THERAPEUTIC GENOME EDITING OF COMPLEX VERTEBRAL MALFORMATION IN CATTLE

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by

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ABSTRACT

Therapeutic Genome Editing of Complex Vertebral Malformation in Cattle

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Autosomal recessive genetic disorders such as Complex Vertebral Malformation (CVM) cause a significant economic burden to dairy producers and impede genetic progress in the dairy industry as a whole. Many of these diseases, including CVM, have homozygous lethal phenotypes, and thus negatively impact the fertility and breeding value of heterozygous carriers. Identification of carriers typically results in culling, and forfeiture of the animal's genetic value irrespective of the lethal recessive. Genome engineering technologies provide an opportunity to rescue the genetic value of carrier animals with economically significant production traits by repairing the disease-causing alleles. This thesis describes the optimization of a workflow for the correction of bovine CVM via SNP modification in primary cells using the CRISPR Cas9 system and ssDNA donor templates. It also attempts to quantify differences in the efficiency of SNP modifications between delivery methods for CRISPR, as well as the location of CRISPR cutting with regard to the mutation, and the length of ssDNA donor homology arms.

DEDICATION

This project is dedicated to my father, who has supported me and enabled me to chase my dreams, and who also provided the inspiration for this project.

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I would like to thank Dr. Charles Long, who has provided valuable guidance for this project, and helped secure funding to make it possible. I would also like to give special thanks to Carlos Pinzon Arteaga, who has been my mentor in genome engineering and life for the past two years. Carlos' teaching and guidance has helped develop me into a better scientist and a better man. Finally, I would like to thank my colleague Diego Fernando Carrillo who has worked alongside me, including many early mornings and late nights, to make this project possible.

NOMENCLATURE

AI	Artificial insemination
Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
CVM	Complex vertebral malformation
DNA	Deoxy-ribonucleic acid
ssDNA	Single-stranded DNA
dsDNA	Double-stranded DNA
DSB	Double stranded break
EBV	Estimated breeding value
EPD	Expected progeny difference
GFP	Green fluorescence protein
HDR	Homology directed repair
HR	Homologous recombination
NHEJ	Non-homologous end joining
ODN	Oligodeoxynucleotide
ssODN	Single-stranded oligodeoxynucleotide
PAM	Protospacer adjacent motif
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic Acid
sgRNA	Single guide RNA
SCNT	Somatic Cell Nuclear Transfer

SLC35A3	Solute carrier family 35, member 3 gene
WT	Wild type

CHAPTER I

INTRODUCTION

Animal Breeding and Genetic Diseases

Like most commercial industries, the global dairy industry has historically focused on maximizing profit. Dairy cows are bred based on traits of economic interest, such as milk production, milk composition, fertility, health, and calving ease [1-3]. The development of improved selection indexes such as estimated breeding value (EBV) and expected progeny difference (EPD) [3] as well as the popularization of artificial insemination (AI) technology in the 1940s [4] stimulated an increased average yearly milk production per cow from 4,167lbs in 1924 to 20,397 lbs in 2007 [5]. Improvements in milk production stagnated for several years, but the publication of the Bos Taurus genome in 2009 [6] and further development of genomic testing and breeding programs helped the number increase to 22,774 lbs by 2016 [5]. These dramatic improvements in cow productivity have resulted in part from breeding programs which favor animals in the upper tier of the productive bell curve. This type of breeding strategy often results in widespread propagation of lethal recessives when top-performing animals are also carrier of these mutations [7, 8]. The recommended to avoid breeding known-carriers of genetic disorders has historically been ignored by both breeders and AI companies [2], and today, many top-performing bulls with thousands of offspring continue to increase the frequency of lethal recessives in the Holstein gene pool [8, 9].

Complex Vertebral Malformation

Complex Vertebral Malformation (CVM) is an autosomal recessive genetic disease that affects Holstein cattle. The disease-causing mutation is encoded by a G>T missense mutation in

the bovine SLC35A3 gene [9]. Animals that are homozygous for this mutation exhibit malformed vertebrae around the cervico-thoracic junction, and are typically aborted [9]. Heterozygote carriers are unaffected, and thus have a chance of passing on the mutation to their offspring. This pattern of inheritance has resulted in an evenly-spread blanket of carriers across the Holstein population, many of which have high EBV [8, 9]. The disease-causing mutation can be traced back to one bull, Carlin-M Ivanhoe Bell (born 1974) [9], and has since reached an allele frequency of 0.0137 in the Holstein gene pool [7].

Therapeutic Genome Editing

The promise of therapeutic genome editing has become increasingly relevant over time. The discovery of targeted endonuclease systems such as zinc-finger nucleases (ZFN) [10-12] and transcription activator-like effector nucleases (TALEN) [13-15] widened scientist's ability to induce targeted double stranded breaks in DNA. However, these protein-guided systems were costly and impractical because they required re-engineering to target each genomic locus. The emergence of CRISPR-Cas9 as a genome engineering tool changed this. Originally described as a component of bacterial immune systems [16], Cas9 provided scientists an inexpensive, modular, and customizable approach to genome editing [17-20]. Further optimization of the CRISPR-Cas9 system for expression in and subsequent engineering of human cells has also increased its applicability to the cells of other mammals [19, 21-23]. This tool has allowed scientists to produce pigs resistant to the Porcine Reproductive and Respiratory Syndrome (PRRS) virus, [24] hornless cattle [25], livestock which produce heavier muscling [26], and cattle free of genetic diseases [27].

The gene-modification workflow for correction of CVM supplies an opportunity to test and optimize a therapeutic SNP modification workflow for cattle. We tested several factors that affect the efficiency of SNP repair when electroporating primary cells with the CRISPR/Cas9

system and ssDNA repair templates [28]. CRISPR/Cas9 (spCas9) is a dsDNA endonuclease guided by two RNA molecules, a crRNA with 20 nucleotides of homology to the desired cut site, and tracrRNA which directs Cas9 cutting activity [28]. Cas9 creates a double stranded break (DSB) with blunt ends three bases upstream of the protospacer adjacent motif (PAM) sequence, which is 5'-NGG-3' [28]. The crRNA and tracrRNA have been fused into a single guide RNA (sgRNA) which further simplifies the enzyme's use in genome engineering [28].

Following DSB formation by these nucleases, the cellular DNA repair machinery recognizes a cut, and activates DNA repair through several pathways. The two most-prominent DNA repair pathways are non-homologous end joining (NHEJ) and homology directed repair (HDR) [29, 30]. NHEJ is an error-prone process that results in random insertions and deletions, often disrupting gene function [30, 31]. When an insertion is desired, as is the case in this project, DNA repair must follow the HDR pathway. HDR, which occurs predominantly in S and G2 phases of the cell cycle, incorporates a DNA strand with homologous sequences flanking the cut site [29, 32]. Because HDR events are rare, many strategies to improve the pathway efficiency have been tested. Donor design principles such as the use of asymmetric ssDNA donors and phosphorothioate modifications which protect donor ends from exonuclease digestion have been show to increase HDR rates [33]. Further, increased rates of HDR have been observed when Cas9 induced DSBs are proximate to the intended integration site [33]. Despite significant advances in the field, genetically modifying primary cells via HDR remains difficult.

Comparisons

In this study, we investigate the effects of several variables on the rate of HDR mediated correction of bovine CVM in primary cells. We first compare CRISPR-Cas9 delivery via ribonucleoprotein complex (RNP) and PX458 plasmid vector, expecting that RNP delivery will

produce more genetically corrected cells because of the high rates of cell death experienced with plasmid electroporation [34]. Within that comparison, two sgRNA guides which target the same DNA strand 7 bases 5' and 17 bases 3' of the CVM mutation respectively are also compared. We expect that the sgRNA cutting more proximal to the CVM mutation will yield more genetically corrected cells based on the mechanism of Cas9 binding and strand release proposed by Richardson et al [35]. Additionally, we compare two designs for ssDNA donors by varying the degree of asymmetry between 3' and 5' homology arms. All of the comparisons are outlined in Table 1. We expect that the ssDNA donor with a longer homology arm 5' of the CVM mutation will produce a higher rate of SNP repair for the CVM locus based on characteristics of the DNA sequence 5' of the mutation.

Table 1. Illustrates the comparisons that will be made for this project. Each group of constructs along with controls will be electroporated into primary cells using standardized conditions.

1. Sg1 RNP + HR67-36	3. Sg1 Plasmid + HR67-36	5. Sg2 RNP + HR67-36	7. Sg2 Plasmid + HR67-36
2. Sg1 RNP + HR91-36	4. Sg1 Plasmid + HR91-36	6. Sg2 RNP + HR91-36	8. Sg2 Plasmid + HR91-36

DNA extracted from Cas9 treated cell colonies will be extracted and Sanger sequenced to compare the rate of SNP correction. These comparisons may be valuable for the establishment of routine therapeutic genome editing protocols for cattle. Following verification that the disease allele has been corrected, the corrected cells will be used to clone a genetically identical, yet disease free version of our cow of interest via the SCNT technique. Further comparisons between clones produced in each of the different methods will be made after cloned calves are born.

CHAPTER II

MATERIALS AND METHODS

Carrier Identification

The cow of interest was confirmed as a CVM carrier by RFLP analysis conducted at Genetic Visions-ST LLC, and carrier status was added to her pedigree.

Tissue Culture

Skin biopsies were taken from the cow's ear by the owner and donated for research purposes. The tissue was minced and digested in collagenase, washed by centrifugation, cultured, and observed for seven days prior to shipping to confirm normal cell division. Four vials containing somatic cells were cryopreserved and shipped overnight in liquid nitrogen. When received, the cells were immediately transferred to a new liquid nitrogen tank for long term storage.

To establish cell culture, one vial was thawed in water at 37ºC for one minute. The cells were re-suspended in 10mL of Gibco™ Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Thermo®) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals) and 1x antibiotic-antimycotic (Anti-Anti™, Thermo®) and were deposited in a T75 tissue culture flask (Falcon). The cells were cultured at 37ºC in a 5% CO_2 and 5% O_2 humidified incubator (Nuaire), and media was replaced after 12 hours to eliminate trace DMSO from the freezing media. Media was subsequently replaced as-needed, and cells were passaged at 80% confluency by splitting in a ratio of no more than 1:3. A third of the cells from each passage were frozen in 10% DMSO, 45% FBS, 45% DMEM/F-12 media using an isopropanol freezing

container (Thermo®). The frozen cells were transferred to liquid nitrogen after 24 hours for long term storage.

Primer Design

Two PCR primer pairs were designed in Benchling®. Primer pair 1 (CVM Fp1 and CVM Rp1) spanning a 1967 nucleotide amplicon and primer pair 2 (CVM Fp2 and CVM Rp2) spanning a 1944 nucleotide amplicon. Both primer pairs were ordered from Invitrogen and were re-suspended in water. PCR optimization was performed, and products were visualized using a 1% Agarose gel (Thermo®) stained with 1µl of GelRed™ (Biotium®).

An additional set of sequencing primers spanning 1047 nucleotides was designed in Benchling® to allow provide superior coverage when performing Sanger sequencing.

Sanger Sequencing

To identify SNPs that could interfere with sgRNA activity, Sanger sequencing was performed. DNA was extracted from primary cells using the DNeasy Blood & Tissue Kit (Quiagen®). DNA was eluted in 25µl of water, and the concentration was measured with a nanoDrop™ spectrophotometer (Thermo®). PCR was performed using HiFi PCR premix (Clonetech®) with 200ng of genomic DNA and 0.1µM of each primer pair in a 25µl reaction. The PCR reaction was conducted at an annealing temperature of 57ºC with two-minute extension time for 35 cycles. The PCR product was purified using QIAquick™ PCR purification kit (Quiagen®) and eluted with water. Sanger sequencing was performed by a technician at the Texas A&M Laboratory for Plant Genome Technologies.

Sanger sequencing was also performed to assess correction of the CVM mutation in cells treated with CRISPR-Cas9.

Oligo Design and Cloning

All oligonucleotides were designed by hand using Benchling®. Once sequencing confirmed that there were no SNPS which might interfere with sgRNA binding, cloning primers were ordered (Thermo®) and resuspended to a 100µM concentration in Milli-Q® water. Primers were cloned into a PX458 (pSpCas9(BB)-2A-GFP) plasmid vector (Addgene, #48138) for electroporation into cells using the Zhang Lab protocol [34]. Precise cloning of both sgRNA into the PX458 vector was confirmed via Sanger sequencing using the LKO 1.5' sequencing primer for the human U6 promoter. Modified synthetic versions of both sgRNAs were ordered from Synthego® and were resuspended to a 100µM concentration in Milli-Q® water prior to dilution of working stocks. Repair templates designs were centered around the CVM mutation (Figure 1) and were ordered from IDT® in ssDNA format with phosphorothioate end protection. Repair templates were diluted to 100µM in Milli-Q® water prior to dilution of working stocks.

Figure 1. Sequence alignment of the Bos Taurus genome displaying ssDNA repair template designs with reference to the SNP encoding CVM.

Electroporation and Monoclonal Isolation

All transfections were carried out using the Neon™ electroporation system (Thermo®). Electroporation was optimized and carried out at 1350 V with 2 pulses of 20ms each. Bovine primary fibroblasts were trypsinized and centrifuged at 250g for 5 minutes, followed by aspiration of supernatant and re-suspension in 10mL of DPBS. A 10µl sample was

taken and stained with 10µl of 0.4% trypan blue, and cells were counted using a hemocytometer. Cells in DPBS were centrifuged again and the supernatant was aspirated. Cells were then re-suspended in Buffer R to reach a concentration of 5E5 cells/µl. Ribonucleoprotein complexes were prepared for electroporation by suspending 1µg of GeneArt™ Platinum™ Cas9 Nuclease (Thermo®) and 500ng of each synthetic sgRNA in Buffer R to a total volume of 8µL, followed by incubating for 10 minutes at room temperature. Plasmids were prepared for electroporation by suspending 1 µg of each plasmid in Buffer R to a volume of 8µl. Both sgRNAs were independently delivered in plasmid and RNP form, along with 10pMol of either ssDNA repair template in a 10µL electroporation.

Following electroporation, cells transfected with RNP were diluted 1:1000, and ~500 cells were plated in recovery media (20% FBS, 80% DMEMF12, and 100µM Y-27632, ROCK inhibitor (Stemcell®) in a 150mm cell culture dish. Cells transfected with plasmids were flow-sorted based on GFP fluorescence, and ~1000 cells were plated in recovery media in a 150mm cell culture dish. Both groups of cells were cultured for approximately 10 days, until distinct monoclonal colonies were observed. Media was aspirated and colonies were marked on the lid of each cell culture dish while being observed under a stereoscope. Plates were then placed in a tissue culture hood, and cloning rings were places around each colony using sterile forceps. A solution of 1% LMP agarose (Sigma®) in 1xDPBS was then distributed dropwise around the cloning rings, holding them in place. Colonies were released by applying 30µl of 0.05% trypsin-EDTA (Invitrogen®) at 37°C, followed by inactivation of trypsin with 100µl of culture media and subsequent pipetting to suspend cells. Colonies were then plated in individual T25 tissue culture flasks (Falcon). Once cells reached confluency, half were used for DNA extraction and

half were frozen in 10% DMSO, 45% FBS, 45% DMEM/F12 media using an isopropyl alcohol freezing container (Thermo®).

Cleavage Assay

To estimate the cutting activity of sgRNA #1 and sgRNA #2 in cells electroporated with RNP and plasmid, a GeneArt® Genomic Cleavage Detection assay (Thermo®) was performed.

Colony Genotyping

DNA was extracted from each group of isolated cells using the DNeasy Blood & Tissue Kit (Qiagen®). DNA was eluted in water and each concentration was measured using the nanoDrop™ system (Thermo®). PCR reactions were carried out using AmpliTaq Gold™ 360 master mix, (Thermo®) 0.1µM of PCR primers, and approximately 250ng of genomic DNA. An annealing temperature of 57.4ºC and extension time of 30 seconds were used, and PCR was run for 40 cycles. PCR products were send to the Texas A&M Institute for Plant Genomics and Biotechnologies for Sanger sequencing.

Statistical Analysis

Each experiment was conducted using at least three independent biological replicates. Error bars represent standard deviation. All statistical analysis was organized for presentation using Prism™ Graphpad®.

CHAPTER III

RESULTS

Carrier Confirmation and Sequence Consideration

The animal's status as a carrier of CVM was confirmed by Sanger sequencing. A double peak on the chromatograph (Figure 2) indicates that one allele is normal (G), while the other is mutated (T). Thus, the animal is a heterozygous carrier of bovine CVM. The chromatograph was also used to confirm that no CRISPR-Cas9 cutting activity would not be impaired by mutated PAM (NGG) sequences. The two sgRNAs used, and their respective cut sites for CRISPR-Cas9 are also displayed within the alignment.

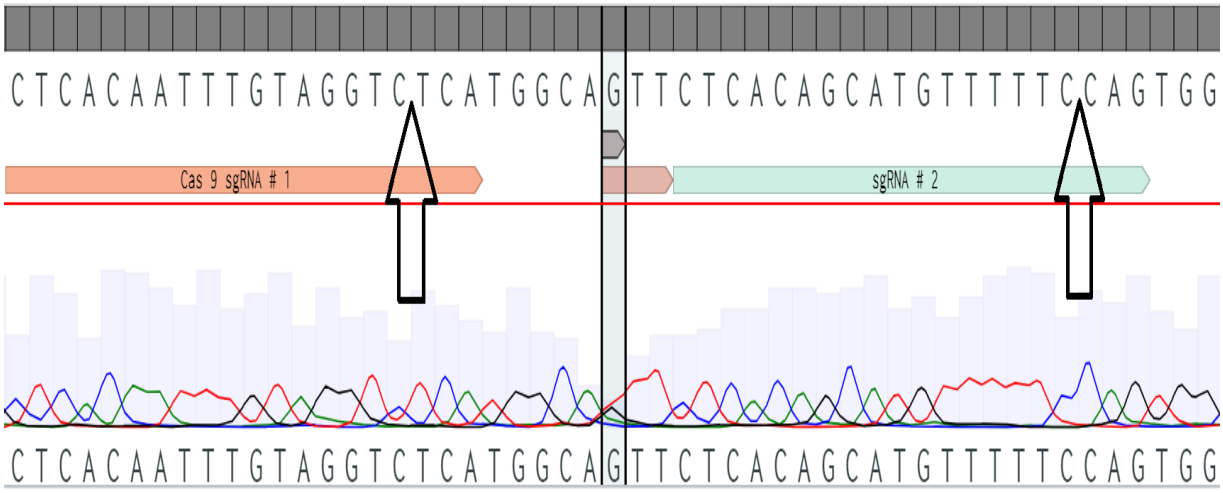

Figure 2. Chromatographs obtained from Sanger sequencing Exon 5 of the Bovine SLC35A3 gene. Double peak for G/T in each chromatograph indicates heterozygosity for the mutant SNP. Arrows indicate the cutting location of sg1 and sg2.

Plasmid Cloning

Following the cloning of sgRNAs into the PX458 (pSpCas9(BB)-2A-GFP) plasmid vector, (Addgene, #48138) Sanger sequencing using the LKO 1.5' sequencing primer confirmed that sgRNAs were correctly cloned into the plasmid. The chromatographs obtained from Sanger sequencing are shown in Figure 3.

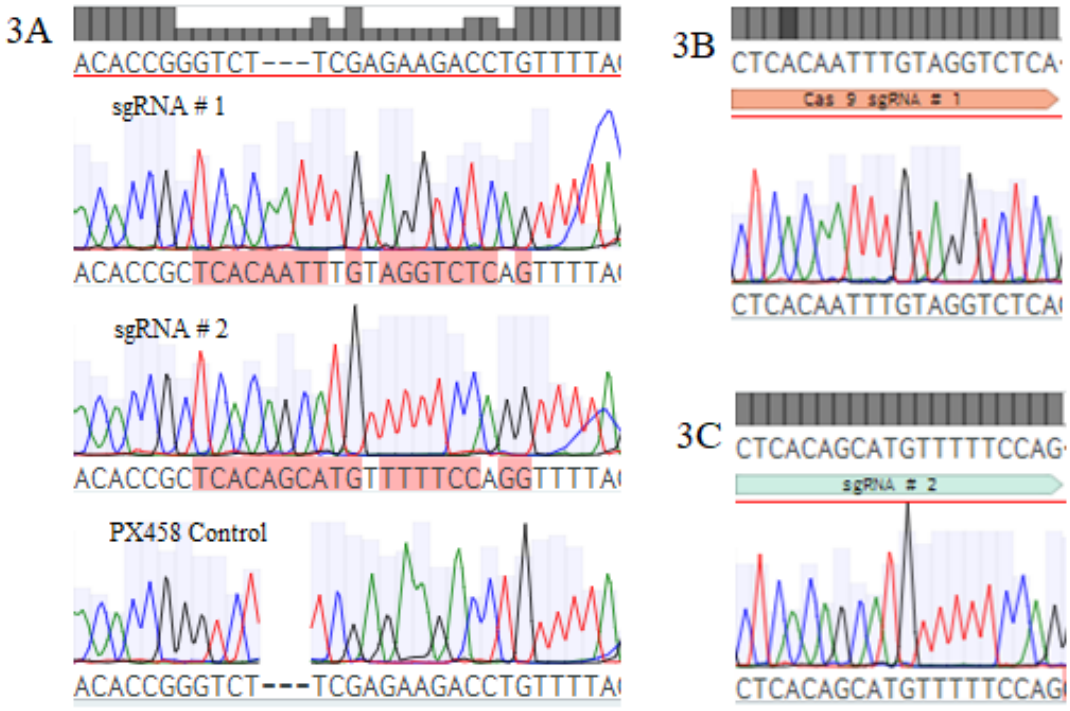

Figure 3. Chromatographs obtained from Sanger sequencing of the PX458 plasmid vector with and without inserts. A) sgRNA #1, sgRNA #2, and an un-treated PX458 plasmid control aligned against the PX458 sequence. B) sgRNA #1 cloned into the PX458 vector, aligned against the sgRNA #1 sequence. C) sgRNA #2 cloned into the PX458 vector, aligned against the sgRNA #2 sequence.

Cleavage Assay

Following electroporation of sgRNA # 1 and sgRNA # 2 in RNP and plasmid formats, cutting efficiency was confirmed and measured using a T7 endonuclease assay and fluorescence based densitometric quantification. The results shown in Figure 4 indicate similar cutting activity between sgRNA #1 and sgRNA #2, as well as between plasmid and RNP formats.

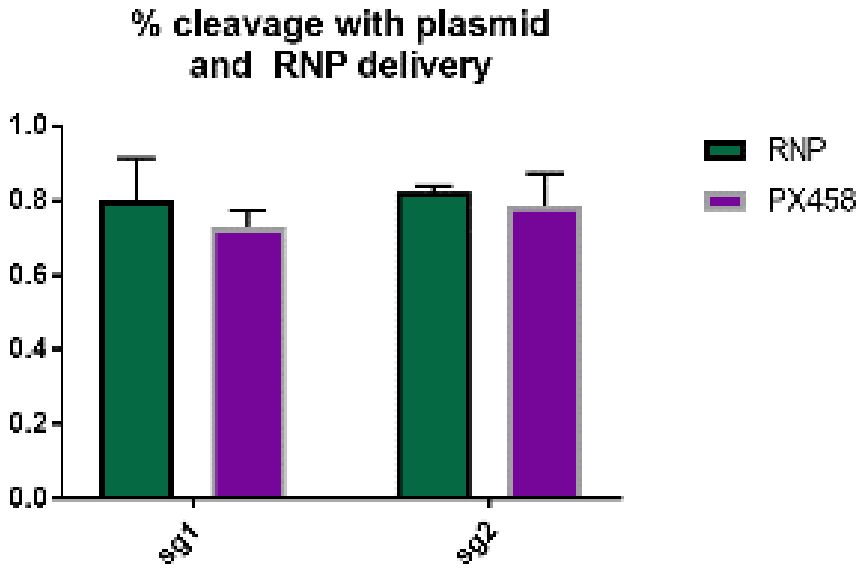

Figure 4. Quantification of data from the cleavage detection assay indicates similar cutting efficiency between sgRNAs and delivery formats. More data is needed in order to draw statistics-based conclusions about cleavage activity between these groups.

Homology Directed Repair Assay

Data from HDR assays will be collected by Sanger sequencing. We expect that sgRNA electroporated into cells in RNP format will generate higher rates of HDR for both guides. We also expect that cells electroporated with sgRNA # 1 will generate higher rates of HDR than cells electroporated with sgRNA # 2 using the same format, based on sgRNA # 1 cutting more proximally to the CVM mutation. Furthermore, we anticipate that HR91-36 will generate higher rates of HDR when paired with sgRNA # 1 because of the template's extension into the intron 5'

to Exon 5, which has lower GC content, and is likely to exhibit more dramatic strand excision based on the mechanism of CRISPR-Cas9 binding established by Richardson et al [35].

CHAPTER IV

CONCLUSION

Thus far, this project has demonstrated the planning and optimization that is required to carry out the therapeutic genome editing of complex vertebral malformation in cattle. We have also designed assays that measure cutting efficiency and the rate of homology directed repair when bovine primary cells are treated with CRISPR-Cas9 that cuts in different locations, is delivered in different formats, and is co-delivered with repair templates of differing lengths. Thus far, cutting efficiency has been confirmed in two sgRNAs which cut 7 bases 5' and 17 bases 3' of the CVM mutation. The cutting activity was also confirmed in both plasmid and RNP deliver formats for CRISPR-Ca9. Comparisons for homology directed repair have not yet been made. However, we expect that cells electroporated with the sgRNA which cuts more proximally (7 bases) to the CVM mutation will yield higher rates of HDR. We also expect that constructs delivered in RNP format will yield higher HDR rates, as RNP delivery is less cytotoxic to cells based on our observations. Further, it is expected that the ssDNA donor with a longer homology arm 5' of the CVM mutation will produce a higher rate of HDR for this locus based on characteristics of the DNA sequence 5' of the mutation. This project demonstrates the principles needed to carry out a therapeutic genome editing project in primary cells. It should be noted that high variability in sequence characteristics at different genomic loci present unique conditions which must be considered and conditions optimized for each site. In the future, additional electroporations will be carried out comparing the previously mentioned variables. Electroporated cell colonies of clonal origin will be isolated, grown, and genotyped until cells

which no longer encode CVM in their genomes are found. These cell colonies will then be used to clone a CVM free cow which retains the genetic merit of our CVM carrier.

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