



Baculovirus-transduced, VEGF-expressing adipose-derived stem cell sheet for the treatment of myocardium infarction



Tsung-Szu Yeh^{a,b,1}, Yu-Hua Dean Fang^{c,d,1}, Chia-Hsin Lu^a, Shao-Chieh Chiu^c,
Chia-Lin Yeh^a, Tzu-Chen Yen^{c,e}, Yelena Parfyonova^{f,g}, Yu-Chen Hu^{a,*}

^a Department of Chemical Engineering, National Tsing Hua University, Hsinchu 300, Taiwan

^b Division of Cardiovascular Surgery, Department of Surgery, Taipei Medical University Hospital, Taipei 110, Taiwan

^c Molecular Imaging Center, Chang Gung Memorial Hospital, Linkou 333, Taiwan

^d Department of Electrical Engineering, Chang Gung University, Taoyuan 333, Taiwan

^e Department of Nuclear Medicine, Chang Gung Memorial Hospital, Linkou 333, Taiwan

^f Laboratory of Angiogenesis, Russian Cardiology Research and Production Complex, Moscow 121552, Russia

^g Department of Biochemistry and Molecular Medicine, School of Medicine, Lomonosov's Moscow State University, Moscow 121552, Russia

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ABSTRACT

Cell sheet technology has been widely employed for the treatment of myocardial infarction (MI), but cell sheet fabrication generally requires the use of thermo-responsive dishes. Here we developed a method for the preparation of adipose-derived stem cell (ASC) sheet that obviated the need of thermo-responsive dishes. This method only required the seeding of rabbit ASC onto 6-well plates at an appropriate cell density and culture in appropriate medium, and the cells were able to develop into ASC sheet in 2 days. The ASC sheet allowed for transduction with the hybrid baculovirus at efficiencies >97%, conferring robust and prolonged (>35 days) overexpression of vascular endothelial growth factor (VEGF). The ASC sheet was easily detached by brief (10 s) trypsinization and saline wash, while retaining the extracellular matrix and desired physical properties. The ASC sheet formation and VEGF expression promoted cell survival under hypoxia *in vitro*. Epicardial implantation of the VEGF-expressing ASC sheet to rabbit MI models reduced the infarct size and improved cardiac functions to non-diseased levels, as judged from the left ventricular ejection fraction/myocardial perfusion. The VEGF-expressing ASC sheet also effectively prevented myocardial wall thinning, suppressed myocardium fibrosis and enhanced blood vessel formation. These data implicated the potential of this method for the preparation of genetically engineered ASC sheet and future MI treatment.

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1. Introduction

Since adult hearts have a limited capacity for regeneration, myocardial infarction (MI) results in massive cardiomyocyte death and formation of a fibrous non-contractile scar that often leads to ultimate heart failure, and remains a major cause of morbidity and mortality worldwide [1]. Injection/infusion of suspended stem cells such as mesenchymal stem cells (MSC) for cardiac repair following MI is appealing, but hypoxia-induced apoptosis and wash-out result in poor cell survival/engraftment, thus compromising the therapeutic effects [2]. This problem can be alleviated by

transplantation of cell sheets, whose extracellular matrix (ECM) and cell-to-cell junctions significantly improve the cell survival and engraftment [2–4]. Regardless of dissociated cells or cell sheet, cytokines and growth factors including vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are secreted to enhance angiogenesis, suppress apoptosis and ameliorate cardiac functions, thus paracrine stimulation of the injured myocardium is considered the major mechanism behind the therapeutic action (for review, see Refs. [2,5]).

To date, cell sheets composed of different cell types, such as skeletal myoblasts [2], adipocytes [1], cardiomyocytes [6] and muscle-derived stem cells [7], have been employed for MI treatment. In addition to these cells, adipose-derived stem cells (ASC) can be readily isolated from liposuction in large quantities and are easy to expand and capable of differentiating into cardiomyocyte, rendering ASC a potential cell type for MI therapy, either in the

* Corresponding author. Tel.: +886 3 571 8245; fax: +886 3 571 5408.

E-mail addresses: ychu@mx.nthu.edu.tw, yuchen@che.nthu.edu.tw (Y.-C. Hu).

¹ These two authors contributed equally to this work.

form of dissociated cells [8–10] or cell sheet [4,11]. Similar to MSC, ASC can secrete various growth factors such as VEGF, which facilitate the repair of damaged cardiac muscle by inducing angiogenesis and protecting cells from death [12]. However, the VEGF level secreted by ASC sheet is insufficient to provide complete restoration of cardiac functions after MI.

Aside from cell therapy, gene therapy holds promise for MI treatment. Injection of adeno-associated virus (AAV) 9 expressing microRNA into the peri-infarct area stimulates cardiomyocyte proliferation and improves cardiac functions in mice [13]. Intramyocardial injection of retrovirus encoding transcription factors can reprogram non-myocytes into cardiac-like cells and reduce adverse ventricular remodeling following MI [14,15]. However, direct injection of these viral vectors may raise safety concerns.

In addition, baculovirus (BV) is an emerging gene delivery vector [16] and can transduce ASC and MSC at efficiencies exceeding 95% [17,18], making it a promising vector for cartilage and bone regeneration [19]. However, BV only mediates transient transgene expression in mammalian cells (<7 days) due to its non-replication nature. To prolong the expression, we developed a hybrid BV system in which one BV expresses a codon-optimized FLP recombinase (FLPo) while the other substrate BV harbors the transgene cassette flanked by two Frt sequences [20]. After co-transduction of rabbit ASC or MSC with the two BV, the expressed FLPo cleaves the Frt-flanking cassette off the BV genome and catalyzes the recombination and formation of episomal DNA minicircles encompassing the transgene cassette. The co-transduction gives rise to FLPo-mediated recombination in 90–95% of ASC and the resultant minicircles effectively prolong the transgene expression to >30 days [20].

Although epicardial implantation of ASC sheet can improve cell viability and ameliorate cardiac performance after MI induction, cell sheets are still subject to hostile apoptotic stress due to the stringent hypoxic environment and limited vascularization [21]. Moreover, cell sheets are generally fabricated by culturing cells on dish surfaces grafted with the temperature-responsive polymer such as poly(*N*-isopropylacrylamide) so that cell sheet detachment can be controlled by simple temperature change [5,22]. However, the thermo-responsive dishes are expensive. For these problems, we hypothesized that genetic modification of the ASC sheet for VEGF expression may improve angiogenesis and augment cardiac functions after acute MI. We also aimed to develop a protocol for simple and cost-effective ASC sheet preparation to obviate the need of thermo-responsive dishes and allow for BV transduction, hoping that the genetically modified ASC sheet can overexpress VEGF to protect ASC and stimulate angiogenesis for myocardial repair. We prepared the ASC sheet using normal culture dish and transduced the ASC sheet with Bac-FLPo and a new hybrid BV that expressed VEGF (Bac-FCVW). Whether the BV-engineered ASC sheet conferred cardioprotective effects and improved cardiac functions was evaluated in an acute MI model in New Zealand white (NZW) rabbits.

2. Materials and methods

2.1. Recombinant BV preparation

All recombinant BV vectors were constructed previously (Fig. S1). Bac-CE expressed the enhanced green fluorescent protein (EGFP) [23] while Bac-FLPo expressed the FLPo recombinase [20]. Substrate BV vector Bac-FCVW carried the Frt-flanking human *vegfr165* gene and was constructed earlier [24]. Bac-FdEC was constructed to harbor the Frt-flanking destabilized *egfp* gene [24]. All BV stocks were produced by infecting insect cell Sf-9 and titered by end-point dilution method as described [23].

2.2. ASC isolation, culture, sheet preparation and transduction

All animal experiments were approved by the National Tsing Hua University Institutional Animal Care and Use Committee. ASC were harvested subcutaneously

from the inguinal fat pads surrounding epididymis of male NZW rabbits (3–4 month old) and subcultured to passage 3 as described [24].

Unless otherwise noted, ASC were subsequently cultured in α -MEM medium (Gibco) containing 20% fetal bovine serum (FBS, Hyclone). For ASC sheet fabrication, cells were seeded to polystyrene 6-well plates (Corning Costar) at 5×10^5 cells/well (confluency >95%). ASC attached to the wells in 3 h and were cultured at 37 °C under normoxic conditions. Two days later, ASC sheet was visible and BV transduction was performed as described previously [25] with minor modifications. Briefly, ASC sheet was gently washed with phosphate-buffered saline (PBS), and a certain amount of virus was diluted with TNM-FH medium (the medium for insect cell culture and BV vector production, Sigma), depending on the multiplicity of infection (MOI) to be used. The virus solution was mixed with NaHCO₃-free α -MEM at a volumetric ratio of 1:4 (total volume = 500 μ l per well) and added to the wells. For mock-transduction control, fresh TNM-FH medium was mixed with NaHCO₃-free α -MEM at a volumetric ratio of 1:4 and added to the cells. Cells were gently shaken on a rocking plate at room temperature for 6 h, and the virus mixture was removed, followed by culturing the ASC sheet at 37 °C in α -MEM medium containing 3 mM sodium butyrate. After 15 h incubation, the medium was replaced by fresh α -MEM medium containing 20% FBS. ASC sheet was then cultured for 35 days with medium exchange in half every 2–3 days.

Alternatively, the transduced ASC sheet was washed with PBS twice and statically incubated with 0.05% trypsin-EDTA (0.5 ml/well, Gibco) for 10 s at 1 day post-transduction (dpt, i.e. 3 days post-seeding). After trypsin removal, ASC sheet was washed twice and incubated in PBS. By gentle shaking, the transparent ASC sheet spontaneously detached from the well. The detached ASC sheet was handled with a forcep for epicardial transplantation (see below), or transferred to a new 6-well plate in the normal incubator (21% O₂) or hypoxic chamber (5% O₂, Billups-Rothenberg Inc.), and cultured for another 3 days. To dissociate the cells as controls, ASC sheet was trypsinized for 2 min with mild shaking. Following the medium addition to inactivate trypsin activities, the cells were seeded to a new 6-well plate and cultured under normoxia or hypoxia as described above.

2.3. Transgene expression and recombination efficiency measurement

To measure the transduction efficiency, ASC sheet was transduced with Bac-CE at 2 days post-seeding. For recombination efficiency determination, ASC sheet was co-transduced with Bac-FLPo (MOI 15) and Bac-FdEC (MOI 150) at 2 days post-seeding. The transduced ASC sheets were dissociated at 1 dpt (3 days post-seeding) and the cells were analyzed by flow cytometry for the percentage of EGFP-positive cells. VEGF expression was measured by ELISA analysis of the culture medium harvested at different time points using the kit (R&D Systems).

2.4. Characterization of ASC sheets

The morphology of cells in the ASC sheet was observed under a microscope. ASC sheet was detached at 3 days post-seeding as described above and transferred to another 6-well plate. As a control, ASC sheet was dissociated and the cells were seeded to another 6-well plate. After attachment and 3 h culture, the ASC sheet and dissociated cells were fixed with 10% formaldehyde, washed with PBS 3 times and immunostained using primary antibodies specific for fibronectin (1:100 dilution, Millipore) or laminin (1:100 dilution, AbD Serotec), and FITC-labeled secondary antibody (1:200 dilution, Abcam). The ASC sheet/dissociated cells were counterstained with DAPI (Vector Laboratories) and observed under a confocal microscope.

2.5. VEGF expression and cell health under hypoxia

The ASC sheet and dissociated cells harvested at 3 days post-seeding were transferred to separate 6-well plates placed in a 37 °C incubator (21% O₂) or a hypoxic chamber (5% O₂) and cultured for 3 days. The medium was withdrawn for VEGF-specific ELISA (R&D Systems). The cell metabolic activity was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [26]. The cell death was also observed by a microscope or assessed using the Live/Dead Assay kit (Invitrogen).

2.6. Animal surgery and MI induction

Twenty NZW rabbits (male, 2.5–3.0 kg, 12–14 week-old) were randomly grouped, anesthetized by intramuscular injection of ZoetisTM (10 mg/kg body weight, Virbac) and Rompun[®] (0.2 mg/kg body weight, Bayer), intubated and ventilated and subjected to thoracotomy. The surgical site was expanded with a retractor to expose the heart. Left anterior descending (LAD) coronary arteries were permanently ligated with the 7-0 PROLENETM suture (Ethicon). Following ligation, one Bac-FLPo/Bac-FCVW-transduced ASC sheet was attached to the ligation site, with 2 more transduced ASC sheets attached to the epicardial surface next to the ligation sites (Bac-FLPo/Bac-FCVW group, *n* = 5). As controls, the rabbits either received mock-transduced ASC sheets (Mock group, *n* = 5) or received no ASC sheets (Untreated group, *n* = 5). The chest was closed with a 3-0 Vicryl and 4-0 Nylon suture and $\approx$ 4 ml saline was injected subcutaneously for fluid supplement. Gentamycin (1 ml/

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