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Efficient generation of targeted and controlled mutational events in porcine cells using nuclease-directed homologous recombination

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ABSTRACT

Background: Nuclease-based genome editing has rapidly sped the creation of new models of human disease. These techniques also hold great promise for the future of clinical xenotransplantation and cell-based therapies for cancer or immunodeficient pathology. However, to fully realize the potential of nuclease editing tools, the efficiency and precision of their application must be optimized. The object of this study was to use nonintegrating selection and nuclease-directed homologous recombination to efficiently control the genetic modification of the porcine genome.
Methods: Clustered randomly integrating spaced palindromic repeats and associated Cas9 protein (CRISPR/Cas9)-directed mutagenesis with a single-guide RNA target was designed to target the alpha-1,3-galactosyltransferase locus (GGTA1) of the porcine genome. A vector expressing a single-guide RNA, Cas9 protein, and green fluorescent protein was used to increase plasmid-delivered mutational efficiency when coupled with fluorescence sorting. Single and double-strand DNA oligonucleotides with a restriction site replacing the start codon were created with variable homology lengths surrounding the mutational event site. Finally, a transgene construct was flanked with 50 base pairs of homology directed immediately 5′ to a nuclease cut site. These products were introduced to cells with a constant concentration of CRISPR/cas9 vector. Phenotype-specific mutational efficiency was measured by flow cytometer. Controlled homologous insertion was measured by Sanger sequence, restriction enzyme digest and flow cytometry.
Results: Expression of a fluorescence protein on the Cas9 vector functioned as a nonintegrating selection marker. Selection by this marker increased phenotype-silencing mutation rates from 3.5% to 82% ($P = 0.0002$). Insertion or deletion mutation increased from 11% to 96% ($P = 0.0007$). Co-transfection with homologous DNA oligonucleotides

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increased the aggregate phenotype-silencing mutation rates up to 22% and increased biallelic events. Single-strand DNA was twice as efficient as double-strand DNA. Furthermore, nuclease-mediated insertion by homology-directed repair successfully drove locus-specific transgene expression in the porcine genome.
Conclusions: A nonintegrating selection strategy based on fluorescence expression can increase the mutational efficiency of the CRISPR/Cas9 system. The precision of this system can be increased by the addition of a very short homologous template sequence and can serve as a method for locus-specific transgene delivery. Together these strategies may be used to efficiently control mutational events. This system may be used to better use the potential of nuclease-mediated genomic editing.

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Introduction

The clustered randomly integrating spaced palindromic repeats and associated Cas9 protein (CRISPR/Cas9) platform has revolutionized the process of creating targeted genetic modification. Capitalizing on the error-prone nature of DNA repair, CRISPR-targeted DNA damage delivers phenotype-altering insertions and deletions. When coupled with the success of somatic cell nuclear transfer (SCNT), this has produced the ability to efficiently create model organisms at an unprecedented pace.[1] Improvements in the efficiency and precision of this platform will increase its ability to develop novel clinical applications.

Recent success using to nuclease editing to efficiently new create model swine has relied on a phenotypic selection strategy of genetically modified cells for use in animal cloning that involves SCNT.[2,3] Occasionally, cell types that are useful in animal cloning cannot be selected on the basis of the desired phenotype ultimately requiring genomic analysis to isolate the desired mutants. In this territory, even small improvements in efficiency can reduce the time and cost of identifying appropriately modified cells. To this end, the targeted introduction of restriction enzyme binding sites into the genome offers an important selection tool; restriction digest of clonal cell populations can reliably identify genomic modification.

Herein we describe a process by which nuclease-driven genomic editing in pigs can be enhanced by a nonintegrating selection strategy. We hypothesized that inclusion of a selectable fluorescent marker on the Cas9 delivery plasmid would increase downstream mutation efficiency without integration of the selection marker into the porcine genome. We endeavored to test the ability of this process to facilitate greater editing precision within the porcine genome. To this end, we describe the introduction of short exogenous DNA sequences to offer seamless homology-driven control over the mutational event and aid in genotype selection. Furthermore, we show that the use of a very short 5′ homology sequences can affect locus-specific transgene delivery to the porcine genome. These methods increase the precision of nuclease-based genome editing. Their application will aid broadening applications for nuclease technology by unfettering their use from phenotypic selection.

Methods

Porcine aortic cell isolation and culture

The 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 (AECs) were isolated from wild-type porcine aorta after euthanasia. The posterior lumbar arteries were ligated, and the aortic lumen was filled with 0.025% of collagenase type IV from *Clostridium histolyticum* (Sigma, St. Louis, MO), placing vascular clamps on proximal and distal ends. This sample was incubated at 37°C for 35 min. Enzyme activity in the perfusate was immediately quenched by the addition of 1/10 volume newborn calf serum. The perfusate was centrifuged at 400 g for 10 min at room temperature. The cell pellet was saved and resuspended in Roswell Park Memorial Institute medium supplemented with 0.02% (wt/vol) ethylenediaminetetraacetic acid. The cell suspension was centrifuged again at 400 g for 10 min at 4°C. The pellet was resuspended in EC culture medium (Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum (vol/vol), 100 μg/mL endothelial cell–specific growth factor, penicillin, streptomycin, and amphotericin B). Cells were maintained in culture at 37° in 5% $C0_2$ on attachment factor–coated cell dishes (Thermo Fisher, Waltham, MA). After 7 d, endothelial cells were purified by staining with chicken anti-Pig CD31 (Invitrogen, Grand Island, NY) and positive sorting using an FACSAria II (Becton Dickinson, San Jose, CA).

Creation of an immortalized AEC line

Porcine immortalized cell lines were generated described previously[4]. Briefly, primary AECs from domestic pigs were isolated as described previously. Cells were maintained in culture at 37° in 5% $C0_2$ on attachment factor–coated cell dishes (Thermo). After a 3-d culture, AECs were infected for 24 h with lentiviral supernatant, containing lentiviral vector in which a complementary DNA expresses the large and small T antigen of SV40 (Applied Biological Materials Inc, Richmond, BC, Canada). Single-cell clones were isolated and amplified. To limit the potential of background colony mutation, all experiments were performed on immortalized cells before 15 passages.

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