RESEARCH REPORT
SWINE HEALTH

Title: Genetic modifications in CD163 that confer complete resistance of pigs to infection with PRRSV – **NPB #16-181**

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Industry Summary: In collaboration with researchers at the University of Missouri, we demonstrated that genetically edited pigs lacking CD163 are completely resistant to infection with porcine reproductive and respiratory syndrome virus (PRRSV). This is the first clear demonstration that PRRS can be prevented. The use of the CD163-modified pig in swine production means that PRRS-specific control measures, including vaccines, diagnostics, and barn filtration, will no longer be needed. As part of normal homeostasis, CD163 participates in the removal of excess hemoglobin from the blood and modulation of the inflammatory response after tissue damage or infection. The purpose of this work is to identify small regions in CD163, which can be removed or altered, which will protect a pig from infection without affecting other important biological functions of CD163. The first part of the project utilized a culture system for testing the susceptibility of cells expressing modifications in CD163. Infection of cultured cells identified three candidate constructs for further development in pigs. All modifications were predicted to produce resistance against common strains of PRRSV. The CRISPR/Cas9 methodology was successfully applied for the construction of CD163-modified embryos. Because this is new technology, several challenges were encountered. Therefore, additional funding was obtained from USDA NIFA to increase the number and efficiency of CD163-edited pigs, which can be used for infection studies.

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Key Findings:

- PRRSV infection can be prevented in cells by making small mutations in CD163
- Modified CD163 shows resistance to PRRSV-1 and PRRSV-2 isolates
- CRISPR-modified pig embryos were successfully prepared and evaluated for mutations

Keywords: porcine reproductive and respiratory syndrome virus, PRRSV, CD163, genetic modification

Scientific Abstract: CD163 is a receptor required for infection of porcine macrophages with porcine reproductive and respiratory syndrome virus (PRRSV). The extracellular region of CD163 possesses nine scavenger receptor cysteine-rich (SRCR) and two proline-serine-threonine (PST) domains. In previous work, we demonstrated that CRISPR-edited pigs lacking the entire CD163 are completely resistant to PRRSV-1 and PRRSV-2 viruses. These results represent the first clear demonstration that PRRS can be prevented. The overall goal of this research is to pursue a further refinement of the CRISPR technology to construct pigs that possess small deletions in CD163 sufficient to confer resistance, but without affecting other CD163 functions. For Objective 1, we employed an *in vitro* system for evaluating the permissiveness of HEK293T (HEK) cells transfected with modified CD163 receptors, prepared from cloned cDNA. HEK cells were transfected with a CD163 plasmid construct fused to enhanced green fluorescent protein (EGFP) and then infected with a PRRSV-2 isolate expressing a red fluorescent protein (RFP). Flow cytometry staining using anti-CD163 mAb, 2A10, was used to monitor the expression of CD163 on the surface of transfected cells. The presence of green and red in the same cell identified the infected transfected cells. CD163 modifications included the insertion of proline-arginine (PR) dipeptides in SRCR-5, single amino acid substitutions, and amino acid deletions. The results for the PR mutations identified several insertions in SRCR-5 that blocked infection. The construction of a computer model of SRCR-5 showed that the four PR insertions exerting the greatest effect on infection formed a potential binding pocket. CD163 proteins possessing a deletion of PSTII or the SRCR4-5 interdomain tetrapeptide, AHRK, were also resistant to infection. Substitution of cysteines for alanines, as the means to disrupt SRCR-5 interdomain disulfide bonds, blocked infection. The results from Objective 1 identified several candidate mutations that were predicted to confer PRRS resistance to pigs. Under Objective 2, which remains ongoing, CRISPR is being used to prepare bi-allelic mutations in the *CD163* gene. The targets selected for editing included the deletion of the N-terminal region of SRCR5 (Construct 1), removal of PST-II by deleting exon 13 (Construct 2), and deletion of AHRK interdomain peptide sequence (Construct 3). Several CRISPR guide sequences were prepared and tested on single cell embryos. Each guide sequence was designed to produce optimal editing efficiency combined with few off-target effects. Sequencing cells from 7 day embryos showed a wide range of editing frequencies; from 1 out of 6 embryos for Guide 1-2 to 8/8 embryos for Guide 1-1. In order to obtain a high frequency of bi-allelic modification, further work is being performed to increase the editing frequencies. The next stage is to implant embryos and infect the resulting litters with PRRSV-1 and PRRSV-2. As a means to complete the project, this work is being supported with additional funding from USDA.

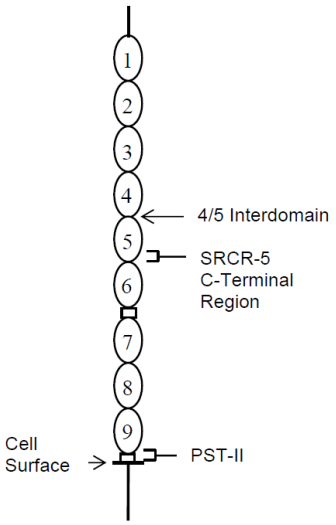

Figure 1. Organization of CD163 and the three sites selected for CRISPR modification.

Introduction: The elimination of PRRSV will have a lasting and indelible impact on the US swine industry. This project takes advantage of the fact that PRRSV possesses a highly restricted host and cell tropism, infecting porcine macrophages that possess the CD163 receptor. As reported by us in Nature Biotechnology in 2015 (CD163 facilitates both entry and replication of porcine reproductive and respiratory syndrome virus. Nature Biotech. http://dx.doi.org/10.1038/nbt.3434), we demonstrated that genetically edited pigs lacking the expression of the *CD163* gene fail to support the replication of PRRSV.

As illustrated in Figure 1, CD163 is a type I membrane protein composed of a signal peptide followed by nine scavenger receptor cysteine-rich (SRCR) domains. A 35-amino-acid proline-serine-threonine (PST)-rich domain, PST-I separates SRCR domain 6 (SRCR 6) and SRCR 7 (see Figure 1). A second PST-rich 12 amino acid peptide sequence, PST-II, connects SRCR 9 with the transmembrane domain, which is followed by a functional internalization motif. In addition to functioning as the PRRSV receptor, CD163 is important for the modulation of inflammation in response to tissue damage and infection. As an anti-inflammatory protein, CD163 decreases oxidative stress by removing excess heme, in the form of hemoglobin-haptoglobin complexes (Jeney et al., 2002). The metabolic degradation of hemoglobin by macrophages yields several potent anti-inflammatory products, including biliverdin, bilirubin, and carbon monoxide (Soares and Bach, 2009). In a pro-inflammatory capacity, the crosslinking of CD163 on the macrophage surface by antibody or bacteria results in the localized release of pro-inflammatory cytokines, (Van den Heuval et al., 1999; Fabriek et al., 2009).

As part of a collaboration between University of Missouri and Kansas State University, we showed that genetically modified pigs that do not express CD163 on macrophages fail to support PRRSV infection (Whitworth et al., 2015). The CD163 modification that resulted in the loss of CD163 expression was accomplished using the genomic editing tool, CRISPR/Cas9. In fact, the CD163 knockout (KO) was constructed by deleting as few as 11 bp out of the 2.7 billion bp that comprise the pig genome. This is the first demonstration of a method for the complete prevention of PRRS in pigs.

One consequence of the CD163 KO mutation was the loss of the capacity to scavenge haptoglobin from the blood, a property of CD163 that maps to SRCR domain 3 (see Figure 1). The overall goal of this project was to further refine the CD163 modification by determining the smallest peptide sequence deletion in CD163 that can block PRRSV infection while preserving the important homeostatic functions of CD163.

Objectives:

Objective 1. *Identify the CD163 protein domains involved in making HEK cells permissive for infection with PRRSV Type 2 viruses.* The purpose is to find the smallest deletion in CD163 that will prevent infection of HEK cells transfected with CD163 plasmid constructs. The data are used to construct pigs that possess the corresponding genetic modifications.

Objective 2. *Determine the biological properties and PRRSV permissiveness of pigs possessing modifications in CD163.* The results from Objective 1 are used to prepare CD163 genetically modified pigs for virus susceptibility. The approach is the application of CRISPR/Cas9 to modify small peptide sequences in the *CD163* gene. The biological function of the modified CD163 will be tested by measuring circulating haptoglobin levels.

Materials & Methods:

Objective 1. The identification of peptide sequences in CD163 was performed using a modification of the *in vitro* system, originally described by Van Gorp. et al. (2010).

Wild-type and modified CD163 DNA constructs fused to EGFP were prepared and cloned into the pcDNA™3.1 mammalian expression vector. HEK cells, which do not support PRRSV infection, were transfected with the CD163 constructs. Successful expression of the CD163-EGFP fusion protein was evident by the presence of green fluorescence (see Figure 2). The permissiveness of transfected cells for PRRSV is tested using recombinant PRRS viruses expressing a red fluorescent protein (RFP). Expression of CD163 on the surface of HEK cells was verified using flow cytometry and anti CD163 antibodies.

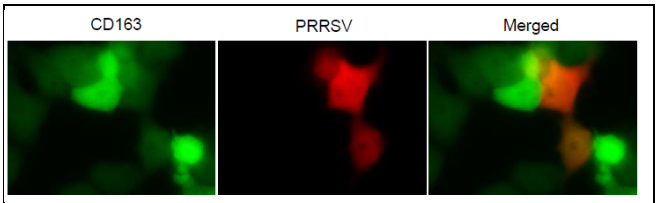

Fig. 2. Co-localization of CD163-EGFP with PRRSV-RFP.

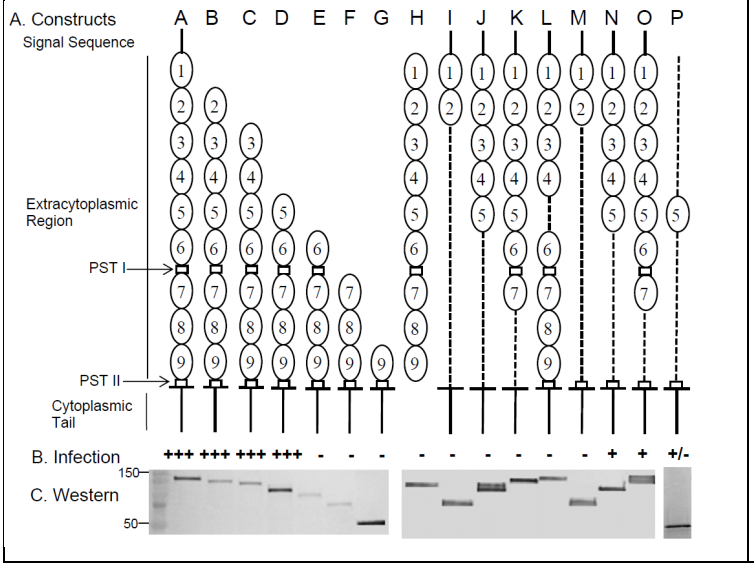

Fig. 3. CD163 constructs used to identify domains involved in PRRSV-2 infection. (A) Deletion mutants used in the transfection of HEK cells. Ovals and squares identify the SRCR and PST domains, respectively. (B) Result for PRRSV infection of transfected HEK cells. Key: (+++), similar to results for wild-type CD163 including numerous large clusters of infected cells; (++), several small clusters of infected cells; (+), multiple single infected cells, but no clusters; (+/-), a few scattered infected cells; (-), no detectable infected cells. (C) Results for Western blots using anti-GFP antibody for the detection of the CD163-EGFP fusion protein.

The first step in the identification of CD163 domains required for PRRSV infection was to test CD163 constructs that possessed large deletions, consisting of the removal of two SRCR domains at a time. The CD163 cDNA constructs possessing truncations from the N-terminal end were prepared using PCR primers that possessed *Kpn*I and *Xba*I restriction sites. PCR amplification of the CD163 cDNA was performed using the GoTaqGreen® Master Mix (Promega) according to manufacturer's instructions. The PCR products with *Kpn*I and *Xba*I were cloned into the *Kpn*I-*Xba*I sites of the pcDNA3.1-EGFP vector. Plasmids were transfected into HEK cells using FuGENE® HD reagent (Promega) according to manufacturer's instructions and viewed for the presence of EGFP expression under a fluorescence microscope. The resulting N-terminally truncated proteins are illustrated in Figure 3A (see constructs B through G). Constructs that possessed domain deletions at the C-terminal end of CD163 incorporated primers that possessed *Pac*I restriction sites. Deletions were made using a long PCR protocol designed to amplify the desired CD163 fragment along with the entire pcDNA3.1-EGFP plasmid. The resulting CD163 deletion constructs retained intact transmembrane and cytoplasmic domains along with an additional *Pac*I site (see constructs I through O in Figure 3A).

Finer mapping within SRCR5 was performed by inserting proline-arginine (PR) dipeptides along the SRCR5 polypeptide sequence. Each PR was inserted by adding a *Sac*II restriction site, in frame, into the CD163 cDNA. The intact pcDNA3.1 CD163-EGFP plasmid was used as PCR template and the entire plasmid amplified using LongAmp® *Taq* DNA Polymerase and the same amplification conditions described above for the construction of the C-terminal CD163 truncation mutants.

Objective 2. Mutations in *CD163* were created in single-cell embryos using the CRISPR/Cas9 technology as previously described in Whitworth et al. (2015). The CRISPR guide RNAs targeting the appropriate sequence

in the *CD163* gene were designed based on software programs that are predicted to produce guides that produce maximum editing efficiency and low off-site targeting. The presence of a specific genetic modification was confirmed by DNA sequencing.

Results:

Infection of HEK cells transfected with CD163 domain deletion mutants. All constructs diagrammed in Figure 3A showed CD163 expression, which was evident by the presence of EGFP fluorescence within 24 hrs after transfection with CD163-EGFP plasmid plasmids. In addition, flow cytometry using labeled anti-CD163 antibodies confirmed that all constructs showed surface expression of CD163 on the surface of HEK cells (data not shown). Western blots stained with anti-GFP antibody confirmed that all CD163-EGFP constructs migrated according to their predicted size (Figure 3C). HEK cells transfected with the full-length CD163-EGFP plasmid (Construct A) served as a positive control for the infection experiments. As shown in Figure 3B, the N-terminal deletion mutants B, C and D, which retained the SRCR5 domain, were positive for infection; whereas; the constructs E, F, G, which lacked SRCR5, were negative for infection. The results for the C-terminal deletions lacking the PST-II domain (constructs I, J, K), were all negative for infection; even the constructs J and K, which retained the SRCR5 domain. Constructs N and O, which were identical to J and K, but retained the PST-II domain were positive for infection. Construct O, which lacked domains 8 and 9, showed reduced infection. Construct P, which possessed only SRCR5 and PSTII as external domains, was positive for infection, but at a reduced level compared to the wild-type CD163. The results showed that SRCR5 and PST-II were required for infection with a PRRSV-2 isolate.

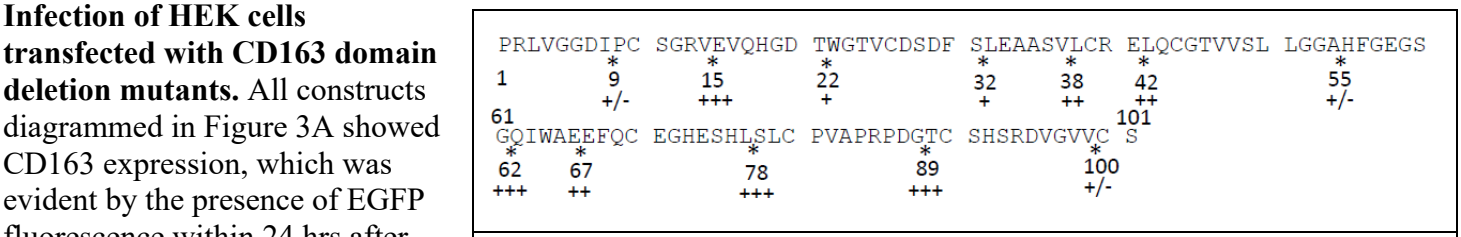

Fig. 4. Location of PR insertions in SRCR5 of porcine CD163. The asterisks show the location of the proline-arginine insertions. Below each construct is the result for infection of transfected HEK cells. The key for the infection results is the same as described in Figure 3.

Effect of PR dipeptide insertions in SRCR5 on PRRSV infection. The approach for probing the regions within SRCR5 important for PRRSV infection incorporated the insertion of single proline-arginine (PR) throughout the SRCR5 polypeptide sequence. Insertions were accomplished by placing *Sac*II restriction sites every 30 bp in the same reading frame as the SRCR5 cDNA sequence. Staining transfected HEK cells with the anti-CD163 mAb, 2A10, confirmed the surface expression of mutant CD163 proteins (data not shown).
The results after infection of transfected HEK cells with PRRSV-RFP identified a wide range of infection rates (see Figure 4). In general, PR insertions placed in the N-terminal half of the SRCR5 polypeptide had a greater impact on infection than mutations placed in the C-terminal half. Three PR insertions, PR-9, PR-55 and PR-100, produced the greatest reduction in infection, with only a small percentage of CD163-EGFP cells showing infection after 48 hrs

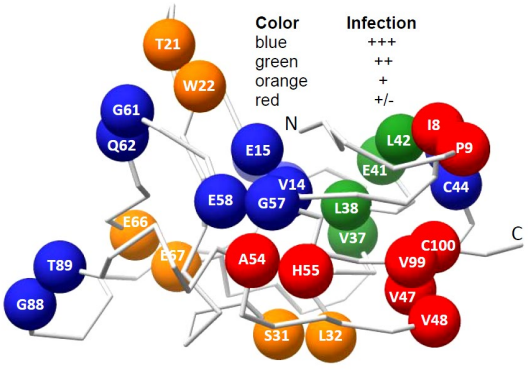

Fig. 5. Predicted location of PR insertions in SRCR5. Each amino acid pair in the ribbon structure identifies the location of each PR insertion. The structures are based on the X-ray crystallography data deposited in RCSB Protein Data Bank (PDB code 5JFB) and viewed using UCSF Chimera (Pettersen et al., 2004).

A more detailed analysis of the effect of the PR-9, PR-55 and PR-100 mutations on PRRSV-2 infection was conducted by performing growth curves. Growth curves for all three mutant CD163 HEK cell constructs were flat, indicating the absence of virus replication. In comparison, the yield on HEK cells transfected

with the wild-type CD163 increased over time; reaching a peak of 3 $\log_{10}$ $TCID_{50}$/ml within 36 hrs after infection (data not shown). Together, these data suggest that PR-9, PR-55 and PR-100 CD163 SRCR5 mutants are resistant to virus infection.

Recently, Ma et al. (2016) resolved the X-ray crystallographic structure of porcine CD163 SRCR5. A computer model of the predicted locations of all the PR insertions within the predicted SRCR5 structure are shown in Figure 5. As illustrated by the different colored balloons, mutations producing a similar effect on infection tended to group together. The red balloons identify a potential binding pocket for the PRRSV-2 virus.

The effect of mutations in the SRCR 4-5 interdomain region on PRRSV-2 infection. The SRCR 4-5 interdomain region is composed of the tertrapeptide sequence, alanine-histidine-arginine-lysine (AHRK). As shown in Table 2, the removal of the AHRK (plasmid construct 2) produced a dramatic reduction in infection. One explanation for this outcome is that the interdomain functions as a spacer, which is required for the proper conformation of SRCR5. However, the same negative effect on infection was found when the HRK sequence was replaced with AAA (see plasmid construct 3). Other substitutions also had an effect on infection. Therefore, it appears that the AHRK peptide sequence likely forms a contact with the virus.

Effect of disulfide bonds on infection. Individual disulfide bonds were removed by replacing the cysteines at positions C1, C3, C5 and C7 with alanines. HEK cells transfected with the wild-type CD163 showed an infection rate of 61.2% compared to 0.8% for the alanine substitution at C1; 1.0% for the alanine substitution at C3; 1.2% for the alanine substitution at C5; and 1.3% for the alanine substitution at C7. These data show that the disruption of any of the disulfide bonds results in a dramatic reduction in infection.

Table 1. Effect of mutations in the SRCR 4/5 region on infection with PRRSV-2

Construct	SRCR4	Interdomain	SRCR5	PRRSV-2
1	KITCS	AHRK	PRLVG	+++
2	.....	----	.....	+/-
3	.....	.AAA	.....	+/-
4	.....	.AA.	.....	+/-
5	.....	..AA	.....	+++
6	.....	.A.A	.....	+/-
7	.....	.A..	.....	+++
8	.....	...R	.....	+++
9	.....	..K.	.....	++
10	.....	.KK.	.....	+/-
11	.....	D...	.....	+++
12	.....	..RAK	.....	+/-
13	.....	.HAR.	.....	+

Design and application of CRISPR guides for the gene editing of pig embryos. Based on the results obtained from the *in vitro* studies, we selected three constructs for preparation of CD163-modified embryos. The three constructs, listed in Table 1, incorporated two guide sequences. Construct 1, incorporated the guides, sgRNA 1-1 and 2-1, which are designed to make a deletion in the N-terminal end of SRCR5. The results in Table showed that sgRNA 1-1 produced an editing frequency of 100%, which makes it a good candidate for a bi-allelic mutation (knocking out PST-II on both chromosomes). However, sgRNA 2-1 produced an editing efficiency of 60% (3 of 5 embryos). Therefore, the incorporation of both guides would be predicted to produce an overall editing efficiency of 60%. Construct 2, which is designed to delete exon 13 (which codes for PST-II), incorporates guide sequences sgRNA 1-2 and 2-2, which produced and editing frequency of 1/6 (17%) and 5/6 (80%). This means that the expected editing efficiency is predicted to be only about 13%. Construct 3 was designed to remove the tetrapeptide, AHRK, located between SRCR 4 and 5. The two guides, sgRNA 1-3 and 2-3, produced editing frequencies of 5/7 (71%) and 5/8 (62%), respectively, for a predicted knockout efficiency of 44%.

Table 2. Frequency of genome editing using the designed sgRNAs in pig embryos: RNA form of CRISPR/Cas9system was injected into one-cell stage embryos and Day 7 embryos were sequenced

Construct	Editing Frequency	Possible Off-Targeting Sites
1. C-Terminal SRCR-5		
sgRNA 1-1	8/8	Non-coding region
sgRNA 2-1	3/5	Diacylglycerolkinase theta and Histone deacetylase 1
2. PST-II		
sgRNA 1-2	1/6	Non-coding region
sgRNA 2-2	5/6	Glycine receptor subunit alpha-3 and Coatomer subunit epsilon
3. Interdomain 4/5		
sgRNA 1-3	5/7	Scavenger receptor cysteine-rich protein domain
sgRNA 2-3	5/8	Cleavage and polyadenylation specificity factor (CPSF)

Discussion:

CRISPR is a powerful technology for creating the genetic means to confer complete resistance to PRRSV infection and other swine diseases. Previous studies by our lab and others show that the complete deletion of CD163 or removal of the CD163 SRCR5 domain is sufficient to protect pigs from PRRSV infection. The current project extends the exploration of CD163 function by seeking the smallest deletion sufficient to confer PRRSV resistance while retaining a functionally intact CD163 protein. The results of Objective 1 identified the SRCR5 (Construct 1), PST-II

(Construct 2) and the SRCR 4/5 interdomain (Construct 3) as important for PRRSV infection. Deletions in the three regions block infection of transfected HEK cells and are predicted to have a similar impact in modified pigs. Furthermore, the observations from these data suggest that there are several domains on the CD163 protein that make contact with proteins on the surface of the PRRS virion.

SRCR5, PST-II and the 5/6 interdomain regions were selected for constructing CRISPR-edited pigs. The goals in constructing CRISPR-modified pigs are to maximize CD163 editing frequencies while minimizing off-target effects. The three constructs, when tested in embryos, showed a wide variation in editing frequencies. Since the genetic modification is dependent on frequency of mutation by each guide sequence, the goal is to obtain 100% efficiency. The highest editing frequency was obtained for guide, sgRNA 1-1, which produced a modification in 8 of 8 embryos (100%). However, when combined with sgRNA 2-1, which produced a modification in 3 of 5 embryos, the probability of obtaining the desired edit is decreased. If, for example, both edited alleles show a frameshift mutation, then the entire CD163 will be knocked out. Guide, sgRNA 2-1 is predicted to potentially edit other genes, which code for diacylglycerolkinase theta and histone deacetylase 1. Current efforts are directed at sequencing the existing edits and on improving the design of the guide sequences to increase the frequency of CRISPR edits.

Several challenges were confronted during the project. One important issue was the long delay in initiating Objective 2, which included the CRISPR guide designs and their use in the genetic editing of pigs. Therefore, we engaged another laboratory to prepare the genetically edited pigs. This change caused another delay but put the project on track. Another limitation was the overall cost of the project. We selected three editing strategies as the means to increase the probability of obtaining a resistant pig. This change increased the cost of the project and added further delays. However, we have obtained additional funding from USDA to complete the project. Based on the current timeline, we should have edited pigs in spring 2020.

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