

## RESEARCH ARTICLE

# Adenovirus-based vascular endothelial growth factor gene delivery to human pancreatic islets

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Islet transplantation is limited by islet graft failure due to poor revascularization, host immune rejection and nonspecific inflammatory response. Delivery of human vascular endothelial growth factor (hVEGF) gene to the islets is likely to promote islet revascularization and survival. We used a bicistronic adenoviral vector encoding hVEGF and CpG-free allele of green fluorescent protein (Adv-GFP-hVEGF) and introduced into human pancreatic islets by transfection. We found that transfection efficiency and apoptosis were dependent on the multiplicity of infection (MOI). Compared to Adv-GFP transfected and nontransfected islets, the levels of hVEGF secreted from Adv-GFP-hVEGF transfected islets were higher and exhibit a linear relationship between hVEGF expression and MOI (10–5000). Persistent, but low level

expression of hVEGF from nontransfected islets was also observed. This may be due to expression of the endogenous hVEGF gene under hypoxic conditions. The levels of DNA fragmentation determined by ELISA of islet lysates were dependent on the MOI of Adv-GFP-hVEGF. On glucose challenge, insulin release from transfected islets was comparable to nontransfected islets. Immunohistochemical staining for hVEGF was very high in Adv-GFP-hVEGF transfected islets. Weak staining was also observed for hCD31 in both transfected and nontransfected islets. These findings suggest that Adv-GFP-hVEGF is a potential candidate for promoting islet revascularization. Gene Therapy (2004) 11, 1105–1116. doi:10.1038/sj.gt.3302267

**Keywords:** human islets; adenoviral vector; real time RT-PCR; hVEGF; DNA fragmentation; immunohistochemistry

## Introduction

Although human islet transplantation offers a great potential for the treatment of type I diabetes, wide clinical application of this approach, however, is currently limited by short supply of cadaver donors, host immune rejection, primary non-function and cell death of islets following transplantation.<sup>1–3</sup> Apoptotic death of transplanted islets is further enhanced by hypoxia experienced immediately following transplantation.<sup>4</sup> To achieve successful islet transplantation, the primary non-function of islets has to be eliminated. Our previous work has resulted in improved recovery and viability of human islets by optimization of islet culture conditions.<sup>5,6</sup>

Islet destruction following transplantation can be prevented by genetically engineering  $\beta$ -cells to (1) promote revascularization, and intercept (2) apoptotic and inflammatory pathways by expressing desired proteins in the vicinity of the islets.<sup>7–9</sup> IL-10 gene expression appears to provide significant improvements in islet function in either aspect and prevents type I diabetes,<sup>10</sup> although simultaneous expression of both IL-4 and IL-10 has been shown to be more effective in this

clinical setting.<sup>11</sup> One of the two genes should initially be a reporter gene for optimization of gene transfer process and for easy assessment of transfection efficiency.<sup>12</sup> Following these lines, Kapturczak *et al*<sup>13</sup> have recently developed a bicistronic adeno-associated virus (AAV) vector driven by cytomegalovirus (CMV) enhancer-chicken  $\beta$ -actin hybrid promoter and an internal ribosome entry site (IRES) element to allow the simultaneous translation of rhodamine and green fluorescent proteins (RFP and GFP) in human and murine pancreatic islets.

Pancreatic islets are a complex cluster of heterogeneous cell types with extensive intra-islet vasculature formed of fenestrated capillary endothelial lining, which gets disrupted during islet isolation, leading to collapse of vasculature, accumulation of endothelial fragments and compromised perfusion in the core of the islets. Hence, unlike whole pancreas transplantation, islet grafts require the process of revascularization to establish adequate microvascular blood supply.<sup>14,15</sup> Isolated islets are reported to revascularize within 10–14 days after transplantation.<sup>16</sup>

Vascular endothelial growth factor (VEGF) is a homodimeric protein comprised of four isoforms of 206, 189, 165 and 121 amino acids resulting from differential splicing. Among them, VEGF<sub>165</sub> is the most abundant and widely distributed form in the majority of cells and tissues.<sup>16</sup> VEGF has two high-affinity receptors:

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tyrosine kinases flt-1 and flk-1/KDR, which are expressed exclusively in endothelial cells. VEGF is an endothelium-specific mitogen with potent angiogenic and vascular permeability-inducing properties.<sup>17,18</sup> Therefore, we recently investigated the possible use of plasmid DNA-based gene delivery approaches for transient expression of human VEGF (hVEGF) to improve neovascularization.<sup>19</sup> We recently constructed a bicistronic plasmid DNA encoding GFP and hVEGF and tested for transfection efficiency and revascularization after transplantation under the kidney capsules of the NOD-SCID mice.<sup>20</sup>

At this stage, it is not yet clear whether high or low levels of hVEGF gene expression are required for revascularization of human islets to improve islet engraftment after transplantation. Since islet is a compact cluster of about 1000 non-dividing cells, it might be difficult to transfect all cells by the available non-viral methods.<sup>19,20</sup> There are also reports of low transfection of lipofectin/pDNA, lipofectAMINE/pDNA, DOSPA:DOPE liposome/pDNA and polyethylenimine (PEI)/pDNA complexes into intact mouse, rat and porcine islets.<sup>21–23</sup> In contrast, adenoviral vectors are known to efficiently transfect non-proliferating cells, such as pancreatic islets.<sup>24,25</sup> In addition, adenovirus vectors can be produced in high titers and there is no risk of insertional mutagenesis as their genomes are not integrated into chromosomes.<sup>25</sup> Therefore, in this study, we have determined the optimal conditions for introducing a bicistronic adenoviral vector encoding both GFP and hVEGF (Adv-GFP-hVEGF) into human islets, with little adverse effect on their cellular and functional viability. We have further demonstrated a linear relationship between hVEGF gene expression and the dose of replication-deficient serotype 5 adenovirus in human islets. More importantly, we show that Adv-GFP-hVEGF

causes little apoptosis to human islets and there is no adverse effect on insulin release in response to glucose challenge at the MOI when high levels of hVEGF gene expression can be achieved.

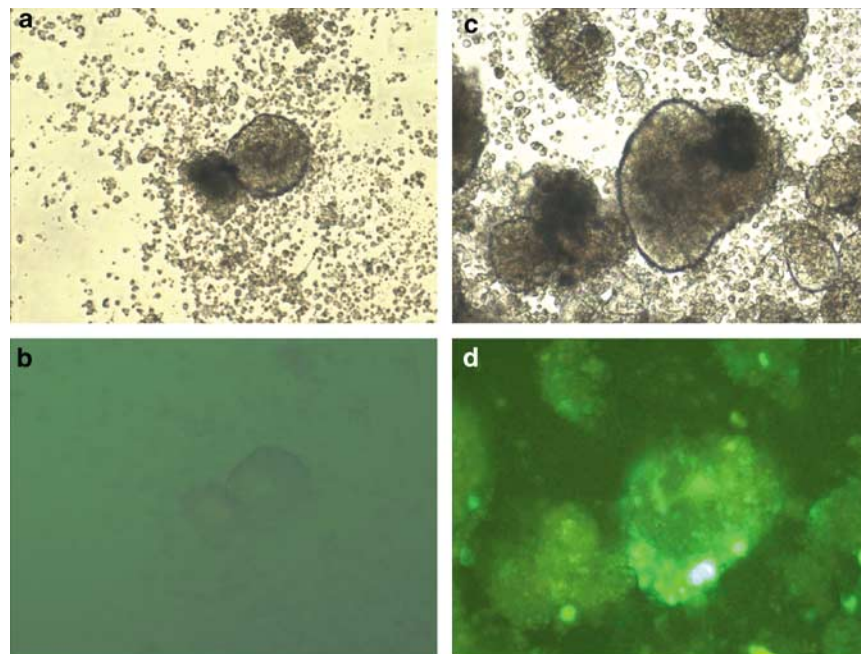
## Results

### Islet isolation and culture

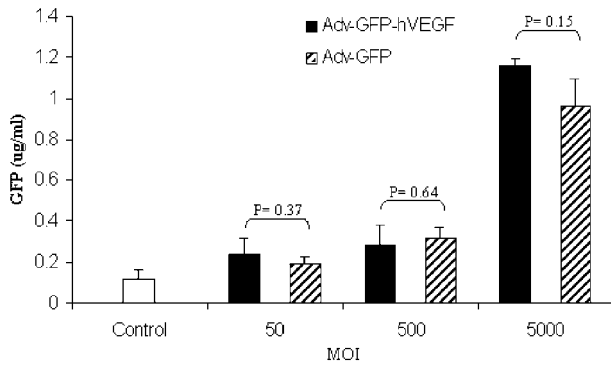
Islets were isolated from heart-beating cadaveric donors and purified as described by Gaber *et al*<sup>5</sup> Since there is a huge variation in different human islet isolations, we checked the *in vivo* islet function after each isolation by engrafting them under the kidney capsules of NOD-SCID mice.<sup>26</sup> These mice lack mature T and B lymphocytes and thus cannot mount adaptive immune responses to reject xenografts of human islets. In this model, islet viability is assessed by measuring human C-peptide and insulin levels in response to glucose challenge. C-peptide levels >1.5 and insulin >5 ng/ml were considered indicative of human islet function in the NOD-SCID mice<sup>5</sup> and these islets were used for transfection experiments.

### Effect of viral dose on transfection efficiency

Since adenovirus infection may damage human islets and interfere with their biologic function,<sup>27</sup> we determined the effect of viral dose on transfection efficiency. Results indicated expression of GFP in islets, while no GFP-positive islets were observed for non-transfected islet samples. Typical fluorescence microphotographs of human islets transfected with Adv-GFP-hVEGF at an MOI of 500 day 3 post-transfection are shown in Figure 1. Panels 'A' and 'B' show the non-transfected and transfected islets under normal light, while 'C' and 'D' show the same sample under fluorescence light.



**Figure 1** Typical fluorescence microphotographs of human islets after transfection with Adv-GFP-hVEGF at MOI 500/islet 3 days post-transfection. Non-transfected islets were used as control. (a) Non-transfected islets under normal light, (b) non-transfected islets under fluorescence light, (c) Adv-GFP-hVEGF transfected islets under normal light, (d) Adv-GFP-hVEGF transfected islets under fluorescence light.



**Figure 2** Fluorometry of human islets after transfection with Adv-GFP-hVEGF and Adv-GFP at different MOI (50, 500, 5000). Islets were collected at day 10 post-transfection, lysed with lysis buffer and GFP concentration was determined by fluorometer. Recombinant GFP was used for preparation of standard curves. The data show an increase in GFP protein expression with increase in MOI.

Non-transfected islets showed no green fluorescence (panel C), whereas transfected islets showed green fluorescence (panel D), which indicate that transfection was successful. We found that the number of GFP-expressing islets is proportional to the viral dose and incubation time post-transfection (data not shown).

Since some proteins may get lost during the extraction process from islets, we also measured the total protein content of the islet lysates by BCA protein assay kit (Pierce Chemical Company, Rockford, IL, USA) to normalize GFP expression. As shown in Figure 2, the transfection efficiency increased with an increase in MOI of both Adv-GFP-hVEGF and Adv-GFP vectors. There was no statistical difference in GFP expression between Adv-GFP-hVEGF and Adv-GFP transfected islets ( $P > 0.05$ ), suggesting no interference by hVEGF expression in the bicistronic vector.

We also determined the effect of incubation time of islets with these adenoviral vectors on their transfection efficiency (data not shown). There was an increase in the intensity and number of GFP-positive islets with increase in the incubation time, 24 h being the highest among all the time tested (3, 6, 12, 24 h). Since our results indicated that prolonged incubation of islets with adenoviral

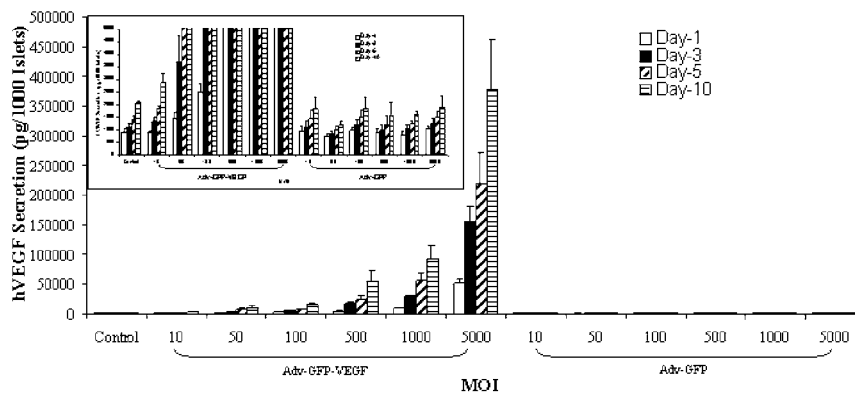
vector is detrimental to the function of the islets, in all subsequent experiments we used 12 h as the optimal time for incubation of islets with these vectors.

### Persistence of hVEGF gene expression

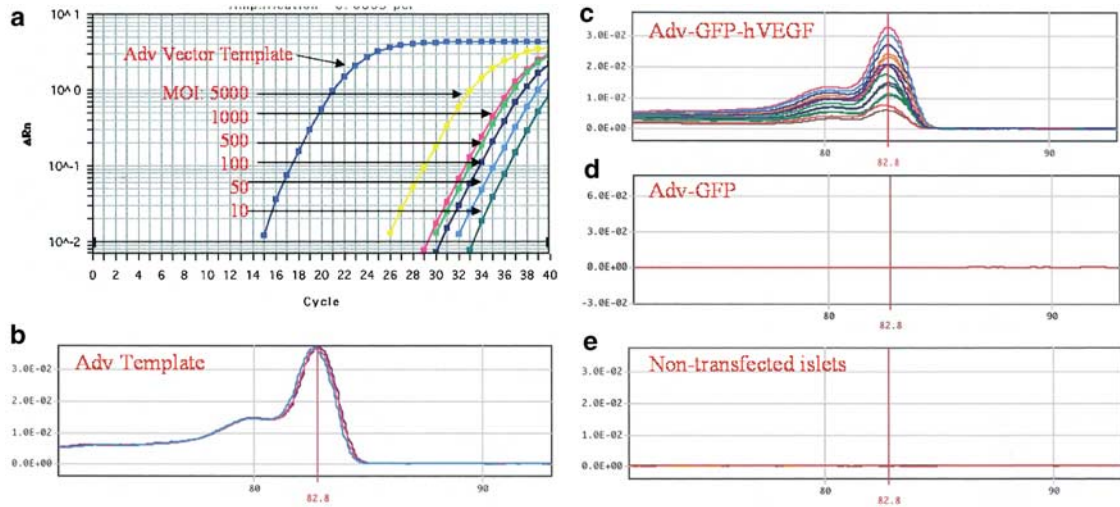
The level and duration of hVEGF expression were determined by ELISA of the culture supernatants at days 1, 3, 5 and 10 post-transfection of human islets. Islets which were not transfected but still incubated with islet growth media were used as controls for determination of endogenously produced hVEGF by human islets. Figure 3 shows the cumulative level of hVEGF protein secreted from the islets into the culture media up to 10 days. Both transfected and non-transfected islets secreted hVEGF and its secretion increased with time compared to Adv-GFP transfected and non-transfected islets. The levels of hVEGF secreted from Adv-GFP-hVEGF transfected human islets were very high and increased with viral dose as well as with incubation time post transfection. At day 10, the cumulative hVEGF secretion of Adv-GFP-hVEGF transfected islets increased 1800 times compared to the control non-transfected islet samples. Our results indicated a linear relationship between viral dose and hVEGF expression, with  $R^2 > 0.997$  for all the days tested. hVEGF secretion profiles from Adv-GFP transfected human islets are the same for different MOI (10, 50, 100, 500, 1000, 5000), and are similar to that of non-transfected islets.

### Real-time RT-PCR

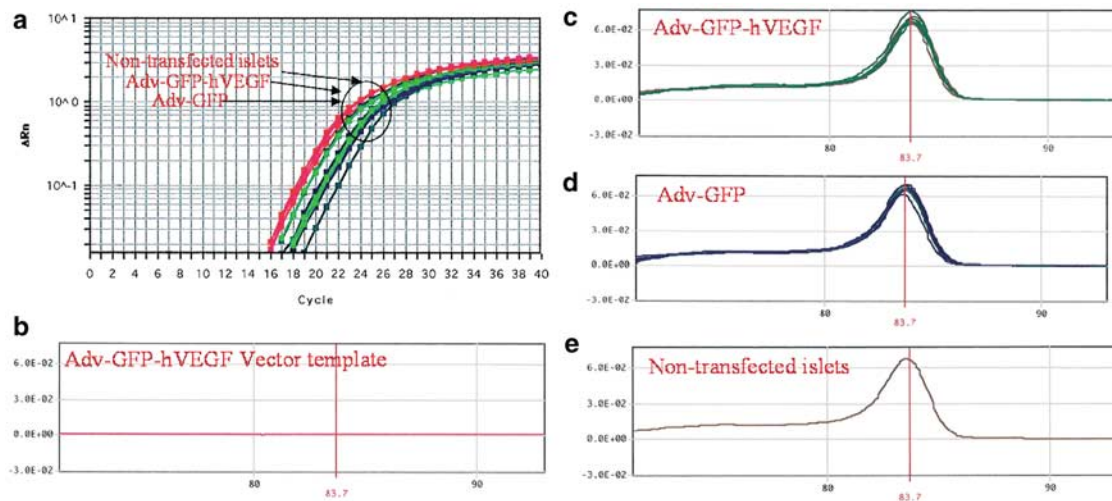
In order to determine the levels of hVEGF transcription, real-time RT-PCR was carried out by determining the threshold cycle ( $C_T$ ), which is defined as the fractional cycle number at which the fluorescence crosses the fixed threshold. The higher the  $C_T$  values, the lower the gene expression. Therefore, we transfected human islets with Adv-GFP-hVEGF or Adv-GFP at different MOI and total RNA was isolated, converted to cDNA and then amplified by real-time PCR using SYBR Green I dye. To rule out the possibility of endogenous hVEGF expression, we used a forward primer (5'-AGC CTC AGA CAG TGG TTC AAA-3') from the Adv-GFP-hVEGF vector, and the reverse primer (5'-ACT TGG CAT GGT GGA GGT A-3') from hVEGF gene. Results are presented in Figure 4a. There was at least 16–20-fold higher hVEGF-



**Figure 3** Time profile of hVEGF expression at protein level in human islets transfected with Adv-GFP-hVEGF (MOI: 10, 50, 100, 500, 1000 and 5000) as compared to Adv-GFP transfected (MOI: 10, 50, 100, 500, 1000 and 5000) and non-transfected control islets. ELISA of cell culture supernatants of separate sets of samples was done at days 1, 3, 5 and 10. The cumulative expression levels were presented in this figure. The data show consistent gene expression over the time period studied. There was an increase in hVEGF protein with increase in MOI.



**Figure 4** Real-time RT-PCR of human islets with Adv-GFP-hVEGF vector-specific primers after transfection with Adv-GFP-hVEGF, Adv-GFP and non-transfection. Change in normalized reporter signal ( $\Delta R_n$ ) was plotted against cycle number (X-axis).  $\Delta R_n$  represents the reporter signal ( $R_n$ ) minus the baseline signal. The threshold cycle ( $C_T$ ) value represents the fractional cycle number at which a significant increase in  $R_n$  (horizontal block line) can be detected. (a) Amplification plot of the Adv-GFP-hVEGF template, islets transfected with Adv-GFP-hVEGF at different MOI (10, 50, 100, 500, 1000, 5000), islets transfected with Adv-GFP at different MOI (10, 50, 100, 500, 1000, 5000), islets without transfection. (b) Melting curve of the amplified PCR products from Adv-GFP-hVEGF vector. (c) Melting curve of the amplified PCR products from islets transfected with Adv-GFP-hVEGF. (d) Melting curve of the amplified PCR products from islets transfected with Adv-GFP. (e) Melting curve of the amplified PCR products from non-transfected islets.

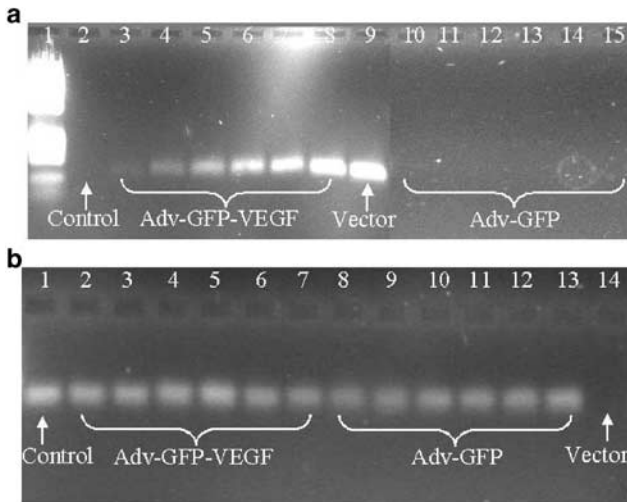


**Figure 5** Real-time PCR of human islets with 18 s ribosomal RNA-specific primers after transfection with Adv-GFP-hVEGF, Adv-GFP and non-transfection. Change in normalized reporter signal ( $\Delta R_n$ ) was plotted against cycle number (X-axis).  $\Delta R_n$  represents the reporter signal ( $R_n$ ) minus the baseline signal. The threshold cycle ( $C_T$ ) value represents the fractional cycle number at which a significant increase in  $R_n$  (horizontal block line) can be detected. (a) Amplification plot of the Adv-GFP-hVEGF template, islets transfected with Adv-GFP-hVEGF at different MOI (10, 50, 100, 500, 1000, 5000), islets transfected with Adv-GFP at different MOI (10, 50, 100, 500, 1000, 5000), islets without transfection. (b) Melting curve of the amplified PCR products from Adv-GFP-hVEGF vector. (c) Melting curve of the amplified PCR products from islets transfected with Adv-GFP-hVEGF. (d) Melting curve of the amplified PCR products from islets transfected with Adv-GFP. (e) Melting curve of the amplified PCR products from non-transfected islets.

specific mRNA for Adv-GFP-hVEGF transplanted islets at an MOI of 1000 compared to MOI of 10, indicating that the transcription levels were proportional to the dose. In contrast, little amplification of hVEGF was observed from the islets transfected with Adv-GFP ( $C_T > 40$ ) or non-transfected islets ( $C_T > 40$ ). The  $C_T$  value of Adv-GFP-hVEGF transfected islets decreased with increase in MOI, which indicated that the higher the MOI the higher the hVEGF gene expression in the transfected islets.

To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis as shown in Figure 4b–d. There was a single

predominant distinct melting temperature ( $T_m$ ) of 82.8°C for vector template control and Adv-GFP-hVEGF transfected islets, while there was no peak in Adv-GFP transfected and non-transfected islet samples. As an internal control for real-time RT-PCR, we used primers specific for 18 s ribosomal RNA. Once the mRNA was converted to cDNA, both transfected and non-transfected islets should give the same PCR products when 18 s ribosomal RNA-specific primers were used. As shown in Figure 5, the extent of amplification was the same for all transfected and non-transfected islet samples, indicating that validation of the real-time RT-PCR techniques.

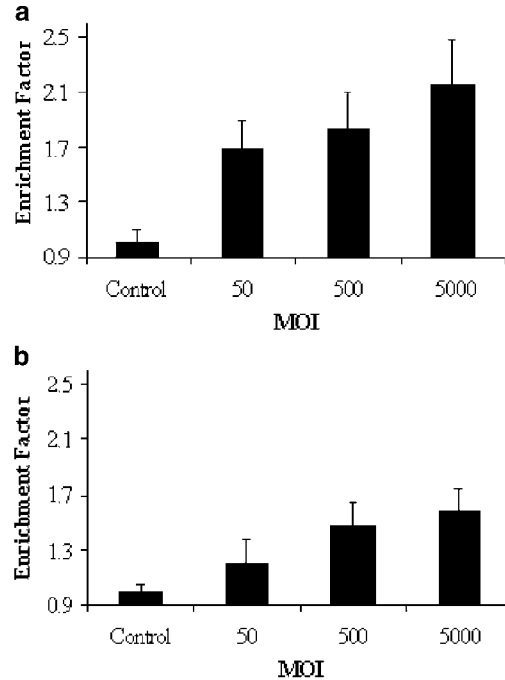


**Figure 6** Agarose gel electrophoresis of real-time RT-PCR products with Adv-GFP-hVEGF specific primers (a) and 18 s ribosomal RNA-specific primers (b). (a) Lane 1: HaeIII marker, lane 2: control (non-transfected islets); lanes 3–8: islets transfected with Adv-GFP-hVEGF (lane 3: MOI 10, lane 4: MOI 50, lane 5: MOI 100, lane 6: MOI 500, lane 7: MOI 1000, lane 8: MOI 5000); lane 9: Adv-GFP-hVEGF vector template; lanes 10–15: islets transfected with Adv-GFP (lane 10: MOI 10, lane 11: MOI 50, lane 12: MOI 100, lane 13: MOI 500, lane 14: MOI 1000, lane 15: MOI 5000). (b) lane 1: non-transfected islets; lanes 2–7: islets transfected with Adv-GFP-hVEGF (lane 2: MOI 10, lane 3: MOI 50, lane 4: MOI 100, lane 5: MOI 500, lane 6: MOI 1000, lane 7: MOI 5000); lanes 8–13: islets transfected with Adv-GFP (lane 8: MOI 10, lane 9: MOI 50, lane 10: MOI 100, lane 11: MOI 500, lane 12: MOI 1000, lane 13: MOI 5000); lane 14: Adv-GFP-hVEGF vector template.

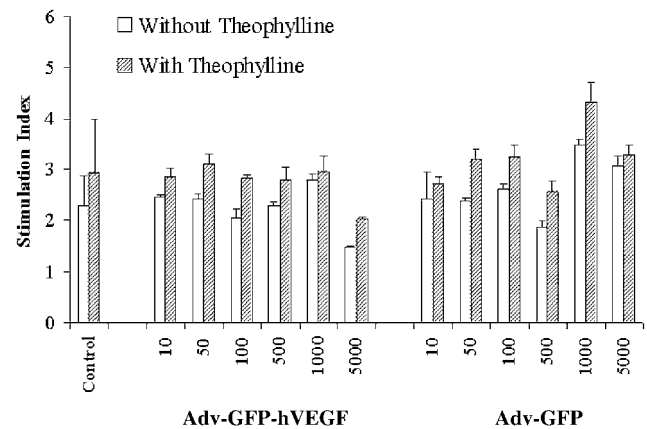
To further confirm the purity of PCR products as well as specific amplification of hVEGF gene in human islets, we carried out agarose gel electrophoresis of real-time RT-PCR products amplified using primers specific for Adv-GFP-hVEGF and 18 s ribosomal RNA. As can be seen in Figure 6b, both transfected and non-transfected islets showed bands of the same size and intensity for 18 s ribosomal RNA products. In contrast, only Adv-GFP-hVEGF transfected islets and vector template showed the same size bands of the PCR products when vector-specific reverse primers were used. The intensity of amplified band increased with increase in MOI, which is consistent with the results obtained by  $C_T$  value (Figure 4). As expected, the islets transfected with Adv-GFP and non-transfected islet samples showed no bands (Figure 6a).

**Effect of MOI of adenovirus on apoptosis and necrosis of human islets**

Transfection of replication-deficient adenoviral vectors has been shown to cause cell damage, especially at high MOI.<sup>27</sup> Therefore, we determined the effect of adenovirus dose on apoptosis and necrosis of human islets. The levels of DNA fragmentation determined by ELISA of islet cell lysates were dependent on the MOI of Adv-GFP-hVEGF and high at high MOI, compared to the untransfected control group (Figure 7). To determine the effect of adenoviral vectors on necrosis of human islets, we determined the level of enrichment factor (EF) of the culture supernatants, which is an indication of necrosis, and was much lower than those of the islet cell extracts, which is indicative of early apoptosis. This suggests that



**Figure 7** DNA fragmentation of human islets transfected with Adv-GFP-hVEGF and non-transfected islets as control. EF was calculated as the ratio of DNA fragments in transfected islets to that in non-transfected islets and 1 was used as the EF for the control samples (non-transfected islets). (a) Enrichment of DNA fragmentation in the cytoplasm of transfected islets; (b) enrichment of DNA fragmentation in the cell culture supernatant of transfected islets.



**Figure 8** *In vitro* static incubation test for human islets 3 days post-transfection with Adv-GFP-hVEGF, Adv-GFP and non-transfected control. Islet function was evaluated by measuring insulin secretion in response to varying levels of glucose concentrations from 60 mg/dl (considered basal stimulation level) to 300 mg/dl (stimulated level) and back to the basal level after theophylline treatment. Increase in secretion of insulin in response to glucose concentration and decrease with reducing concentration was indicative of metabolically healthy and functional islets.

its effect on necrosis was not as significant as on apoptosis.

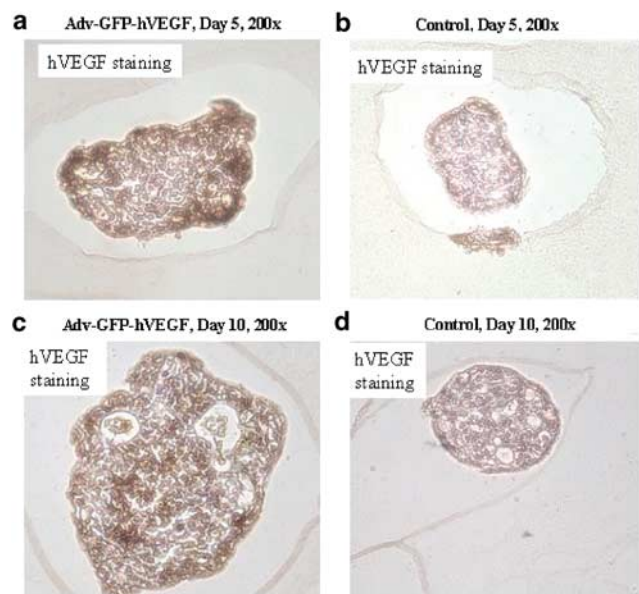
**Glucose-stimulated insulin release from transfected islets**

To determine whether insulin secretion was adversely affected by adenovirus infection, *in vitro* islet function

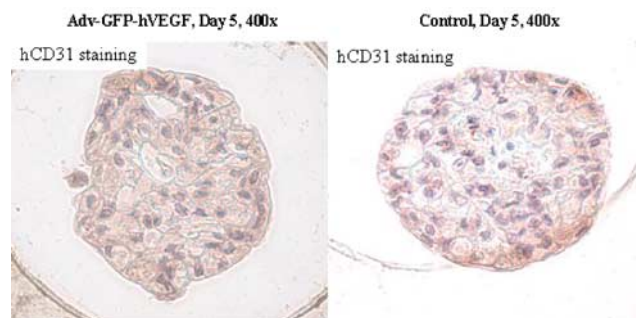
tests by static incubation of human islets were carried out after infection with Adv-GFP-hVEGF, Adv-GFP and non-transfected control. Insulin release by infected islets after 1 h incubation sequentially with MEM media containing basal (60 mg/dl) and stimulated (300 mg/dl) levels of glucose was compared to determine the stimulation index of the islets. In all groups, there was increase in the amount of insulin released when islets were incubated with both theophylline and glucose. This is indicative of the capacity of islets to respond to glucose concentration. As shown in Figure 8, the stimulation indices of islets treated with adenovirus were comparable with the non-transfected islets. In all groups, there was a further increase in the amount of insulin released when islets were incubated with both theophylline and glucose. The data indicated the maintenance of islet function over this period of time. The results suggest that transfection of human islets with Adv-GFP-hVEGF complexes does not alter the islet  $\beta$ -cell physiological response to changing glucose concentrations.

#### Effect of hVEGF on CD31 expression and cytoprotection

To determine whether hVEGF gene expression enhances the expression of human platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) by human islets in culture, we carried out immunohistochemical staining of the transfected islets with mouse anti-human CD31 antibodies, while staining with mouse anti-hVEGF antibodies was used as controls. As shown in Figure 9, positive staining of hVEGF was much higher in the transfected islets compared to non-transfected ones, suggesting efficient hVEGF gene expression after transfection of the islets with Adv-GFP-hVEGF. There was also weak positive staining for hCD31 in both transfected and non-transfected islet samples (Figure 10).



**Figure 9** Immunohistochemical staining of islets for hVEGF of human islets at days 5 and 10 post-transfection with Adv-GFP-hVEGF. These pictures were taken at  $\times 200$  magnification. Adv-GFP-hVEGF transfected islets: (a) day 5 and (c) day 10; non-transfected control: (b) day 5 and (d) day 10.



**Figure 10** Immunohistochemical staining of islets for human CD31 (also known as PECAM-1), an endothelial cell marker at days 5 post-transfection with Adv-GFP-hVEGF. These pictures were taken at  $\times 400$  magnification.

To examine the potential cytoprotective effects of hVEGF, islets were incubated with a mixture of TNF- $\alpha$  and IFN- $\gamma$  at 12 h after transfection with Adv-GFP-hVEGF. Islet samples were collected at days 5 and 10 for islet function test and immunohistochemical staining of insulin. The culture supernatants were also collected for assays of nitric oxide (NO) in terms of nitrite (NO<sub>2</sub>) using the Griess reagent system. The islet samples were used for measurement of glucose-stimulated insulin release. There was no increase in NO release in the presence of these cytokines. Similarly, the presence of 500 Units/ml of TNF- $\alpha$  and IFN- $\gamma$  had little effect on insulin release, as in all the transfected islet samples there was increase in insulin release with increase in glucose concentration from 60 to 300 mg/dl (data not shown).

#### Discussion

Pancreatic islet survival and long-term function following transplantation is often antagonized by the lack of re-establishment of capillary networks within the islets, which in turn exacerbates further immune destruction of transplanted islets.<sup>15</sup> Moreover, diabetes has been shown to impair angiogenesis, as evidenced by the reduction in neovascularization in the NOD mice due to decrease in VEGF in the ischemic tissues.<sup>28</sup> Vasir *et al*<sup>29</sup> reported that angiogenesis is delayed in diabetic recipients and thus islets transplanted into the diabetic milieu do less well than those placed into a normoglycemic environment. With this interest in mind, we recently used a bicistronic plasmid vector pCMS-EGFP-hVEGF for multiple gene delivery to human pancreatic islets.<sup>20</sup> The expression of EGFP as a reporter gene in addition to hVEGF as a functional transgene from this bicistronic vector could aid in the easy assessment of transfection efficiency and the optimization of gene transfer process. We selected hVEGF as our model therapeutic gene, as hVEGF is a human endothelial-specific growth factor, and thus has less propensity to cause unwanted side effects like transforming growth factor  $\beta$ .<sup>20</sup> Rivard *et al*<sup>28</sup> demonstrated the restoration of neovascularization in NOD mice after intramuscular injection of replication-deficient adenovirus encoding for murine VEGF. Furthermore, VEGF is also known to enhance vascular permeability *in vivo*, which might be valuable to initial islet survival and angiogenesis since islets rely on diffusion-mediated

oxygen, nutrient and metabolite transport during the initial few days of transplantation.<sup>30</sup> More recently, Linn *et al*<sup>31</sup> have demonstrated that the addition of recombinant VEGF to the culture medium increases proliferation of islet endothelial cells and causes substantial cord formation in a fibrin gel model. In a separate study, these authors further demonstrated that the survival of pancreatic cell lines depends on their ability to produce VEGF under anoxic conditions.<sup>32</sup>

In our recent study, we demonstrated a low level of hVEGF secretion when LipofectAMINE/pCMS-EGFP-hVEGF complexes were transfected into human islets at a dose of 5 µg DNA/2000 islets. Our immunohistological results suggested the involvement of both donor and host endothelial cells in islet revascularization after transplantation.<sup>20</sup> However, at this stage, it is not clear whether a high or low level of hVEGF gene expression is required for islet graft improvement after transplantation. Therefore, in this study, we have tested Adv-GFP-hVEGF vector for hVEGF gene delivery to human islets, since adenoviral vectors are known for efficient gene delivery to differentiated and non-proliferating cells, such as isolated pancreatic islets.<sup>8,33</sup>

Variation in vector MOI, vector promoters, concentration of vector-containing medium and the duration of vector incubation time are known to affect the transfection efficiency of adenoviral vectors in several cell types.<sup>34,35</sup> Therefore, as a first attempt, we sought to optimize the transfection conditions of human islets with Adv-GFP-hVEGF. The GFP intensity of islets after transfection with Adv-GFP-hVEGF increased with increase in MOI from 50 to 5000 (Figure 2), suggesting efficient transfection at high MOI. Since prolonged incubation of human islets with adenoviral vectors may induce apoptosis<sup>27</sup> and cause loss or damage of endothelial cells,<sup>36</sup> we decided not to co-culture islets beyond 12 h, even though transfection efficiency increased with increase in co-culturing time of the islets.

Both transfected and non-transfected islets secreted hVEGF persistently up to 10 days (Figure 3). In general, MOI is defined as plaque-forming unit (PFU) per cell. However, in this study, MOI was defined as PFU per islet. Considering that islet is a cluster of ~1000 cells, even the highest MOI (5000) we used in this study is similar to 5 PFU per cell. Very low but persistent levels of hVEGF expression were also seen with Adv-GFP transfected and non-transfected islets. Unlike the Adv-GFP-hVEGF transfected samples, there was no effect of MOI on hVEGF expression profiles of Adv-GFP transfected human islet samples. This could be explained by the fact that VEGF is a naturally secreted protein by the islets and Adv-GFP does not contain hVEGF gene, all hVEGF secreted from Adv-GFP transfected islets come from endogenous hVEGF expression, whose level is similar to that of non-transfected islets. Prolonged hVEGF expression is partly due to the upregulation of hVEGF gene expression under hypoxia experienced by the islets during cultures, which is in good agreement with the literature.<sup>16</sup>

Ribosomal RNA (rRNA) comprises the majority of total RNA and thus 18 s rRNA can be used as an internal control gene in quantitative real-time PCR experiments. Schmittgen and Zakrajsek have demonstrated that 18s rRNA was a more suitable internal control gene than other commonly used housekeeping genes, such as β-

actin and glyceraldehydes 3 phosphate dehydrogenase (GAPDH).<sup>37</sup> Thus, we used 18S rRNA-specific primers for real-time PCR of all samples. Figure 5a showed similar rapid amplification for all transfected and non-transfected islet samples, indicating that both the extraction of mRNA and the reverse transcription of mRNA to cDNA were successful for all islet samples. Melting curve analysis of both transfected and non-transfected islet samples showed a single predominant distinct melting temperature (T<sub>m</sub>) of 82.8°C. Our real-time RT-PCR results (Figure 4) are in good agreement with the work of Simpson *et al*,<sup>38</sup> who measured retinal VEGF mRNA by real-time RT-PCR using SYBR Green I. We also confirmed the purity and size of real-time RT-PCR products by gel electrophoresis. Both transfected and non-transfected islet samples had bands of the same size and intensity for 18 s rRNA products. In contrast, vector-specific products had viral dose-dependant band intensities, which is consistent with real-time PCR amplification plots shown in Figure 4a.

Replication-deficient (ΔE1, ΔE3) adenoviral vectors were chosen in this study because our goal was to achieve transient hVEGF gene expression, while avoiding other potential side effects and *in vivo* virus replication. However, there is still possibility that these adenoviral vectors can induce apoptosis and necrosis of human islets after transfection with Adv-GFP-hVEGF. Indeed, there are several reports to suggest that replication-deficient adenoviral vectors can illicit immune responses and inflammation if present in high concentrations.<sup>39</sup> Since the transfection efficiency is dependent on the concentration of virus, there is tendency to use high MOI to achieve high transfection efficiency. In this study, we used cellular DNA fragmentation ELISA kit to determine the effect of viral dose on apoptosis and necrosis of human islets. As reported by Weber *et al*,<sup>27</sup> EF of 1 was used for the control islet samples, whereas EFs of transfected islet samples were calculated as  $EF = (OD_{\text{transfected islets}} - OD_{\text{blank}}) / (OD_{\text{non-transfected islets}} - OD_{\text{blank}})$ . As shown in Figure 7, Adv-GFP-hVEGF seems to have some early apoptotic effect at very high MOI (5000), whereas there is little effect on necrosis, which represents cell death due to overwhelming cellular injury. Unlike apoptosis, necrosis is characterized by swelling and lysis of the nucleus and cellular membrane as well as random digestion of DNA.<sup>40</sup> Our results are in consistent with the work of Weber *et al*, who also found some apoptotic effects of adenovirus on rat islets. However, these authors did not report the effect of adenovirus on necrosis of rat islets since they did not use the culture medium to determine the EF.

Insulin release upon glucose stimulation is a measure of islet function, which is a prerequisite to any gene therapy program for diabetes treatment by islet transplantation.<sup>41</sup> Islet function is routinely determined by static glucose stimulation where islets are sequentially incubated with 60 mg/dl glucose (basal level), then with 300 mg/dl glucose (stimulated level) and eventually with 60 mg/dl glucose (basal level) (basal→stimulated→basal). Since theophylline is a known potent secretagogue for insulin, we also co-cultured islets with glucose (300 mg/dl) and theophylline (180 mg/dl). As shown in Figure 8, the stimulation index was found to be comparable for transfected and non-transfected cultures. The co-culture of theophylline increased the stimulation index for all islet samples, which is in good agreement

with the literature.<sup>42</sup> Furthermore, reduction of glucose concentration back to basal level has resulted in reduction in insulin response to basal level in all cases, indicating that the islets do remain dynamically responsive to glucose concentration. Our results for the *in vitro* islet function test are in good agreement with the work of Dobson *et al*,<sup>26</sup> who demonstrated that the transfection of human islets with the adenovirus did not affect graft function in NOD-SCID mice recipients, as human insulin secretion from the transfected and non-transfected grafts did not differ. This is also consistent with the results reported by others showing that transfection of islets with adenoviral vectors does not inhibit islet graft function in allograft or xenograft models.<sup>27</sup>

Platelet endothelial cell adhesion molecule-1 (PECAM-1/hCD31) is known to express on the surface of vascular endothelium and is implicated in angiogenesis and vasculogenesis.<sup>43</sup> To determine whether transfection with Adv-GFP-hVEGF enhances hCD31 expression in the human islets in culture, we performed immunohistochemistry for hCD31 using mouse anti-human CD31 antibodies at days 5 and 10 post-transfection. Positive staining for hVEGF was much higher for Adv-GFP-hVEGF transfected islets (Figure 9). In contrast, hCD31 staining was weak and therefore we could not use this method to detect the difference in hCD31 staining between transfected and non-transfected islets (Figure 10). Islet endothelial cells are reported to disappear or dedifferentiate during culture, but islet vasculature appears to be re-built after transplantation. We and others have shown that after transplantation newly formed microvessels grow from the microvasculature of the host into the tissue of the graft. The endothelium of revascularized islets originates from the host vascular bed.<sup>20,44</sup> Moreover, Furuya *et al*<sup>45</sup> demonstrated that the positive staining of hCD31 is high in freshly isolated islets, but significantly decreased in culture and almost disappear after 7 days of culturing. In the freshly isolated islets islet isografts, hCD31-positive cells remained viable immediately after transplantation. Therefore, we plan to determine the effect of hVEGF proteins on hCD31 expression after transplantation of human islets transfected with Adv-GFP-hVEGF under the kidney capsule of NOD-SCID mice.

In order to determine the cytoprotective effect of hVEGF, we also transfected human islets with Adv-GFP-hVEGF in the presence or absence of pro-apoptotic cytokines, such as TNF- $\alpha$  and IFN- $\gamma$ . At days 5 and 10 post-transfection, 1 ml of the culture supernatants was collected for nitric oxide (NO) assay in terms on nitrite (NO<sub>2</sub><sup>-</sup>), using the Griess Reagent system.<sup>46</sup> The islet samples were used for glucose-stimulated insulin release.<sup>19</sup> There was no increase in NO release in the presence of these cytokines. Similarly, the presence of 500 Units/ml of TNF- $\alpha$  and IFN- $\gamma$  had little effect on insulin release, as in all the transfected islet samples there was increase in insulin with increase in glucose concentration from 60 to 300 mg/dl (data not shown). Our results suggest that hVEGF gene expression protects islets from the apoptotic effect of TNF- $\alpha$  and IFN- $\gamma$ . There is also a report on the preservation of islet structure and function when encapsulated in the presence of hVEGF.<sup>47</sup> VEGF has been shown to activate *Raf* kinases, resulting in protection in human endothelial cells. Arnush *et al*<sup>48</sup> demonstrated that the apoptotic effect of TNF- $\alpha$  and IFN-

$\gamma$  is greatly dependant on their concentration and presence of IL-1 $\beta$ . Moreover, the cytoprotective effect of hVEGF is likely to be affected by the age of islets used. Therefore, we plan to do similar experiments using high concentration of TNF- $\alpha$ , IFN- $\gamma$  and IL-1 $\beta$  as a mixture using freshly isolated islets.

In summary, we have demonstrated that bicistronic adenoviral vector, Adv-GFP-hVEGF, can efficiently transfect human islets with little side effect on their cellular and functional viability. hVEGF expression persisted up to 10 days post-transfection and there was a linear relationship between viral dose and hVEGF expression. Compared to LipofectAMINE/pCMV-EGFP-hVEGF complexes, Adv-GFP-hVEGF efficiently transfected human islets. Therefore, we expect significant improvement in revascularization and prolongation of islet survival. We are in process of testing Adv-GFP-hVEGF *in vivo*.

## Materials and methods

### Materials

Adenoviral vector encoding GFP and hVEGF (Adv-GFP-hVEGF, commercially known as AdenoVec-hVEGF) and Adv-GFP (commercially known as AdenoVec-Null) were purchased from InvivoGen (San Diego, CA, USA). 293 T cell line was purchased from ATCC (Manassas, VA, USA). Agarose, TBE buffer and anti-mouse IgG were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) and Promega (Madison, WI, USA), respectively. Fetal bovine serum (FBS) and L-glutamine were purchased from Mediatech Cellgro<sup>®</sup> (Herndon, VA, USA), penicillin/streptomycin, gentamycin, phosphate-buffered saline (PBS), 0.25% (w/v) trypsin-EDTA and DMEM were purchased from GIBCO-BRL (Gaithersburg, MD, USA). ITS was purchased from Collaborative Biomedical Products (Bedford, MA, USA), which contains the following components: insulin (6.25  $\mu$ g/ml), transferrin (6.25  $\mu$ g/ml), selenious acid (6.25 ng/ml), linoleic acid (5.35  $\mu$ g/ml), and albumin (1.25 mg/ml). Human insulin was procured from Alpco Diagnostics (Windham, NH, USA). Recombinant Human VEGF<sub>165</sub> and hVEGF ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). RNA STAT 60 was purchased from Tel Test Inc. (Friendswood, TX, USA). SYBR Green real-time PCR master mix and reverse transcriptase reagents were obtained from Applied Biosystems (Foster City, CA, USA). Cell Death Detection ELISA<sup>PLUS</sup> kit was purchased from Roche Diagnostics Corp. (Indianapolis, IN, USA). IFN- $\gamma$  and TNF- $\alpha$  were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA) and Griess Reagent System from Promega (Madison, WI, USA). Mouse anti-human CD31 was purchased from Biosource International, Inc. (Camarillo, CA, USA) and mouse anti-human hVEGF was purchased from Dako Cytomation (Carpinteria, CA, USA). DAB substrate solution was purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

### Methods

#### Amplification and titration of adenoviral vectors

Replication-deficient serotype 5 adenoviral vector encoding a CpG-free allele of the green fluorescent protein

(GFP) and hVEGF (Adv-GFP-hVEGF, commercially known as AdenoVec-hVEGF) as well as Adv-GFP (commercially known as Adv-Null) were purchased as a lyophilized adenoplasmid from InvivoGen (San Diego, CA, USA). Replication-deficient vectors were chosen because the goal was to achieve transient gene expression, while keeping other potential side effects and *in vivo* virus replication minimized. GFP is cloned in the first transcription unit under the control of the FerH composite promoter, whereas hVEGF cloned in the second transcription unit driven by the FerL composite promoter. Adv-GFP, which does not contain hVEGF gene, was used as a control. These E1–E3 deleted adenoplasmids were mixed with LipofectAMINE at 5/1 w/w and the complexes were used for transfection of human embryonic kidney cell line 293 cells at a dose of 1.5 µg DNA/1 × 10<sup>6</sup> cells per well in a six-well plate. Cells were incubated at 37°C in a CO<sub>2</sub> incubator and split into three 100 mm culture dishes at 48 h post-transfection. Following observation of small plaques under microscopy at 7–10-day post-transfection, cells were lysed by four cycles of freeze/thaw in the dry ice-methanol bath and the 37°C water bath (cell suspension was vortexed after each thaw), centrifuged at 12 000 g for 10 min, and supernatant was used as the viral stock. Adenoviruses were amplified by infection of 293 cells with viral stock and the viral titer was determined using Tissue Culture Infectious Dose 50 (TCID<sub>50</sub>) method, which is based on the development of cytopathic effect.

#### *Pancreas gland procurement, islet isolation and purification*

Pancreas were obtained from heart-beating cadaveric multiorgan donors with appropriate consent through the efforts of the following agencies: IBS International Bioresearch Center (Tucson, AZ, USA), Tennessee Donor Services, the Mid-South Transplant Foundation, Life Resources Regional Donor Center, and the United Network for Organ Sharing. The pancreata were perfused *in situ* by aortic flushing with University of Wisconsin (UW; Viaspan Dupont Pharma, Wilmington, DE, USA) solution; pancreata were then placed in a container of cold UW solution and packaged in wet ice for shipping to our Islet Processing Laboratory.

On arrival at our facility, the pancreas were passed into an isolator module (CBC Ltd, Madison, WI, USA), described previously.<sup>5</sup> The organ was trimmed and weighed to determine the appropriate size digestion chamber and collagenase volume to use for distension. Collagenase solution was prepared in Hank's balanced salt solution (HBSS; Mediatech, Inc., Herndon, VA, USA) supplemented with 0.2 mg/ml DNase, 20 mg/dl calcium chloride and HEPES. The pH was adjusted to 7.7–7.9. Once dissolved, the collagenase was sterile filtered, warmed to 37°C, and passed into the isolator module; then, the pancreas was distended intraductally. The chamber was closed and the heating circuit started. The digestion mix was brought up to 35–37°C and maintained at this temperature until tissue collection began, at which time the heating circuit was disabled. Digested tissue was collected into cold islet dilution solution (IDS) supplemented with 20% human albumin. The tissue digest was centrifuged at 400 g at 4°C for 5 min.

Islet purification was performed on the COBE 2991 Cell Processor (COBE BCT, Lakewood, CO, USA) using OptiPrep (Nycomed Pharma AS, Oslo, Norway) and a discontinuous gradient system. Following purification, islet tissue was rinsed with IDS and re-suspended for counting in CMRL containing 1% ITS, 1% L-glutamine, 1% AB/AM, 16.8 µM/l ZnSO<sub>4</sub> buffered with HEPES to a pH of 7.4.

#### *Transfection of human islets*

Aliquots from human islet isolations were cultured in a serum-free media (SFM) containing 1% ITS, 1% L-glutamine, 1% albumin and 16.8 µM/l zinc sulfate at 37°C in a 5% CO<sub>2</sub> incubator with media change at weekly intervals. Human pancreatic islets were transfected using Adv-GFP-hVEGF, while Adv-GFP transfected and non-transfected islets were used as controls. To determine the optimal multiplicity of infection (MOI, infectious units/islet), 1000 islets in 150 µl media were co-cultured in 48-well plates with 50 µl Adv-GFP-hVEGF or Adv-GFP at MOI 10:1, 50:1, 100:1, 500:1, 1000:1 and 5000:1. After incubation for 12 h, transfected islets were washed with fresh medium and transferred to a 24-well plate with 2 ml fresh medium each well. GFP expression by transfected islets was also determined by fluorescence microscopy as well as by fluorometry of the cell extract of the islets lysed by cell lysis buffer (Promega, Madison, WI, USA). Total protein of the cell extracts of the islet lysates was also determined by bicistronic acid (BCA) protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Triplicate supernatants from each group were withdrawn at days 1, 3, 5 and 10, and analyzed for hVEGF concentration by ELISA as per vendor protocol (R&D Systems, Minneapolis, MN, USA).

#### *Quantitative real-time RT-PCR for evaluation of mRNA expression*

Expression of hVEGF in human islets was assessed at mRNA level by real-time RT-PCR. Islets were pelleted 72 h post-transfection by centrifugation at 500 g for 10 min. Total mRNA was isolated from cell pellets by a guanidine isothiocyanate method using RNA STAT 60 reagent as per the vendor protocol (Tel Test Inc., Friendswood, TX, USA). RNA concentration was measured by UV spectrophotometry using Biomate 3 spectrophotometer (ThermoSpectronic, Waltham, MA, USA). Extracted RNA (1 µg) was converted to cDNA using MultiScribe Reverse Transcriptase Reagent and random hexamers (Applied Biosystems, Inc., Branchburg, NJ, USA) by incubation at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and enzyme inactivation at 95°C for 5 min. In all, 5 µl of the extracted cDNA was amplified by real-time PCR using SYBR Green-I dye universal master mix on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA). To determine the level of hVEGF expression by Adv-GFP-hVEGF while excluding hVEGF expression resulting from endogenous production or stimulation by the adenoviral vectors, vector-specific forward primer (5'-AGC CTC AGA CAG TGG TTC AAA-3') was used for both Adv-GFP-hVEGF and Adv-GFP, while hVEGF gene-specific reverse primer with the following sequence: 5'-ACT TGG CAT GGT GGA GGT A-3', which is designed based on the Gene

Bank sequence#: AY047581 (hVEGF<sub>165</sub>). The PCR conditions included denaturation at 95°C for 10 min, followed by 40 cycles of amplification by sequential denaturation at 95°C for 15 s and primer annealing as well as strand extension at 60°C for 1 min.

To confirm amplification specificity, the PCR products were subjected to a melting-curve analysis and agarose gel electrophoresis. The expressed hVEGF mRNA level was quantified using standard curve and normalized to the total amount of cDNA used. Threshold cycle number was compared between Adv-GFP-hVEGF and Adv-GFP with the same parameters, using non-transfected samples as controls.

#### Detection of DNA fragmentation by ELISA

To assess the effect of viral dose of transfected human islets with adenoviral vectors on their cell death by necrosis and apoptosis, DNA fragmentation of the transfected islets was determined using Cell Death Detection ELISA<sup>Plus</sup> as described by the manufacturers (Roche Diagnostics Corp., Indianapolis, IN, USA). This ELISA kit allows the determination of cytosolic histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of the cell lysates after induced cell death. These islets were then re-suspended in 0.2 ml lysis buffer, incubated for 30 min at room temperature, centrifuged at 500 g for 10 min and the cell extracts were applied to a microtiter plate coated with anti-histone antibody. To analyze necrosis, supernatants of transfected and non-transfected control islets were removed carefully and stored at 4°C. The immunoreagent was prepared by mixing 4320 µl of incubation buffer, 240 µl of anti-histone-biotin and 240 µl of anti-DNA-Peroxidase (anti-DNA-POD). In all, 20 µl of the cell extracts and 80 µl of the immunoreagents were added to streptavidin-coated microplate, the plate covered with an adhesive foil and incubated for 2 h under gently shaking (250 rpm) at room temperature. A volume of 100 µl of peroxidase substrate 2,2'-azino-di-[5-ethylbenzthiazoline sulfonate (ABTS) solution was added to each well, incubated for 10–20 min to allow color development and measured at 405 nm against ABTS solution as a blank (reference wavelength approx. 450 nm). The results were expressed as EF, which represents the ratio of optical densities (OD) for experimental conditions and control according to the formula,  $EF = (OD_{\text{transfected islets}} - OD_{\text{blank}}) / (OD_{\text{non-transfected islets}} - OD_{\text{blank}})$ .

#### In vitro islet function after transfection

The *in vitro* function of human islets after transfection with Adv-GFP-hVEGF was determined by static incubation as described before.<sup>19</sup> Non-transfected islets were used as controls. Transfection media was removed by centrifugation at 400 g and the islets were sequentially incubated at 37°C for 1 h with MEM media containing low (0.6 mg/ml, basal), high (3 mg/ml, stimulated) and glucose plus theophylline (1.8 mg/ml), and again back at the basal glucose levels for 1 h each at 37°C in 5% CO<sub>2</sub> incubator. After incubation, supernatants were collected and analyzed for insulin release by ELISA (Alpco Diagnostics, Windham, NH, USA). Insulin secretion was expressed as µU/ml and the ratio of insulin level at 300 mg/dl glucose to that at 60 mg/dl was used to calculate the stimulation index (SI).

#### Immunohistochemistry

Following transfection with Adv-GFP-hVEGF, islets were washed with PBS, fixed in 4% formaldehyde for 45 min and then washed with PBS. Agar solution (2%) was prepared by boiling water. Islets were gently suspended in 0.2 ml of warm agar and promptly put in refrigerator, a solid gelled agar containing islets was formed. The gelled agar was removed from the tube and stored in 70% ethanol at room temperature until further processing. Islet embedded agar was dehydrated by serial incubation in 95% ethanol and then in 100% ethanol, twice for 20 min each. The samples were then incubated in xylene for 60 min, embedded in paraffin in biopsy cassettes and stored at room temperature. Sections of 7 µm thickness were then prepared from paraffin-embedded blocks on a microtome, floated on warm water and picked up on superfrost glass slides. Samples were fixed on glass slides overnight in a 37°C oven, followed by 1 h of fixation at 60°C. Samples were deparaffinized by incubation in xylene for 5 min thrice and then hydrated in 100% ethanol, followed by 95% ethanol, 70% ethanol and finally in water. All samples were put in peroxidase (0.8% v/v) for 20 min and then washed with water for 1 min. The samples were incubated at 90–94°C in diluted antigen unmasking solution for 10 min and then cooled down to 40–50°C. The slides were then transferred into PBS buffer for 5 min and circled each section with PAP PEN. One drop of diluted rabbit serum was added to each section and incubated for 30 min. Diluted mouse anti-h VEGF or CD-31 was then added to each section and incubated for 1 h. After washing with PBS for 2 min, diluted anti-mouse IgG was added to each section and incubated for 30 min. The slides were then washed with PBS for 5 min, incubated in DAB substrate solution for 5 min and washed with PBS for 5 min. Slides were stained with hematoxylin for 1 min and dehydrated by serial incubation in the following solvents thrice for 2 min each: 75% ethanol, 95% ethanol, 100% ethanol and xylene.

#### Cytoprotective effect of hVEGF

In all, 1000 islets per well of 48-well plates were transfected with 50 MOI of Adv-GFP-hVEGF. At 12 h post-transfection, islets were washed and incubated at 37°C with 500 Units/ml each of TNF-α and IFN-γ. At days 5 and 10, 1 ml of the culture supernatants was collected from each well for assays of nitric oxide (NO) in terms of nitrite (NO<sub>2</sub><sup>-</sup>) using the Griess Reagent System.<sup>46</sup> The islet samples were used for determining glucose-stimulated insulin release.<sup>19</sup>

#### Statistical analysis

The statistical significance of differences between groups was determined by applying one-way analysis of variance (ANOVA). Statistical significance was set at  $P < 0.05$ . Results are expressed as the mean ± SD.

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