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Induction of Efficient Differentiation and Survival of Porcine Neonatal Pancreatic Cell Clusters Using an EBV-based Plasmid Expressing HGF

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Porcine neonatal pancreatic cell clusters (NPCCs) have been actively studied as a source of pancreatic stem cell transplantation for the treatment of diabetes. In this study, the hepatocyte growth factor (*HGF*) gene was cloned in an Epstein–Barr virus (EBV)-based plasmid vector (pEBVHGF) and the effects of the HGF expression on the survival and differentiation of NPCCs were analysed. For comparison, pHGF was constructed by deleting EBNA-1 and OriP from pEBVHGF. The expression of HGF, as measured by ELISA, lasted longer when pEBVHGF was used than when pHGF was used. C-Met phosphorylation co-related with the expression of HGF in the transfected NPCCs. Immunocytochemistry experiments showed that NPCCs showed a higher and longer expression of insulin when they were transfected with pEBVHGF than with pHGF. Moreover, a greater number of NPCCs survived for a longer period after they were transfected with pEBVHGF than when they were transfected with pHGF. Taken together, these results indicate that transfecting NPCCs with the *HGF* gene using an EBV-based plasmid is a more effective method of inducing differentiation to beta cells and enhancing survival than using a conventional plasmid. Therefore, it may be possible to use EBV-based plasmids to modify pancreatic stem cells for xenotransplantation.

Key words: Epstein–Barr virus-based plasmids, hepatocyte growth factor, insulin, porcine neonatal pancreatic cell clusters.

Abbreviations: DAPI, 4′6-diamidino-2-phenylindole; DMEM, Dulbeco's Modified Eagle Medium; EBNA-1, EBV nuclear antigen 1; EBV-based plasmid, Epstein-Barr virus-based plasmids; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GFP, green fluorescence protein; HBSS, Hank's balanced salt solution; HGF, hepatocyte growth factor; IFA, immuno-fluorescence assay; NPCCs, porcine neonatal pancreatic cell clusters; OriP, EBV replication origin.

Type 1 diabetes is an autoimmune disease that is caused by the destruction of beta cells in the Langerhans islets of the pancreas (*1*). This loss of cells leads to elevated blood glucose level, which in turn causes many complications (*2*). Diabetes is treated either by regularly supplying insulin *via* injections or by transplanting the pancreas or islets to replace the beta cell mass (*3*, *4*). However, because there is an insufficient supply of pancreases and islets which are available for transplantation, pigs have been considered as a source for xenotransplantation (*5*). The organs of pigs are morphologically and physiologically similar to those of humans, and porcine insulin has been used to safely treat diabetes in humans.

Porcine neonatal pancreatic cell clusters (NPCCs) are primarily comprised of pancreatic ductal cells, which are considered to be pancreatic stem cells because they have the potential to proliferate and differentiate to beta cells, as well as to react to glucose stimulation in the culture (*6*, *7*). In a study performed in Mexico, transplantation of NPCCs to type I diabetes patients induced insulin production for 4 years without administration of immune suppressors (*8*). However, it takes a long time for NPCCs to differentiate to beta cells, and they do not survive long enough after transplant.

Hepatocyte growth factor (HGF) is a multi-functional factor that is derived from the mesenchyme and expressed in pancreatic islets (*2*, *9*, *10*). HGF is expected in many organs such as placenta, kidney, lung and ovary (*11–13*). HGF facilitates tissue repair and organ regeneration after injury by bindings to the c-Met receptor. HGF also functions as an insulinotropic factor in the pancreas (*2*, *10*, *14–16*), and HGF as well as receptor c-Met are highly expressed during pancreas development (*10*). Furthermore, HGF enhances viability and differentiation of ductal cells to beta cells (*17–19*), therefore manipulating NPCCs to express HGF prior to transplantation would be beneficial.

To maximize the beneficial effect of HGF, it is plausible to use a vector that can express HGF for a longer period than a conventional plasmid. Previously, we showed that NPCCs expressed GFP for more than 17 days when they were transfected with the *GFP* gene

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cloned in an Epstein–Barr virus (EBV)-based plasmid (*20*, *21*). This prolonged gene expression in the porcine cells was not due to integration of the EBV-based plasmid into the host DNA, but due to its self-replication as an episome (*22*, *23*). These results indicate that the EBV-based plasmid is an excellent candidate vector for transfection of HGF into NPCCs.

In this study, we compared the efficiency of an EBV-based plasmid with that of a conventional plasmid at expressing HGF in NPCCs. pEBVHGF was constructed by cloning the *HGF* gene in the EBV-based plasmid, pCEP4, and then its effect on the survival and differentiation of NPCCs was examined. pHGF that had been prepared from pEBVHGF by deleting EBNA-1 and OriP was used as a conventional plasmid control.

MATERIALS AND METHODS

Materials—Hank's balanced salt solution (HBSS), dissociation medium (Sigma Chemical, St Louis, MO, USA), collagenase P (Boehringer-Mannheim, Indianapolis, IN, USA) and Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) were used to isolate and culture cells from neonatal pig pancreases. Nicotinamide (1,220 mg/l), HEPES (2,380 mg/l), bicarbonate (2,000 mg/l), L-glutamine (600 mg/l), fetal bovine serum (FBS; 100 ml/l) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, Sigma Chemical) were added to DMEM (8 mM glucose). Polyclonal guinea pig anti-insulin (Zymed, San Francisco, CA, USA) and rhodamine-conjugated anti-guinea pig IgG (Jackson Immunoresearch, Inc., West Grove, PA, USA) were used in the immunofluorescence detection of insulin. For immunofluorescence staining of activate c-Met, rabbit polyclonal anti-c-Met phosphospecific antibody (Invitrogen) was used.

Preparation of Porcine NPCCs—NPCCs were prepared using the technique described by Korbutt *et al*. (*24*) with a slight modification. Briefly, porcine neonatal pancreas was obtained from 1- to 3-day-old pigs that had been anaesthetized with Ketamin and Rumpun. The pancreases were harvested and minced into 2-mm^3 fragments in M199 using scissors, and then washed three times with cold HBSS. Next, the minced pancreas was digested with 2.5 mg/ml of collagenase P at 37°C with gentle shaking, washed three times with HBSS at 4°C and then re-suspended in 10–30 ml of Ham's F10 medium. The cell suspension was seeded in 150×15 mm^2 bacteriological plates (Nalge Nunc International, Rocskilde, Denmark) and cultured at 37°C in a humidified atmosphere under 5% CO_2. After 12 h, 25 ml of Ham's F10 medium was added and the cells were incubated overnight. Next, the cells were collected and suspended in 10 ml of dissociation medium (Sigma Chemical). The cell clusters were broken apart by repeated gentle aspiration with a pipette. The separated NPCCs were transferred into 12-well plates at a density of 5×10^5 cells/well and maintained in DMEM medium containing 10% FBS at 37°C under 5% CO_2. Unattached cells were removed after 24 h.

Plasmid Construction—To transfect the NPCCs, pEBVHGF was constructed by cloning the *HGF* gene in the EBV-based plasmid, pCEP4 (Fig. 1). pCEP4 carries

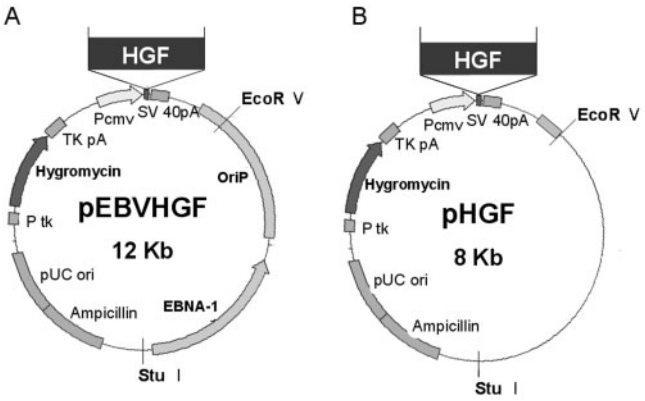

Fig. 1. **Schematic representation of pEBVHGF and pHGF.** The EBV-based plasmid contains nuclear antigen-1 (EBNA-1) and the origin of EBV replication (oriP). The resistance gene for hygromycin and ampicillin, as well as a prokaryotic plasmid origin of replication are also present in both the plasmids.

the EBV origin of replication (oriP), the EBV nuclear antigen, EBNA-1, and the hygromycin-resistant gene. A conventional control plasmid, pHGF, was also constructed by cutting the OriP and EBNA-1 sequences out from pEBVHGF. DNA was purified using CsCl gradient.

Transfection—Transfection was performed 1 day after the NPCCs were isolated. pEBVHGF or pHGF were individually mixed with Opti-MEM (Invitrogen), and then Lipofectamine 2000 (Invitrogen) and the DNA solution were combined and incubated for 30 min at room temperature. The complexes were diluted with serum-free medium and added to the cells, which had been rinsed with serum-free medium. After 5 h of incubation at 37°C under 5% CO_2, the medium was replaced with complete growth medium for further culture.

Hygromycin Selection—Cells that had been transfected with pEBVHGF or pHGF were selected by treatment with 50 μg/ml of hygromin B (Sigma Chemical) 2 days after transfection.

Sandwich Enzyme-linked Immunosorbent Assay (ELISA)—The amount of HGF in the conditioned medium of pEBVHGF or pHGF transfected NPCCs was measured by ELISA. Briefly, a 96-well MaxiSorb plate (Nalge Nunc International) was incubated with 500 ng/ml of monoclonal anti-human HGF antibody (# BAF 694, R&D Systems Inc, Minneapolis, MN, USA) for 12 hours at 4°C. The plates were washed four times with PBS (PH 7.4) containing 0.05% Tween-20 and blocked with 200 μl of PBS containing 1% BSA at room temperature for 2 h.

Next, aliquots of serially diluted human standard HGF or 50 μl of culture supernatant samples were added to the wells, and the plates were then incubated for an additional 2 h at room temperature. After extensive washing, biotinylated anti-human HGF antibody (# BAF 294, HG R&D Systems Inc) at a dilution of 1:1,000 was added and the plates were incubated for another 2 h. Following washing, the plates were incubated with 50 μl of streptavidin HRP (# DY 998, R&D Systems Inc) at a dilution of 1:1,000, and subsequently with TMB solution (# 421101, BioLegend, San Diego, CA, USA). The plates were then allowed to stand for 15 min at room temperature. The reaction was

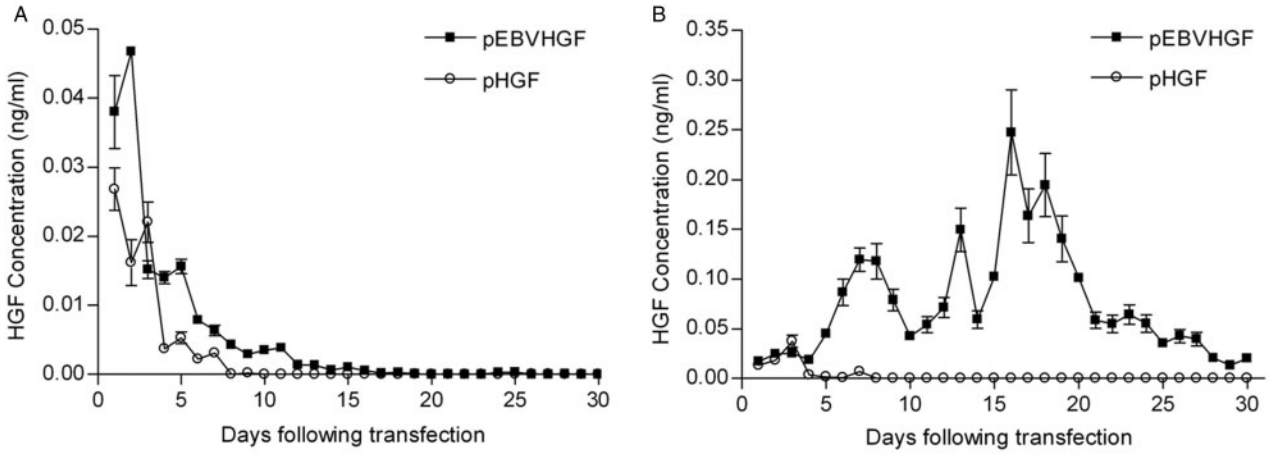

Fig. 2. **Prolonged HGF expression using an EBV-based plasmid.** NPCCs plated in a 12-well plate were transfected with pEBVHGF or pHGF using Lipofectamine 2000. Transfected NPCCs were then cultured in the absence (A) or presence (B) of 50 μg/ml of hygromycin and the amount of HGF secreted into the medium was measured using ELISA. The results were normalized by the number of cells present at the time of sample collection. Results are the mean ± S.E. of three or four independent experiments conducted in duplicate.

stopped by adding 25 μl of 2 M H_2SO_4. The absorbance at 405 nm was measured using a microplate reader (Spectra MAX 250 ELISA Reader, Molecular Devices Co, Sunnyvale, CA, USA).

Immunocytochemistry—After being transfected with pEBVHGF or pHGF on a coverslip, NPCCs were cultured with or without hygromycin selection. For insulin staining, the cells were washed four times with PBS and then fixed with 4% paraformaldehyde. Next, the cells were washed and incubated with 1% normal donkey serum (# 71413, Jackson Immunoresearch) for 30 min at room temperature to block non-specific binding. The cells were then incubated overnight with polyclonal guinea pig anti-insulin antibody (# 18-0067, Zymed, San Francisco, CA, USA) at 4°C. The next morning, primary antibody solution was removed and the cells were washed twice with PBS. The cells were then stained with rhodamine-conjugated affinipure donkey anti-guinea pig IgG (#70907, Jackson Immunoresearch). For the detection of phosphorylated c-Met, the cells were fixed with cold methanol and incubated with the anti-human phosphospecific-$Met^{Y1230/1234/1235}$ (1: 50) and then with Cy-3-conjugated anti-rabbit IgG (1: 1,000) (*25*, *26*). To visualize the cell nuclei, the cells were also stained with 4'6-diamidino-2-phenylindole (DAPI). After washing, the slides were mounted with an anti-fading reagent and observed using a fluorescence microscope. The ratio of beta cells in the NPCCs was calculated by dividing the insulin positive cell counts by the total DAPI positive cell counts.

Statistical Analysis—The results are presented as the mean ± SE of at least three independent experiments. Analysis of variance was used to compare different groups. Statistical significance was determined using Student's *t*-tests and $P < 0.05$ was assumed to be significant.

RESULTS

The Efficacy of Gene Transfection Using Lipofectamine—To establish the optimal transfection conditions, NPCCs (5×10^5 cells/well) were inoculated in 12-well plates and transfection was conducted using varying amounts of Lipofectamine 2000 and pEBVGFP (data not shown). The optimal transfection was observed when 2 μl of Lipofectamine 2,000 and 0.4 μg/μl of pEBVGFP were used (data not shown). Subsequent transfection was then performed by using an equi-molar amount of pEBVHGF or pHGF based on the optimal transfection conditions established for pEBVGFP.

Selection of Transfected Cells Using Hygromycin Treatment—After transfection with pEBVGFP or pGFP, the NPCCs were treated with various concentrations of hygromycin. Apoptosis was induced in both the non-transfected and transfected cells when concentrations of hygromycin >70 μg/ml were used. However, selection was not efficient when concentrations <40 μg/ml were used (data not shown). Therefore, 50 μg/ml of hygromycin was used to select the transfectants. The cells selected with hygromycin after pEBVHGF transfection grew well for ~20 days, after which point they began to die slowly (data not shown). In contrast, the number of cells began to decrease 3–5 days after pHGF transfection, and only a few cells survived after 30 days, even when hygromycin was used for selection (data not shown).

Prolonged HGF Expression Using an EBV-based Plasmid—The NPCCs were transfected with either pEBVHGF or pHGF to compare the expression patterns of HGF obtained with the two plasmids. Beginning 2 days after transfection, the culture supernatant was collected daily and the amounts of HGF were analysed by ELISA. When hygromycin selection was not used, HGF was expressed in NPCCs for up to 6 and 16 days after pHGF and pEBVHGF transfection, respectively (Fig. 2A). However, when the transfected cells were selected for with hygromycin, HGF expression was detectable for up to 30 days in the NPCCs that had been transfected with pEBVHGF, whereas HGF expression was only observed for 7 days in NPCCs that had been transfected with pHGF (Fig. 2B).

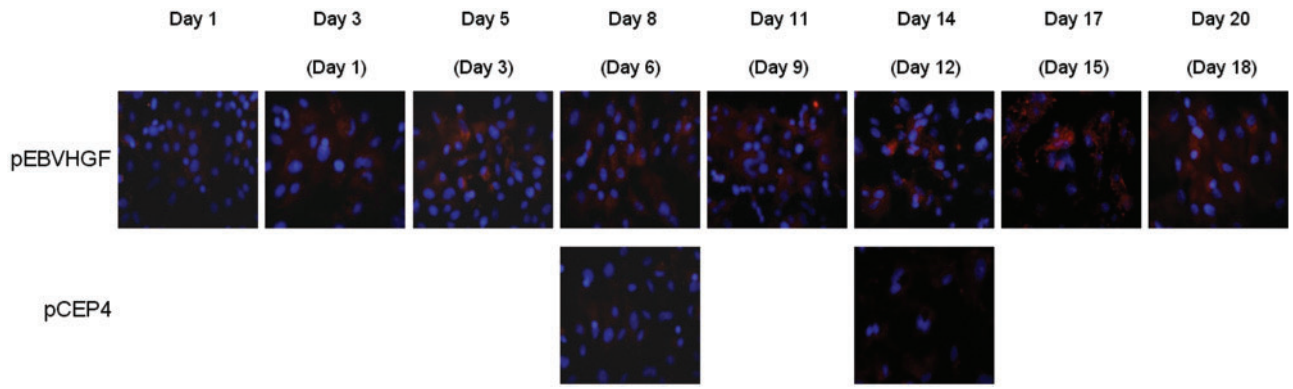

Fig. 3. **Phosphorylation of c-Met in the NPCCs transfected with pEBVHGF.** NPCCs were transfected with appropriate plasmids and then cultured with 50 μg/ml of hygromycin. Days represent the duration after transfection, and the days in the parenthesis denote the duration of hygromycin selection. Phosphorylated c-Met was stained with a cy-3-conjugated antibody and detected using fluorescence microscope (Zeiss) (400 × objective). Subcellular localization of phospho-Met is shown in red, while nuclear staining is shown in blue.

Phosphorylation of c-Met, in the NPCCs Transfected With pEBVHGF—To test whether the expressed HGF in the transfected NPCCs signals through the HGF receptor c-Met, the phosphorylation status of c-Met was analysed by immunofluorescence assay. Phosphorylated c-Met was hardly detectable in NPCCs one day after pEBVHGF transfection (Fig. 3). As the transfectants were selected with hygromycin, the puntate staining of phosphorylated c-Met increased continually up to 14–17 days after transfection and then began to decrease. The puntate signals began to be detected mainly from 3 days after pEBVHGF transfection in the perinuclear region of the cells. In contrast, no positive signal was observed in the control NPCCs at 8 and 14 days after transfection with the empty vector, pCEP4 (Fig. 3).

Insulin Expression Following Transfection With pEBVHGF—To assess the differentiation of ductal cells to beta cells following HGF expression, insulin expression was examined by immunocytochemistry. Some of the NPCCs that had not been transfected or that had been transfected with an empty vector (pCEP4) expressed insulin immediately after isolation (data not shown and Fig. 4A). However, the endogenous insulin expression decreased gradually, with few insulin positive cells being observed 7 days after transfection (Fig. 4A and B). The ratio of insulin positive cells in the NPCCs transfected with pHGF was similar with what was observed in the NPCCs transfected with pCEP4. When the cells were transfected with pEBVGFP, the level of insulin expressing cells was the highest among the three groups of cells and insulin positive cells were detected for up to 9–11 days after transfection (Fig. 4A and C).

The insulin positive cells among the NPCCs transfected with pHGF decreased rapidly and disappeared after 5–9 days, even after being subjected to hygromycin selection. Conversely, when the NPCCs transfected with pEBVGFP were selected for with hygromycin, the number of insulin positive cells decreased rapidly for the initial 5 days, but then increased slowly and was maintained at 20–30% of the initial levels for 9–13 days after transfection. The proportion of insulin positive cells then decreased further, but remained at ~5–10% for up to 27 days after transfection (Fig.4B and D).

The Effect HGF Gene Expression Using an EBV-Based Plasmid on the Viability of NPCCs—The viability of the NPCCs transfected with pEBVHGF and pHGF were compared by staining the cells with DAPI. When hygromycin selection was not used there was little difference in the number of DAPI-positive cells observed between the two transfectants (data not shown). Fibroblasts occupied the majority of the well space from 7 days after transfection and very few ductal cells were detected after 10 days, unless hygromycin was used for selection (data not shown).

Under hygromycin selection, the number of DAPI-stained cells was significantly higher for the pEBVHGF transfected NPCCs than the pHGF transfected NPCCs. Furthermore, many cells with ductal cell morphology grew well for over 20 days under hygromycin selection when the pEBVHGF transfected NPCCs were evaluated. However, the number of NPCCs transfected with pHGF decreased gradually, even when the cells were selected using hygromycin treatment (Fig. 5).

DISCUSSION

When the cells were not selected with hygromycin treatment, HGF expression was lost rapidly after transfection with pEBVHGF, although this plasmid can self-replicate in NPCCs. We reported that over 70% of the NPCCs were ductal cells and that they were the majority of cells transfected when an EBV-based plasmid was used (*23*). However, over time, the proportion of fibroblasts became greater than that of the ductal cells because fibroblasts grow much faster than ductal cells. Therefore, without hygromycin treatment, rapidly growing un-transfected fibroblasts would impede the growth of ductal cells that were transfected with pEBVHGF.

Conversely, HGF was expressed for 30 days when the same cells were maintained under hygromycin selection. This may have occurred because hygromycin treatment removed the majority of the un-transfected fibroblasts, allowing the transfected ductal cells to grow. Under this

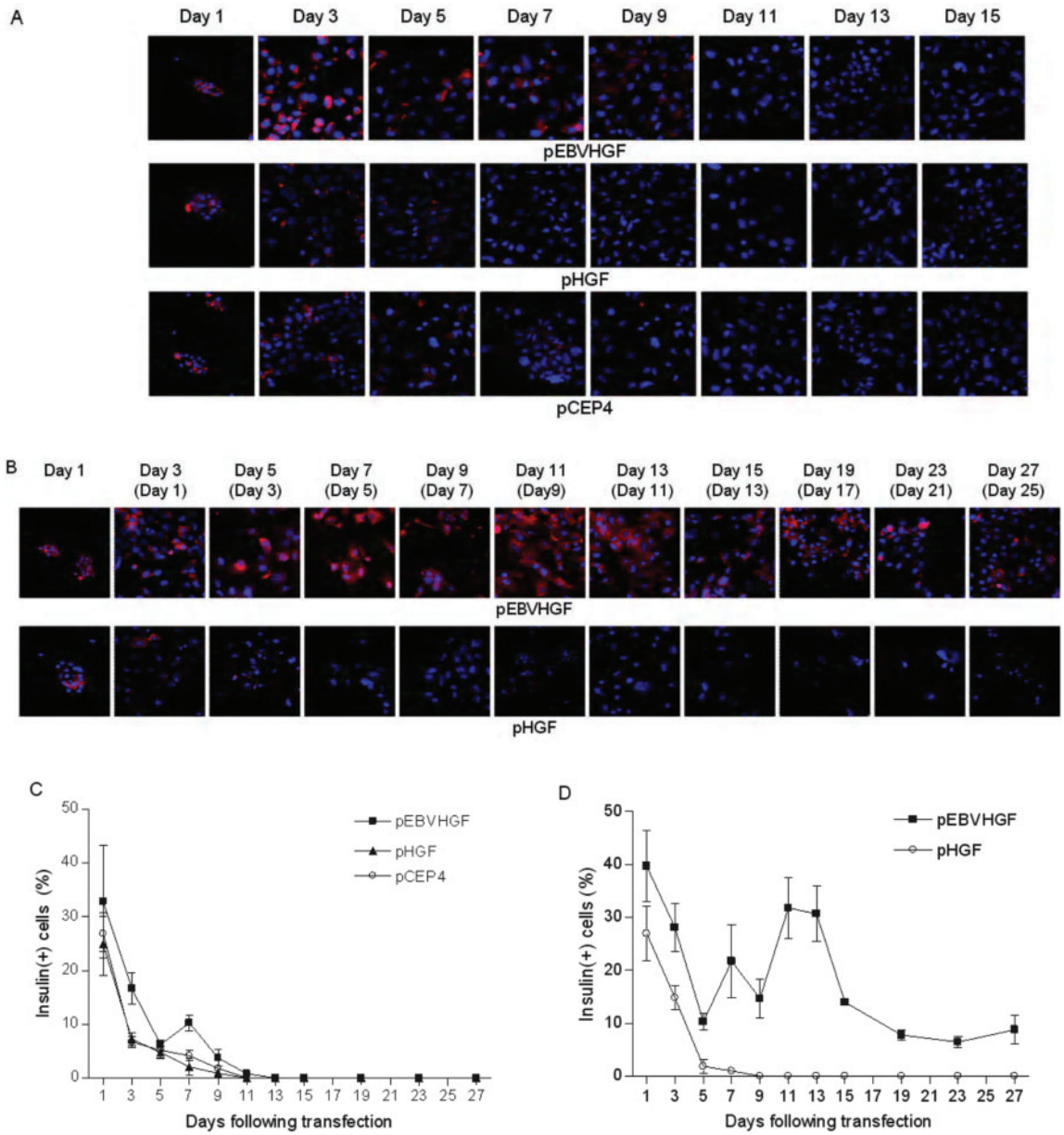

Fig. 4. **Insulin expression following pEBVHGF transfection.** NPCCs were transfected with each plasmid and then cultured in complete media without (A, C) or with (B, D) 50 μg/ml of hygromycin. A and B are the representative results of immunocytochemical staining, while C and D are the summarized results of four independent immunochemical staining experiments. Days represent the duration after transfection. Days in the parenthesis denote the duration of hygromycin selection. The cells were observed using a confocal microscope (BioRad) (400 × objective).

condition, the self-replicating ability of the EBV-based plasmid would have become evident resulting in extended *HGF* gene expression. In contrast, *HGF* gene expression was lost rapidly in NPCCs that had been transfected with pHGF, regardless of hygromycin selection. pHGF also has the hygromycin selection marker, but it does not have EBNA-1 or Ori P. Thus, this conventional plasmid would be lost rapidly even under the hygromycin selection because it cannot self-replicate in NPCCs. This was reflected by a significantly greater amount of NPCCs that had been transfected with pEBVHGF being observed after 15–27 days of hygromycin selection than the cells that had been transfected with pHGF (Figs 4 and 5).

The biological activities of HGF are mediated by a specific receptor c-Met, a transmembrane receptor tyrosine kinase encoded by c-Met (*27*, *28*). NPCCs which were transfected with pEBVHGF, expressed HGF and showed c-Met phosphorylation. c-Met phosphorylation recruits numerous adaptors and begins signalling cascade. After HGF stimulation, c-Met undergoes a rapid endocytosis and transport through an early endosomal compartment to a late perinuclear compartment as well as to the Golgi apparatus (*29*, *30*). In our results,

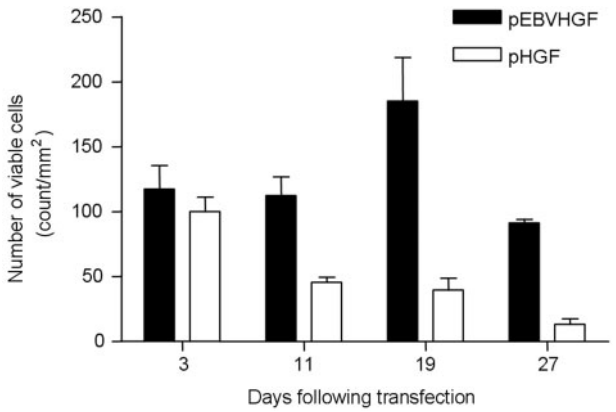

Fig. 5. **Viability of the NPCCs following pEBVHGF transfection.** NPCCs were transfected with pEBVHGF (black bar) or pHGF (white bar) and then selected using 50 μg/ml of hygromycin from 2 days after transfection. The viability of the NPCCs was determined by counting DAPI positive cells at different time points. Data are presented as the mean ± S.E. ($n = 5$).

phosphorylated c-Met staining located indeed to the perinuclear region (Fig. 3). Furthermore, the time course of c-Met phosphorylation approximately followed that of HGF expression, suggesting that the expressed HGF functioned through c-Met.

Theoretically, HGF expression would induce differentiation of ductal cells to beta cells, which would result in insulin expression. Therefore, HGF expression is expected to precede insulin expression. However, the levels of HGF expression and c-Met phosphorylation peaked 13–17 days after transfection, whereas the expression of insulin was highest after 11–13 days. This may be due to the method we used to calculate the level of HGF. The level of HGF expression was normalized using the total number of cells present at the time of sample collection. As it took several days to select out un-transfected cells by hygromycin treatment, HGF expression which was normalized by cell number is expected to be low immediately after transfection. However, with time, the un-transfected cells would undergo apoptosis, leaving mainly transfected cells. This is reflected as a high HGF expression 6–19 days after transfection. After this time, the transfected ductal cells in the NPCCs would undergo apoptosis because they are primary cells, resulting in reduced HGF expression.

Insulin expression was examined using IFA. The expression of insulin by NPCCs that had been transfected with pEBVHGF showed two peaks. The first peak may reflect endogenous insulin secreted by the nascent NPCCs, as it was observed in both un-transfected and empty vector transfected cells. Other investigators also reported insulin secretion from freshly isolated NPCCs for up to days 6 (*31*). The second peak might have originated from the transfected plasmids.

The effect of HGF expression on the viability of the cells also confirmed the benefit of using an EBV-based plasmid instead of a conventional plasmid. HGF is known to enhance cell viability by affecting both apoptosis and proliferation (*32–34*). However, it is not clear whether the increased cell viability is due to an HGF effect on apoptosis and/or the proliferation of beta cells in our results.

Overall, our results support that delivery of the *HGF* gene to NPCCs using an EBV-based plasmid which was able to induce differentiation to beta cells more efficiently and further improve cell viability when compared to a conventional plasmid. In addition, our results suggest that HGF initiated signalling cascade through the activated receptor to stimulate insulin expression and secretion in the NPCCs transfected with pEBVHGF. The NPCCs transfected with useful genes by EBV-based plasmids would facilitate the development of a successful xenotransplantation for the treatment of diabetes.

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REFERENCES

1. Dai, C., Li, Y., Yang, J., and Liu, Y. (2003) Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice. *J. Biol. Chem.* **278**, 27080–27087
2. Vasavada, R.C., Gonzalez-Pertusa, J.A., Fujinaka, Y., Fiaschi-Taesch, N., Cozar-Castellano, I., and Garcia-Ocaña, A. (2006) Growth factors and beta cell replication. *Int. J. Biochem. Cell. Biol.* **38**, 931–950
3. Ryan, E.A., Lakey, J.R., Rajotte, R.V., Korbutt, G.S., Kin, T., Imes, S., Rabinovitch, A., Elliott, J.F., Bigam, D., Kneteman, N.M., Warnock, G.L., Larsen, I., and Shapiro, A.M. (2001) Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* **50**, 710–719
4. Shapiro, A.M., Lakey, J.R., Ryan, E.A., Korbutt, G.S., Toth, E., Warnock, G.L., IV, Kneteman, N.M., and Rajotte, R.V. (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.* **343**, 230–238
5. Weir, G.C., Quickel, R.R., Yoon, K.H., Tatarkiewicz, K., Ulrich, T.R., Hollister-Lock, J., and Bonner-Weir, S. (1997) Porcine neonatal pancreatic cell clusters (NPCCs): a potential source of tissue for islet transplantation. *Ann. Transplant.* **2**, 63–68
6. Tsuchiya, M., Tsuchiya, K., Iwami, Y., and Ohgawara, H. (2003) Analysis of gene expression and insulin secretion by monolayer-forming adult porcine pancreatic endocrine cells. *Pancreas* **26**, 71–75
7. Yoon, K.H., Quickel, R.R., Tatarkiewicz, K., Ulrich, T.R., Hollister-Lock, J., Trivedi, N., Bonner-Weir, S., and Weir, G.C. (1999) Differentiation and expansion of beta cell mass in porcine neonatal pancreatic cell clusters transplanted into nude mice. *Cell Transplant.* **8**, 673–689
8. Valdés-González, R.A., Dorantes, L.M., Garibay, G.N., Bracho-Blanchet, E., Mendez, A.J., Dávila-Pérez, R., Elliott, R.B., Terán, L., and White, D.J. (2005) Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. *Eur. J. Endocrinol.* **153**, 419–427
9. Zarnegar, R. and Michalopoulos, G.K. (1995) The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J. Cell. Biol.* **129**, 1177–1180
10. Otonkoski, T., Cirulli, V., Beattie, M., Mally, M.I., Soto, G., Rubin, J.S., and Hayek, A. (1996) A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology* **137**, 3131–3139
11. Shimabukuro, K., Ichinose, S., Koike, R., Kubota, T., Yamaguchi, M., Miyasaka, M., and Aso, T. (2001)

Hepatocyte growth factor/scatter factor is implicated in the mode of stromal invasion of uterine squamous cervical cancer. *Gynecol. Oncol.* **83**, 205–215
12. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Molecular cloning and expression of human hepatocyte growth factor. *Nature* **23**, 440–443
13. Matsumoto, K. and Nakamura, T. (1997) Hepatocyte growth factor (HGF) as a tissue organizer for organogenesis and regeneration. *Biochem. Biophys. Res. Commun.* **239**, 639–644
14. Otonkoski, T., Beattie, G.M., Rubin, J.S., Lopez, A.D., Baird, A., and Hayek, A. (1994) Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. *Diabetes* **43**, 947–953
15. Hayek, A., Beattie, G.M., Cirulli, V., Lopez, A.D., Ricordi, C., and Rubin, J.S. (1995) Growth factor/matrix-induced proliferation of human adult beta-cells. *Diabetes* **44**, 1458–1460
16. Izumida, Y., Aoki, T., Yasuda, D., Koizumi, T., Suganuma, C., Saito, K., Murai, N., Shimizu, Y., Hayashi, K., Odaira, M., Kusano, T., Kushima, M., and Kusano, M. (2005) Hepatocyte growth factor is constitutively produced by donor-derived bone marrow cells and promotes regenerationof pancreatic beta-cells. *Biochem. Biophys. Res. Commun.* **333**, 273–282
17. Lopez-Talavera, J.C., Garcia-Ocaña, A., Sipula, I., Takane, K.K., Cozar-Castellano, I., and Stewart, A.F. (2004) Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. *Endocrinology* **145**, 467–474
18. Garcia-Ocaña, A., Takane, K.K., Syed, M.A., Philbrick, W.M., Vasavada, R.C., and Stewart, A.F. (2000) Hepatocyte growth factor overexpression in the islet of transgenic mice increases beta cell proliferation, enhances islet mass, and induces mild hypoglycemia. *J. Biol. Chem.* **275**, 1226–1232
19. Garcia-Ocana, A., Takane, K.K., Reddy, V.T., Lopez-Talavera, J.C., Vasavada, R.C., and Stewart, A.F. (2003) Adenovirus-mediated hepatocyte growth factor expression in mouse islets improves pancreatic islet transplant performance and reduces beta cell death. *J. Biol. Chem.* **278**, 343–351
20. Middleton, T. and Sugden, B. (1994) Retention of plasmid DNA in mammalian cells is enhanced by binding of the Epstein–Barr virus replication protein EBNA1. *J. Virol.* **68**, 4067–4071
21. Mazda, O. (2002) Improvement of nonviral gene therapy by Epstein–Barr virus (EBV)-based plasmid vectors. *Curr. Gene Ther.* **2**, 379–392
22. Min, K.A., Oh, S.T., Yoon, K.H., Kim, C.K., and Lee, S.K. (2003) Prolonged gene expression in primary porcine pancreatic cells using an Epstein-Barr virus-based episomal vector. *Biochem. Biophys. Res. Commun.* **305**, 108–115
23. Son, J.K., Oh, S.T., Cho, S.K., Yoon, K.H., and Lee, S.K. (2006) Mechanism of prolonged gene expression by Epstein–Barr virus-based plasmid in porcine cells. *Xenotransplantation* **13**, 560–565
24. Korbutt, G.S., Elliott, J.F., Ao, Z., Smith, D.K., Warnock, G.L., and Rajotte, RV. (1996) Large scale isolation, growth and function of neonatal porcine islets. *J. Clin. Invest.* **97**, 2110–2129
25. Inoue, T., Kataoka, H., Goto, K., Nagaike, K., Igami, K., Naka, D., Kitamura, N., and Miyazawa, K. (2004) Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci.* **95**, 803–808
26. Wen, J., Matsumoto, K., Taniura, N., Tomioka, D., and Nakamura, T. (2004) Hepatic gene expression of NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice. *Cancer Gene Ther.* **11**, 419–430
27. Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M., Kmiecik, T.E., Vande Woude, G.F., and Aaronson, S.A. (1991) Identification of the hepatocyte growth factor receptor as the c-Met proto-oncogene product. *Science* **251**, 802–804
28. Naldini, L., Vigna, E., Narsimhan, R.P., Gaudino, G., Zarnegar, R., Michalopoulos, G.K., and Comoglio, P.M. (1991) Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the protooncogene c-MET. *Oncogene* **6**, 501–504
29. Hammond, D.E., Urbé, S., Vande Woude, G.F., and Clague, M.J. (2001) Down-regulation of MET, the receptor for hepatocyte growth factor. *Oncogene* **20**, 2761–2770
30. Kermorgant, S., Zicha, D., and Parker, P.J. (2003) Protein kinase C controls microtubule-based traffic but not proteasomal degradation of c-Met. *J. Biol. Chem.* **278**, 28921–28929
31. Ohgawara, H., Iwanaga, T., Yui, R., Nishjima, S., and Hirata, Y. (1987) Monolayer-forming islet cell culture from neonatal pig pancreas: usuing sequential treatment with EDTA-dispase and monoiodoacetic acid for preparation and purification. *Tohoku J. Exp. Med.* **153**, 375–382
32. Datta, S.R, Brunet, A., and Greenberg, M.E. (1999) Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905–2927
33. Federici, M., Hribal, M., Perego, L., Ranalli, M., Caradonna, Z., Perego, C., Usellini, L., Nano, R., Bonini, P., Bertuzzi, F., Marlier, L.N., Davalli, A.M., Carandente, O., Pontiroli, A.E., Melino, G., Marchetti, P., Lauro, R., Sesti, G., and Folli, F. (2001) High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. *Diabetes* **50**, 1290–1301
34. Nicholson, K. M. and Anderson, N. G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal* **14**, 381–395