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Research article

LABELING AND TRACKING HUMAN AMNIOTIC EPITHELIAL CELLS WITH GREEN FLUORESCENT PROTEIN IN AN ADENO-ASSOCIATED VIRUS VECTOR

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Abstract: Human amniotic epithelial cells (hAECs) are a recently identified type of stem cell. Thanks to their ready availability and the lower risk of teratoma formation, hAECs have been studied and tested for a variety of human disease treatments and tissue reconstruction efforts. This aim of this study was to establish a stable tracking system to further monitor hAECs *in vivo* after transplantation. hAECs were isolated from the placentas of patients who visited the Hunan Province Maternity and Child Care Hospitals between Jan 2008 and Jan 2009. Using the classic transfection/infection technique, we successfully introduced green fluorescent protein (GFP) into cultured hAECs with an adeno-associated virus (AAV) vector. The initial preparation of the AAV-GFP virus stock was titrated using HT1081 cells, and further used for the infection of hAECs. GFP^+ hAECs preserve the capacity of differentiation into hepatocyte-like cells with the expression of cytokeratin-18 (CK18) and albumin (ALB). AAV-GFP virus-infected hAECs were transplanted through the spleen into severe combined immune deficiency (SCID) mice via hepatectomy. Four weeks later, the GFP and human albumin expressions were examined in multiple organs through immunoflourence staining. In culture, over 50% of the hAECs

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Abbreviations used: AAV – adeno-associated virus; ALB – albumin; CK18 – CK18 cytokeratin; Dex – dexamethasone; FBS – fetal bovine serum; GFP – green fluorescent protein; hAEC – human amniotic epithelial cell; hESC – human embryonic stem cell; HGF – hepatocyte growth factor; IGF – insulin-like growth factor

were GFP-positive 3 days after infection. Following transplantation, AAV-GFP-infected hAECs survived and continued to express GFP in the host for up to 4 weeks. These cells were primarily found in the spleen and liver, expressing human albumin. This study provides a feasible and stable system to track hAECs. It may prove useful to further identify their biological characteristics after transplantation and to elucidate their beneficial roles for therapeutic purposes.

Key words: Human amniotic epithelial cells, Adeno-associated virus, Green fluorescent protein, Transplantation, Tracking system

INTRODUCTION

Human amniotic epithelial cells (hAECs) are derived from epiblast cells, and form the epithelial lining of the amniotic cavity. Since the amniotic epithelium develops prior to gastrulation, hAECs were thought to retain the features of pluripotent stem cells [1]. It was recently confirmed that hAECs expressed human embryonic stem cell-associated (hESC-associated) makers [2]. However, unlike hESCs, hAECs are found in unlimited quantities, they are easily accessible, and there is less of a risk of teratoma formation [2, 3]. Therefore, as a new source of stem cells hAECs have garnered great interest in the stem cell research focused on human disease treatment and tissue repair.

Initially, amniotic membranes were used in ocular surface reconstruction because of their transparency [4, 5], and in the treatment of skin injury resulting from ulcerations and burns [6]. In 2000, hAECs were isolated by Terada *et al.* [7] and used for cell therapy. Recently, hAECs were further characterized [3] and tested for potential applications in other diseases and in the treatment of injuries. It was previously shown that hAECs expressed a hepatocyte-related gene and possessed some of the intracellular features and functional properties of hepatocytes [2]. It was proposed that hAECs may be a candidate as seed cells for liver regeneration in the cases of certain hepatic diseases and as a gene-delivery vehicle [8]. For example, hAECs with overexpression of b-glucuronidase (GUSB) were transplanted into the spleen for the treatment of congenital lysosomal storage disorders [9]. In addition, Wei *et al.* [10] showed that hAECs were capable of normalizing the blood glucose level in a murine diabetic model. Furthermore, Miki *et al.* [3] demonstrated that hAECs expressed the insulin gene and produced insulin protein following nicotinamide stimulation, implying a therapeutic potential for the treatment of type I diabetes mellitus. Finally, it was previously found that hAECs synthesized and secreted several neurotrophic factors and had a possible involvement in neural formation [11-13]. These factors were applied to the spinal cord in a nerve injury model and showed beneficial effects, and a comparable outcome to a neural tissue graft transplantation [14-15]. In conclusion, hAECs are a potentially therapeutic cell type in human disease treatment and tissue repair [16].

It remains controversial whether hAECs preserve their stem cell property or differentiate into a certain type of functional cells over time in the host [16]. To further study post-transplantation hAECs *in vivo*, a long-term tracking system is needed. Dye labeling [14] and LacZ maker gene transduction [13] have been used to monitor these cells, but the diminishing of the color with time and additional staining processes prevent the direct monitoring of these cells *in vivo* and their immediate analysis *ex vivo*. Establishing a stable tracking approach would provide critical information to understand their survival, differentiation and other functional properties *in vivo*, as well as to evaluate the side effects for the application of tissue repair. In this study, we used an adeno-associated virus (AAV) vector to introduce green fluorescent protein (GFP) into cultured hAECs, and successfully tracked these cells *in vivo* for 4 weeks after transplantation into mice.

MATERIALS AND METHODS

The collection, purification and culture of hAEC

Placentas were collected from patients who visited the Hunan Province Maternity and Child Care Hospitals between Jan 2008 and Jan 2009. All of the placentas were subjected to screening tests for HIV, HAV, HBV and syphilis. Placentas with a positive result for any of the above tests were excluded. Written informed consent was obtained from all of the patients. This study was approved by the Central South University Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

The primary hAECs were prepared according to the method of Miki *et al.* [3]. The amniotic membrane was separated from the placenta and rinsed with normal saline solution to remove blood stains and tissue debris. When it had been completely cleaned, the amniotic membrane was cut into small pieces to fit the cell culture dish and digested according to the following protocol. Trypsin-EDTA (concentration 0.05%, 0.5-1 ml/cm^2) was added to a 100-mm dish, and the amniotic membrane pieces were placed into the dish such that they were completely covered by the solution. The dish was incubated at 37ºC for 15-30 min in a cell culture incubator (DNP-9160BS-Ⅲ, Yuejing, China). The digestion was checked under a microscope (E80i, Nikon, Japan) and stopped when the cells became round. The amniotic membrane was rinsed with the digestion solution a few times, then rinsed with an equal volume of DMEM (Gibco, USA) and PBS. The resulting cell suspension was collected in a 50-ml tube and centrifuged at 1500-2000 rpm for 5 min. The amniotic epithelial cells were resuspended in complete DMEM medium and seeded at 2×10^7 cells per 100-mm cell culture dish with DMEM. The cells were cultured in the CO_2 incubator and supplied with fresh media every three days. When the cells reached 80-90% confluence, they were treated with trypsin and prepared into a single cell suspension. After washing, the cells were counted and re-seeded in a dish for continuous culture.

Preparation of AAV-GFP virus

AAV-GFP plasmid was purchased from Stratagene (USA) and amplified in DH5a competent cells, which are routinely maintained in our laboratory. Plasmids were purified using a commercial kit and stocked at 1 μg/μl for the transduction of HEK 293 cells (Qiagen GmbH, Germany), which contain the necessary gene for the AAV-GFP virus package. One day prior to the transfection, HEK 293 cells were seeded at a concentration of 10×10^6 cells in 10 ml complete DMEM medium, and cultured overnight. Upon transfection, 1 mg/ml plasmid stock was taken and adjusted into a volume of 1600 μl, then 150 μl calcium phosphate was added to the same tube, and the whole was mixed well and incubated at room temperature for 20 min. The resultant mixture was slowly added to the HEK 293 cell culture. The dish was gently moved and swirled to obtain an even virus distribution in the culture. The cells were further cultured for 16 h. The supernatant was discarded and replaced with fresh medium. After a 2-day culture, the cells were examined for GFP^+ cells under a fluorescence microscope. The cells were harvested and lysed using freezing (-70ºC) and thawing (37ºC) twice. The resultant mixture was centrifuged at 10,000 g for 10 min. The supernatant with virus was collected and stored at -70ºC for the future infection of target cells.

Titration of the AAV-GFP virus, FACS analysis, and infection of the target cell

The AAV-GFP virus stock was titrated using HT1081 cell culture. HT1081 cells were trypsinized, prepared into a single cell suspension, and seeded at a concentration of 3×10^5/ml in a 6-well plate. When the cells reached 70% confluence, the supernatant was carefully removed and replaced with a medium containing one of a range of AAV-GFP virus concentrations (which had been diluted from the stock): 0, $1{:}10^8$, $8{:}10^4$, $4{:}10^3$, $2{:}10^2$, 1:10 and 1:1. The cells were further cultured for 3 days and were examined for GFP expression under an inverted fluorescence microscope and photographed with a digital camera system (E80i, Nikon, Japan). The cells were separately collected and subjected to FACS analysis. The frequency of GFP-positive cells was determined using a FacsCalibur flow cytometry device (Becton Dickinson, USA) and Flowjo analysis software (TreeStar, USA). The titration of the virus was calculated by dividing the number of GFP^+ cells by the total number of analyzed cells, and multiplied by the dilution of the virus. The hAEC was infected with the original preparation of AAV-GFP, using the same protocol for the infection of HT1081 cells.

Induction of hAEC differentiation *in vitro* and analysis for hepatocyte markers

After a 3-day culture, AAV-GFP-infected hAECs were prepared into a single cell suspension. GFP^+ hAECs were sorted with a FACS sorter and seeded in Low-DMEM medium (Gibco, USA) with a supplement of hepatocyte growth factor (HGF, 20 ng/ml, R&D system, USA), insulin-like growth factor (IGF, 10 ng/ml, R&D system, USA), dexamethasone (Dex, 100 nM, Santa Cruz Biotechnology, USA), and 10% fetal bovine serum (FBS, Gibco, USA). The

culture medium was changed every 3 days. The induction period was 14 days. The cells were collected on days 10, 12 and 14 post-induction, stained for cytokeratin-18 (CK18) and albumin (ALB; Santa Cruz, USA), and analyzed using FACS.

Animal model of partial hepatectomy and cell transplantation

Six- to eight-week old SCID mice (C57B6 background) were purchased from the Shanghai Animal Center (Shanghai China). The mice were housed at 25ºC ± 2ºC with a 12-h light/dark cycle in microisolater cages contained within a laminar flow system to maintain a pathogen-free environment. All of the surgical procedures were completed in accordance with the guidelines on the care and use of laboratory animals for research purposes issued by the Central South University Xiangya Medical School's Animal Care and Use Committee. The mice were anesthetized with sodium pentobarbital (i.p.) for all of the surgical procedures.

Using aseptic techniques, the liver was exposed and the left and middle lobes (approximately 70%) were removed using the routine silk sutures ligation method. The spleen was exposed and its blood flow was blocked at the splenic hilus with a toothless hemostatic clamp. Depending on the group of mice, cells or PBS were slowly injected at the inferior pole using a BD insulin injection syringe. Following the hemostatic treatment, the hemostatic clamp was released and the spleen was returned to its anatomic location. The incision was sutured layer by layer. Penicillin was given to all of the mice daily (i.m. 20 wU) for five days post-operation to prevent infection.

Transplantation of AAV-GFP-infected hAECs

Forty severe combined immune deficiency (SCID) mice were randomly divided into three groups. The first group (n = 20) underwent a 2/3 hepatectomy plus inoculation with 5×10^6 AAV-GFP virus-infected hAECs at the inferior end of the spleen. The second group (n = 10) received an inoculation of 5×10^6 AAV-GFP virus-infected hAECs at the inferior end of the spleen without the hepatectomy. The third group (n = 10) underwent the 2/3 hepatectomy with inoculation with 0.2 ml normal saline instead of with hAECs.

Examination of transplanted GFP^+ cells

GFP^+ cells in the host were examined 4 weeks after transplantation. The mice were euthanized and infused with normal saline until the liver became pale. The liver, spleen, heart, lungs, brain and kidneys were collected, cryostat sectioned at a thickness of 2 μm, and examined for GFP^+ cells under a fluorescence microscope. A fraction of the tissues from the above organs was also formalin-fixed, paraffin-embedded, continuously sectioned for H&E (hematoxylin and eosin) staining and histological analysis, and for antibody staining and immunohistochemistry analysis.

Immunofluorescence staining for human albumin

After being washed with phosphate buffered saline (PBS) 3 times (3 min per wash), the sections were permeabilized with 0.1% Triton-100 in PBS for 15 min at room temperature. Non-specific staining was blocked using 10% normal goat serum albumin in PBS for 25 min. The sections were incubated overnight with rabbit anti-human albumin antibody (1:200, Rabbit IgG; Santa Cruz, USA). The sections were washed extensively in PBS and incubated with the secondary antibody, goat anti-rabbit IgG (1:200, R&D Systems, USA) at room temperature for 30 min. After being washed, the slides were viewed using a Nikon microscope equipped with a digital camera system (Qimaging Retiga 2000R, USA).

Statistical analysis

The SPSS program (version 12.0, SPSS Inc., USA) was used for the statistical analysis. The quantitative data is expressed as the means ± SD. The Student t-test was used for group comparisons. The differences were considered significant when $p < 0.05$.

RESULTS

Preparation of the AVV-GFP virus

Owing to a lack of polymerase and adenovirus helper factor, the AVV vector relies on cellular polymerases for genome replication and a third plasmid to provide the necessary factors. HEK293 cells with the necessary factors for the AVV virus were commonly used as AAV-GFP production cells. We used HEK293 cells for the preparation of the AVV-GFP virus. As shown in Fig. 1, following transfection with the AVV-GFP plasmid, GFP expression was detected in the majority of HEK293 cells. The virus particles were harvested through lysis of the cells using the freezing and thawing method.

Measurement of AVV-GFP titration and infection of hAECs

To titrate the aforementioned AVV-GFP virus, we infected HT1081 cells with the AVV-GFP virus at a variety of concentrations, prepared by diluting the original virus stock at the following ratios: $1:10^8$, $8:10^4$, $4:10^3$, $2:10^2$, 1:10 and 1:1. As shown in Fig. 2A, the higher virus concentrations resulted in more GFP^+ cells in the culture. The quantitative results of FACS analysis indicated that the original virus stock achieved a 58.2% infection rate (Fig. 2B, plot f) and the dilution led to a decrease in the GFP^+ cell percentage (Fig. 2B, plot a-e). Using the original virus stock, we infected the cultured hAECs, and over 50% of the hAECs expressed GFP (Fig. 3B). To estimate the survival and proliferation of hAECs following infection, we counted the total number of viable hAECs on days 1, 3, 5 and 7 after infection. The results are summarized in Tab. 1. In comparison to the non-infection control, hAECs following AAV-GFP infection exhibited the same cell survival and proliferation pattern, suggesting that AAV-GFP infection did not influence the hAEC survival and division capabilities.

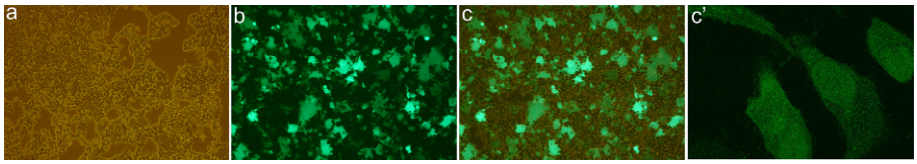

Fig. 1. The package of the AAV-GFP virus in the HEK293 cells. HEK293 cells were transfected with the AAV-GFP plasmid and examined under regular light (a) and fluorescence (b) microscopes 2 days after transfection. GFP expression was observed in the majority of the HEK 293 cells (c, merged image of a and b). The morphology of the infected HEK293 cells at high power is shown in micrograph c'.Original magnifications: 100× for a-c and 800× for c'.

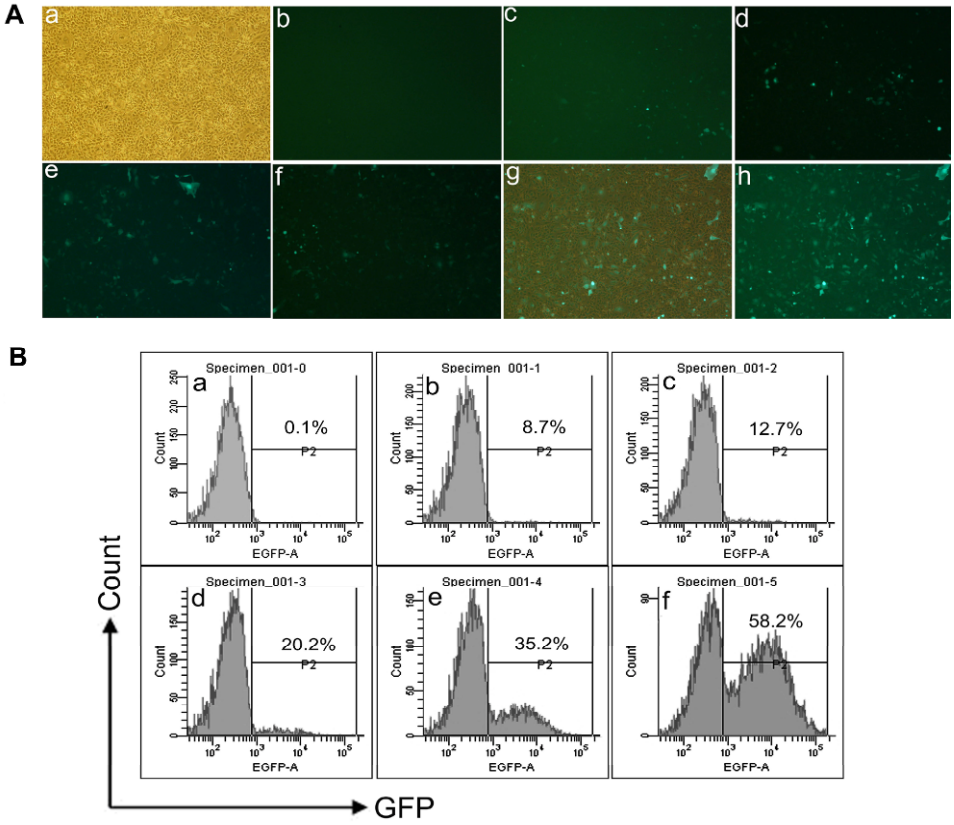

Fig. 2. Titration of the AAV-GFP virus by infecting HT1081 cells. HT1081 cells were infected with the AAV-GFP virus at a series of dilutions from the original preparation. A – The GFP^+ cells were examined under a microscope. Micrographs a and b are representative photographs of the negative control cells under regular light and fluorescence microscopes. Micrographs c-h correspond to virus dilutions of $1:10^8$, $8:10^4$, $4:10^3$, $2:10^2$, 1:10 and 1:1. Original magnifications: 40× for a-h. B – Cells in each well were separately collected and subjected to FACS analysis. Plots a-f correspond to virus dilutions of $1:10^8$, $8:10^4$, $4:10^3$, $2:10^2$, 1:10 and 1:1 with a positive GFP percentage of 0.1 ± 2.1%, 8.7% ± 1.3%, 12.7% ± 2.1%, 20.2% ± 3.1%, 35.2% ± 2.6%, and 58.2% ± 1.3% (n = 3).

Tab. 1. The total number of viable hAECs in culture with or without AAV-GFP infection.

Day post-infection	0	1	3	5	7
hAECs without infection	$1x10^4$/ml	$1x10^4$/ml	$2.5x10^4$/ml	$5.0x10^4$/ml	10^5/ml
hAECs with AAV-GFP infection	$1x10^4$/ml	$1x10^4$/ml	$2.4\ x10^4$/ml	$4.9x10^4$/ml	10^5/ml

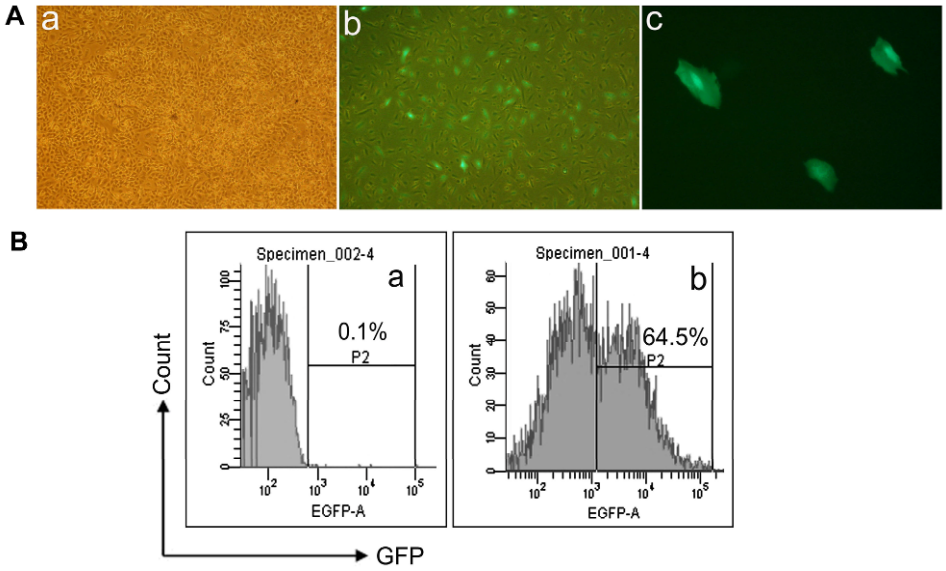

Fig. 3. The infection of hAECs with the AAV-GFP virus. A – Fluorescence microscope images of the primary culture of the hAECs (a) and the hAECs on day 3 post-infection (b). The morphology of the infected hAECs at high power is shown in micrograph c. Original magnifications: 100× for a-b and 400× for c. B – Representative FACS results for hAECs without (a) or with (b) AVV-GFP infection.

Differentiation of hAECs into hepatocyte-like cells *in vitro*

To estimate whether hAECs preserve the capacity to differentiate following AVV-GFP infection, we isolated GFP^+ cells using a FACS sorter, and continued to culture them in a medium with differentiation-inducing factors, as described in the Materials and Methods section. These cells were collected on days 10, 12 and 14 post-induction, stained for CK18 and ALB, and analyzed using FACS. As shown in Fig. 4, the frequency of CK18- and ALB-positive hAECs continuously increased from day 10 to 14 post-infection, suggesting AVV-GFP infected hAECs can differentiate into hepatocyte-like cells.

Examination of GFP^+ hAECs following transplantation into mice

To determine whether GFP is continuously expressed *in vivo*, and at a sufficient level for tracking hAECs, we inoculated these cells into SCID mice by injecting them into the animals' spleens. Four weeks following cell injection, we examined the GFP^+ cells in the host organs. GFP^+ cells were detected in the

spleens and livers that had previously been injured (micrograph b in Fig. 4A and B, respectively). By contrast, GFP^+ cells were not found in livers that had not previously been injured (data not shown) or had not received hAECs.

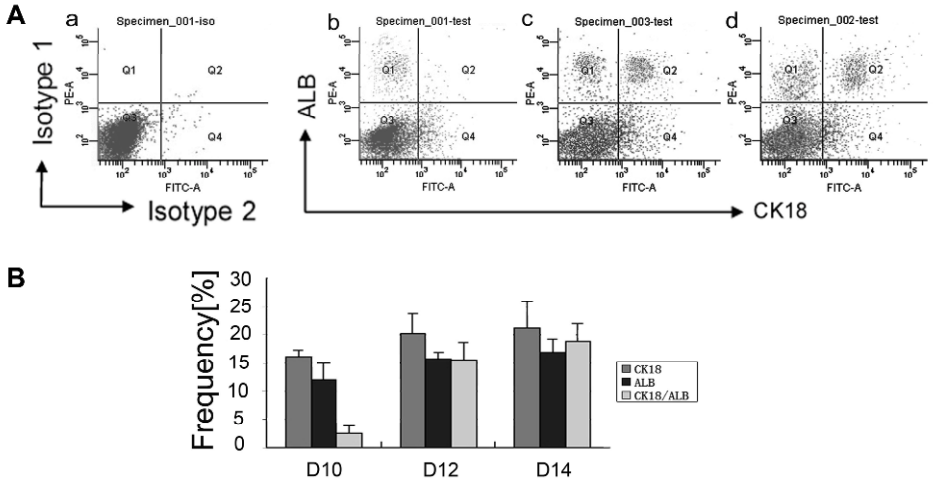

Fig. 4. Differentiation of hAECs into hepatocyte-like cells *in vitro*. GFP^+ hAECs were sorted and subjected to the induction of differentiation. The cells were collected on different days, stained for ALB and CK18, and analyzed using FACS. A – Isotype controls for ALB and CK18 were used for gate setting (a). ALB and CK18 expressions in the hAECs were analyzed on days 10 (b), 12 (c) and 14 (d) post-induction. Q1 = ALB-positive cells, Q2 = ALB and CK18 double-positive cells, Q3 = CK18-positive cells. B – The bar graphs show the frequencies of the cells in Q1-Q3 in panel A. D10, D12 and D14 on the x-axis correspond to plots b-d, respectively (the data is from triplicate samples).

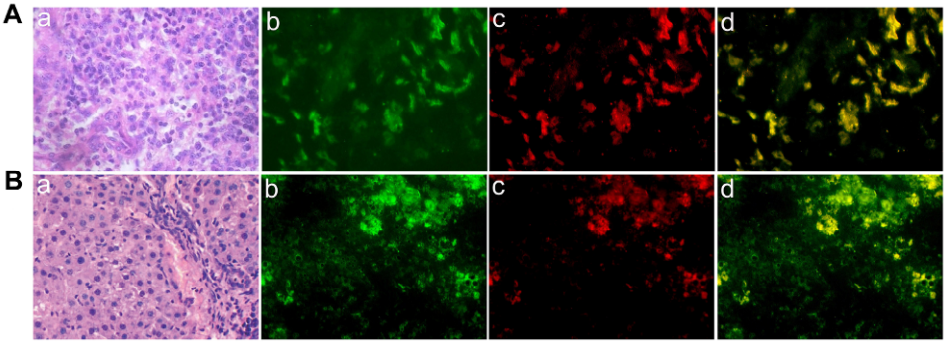

Fig. 5. Tracking hAECs infected with AAV-GFP in the host tissue. Four weeks after the transplantation of AAV-GFP-infected hAECs, the spleens (A) and livers (B) were collected from the host mice and subjected to H&E staining (a), fluorescence microscope examination (b), and immune-fluorescence staining for human albumin (c). Co-localization of GPP and human albumin expression (d) was observed in both the liver and spleen sections. Original magnifications: 400×for the spleens (a) and livers (b), 200x for (b-d).

In addition, GFP^+ cells were not found in the hearts, kidneys, lungs and brains (data not shown). Furthermore, staining the livers and spleens with anti-human albumin antibody indicated that the human albumin signal co-localized with GFP, suggesting that GFP reports the hAECs (Fig. 5A and B, micrographs c and d, respectively).

Pilot study for the risk of GFP^+ hAEC transplantation

To evaluate the risk of introduction of the AVV-GFP virus into hAECs, we examined the tumor tissue and monitored mouse survival for up to 4 weeks after transplantation. Histological analyses of the hearts, kidneys, lungs, brains, spleens and livers of all of the mice were carried out via extensive microscopic examination of the tissue at a thickness of 2 μm. No tumor tissue was found in any of these organs (data not shown). A visual inspection of the pleural and peritoneal cavities did not find any suspicious masses. Mouse death occurred in the mouse groups that had undergone a partial hepatectomy (2 in each group), but not in the control group. We speculate that these deaths resulted from complications due to the hepatectomy, rather than from the hAEC transplantation.

DISCUSSION

In the past decade, human amniotic membrane (hAM) cells have been actively studied and used for ocular surface reconstruction, skin repair after burns, and diabetes and lysosomal storage disorder treatment [4-6, 9, 10, 17]. hAEC, the major type of stem cell in the hAM, is a potentially therapeutic cell in human disease treatment with several advantages [16]. First, its collection and preparation is feasible, simple and non-invasive. There is no ethical issue to debate when using hAEC. In addition, purified hAEC can be cultured and expanded [2], and may be cryopreserved, which provides an important opportunity to convert a “useless and discarded” tissue into a valuable source of possible cell transplants for the future. Furthermore, compared to hESC, hAEC have less risk of teratoma formation [2, 3]. Lastly, it has been shown that hAECs have a low immunogenicity, have a very low risk of rejection, and survive for a long time after host transplantation [2, 3]. These features of hAECs have been well studied and documented in the literature. However, owing to the lack of a tracking system that can be used to identify the transplanted hAECs over a long period, their behaviour following transplantation has not been well characterized.

Adeno-associated virus is the smallest virus known. It infects both proliferating and non-dividing cells with very low immunogenicity, and is not associated with any known diseases. Consequently, it has been widely used for gene therapy [18]. AAV-GFP virus is used for cell labeling because of its high efficiency of infection, stable expression of GFP *in vivo*, and ease of detection [19]. In this study, using the classic transfection/infection system, we successfully labeled hAECs with the AVV-GFP virus and further examined GFP expression in the

host after transplantation. Our results showed that hAECs can be infected with the AVV-GFP virus and tracked by the expression of GFP following transplantation.

Furthermore, we evaluated the impact of the infection of the AVV-GFP in the hAECs. Our results show that it had minimal cytotoxicity and did not alter the characteristics of the hAECs. The morphology of the infected hAECs did not change, and these cells survived in the host. In addition, GFP^+ hAECs preserved the capacity of differentiation into hepatocyte-like cells, with the expression of CK18 and ALB. Furthermore, AAV-GFP-infected hAECs stably expressed GFP in the host following transplantation, and survived for up to 4 weeks. These cells were primarily found in the spleen and liver when they were injected into the spleen. Lastly, these cells maintain hepatocyte-like features such as the expression of human albumin. This study provides a feasible and stable system to track hAECs, which may be useful to further identify their biological characteristics and to elucidate their beneficial roles in cell therapy.

While tail-vein injection is the most widely used method to deliver cells into a host, it is believed that injecting cells into the spleen is the best route to transplant cells into the liver, as the injected cells can reach the liver through the portal vein [20]. In this study, we transplanted our cells through this route and found that GFP^+ cells were present in the injured liver on day 28 after transplantation. By contrast, the transplanted hAECs were not found in livers that had not undergone resection. This finding is consistent with the results of Yovchev *et al.* [21], supporting the hypothesis that injury of the liver is one of the prerequisite conditions that may induce necessary factors for hAEC survival, migration into the liver, and the exhibition of hepatocyte-like features. However, whether the transplanted hAECs will differentiate into mature hepatocytes remains unclear. In further studies, we will track hAECs with GFP-labeling following transplantation and investigate their biological features such as proliferation, differentiation, and safety in the host.

In this study, we could not monitor these cells for more than 4 weeks after transplantation. Some of the animals died after transplantation, but this appears to have been caused by bleeding and other surgery-related complications, rather than by inoculation with hAECs, since no tumor tissues or other hAEC-derived pathological changes were found in the deceased or surviving mice. It is suggested that AAV-GFP infection did not increase the risk of tumors.

In summary, hAECs can be successfully isolated, cultured and passed using the modified protocol in this study. The hAECs infected with the AAV-GFP virus stably express GFP without any change in morphology, survival and proliferation. GFP^+ hAECs survived and migrated to the injured liver following injection into the spleen, suggesting that this labeling system is a reliable and safe tool to monitor and study hAECs *in vivo*.

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