Heliyon

ELSEVIER

Received:
16 January 2018
Revised:
14 March 2018
Accepted:
15 May 2018

Cite as: Tomoko Tanaka, Daibo Kojima, Toshiyuki Mera, Masahito Matsumoto, Yohichi Yasunami, Toshihiko Yanase. Expansion of transplanted islets in mice by co-transplantation with adipose tissue-derived mesenchymal stem cells. Heliyon 4 (2018) e00632.
doi: 10.1016/j.heliyon.2018.e00632

Expansion of transplanted islets in mice by co-transplantation with adipose tissue-derived mesenchymal stem cells

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Abstract

The shortage of donor islets is a significant obstacle for widespread clinical application of pancreatic islet transplantation. To investigate whether adipose tissue-derived mesenchymal stem cells (ADSCs) induce expansion of transplanted islets, we performed co-transplantation experiments in a mouse model. Streptozotosin (STZ)-induced diabetic mice transplanted with 50 syngeneic islets remained hyperglycemic. However, hyperglycemia was ameliorated gradually when 50 islets were co-transplanted with ADSCs but not separately grafted into the contralateral kidney. Insulin and proinsulin contents of 120-day grafts containing 50 islets co-transplanted with ADSCs were significantly increased compared with those of 50 isolated islets. The Ki67-positive ratios in islets of the

https://doi.org/10.1016/j.heliyon.2018.e00632

naïve pancreas, at 30 and 120 days grafts were 0.23%, 2.12%, and 1.52%, respectively. Ki67-positive cells were predominantly Pdx1+ and insulin+ cells. These results demonstrate that co-transplantation with ADSCs induces proliferation of transplanted islets in mice, suggesting a potential solution for the low efficiency of islet transplantation.

Keywords: Endocrinology, Stem cell research

1. Introduction

Islet transplantation supplements insulin from transplanted β-cells. The clinical outcomes of islet transplantation have been improved by the development of isolation protocols and immunosuppressive medications after transplantation (Barton et al., 2012; Bellin et al., 2012; Matsumoto et al., 2004; McCall and Shapiro, 2012; Shapiro et al., 2000, 2006). However, to achieve long term insulin independency, recipients require sequential transplants, and the shortage of donor islets is an obstacle for the clinical application of islet transplantation. To overcome this problem, we need novel procedures to enhance the engraftment of islets and expand transplanted islet cells.

Mesenchymal stem cells (MSCs) in adult tissues, such as bone marrow and adipose tissues, have multipotency to differentiate into osteoblasts, adipocytes, and chondrocytes (Pittenger et al., 1999). In fact, bone marrow or adipose tissue-derived BMCs can differentiate into steroidogenic cells by forced expression of the transcription factor (SF-1) (Gondo et al., 2004, 2008; Tanaka et al., 2007). In addition, MSCs secrete cytokines and growth factors, and have immunosuppressive and cytoprotective effects. It has been reported that co-transplantation of MSCs avoids rejection of allogenic islets by immunosuppressive effects (Berman et al., 2010; Ding et al., 2009; Solari et al., 2009). Moreover, MSCs produce vascular endothelial growth factor and promote revascularization of transplanted islets (Cao et al., 2016; Figliuzzi et al., 2009; Ito et al., 2010; Oh et al., 2013; Rackham et al., 2011). Thus, MSCs are also promising feeder or supportive cells in cell transplantation therapy.

Adipose tissue-derived mesenchymal stem cells (ADSCs) have many of the same cell surface markers as bone marrow-derived MSCs (Zuk et al., 2002). The characteristics of ADSCs are almost the same as those of bone marrow-derived MSCs in terms of multipotency, ex vivo expansion, and possible cryopreservation. ADSCs have several advantages, less inversion, and higher availability than bone marrow-derived MSCs (Zuk et al., 2002).

Several previous studies have demonstrated that co-transplantation with MSCs prevents early graft loss by promoting angiogenesis (Eberhard et al., 2010; Figliuzzi et al., 2009; Ito et al., 2010; Johansson et al., 2008; Oh et al., 2013; Rackham

https://doi.org/10.1016/j.heliyon.2018.e00632

et al., 2011) and exerting anti-inflammation effects associated with increased regulatory T-cells (Berman et al., 2010; Ding et al., 2009; Solari et al., 2009). In addition, anti-apoptotic effects of MSCs on grafted islets have been reported (Borg et al., 2014; Scuteri et al., 2014). Importantly, MSCs have been suggested to differentiate toward insulin-producing cells *in vitro* (Chandra et al., 2009; Timper et al., 2006). The microRNA miR-375, which is abundant in pancreatic β-cells, has been recently shown to promote insulin production from ADSC-derived islet like-clusters *in vitro* (Piran et al., 2017). However, in previous reports concerning islet cell transplantation experiments *in vivo* (Berman et al., 2010; Ding et al., 2009; Eberhard et al., 2010; Figliuzzi et al., 2009; Ito et al., 2010; Johansson et al., 2008; Oh et al., 2013; Rackham et al., 2011; Solari et al., 2009), an essential issue has remained unclear: whether grafted islet cells proliferate in the presence of MSCs or MSCs themselves differentiate into islet cells and proliferate.

In this study, we examined the effects of ADSCs on transplanted islets and demonstrated that co-transplantation with ADSCs not only enhances the engraftment of islets but also induces the expansion of transplanted islet cells.

2. Materials and methods

2.1. Mice

All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Animal Care and Use Committee of Fukuoka University. Male C57BL/6 mice and mouse insulin I promoter (MIP)-green fluorescent protein (GFP) transgenic mice expressing GFP under the control of the MIP (Hara et al., 2003) were purchased from Charles River Japan and Jackson Laboratory, respectively. Mice were maintained under specific pathogen-free conditions and used for experiments at 8−16 weeks of age.

2.2. ADSCs

ADSCs were prepared from C57BL/6 subcutaneous fat as described previously (Gondo et al., 2008) and cultured for 2 weeks in alpha-minimum essential medium containing 20% horse serum and 1% antibiotic antimycotic (Gibco) in a 5% CO_2 incubator at 37 °C. After four times passage of the culture, the adherent cells were used as ADSCs. For characterization of ADSCs, cell surface markers were analyzed by a flow cytometer using a Mouse Multipotent Mesenchymal Stromal Cell Marker Antibody Panel (R&D systems, Inc., MN). To test their capability for osteoblastic differentiation, ADSCs were cultured under a previously described condition (Gondo et al., 2004) and then stained with an anti-osteopontin antibody (R&D systems, Inc.). Induction of adipogenesis followed by Oil-Red O staining was performed using an Adipogenesis Assay Kit (Cayman Chemical Company, MI).

https://doi.org/10.1016/j.heliyon.2018.e00632

2.3. Islet isolation and transplantation

Islets of C57BL/6 mice were isolated (Okeda et al., 1979; Sutton et al., 1986) and cultured overnight. ADSCs were peeled off from culture dish using TrypleExpress (Gibco), and counted. Before transplantation, hand-picked islets (average size was 150 μm) and ADSCs were mixed in a 1.5 ml tube and centrifuged for 5 min at 1,200×g, and the precipitants were suspended in a small volume of medium. Islets with or without ADSCs were transplanted under the kidney capsule of streptozotocin (STZ)-induced diabetic mice injected with 180 mg/kg STZ (Sigma Aldrich) at 3 days before transplantation. We used C57BL/6 male for the recipients. After transplantation, their body weight and non-fasting blood glucose concentration were measured twice a week, and the recipients were not supplemented with exogenous insulin. At 30 or 120 days after transplantation, the left kidney bearing the grafts was removed and the morphology and hormone contents were examined. Hyperglycemia was defined as >400 mg/dL blood glucose. When <200 mg/dL was detected twice consecutively, the blood glucose level was considered as normalized.

2.4. Intraperitoneal glucose tolerance test (ipGTT)

Mice were fasted for 15 h before the ipGTT and then intraperitoneally injected with 1 g/kg glucose. After the injection, the blood glucose levels and plasma insulin concentrations were measured at 0, 30 and 120 min. Plasma insulin was measured by an Ultra Sensitive Mouse Insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

2.5. Hormone content measurements

Extracts of the kidney bearing the grafted islets and isolated islets were prepared as described previously (Ueki et al., 1995). Insulin and pro-insulin contents were measured with a Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc.) and Rat/mouse proinsulin ELISA (Mercodia Developing Diagnostics, Uppsala, Sweden), respectively.

2.6. Morphological analysis

The graft-bearing kidney and isolated islets were fixed in 10% formaldehyde or 4% paraformaldehyde, processed, and embedded in paraffin. Sections were stained with eosin and nuclei were counterstained with hematoxylin. Immunohistochemical staining was performed using the antibodies listed in Table 1. For electron microscopic observation, grafted and isolated islets were fixed and observed as described previously (Nagai et al., 1995).

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Table 1. Antibodies for immunostaining.

Gene name	Supplier/catalogue number
Insulin	Dako/A0564
Glucagon	Thermo/PA1-37768
Somatostatin	abcam/ab103790
PP	abcam/ab103790
Ki67	abcam/ab66155
Pdx1	abcam/ab47383, DSHB/F6A11-6
Ngn3	abcam/ab38548
GFP	abcam/ab6556, ab13970
Pax4	ThermoFisher/PA1-108
Nkx6.1	DSHB/F55A10-c

2.7. RT-PCR

Total RNA isolated from the graft-bearing kidney or pancreas was reverse transcribed by a QuantiTect Reverse Transcription Kit (Qiagen). PCR was performed using KOD-FX (Toyobo) at 95 °C for 10 min, 95 °C for 10 sec, and 60 °C for 20 sec (40 cycles). GFP primers were 5′-CAACAGCCACAACGTCTATATCACC-3′ and 5′-ATGTTGTGGCGGATCTTGAAG-3′, insulin primers were 5′-TCAAG-CAGCACCTTTGTGGTT-3′ and 5′-TCCACCCAGCTCCAGTTGT-3′, and β-actin primers were 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and 5′-ATGGAGC-CACCGATCCACA-3′.

2.8. Statistics

All data are expressed as means ± standard error of the mean (SEM) and were obtained from at least three individual experiments. Statistical comparisons were performed using the Student's *t*-test, One-way ANOVA and Two-way ANOVA (GraphPad Prism). *P*-values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Co-transplantation of 50 islets and ADSCs into diabetic mice leads to normoglycemia

We first transplanted syngeneic islets alone under the kidney capsule of diabetic mice induced by the iv injection of STZ (180 mg/kg) at 3 days before transplantation. Recipient mice without islet transplantation remained hyperglycemic (Fig. 1A). Hyperglycemia of mice received with 200 islets was ameliorated by 2

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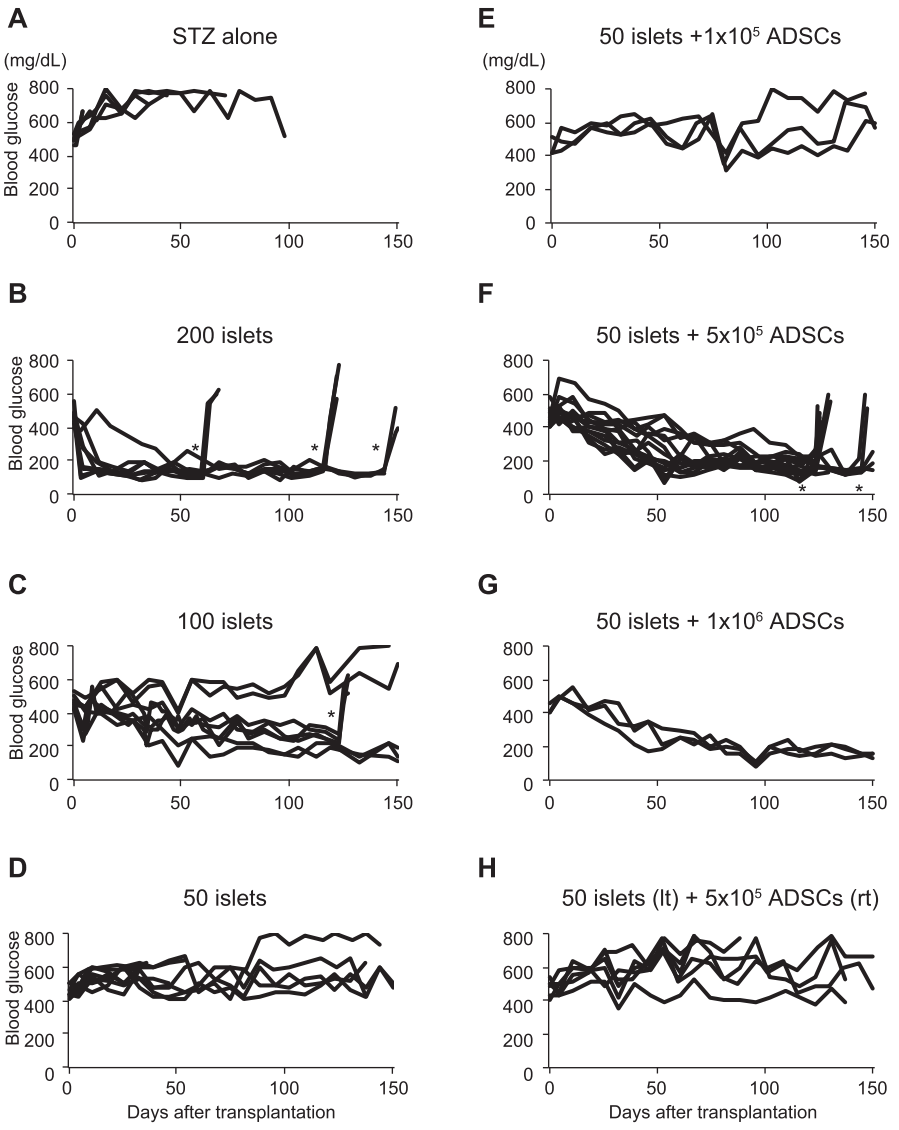

Fig. 1. Recipients co-transplanted with 50 islets and ADSCs become normoglycemic in an ADSC dose-dependent manner. Hyperglycemia was induced by STZ injection, followed by monitoring non-fasting blood glucose levels (n = 8) (A). Non-fasting blood glucose levels of diabetic recipient mice transplanted with 200 (n = 8), 100 (n = 7), or 50 (n = 9) syngeneic islets were monitored (B,C). A total of 50 islets were co-transplanted with 1×10^5 (n = 3), 5×10^5 (n = 14), or 1×10^6 (n = 4) (E–G). Blood glucose levels of mice transplanted with 50 islets and 5×10^5 ADSCs into left and right kidneys, respectively (n = 6) (H). Asterisk indicates removal of the kidney bearing the graft after normalization of blood glucose levels.

weeks after transplantation (n = 8), and removal of the kidney bearing the graft promptly made recipient mice hyperglycemic again (Fig. 1B). A total of five out of seven recipients transplanted with 100 islets became normoglycemic, and two recipients remained hyperglycemic (Fig. 1C). All recipients transplanted with 50 islets remained hyperglycemic (n = 9) (Fig. 1D). Therefore, we used 50 islets in the following experiments to examine the effects of ADSCs.

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ADSCs obtained from subcutaneous white adipose tissue had a spindle shape and differentiated into mature adipocytes and osteoblasts (Fig. 2A–C). They were positive for Sca-1, CD106, CD29, and CD44 but negative for CD45, CD11b, and CD31 (Fig. 2D). To examine the effect of co-transplantation with ADSCs, we combined 50 islets with 1×10^5, 5×10^5 or 1×10^6 ADSCs and transplanted them into STZ-induced hyperglycemic mice. Recipients co-transplanted with 1×10^5 ADSCs remained hyperglycemic (n = 3) (Fig. 1E). In contrast, recipients co-transplanted with 5×10^5 or 1×10^6 ADSCs gradually became normoglycemic by 30 days after transplantation in a cell number-dependent manner (Fig. 1F and G). They became hyperglycemic again after removal of the kidney bearing the graft at 120 days after transplantation.

We transplanted 1×10^6 ADSCs alone into STZ-induced hyperglycemic mice, but the recipients remained hyperglycemic (data not shown). When islets and ADSCs were respectively transplanted into left and right kidneys, the recipients remained hyperglycemic (Fig. 1H), indicating that the effect ADSCs was not systemic but local. These data clearly demonstrate that ADSCs enhance engraftment of transplanted islets, resulting in normoglycemic recipients.

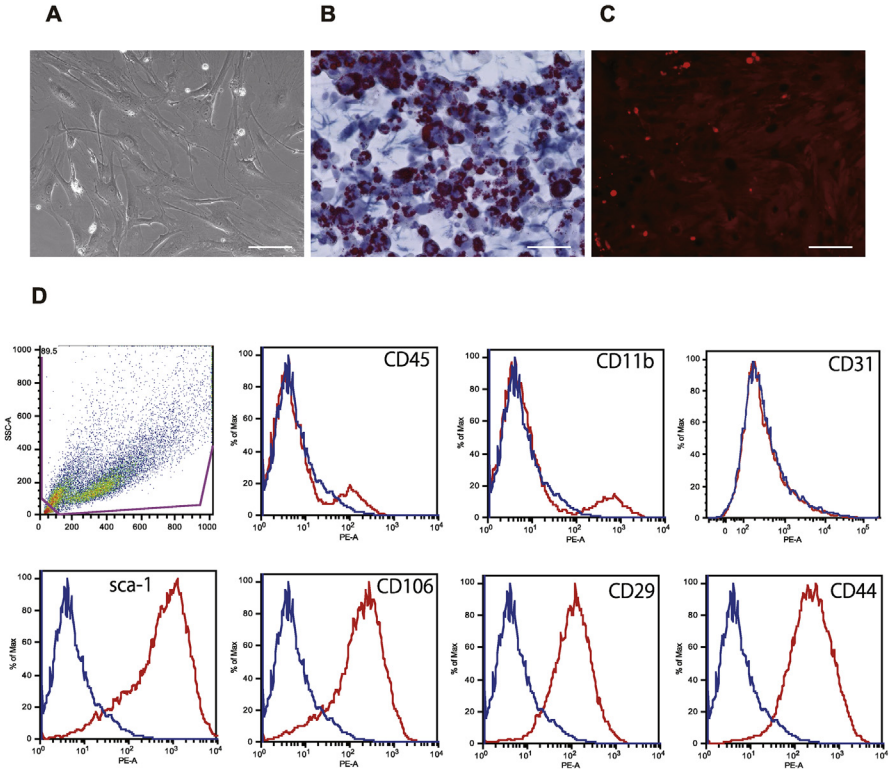

Fig. 2. Characteristics of ADSCs. ADSCs observed with a phase-contrast microscopy (A). Bar represents 100 μm. Oil-Red O-stained ADSCs after adipogenic differentiation (B). Bar represents 50 μm. After osteogenic differentiation, ADSCs were immnostained with anti-osteopontin antibody (C). Bar represent 100 μm. Expression patterns of cell surface markers of ADSCs were analyzed by flowcytometry (D). Blue line and red line show isotype control and surface marker, respectively.

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To confirm insulin secretion from the grafted islets in response to blood glucose levels, we performed an ipGTT before and after the removal of the transplanted islets at 120 days after transplantation. Most of mice transplanted 50 islets alone were dead within 30 days because of hyperglycemia, and we performed ipGTT using naïve mice as control. As a result, blood glucose levels of grafted animals at 30 and 120 min significantly increased compared to naïve mice. However, AUC was not significantly different between naive and grafted animals. Those results suggested that glucose tolerance of grafted animals was partially recovered, before nephrectomy. After nephrectomy, the glucose tolerance of co-transplanted mice was significantly lower than that of naive mice (Fig. 3A and B). Before nephrectomy, the plasma insulin level of co-transplanted mice was clearly detectable in response to glucose injection, whereas it became undetectable after nephrectomy (Fig. 3C). These data demonstrated that exogenous insulin from the grafted islets controlled recipient blood glucose levels in response to the increase of glucose.

3.2. Increases of insulin granules and hormone contents

Macroscopically, the grafted islets were seen in adipose-like tissue under the kidney capsule with neovascularization at 120 days after transplantation (Fig. 4A). Microscopically, the grafted islets were surrounded by fibroblast-like cells with adipose tissue (Fig. 4B). Immunohistochemical analyses revealed that islet cells were positive for insulin, glucagon, somatostatin, and pancreatic polypeptide (PP) (Fig. 4C–F). Of note, α-cells in transplanted islets were distributed inside of the

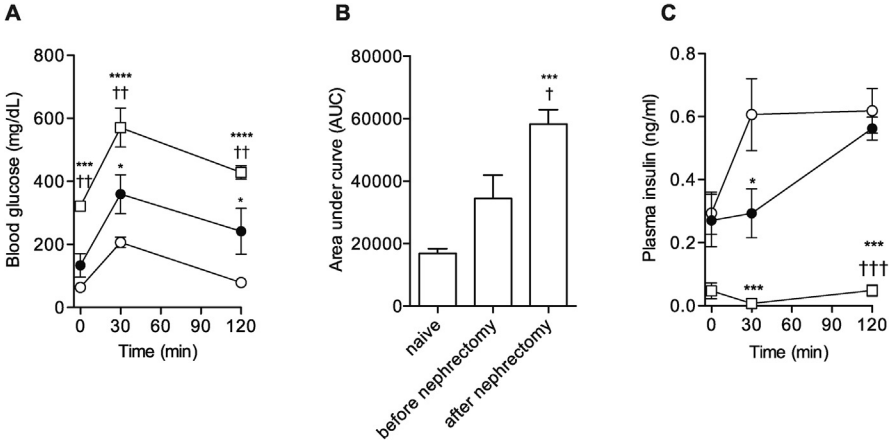

Fig. 3. Intraperitoneal glucose tolerance test (ipGTT) before and after removal of the graft-bearing kidney of mice transplanted with 50 islets and 5×10^5 ADSCs. Open circles, naïve mice (n = 4); black circles, 120-day normoglycemic mice co-transplanted with islets and ADSCs before removal of the graft-bearing kidney (n = 4); open squares, 5 days after removal of the kidney. A–C show blood glucose levels, the area under the curve, and plasma insulin, respectively. Values are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. naïve, †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ vs. before nephrectomy.

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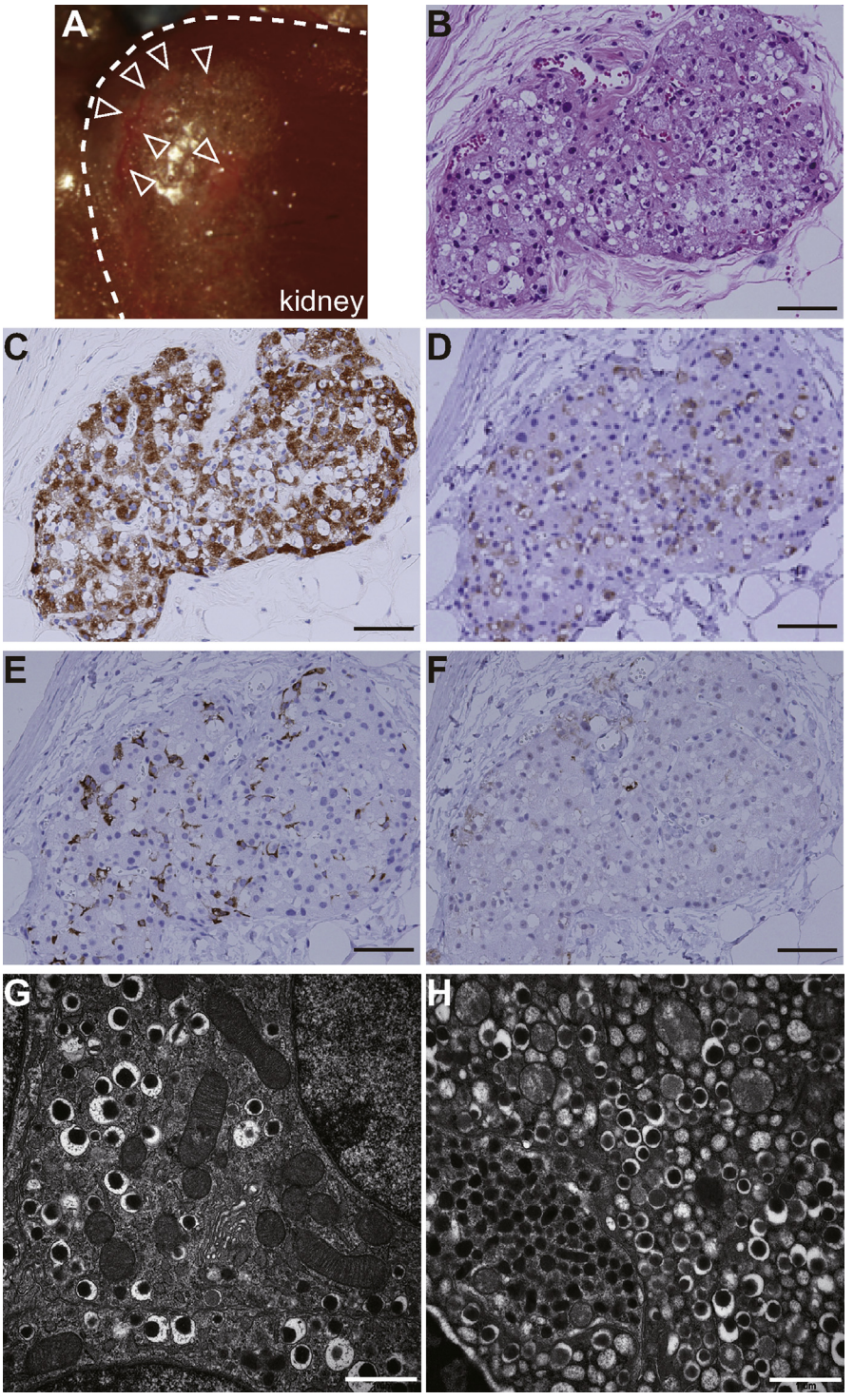

Fig. 4. Macroscopic images of the graft and immunohistochemical staining for insulin, glucagon, somatostatin, and PP. At 120 days after co-transplantation of 50 islets and ADSCs, the graft was observed macroscopically. Dotted lined area and arrow heads indicate kidney and graft islets, respectively (A). Section of the graft stained with hematoxylin and eosin (B), and serial sections immunostained with antibodies against insulin (C), glucagon (D), somatostatin (E), and PP (F). Bars represent 50 μm in B—F. Electron micrograph of isolated islets (G) or a β-cell in the graft islet at 120 days after co-transplantation (H), Bars represent 1 μm.

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islets, which is dissimilar to the peripheral localization of α-cells that are observed in pancreatic islets of naïve mouse (Fig. 4D).

Next, to examine the cellular structure of β-cells, we performed electron microscopic observations. Compared with isolated islets, the number of insulin granules in the graft was increased dramatically, and the rough endoplasmic reticulum was developed in β-cells (Fig. 4G and H). In the grafted β-cells, insulin granules were densely observed, suggesting an increase of insulin production from each β-cell. Then, we measured the insulin and pro-insulin contents of the grafted islets for comparison with isolated islets. The insulin contents of 50 isolated and grafted islets were 8.66 ± 0.62 μg and 12.1 ± 0.80 μg, respectively ($P = 0.0060$) (Fig. 5A). Pro-insulin contents of 50 isolated and grafted islets were 12.7 ± 2.59 ng and 25.8 ± 3.07 ng, respectively ($P = 0.0097$) (Fig. 5B), indicating that the hormone contents of grafted islets were increased significantly. Next, we examined whether such increases of hormone contents in the graft occurred in normoglycemic recipients, we transplanted 50 islets with ADSCs into the normoglycemic mice and compared their hormone contents with those of hyperglycemic recipients. Insulin contents of the grafts in hyperglycemic and normoglycemic recipients were 11.6 ± 0.84 μg and 7.31 ± 1.23 μg, respectively ($P = 0.0277$) (Fig. 5C). The increase of hormone contents by co-transplantation with ADSCs was not seen in the grafts of

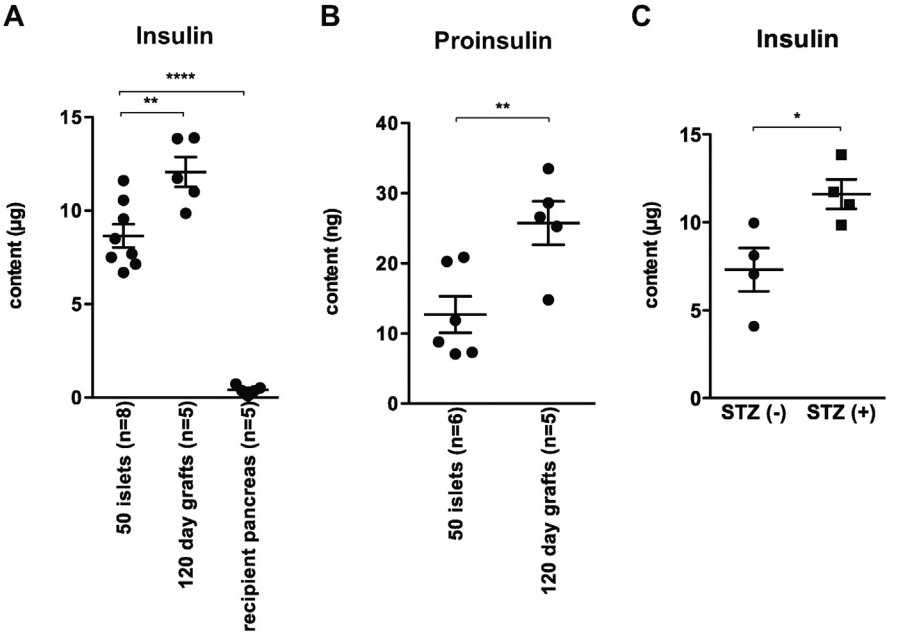

Fig. 5. Insulin and pro-insulin contents of grafted islets. Peptide hormones were extracted from isolated islets, the kidney bearing the graft at 120 days post-transplantation, and the recipient pancreas, and insulin contents were measured (A). Pro-insulin contents of isolated islets and the kidney bearing the graft (B). To examine the effects of recipient blood glucose levels on the hormone contents of grafts, at 120 days after transplantation, insulin contents of the graft co-transplanted with 50 islets and ADSCs into normoglycemic recipient mice [STZ (−)] and STZ-induced diabetic mice [STZ (+)] were compared (C). Values are expressed as the mean ± SEM. $*P < 0.05$.

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normoglycemic recipient mice, suggesting that blood glucose levels of recipients might be a possible factor in the increase of hormone contents.

We hypothesized that the increase of hormone contents was caused by the expansion of transplanted islet cells. To confirm this hypothesis, we performed immunostaining with anti-insulin and anti-Ki67 (proliferation marker) antibodies. In pancreatic islets, Ki67-positive cells were rarely detected (Fig. 6B), whereas several Ki67-positive cells were detected in the grafted islets, and most of them were positive for insulin

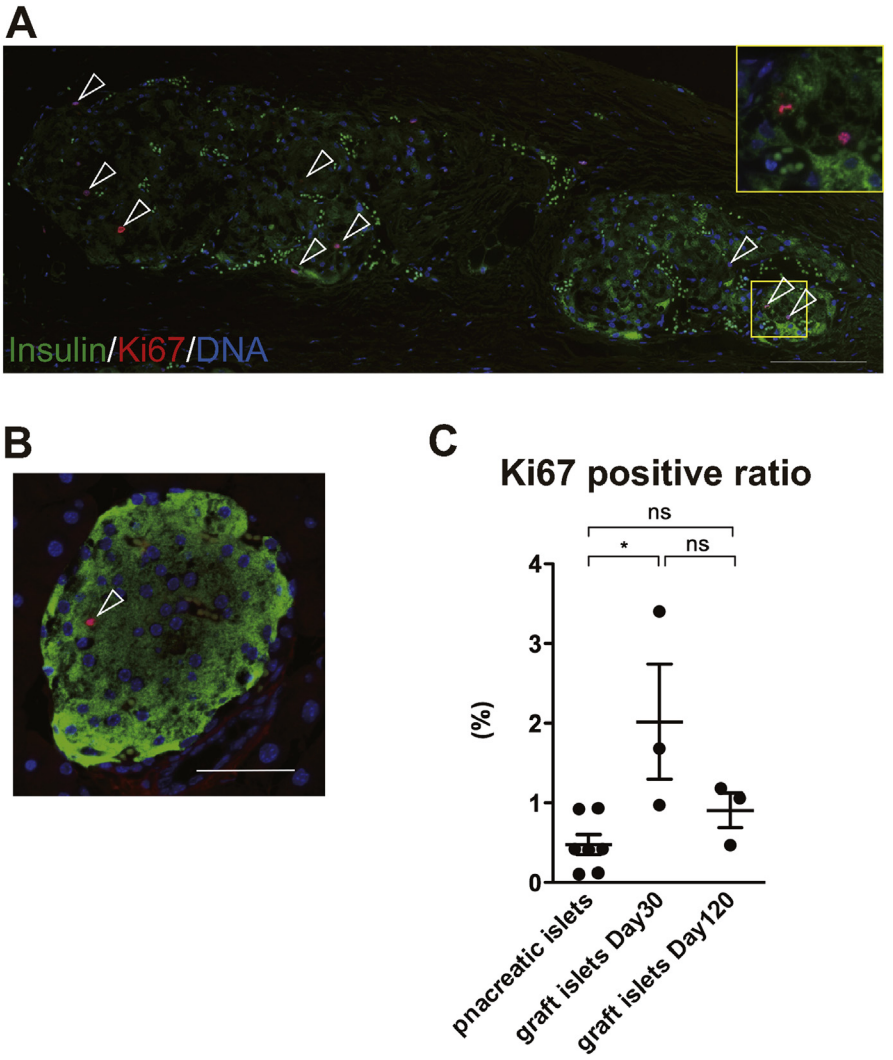

Fig. 6. Ki67- and insulin-positive cells in grafted islets and the Ki67-positive ratio. Grafted and pancreatic islets were double stained with anti-insulin and -Ki67 antibodies, and their nuclei were counterstained with DAPI (A, B). Bars represent 100 μm and 50 μm in A and B, respectively. Numbers of Ki67- and DAPI-positive cells in islets of the pancreas of naïve mouse (n = 7) and grafted islets at 30 days (n = 3) or 120 days (n = 3) after transplantation were counted, and the Ki67-positive ratio was calculated (C). Arrowheads show Ki67-positive cells. Values are expressed as the mean ± SEM. Bars represent 100 μm and 50 μm in A and B. *$P < 0.05$ vs. naïve pancreatic islets. ns, not significant.

https://doi.org/10.1016/j.heliyon.2018.e00632

(Fig. 6A). We counted the Ki67-positive cells in the graft islets and calculated the ratio of Ki67-positive cells to DAPI-positive cells in the islets. As a result, the Ki67-positive ratio in pancreatic islets, grafted islets at 30 days post-transplantation and graft islets at 120 days post-transplantation were 0.47 ± 0.13% (n = 6), 2.02 ± 0.72% (n = 3), and 0.90 ± 0.22% (n = 3), respectively (Fig. 6C). Ki67-positive cells in grafted islets with ADSCs post 30 days were significantly increased compared with those in pancreatic islets (P = 0.0119).

Because most Ki67-positive cells were also positive for insulin, we next performed double immunostaining using anti-Pdx1, -glucagon, -somatostatin and -PP antibodies to assess which endocrine cells were positive for Ki67 in the grafted islets. The double staining revealed that most Ki67-positive cells were also positive for Pdx1. Several Ki67-positive cells were weakly positive for Nkx6.1 (Sander et al., 2000), and double positive cells for glucagon, somatostatin, or PP were not detected (Fig. 7). We also performed immunostaining for the progenitor marker Ngn3 (Gradwohl et al., 2000; Gu et al., 2002) and Ngn3 downstream transcriptional factor Pax4 (Sosa-Pineda et al., 1997), but neither Ngn3- or Pax4-positive cells were not detected in the graft islets (Fig. 7). Those data suggested that the Ki67-positive cells

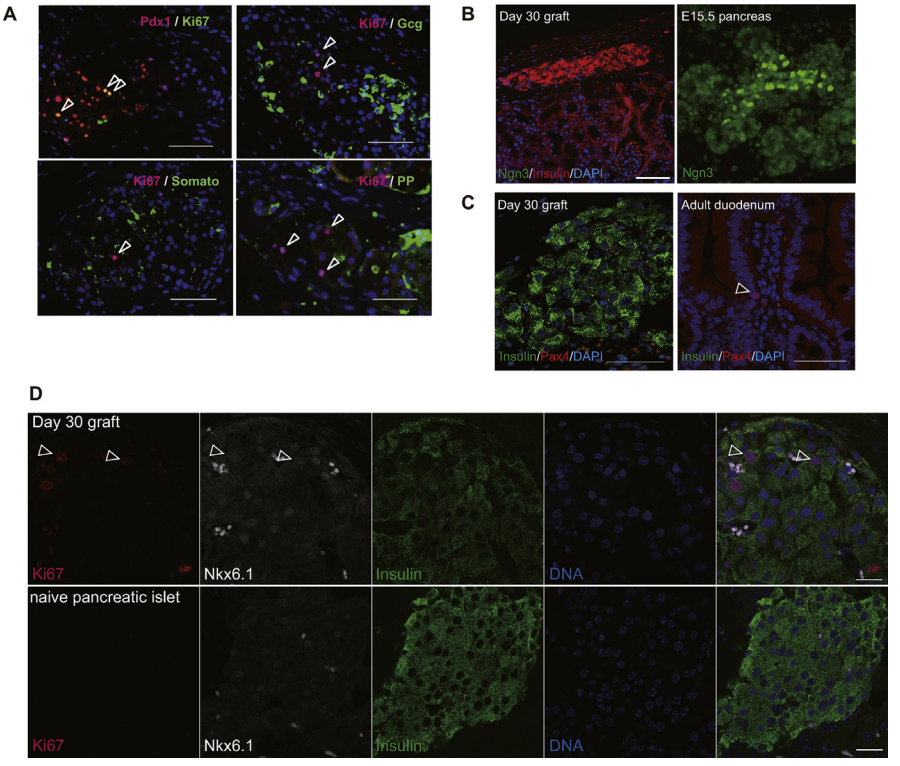

Fig. 7. Ki67-positive cells express Pdx1. A, Grafts at 30 days after co-transplantation were double stained for Ki67 and Pdx1, glucagon, somatostatin, or PP. Nuclei were counterstained with DAPI. Bars represent 50 μm. Arrowheads show Ki67-positive cells. At 30 days after transplantation of 50 islets and ADSCs, the graft sections were immunostained with antibodies against Ngn3 (A), Pax4 (B), and Nkx6.1 (C). Arrowheads indicate positive cells. Bars represent 50 μm and 20 μm in B and C, and D, respectively.

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were derived from β-cells and co-transplantation with ADSCs did not induce β-cell neogenesis but induced proliferation of β-cells in the graft.

3.3. β-cells are derived from donor islets

Several groups have reported that MSCs can differentiate into insulin-producing cells (Chandra et al., 2009; Piran et al., 2017; Timper et al., 2006). To examine the possibility of β-cell differentiation from ADSCs or recipient cells, we co-transplanted wild-type islets with ADSCs derived from MIP-GFP transgenic mice.

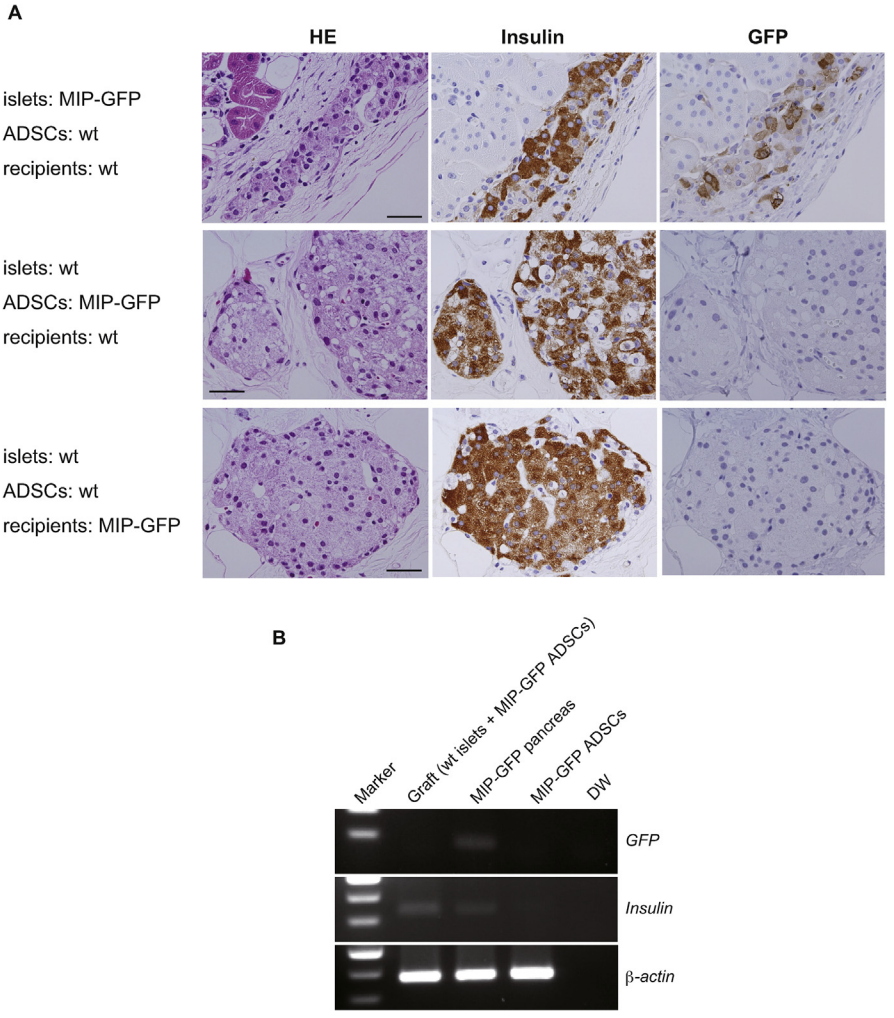

Fig. 8. β-cells in the graft are derived from grafted islets. (A, upper) MIP-GFP islets and wild-type ADSCs were transplanted into wild-type recipient mice. At 120 days after transplantation, graft sections were stained with an anti-GFP antibody, and GFP-positive cells were detected in the graft. (A, middle) Wild-type islets and ADSCs derived from MIP-GFP mice were co-transplanted. GFP-positive cells were not detected. (A, lower) Recipient mice were MIP-GFP. GFP-positive cells were not detected in the grafted islets. Bars represent 20 μm. (B) Total RNA was isolated from the kidney containing the graft, and GFP expression levels were examined by RT-PCR. GFP was not detected in the graft consisting of co-transplanted wild-type islets and MIP-GFP ADSCs. Full image of agarose electrophoresis is shown in Supplementary Fig. 1.

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Post 120 days of transplantation, GFP expression levels in the grafts were examined by immunostaining and RT-PCR. As a result, expression of GFP was not detected in both protein and mRNA levels (Fig. 8, Supplementary Fig. 1). When we transplanted wild-type islets and wild-type ADSCs into diabetic MIP-GFP mice, GFP-positive cells were not detected. These data clearly indicated that the β-cells were derived from the donor islets.

4. Discussion

In this study, we clearly demonstrated that diabetic recipient mice transplanted with 50 islets alone remained hyperglycemia, although diabetic recipients transplanted 50 islets and ADSCs gradually became normoglycemic, suggesting that ADSCs promote engraftment of transplanted islets. The enhancing effect of ADSCs on grafted islet cell function was not systemic but local since the contralateral transplantation of ADSCs was ineffective.

In the graft islets transplanted with ADSCs, the number of insulin granules was increased dramatically, and insulin and proinsulin contents of the graft were increased, suggesting that β-cell function was promoted by co-transplantation of ADSCs. In addition, the result of immunostaining, the ratio of Ki67-positive cells in the grafted islets at 30 days after transplantation was significantly higher than that of islets in the pancreas of naïve mouse. Most Ki67-positive cells were positive for insulin and Pdx1. These data suggested that co-transplantation with ADSCs induced β-cell replication. Moreover, the proliferated β-cells were found to be derived from the donor islets but not from co-transplanted ADSCs or recipients based on the finding with use of MIP-GFP Tg mice. Taken together, we showed that ADSCs enhance the proliferation of islet cells in grafted tissue by co-transplantation.

During the first 30 days after co-transplantation, recipient mice were still in the hyperglycemic state, but they gradually became normoglycemic from 45 days after transplantation. The Ki67-positive ratio of grafted islets at 30 days was significantly increased compared with that of pancreatic islets. Conversely, at 120 days, when the recipient became normoglycemic, the Ki67-positive ratio of the grafted islets was not different from that of pancreatic islets. Moreover, when we co-transplanted islets and ADSCs into non-diabetic normoglycemic recipient, there was no increase in insulin content of the grafts compared with 50 donor islets. These findings suggest that the hyperglycemic condition may be essential not only for promoting β-cell function but also for β-cell replication produced by co-transplantation of ADSCs. Indeed, it has been demonstrated that differentiated β-cell could be redifferentiated by stimulation with high glucose level in the medium for 2 days during which the expression of insulin, Pdx-1 and MafA significantly increased over time (Russ et al., 2011). In addition, hyperglycemia has been also reported to stimulate an increase in β-cell number (Otonkoski et al., 1994; Zhang et al., 2016).

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The mechanism of pancreatic β-cell regeneration remains unclear and has been controversial. In general, β-cell regeneration was thought to be mediated by both β-cell neogenesis and the proliferation of existing β-cells (Gu et al., 1995). β-cell neogenesis is new β-cell formation from other cells such as acinar and ductal compartments as suggested by experiments of injury-induced regeneration in the pancreas (Westphalen et al., 2016). Although the actual propensity for acinar cells to contribute to the endocrine lineage is limited, acinar cells poses intrinsic plasticity that directs them toward a β-cell-like phenotype by the introduction of three genes, Pdx-1, Ngn3, and MafA (Zhou et al., 2008). Acinar cells can be converted to ??-like and α-like cells by overexpression of Ngn3 and Ngn3 plus MafA, respectively (Li et al., 2014). However, studies employing 50−70% pancreatomy have demonstrated that β-cells regenerate almost exclusively by self-duplication of existing β-cells and not through neogenesis (Meier et al., 2008; Nir et al., 2007). Recently, functional heterogeneity of β-cells and the presence of a population of proliferative and mature β-cells have been reported (Bader et al., 2016). Our study clearly indicated that, at least in transplantation experiments, β-cells were derived from donor islets but neither from co-transplanted ADSCs nor from recipients.

It has been reported that proliferation of β-cells can be induced under physiological conditions and by several stimuli (Dor et al., 2004). Insulin, insulin-like growth factor-1, interleukin (IL)-6, serotonin, incretins, hepatocyte growth factor (HGF) (Alvarez-Perez et al., 2014; Mellado-Gil et al., 2011), platelet-derived growth factor (PDGF) (Chen et al., 2011), and microRNAs are reported to enhance β-cell proliferation (Filios and Shalev, 2015). Among them, MSCs secrete IL-6, HGF, and PDGF, although we did not examine the roles of these factors in our experiments. The extracellular matrix (ECM) provided by ADSCs might be important for graft islets. ECM, integrin β1 (Diaferia et al., 2013), connective tissue growth factor (Riley et al., 2015), and tissue inhibitor of metalloproteinases-1 (Kono et al., 2014) have been reported to be related to β-cell proliferation and suppress apoptosis of graft islets.

Interestingly, in the co-transplantation experiment, the localization pattern of α-cells was dissimilar to that of pancreatic islets. The possibility that proliferation of α-cells caused different localization patterns of α-cells appears to be unlikely because we did not find double staining of Ki67 and glucagon or somatostatin. At least in our experiments, the proliferation of the grafted islets appears to be restricted to β-cells only. Trans-differentiation of α- or δ-cells into β-cells (Chera et al., 2014) may be another possibility, but it is very difficult to examine this possibility by *in vivo* transplantation experiments. Further studies are needed to clarify how β-cells expand by co-transplantation with ADSCs.

In this study, we used mouse inguinal subcutaneous white adipose tissue as a source of MSCs. Considering clinical islet transplantation, the preparation of stem cells from adipose tissue is more suitable than from bone marrow because adipose tissue is easy to access with better recovery of cells (Gondo et al., 2008). However, it is of

https://doi.org/10.1016/j.heliyon.2018.e00632

note that ADSC-stimulated expansion of the co-transplanted islet was observed in STZ-induced and syngeneic conditions and thus, the simple application of our system to autoimmune diabetes may not be possible.

5. Conclusions

We examined the effects of ADSCs on transplanted islets and demonstrated that co-transplantation with ADSCs not only enhances engraftment of islets but also induces β-cell expansion of transplanted islet cells in a syngeneic mice transplantation model.

Declarations

Author contribution statement

Tomoko Tanaka: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Daibo Kojima, Toshiyuki Mera, Masahito Matsumoto: Performed the experiments.

Yohichi Yasunami: Conceived and designed the experiments.

Toshihiko Yanase: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by a KAKENHI grant (No. 16K09815) from the Japan Society of the Promotion of Science (TT), the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (S1512005) (YY), and funds (No. 147014) from the Central Research Institute of Fukuoka University (YY). The Department of Bioregulatory Science of Life-related Diseases of Fukuoka University (Fukuoka, Japan) was supported financially by a donation from MSD K. K. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of manuscript.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2018.e00632.

Acknowledgements

The authors thank Mrs. Fusako Ogata, Mrs. Yuriko Hamaguchi and Mr. Masaru Segawa for immunostaining and hormone content measurements, and electron microscopic observations, respectively. This work was supported by KAKENHI (No. 16K09815) form Japan Society of the Promotion of Science (TT), MEXT-Supported Program for the Strategic Research Foundation at Private Universities (S1512005) (YY) and by funds (No. 147014) from the Central Research Institute of Fukuoka University (YY). We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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