Minimum length of homology in a donor DNA to facilitate homologous recombination during ZFN mediated gene targeting

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Introduction

Zinc-finger nucleases (ZFNs) can induce targeted mutations by causing double strand breaks at a specific location on a chromosome (Urnov et al., 2005). CMP-Neu5Ac hydroxylase (CMAH) is widely expressed on the endothelial cells of many mammals except humans (both of these genes are pseudo genes in humans). Therefore, this epitope is a potential porcine target for human endogenous antibodies. Previously we produced CMAH knock-out (KO) Minnesota miniature pigs using ZFNs with a donor DNA containing only 800 bp homology on each arm to induce homologous recombination (HR) (Figure 1). The length of homology in the donor DNA was much smaller than conventional targeting vectors. The next question we wanted to address was to identify a minimum length of homology needed to induce HR during ZFN-mediated targeting. Identifying minimum length of homology would help us design donor DNAs for ZFN-mediated KOs and knock-ins.

Materials & Methods

• Production of small donor DNAs: To identify a minimum length of homology needed to induce HR during ZFN-mediated targeting. Donor DNAs with a series of homology lengths were generated by first annealing two oligonucleotides containing a short homology, then PCR to extend the homology (Figure 2). ZFN binding sites were modified and an in-frame stop codon was introduced to the donor DNA; 35 (A), 85 (B), and 125 (C) bp homology on each arm.

• Gene targeting by electroporation: For gene targeting, 1 million cells were transfected with ZFN constructs with each donor DNA; 200ng each. The cells were electroporated with the constructs at 490 V, 1 msec, 3 pulses using a BTX Electro Cell Manipulator (Harvard Apparatus, Holliston, MA). ZFNs alone served as control. The ZFN used in the study is shown in Figure 3.

• Quantitative real-time PCR: To identify the rate of gene targeting induced by HR, DNA was isolated from the transfected cells after three days. Then 50ng of DNA was used for the PCR analysis. Quantitative real-time PCR was then conducted using IQ SYBR Green Supermix (Bio-Rad Laboratories). The PCR was performed on a MyiQ single color real-time thermal cycler (Bio-Rad). The program used for amplification included an initial temperature of 94°C for 2 min followed by 40 cycles of 5 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. Real time fluorescence data was collected during the extension time. Differences in gene targeting were compared by ANOVA and p<0.05 was considered significant. Three biological replicates with two technical replication were applied.

Materials & Methods

Results

• The highest targeting efficiency was observed when the donor DNA with 125 bp homology on each arm was used; 10 times higher compared to the donor with 35 bp homology. No recombination event was detected in the negative control as we expected. When the PCR products were loaded on a gel, we could not detect a visible PCR product from the 35 bp homology group. Also, highest intensity was detected from PCR product from the donor with the longest homology (donor C).

Conclusions

• Our results suggest that a minimum of 85 bp on each side of donor DNA is needed to successfully facilitate HR during ZFN-mediated targeting and longer homology can be more effective. Further studies may suggest more specific requirement of homology length during ZFN-mediated targeting.

• Use of donor DNA can introduce a specific mutation rather than KOs caused by ZFN-mediated NHEJ. This strategy can be used to induce substitution of amino acids.

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References
