Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Crispr/Cas and recombinase-based human-to-pig orthotopic gene exchange for xenotransplantation

Rafael Miyashiro Nunes dos Santos, MD,[a,b] *Luiz Augusto Carneiro D'Albuquerque, MD, PhD,*[b] *Luz M. Reyes, PhD,*[c] *Jose L. Estrada, DVM, PhD,*[c] *Zheng-Yu Wang, PhD,*[c] *Matthew Tector, PhD,*[c] *and A. Joseph Tector, MD, PhD*[c,*]

[a] *Department of Surgery, Indiana University School of Medicine, Indianapolis, Indiana*
[b] *Digestive Organs Transplant Division, Gastroenterology Department, Sao Paulo University School of Medicine, São Paulo, Brazil*
[c] *Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama*

ARTICLE INFO

Article history:
Received 3 October 2017
Received in revised form
13 February 2018
Accepted 20 March 2018
Available online xxx

Keywords:
CRISPR
Cas9
Xenotransplantation
Thrombomodulin
Gene replacement

ABSTRACT

Background: Tools for genome editing in pigs are improving rapidly so that making precise cuts in DNA for the purposes of deleting genes is straightforward. Development of means to replace pig genes with human genes with precision is very desirable for the future development of donor pigs for xenotransplantation.
Materials and methods: We used Cas9 to cut pig thrombomodulin (pTHBD) and replace it with a plasmid containing a promoterless antibiotic selection marker and the exon for human thrombomodulin. PhiC31 recombinase was used to remove the antibiotic selection marker to create porcine aortic endothelial cells expressing human instead of pTHBD, driven by the endogenous pig promoter.
Results: The promoterless selection cassette permitted efficient enrichment of cells containing correctly inserted transgene. Recombinase treatment of selected cells excised the resistance marker permitting expression of the human transgene by the endogenous pTHBD promoter. Gene regulation was maintained after gene replacement because pig endogenous promoter was kept intact in the correct position.
Conclusions: Cas9 and recombinase technology make orthotopic human for pig gene exchange feasible and pave the way for creation of pigs with human genes that can be expressed in the appropriate tissues preserving gene regulation.

Introduction

Genome editing and somatic cell nuclear transfer (SCNT) open the possibility to create pigs whose tissues could be used for clinical xenotransplantation to end the shortage of donor organs.[1-5] Genome editing in pigs must be able to remove antigens to eliminate xenoreactive antibodies as a barrier to clinical application and insert human genes that may confer a

* *Corresponding author*. Department of Surgery, University of Alabama at Birmingham, LHRB 728, 1720 2nd Avenue South, Birmingham, AL 35294. Tel.: +1 205 934 5747; fax: +1 205 934 0135.
E-mail address: jtector@uab.edu (A.J. Tector).
0022-4804/$ – see front matter © 2018 Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.jss.2018.03.051

survival advantage for a pig organ transplanted into a human (e.g., immunological and or coagulation regulatory genes).[6]

Current nuclease-based genome editing technology using zinc-finger nucleases, transcription activator-like effector nucleases, or Cas9 has made it possible to delete genes in pigs with great precision.[2-5,7-10] Using Cas9, pigs with three xenoantigen deletions, GGTA1, CMAH, and B4GALNT2 were created. Crossmatches performed with peripheral blood mononuclear cells from these pigs with human serum revealed that many of the waitlisted patients have a negative crossmatch, eliminating xenoreactive antibodies as an immediate barrier to the clinical application of xenotransplantation.[11] Many highly sensitized patients still have a positive crossmatch to the triple knock out (KO) pigs, and data using crossmatches with cells from class I Swine Leukocyte Antigen KO pigs show that some HLA antibodies cross react with Swine Leukocyte Antigen, making these antigens a target for genome editing. Now that the humoral barrier to xenotransplantation has been lifted for many patients, the ability to add genes of interest will take on increased importance for xenotransplantation.

The introduction of human transgenes in donor pigs first started in 1994 with the creation of the human decay accelerating factor transgenic pig. This pig was created using random integration with a selection cassette that included a viral promoter, an antibiotic selection marker, and the CD55 transgene.[12] In an ideal situation, genome editing with gene insertion would include the ability to insert the gene in a precise location where it will be expressed[1]: in appropriate tissues and cells,[2] at appropriate levels,[3] with appropriate regulation, and[4] with minimal excess DNA added (no selection markers).[10] Our goal of inserting transgenes into an endogenous promoter is to create stable expression that is regulated in response to physiologic needs of the organ. Thus far, using foreign promoters to express exogenous DNA in animals has been challenged by inconsistent transgene expression with little control over transcriptional activity.

The experiments in the present work describe an efficient two-step strategy to generate cell lines with pig-to-human gene substitution in 14 d. The first step uses the CRISPR/Cas9 system to accomplish the targeted integration of a promoterless transgene cassette behind an endogenous pig promoter. The cassettes are engineered to express an antibiotic resistance gene that is active when properly oriented behind a pig promoter enabling rapid selection transgene-positive cells. Recombinase sites flanking the antibiotic resistance gene enable its removal leaving only the transgene under control of the endogenous pig promoter.

Materials and methods

Aortic cell isolation and culture

All animals used in this study were approved by the Institutional biosafety and Institutional Animal Care and Use Committee of Indiana University School of Medicine. Porcine primary aortic endothelial cells (pAECs) were isolated from pigs procured during general anesthesia. pAECs were used because they express pig thrombomodulin (pTHBD) and can undergo many doublings in cell culture without evidence of senescence or differentiation. This expression made it possible to study gene activity *in vitro* after the gene swaps (described below) had been performed. The posterior lumbar arteries were ligated and the aortic lumen was filled with 0.025% *Clostridium histolyticum*'s type IV collagenase (Sigma, St. Louis, MO), placing vascular clamps on proximal and distal ends. This sample was incubated at 37°C for 45 min. Enzyme activity was quenched by addition of 1/10 volume FBS. After enzyme inactivation, the sample was centrifuged at 400 g for 5 min. The cell pellet was resuspended in Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum, 100 μg/mL Corning endothelial cell growth supplement (Fisher Health Care), 1% penicillin + streptomycin (Invitrogen), and 1% amphotericin (Fisher Scientific) and cultured in the same media. Primary human aortic endothelial cells (hAECs) were purchased from American Type Culture Collection.

Transfection optimization

The Neon transfection system (Life Technologies, Grand Island, NY) was used. Two hundred fifty thousand cells were transfected with 400 ng of pEGFP-N1 plasmid (Clontech) and cultured for 24 h. After this period, cells were harvested and transfection efficiency was measured based on fluorescence using a BD Accuri C6 flow machine (BD Biosciences, San Jose, CA) and visual mortality rate based on cells attached/cells floating. The transfection optimization parameters on Neon transfection system was used (Supplemental Table 1). The best parameters of transfection were matched with visual mortality rate, and if small mortality rate was noted for that parameter, voltage was increased until most cells were killed (Supplemental Table 2). Fluorescence was measured 24 h after transfection.

Gene swap

For generation of CRISPR/Cas9, P × 330 plasmid[13] from Addgene (plasmid #42230, A gift from by Feng Zhang) was used. Two plasmids were created to express genomic RNA (gRNA) that flank the coding sequences of pTHBD (NCBI accession NM_001130732.1), 5′—AGGAGCAGAACGCGGAGCA—3′, and 5′—AACCTTCTAACCTAACCGGT—3′. These plasmids were simultaneously introduced into pAEC with a promoterless selection/transgene cassette to achieve gene swapping. In the first step of the procedure, 1×10^6 pAECs were transfected with 2.5 μg of each CRISPR plasmid in association with 2 μg of linearized insert containing the promoterless selection marker surrounded by recombinase sequences followed by a promoterless human thrombomodulin (hTHBD) gene (Fig. 1A). After initial transfection, cells were cultured for 2 d in media without antibiotic, and after that period, they were submitted to hygromycin antibiotic selection for 8 d. Removal of the selection marker was achieved by transfection of 1×10^6 antibiotic-selected cells with a codon optimized PhiC31 recombinase containing a nuclear localization signal (4 μg) driven by the cytomegalovirus promoter modified from Addgene plasmid pPGKphiC31obpA. (pPGKPhiC31obpA was a