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Gene Targeting With Zinc Finger Nucleases to Produce Cloned eGFP Knockout Pigs[†]

JEFFREY J. WHYTE^{1,2,3}, JIANGUO ZHAO^{2,3}, KEVIN D. WELLS^{2,3}, MELISSA S. SAMUEL^{2,3}, KRISTIN M. WHITWORTH³, ERIC M. WALTERS², M. HAROLD LAUGHLIN¹, and RANDALL S. PRATHER^{2,3,*}

¹Department of Biomedical Sciences, University of Missouri, Columbia, Missouri

²National Swine Resource & Research Center (NRSSC), University of Missouri, Columbia, Missouri

³Division of Animal Sciences, University of Missouri, Columbia, Missouri

We report using zinc finger nucleases (ZFNs) and somatic cell nuclear transfer (SCNT) to generate pigs with a knockout (KO) mutation of an enhanced green fluorescent protein (eGFP) transgene. ZFNs are synthetic modular proteins composed of a FokI endonuclease domain linked to a sequence-specific zinc finger DNA-binding domain. Pairs of ZFNs bind to the target region, allowing FokI dimerization and subsequent DNA cleavage. Mutation of an eGFP transgene by using ZFNs has been demonstrated in rats (Geurts et al., 2009); however, there are no reports of ZFN-mutated livestock offspring. Using ZFNs to increase the efficiency of gene modification may advance the production of agriculturally and clinically relevant animal models, particularly in species where modification is difficult. All animal procedures followed an approved IACUC protocol. Adult porcine ear fibroblasts hemizygous for the eGFP transgene (Whitworth et al., 2009), from a grandson of the founder animal, were cultured to 75% confluency, trypsinized, and cotransfected (Ross et al., 2010) with a pair of ZFN plasmids that bind to opposing strands at the eGFP target site (Sigma–Aldrich CompoZr®) and a red fluorescent CAG-tomato plasmid as a transient selectable fluorophore reporting transfection efficiency. Transfected fibroblasts were cultured for 96 h and selected for red fluorescence by automated cell sorting (FACS; Fig. 1A,B) and seeded into 96-well plates (100 cells/well) based on CAG-tomato expression (2% of total cells sorted). A second round of FACS using negative selection of eGFP fluorescence enriched for eGFP KO cells (~5% of sorted cells). PCR of genomic DNA from fibroblasts in wells containing predominantly non-green cells was used to amplify a fragment bracketing the ZFN targeting site. The PCR product was cloned into *E. coli*, and 16 colonies showed mutations by Sanger sequencing. These mutations at the ZFN cleavage site included a 6-bp deletion, a 333-bp deletion, and a 222-bp deletion replaced with a 113-bp inversion of the deleted sequence (Supplemental Information <http://>

[†]Jeffrey J. Whyte and Jianguo Zhao contributed equally to this work.

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*Corresponding author: 920 East Campus Drive, E125 ASRC, Columbia, MO 65211-5300., pratherr@missouri.edu.

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animalsciences.missouri.edu/faculty/prather/). Fibroblast colonies determined to carry ZFN-induced mutations were used as donor cells for SCNT, and embryo transfer (n = 3; Whitworth et al., 2009). One Day-12 embryo collection recovered 7 of 9 embryos that did not fluoresce. Quality sequence was obtained from four embryos, and confirmed that the two embryos that fluoresced had an intact eGFP and two embryos that did not fluoresce were mutated. One of seven piglets recovered via Cesarean section at term for the other two pregnancies fluoresced (Fig. 1C). DNA sequencing confirmed that this piglet had unaltered sequence at the predicted ZFN cut site, while the non-fluorescing piglets had various deletions and insertions. While a direct comparison to a 12% mutation rate in the rat made by microinjection (Geurts et al., 2009) cannot be made because of our FACS preselection and SCNT methods, six out of seven founders being mutated is consistent with their results. This study establishes ZFN-based gene modification in a large animal model. The methods used are anticipated to be useful in selective knockout of endogenous swine genes of agricultural and biomedical importance without introducing any transgenic sequence into the genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A: Control porcine ear fibroblasts; eGFP and Hoechst DNA stain fluorescence. **B:** Porcine ear fibroblasts treated with eGFP-specific ZFNs; eGFP and Hoechst DNA stain fluorescence. GFP fluorescence is absent in a high proportion of the treated fibroblasts after FACS sorting. **C:** Knockout (**left**) and wild type (**right**) eGFP piglets derived from porcine ear fibroblasts treated with eGFP-targeting ZFNs (ambient, **C**, and UV light exposure, **C'**).