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Prolonged gene expression in primary porcine pancreatic cells using an Epstein–Barr virus-based episomal vector

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Abstract

Epstein–Barr virus (EBV)-based plasmids containing the origin of replication (oriP) and EBV nuclear antigen 1 (EBNA-1) are well known for the stable episomal maintenance in human cells. In order to clarify whether an EBV-based plasmid can be maintained stably in the porcine pancreatic cells which are the primary candidate sources of islet xenotransplantation, we constructed pEBVGFP encoding the green fluorescent protein (GFP). Monolayer culture of the porcine neonatal pancreatic cells was lipofected with pEBVGFP or pGFP which was derived from pEBVGFP by deleting out oriP and EBNA-1. pEBVGFP significantly prolonged GFP expression not only in human cell lines but also in the primary porcine pancreatic cells compared with pGFP. Interestingly, the duct cells that are believed as the pancreatic precursor cells were preferentially transfected and conveniently enriched among the mixed primary cell populations using a hygromycin B selection. To our knowledge, this is the first report suggesting the potential application of an EBV-based plasmid for the extended gene expression in the primary porcine pancreatic duct cells.

Keywords: Epstein–Barr virus; Episomal vector; Porcine; Pancreas; Duct cells; Diabetes mellitus; Green fluorescent protein

Epstein–Barr virus (EBV) is a γ herpesvirus with a large 172-kb double-stranded DNA genome. After B cell infection, EBV retains its genome as an episome in the nucleus to maintain a latent infection [1]. Previous studies have demonstrated that the persistent maintenance of EBV genome is mediated by the replication origin of the latent viral DNA (oriP) and EBV nuclear antigen 1 (EBNA-1) [2,3]. OriP comprises a family of repeat and a dyad symmetry sequence [4,5]. Both elements have multiple binding sites for EBNA-1 and are essential for the replication and nuclear retention of EBV genome [6–8]. EBV-based plasmids containing the oriP and EBNA-1 sequences are maintained as episomes in human cells because they replicate once per each cell cycle and segregate into the daughter cells [9]. Animal cells are typically resistant to infection by EBV as no known EBV receptor(s) are expressed on the surface of the cells. Among animal cells, rodent cells have received most attention regarding whether they can be infected with EBV because rodents are the most widely used animal models. Yates et al. [2] reported that EBV episomes did not replicate in three different rodent cell lines while they replicated in monkey and dog cell lines as well as in human cell lines. Later, other researchers observed that EBV-based plasmids could replicate in some rodent cell lines if the cells were grown in the selection media for the introduced plasmids [10,11]. Furthermore, rodent cells transfected with human CD21 and HLA class II were infected with recombinant EBV and kept the EBV genome for extended periods under the selection [12,13]. These suggest that strictly limited host range of EBV is mainly caused by the lack of EBV receptor on the animal cell surface and that the intracellular factors of rodent cells can help the replication and segregation of the EBV genome at least under the selection pressure.

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The pig has been studied extensively as a potential source of xenogeneic islets since porcine insulin is structurally similar to human insulin and has been used safely for many years to treat diabetes [14–17]. In previous studies including ours, porcine neonatal pancreatic cell clusters (NPCCs) cultured in suspension consisted of approximately 60% duct cells, and more than 90% of these developed into insulin-producing cells after transplantation into nude mice [18–20]. After NPCC transplantation, there is a 6–12 week delay before the reversal of hyperglycemia [20]. This delay presumably reflects the proliferation and differentiation process of the grafted precursor cells before they become fully matured β-cells. Furthermore, porcine pancreatic duct cells strongly express the Galα1,3Gal epitope that is a target of the hyperacute xenorejection [21]. However, the Galα1,3Gal epitope is not expressed on mature islets [21,22]. So if we want to use the porcine NPCCs in a clinical setting, appropriate strategies for the maturation of porcine pancreatic duct cells in vitro should be established. If some genes that regulate the growth and differentiation of duct cells into mature β-cells could be transferred into the cells before transplantation, a faster relief from hyperglycemia without hyperacute rejection would be achieved. Thus, a safe and efficient gene delivery is required to further enhance the value of the neonatal porcine pancreatic cells as xenotransplantation sources. A plasmid, which can be retained and/or replicate in the nucleus of the porcine cells, would extend gene expression capacity and duration. So far, it is not clear whether an EBV-based plasmid can be maintained stably in porcine cells, especially in primary porcine cells. Therefore, we constructed an EBV replicon vector (pEBVGFP) containing the enhanced green fluorescent protein (EGFP) coding sequence and examined the duration of GFP expression in the primary porcine neonatal pancreatic cells transfected with pEBVGFP. GFP enabled us a straightforward detection of the transgene expression by fluorescence microscopy or fluorescence-activated cell sorting (FACS) analysis [23,24].

Materials and methods

Plasmid constructions. EBV-based plasmid (pEBVGFP) expressing the green fluorescent protein (GFP) was constructed by subcloning the GFP gene of pEGFP-C1 (Clontech, Palo Alto, CA) into pCEP4 (Invitrogen, San Diego, CA). pCEP4 carries the EBV origin of replication (oriP), the EBV nuclear antigen EBNA-1, and the hygromycin resistant gene. pEGFP-C1 was cut with *Nhe*I and *Bam*HI to release an 800-bp fragment containing the GFP sequence (Fig. 1). This GFP fragment was ligated with the pCEP4 digested with *Nhe*I and *Bam*HI. A control plasmid, pGFP, was constructed from pEBVGFP by deleting the oriP and EBNA-1 sequences out using *Bbs*I. Successful cloning of pEBVGFP and pGFP was confirmed by DNA sequencing using the selective primers for each construct. The sequencing primers used for pEBVGFP were 5′CAGAGCTCGTTTAGTGAACCG3′ for the upstream position and 5′GTGGTTTGTCCAAACTCATCA3′ for the downstream position. The sequencing primers used for pGFP were 5′AATCCCTTCAGTTGGTTGGT3′ for the upstream position and 5′GAGCAAAAACAGGAAGGCAA3′ for the downstream position. These constructs were amplified in *Escherichia coli* DH5α or TOP10 and purified using a Qiagen Plasmid Mega-Prep Kit (Qiagen, Valencia, CA).

Cell culture. HepG2 (human hepatocellular carcinoma cells) and HeLa (human cervical carcinoma cells) cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL, Invitrogen, San Diego, CA), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and Fungizone (Gibco-BRL). All the cells were maintained in 37 °C incubator with a 5% CO_2 water-saturated atmosphere.

Monolayer culture of porcine neonatal pancreatic cells. The porcine neonatal pancreatic cells were prepared from 1 to 3-day-old pigs using a previously described method with a slight modification [18,19]. Each pancreas was minced into 1–2 mm pieces and digested with collagenase P (Boehringer–Mannheim, Indianapolis, IN) in a shaking water bath. After digestion, the cell suspension was washed with HBSS solution and resuspended in Hams' F10 (Gibco-BRL). The cell suspension was incubated overnight in 150×15 mm bacteriological plates (Nalge

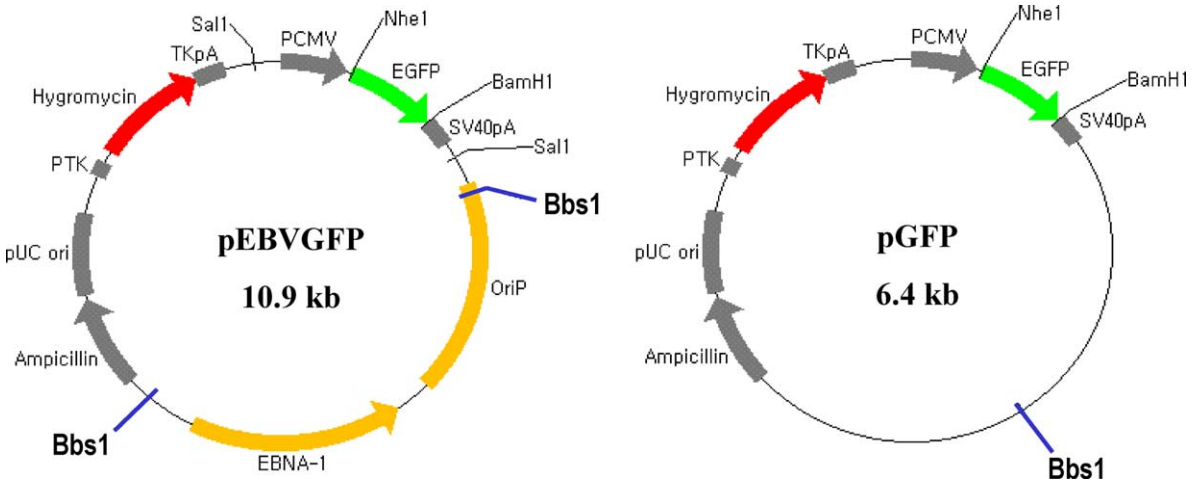

Fig. 1. Schematic representation of pEBVGFP and pGFP. pEBVGFP is an EBV-based plasmid carrying a GFP reporter gene driven by cytomegalovirus (CMV) promoter, EBV nuclear antigen (EBNA-1) gene, and the origin of EBV replication (oriP). The genes for hygromycin and ampicillin resistance and a prokaryotic plasmid origin of replication are also present. pGFP was derived from pEBVGFP by deleting out the oriP and EBNA-1 sequences.

Nunc International, Rochester, NY) at 37 °C incubator with a 5% CO_2 water- saturated atmosphere. Next morning, the NPCCs were collected and suspended in a 50-ml tissue culture flask containing a dissociation medium (Sigma, St. Louis, MI). Cell clusters were broken apart by repeated gentle aspiration with a pipette. Then, the separated single cells were transferred into 12-well plates (Nalge Nunc International) at a density of approximately 5×10^5 cells/well. The primary porcine cells were maintained in a medium containing RPMI-1640 with 10% FBS and incubated at 37 °C, 5% CO_2. After 24 h, the unattached cells were removed.

In vitro transfection and hygromycin selection. Transfection was performed when the cells were 60–70% confluent. Lipofectamine (Invitrogen) and DNA were separately mixed with Opti-MEM (Invitrogen). Transfection complex was formed by combining Lipofectamine with the DNA solution and incubating for 30 min at room temperature. The complexes mixed with a serum-free medium were added to the cells rinsed with a serum-free medium. After 5 h of incubation at 37 °C, the medium was replaced with a complete growth medium for further culture. The cells transfected with pEBVGFP or pGFP were selected by hygromycin B (Sigma) treatment from 2 days after transfection.

FACS analysis. The transfected cells were harvested by treating with trypsin–EDTA. After incubation, a medium containing 10% FBS was added and the cell suspension was centrifuged at 4400 rpm for 5 min. The cells were washed with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde for 15 min at 4 °C. The fixed cells were washed twice with PBS and then resuspended in a FACS buffer (PBS containing 0.5% bovine serum albumin and 0.01% sodium azide). FACS analysis was performed using a FACSVantage SE (Becton–Dickinson, California, USA) flow cytometer with an argon-ion laser producing 10-mW of 488-nm light for excitation. The green fluorescence was measured through a 530/30-nm bandpass filter (Becton–Dickinson). Data for 10,000 fluorescent events were obtained by recording forward scatter, side scatter, and green fluorescence (FL1).

Confocal microscopy. At various time points after transfection, the cells were observed with a confocal microscope (Bio-Rad MRC-1024, Bio-Rad Laboratories, California, USA) supplemented with an argon–krypton laser and equipped with a 200 or 100× objective. The excitation wavelength was 488 nm.

Results

Prolonged GFP expression by an EBV-based plasmid in human cell lines

First, optimal transfection condition was assessed for HepG2 cells using Lipofectamine and pEGFP-C1. The cells were seeded on 24-well plates at a density of 1×10^5 cells/well and transfected when they became 60–70% confluent. Increasing amounts of Lipofectamine (0.4, 0.8, 1.2, 1.6, or 2.0 μl) with a fixed amount (0.4 μg) of pEGFP-C1 were used for transfection following manufacturer's recommendation. Flow cytometric analysis of the cells was carried out 24 h after transfection (Fig. 2). Ten to forty two percent of the cells expressed GFP depending on the amount of Lipofectamine. Maximum transfection efficiency was achieved when 1.2 μl of Lipofectamine was used (DNA:Lipofectamine = 1:3 (w/v)). For HeLa cells, optimal transfection was achieved also when DNA:Lipofectamine was 1:3 (w/v) (data not shown). Cells were transfected under this condition for later experiments. To confirm prolonged GFP expression from EBV-based plasmid, confocal microscopy was carried out for HepG2 cells transfected with pEBVGFP, pGFP, or pEGFP-C1 (Fig. 3). One day after transfection, percent of GFP expressing cells was highest for pEGFP-C1 and lowest for pEBVGFP. This may be due to the fact that for each DNA, the same weight of DNA (0.4 μg each) was used for transfection. As the sizes of pEGFP-C1, pGFP, and pEBVGFP were 4.7, 6.4, and 10.9 kb, respectively, the molar ratio for pEGFP- C1:pGFP:pEBVGFP used for transfection was 1:0.73:0.43. Thus, less than half the amounts of pEBVGFP molecules were used for the transfection in our experiments compared with pEGFP-C1. The percent of GFP-expressing cells transfected with pGFP or pEGFP-C1 decreased from 4 days after transfection and continued to decrease. By 12 days after transfection, only a few cells expressed GFP. These GFP expressing cells were extinct by 17 days after transfection. On the contrary, HepG2 cells transfected with pEBVGFP showed increased GFP expression 4 days after transfection. The percent of GFP expressing cells decreased slowly from 7 days after transfection but maintained above the initial expression level up to 17 days. For these cells, GFP expression was detected for 26 days at least. Similar experiments were performed using HeLa cells and

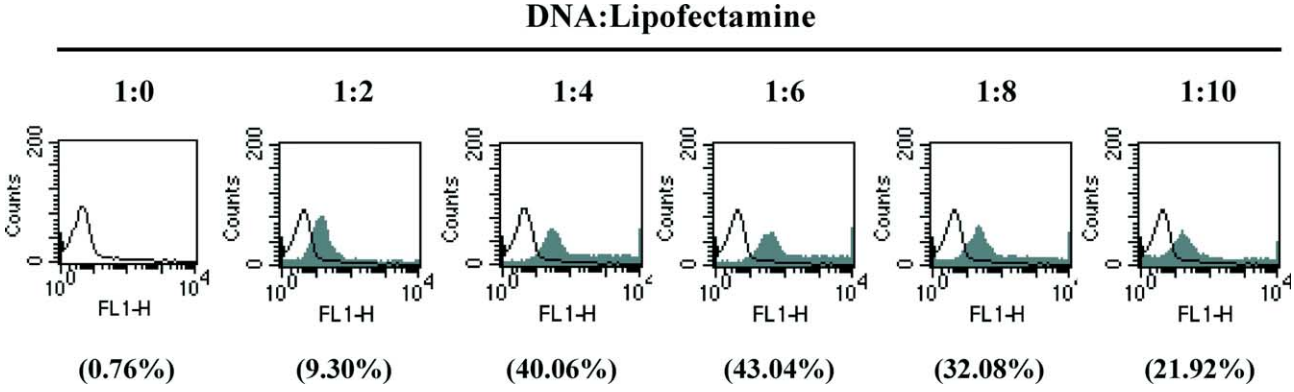

Fig. 2. FACS analysis of GFP gene expression in the HepG2 cells transfected with various compositions of the DNA/Lipofectamine complex. Viable cells were gated and analyzed for GFP expression 24 h after the transfection. Values within the parentheses indicate the percentage of GFP expressing cells.

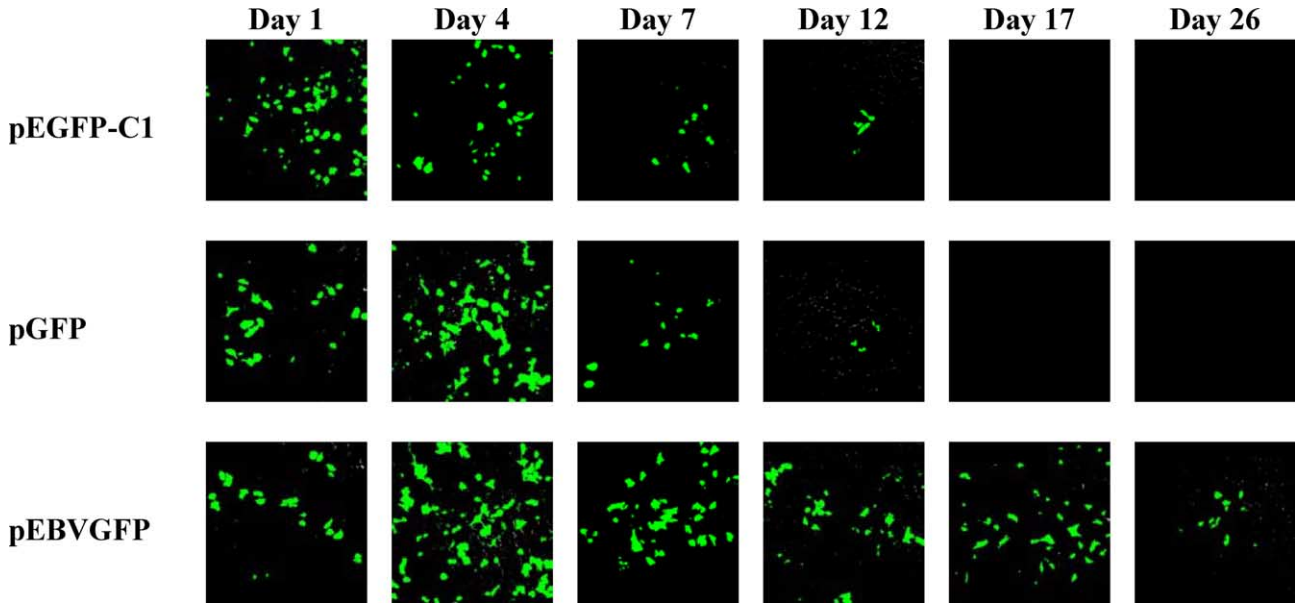

Fig. 3. Prolonged GFP expression by an EBV-based plasmid in human cell lines. HepG2 cells were transfected with pEBVGFP, pGFP, or pEGFP-C1 using Lipofectamine. GFP expression was examined by a confocal microscope at different time points (200× objective).

almost identical results were obtained (data not shown). These results confirmed that the newly constructed EBV-based plasmid provided a prolonged gene expression in human cells as previously reported.

Selection of the transfected human cells by hygromycin treatment

pEBVGFP and pGFP contained the hygromycin selection marker gene. This eukaryotic resistant gene was necessary because the retention of an EBV-based plasmid is known to be imperfect and results in slow loss of the vector over time. To enrich the transfected cells and further extend the duration of GFP expression, the cells transfected with pEBVGFP or pGFP were selected using hygromycin. From 2 days after transfection, HepG2 cells were cultivated with increasing concentrations of hygromycin (Fig. 4). The cells were sensitive to 150 μg/ml or higher concentrations of hygromycin. The optimal hygromycin concentration for the HepG2 transfectants was 300 μg/ml. The HepG2 cells transfected with pEBVGFP showed enrichment of the GFP expressing cells but the cells transfected with pGFP did not. In addition, the pEBVGFP transfected HepG2 cells showed much prolonged GFP expression, resulting in GFP detection for the entire observed period under the hygromycin selection. The HeLa cells transfected with pEBVGFP or pGFP were also cultivated in the presence of hygromycin in a similar method. HeLa cells were less sensitive to hygromycin than HepG2 cells and higher concentrations of hygromycin were used for the selection (data not shown). While the HeLa cells transfected with pEBVGFP were easily enriched by treating with higher than 400 μg/ml hygromycin, the cells transfected with pGFP were not selected under the same condition. The easy enrichment of cells transfected with pEBVGFP using hygromycin might account for the long-term episomal replication of the EBV-based plasmid outside the chromosome compared with the conventional plasmids. Occasionally a few cells showed hygromycin resistance among pGFP transfected cells (data not shown). These cells might reflect the integration of the plasmid into the genomic DNA of the cells.

Prolonged GFP expression by an EBV-based plasmid in primary porcine cells

The primary porcine pancreatic cells were seeded on 12-well plates at a density of 5×10^5 cells/well. After 24 h, the unattached cells which were usually more than half of the seeded cells were removed by replacing the medium. Two days after seeding, transfection was performed using different amounts of DNA and Lipofectamine to find the optimal transfection conditions for the neonatal porcine cells. The optimal transfection of pEBVGFP was achieved using 0.8 μg DNA and 4 μl of Lipofectamine (data not shown). The GFP expressing cells transfected with pGFP began to decrease from 4 days after transfection, continued to decrease, and completely disappeared by 15 days after transfection. On the contrary, the GFP expression decreased much more slowly and lasted longer in the cells transfected with pEBVGFP (Fig. 5). From 2 days after the transfection, the primary porcine cells were cultured in a medium containing hygromycin (30 or 50 μg/ml) to select the transfectants. Using 30 or 50 μg/ml hygromycin,

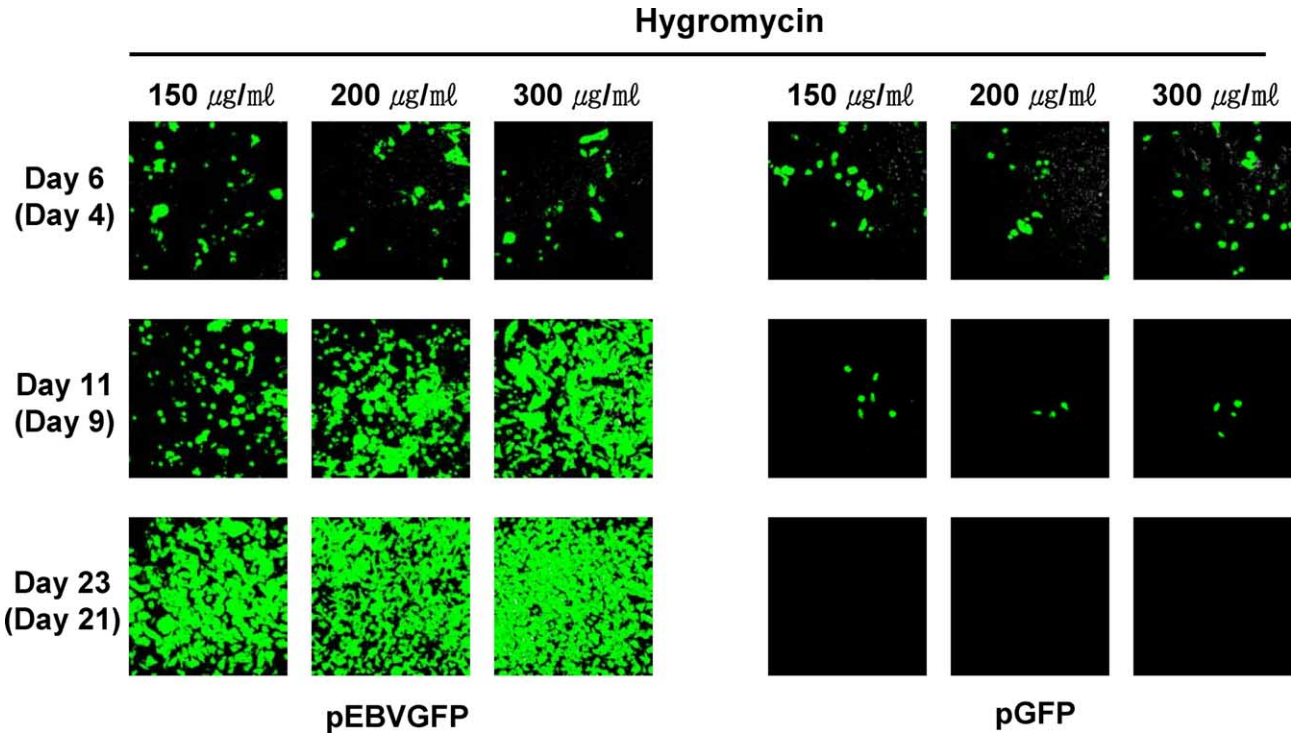

Fig. 4. Selection of the transfected human cells by hygromycin treatment. HepG2 cells were transfected with either pEBVGFP or pGFP using Lipofectamine. Days shown in the figures represent the duration after transfection. Days in the parentheses denote the duration of hygromycin selection. The cells were passaged and cultured in the media containing increasing concentrations of hygromycin. The GFP expressing cells were observed using a confocal microscope (200× objective).

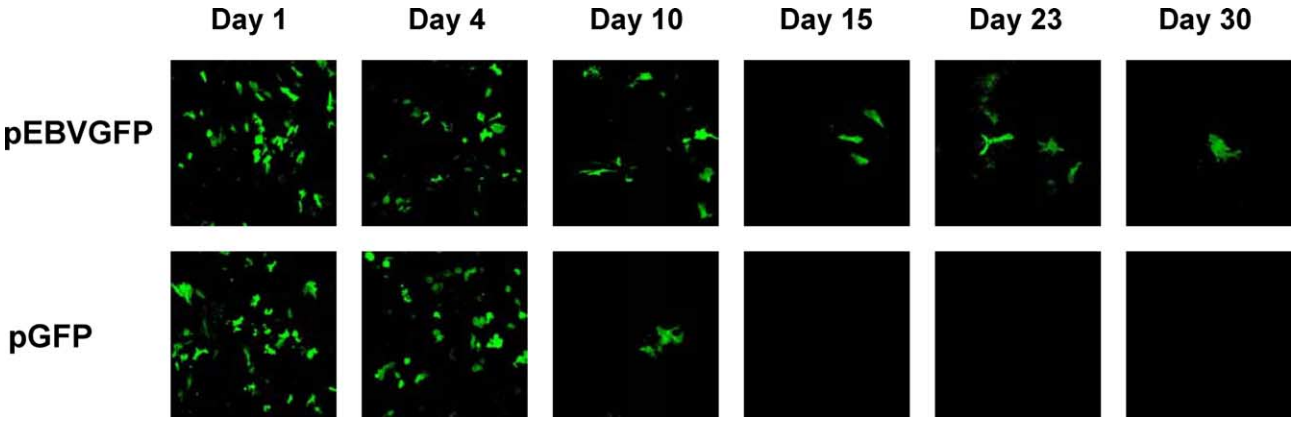

Fig. 5. Prolonged GFP expression by an EBV-based plasmid in primary porcine neonatal pancreatic cells. The cells were transfected with 4 μl Lipofectamine complexed with 0.8 μg pEBVGFP or pGFP. The cells were analyzed by a confocal microscope 1, 4, 10, 15, 23, and 30 days after transfection (100× objective).

the GFP expressing cells were enriched when pEBVGFP was transfected (Fig. 6). The GFP expression was prolonged by a hygromycin selection up to 30 days after the pEBVGFP transfection. But due to the primary cell's nature, the entire cells were gradually lost after 30 days unlike the cell lines. In the primary porcine cells transfected with pGFP, the GFP expressing cells were completely lost by 15 days after transfection even under the hygromycin selection.

Preferential transfection of duct cells among mixed primary neonatal porcine pancreatic cells

Just before transfection, over 70% of the primary neonatal porcine pancreatic cells were duct cells (data not shown) while some endocrine cells and fibroblasts were also observed as previously reported by our group [25]. When the mixed cell population was transfected, the major cells transfected were duct cells rather than the

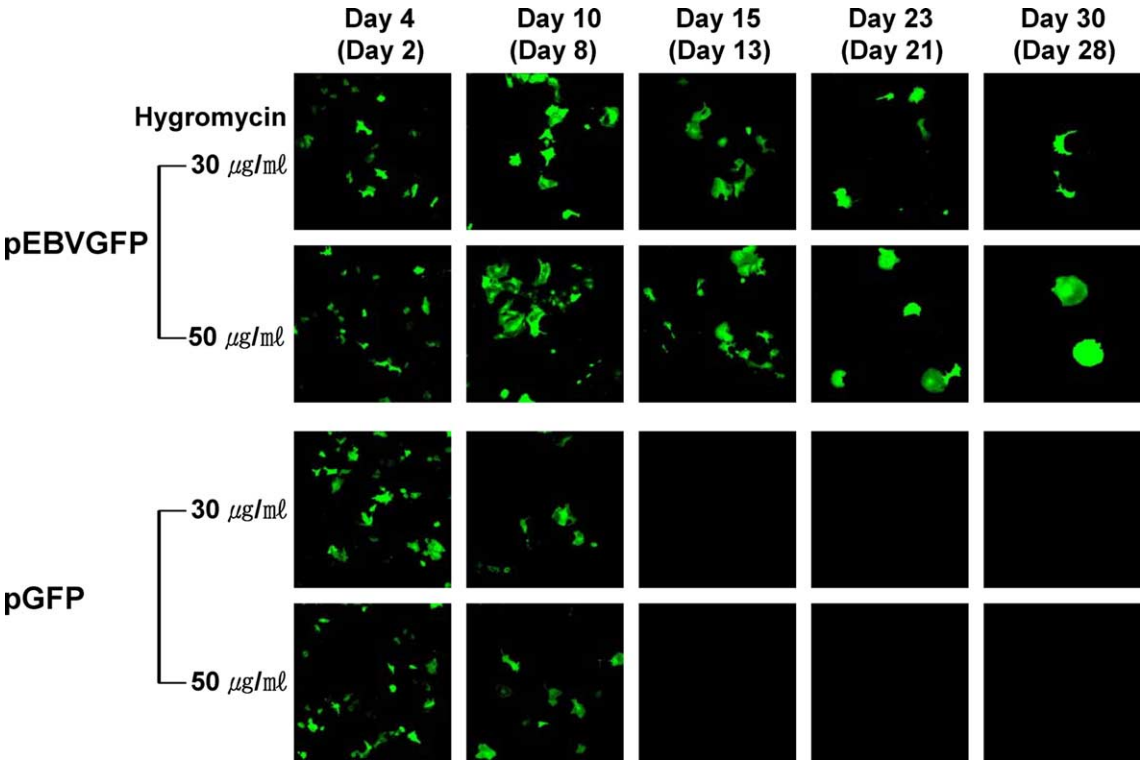

Fig. 6. Selection of the transfected primary porcine neonatal pancreatic cells by hygromycin treatment. The cells were cultured in a medium containing 30 or 50 μg/ml hygromycin. Days shown in the figures represent the duration after transfection. Days in the parentheses denote duration of hygromycin selection. The GFP expressing cells were observed using a confocal microscope (100× objective).

other types of cells by judging from their morphology and the growing pattern (Fig. 7). Without a selection, the number of duct cells transfected with pEBVGFP increased between 4 and 10 days after transfection but decreased rapidly after 10 days. Meanwhile, the untransfected fibroblasts grew rapidly and occupied almost all the growing area of the flask by 10 days after the transfection. Later than that, the fibroblasts grew on top of each other forming large islands of cell patches. But when the cells were treated with hygromycin to select out the untransfected cells, almost all the fibroblasts were detached from the culture plate leaving the transfected duct cells behind. However, even under the selection, the number of transfected cells began to decrease gradually later than 10 days after transfection. From 15 days after transfection, only a small number of duct cells were alive and almost all of them expressed GFP.

Discussion

In this study, we investigated whether an EBV-based plasmid provides an enhanced and prolonged gene expression in the primary porcine cells as well as in human cell lines. As expected, pEBVGFP provided much prolonged GFP expression in the human cell lines than the conventional plasmids, pGFP or pEGFP-C1. We also found that the EBV-based plasmid prolonged gene expression in the primary porcine cells. Recent study showed that several cell phenotypes were recognized in the monolayer culture of porcine pancreatic cells: most frequently found duct epithelial cells with characteristic intermediate filaments, small oval-shaped endocrine cells, and large spindle-like or small comma-shaped fibroblasts [25,26]. The cells used in our study also showed mixed morphology of duct cells, endocrine cells, and small number of fibroblasts just after isolation. Interestingly, duct cells were preferentially transfected using DNA/Lipofectamine complex. The favored transfection of duct cells among the mixed cell population is not unusual as it is widely known that a transfection of mixed cell cultures results in the differential uptake and expression of DNA by different cell types [27]. We observed that without a selection, fibroblasts overgrew duct cells fast and covered the whole flask within 10 days. This may be due to the fact that the speed of the duct cells reaching senescence is faster than that of the fibroblasts [28]. Another possible explanation is that the speed of the duct cell replication is much slower than that of the fibroblasts. These observed growth patterns of the duct cells and the fibroblasts correlate well with what other investigators found [29,30]. Even when the transfectants were selected, the

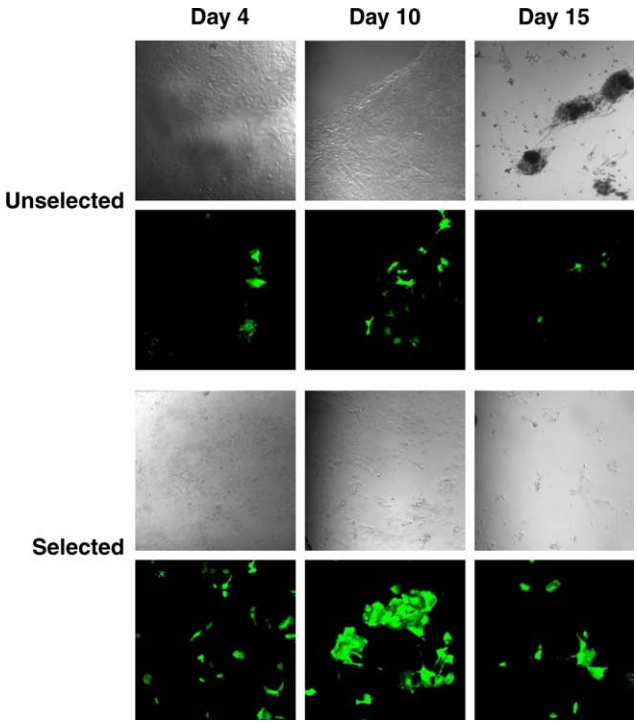

Fig. 7. Preferential transfection of duct cells among the mixed primary porcine neonatal pancreatic cells. Without a selection, the fibroblasts became confluent and piled up, so that a few duct cells expressing GFP remained. Under the selection, the untransfected fibroblasts were removed, resulting in the enrichment of GFP expressing duct cells. Days shown in the figures represent the duration after transfection. For selection, hygromycin B was added to the medium 2 days after transfection. The cells were observed using a confocal microscope (100× objective). All the transmission images were taken at the same magnification on the focus as the fluorescence images.

cells were lost gradually as shown in Figs. 6 and 7. The duct cells would have grown and differentiated into β-cells successfully using the factors available in vivo if the transfected duct cells were transplanted in an animal as observed previously [18,20,31]. Even in vitro, the duct cells may have grown and differentiated into β-cells if the EBV-based plasmid carried a gene encoding an appropriate growth/ differentiation factor rather than the GFP gene. Otonkoski et al. [32] reported that formation of the islet-like cell clusters of primary human fetal pancreatic cells was stimulated by hepatocyte growth factor/scatter factor (HGF/SF), basic fibroblast growth factor (FGF)-2, keratinocyte growth factor (FGF-7), and insulin-like growth factor-II (IGF-II). These are good candidate genes to be tested in primary neonatal porcine pancreatic cells for the potential to stimulate growth and differentiation. In addition, the EBV-based vectors encoding a marker gene will be valuable to monitor the status of the grafted duct cells after transplantation for a longer period.

The prolonged gene expression of pEBVGFP in the primary porcine cells would reflect the sum of plasmid replication and retention [2–4]. The efficiency of EBV plasmid replication in cells can be determined by Southern blot analysis using the different methylation status of input and replicated DNA in the mammalian cells [33]. Experiments to check if the autonomous replication of pEBVGFP contributed to the extended GFP expression in the primary porcine cells were not possible because we failed to collect enough cells to answer the question. The primary porcine cells grew for several days just after transfection but gradually lost during the selection process due to the primary cell's nature. Experiments testing whether pEBVGFP could autonomously replicate in porcine cells are now undertaken using porcine cell lines to get enough cells needed for analysis.

In summary, our study showed that the primary porcine neonatal pancreatic cells were efficiently transfected with pEBVGFP using Lipofectamine. The pEBVGFP ensured much longer expression of GFP in the transfected primary porcine cells than the conventional plasmid, pGFP. Compared with the mixed fibroblasts, pancreatic duct cells were preferentially transfected and selected by hygromycin treatment making it convenient to enrich the useful duct cells. This favored transfection of duct cells and the effectiveness of the EBV-based plasmid vector in these cells make the primary porcine pancreatic cells more attractive as xenotransplantation sources for the treatment of insulin-dependent diabetes.

Acknowledgments

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