells started expressing endoderm markers like HNF-3 beta, TCF-2 and Sox-17 as compared to undifferentiated h-ASCs (Figure 4A a–c). qRT-PCR analysis of day2-ICAs showed increased transcript abundance of the endoderm markers HNF-3 beta/FoxA2 (103 fold), CK-19 (10 fold) and Sox-17 (2 fold) as compared to undifferentiated h-ASCs (Figure 4B) The highest mRNA expression levels of Sox17, a definitive endoderm marker was observed by day 5.

Pancreatic Endoderm differentiation of ICAs derived from h-ASCs

In the next stage, to achieve pancreatic endoderm differentiation day2-ICAs were exposed to SFM supplemented with taurine (SFM-B), a non-essential amino acid involved in the development of pancreatic β-cells [24,25]. After 2 days of incubation in SFM-B, in the final stage of differentiation, ICAs were exposed to differentiation media supplemented with β-cell maturation factors.
Figure 2. In vitro characterization of clonally expanded h-ASCs for mesenchymal markers. Representative immunostaining of cytoskeletal protein vimentin (A), alpha smooth muscle actin (B), nestin (C), fibronectin (D) and cell proliferation marker Ki-67 (E) with immunofluorescence staining. Negative control staining with fluorescence conjugated secondary antibodies shown (F). Cell nuclei were stained with DAPI. Scale bar, 20 μm. h-ASCs exhibited competence to differentiate into adipogenic, osteogenic and chondrogenic lineages in vitro upon specific induction as confirmed by Oil red O (G), Saffranin-O (H) and Alizarin-red (I) staining respectively.

doi:10.1371/journal.pone.0020615.g002

Figure 3. Differentiation strategy for h-ASCs derived ICAs, day 14 ICAs stained positive with islet specific DTZ staining. h-ASCs in monolayer when exposed to SFM A undergo cell clustering and aggregation. Subsequent exposure and incubation of h-ASCs in SFM B on day 3 and SFM C on day 5 results in the formation of mature Islet like cell aggregates (ICAs). By day 14, the ICAs become fully differentiated and mature and stain positive for DTZ. Abbreviations: SFM, Serum free media; ITS, Insulin-transferrin-selenium; BSA, Bovine serum albumin, GLP, Glucagon Like Peptide, DTZ, dithizone.

doi:10.1371/journal.pone.0020615.g003
like GLP-1 and nicotinamide (SFM-C) and maintained for additional 7–8 days. A significant up regulation in the expression of pancreatic endoderm markers as well as key transcription factors involved in pancreas development like PDX-1, Ngn3, NeuroD, Pax-4, Nkx2.2, Nkx6.1, Pax-6, Isl-1 and glucose transporter Glut-2 were observed in d14- ICAs over undifferentiated h-ASCs (Figure 5).

Transcript levels of PDX-1, Ngn-3, Pax-4 and Isl-1 in d14 ICAs were comparable to that of 12 week old human fetal pancreas. By day-14 of differentiation the ICAs stained positive for DTZ, a zinc-chelating agent known to selectively stain pancreatic beta cells (Figure 3).

The mature day-14 ICAs showed enhanced transcript levels of insulin, glucagon, somatostatin, pan-polypeptide and ghrelin as compared to undifferentiated h-ASCs. The transcript abundance of pancreatic hormones in mature ICAs was lower than that of human fetal pancreas (12 week). Undifferentiated h-ASCs consistently exhibited transcripts of ghrelin (Figure 6B). Similar to our earlier observation with murine ASCs derived ICAs (23), the Day 14 human ICAs also showed co-expression of insulin and somatostatin (Figure S2A). The mature ICAs also showed proliferative potential depicted by Ki-67 expression (Figure S2B).

The gene profiling studies for adipose tissue-specific markers during ICA differentiation showed that the transcript level of
leptin and adiponectin in mature ICAs were comparable to that of h-ASCs (Figure S3A). Immunostaining of h-ASCs and d14-ICAs showed low levels of leptin expression (Figure S3B).

**Static stimulation and total C-peptide content of mature ICAs**

In this study, day-14 h-ASCs derived ICAs were first incubated with 5.5 mM glucose for 1 h to measure the basal level of c-peptide release which is a direct indicator of the amount of de novo insulin released. The same ICAs were subsequently incubated for additional 1 h in 22 mM glucose to measure glucose-stimulated C-peptide release (n = 3). Total C-peptide content of day 14 ICAs measured up to 47.68±15.20 pM per 60 min when exposed to 5.5 mM glucose (p = 0.070). When stimulated with 22 mM glucose, the total C-peptide content of ICAs increased to 80.61±8.92 pM per 60 minute (p = 0.05) confirming their in vitro functionality and ability to respond to...
glucose (Figure 8A). Total intracellular C-peptide content of day 14 differentiated ICAs was calculated and value was compared with undifferentiated h-ASCs (equal number of starting cells (2×10^6 cells) were taken to compare the two values). It was observed that h-ASC derived day 14 ICAs contained up to 1.76±0.45 mg/l (582.56±148.95 pM) (p = 0.02) as compared to 0.051±0.006 mg/l (p = 0.005) of C-peptide in undifferentiated h-ASCs (n = 3) (Figure 8B). However in day-14 ICAs cell numbers are potentially not equal and therefore this makes direct comparison of C-peptide content difficult.

Mature ICAs show hypoglycemic effect in experimental diabetic mice

The physiological competence of mature day-14 ICAs to maintain glucose homeostasis in vivo was evaluated by transplantation of ICAs in STZ induced diabetic mice. ICAs (1000–1200) encapsulated in calcium-alginate were packed into biocompatible capsules of PU-PVP-IPN [26] and transplanted into the peritoneal cavity of diabetic mice (n = 6). By 2–3 weeks, mice transplanted with mature ICAs showed reduction in blood glucose levels. The mice maintained lowering of blood glucose (180–190 mg/dl) upto eight week of follow up. However, it was observed that normoglycemia was not restored in these mice. Transplantation of undifferentiated h-ASCs (n = 6) to STZ-induced diabetic mice also showed lowering of blood glucose (250 mg/dl) which was maintained but failed to restore normoglycemia within the stipulated time (Figure 8C). To determine whether the ICAs could regulate blood glucose levels independently of the endogenous pancreatic β-cells, we measured level of human as well as mouse C-peptide in blood serum of all experimental mice. We measured up to 1155±165 pM (3.49±0.50 mg/l) serum level of human C-peptide in mice transplanted with day 14 ICAs on day 28 post transplantation. The level of human C-peptide in mice transplanted with undifferentiated h-ASCs (2×10^6), 26th days post transplantation measured to 1009±383 pM (3.05±1.16 mg/l) The mouse C-peptide concentration was found to be very low to undetectable (~0.002 mg/l) in control diabetic mice (Figure 8D). ICAs retrieved from the transplanted mice after 4 weeks showed good cellular aggregation and viability (data not shown). The ICAs and undifferentiated h-
ASCs retrieved from the transplanted mice showed insulin and C-peptide expression (Figure S4A and B).

Discussion

In this study we assessed the potential of h-ASCs to produce functional pancreatic hormone producing islet like cell aggregates from resected human fat samples. Our isolation protocol for Stromal vascular fractions consistently yielded multipotent mesenchymal cells expressing CD29+/CD44+/CD90+ surface antigen profile. These cells demonstrated high competence to differentiate to adipogenic, osteogenic and chondrogenic lineages. h-ASCs isolated in culture differed in its expression of surface antigens from murine ASCs isolated by similar procedure. h-ASCs distinctly showed high expression of CD90 and low expression level of Sca-1 as against m-ASCs [23]. The surface antigen profile differed from human BM derived mesenchymal cells as well indicating that despite the similarities in morphology, mesenchymal cells isolated from different sites/tissue of origin possess distinct molecular signatures and identities. In accordance with earlier reports, we observed a stringent dependence of h-ASCs on FGF for proliferation and survival in vitro [27]. Subsequently all media and differentiation cocktails were supplemented with 2 ng/ml FGF.

To achieve differentiation to pancreatic lineage, specific stepwise conditions were formulated as per earlier reported protocol.

Figure 7. Confocal immunocytochemistry of day14 ICAs shows the expression of pancreatic endoderm markers. Confocal optical sections of ICAs stained for PDX-1(a), C-peptide (b), Insulin (c), Somatostatin (d), and Glut 2 (e). Nuclei are stained by DAPI (4', 6-diamidino-2-phenylindole). (Scale bar = 20 μm).
doi:10.1371/journal.pone.0020615.g007
We achieved pancreatic endoderm differentiation of the mesodermic h-ASCs with activin A, sodium butyrate, 2-mercaptoethanol, ITS and 2 ng/ml FGF (SFM-A). We found that addition of FGF stimulate the initial process of cell aggregation and cluster formation, an important step in the development and differentiation of pancreas via FGF-receptor on h-ASCs [28]. Definitive endoderm differentiation was identified by the expression of Hnf3b/Foxa2 in (~80%) [29], TGF-2 [30] and Sox-17 [31] in 50%-60% cells in day 2 differentiated cells. Increased mRNA expression of Hnf3β, CK-19 and Sox-17 in comparison to undifferentiated h-ASCs, further confirmed their definitive endoderm differentiation. Sox-17 and Hnf3β expressions were highest during day-5 of differentiation. The expression of these genes occurs in a similar pattern as observed during pancreatic development. The low cell attachment culture dish and initial high plating density (2×10^6 cells generated around 400–600 ICAs) proved to be highly efficient in helping cells to form 3D aggregates similar to our earlier observations with murine ASCs. By 24–36 h of initial plating, h-ASCs formed clusters (ICAs) with compact cellular aggregation.

Previous studies have shown that supplementation of taurine, a non essential amino acid modulate glucose homeostasis and islet function [24,25]. The taurine supplemented with nicotinamide and GLP-1 in the differentiation cocktail resulted in the up regulation of a number of genes (PDX1, Ngn3, NeuroD, Pax4, Nkx2.2, Nkx6.1, Pax-6, Isl-1 and pancreas specific glucose transporter Glat-2) involved in pancreatic beta cells development cascade. Additionally, detectable level of transcripts of two pancreatic progenitor specific transcription factors Nkx2.2/ Nkx6.1 and Isl-1 in undifferentiated h-ASCs make it more suitable candidate stem cells to differentiate into pancreatic lineage. Nkx2.2 and its down stream factor Nkx6.1 participate in the
major pathway of β cell formation in the pancreas and knockout mice of these factors develop with reduced number of mature β cells [32].

Mature ICAs showed transcript abundance and expression of pancreatic endocrine hormones. We observed that cells within the differentiated ICAs co-express insulin and somatostatin which is in concordance with earlier reports on newly differentiated mid-gestational human fetal pancreas cells which co-express multiple hormones [33,34]. Glucose static stimulation index of mature d14 ICAs measured up to 1.69 for C-peptide. Total intracellular human C-peptide content in d14 ICAs was 1.76±0.45 µg/l. As our differentiation cocktail includes ITS, to rule out any possibilities that insulin might be absorbed by cells during in vitro culture [35,36], we always calculated the level of human c-peptide for de novo synthesis of insulin. Previous reports on the differentiation potential of human ASCs by Timper et al and Lee et al [21,22] although novel, did not demonstrate in vitro/ invivo functionality of the differentiated cells. To the best of our knowledge this is the first study demonstrating in vitro/in vivo functionality of ICAs differentiated from h-ASCs.

STZ induced mice were considered diabetic when the blood glucose levels were higher than 200 mg/dl. For in vivo transplantation studies we used PU-PVP-IPN biocompatible capsules as an immune-isolatory devise. Our initial studies have shown that a minimum of 1000–1200 ICAs/capsule/mice is required to achieve significant hypoglycemia in STZ induced diabetic mice. The capsule exhibited biocompatibility and did not evoke any immune rejection in the implanted mice. The porosity of the capsule is such that it easily allows nutrient exchange between the packed ICAs and the peritoneal cavity. By three weeks, all the mice transplanted with ICAs, showed lowering of blood glucose levels and maintained the status for two months of tracking. However, transplanted ICAs could not bring down blood glucose to normal physiological levels. This may be circumvented by optimizing the number of ICAs to be transplanted to experimental mice. To determine whether the h-ASCs derived ICAs could regulate blood glucose levels independently of the mice endogenous pancreatic β cells; we have estimated human C-peptide level in serum of experimental mice. Random serum levels of human C-peptide after four weeks of transplantation was measured up to 1155±165 pM (3.49±0.50 µg/l) (n = 3), is comparable with the earlier reports with human embryonic stem cells [37] where they found serum levels of human C-peptide up to 852±263 pM fasting and 2361±565 pM in glucose stimulated. It was interesting to observe that transplantation of undifferentiated h-ASCs to STZ induced diabetic mice showed moderate lowering of blood glucose levels, which also reflected in the serum levels of human C-peptide1009±383 pM (3.05±1.16 µg/l). This shows that the autocrine and paracrine factors of regenerating pancreas and hyperglycemic local diabetic micro-environment of mice may contribute to a small extend, the differentiation of ASCs as earlier reported for human bone marrow MSCs [38]. This observation calls for detailed studies to explore the potential of h-ASCs to differentiate into pancreatic lineage and regulate glucose homeostasis with minimum manipulation. We also checked the pattern of some of adipokines (leptin and adiponectin) during the course of differentiation. It was found that the after initial down regulation of these genes they reappear at the end of differentiation. Leptin and adiponectin are considered to be good adipokines that play important role in glucose homeostasis and fatty acid oxidation. The role of adipokines in glucose metabolism is a highly studied subject. Earlier reports show that in vitro chronic exposure of human islets to leptin induced IL-1beta, leading to impair beta cell function [39]. However recent studies demonstrate that Leptin monotherapy improves several of the metabolic imbalances caused by insulin deficient type 1 diabetes in rodents by CNS dependent mechanism [40]. It would be interesting to find out whether these adipokines play any regulatory role in glucose homeostasis in h-ASC derived ICAs. Scanning electron microscopy based ultra-structural comparison of these 3D ICAs revealed that their surface topography and arrangement between cells is very similar to that of normal pancreatic islets [41]. All the four clones used in this study exhibited equal frequency of in vitro differentiation and maturation to ICAs. The inherited expression of Idl-1/Pax-6/Nks6.1/Somatostatin transcripts in h-ASCs, its ease in availability and autologous origin, make it an eminent candidate for cell replacement therapy in diabetes. To the best of our knowledge this is the first detailed report demonstrating the differentiation potential of human ASCs to glucose responsive pancreatic hormone expressing islet like cellular 3D aggregates. We anticipate that our work would add on to the ongoing research to find alternative sources of islets for cell replacement therapy in type 1 diabetes.

Conclusion
Our findings present evidence that h-ASCs could be induced to differentiate into physiologically functional islet like cell aggregates, which may provide a source of alternative islets for cell replacement therapy in type 1 diabetes.

Materials and Methods

ASCs Cell Isolation and Expansion
Resected fat tissue samples were obtained during different abdominal surgeries from female donors (20 to 40 years old). Collection of the sample was approved by the institutional review board (IEC-Institutional ethical committee, NCCS, National Center for Cell Science, Pune) and written consent was obtained from all donors. Adipose tissue samples (n = 6) were washed and digested in PBS supplemented with 2% bovine serum albumin (BSA) and 0.2% collagenase-type-II [Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com] pre-warmed to 37°C for 45 minutes. The cells were centrifuged (400 g for 4 minutes) at room temperature (RT) in 1–2 ml sterile PBS containing 2% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich). Supernatant containing mature adipocytes were discarded. The pellet containing stromal vascular fraction was resuspended in expansion medium.

Expansion medium. Dulbecco’s modified eagle’s medium [DMEM]/Ham’s F-12 (GIBCO) with 10% FBS, antibiotics and supplemented with 2 ng/ml Fibroblast Growth Factor (FGF, Sigma-Aldrich #F0291). The cells were plated at density of 2×105/cm2 into T25 culture flasks.

Serial dilution method was used to generate single-cell clonogenic culture. Briefly, 104 isolated human ASCs (h-ASCs) were suspended in 1 ml culture medium. Repeated serial dilution was carried out to achieve a final dilution of 100 cells in 10 ml medium. For single-cell culture, 100 µl of the diluted cell suspension was transferred into each well of a 96-well plate containing 100 µl of culture medium.

In Vitro Differentiation of mE-ASCs to ICAs
Differentiation was carried out in three stages as reported earlier [23]. Cells were counted for initial seeding density and 1×106 cells/cm2 were resuspended in SFM-A and plated to ultra-low attachment tissue culture plates (Corning, Fisher scientific) or small glass petri plate (8×106 Cells in 2” glass petri plate makes ~800–1200 ICAs). SFM-A contained DMEM/F12 (1:1) (GIBCO) with 17.5 mM glucose, 1% BSA Cohn fraction V, fatty acid free.
(#A8906, Sigma-Aldrich), 1× Insulin-transferrin-selenium (ITS, 5 mg/L insulin+5 mg/L transferrin+5 mg/L selenium), 4 nM activin A, 1 mM sodium butyrate, 50 μM 2-mercaptoethanol and 2 ng/ml Fibroblast Growth Factor. The cells were cultured in this media for 2 days. On 3rd day media was changed to SFM-B that contained DMEM/F12 (1:1) with 17.5 mM glucose, 1% BSA, ITS and 0.3 mM Taurine. The cell aggregates were cultured in this medium for another 2 days and shifted to SFM-C on 5th day.

SFM-C contained DMEM/F12 (1:1) with 17.5 mM glucose, 1.5% BSA, ITS, 3 mM Taurine, 100 mM Glucagon-like peptide 1 (GLP-1) (amide fragment 7–36, Sigma Aldrich), 1 mM nicotineamide and 1× non-essential amino acids (NEAA). The cell aggregates were fed with fresh SFM-C medium every 2 days for another 12–14 days. All chemicals were purchased from Sigma Aldrich unless otherwise indicated.

Quantitative Real-Time Polymerase Chain Reaction

Tissue/Cells samples were frozen in Trizol (Invitrogen, Carlsbad, CA). Total RNA was isolated from duplicate and triplicate samples as per the manufacturers’ instructions, measured on ND-100 spectrophotometer (Nanodrop Techno lics, Wilmington, DE) and 2 μg of RNA was used for cDNA synthesis per 20 μl reaction. cDNA was amplified using Reverse Transcription System Kit (Invitrogen). Primary antibodies were incubated overnight at 4°C, washed with PBS and then incubated with the secondary antibodies at RT for 1 h. Slides were washed in PBS and mounted with Vectashield (Vector-Laboratories, CA, http://www.vectorslab.com). DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) was used to visualize nuclei. The sources of antibodies and dilutions used are summarized in supplementary Table S1. Confocal images were captured using a Zeiss-LSM 510 laser scanning microscope using a 63×/1.3 oil objective with optical slices, ~1–2 μm. All results are representative fields confirmed from at least 5 different experiments.

In Vitro multilineage Differentiation Studies

Adipogenesis, chondrogenesis and osteogenesis for h-ASCs were carried out in the appropriate induction media according to the manufacturer’s protocol (Cambrex, MD http://www.cambrex.com). The differentiation phenotype was documented using oil red-O for adipocytes, Safranin-O staining for chondrocytes and Alizarin staining for osteocytes. Dithizone (DTZ) (Sigma-Aldrich) stain of 10 mg/ml in DMSO (dimethyl sulphoxide) concentration was used to stain islet like cell aggregates (ICAs).

Total insulin content and release assays

For glucose stimulated insulin release assay, about 200–300 day-14 ICAs were handpicked in eppendorf tube. ICAs were then washed three times with PBS and incubated with freshly prepared KRBH buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.1 mM MgCl₂, 25 mM NaHCO₃, with 10 mM HEPES buffer and 0.1% BSA) without glucose for 3 to 6 h. ICAs were incubated with 100 μl KRBH buffer containing 3.5 mM glucose for 1 h at 37°C. The supernatant were collected and the same ICAs were further incubated 22 mM glucose for additional 1 h at 37°C. The human C-peptide concentrations were measured using (Ultra sensitive human C-peptide EIA kit, Merckodia, Uppsala, Sweden), mouse C-peptide concentrations were measured using mouse C-peptide-I+II EIA kit (#YK013, Yanaihara Institute Inc., Japan, www.yanaihara.com). Total Intracellular C-peptide was extracted by incubating ICAs/h-ASCs overnight in acid ethanol (18 ml 10 M HCl/liter 70% ethanol) at 4°C. The total C-peptide content of ICAs was estimated after sonicating the ICA pellet in 200 μl acid/ethanol. The conversion factor for human C-peptide is 1 μg/1 corresponds to 331 pmol/l (pM).

In-vivo transplantation studies

Male Swiss albino mice aged 8–10 weeks were used for transplantation studies. The study was conducted adhering to the institution’s guidelines for animal husbandry and has been approved by IAEC-NCCS/PCPSEA (Institutional animal ethical committee-NCCS/Committee for the Purpose of Control and Supervision of Experiments on Animals. Approval number: IACUC-Institutional Animal Care and Use Committee, EAF-Experimental Animal Facility/2006/B-56). Streptozotocin (STZ, Sigma-Aldrich) was injected intraperitoneally at 160 mg/kg of body weight, freshly dissolved in citrate buffer (pH 4.5). Blood glucose (BG) was measured by ACCU-CHEK (Roche, www.roche.com) from snipped tail. Only mice with BG levels stably above 200 mg/dl after the STZ injection were used for transplantation studies. For transplantation, around 1000–1200 day-10 ICAs were washed with PBS, suspended in 100 μl of sodium-alginolate solution (1-2% w/v Alginic acid; Sigma-Chemical, USA, in 0-85% saline), packed into a biocompatible capsule (Polyurethane-Poly vinyl pyrolidone-Interpenetrating network (PU-PVP-IPN), developed by SCTIMST, Kerala, India [23]). The ICAs-alginat capsule were sealed and dipped into 0-1N acetic-acid and CaCl₂ (0-15 M in distilled water) solution for gelling. These encapsulated ICAs were then implanted into the peritoneal cavity of diabetic mice. Fasting blood glucose levels
were measured regularly using a glucometer after the mice were fasted for 6 h.

Scanning Electron microscopy (SEM)
The differentiated ICAs were incubated in 2.5% phosphate buffer (0.1 M, pH 7.4) glutaraldehyde solution for 24 hrs. The tissue was post fixed in 1% OsO4 for 2 h at 4°C, dehydrated and dried. Specimens were then glued onto stubs, covered with gold in an S150 sputter coater and examined with a Hitachi S4000 field emission (Hitachi Ltd. Tokyo, Japan) scanning electron microscope operating at 10 kV. (IIT, Mumbai).

Statistical Analysis
Values are expressed as mean fold change ± S.E.M. from three different experiments unless otherwise indicated. Statistical analysis was done using Student’s two tailed t-test to determine the significance between different conditions. Prism4, Graphpad Software, San Diego, (http://www.graphpad.com) was used for all analysis.

Supporting Information
Figure S1  FACS based surface phenotypic characterization of h-ASCs. h-ASCs clones at passage 4 (n = 4), cells were harvested and labeled with antibodies against CD44-PE, CD13-PE, CD73-PE, CD90-PE, CD105-PE, CD59-PE, CD166-PE, and CD117-PE and Isotype control PE, CD73-PE, CD90-PE, CD105-PE, CD59-PE, CD166-PE, and CD117-PE. We wish to thank Dr. Satish Patki, Patki Hospital, Kollapur (Maharashtra, India) for providing resected human fat samples. We thank Swapnil Walke for FACS analysis and Ashwini Atre for assistance with confocal microscopy.

Author Contributions
Conceived and designed the experiments: VC RRB. Performed the experiments: VC SG AKJ SM. Analyzed the data: VC SG. Contributed reagents/materials/analysis tools: RRB JRB PDN. Wrote the paper: VC

References
1. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, et al. (2000) Ilet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid free immunosuppressive regimen. N Engl J Med 343: 230–238.
2. Berner T, Tosco C (2006) Monitoring of the islet graft. Diabetes Metab 32: 503–512.
3. Shapiro AM, Ricordi C, Hering BJ, Aschincloss H, Linfaldslad R, et al. (2006) International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355: 1318–1330.
4. Fung S, Barr M, Roberts J, Oberbauer R, Kaplan B (2006) Developments in clinical islet, liver thoracic, kidney and pancreas transplantation in the last 5 years. Am J Transplant 6: 1759–1767.
5. Lumskey N, Blondel O, Laeng P, Velasco I, Ravin R, et al. (2001) Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 292: 1389–1394.
6. D’Amour KA, Bang AG, Eliazaer S, Kelly OG, Agudlick AD, et al. (2006) Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 11: 1392–1401.
7. Jiang J, Au M, Lu K, Edsperter A, Korbutt G, et al. (2007) Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 25: 1940–1953.
8. Tateishi K, He J, Tarasova O, Liang G, D’ Alessio AC, et al. (2006) Generation of insulin-secreting islet-like clusters from human skin fibroblasts. J Biol Chem 281: 31601–31607.
9. Mader R, Chen S, Saito M, Landsberg T, Yagasaki L, et al. (2006) Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci U S A 103: 15768–15773.
10. Xie QP, Huang H, Xu B, Dong X, Gao SL (2009) Human bone marrow mesenchymal stem cells differentiate into insulin-producing cells upon microenvironmental manipulation in vitro. Differentiation 77: 403–491.
11. Noguchi H, Naziruddin B, Shimoda M, Fujita Y, Chugo D, et al. (2010) Induction of insulin-producing cells from human pancreatic progenitor cells. Transplant Proc 42: 2081–2083.
12. Seaberg RM, Smalder SR, Kieffer TJ, Enoklopopov G, Aggar Z, et al. (2004) Identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22: 1115–1124.
13. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, et al. (2002) In vitro trans differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 99: 8076–8083.
14. Sun B, Roh KH, Lee SR, Lee YS, Kang KS (2007) Induction of human umbilical cord blood derived stem cells with embryonic stem cell phenotypes into insulin producing islet-like structure. Biochem Biophys Res Commun 354: 919–923.
15. Chao KG, Chao KF, Fu YS, Liu SH (2006) Islet-like clusters derived from mesenchymal stem cells in Wharton’s Jelly of the human umbilical cord for transplantation to control type 1 diabetes. PLoS One 3: e1451.
16. Chang CM, Kao CL, Chang YL, Yang MJ, Chen YC, et al. (2007) Placenta-derived multipotent stem cells induced to differentiate into insulin-positive cells. Biochem Biophys Res Commun 357: 414–420.
17. Zook PA, Zhu M, Ashjian P, De Ugarte DA, Huang JJ, et al. (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 12: 4727–4729.
18. Tran TT, Kahro CR (2010) Transplantation of adipose tissue and stem cells: role in metabolism and disease. Nat Rev Endocrinol 6: 195–213.
19. Rabe K, Lehlke M, Parhofer KG, Broedl UC (2008) Adipokines and insulin resistance. Mol Med 14: 741–751.
20. Antonina-Parente B, Feve B, Fellahi S, Bastard JP (2008) Adipokines: the missing link between insulin resistance and obesity. Diabetes Metab 34: 2–11.
21. Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, et al. (2006) The link between insulin resistance and obesity. Diabetes Metab 32: 2081–2083.
22. Lee J, Han DJ, Kim SC (2008) In vitro differentiation of human adipose tissue-derived stem cells into cells with pancreatic phenotype by regenerating pancreas extract. Biochem Biophys Res Commun 357: 547–551.
23. Chandra V, G S, Phadnis S, Nair PD, Bhonde RR (2009) Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. Stem Cells 27: 1941–1953.

Figure S1  FACS based surface phenotypic characterization of h-ASCs. h-ASCs clones at passage4 (n = 4), cells were harvested and labeled with antibodies against CD44-PE, CD13-PE, CD73-PE, CD90-PE, CD105-PE, CD59-PE, CD166-PE, and Isotype control PE, CD73-PE, CD90-PE, CD105-PE, CD59-PE, CD166-PE, and CD117-PE. We wish to thank Dr. Satish Patki, Patki Hospital, Kollapur (Maharashtra, India) for providing resected human fat samples. We thank Swapnil Walke for FACS analysis and Ashwini Atre for assistance with confocal microscopy.

Figure S2  Characterization of day 14 ICAs. Immunostaining of day 14 ICAs for the expression of insulin and somatostatin (A), proliferation marker Ki-67 and insulin (B). The nuclei of the cells were stained with DAPI (4’, 6-diamidino-2-phenylindole) (Scale bar = 20 μm).

Figure S3  Expression of adipose tissue specific markers during the course of differentiation. SYBR-Green based qRT-PCR analysis was carried out for day5 (Dif-d5) and day10 (Dif-d10) ICAs for adipose tissue specific markers like leptin, and adiponectin compared with undifferentiated h-ASCs (Undif). Relative levels of gene expression were normalized to the GAPDH mRNA level(A). h-ASCs and day 14 ICAs are stained for the adipokine leptin (B). The nuclei of the cells were stained with DAPI (4’, 6-diamidino-2-phenylindole) (Scale bar = 20 μm).

Table S1  List of primers and antibodies used in this study.

Acknowledgments
We wish to thank Dr. Satish Patki, Patki Hospital, Kollapur (Maharashtra, India) for providing resected human fat samples. We thank Swapnil Walke for FACS analysis and Ashwini Atre for assistance with confocal microscopy.

Author Contributions
Conceived and designed the experiments: VC RRB. Performed the experiments: VC SG AKJ SM. Analyzed the data: VC SG. Contributed reagents/materials/analysis tools: RRB JRB PDN. Wrote the paper: VC SG RRB.

Generation of Islets from Human Adipose Stem Cells

PLoS ONE | www.plosone.org 11 June 2011 | Volume 6 | Issue 6 | e20615

www.plosone.org
24. Boujendar S, Arany E, Hill D, Remacle C, Reusens B (2003) Taurine supplementation of a low protein diet fed to rat dams normalizes the vascularization of the fetal endocrine pancreas. J Nutr 133: 2820–2825.

25. Cherif H, Reusens B, Ahn MT, Hoet JJ, Remacle C (1998) Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. J Endocrinol 159: 341–348.

26. George S, Nair PD, Rashid MV, Bhonde RR (2002) Nonporous polyurethane membranes as islet immunosolation matrices–biocompatibility studies. J Biomater Appl 16: 327–340.

27. Rider DA, Dombrowski C, Sawyer AA, Ng GH, Leong D, et al. (2008) Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells. Stem Cells 26: 1598–1608.

28. Hardikar AA, Marcus-Samuels B, Geras-Raaka E, Raaka BM, Gershengorn MC (2003) Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates. Proc Natl Acad Sci U S A 100: 7117–7122.

29. Sasaki H, Hogan BL (1993) Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. Development 118: 47–59.

30. Barbacci E, Reber M, Ott MO, Beullat C, Hueta F, et al. (1999) Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. Development 126: 4795–4805.

31. Kanai-Azuma M, Kanai Y, Gad JM, Tajima Y, Taya C (2002) Depletion of definitive gut endoderm in Sox17-null mutant mice. Development 129: 2367–2379.

32. Schider JC, Jensen PB, Taylor DG, Becker TC, Knop FK, et al. (2005) The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A 102: 7297–7302.

33. De Kriger RR, Aanstoot HJ, Krausenburg G, Reinhard M, Visser WJ, et al. (1992) The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev Biol 153: 360–373.

34. Polak M, Bouchareb-Banaei L, Scharfmann R, Czernichow P (2000) Early pattern of differentiation in the human pancreas. Diabetes 49: 225–232.

35. Rajagopal J, Anderson WJ, Kume S, Martinez OI, Methon DA (2003) Insulin staining of ES cell progeny from insulin uptake. Science 299: 363.

36. Hansson M, Toming A, Frandsen U, Petri A, Rajagopal J (2004) Artificial insulin release from differentiated embryonic stem cells. Diabetes 53: 2603–2609.

37. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, et al. (2008) Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 26: 443–452.

38. Phadnis SM, Joglekar MV, Dalvi MP, Muthyala S, Nair PD (2011) Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage in vivo. Cytotherapy 13: 279–293.

39. Maedler K, Sergeev P, Elshe JA, Mathe Z, Bosco D, et al. (2004) Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. Proc Natl Acad Sci U S A 101: 8138–8143.

40. Fujikawa T, Ghaun JC, Sakata I, Ramadori G, Coppari R (2010) Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. Proc Natl Acad Sci U S A 107: 17291–17296.

41. Morini S, Braun M, Oueri P, Cicalero L, Elias G, et al. (2006) Morphological changes of isolated rat pancreatic islets: a structural, ultrastructural and morphometric study. J Anat 209: 381–392.