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ORIGINAL ARTICLE

Effect of hypoxia-inducible VEGF gene expression on revascularization and graft function in mouse islet transplantation

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Summary

For gene transfer strategies to improve islet engraftment, vascular endothelial growth factor (VEGF) expression should be regulated in a way that matches the transient nature of revascularization with simultaneously avoiding undesirable effects of overexpression. The aim of this study was to investigate the effects of hypoxia-inducible VEGF gene transfer using the RTP801 promoter on islet grafts. We implanted pSV-hVEGF transfected, pRTP801-hVEGF transfected or nontransfected mouse islets under the kidney capsule of streptozotocin-induced diabetic syngeneic mice. Human VEGF immunostaining of day 3 grafts revealed that the pRTP801-hVEGF transfected group had higher hVEGF expression compared with the pSV-hVEGF transfected group. BS-1 staining of day 3 grafts from the pRTP801-hVEGF transfected group showed the highest vascular density, which was comparable with day 6 grafts from the nontransfected group. In 360 islet equivalent (IEQ)-transplantation which reverted hyperglycemia in all mice, the area under the curve of glucose levels during intraperitoneal glucose tolerance test 7 weeks post-transplant was lower in mice transplanted with pRTP801-hVEGF transfected grafts compared with mice transplanted with nontransfected grafts. In 220 IEQ-transplantations, diabetic mice transplanted with pRTP801-hVEGF islets became normoglycemic more rapidly compared with mice transplanted with pSV-hVEGF or nontransfected islets, and diabetes reversal rate after 50 days was 90%, 68%, and 50%, respectively. In conclusion, our results indicate that regulated overexpression of hVEGF in a hypoxia-inducible manner enhances islet vascular engraftment and preserves islet function overtime in transplants.

Introduction

Pancreatic islet transplantation is a potentially effective therapy for insulin deficient diabetes. Currently, allogeneic islet transplantation from a single donor usually fails to achieve insulin independence in a diabetic recipient because of an early and profound loss of transplanted islets [1,2]. The mechanisms involved in early post-transplant graft loss include hypoxic and inflammatory insults from the stressful process of islet isolation and perturbation of the graft microenvironment [3–5]. From these, the majority of islets rapidly fail to engraft and undergo cell death. During the process of islet isolation, the islet capillary networks are destroyed, and revascularization takes place over a period of 7–14 days [6–9]. Thus, it is evident that islets are exposed to hypoxic conditions during transplantation and engraftment. To improve survival rates of transplanted islets, *ex vivo* angiogenic gene

transfer strategies have been investigated [10–18]. Among the angiogenic genes, delivery of vascular endothelial growth factor (VEGF) to islets using adenoviral vectors or nonviral carriers [13–18] has been shown to increase the survival of transplanted islets.

Vascular endothelial growth factor gene therapy has been widely investigated for its use in ischemic disease therapy because it is the most efficient angiogenic factor. However, the safety issues surrounding VEGF overexpression, such as the role of VEGF in inducing aberrant leaky vessels, or proliferation of endothelial cells and hemangiomas [19–22], have not been completely addressed for clinical applications. In islet transplantations, to allow rapid and adequate angiogenesis in islets and to avoid undesirable effects of unregulated VEGF overexpression, the expression profile of VEGF within islet grafts should match the transient nature of islet revascularization.

Previously, we developed an RTP801 promoter-mediated hypoxia-inducible gene expression system [23]. The RTP801 promoter induced gene expression under hypoxic conditions in various types of cells *in vitro*, and in ischemic myocardium, injured spinal cord, and ischemic cavernosum *in vivo* [24–26]. In isolated rat islets, delivery of pRTP801-hVEGF using nonviral carriers also induced the hVEGF expression under hypoxic conditions [27,28].

In this study, we investigated the effects of hypoxia-inducible human VEGF (hVEGF) gene transfer, using the RTP801 promoter, on revascularization of transplanted islets and graft function over time, in a syngeneic mouse transplantation model.

Materials and methods

Islets isolation

Male inbred Balb/c mice, aged 9–10 weeks, were purchased from Koatech (Pyungtaek, Korea). Mouse islets were isolated and purified from 12-week-old mice by digesting pancreatic tissues with 1 mg/ml collagenase P (Roche, Mannheim, Germany) followed by Ficoll (Biochrom AG, Berlin, Germany) gradient purification. The isolated islets were cultured free-floating in Medium 199 (Gibco, Grand Island, NY, USA) supplemented with 10% FBS in a 5% CO_2 incubator. Individual islets were hand-picked under an inverted microscope and quantified by dithizone staining in duplicate using a standard islet diameter of 150 μm as 1 islet equivalent (IEQ).

Islet culture, transfection of plasmids and ELISA of hVEGF

The construction of pSV-hVEGF and pRTP801-hVEGF was described previously [27]. Effectene (Qiagen, Valencia, CA, USA) was used as a gene carrier. Transfection conditions were optimized based on the manufacturer's instructions and our previous studies [14,28]. Briefly, isolated mouse islets, maintained in Medium 199 supplemented with 10% FBS in a 5% CO_2 incubator, were washed with serum-free Medium 199 and transfected with Effectene-pSV-hVEGF or Effectene-pRTP801-hVEGF complexes at a dose of 2 μg plasmid DNA in 1 ml of modified serum-free OPTI-MEM (Gibco, Grand Island, NY, USA) at 37 °C in a 5% CO_2 incubator. After 4 h, the transfection mixtures were removed and fresh Medium 199 containing 10% FBS was added. The islets were then incubated in either normoxic (20% oxygen) or hypoxic (1% oxygen) conditions at 37 °C for 12 h. For hypoxic condition, mixed gas of 1% O_2, 5% CO_2, and balanced N_2 was used in a hypoxia chamber. Human VEGF was measured in culture media using a hVEGF ELISA kit (R&D systems, Abingdon, UK). For transplantation experiments, transfected mouse islets were incubated in Medium 199 containing 10% FBS for 4 h before transplantation.

Islet transplantation

Mice were fed standard rodent chow in a barrier animal facility under a 12 h light/dark cycle and used for this study in compliance with the guidelines from the Institutional Animal Care Committee at Hallym University. At 12 weeks of age, diabetes was induced by a single intraperitoneal injection of 200 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO, USA) 3–5 days before transplantation. Mice were considered diabetic when their blood glucose levels were ≥300 mg/dl for two consecutive days. Nontransfected, pSV-hVEGF transfected, or pRTP801-hVEGF transfected islets were transplanted as pellets under the left kidney capsule of diabetic syngeneic mice. Based on our previous report [14] which showed that STZ-induced diabetic Balb/c mice transplanted with 300, 200, or 100 syngeneic IEQ under the kidney capsule were reverted to normoglycemia in 90%, 50%, and 10%, respectively, we implanted 360 IEQ as a sufficient number and 220 IEQ as a marginal number to cure diabetes. After transplantation, nonfasting blood glucose levels were measured 3 days per week for 50 days. Reversal of diabetes was defined as the consistent reversal of hyperglycemia to <200 mg/dl. In 360 IEQ-transplanted mice, intraperitoneal glucose tolerance tests were performed 7 weeks after transplantation. Mice fasted for 6 h were injected intraperitoneally with a bolus of 50% glucose solution at 1 g/kg BW. Blood glucose levels were measured at the indicated times for 2 h after glucose infusion. At 50 days post-transplant, left nephrectomy was performed in the cured mice to confirm a return to hyperglycemia. To study

angiogenesis in early islet grafts, nontransfected or transfected 360 IEQ was transplanted into diabetic recipients and left nephrectomy was performed 3 or 6 days after transplantation.

Histochemistry of islet grafts

The graft-bearing kidneys retrieved 3 or 6 days after transplantation were fixed in a 10% formaldehyde solution, processed, and embedded in paraffin. The sections (4 μm thick) were stained with lectin *Bandeiraea simplicifolia* (BS-1; Sigma-Aldrich, St. Louis, MO, USA) which stains mouse islet endothelial cells [29], and were counterstained with hematoxylin. The fraction of stained blood vessels in islet grafts was quantified under a light microscope using a direct point-counting method, as described previously [11]. Briefly, a grid with 121 intersections was placed onto each tissue section under a light microscope (×400) and the number of intersections overlapping islet endothelial cells (stained with BS-1) was counted. In each graft, ≥10 tissue sections stained with BS-1 from all parts of the islet grafts were evaluated. To confirm hVEGF expression in early islet grafts, the sections were also stained with mouse anti-human VEGF 165 antibodies (1:100 dilution; BD Biosciences, San Jose, CA, USA) and streptavidin–biotin complexes.

Statistical analysis

All values are expressed as mean ± SEM. Statistical significance was calculated using a Student's *t*-test, or for comparisons involving more than two groups, a one-way analysis of variance (ANOVA). The Kaplan–Meier log-rank test was used to determine significance in the marginal islet mass transplantation experiments. A *P*-value of <0.05 was considered to be significant. All statistical analyses were performed using the MEDCALC program (Mariakerke, Belgium).

Results

Hypoxia-inducible hVEGF expression *in vitro*

Mouse islets were transfected with pSV-hVEGF or pRTP801-hVEGF and incubated under normoxia or hypoxia conditions for 12 h. The pSV-hVEGF vector drives hVEGF expression using the SV40 promoter, while the pRTP801-hVEGF vector drives hVEGF expression using the RTP801 promoter, which has hypoxia-inducible promoter activity. Human VEGF expression was significantly upregulated in the islets transfected with pRTP801-hVEGF under hypoxia conditions (Fig. 1). However, this effect was not observed in the islets transfected with pSV40-hVEGF.

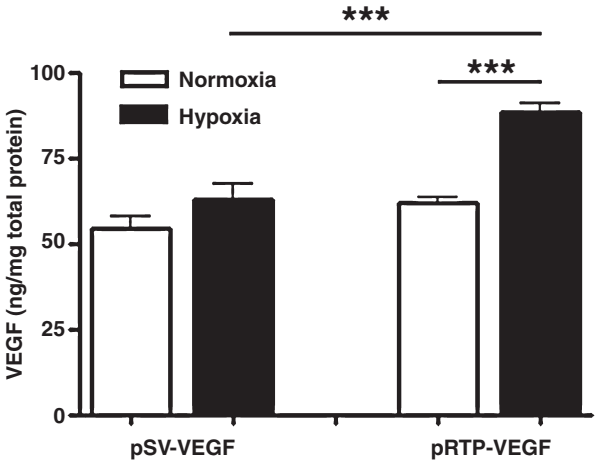

Figure 1 Human VEGF expression from pSV-VEGF or pRTP801-VEGF. pSV-VEGF or pRTP801-VEGF was transfected into mouse islets. The islets were exposed to normoxia or hypoxia for 12 h. Human VEGF was measured in culture media using ELISA. ***$P < 0.001$.

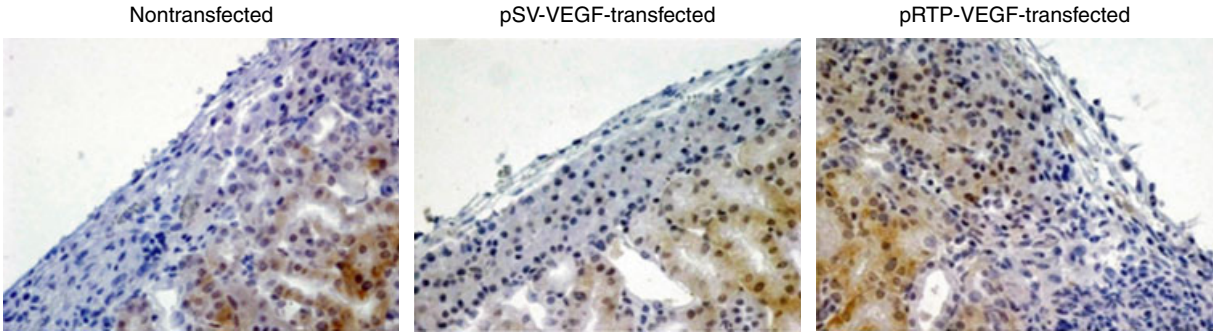

Figure 2 Human VEGF immunostaining of day 3 islet grafts. pRTP801-hVEGF-transfected islet grafts showed higher hVEGF expression compared with pSV-hVEGF-transfected islet grafts, while hVEGF expression was not detected in nontransfected islet grafts.

Human VEGF expression and vascular density in early islet grafts

To determine whether the local production of hVEGF in a hypoxia-inducible manner in islets is beneficial for islet graft revascularization in early islet grafts, pSV-hVEGF transfected, pRTP801-hVEGF transfected, or nontransfected 360 IEQs were transplanted under the kidney capsule of chemically induced diabetic syngeneic mice. The islet grafts retrieved 3 or 6 days after transplantation were stained with anti-hVEGF165 antibody or BS-1 which stains microvascular endothelial cells in formalin-fixed and paraffin-embedded tissue sections from rodents. Human VEGF immunostaining of day 3 grafts revealed that the pRTP801-hVEGF transfected group had higher hVEGF expression compared with the pSV-hVEGF transfected group (Fig. 2), while hVEGF expression was not detected in nontransfected islet grafts. BS-1 positive cells were very scarce in day 3 grafts from the nontransfected group (Fig. 3a and b), but were more abundant in day 6 grafts. Day 3 grafts from the pRTP801-hVEGF transfected group had a higher number of BS-1 positive cells compared with day 3 grafts from the nontransfected, or pSV-hVEGF groups, which was comparable with day 6 grafts from the nontransfected group. In day 6 grafts, vascular density was also higher in the pRTP801-hVEGF transfected group compared with the pSV-hVEGF transfected group (Fig. 3a).

Islet graft survival and function

To examine whether hypoxia-inducible hVEGF expression not only benefits revascularization in early grafts but also improves islet graft survival and function over time, pSV-hVEGF transfected, pRTP801-hVEGF transfected, or nontransfected islets were transplanted under the kidney capsule of diabetic syngeneic mice. Transplantation outcomes of 360 or 220 IEQ grafts were evaluated among groups for 50 days post-transplant. Hyperglycemia in all recipient mice was corrected after transplantation of 360 IEQ, and developed again after nephrectomy of the graft-bearing kidney 50 days after transplantation (Fig. 4a). While there was no difference in the diabetes reversal rate among the groups, for the intraperitoneal glucose tolerance test which was performed 7 weeks post-transplant (Fig. 4b), the area under the curve (AUC) of glucose levels for 2 h were significantly lower in mice that received pRTP801-hVEGF grafts compared with mice that received nontransfected grafts (Fig. 4c). Next, to determine whether there is a difference in the diabetes reversal rate among groups we implanted fewer islets. Diabetic mice transplanted with pRTP801-hVEGF transfected 220 IEQ became normoglycemic more rapidly compared with mice

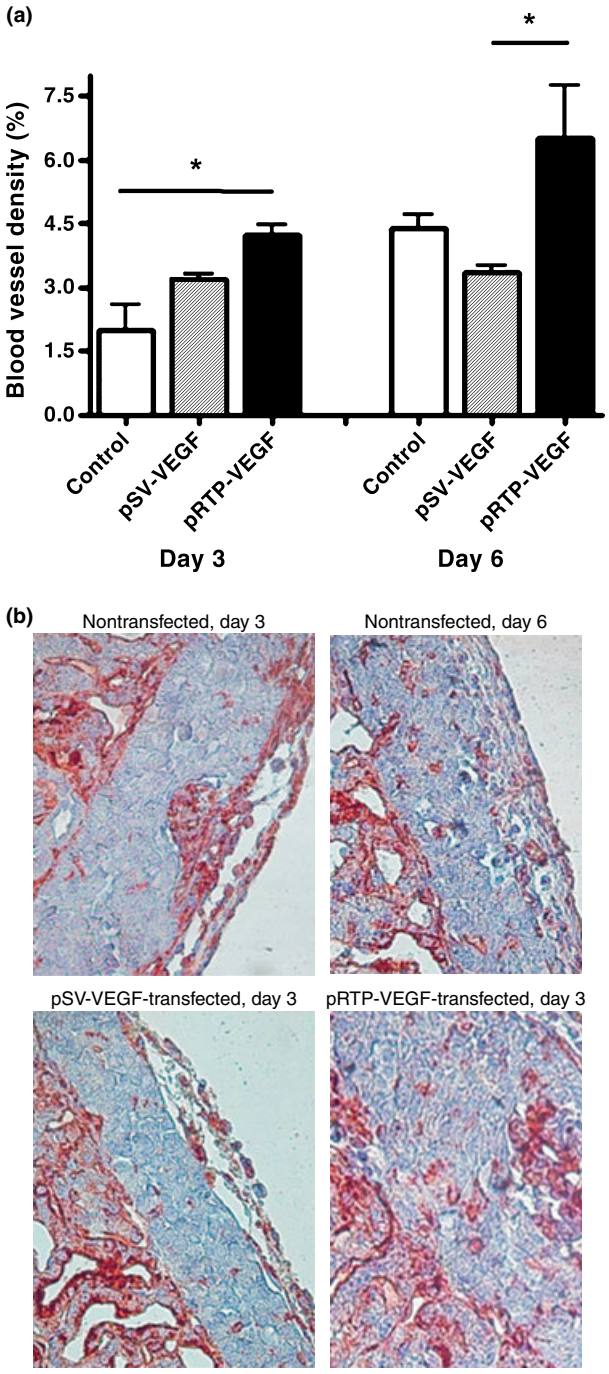

Figure 3 BS-1 staining of day 3 or day 6 islet grafts. (a) Blood vessel density, the fraction of stained blood vessels in islet grafts, obtained by a direct point-counting method. $*P < 0.05$. (b) BS-1 positive cells were very scarce in day 3 grafts of the nontransfected group, while the number of BS-1 positive cells increased in day 6 grafts. Compared with day 3 grafts from nontransfected or pSV-hVEGF transfected group, those of pRTP801-hVEGF transfected group had a higher number of BS-1 positive cells, which was comparable with day 6 grafts of nontransfected grafts.

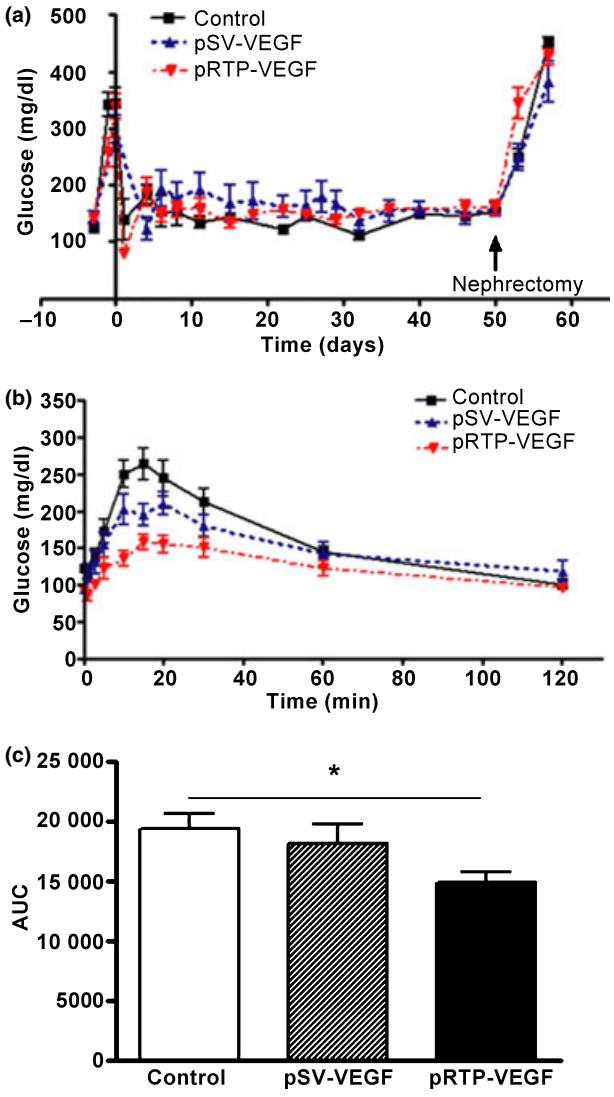

Figure 4 Blood glucose control by 360 IEQ grafts. (a) Blood glucose levels of diabetic mice transplanted with 360 IEQ of nontransfected control (n = 8), pSV-hVEGF-transfected (n = 7), and pRTP801-hVEGF-transfected (n = 7) groups for 50 days post-transplant. (b) Blood glucose levels during intraperitoneal glucose tolerance tests performed at 7 weeks post-transplant. (c) The area under the curve (AUC) of glucose levels for 2 h during the intraperitoneal glucose tolerance test (*P < 0.05).

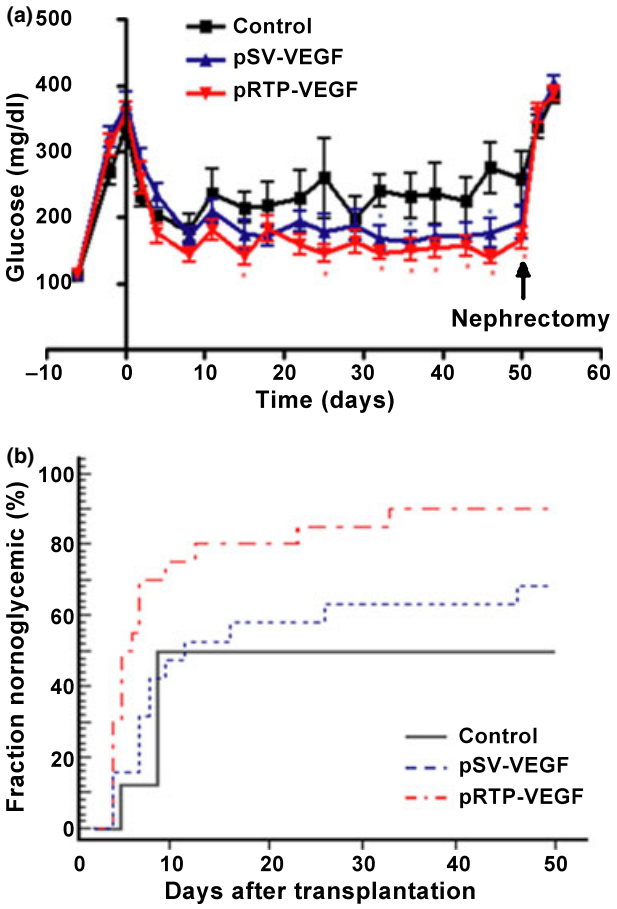

Figure 5 Blood glucose control by 220 IEQ grafts. (a) Blood glucose levels of diabetic mice transplanted with 220 IEQ of nontransfected control (n = 8), pSV-hVEGF-transfected (n = 19), and pRTP801-hVEGF–transfected (n = 20) groups for 50 days post-transplant. P < 0.05 vs. control. (b) The fraction of normoglycemic mice at different time points for 50 days after transplantation is shown. Groups differed in a Kaplan–Meier analysis (P < 0.05).

transplanted with pSV-hVEGF transfected or nontransfected 220 IEQ, indicating better islet engraftment (Fig. 5a and b). After 50 days, islet grafts containing nontransfected 220 IEQ reversed hyperglycemia in only 50% of the diabetic mice. However, upon hVEGF overexpression, islet grafts containing pSV-hVEGF transfected 220 IEQ reversed hyperglycemia in 68% of the recipients, and islet grafts containing pRTP801-hVEGF transfected 220 IEQ were able to restore normoglycemia in 90% of the recipients. Based on the Kaplan–Meier analysis, the trend of curing the diabetic mice by islet grafts differed among groups (P < 0.05).

Discussion

In this study, we evaluated the effect of hypoxia-inducible hVEGF expression by the RTP801 promoter in mouse islets *in vitro* and *in vivo*. First, we observed that hVEGF expression was significantly induced under hypoxic conditions for 12 h through the delivery of pRTP801-hVEGF in mouse islets *in vitro*. This finding is in agreement with our previous reports which showed that hVEGF expression was upregulated in rat islets transfected with pRTP801-hVEGF under hypoxic conditions [27,28]. Next, when we evaluated *in vivo* hVEGF expression in early islet

grafts using immunostaining, we found that the pRTP801-hVEGF transfected islet grafts had higher hVEGF expression compared with the pSV-hVEGF transfected grafts 3 days post-transplant, while hVEGF expression was not detected in nontransfected islet grafts. These results suggest that hypoxia during immediate post-transplant periods enhances hVEGF expression in pRTP801-hVEGF transfected islet grafts.

To determine whether this local production of hVEGF in a hypoxia-inducible manner is beneficial for islet graft revascularization following transplantation, the islet grafts retrieved 3 or 6 days after transplantation were stained with BS-1 which is a reliable marker for rodent microvascular endothelial cells and consistently stains newly formed blood vessels in islet engraftment process [29,30]. BS-1 positive cells were very scarce in day 3 grafts of nontransfected group, and showed a slight increase in number in day 6 grafts. This low vascular density in early islet grafts was in concordance with previous reports [6,31]. Compared with day 3 grafts from the nontransfected or pSV-hVEGF groups, grafts from the pRTP801-hVEGF transfected group had a higher abundance of BS-1 positive cells, which was similar to day 6 grafts of nontransfected grafts. These results indicate that enhanced hVEGF expression following transfection of pRTP801-hVEGF accelerated the revascularization process during immediate post-transplant periods. In day 6 grafts, vascular density was also higher in the pRTP801-hVEGF transfected group compared with the pSV-hVEGF transfected group.

Many reports have demonstrated that only a small fraction of transplanted islets successfully engrafted [2,4]. It has been shown that apoptosis, caused by either inflammatory or hypoxic damages, begins during the islet isolation process, peaks around post-transplant days 2–3, and continues for approximately 10–14 days until revascularization of implanted islets is completed [3,5]. Considering the time course of islet apoptosis and the graft revascularization process, it is conceivable that rapid angiogenesis by pRTP801-hVEGF delivery favors early islet survival and engraftment which may lead to sustained beta-cell mass and function. Thus, we examined whether hypoxia-inducible hVEGF expression not only benefits revascularization in early grafts but also improves islet graft survival and function over time. In 360 IEQ-transplantations, which reverted hyperglycemia in all recipient mice, the AUC of glucose levels during intraperitoneal glucose tolerance tests 7 weeks post-transplant were significantly lower in mice transplanted with pRTP801-hVEGF transfected grafts compared with mice transplanted with nontransfected grafts, suggesting that hypoxia-inducible hVEGF expression improved the glucose clearance rate in islet grafts. This positive finding prompted us to implant smaller numbers of islets to determine if there is a difference in the diabetes reversal rate between the groups. Interestingly, hyperglycemia of diabetic mice was ameliorated more rapidly following transplantation of pRTP801-hVEGF transfected 220 IEQ compared with mice transplanted with pSV-hVEGF transfected or nontransfected 220 IEQ, indicating better islet engraftment. After 50 days, the diabetes reversal rate was also higher in the pRTP801-hVEGF transfected group, demonstrating a sustained and beneficial effect on beta cell mass and function. Collectively, the improved transplantation outcome in the pRTP801-hVEGF group suggests that VEGF overexpression in islet grafts, which matches the natural islet revascularization process, allows rapid angiogenesis and enhances early islet engraftment, which in turn results in long-term functional graft mass. Although the pSV-hVEGF overexpresses hVEGF in islets, it did not show higher hVEGF expression not only under hypoxia but also under normoxia compared with pRTP801-hVEGF in rat islets [27,28] or mouse islets (Fig. 1). Thus, in this study, we could not address the issue that hypoxia-inducible hVEGF gene transfer, using the RTP801 promoter, avoids side effects of unregulated VEGF overexpression.

An efficient and safe vector system is the bottle neck of islet gene therapy. In this study, we delivered pRTP801-hVEGF to mouse islets using Effectene as a gene carrier. Although nonviral vectors including Effectene are relatively safe and conceivable for clinical applications compared with viral vectors, they have very low transfection efficiencies. We recently demonstrated that Effectene showed relatively high gene-delivery efficiency for pancreatic islets compared with other classes of nonviral gene delivery systems [28]. Depending on the target gene and the purpose of gene transfer, transient gene expression in a localized population of islet cells may be sufficient. Considering the still low transfection efficiency of nonviral carriers in islets, the delivery of genes that encode secretory proteins, like hVEGF, is more feasible in islets than genes that encode residing proteins, because the latter must be delivered to most of islet cells to be effective. Transient overexpression of hVEGF is desirable for islet transplantation because this method matches the transient nature of islet revascularization and avoids side effects of sustained overexpression. For these reasons, we adopted Effectene as a gene carrier in this hypoxia-inducible VEGF expression system in islets.

In conclusion, the delivery of pRTP801-hVEGF induces hVEGF expression specifically under hypoxic conditions in islets, enhances islet vascular engraftment, and further preserves islet mass and function over time in transplants. These results suggest that inducing timely and regulated overexpression of hVEGF genes in a hypoxia-inducible manner may be a useful strategy for *ex vivo* gene therapy

in clinical islet transplantation to improve transplant outcomes.

Authorship

BWL: designed research and wrote the paper. ML: contributed important reagents and performed the research. HYC, SL and SJL: performed the research. JGK and CSK: collected and analyzed the data. HJY: wrote the paper. SHI: designed the research, performed the research and wrote the paper.

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