Treatment of Type 1 Diabetes with Adipose Tissue-Derived Stem Cells Expressing Pancreatic Duodenal Homeobox 1

Guiting Lin 1, Guifang Wang 1, Gang Liu 1, Li-Jun Yang 2, Lung-Ji Chang 3, Tom F. Lue 1, and Ching-Shwun Lin 1

1Knuppe Molecular Urology Laboratory, Department of Urology, School of Medicine, University of California, San Francisco, CA 94143-0738, USA
2Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32610-0275, USA.
3Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida 32610-0266, USA.

Abstract

Due to the limited supply of donor pancreas, it is imperative that we identify alternative cell sources that can be used to treat diabetes mellitus. Multipotent adipose tissue-derived stem cells (ADSC) can be abundantly and safely isolated for autologous transplantation and therefore is an ideal candidate. Here, we report the derivation of insulin-producing cells from human or rat ADSC by transduction with the pancreatic duodenal homeobox 1 (Pdx1) gene. RT-PCR analyses showed that native ADSC expressed insulin, glucagon, and NeuroD genes that were upregulated following Pdx1-transduction. ELISA analyses showed that the transduced cells secreted increasing amount of insulin in response to increasing concentration of glucose. Transplantation of these cells under the renal capsule of streptozotocin-induced diabetic rats resulted in lowered blood glucose, higher glucose tolerance, smoother fur, and less cataract. Histological examination showed that the transplanted cells formed tissue-like structures and expressed insulin. Thus, ADSC expressing Pdx1 appear to be suitable for treatment of diabetes mellitus.

Keywords

adipose tissue-derived stem cells; streptozotocin-induced diabetes mellitus; Pdx1 transduction; insulin; beta cells

Introduction

Diabetes mellitus (DM), which causes about 5 percent of all deaths globally each year (http://www.who.int/en), affects 246 million people worldwide presently and will affect 380 million by 2025 (1). DM consists of two diseases, type 1 and type 2, with distinct etiology. Type 1 DM, which accounts for 5–10% of all DM cases, is caused by autoimmune destruction of the pancreatic β-cells, resulting in a reduction of insulin production. In contrast, Type 2 DM is initially caused by insulin resistance and later progresses to β-cell dysfunction that also results in a reduction of insulin production (2,3). Initial treatment for type 2 DM is usually a combination of diet, exercise, and drugs that stimulate insulin secretion from β-cells, reduce...
hepatic glucose output, or increase insulin sensitivity in target cells. However, as the disease progresses further, β-cell mass decreases (due to increased apoptosis and reduced neogenesis/replication) (4,5), these treatments become insufficient to achieve or sustain the desired blood glucose level. Therefore, the majority of type 2 diabetic patients will require insulin therapy, typically between 5 and 10 years after the initial diagnosis (6).

It is well established that the risk of diabetic complications is dependent on the degree of glycemic control in diabetic patients. However, while aggressive insulin therapy that maintains glucose levels near the normal range reduces the risk of secondary complications, patients often find such control difficult to achieve and suffer an increased risk of hypoglycemia (7). This is due to the fact that external insulin injection cannot mimic the physiological control that pancreatic β cell-derived insulin secretion exerts on the body’s glycemia. By contrast, replacement of a patient’s islets of Langerhans by whole pancreas transplantation or by islet transplantation is the only treatment capable of achieving normoglycemia (8,9). However, due to the scarcity of donor pancreases and the necessity of patients undergoing life-long immunosuppression, this therapy can be afforded to only a limited number of patients (8,9). Thus, a renewable source of a physiologically competent substitute for primary human pancreatic islets is highly desirable.

Many alternative strategies have been considered for the replacement of damaged β–cells, including the use of pancreatic β–cell lines, regenerating native islet cells, embryonic stem cells (ESC), and bone marrow stem cells (BMSC). Through the manipulation of culture conditions BMSC have been shown to produce low levels of insulin (10–13). However, the low-level insulin production and the unlikelihood of reproducing the culture conditions in vivo make this approach unfit for clinically applications. To overcome these shortcomings, Li et al (14) and Karnieli et al (15) separately sought to generate stable insulin-producing cell lines by transducing BMSC with the pancreatic duodenal homeobox 1 (Pdx1) gene. The Pdx-1-transduced BMSC expressed several islet-specific genes, produced and released insulin in a glucose-regulated manner, and, when transplanted into streptozotocin-induced diabetic mice, reduced blood glucose levels.

Adipose tissue-derived stem cells (ADSC) are Isolated from the stromal vascular fraction (SVF) of adipose tissue and bear a strong resemblance to BMSC (16–18). Unlike BMSC, however, ADSC can be obtained in large quantities at low risks (19,20). In addition to being more abundant and easily accessible, the adipose tissue yields far more stem cells than bone marrow on a per gram basis (5,000 vs. 100–1,000) (19). Therefore, it is reasonable to expect that ADSC may become the preferred choice of ASC for future clinical applications. In regard to their potential as a surrogate β–cell source, two recent papers (21,22) have shown that, through the manipulation of the culture media, ADSC could become C-peptide-positive cells, suggesting the potential of ADSC as a surrogate β–cell source. However, these two studies did not describe whether the induced ADSC secreted insulin or whether they were responsive to glucose challenge. Furthermore, similar to the situations with BMSC (10–13), the transient nature of induction makes this approach not clinically applicable. As such, the present study sought to generate stable insulin-producing cells by transducing ADSC with Pdx1 gene. Using both human and rat ADSC, we show that the Pdx1-transduced cells secreted insulin, were responsive to glucose challenge, and were capable of reducing glucose levels in STZ-induced diabetic rats.

Materials and Methods

ADSC isolation and culture

We have previously described the isolation of human and rat ADSC (23,24). Human tissues were obtained during routine abdominoplasty following informed patient consent and
according to the guidelines set by our institution’s Committee on Human Research. Rat tissues were obtained with approval from our Institutional Animal Care and Use Committee.

**Lentiviral transduction**

Lentiviral constructs containing mouse Pdx1, Pdx1-VP16 (PV16, a genetically modified form of Pdx1), or green fluorescence protein (GFP, as control) have been described previously (25,26). These constructs were transduced into ADSC overnight at a multiplicity of infection (MOI) of 20. After two days of recovery, the transduced cells were switched to differentiation medium (DMEM with 23 mM glucose), grown to confluence, and split 1:3 to approximately 5×10^5 cells per 10-cm dish. They were thereafter split 1:3 every 7–10 days.

**Measurement of insulin secretion**

Cells grown in a 6-well plate (6×10^4 cells/well) were washed with PBS 3 times and then incubated in KRB buffer (120 mM NaCl, 2.5 mM CaCl_2, 1.1 mM MgCl_2, 25 mM NaHCO_3, and 0.1% BSA) for 1h. Glucose was then added to final concentrations of 0, 5.6, 16.7, 23, and 33 mM. After another hour of incubation, the KRB buffer was collected and analyzed with a commercial ELISA kit for rat insulin (Linco Research, St. Charles, MO). These experiments were done in triplicate.

**Establishment of type 1 diabetic rat model**

Streptozotocin (STZ) is known to selectively destroy pancreatic β-cells (27) and has been used in our previous study to establish a type 1 diabetic rat model (28). In the present study, 2-month-old female Sprague-Dawley rats were made hyperglycemic by intraperitoneal injection of 60 mg of STZ (in 20 mM citrate buffer) per kg of body weight. Control rats were injected with 20 mM citrate buffer. Afterwards, body weight and blood glucose levels were monitored weekly with samples obtained from the tail vein using Accutrend strip (Roche Diagnostics, Indianapolis, IN). When blood glucose reached 300 mg/dl (in ~7 days), the STZ-treated rats were subjected to injection with IP-ADSC or saline under the renal capsule.

**Cell transplantation under renal capsule**

Rats were anesthetized by exposure to 1–3% isoflurane, and a 2-cm incision was made through the skin and muscle of the left flank. The wound was rinsed with 1 ml PBS containing penicillin/streptomycin), and the kidney was externalized. A small lateral cut was made in the capsule, and 2×10^6 IP-ADSC in 1 ml PBS or PBS only was dispensed beneath the capsule. The kidney was returned to the abdominal cavity, and the incision closed using surgical clips. The injection site was marked with a 7-0 nylon suture for identification.

**Glucose tolerance test**

Intraperitoneal glucose tolerance testing (IPGTT) was performed as described by the Animal Models of Diabetes Complications Consortium (www.amdcc.org). Rats were fasted for 7 h and then injected intraperitoneally with 1 mg of glucose in saline per gram of body weight. Blood glucose levels were monitored for 2 h at 30-min intervals in samples obtained from the tail vein.

**Immunocytochemistry and fluorescence microscopy**

Cells were seeded onto a coverslip inside each well of a 6-well plate at 40–60% confluence in DMEM. The next day, the cells were rinsed with PBS and fixed with ice-cold methanol for 5 min. The cells were rinsed with PBS again and permeabilized with 0.05% triton X-100 for 8 min. After another PBS rinse, the cells were incubated with 5% horse serum for 1 h and then with anti-insulin antibody (Abcam Inc., Cambridge, MA, 1:500) for 1 h. After 3 rinses with
PBS, the cells were incubated with Texas red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. After 3 rinses with PBS, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 µg/ml, Sigma-Aldrich, St. Louis, MO) for 5 min. The stained cells were examined with Nikon Eclipse E600 fluorescence microscope and the images recorded with Retiga 1300 Q-imaging camera.

**Histology and Immunofluorescence**

Tissue samples were fixed in cold 2% formaldehyde and 0.002% saturated picric acid in 0.1 M phosphate buffer, pH 8.0, for 4 hours followed by overnight immersion in buffer containing 30% sucrose. The specimens were then embedded in OCT Compound (Sakura Finetic USA, Torrance, CA) and stored at −70 °C until use. Fixed frozen tissue specimens were cut at 10 microns, mounted onto SuperFrost-Plus charged slides (Fisher Scientific, Pittsburgh, PA) and air dried for 5 min. These slides were stained with hematoxylin and eosin (HE staining) for general histological examination. For immunofluorescence examination, the slides were placed in 0.3% H2O2/methanol for 10 min, washed twice in PBS for 5 min and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 min at room temperature. After draining this solution from the tissue section, the slides were incubated overnight at 4°C with anti-insulin antibody (Abcam Inc., Cambridge, MA, 1:500) for 1 h. Control tissue sections were similarly prepared except no primary antibody was added. After rinses, the sections were incubated with Texas red-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by staining with 4',6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 µg/ml, Sigma-Aldrich, St. Louis, MO).

**Western blot analysis**

Cells were lysed in buffer containing 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, aprotinin (10 µg/ml), leupeptin (10 µg/ml), and PBS. Cell lysates containing 20 µg of protein were electrophoresed in SDS–PAGE and then transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. Detection of protein on the membrane was performed with the ECL kit (Amersham Life Sciences Inc., Arlington Heights, IL) using anti-Pdx1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The resulting images were analyzed with ChemiImager 4000 (Alpha Innotech Corporation, San Leandro, CA) to determine the integrated density value (IDV) of each protein band. Before reprobing with anti-b-actin antibody, the membrane was stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 10 mM 2-mercaptoethanol at 56 °C for 30 min and then washed 4 times in 1× TBST.

**RT-PCR analysis**

Cells were homogenized in Tri-Reagent RNA extraction solution (Molecular Research Center, Cincinnati, OH). The extracted RNAs were further treated with DNase I to remove traces of contaminating DNA. Quantity and integrity of RNAs were examined by spectrophotometer and agarose gel electrophoresis, respectively. The RNAs were reverse-transcribed into a “library” of complementary DNAs (cDNAs) using SuperScript reverse transcriptase and its accompanying reagents (Invitrogen, Carlsbad, CA). Briefly, 2.5 µg of RNA were annealed to 0.4 µg of oligo-dT primer in a 12-µl volume. Four µl of 5× buffer, 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP, and 1 µl of reverse transcriptase were then added to bring the final reaction volume to 20 µl. After one hour of incubation at 42 °C, the mixture was incubated at 70 °C for 10 min to inactivate the reverse transcriptase. Eighty µl of TE buffer were then added to make a 5× diluted library. A portion of this library was further diluted to various concentrations (up to 100× dilution). One µl of each dilution was then used in a 10-µl polymerase chain reaction (PCR) to identify the optimal input within the linear amplification range. In addition to the 1-
μl diluted library, the PCR mixture consisted of 10 ng of each of a primer pair (Table 1) and reagents supplied with the Taq polymerase (Invitrogen). PCR was performed in DNA Engine (MJ Research, Inc., Watertown, MA) under calculated temperature control. The cycling program was set for 35 cycles of 94 °C, 5 sec; 55 °C, 5 sec; 72 °C, 10 sec, followed by one cycle of 72 °C, 5 min. The PCR products was electrophoresed in 1.5% agarose gels in the presence of ethidium bromide, visualized by UV fluorescence, and recorded by a digital camera connected to a computer.

Statistical analysis

Data were analyzed with Prism 4 (GraphPad Software, Inc., San Diego, CA) and expressed as means ± standard deviation. Student-t test was used to compare between two groups (e.g., treated and control). One-way ANOVA analysis of variance was used to compare among 3 or more groups. Differences with P< 0.05 were considered significant.

Results

Characteristics of human and rat ADSC cell lines

We have previously reported the isolation and characterization of human and rat ADSC (23, 24). Adherence of freshly isolated cells to plastic culture dishes allowed the selection of morphologically homogeneous populations of ADSC lines. These adherent cells were fibroblastic in shape and grew at rate of about one doubling every 3 days. At the 3rd passage they were mostly negative for endothelial marker CD31 and for hematopoietic marker CD34. They also expressed very low levels of stem cell markers Oct4, SSEA1, and telomerase. However, despite the lack of obvious stem cell markers, both of these human and rat ADSC were capable of differentiating into endothelial and neuron-like cells (24,29).

Transduction and expression of Pdx1 gene

In the present study we chose 2 human and 5 rat ADSC lines as candidates for transduction with Pdx1. Additionally, the 2 human ADSC lines were also transfected with Pdx1-VP16 (PV16), which is a genetically modified form of Pdx1 (25,26). Transduction with GFP served as a negative control as well as for the determination of transduction efficiency, which was found to be greater than 95% (percentage of cells displaying green fluorescence). One week after transduction the Pdx1- and PV16-transduced cells, but not the GFP-transduced cells, exhibited a morphology suggesting the secretion of insulin granules (Fig. 1), which was subsequently confirmed by immunofluorescence staining (Fig. 1). RT-PCR and Western blot analyses also confirmed Pdx1 expression in Pdx1- and PV16-transduced cells but not in GFP-transduced cells (Fig. 2). Finally, ELISA analysis showed the static production of insulin in Pdx1-transduced cells (Fig. 2); hence, insulin-producing ADSC (IPADSC).

Expression of Pdx1-associated genes

Pdx1 controls the expression of several key genes during pancreatic development, including insulin, glucagon and NeuroD genes. RT-PCR analysis showed that human ADSC expressed glucagon and NeuroD constitutively (Fig. 3). They also expressed low levels of insulin, which were upregulated in IPADSC. Control rat ADSC expressed insulin, glucagon, and NeuroD at lower levels than IPADSC did. The specificity of expression of these three genes in control and IPADSC was confirmed by the lack of such expression in rat urethral smooth muscle cells (Fig. 3).

Increased insulin production in response to glucose challenge

As mentioned above, Pdx1 transduction resulted in the generation of IPADSC, which released approximately 30 ng/dl of insulin into the culture medium (Fig. 2). Quantitative analysis
showed that these cells produced increasing levels of insulin in response to increasing concentrations of glucose (Fig 4).

**Treatment of diabetic rats**

Type 1 DM rats were established by intraperitoneal injection of STZ. One week after STZ injection, these rats had blood glucose levels in the range of 300 to 400 mg/dl, while control rats injected with citrate buffer only had normal blood glucose levels. Ten of the STZ-treated rats were subsequently treated with IPADSC while the other 10 STZ-treated rats were treated with saline. Treatment was done by transplantation of approximately 2 million rat IPADSC or injection of saline under renal capsule. These rats’ fast blood glucose levels and body weight were then monitored weekly for 7 weeks. As shown in Fig. 5A, throughout the entire course IPADSC-treated rats had lower blood glucose levels than saline-treated rats (P<0.05). Body weights of IPADSC-treated rats were also better than those of saline-treated rats although the difference was not statistically significant (P>0.05) (Fig. 5B). At the end of the 7th week, all rats were examined for fur appearance and extent of cataract, tested for glucose tolerance, and then sacrificed for histological assessment. The results showed that IPADSC-treated rats had healthier-looking (less scruffy) fur and lesser extent of cataract than saline-treated rats (Fig. 6). IPADSC-treated rats also had higher levels of glucose tolerance (Fig. 7). Finally, histological examination of the transplanted kidneys showed the presence of transplanted cells, which were stained positive for insulin (Fig. 8).

**Discussion**

Recent progress in islet transplantation points to the possibility of restoring normoglycemia in type 1 DM patients. However, the limited supply of primary human islets demands the identification of alternative β-cell sources. Toward fulfilling this demand, several recent studies have demonstrated the generation of insulin-producing cells from liver, intestinal, and stem cells. Among various types of stem cells, ESC and BMSC are the best studied, but ADSC, due to their abundance, ease of isolation, and lack of ethical concerns, are attractive alternatives. Indeed, their potential as surrogate pancreatic cells has been demonstrated in two recent studies (21,22). In the present study we further investigated the differentiation of ADSC into insulin-producing cells and their therapeutic potential.

We initially chose two human ADSC lines for the study because they are more clinically relevant than non-human ADSC. Transduction of these cells with Pdx1 or PV16 resulted in the appearance of insulin-like granules in the cytoplasm and culture medium. ELISA analysis confirmed the secretion of insulin by the Pdx1- and PV16-transduced cells. RT-PCR analysis, however, showed that native human ADSC expressed insulin mRNA, albeit at lower levels than transduced ADSC. This is in agreement with a previous study, which also showed the expression of insulin mRNA in native human ADSC (22). Repeated experiments also showed that human ADSC expressed two other Pdx1-regulated genes, namely glucagon and NeuroD, and this again is in agreement with the study by Lee and Kim (22). The significance of the expression of pancreas-associated genes in native ADSC is presently unknown.

We subsequently transplanted the Pdx1-transduced human ADSC in STZ-induced diabetic rats. The transplanted rats did not receive immunosuppressive drugs because ADSC have been shown to possess immunosuppressive properties (30,31) and to survive in xenotransplantations (32,33). However, because the diabetic rats treated with human IPADSC showed only modest improvement in blood glucose levels (possibly due to insufficient number of transplanted cells and/or immune reaction, data not shown), we decided to switch to rat ADSC.

Similar to the situation with human ADSC, Pdx1-transduced rat ADSC also produced insulin-like granules in the cytoplasm and in the culture medium. Pdx1 transduction apparently
upregulated the expression of insulin, glucagon, and NeuroD mRNA in rat ADSC. However, because untransduced rat ADSC also expressed low levels of these mRNAs, we tested the specificity of the RT-PCR analysis by examining rat urethra smooth muscle cells (RUSMC). The results showed that RUSMC did not express any of these 3 genes. Thus, both human and rat ADSC appeared to express pancreatic genes at the transcriptional level although they do not produce insulin at significant levels as shown in the present study and previously published studies (21,22).

With Pdx1 transduction, rat ADSC produced increasing amounts of insulin in response to increasing concentrations of glucose. Transplantation of these cells into STZ-induced diabetic rats resulted in modest improvement of blood glucose levels. Surprisingly, despite still having high blood glucose levels, the treated rats showed signs of improved health such as smoother fur and lesser extent of cataract. Since the treated rats were still diabetic, we wonder whether the improved health was due to the transplanted cells’ other beneficial effects. Indeed, it has been shown that ADSC are capable of producing anti-inflammatory cytokines and angiogenic factors, which could potentially improve the diabetes-associated inflammatory and ischemic conditions (34). On the other hand, the modest improvement in blood glucose level could possibly be explained by the suboptimal number and/or the functional immaturity of the transplanted cells. While these explanations remain speculative, we believe that the data presented in this study do point to the possibility of using ADSC as a cell-based treatment for type 1 DM.

Acknowledgments

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References


Figure 1. Generation of insulin-producing cells

Human ADSC were transduced with GFP (control), Pdx1 or Pdx1-VP16 (PV16). Phase contrast microscopy showed that the Pdx1- and PV16-transduced cells had insulin-like granules in the cytoplasm and culture media. Immunofluorescence microscopy showed that the Pdx1- and PV16-transduced cells stained positive for insulin (red). Nuclear staining with DAPI (blue) was used to locate cells.
Figure 2. Verification of Pdx1 and insulin expression

Human and rat ADSC were transduced with GFP (control), Pdx1 or Pdx1-VP16 (PV16). Expression of Pdx1 in these cells was examined by western blotting (with β-actin serving as control, Panel A) and RT-PCR (Panel B). Static insulin production by human ADSC (in DMEM with 23 mM glucose) was further examined by ELISA (Panel C).
Figure 3. Examination of pancreatic gene expression

Human and rat ADSC were untransduced (C) or transduced with GFP or Pdx1. These cells and rat urethra smooth muscle cells (RUSMC) were examined by RT-PCR for the expression of Pdx1, insulin, glucagon, and NeuroD (with β-actin serving as control, Panel A). Statistical analyses of the results for the human and rat cells are presented in Panels B (n=3) and C (n=5), respectively. Asterisks indicate significant differences (P<0.05) between Pdx1-transduced cells and untransduced cells.
Figure 4. Insulin production in response to glucose concentration
Pdx1-transduced cells were incubated in buffer containing the indicated concentrations of glucose. One hour later the amount of insulin in the buffer was assessed by ELISA. Asterisks indicate significant differences (P<0.05) as compared to insulin production at 0 mM of glucose.
Figure 5. Changes in blood glucose levels and body weight
Thirty rats were randomly and equally divided into 3 groups. The first group (Control) received intraperitoneal injection of 20 mM citrate buffer. The second and third groups both received intraperitoneal injection of 60 mg of STZ (in 20 mM citrate buffer) per kg of body weight. One week later the second group (Saline) received saline treatment while the third group (IPADSC) received IPADSC treatment. All rats were monitored weekly for body weight and fast blood glucose levels. Asterisks indicate significant differences (P<0.05) between IPADSC-treated and saline-treated rats.
Figure 6. Changes in fur appearance and extent of cataract
Representative photographs of IPADSC-treated and saline-treated rats are shown in the upper and lower panels for fur appearance and extent of cataract, respectively.
At the end of the 7th week post-treatment, rats fasted for 7 h received intraperitoneal injection of 1 mg of glucose per gram of body weight. Blood glucose levels were then monitored for 2 h at 30-min intervals in samples obtained from the tail vein. Asterisks indicate significant differences (P<0.05) between IPADSC-treated and saline-treated rats.

Figure 7. Glucose tolerance

At the end of the 7th week post-treatment, rats fasted for 7 h received intraperitoneal injection of 1 mg of glucose per gram of body weight. Blood glucose levels were then monitored for 2 h at 30-min intervals in samples obtained from the tail vein. Asterisks indicate significant differences (P<0.05) between IPADSC-treated and saline-treated rats.
Figure 8. Identification of transplanted cells
At the end of the 7th week post-treatment, rats were sacrificed and their kidneys harvested for histological examination. HE staining was used to examine the subcapsular space for the presence of transplanted cells. Immunofluorescence (IF) staining was used to identify cells expressing insulin. Boxed areas in the 20x photos are injection sites and are enlarged in the respective 100x photos. The boxed areas in the 100x photos are further enlarged in the respective 400x photos. Note the tissue-like structures in the subcapsular space of the IPADSC-treated kidney. No such structure was visible in the saline-treated kidney. The IF photos were taken from 3 IPADSC-treated kidneys. Note the presence of insulin-positive cells (red). Blue fluorescence is DAPI staining for nuclei.
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