Biomaterials 33 (2012) 7261–7271

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Safeguarding pluripotent stem cells for cell therapy with a non-viral, non-integrating episomal suicide construct

Qingguo Zhao, Baisong Lu*, Sunil K. George, James J. Yoo, Anthony Atala**

Wake Forest Institute for Regenerative Medicine, Wake Forest University Health Sciences, Winston-Salem, NC 27157, USA

ARTICLE INFO

Article history:
Received 30 April 2012
Accepted 22 June 2012
Available online 12 July 2012

Keywords:
Cell culture
DNA
Genetic engineering
Stem cell
Transplantation

ABSTRACT

Pluripotent stem cells provide an unlimited cell source for cell therapy. However, residual pluripotent stem cells after differentiation can form tumors. Modifying stem cells with suicide constructs through integrating plasmid DNA and viral vectors has been attempted to specifically eliminate residual pluripotent stem cells after differentiation. However, integration of foreign DNA has the potential of insertional mutagenesis, position effects and silencing. Scaffold/matrix attachment region (S/MAR)-based plasmid DNA can be maintained extra-chromosomally, offering a safer alternative to integrating vectors for this purpose. Here, we report the design of an S/MAR-based suicide construct capable of episomal maintenance and specifically killing pluripotent stem cells but not differentiated cells in the presence of ganciclovir. Treating cells differentiated from episomal suicide construct-modified stem cells with ganciclovir reduces the tumor formation risk after cell transplantation. Tumors formed by such modified pluripotent stem cells could be inhibited by ganciclovir administration. This episomal suicide construct enables negative selection of residual pluripotent stem cells *in vitro* and control of tumors formed from residual pluripotent stem cells *in vivo*.

1. Introduction

Pluripotent human embryonic stem cells and induced pluripotent stem cells provide limitless cell sources for tissue regeneration [1–4]. However, both confer a risk of forming tumors, which must be addressed before their clinical applications are feasible. While in most cases embryonic stem cells and induced pluripotent stem cells will be differentiated into target cells before they are transplanted to patients, the risk of tumor formation is still significant, since residual pluripotent stem cells will remain even after selection based on cell surface markers. Several groups observed teratoma formation after transplantation of differentiated cells from pluripotent stem cells, due to the presence of a low percentage of undifferentiated pluripotent stem cells [5–7]. Even though 100% of the stem cells are differentiated, *in vivo* dedifferentiation into pluripotent stem cells is still possible [8]. Thus, eliminating residual pluripotent stem cells from differentiated cells *in vitro* and *in vivo* will reduce the risk of tumor formation and is highly desirable [9].

Genetic modification of pluripotent stem cells by suicide genes has been explored to reduce the risk of tumorigenicity. Teratomas formed by embryonic stem cells constitutively expressing the herpes simplex virus thymidine kinase (*tk*) gene were inhibited by ganciclovir delivery [10–12]. The pluripotent stem cell-specific OCT4 or NANOG promoter was subsequently used to selectively remove undifferentiated cells from differentiated cells [13–15]. Other toxic proteins, such as α-1,3-galactosyltransferase and inducible caspase-1, have also been tested to eliminate malignant cells [16,17]. In all these studies, transgenes were integrated into the genome by plasmid or lentiviral integration. This again raises safety concerns due to the possibility of inactivation of tumor suppressor genes or activation of oncogenes [18]. In addition, integrated genes are subject to position effects and silencing [19], making their expression unreliable and unpredictable. Efforts are being made to identify and validate genomic safe harbours for transgene integration to minimize the risks described above [20]. Here we explore an alternative strategy for delivering the transgene by using a non-viral, non-integrating episomal construct.

* Corresponding author. Tel.: +1 3367131328; fax: +1 3367137290.
** Corresponding author. Tel.: +1 3367165701; fax: +1 3367137290.
E-mail addresses: blu@wakehealth.edu (B. Lu), aatala@wakehealth.edu (A. Atala).

0142-9612/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2012.06.038

Plasmid vectors containing the scaffold/matrix attached regions (S/MAR) of the human interferon-β gene can maintain their state as episomal DNA in cells of various species, and the plasmid DNA replicates during cell division if the S/MAR sequence is transcribed [21–24]. The vector replicates once per cell cycle during early S-phase, with the origin recognition complex assembled at various regions on the vector DNA [25]. The origin recognition complex stably interacts with metaphase chromosomes, which leads to stable episomal maintenance [26].

Since S/MAR-based vectors do not integrate into the genome of mammalian cells and mediate long-term gene transfer, they should carry reduced risks of insertional mutagenesis, silencing and variegation. However, in currently available S/MAR-based vectors, the S/MAR sequence is transcribed by the same promoter driving the expression of target genes, so that they can only maintain an episomal state in cells expressing the target gene. Here, we describe the design of a new S/MAR-based episomal plasmid able to maintain the episomal state independent of the expression of the target gene. We further made an episomal suicide construct where the thymidine kinase (*tk*) gene is only expressed in pluripotent stem cells. We explored the use of this construct for selective elimination of residual pluripotent stem cells from differentiated target cells to safeguard pluripotent stem cells in cell therapy.

2. Materials and methods

2.1. Plasmids

pEPI-eGFP was a kind gift from Dr. Hans-Joachim Lipps (Universität Witten/Herdecke). pS/MAR-GFP1 and pS/MAR-GFP2 were constructed based on the backbone of pEGFP-C2 (BD Biosciences, Franklin Lakes, NJ). pEGFP-C2 was cut with *Ase*I and *Bgl*II to delete the CMV promoter and EGFP coding sequences to obtain plasmid pEGFP-C2Δ. Then the *Sex*AI–*Stu*I fragment of pEGFP-C2Δ (containing the SV40 origin of replication and SV40 early promoter sequences) was replaced by a *Sex*AI–*Sma*I fragment of the human EF1α promoter sequence amplified from pWPXL (Addgene, Cambridge, MA) with the primers EF1-F (GAG ACC AGG TCG TGA GGC TCC GGT GCC CGT CAG TGG) and EF1-R (ATA CCC GGG CAC GAC ACC TGA AAT GGA AGA AA), resulting in the plasmid pEGFP-C2Δ/EF1. The 2 kb S/MAR sequence [27] (available at http://smartdb.bioinf.med.uni-goettingen.de/cgi-bin/SMARtDB/getSMAR.cgi?SM00000002) was amplified from pEPI-eGFP by the primers S/MAR-F (actccgggagATCTAAATAAACTTATAAATTGTGAG) and S/MAR-R (actccgggaGAATTCTATCAAATATTTAAAGAAAAAAAAAATTG) and inserted into the *Pfo*I site of pEGFP-C2Δ/EF1 (downstream of the neomycin-resistance gene and upstream of polyadenylation signals), making the plasmid pS/MAR. The *Apa*LI–*Msc*I fragment from pS/MAR was replaced by the *Apa*LI–*Msc*I fragment of pEGFP-C2 (consisting of a CMV promoter and EGFP cDNA) to make plasmid pS/MAR-GFP1. Finally, a *Sal*I–*Sma*I fragment from pWPXL containing the EF1α promoter and EGFP coding sequences was inserted between the *Sal*I/*Sma*I sites of pS/MAR to obtain pS/MAR-GFP2. pSuicide was constructed by one-step ligation of the following three fragments: the *Kpn*I-digested pS/MAR, the *Kpn*I/*Bgl*II-digested human OCT4 promoter sequences from pOCT4-luc [3], and the *Kpn*I/*Bgl*II-digested herpes simplex virus thymidine kinase (tk) cDNA amplified from pLG1 (a kind gift from Dr. Gan Lin at University of Rochester) by the primers TK-F1 (ACAAGATCTACCATGGCTTCGTACCCCTGCCATC) and TK-R1 (ACGGTACCTCAGTTAGCCTCCCCCATCTCC).

2.2. Cell culture and transfection

CHO-K1 cells were cultivated in F-12 Nutrient Mixture medium containing 10% FBS. HeLa and NCCIT cells were cultivated in DMEM supplemented with 10% FBS and 1% glutamine. For cell transfection, 5×10^4 cells were seeded into one well of a 12-well plate, and overnight transfections were performed using 0.5 μg vector DNA and 2 μl Fugene 6 (Roche Applied Science, Germany) transfection reagent per well, following the manufacturer's instructions. To obtain clones, transfected cells were selected with medium containing 500 μg/ml G418 (Sigma–Aldrich, St. Louis, MO) for 2–3 weeks before colonies were picked. Individual clones were checked for episomal DNA by Southern blotting, as described below.

2.3. Flow cytometry analysis of GFP expression

Cells were trypsinized, fixed in 2% paraformaldehyde for 10 min at room temperature and washed with PBS buffer. GFP expression was analyzed using a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA) with CellQuest software.

2.4. Detection of episomal DNA in cells by Southern blotting

Extra-chromosomal DNA was isolated from stable transfected CHO and NCCIT cells using a modified HIRT procedure [28]. Briefly, after removing the medium, cells on a 100-mm dish were directly washed in D-PBS, then lysed by adding 1 ml of Hirt buffer (10 mmol/L EDTA, pH 7.5 and 0.6% SDS). After incubating at room temperature for 20 min, the viscous lysate was transferred into an Eppendorf tube. A 5 mol/L of NaCl was used to create a final concentration of 1 mol/L NaCl in the lysed cells, and the sample was mixed gently. The lysate was incubated at 4 °C for more than 8 h, and centrifuged at 17,000 *g* for 30 min at 4 °C. The supernatant was extracted with 25:24:1 phenol:chloroform:isoamyl alcohol. DNA was precipitated by adding an equal volume of isopropanol and washed with 70% ethanol. The pellet of DNA was dissolved in TE buffer by incubating for 1 h at 65 °C.

Southern blotting was carried out with the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) according to the manufacturer's recommendations. pEGFP-C2, from which pSMAR-GFP2 and pSuicide were derived, was used as the template for probe preparation. One μg plasmid DNA was labeled with Digoxigenin-11-dUTP using DIG-High prime by incubating for up to 20 h at 37 °C. The yield of DIG-labeled DNA was determined by comparing a series of dilutions of DIG-labeled DNA to the DIG-labeled control DNA.

Extra-chromosomal DNA isolated from two 100-mm dish cells was digested with *Bam*HI (for pSMAR-GFP2) or *Eco*RV (for pSuicide) before being loaded on a 0.8% agarose gel. DNA gel electrophoresis, transfer, and fixation were performed according to standard Southern blotting protocols. Hybridization was carried out at 50 °C overnight in 5 ml hybridization solution containing 25 ng/ml DIG-labeled probe DNA. After post-hybridization washes, anti-Digoxigenin-AP immunological detection was performed and the hybridization signal was acquired by exposure to an LAS-3000 imager (Fujifilm Co., Japan) for 2–5 min at room temperature.

2.5. Cell survival under ganciclovir treatment

NCCIT and HeLa cells with and without pSuicide episomal DNA were treated with and without 2 μmol/L ganciclovir. Cell numbers and morphology were compared 7 days after treatment.

2.6. NCCIT cell differentiation and elimination of remaining OCT4-expressing NCCIT cells

NCCIT cells were directly cultivated in standard DMEM medium containing 20 μmol/L retinoic acid to induce differentiation. NCCIT(pSuicide) cells were similarly cultured but included 400 μg/ml G418 in the differentiation medium. The differentiation medium was changed every other day. About 10 days after differentiation, the cells were split, counted, and divided into two aliquots. One aliquot of cells was maintained in differentiation medium containing 2 μmol/L ganciclovir for 7 days (for *in vitro* immuno-analysis) or 14 days (for *in vivo* tumor formation). As a negative control, counterpart cells were grown in differentiation medium containing 0.1% DMSO in which ganciclovir is dissolved.

2.7. Western blotting assay of OCT4 and thymidine kinase expression

Cells were lysed in RIPA buffer with protease inhibitors (0.5 mmol/L PMSF and 1× Complete Protease Inhibitor Cocktail from Roche) for SDS-PAGE and Western blotting analyses. Monoclonal anti-β-actin antibody was from Sigma and used at 1:5000 (St Louis, MO). Rabbit anti-HSV thymidine kinase antiserum was obtained from Dr. William Summers of Yale University (1:5000); anti-OCT4A antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:1000. Horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000) were purchased from Pierce (Rockford, IL). Chemiluminescent reagents from Pierce were used to visualize the protein signals on an LAS-3000 imaging system (Fujifilm). The Integrated Density function (Image J software) was used to quantify the expression of individual proteins after normalized by β-actin.

2.8. Immunofluorescent analysis of differentiated NCCIT cells

OCT4 immunofluorescent staining was performed as described previously [29]. Briefly, differentiated NCCIT cells growing on chamber slides were fixed in 4% PFA for 15 min at room temperature and permeabilized in 0.2% Triton X-100/PBS for 10 min. After blocking with 10% horse serum, cells were incubated with 3% horse serum diluted (1:50) OCT4 antibody (Santa Cruz Biotechnology) for 1 h at room temperature, followed by three washes in PBS-T (0.1% Tween 20 in phosphate-buffered saline). Cells were incubated with 1:200 diluted Texas Red-conjugated anti-mouse secondary antibody for 1 h at room temperature, and then washed again 3 times in PBS-T. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma–Aldrich). Images were captured under a fluorescent microscope.