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Efficient Gene Transduction of Dispersed Islet Cells in Culture Using Fiber-Modified Adenoviral Vectors

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To establish novel islet-based therapies, our group has recently developed technologies for creating functional neo-islet tissues in the subcutaneous space by transplanting monolithic sheets of dispersed islet cells (islet cell sheets). Improving cellular function and viability are the next important challenges for enhancing the therapeutic effects. This article describes the adenoviral vector-mediated gene transduction of dispersed islet cells under culture conditions. Purified pancreatic islets were obtained from Lewis rats and dissociated into single islet cells. Cells were plated onto laminin-5-coated temperature-responsive polymer poly(*N*-isopropylacrylamide)-immobilized plastic dishes. At 0 h, islet cells were infected for 1 h with either conventional type 5 adenoviral vector (Ad-CA-GFP) or fiber-modified adenoviral vector (AdK7-CA-GFP) harboring a polylysine (K7) peptide in the C terminus of the fiber knob. We investigated gene transduction efficiency at 48 h after infection and found that AdK7-CA-GFP yielded higher transduction efficiencies than Ad-CA-GFP at a multiplicity of infection (MOI) of 5 and 10. For AdK7-CA-GFP at MOI = 10, 84.4 ± 1.5% of islet cells were found to be genetically transduced without marked vector infection-related cellular damage as determined by viable cell number and lactate dehydrogenase (LDH) release assay. After AdK7-CA-GFP infection at MOI = 10, cells remained attached and expanded to nearly full confluency, showing that this adenoviral infection protocol is a feasible approach for creating islet cell sheets. We have shown that dispersed and cultured islet cells can be genetically modified efficiently using fiber-modified adenoviral vectors. Therefore, this gene therapy technique could be used for cellular modification or biological assessment of dispersed islet cells.

Key words: Dispersed islet cell; Fiber-modified adenoviral vector; Islet transplantation; Tissue engineering; Gene therapy

INTRODUCTION

Clinical islet transplantation, in which isolated islets are transplanted into a portal circulation to bring about engraftment into the liver microcirculation, has led to substantial therapeutic effects (32,38). However, the liver is a suboptimal site for islet engraftment (24). In addition, intravascularly transplanted islets may lead to instant inflammatory reactions (2) and continuous immune responses (35,38). Islets are cell clusters of a relatively large diameter (50–400 μm); therefore, central necrosis and apoptosis likely occur immediately after transplantation (3,12). It is desirable to develop an alternative islet-based transplantation procedure that avoids these complex cell death processes following islet transplantation.

A potential approach to increasing the longevity of transplanted islet cells is transplanting them into extrahepatic sites. Another approach may be the use of dispersed islet cells to form neo-islet tissues smaller than the original islets. To meet both requirements, our group recently established the technology to create monolithic cell sheets of dispersed islet cells (30,36,39,40,46). Dispersed islet cells were plated onto laminin-5-coated temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm)-immobilized plastic dishes and cells favorably attached and expanded to

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nearly full confluency (30,40). Treating the culture dishes at 20°C for a short period of time (20 min) leads to natural cell detachment from the culture surfaces, thereby allowing harvesting of the cultured islet cells as a monolithic cell sheet. The islet cell sheets were found to retain cell-to-cell connections (desmosome junctions and gap junctions), demonstrating functional tissue-like characteristics (30,40). Thus, simple transplantation of islet cell sheets resulted in the formation of neo-islet tissues in subcutaneous sites (36,40). These created neo-islet tissues are sufficiently functional to normalize the hyperglycemic status of diabetic recipient mice (36).

To overcome the loss of islet grafts due to cytotoxicity, such as hypoxia and immune or inflammatory responses (12), gene therapy-based approaches have been empirically investigated as a promising intervention for enhancing functionality and/or engraftment rate of transplanted islets (10,15,48). Among various available viral vectors, adenoviral (Ad) vectors have been widely used because they can be genetically transduced at high levels with transient gene expression. However, no protocol for Ad vector-mediated gene therapy using dispersed single islet cells adhered to culture surfaces has yet been reported. In the present study, we conducted a gene transduction investigation of dispersed islet cells under culture conditions using Ad vectors. The vector infection protocol was optimized by assessing whether the use of conventional or fiber-modified Ad vectors, vector dose, and fetal bovine serum (FBS) concentration of the culture medium is suitable for cellular modification or biological assessment of dispersed islet cells.

MATERIALS AND METHODS

Animals

Male Lewis rats purchased from Charles River Laboratories (Yokohama, Japan) were housed in cages in a temperature-controlled room with a 12-h light/12-h dark cycle and were used as islet donors at 10–12 weeks of age. All animal experiments were performed in accordance with the institutional guidelines set forth by the Animal Care Committee of Tokyo Women's Medical University and Fukushima Medical University.

Islet Isolation and Single Cell Purification

Islets of Langerhans were isolated by static digestion of the pancreas in situ using collagenase (Liberase TL; Roche, Penzberg, Germany), followed by Ficoll (Ficoll 400; Sigma-Aldrich, St. Louis, MO, USA) discontinuous density gradient centrifugation. Isolated islets were cultured overnight in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich) containing 10% FBS (Nichirei Biosciences, Tokyo, Japan) and 5.5 mM glucose at 37°C and were subsequently treated with trypsin–ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Carlsbad, CA, USA) for dispersion into single islet cells (30,36,39,40,46).

Ad Vector Preparations

Replication-defective recombinant adenoviral (Ad5) vectors expressing green fluorescent protein (GFP) were constructed using an improved in vitro ligation method as described previously (25,26). The CAG promoter (cytomegalovirus immediate-early enhancer, a modified chicken β-actin promoter) (29)-driven GFP-expressing plasmid, pHMCA-GFP, was digested with I-*Ceu*I and PI-*Sce*I and inserted into I-*Ceu*I/PI-*Sce*I-digested pAdHM4 (26) or pAdHM41-K7 (20), resulting in pAd-CA-GFP or pAdK7-CA-GFP, respectively. Ad vectors (Ad-CA-GFP and AdK7-CA-GFP) were generated and purified as described previously (42). The vector solutions were concentrated using a Virakit (Virapur, San Diego, CA, USA) according to the manufacturer's protocol. Virus particle (VP) titer was determined spectrophotometrically by measuring the genome DNA content (23). The infectious titer [plaque forming unit (PFU)] was calculated using the method of Kanegae et al. (17). The ratios of VP to PFU were 1:20 for Ad-CA-GFP and Adk7-CA-GFP. The multiplicity of infection (MOI) was calculated from PFU titer.

Dispersed Islet Cell Culture and Ad Vector-Mediated Gene Transduction

At a density of 0.57×10^6 cells/cm^2, dispersed islet cells resuspended in RPMI-1640 with 10% FBS were plated onto temperature-responsive polymer PIPAAm (Kojin, Tokyo, Japan)-coated culture dishes (UpCell, 24 well; CellSeed, Tokyo, Japan). Prior to the cell plating, the PIPAAm-grafted culture surfaces had been coated with rat laminin-5 (0.24 μg/cm^2; Millipore, Billerica, MA, USA) for enhancing cellular attachment of dispersed islet cells (31,40). After replenishment with new RPMI-1640 medium with 2% FBS at 0 h, Ad vectors (Ad-CA-GFP or AdK7-CA-GFP) were inoculated at different vector doses (MOI) and incubated at 37°C for 1 h. After the infection, islet cells in culture were extensively washed with RPMI-1640 with 10% FBS. The Ad vector-treated islet cells in monolayer format were cultured either for the determination of islet cell transduction efficiency or for vector-mediated cellular toxicity analyses. For the determination of transduction efficiency, cells were additionally cultured for 47 h with RPMI-1640 with 10% FBS medium and harvested using 0.25% trypsin-EDTA for fluorescent-activated cell sorting (FACS) analyses. At 96 h, harvested cells were also assessed for cellular toxicity assay using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) (Fig. 1).

FACS Analysis

At 48 h, pancreatic islet cells infected with conventional adenoviral vectors (Ad-CA-GFP) or fiber-modified adenoviral vectors (AdK7-CA-GFP) were collected by 0.25% trypsin–EDTA treatment. Propidium iodide (PI;

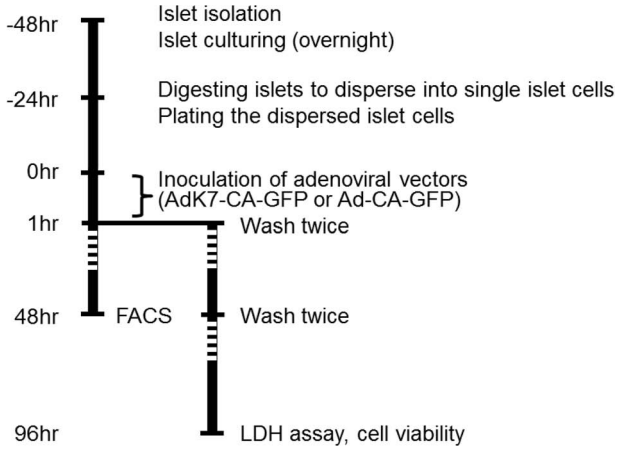

Figure 1. Scheme of the experimental procedure. Pancreatic islets were isolated from male Lewis rats 10- to 12-weeks old and were cultured overnight in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and 5.5 mM glucose at 37°C. Dispersed islet cells were harvested by treatment of the cultured islets with trypsin-ethylenediaminetetraacetic acid (EDTA) and were plated onto laminin-5-coated temperature-responsive culture dishes (24 h). At 0 h, a fully confluent monolayer of islet cells was obtained and was infected with adenoviral (Ad) vectors. Briefly, the medium was replenished with RPMI-1640 medium containing 2% FBS, and then the cells were exposed to normal Ad-CA-green fluorescent protein (GFP) or fiber-modified (AdK7-CA-GFP) vectors at multiplicities of infection (MOIs) of 0–30 in 100 µl of the medium for 1 h at 37°C. Following two washes with RPMI-1640 with 10% FBS, the islet cells were incubated in the medium for either 48 h for fluorescence-activated cell sorting (FACS) analysis or 96 h for the assessment of cytotoxicity [lactate dehydrogenase (LDH) assay]. The medium for culturing for 96 h was renewed at 48 h following washing of the cells twice with the medium.

Sigma-Aldrich) solution (1 µl/ml of 1 mg/ml) was added to the cell suspension, mixed, and incubated in the dark until analysis. PI-positive cells were excluded, and gene transduction efficiency was determined as the number of GFP-positive cells in the islet cells using a FACSCanto II flow cytometer (Becton Dickinson Bioscience, San Jose, CA, USA). A minimum of 10,000 events was collected for each analysis. Data acquisition and analysis were performed with FLOWJO software (Tree Star, San Carlos, CA, USA).

Cell Counting

At 96 h, the number of viable islet cells remaining on the PIPAAm surfaces was determined by CCK-8 (41). At the end of the culture period, cells were incubated with new RPMI medium with one tenth of the CCK-8 solution. After 1.5 h of incubation, the number of viable cells was determined by measurement of O.D. at 450 nm with a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Lactate Dehydrogenase (LDH) Assay

At 96 h, after a 24-h incubation period with new RPMI medium, the culture medium was collected and assayed for analysis of LDH concentration using FUJI DRI-CHEM LDH slides (FUJIFILM, Tokyo, Japan) according to the manufacturer's protocol. The linearity of this assay between 50 and 900 U/L was stated by the manufacturer. The linearity between 30 and 50 U/L was confirmed using serially diluted standard samples in a preliminary experiment (data not shown).

Statistical Analysis

GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Statistical analysis of cytotoxicity and LDH assay was performed by one-way analysis of variance (ANOVA) and Kruskal–Wallis test followed by Dunn's multiple comparisons test to identify groups differing from the normal control group (MOI = 0). The statistical analysis of FACS results was performed by two-way ANOVA followed by Sidak's multiple comparisons test. A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Culturing Dispersed Islet Cells on PIPAAm-Laminin-5-Coated 24-Well Culture Dishes

In our previous studies, plating rat dispersed islet cells at a density of 0.57×10^6 cells/cm^2 on PIPAAm-grafted and rat laminin-5-coated 35-mm culture dishes allowed the cells to attach and reach nearly 100% confluency status at 0 h (30,36,40). In the present study, we used 24-well culture dishes whose surfaces were modified in the same manner and found that plating rat dispersed islet cells at a density of 1×10^6 cells/well resulted in acceptable cell attachment. The attached islet cells expanded favorably and were close to 100% confluent by 24 h after cell plating. We confirmed by light microscopic observation that the dispersed islet cells formed monolayer on the culture surfaces and so we performed Ad vector-mediated gene transduction studies.

Ad Vector-Mediated Gene Transduction for Dispersed and Cultured Islet Cells in Monolayer Format on PIPAAm Surfaces

In a preliminary experiment, we conducted Ad vector (Ad-CA-GFP) infection of islet cells by inoculating vectors (MOI = 5 and 10) into FBS-free RPMI-1640 medium for 1 h and found that most of the cells detached from the culture surface. Therefore, we used 2% FBS-containing RPMI-1640 medium for the culture medium during the vector infection period.

To determine the optimal Ad vector dose for Ad vector-mediated gene transduction into dispersed and cultured islet cells in monolayers on the PIPAAm surfaces, increasing

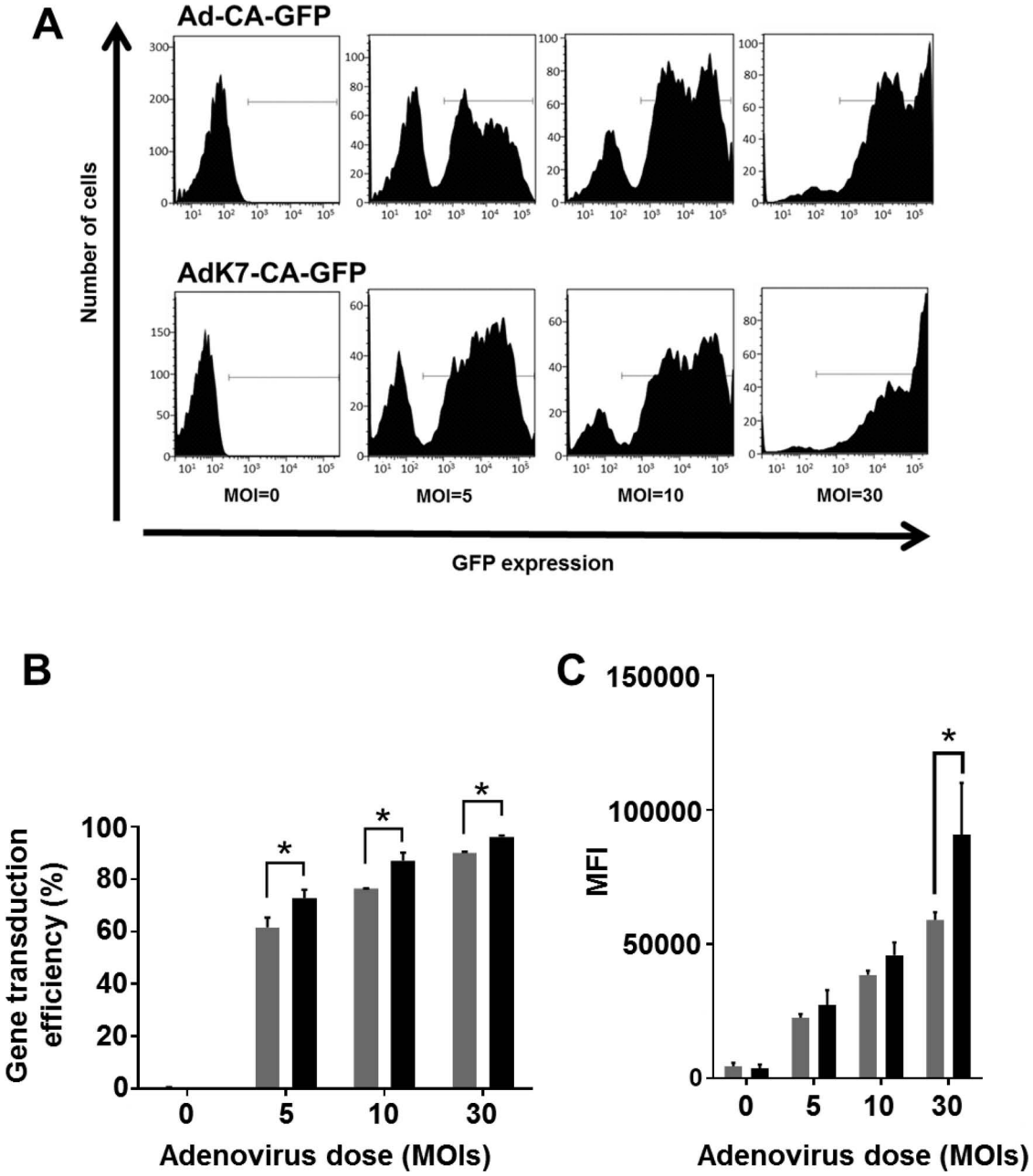

Figure 2. Transduction efficiency of GFP gene into dispersed islet cells by adenoviral vectors. (A) Representative histograms of GFP expression in dispersed islet cells infected with adenoviral vectors. Dispersed islet cells in 24-well plates were infected with Ad-CA-GFP (top) or AdK7-CA-GFP (bottom) at the indicated MOIs. The cells were further cultured in RPMI-1640 with 10% FBS for 47 h and were analyzed by flow cytometry. (B, C) The percentage (B) and mean fluorescence intensity (MFI) (C) of GFP-positive islet cells were determined by flow cytometry. Data are presented as means ± standard deviation ($n = 3$). The asterisks indicate significant differences between Ad-CA-GFP (gray bars) and AdK7-CA-GFP (black bars) determined by Sidak's multiple comparisons test ($p < 0.05$).

doses of Ad vector (AdK7-CA-GFP or Ad-CA-GFP) at MOIs of 5–30 were inoculated into the culture medium at 0 h. The vector infection period was set for 1 h in this study, and after rinsing steps we cultured islet cells for an additional 47 h to assess the gene transduction efficiency. As shown in Figure 2, AdK7-CA-GFP showed significantly higher transduction efficiencies than Ad-CA-GFP at all MOIs. MOIs of 10 or 30 of AdK7-CA-GFP achieved >80% transduction efficiencies. Ad vectors are known to produce some level of cytotoxicity, so we assessed the cell viability of the AdK7-CA-GFP-treated islet cells by counting the viable cells remaining attached at 96 h. Cell number was stably maintained between MOI = 0 and 10, whereas MOI = 30 resulted in the attachment of significantly fewer

cells than MOI = 0 (Fig. 3A). We also assessed LDH activity (a marker for cellular damage) in the culture medium at 96 h. No significant difference was observed between the MOI = 0 and 10 groups, whereas significantly higher LDH activities were detected in the MOI = 30 group than in the MOI = 0 group (Fig. 3B).

Morphological Changes of the Ad Vector-Treated Dispersed and Cultured Islet Cells in Monolayer Format on PIPAAm Surfaces

Finally, we assessed the morphological features of AdK7-CA-GFP-treated islet cells (at 96 h). As shown in Figure 4, islet cells of MOI = 0, 5, and 10 groups showed favorable attachment and expansion on the laminin-5-coated PIPAAm culture surfaces, and maintained nearly full confluency. In contrast, islet cells of MOI = 30 group showed approximately 60% confluency.

From these findings, we conclude that the optimally efficient Ad vector-mediated gene transduction to disperse and cultured islet cells in monolayer format would employ AdK7 vector at MOI = 10 in 2% FBS-containing medium.

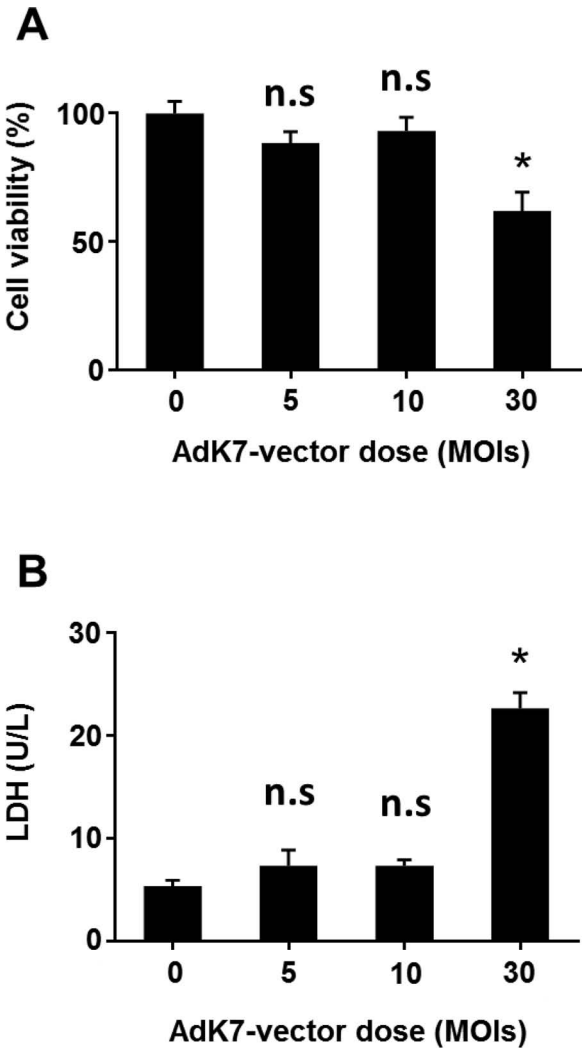

Figure 3. Assessment of cytotoxicity of AdK7-CA-GFP on dispersed islet cells. (A, B) Cytotoxicity of AdK7-CA-GFP was assessed by cell viability (A) and LDH assay (B). Data are presented as means ± standard deviation ($n = 3$). Asterisks indicate a significant difference determined by Dunn's multiple comparisons test ($p < 0.05$, compared with MOI = 0). n.s., not significant compared with MOI = 0.

DISCUSSION

We have described a simple approach for the genetic modification of dispersed and cultured islet cells in monolayer format using fiber-modified Ad vectors. Cytotoxicity and GFP expression studies showed that more than 80% of the cultured islet cells were efficiently transduced with relatively low dose of AdK7 vectors (MOI = 10) in culture medium with 2% FBS for 1 h. No marked cellular damages associated with this Ad vector treatment were confirmed by morphological stability, persistence of cell attachment on culture dishes, and LDH activity of culture medium. More importantly, the Ad vector-infected dispersed islet cells remained attached and showed nearly full confluency on laminin-5-coated PIPAAm culture surfaces, which is an important feature for creating functional islet cell sheets. The present study showed that dispersed islet cells could be genetically modified efficiently using the Ad vector under culture conditions.

Viral vector-based genetic modifications of dispersed islet cells have been experimentally performed using Ad (1,44), adeno-associated viral (AAV) (22,34), and lentiviral (LV) (7,16,21) vectors. Prasad et al. (34) reported that AAV vectors provide high safety profiles and long-term transgene expression for islet cells. However, relatively low efficient transduction efficiency (<50%) limits their application for islet cell functional modification (34). LV vectors allow efficient transgene integration into host genomes of dispersed islet cells, thus providing stable and persistent transgene expression (7,16,21). Thus, in cases when genetic modification period will be limited to specific situations (such as during a culture period or a peritransplantation period until engraftment), the LV vector may not be appropriate (37). In contrast, because Ad vectors normally are highly infectious to target cells at low MOI and are present in episomal form, transgene expression occurs at high levels and is limited to a short term (14,47). In view of these pros and cons of viral vectors, the present study investigated a procedure for Ad vector-based genetic modification of dispersed islet cells.

We have recently established that Ad vector infusion into celiac artery of living mice provides efficient gene transduction to islet clusters within the pancreas (28). In contrast, Ad vector-mediated gene transduction to islet clusters in suspension condition has been partially successful, although researchers have been modified the infection

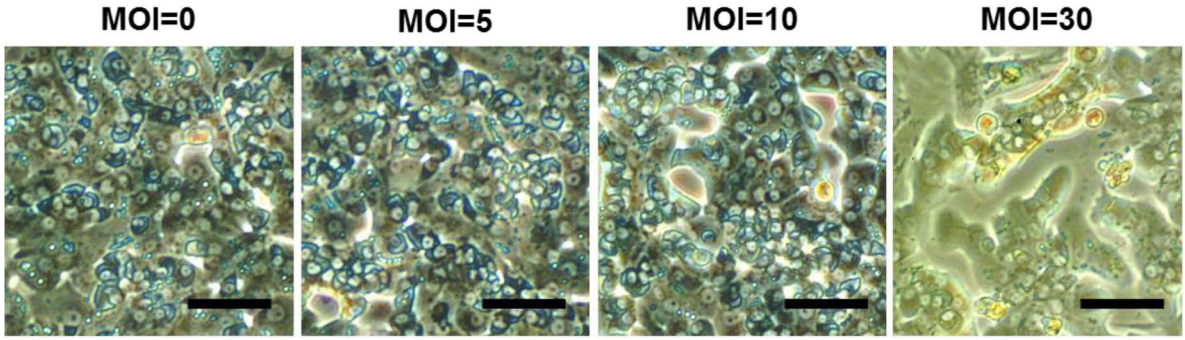

Figure 4. Islet cell sheet morphology after fiber-modified adenoviral vector infection. Following infection with AdK7-CA-GFP at the indicated MOIs, the islet cells were incubated in RPMI 1640 with 10% FBS for 96 h and subjected to phase-contrast microscopic analysis. Scale bars: 50 μm.

protocol, including Ca^{2+}-free treatment prior to the vector infection (8,11,18,28,45). These in suspension vector infection procedures provide high levels of gene transduction of islet cells located in the mantle area of islet clusters; however, cells in the core area are difficult to transduce (4). It is generally accepted that endocrine cells are not randomly distributed within islet clusters. β-Cells compose the core of the islets, and non-β-cells, including α-cells and δ-cells, form the mantle region (5). Considering this unique topographic arrangement of β-cells and non-β-cells and predominant gene transduction to the mantle region, the Ad vector-mediated conventional gene transduction approach is more appropriate for non-β-cells than for β-cells. For further advancing islet-based therapies, an alternative method that enables β-cells to be efficiently transduced should be developed. To this end, our Ad vector-mediated gene transduction protocol, including dispersing and monolayer culturing processes, successfully achieves efficient gene transduction to β-cells as well as non-β-cells. Our previous study showed that islet cell sheets made of monolithic cultured islet cells are transplantable (40) and engraftable (36); therefore, our gene transduction protocol could add valuable functional modification steps for advancing dispersed islet cell-based therapy.

One disadvantage with the use of the Ad vectors is the low efficiency of Ad vector-mediated gene transfer to cells expressing low levels of the coxsackievirus and adenovirus receptor (CAR) (13,33). To overcome this limitation, researchers have developed capsid or fiber-modified Ad vectors for widening the viral tropism (9,13,27). Therefore, we and other researchers created the AdK7 vectors, in which a peptide with seven tandem lysine residues (K7) was inserted in the fiber knob that enables targeting of heparan sulfates on the cellular surface (6,43). AdK7 vectors have been confirmed to show a higher transduction rate than the conventional Ad vectors in bone marrow stromal (43), skeletal muscle (6), and trophoblast cells (19). In agreement with these reports, the present study clearly showed the advantage of the use of AdK7 vectors for the dispersed and cultured islet cells on laminin-5-coated PIPAAm surfaces. We have confirmed that human dispersed islets may be maintained in a monolithic format on laminin-5-coated PIPAAm surfaces, and so we plan to investigate AdK7 vector-mediated gene transduction to human dispersed islets for advancing their clinical application. In addition, the application of Ad vectors carrying anti-inflammatory or antiapoptotic genes using the dispersed islet cell-based tissue engineering approach will be the subject of future experiments.

In summary, the present study demonstrated efficient gene transduction of dispersed and cultured islet cells using fiber-modified AdK7 vectors at MOI = 10. These genetically transduced islet cells did not show marked cellular damage and were capable of nearly full confluency. These findings clearly represent an important step in advancing dispersed islet cell-based therapies, including islet cell sheet-based tissue engineering approaches, for achieving favorable therapeutic outcomes for diabetic mellitus patients.

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