FIBROBLAST GROWTH FACTOR-8 CRISPR/CAS9 MEDIATED GENE TARGETING IN SWINE

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Abstract

Abnormal development of the arterial pole of the heart accounts for more than 30% of human congenital heart defects. Additionally, normal remodeling of the outflow tract (OFT) plays a critical role in the maturation of the arterial pole. Models of early cardiac disease in children such as these typically require immediate surgical intervention to resolve abnormalities. Unfortunately, the first opportunity to practice surgical skills and treatments for early cardiac malformations often occurs on newborn children. Thus, there is a clinical and research need for a model of this condition. Fibroblast Growth Factor-8 (FGF8) is expressed in the anterior portion of the second heart field, and is included in a number of cells identified as cardiac progenitor cells. These cells eventually contribute to the arterial pole of the heart. Recently, FGF8 was shown to be required for normal cardiac development. The loss of FGF8 showed phenotypic defects in disrupted right ventricle development as well as disruption of the OFT. Using the CRISPR/Cas9 system in conjunction with a targeting construct, an FGF8 homologous knockout was produced in a swine model. A conditional knock-out was designed using a targeting vector that was homologous to FGF8. An FRT-flanked neomycin cassette was located in the intron between exons 3 and 4. This vector also places loxP sites to flank exons 4 and 5, for conditional Cre-mediated deletion. To facilitate genome cleavage in the absence of vector cleavage, a CRISPR guide RNA was designed to target the sequence that is disrupted by the neomycin cassette in the targeting vector. Using the CRISPR/Cas9 system with the targeting construct, two targeted colonies were produced and used in somatic cell nuclear transfer. The pregnancies resulted in one stillborn piglet that displayed an FGF8 null phenotype. Our preliminary analysis revealed right ventricular hypoplasia and OFT hypoplasia; data consistent with reduced FGF8. In conclusion, targeting in combination with the CRISPR/Cas9 system was accomplished creating a congenital heart defect model.

Objective

The objective of this project was to create a pig heterozygous for an FGF8 insert flanked by two loxP sites (Fig.1, pALFGF8). Simultaneously, a plasmid was created (Fig.2, pMLT5) to disrupt ISL-1 with insertions between exons 1 and 3 of the gene. Included in the ISL-1 plasmid was Cre for a conditional deletion of FGF8 exons 4 and 5. The intended phenotype of this construct would provide normal piglet growth with retarded cardiac development, allowing for a biomedical research model. This multi-step targeting event was shortened through our current production of a homozygous FGF8 null piglet.

Materials and Methods

FGF8 Targeting Construct (pALFGF8)

Left Arm: 12.5 kb FGF8 exon 3 genomic fragment; FRT-RNA Pol II Promoter; NEO-FRT-loxP
Right Arm: 12.5 kb FRT-RNA Pol II Promoter; NEO-FRT-loxP-FGF8 genomic fragment; exons 4, 5, loxP-FGF8 exon 6-amp

Assay

(Fig.3-5) Left arm primers lie in exons 3 and 4 of FGF8, flanking the beginning of the plasmid and following neomycin. Right arm primers lie in exons 3 and 6 of FGF8 flanking the beginning of the neomycin insert and an additional 533 bp of sequence in exon 6.

Fibroblast Cell Culture & Transfections

- Electroporation-Based Method
- Stable Integration
- 1 μg linearized DNA: pALFGF8
- 3 μg CRISPR DNA: CRISPR 1.4
- 400,000 fibroblast cells
- 400V, 3 pulses
- 14 days culture
- 38.5°C
- 5% O2, 5.5% CO2

CRISPRs

Traditional methods of plasmid transfection failed to produce an inserted product of expected size. Therefore the use of the CRISPR/Cas9 system was introduced to induce genome cleavage. The target sequence used for CRISPR oligo design was located in exon 4 flanking the insertion sites of the plasmid. There were four total CRISPR oligos designed, only two of which produced cleavage sites. One CRISPR oligo produced all three targeted colonies, CRISPR 1.4 5gctcgtgcgtgatattgc.

Summary

The addition of the CRISPR/cas9 system produced endogenous genomic cleavage of the target sequence in porcine FGF8. The induced site specific cleavage allowed for insertion of constructed plasmid followed by homologous recombination. This targeted event was unison when transfecting donor DNA alone (Fig.A-A-C). The amount of donor DNA to CRISPR proved to be most beneficial at a ratio of 1:3. To date, 3 targeted colonies have been produced with 4 total pregnancies. One sow has made it to term producing one still born piglet (161-1). This piglet showed expected physical characteristics of an FGF8 null phenotype.

Further Work

Additional pALFGF8 heterozygous colonies were transfected (SCNT) and are currently mid-gestation. Simultaneously, pMLT5 was targeted and transferred into a surrogate sow, which is also mid-gestation. A cross between the piglets produced from each pregnancy could provide piglets with the intended cardiac specific deletion of exons 4 and 5 of FGF8 in swine.

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