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Genus

Gene Editing as Applied to Prevention of Porcine Reproductive and Respiratory Syndrome

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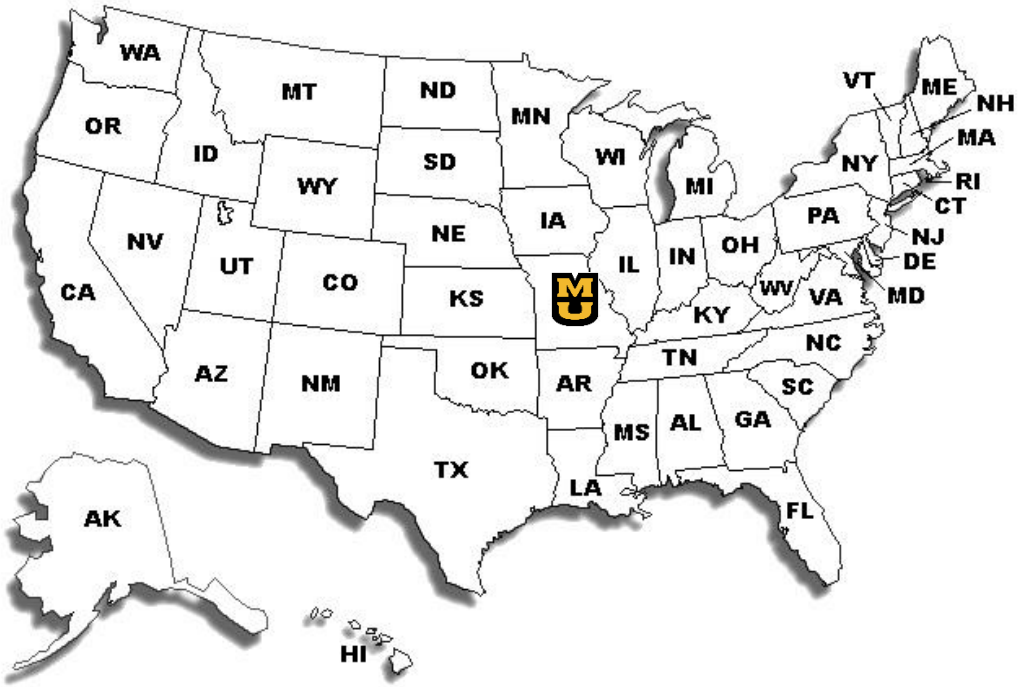
Division of Animal Sciences

National Swine Resource and Research Center

University of Missouri-Columbia, Columbia, Missouri USA


Mizzou


RESPECT
RESPONSIBILITY
DISCOVERY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Overview

- CRISPR/Cas9 overview
- Success Rate
  - *$CD163^{-/-}$ PRRSV resistance?*
  - *Protection from both Type 1 and Type 2 viruses?*
- Reproductive PRRS
  - *Does a $CD163^{-/-}$* sow protect wild type fetuses?
- Other Disease Resistance Models

Porcine Reproductive and Respiratory Syndrome (PRRS)

- The PRRS virus (PRRSv) was first detected in the USA in 1987 (Keffaber et al '89) and in Europe in 1990 (Wensvoort et al '91)
- PRRSv replicates in macrophages
  - Respiratory disease in young pigs
  - Reproductive disease in sows/boars
- It also predisposes infected pigs to other bacterial and viral pathogens

Economic Cost

- Costs $660,000,000 annually in North America (Holtkamp et al ‘13)
- Costs €1,500,000,000 annually in Europe (European PRRSpecive ‘15)
- Translates to ~$6,000,000 each day!
- Doesn’t include Asia

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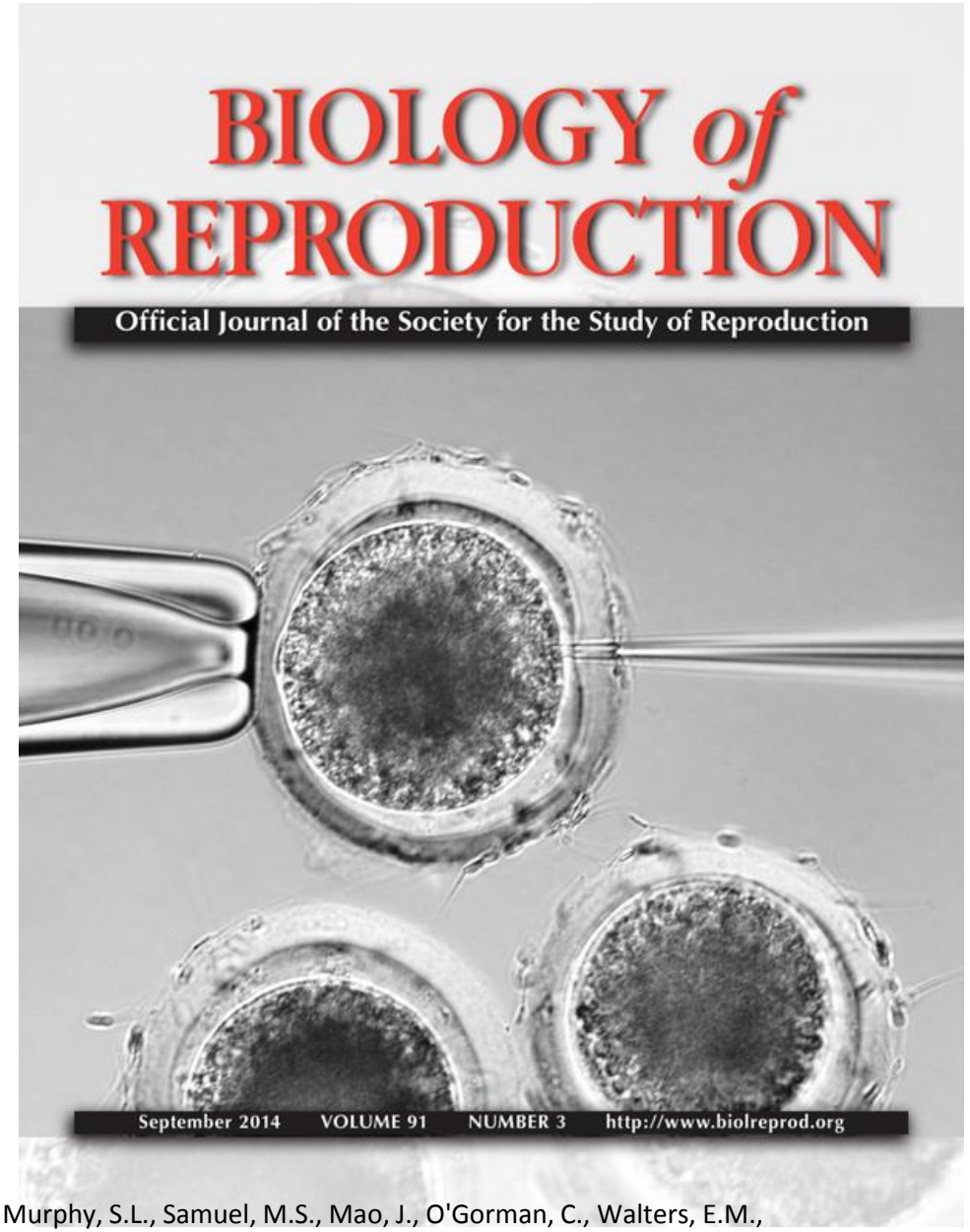
Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos
Whitworth et al., 2014



Whitworth, K.M., Lee, K., Benne, J.A., Beaton, B.P., Spate, L.D., Murphy, S.L., Samuel, M.S., Mao, J., O'Gorman, C., Walters, E.M., Murphy, C.N., Driver, J., Mileham, A., McLaren, D., Wells, K.D., and Prather, R.S. (2014). Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biol Reprod 91, 78.

Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus

To the Editor:

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important disease of swine in North America, Europe and Asia, costing producers in North America more than $600 million annually[1]. The disease syndrome was first recognized in the United States in 1987 and described in 1989 (ref. 2). The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), was subsequently isolated and characterized in Europe in 1991 (ref. 3). Vaccines have been unable to control the disease. It has been suggested that CD163 is the receptor for entry of PRRSV into cells[4]. Thus, we hypothesized that pigs with defective CD163 would be immune to PRRSV. Previously we used CRISPR-disease syndrome and porcine circovirus–associated disease, and can establish a lifelong subclinical infection[6]. In 2006, a more severe form of the disease, called highly pathogenic PRRS, decimated pig populations throughout China[7]. Although genetic selection for natural resistance is an option, success to date has been limited, possibly due to the genetic diversity of the virus[8].

It had been proposed that PRRSV infects alveolar macrophages using the surface protein SIGLEC1 (CD169) as the primary viral receptor[4]. In this proposed model, after binding to CD169 and being taken up into the cell by receptor-mediated endocytosis, the virus is uncoated by CD163 in the endosome, and the viral genome is released into the cytoplasm. However, when homologous recombination and somatic cell nuclear transfer) were infected with PRRSV and compared with infected wild-type pigs, no difference in virus replication was found[9]. To test the role of CD163 in infection, we previously created 45 live-born piglets with insertions ranging from 1 bp to 2 kb, deletions from 11 bp to 1.7 kb, as well as a partial domain swap in *CD163* using CRISPR-Cas9 technology[5].

One founder male and one founder female, both of whom had mutations in exon 7 of *CD163*, were bred to produce offspring (**Supplementary Methods**). The founder male (67-1) possessed an 11-bp deletion in exon 7 on one allele. The other allele had a 2-bp addition in exon 7 and a 377-bp deletion in the preceding intron and was

Whitworth, K.M., Rowland, R.R., Ewen, C.L., Trible, B.R., Kerrigan, M.A., Cino-Ozuna, A.G., Samuel, M.S., Lightner, J.E., McLaren, D.G., Mileham, A.J., Wells, K.D., and Prather, R.S. (2016). Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol 34, 20-2.

AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERA

Replacement of Porcine CD163 Scavenger Receptor Cysteine-Rich Domain 5 with a CD163-Like Homolog Confers Resistance of Pigs to Genotype 1 but Not Genotype 2 Porcine Reproductive and Respiratory Syndrome Virus

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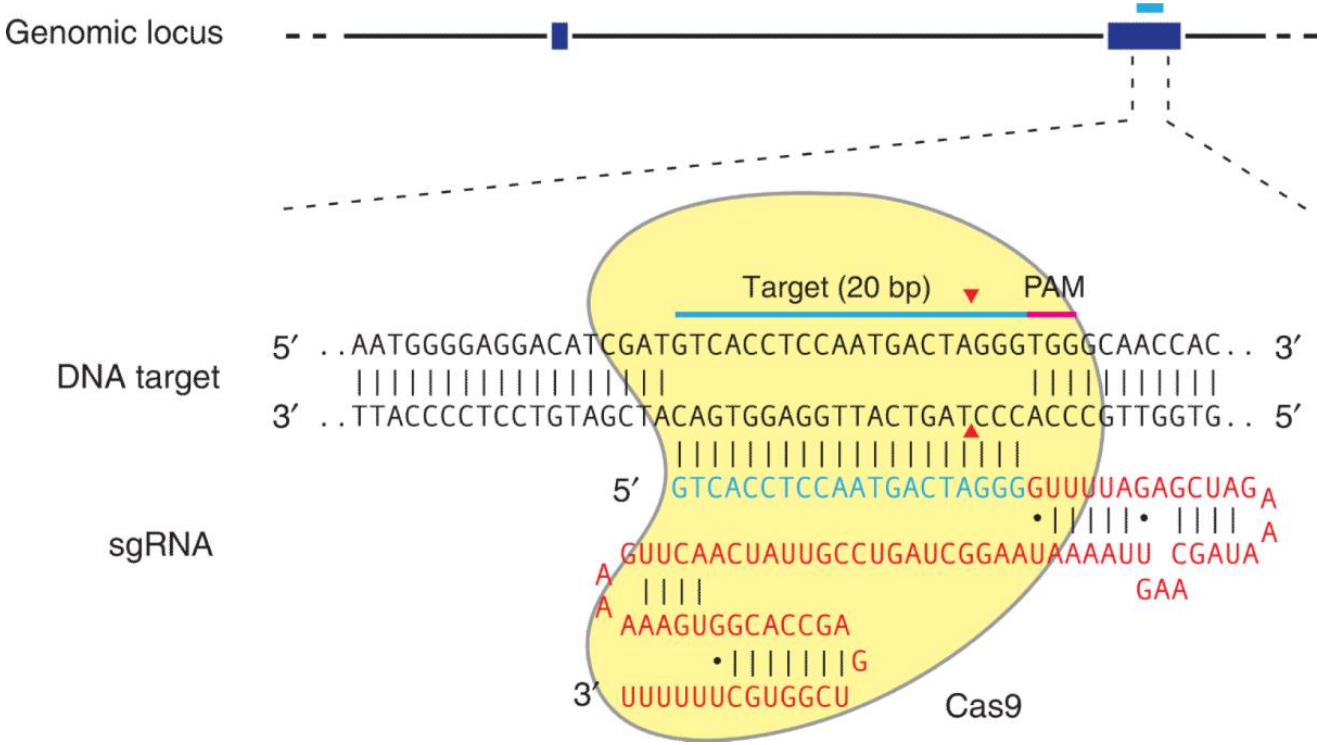
Pubmed search Whitworth AND Prather

CRISPR-Cas9

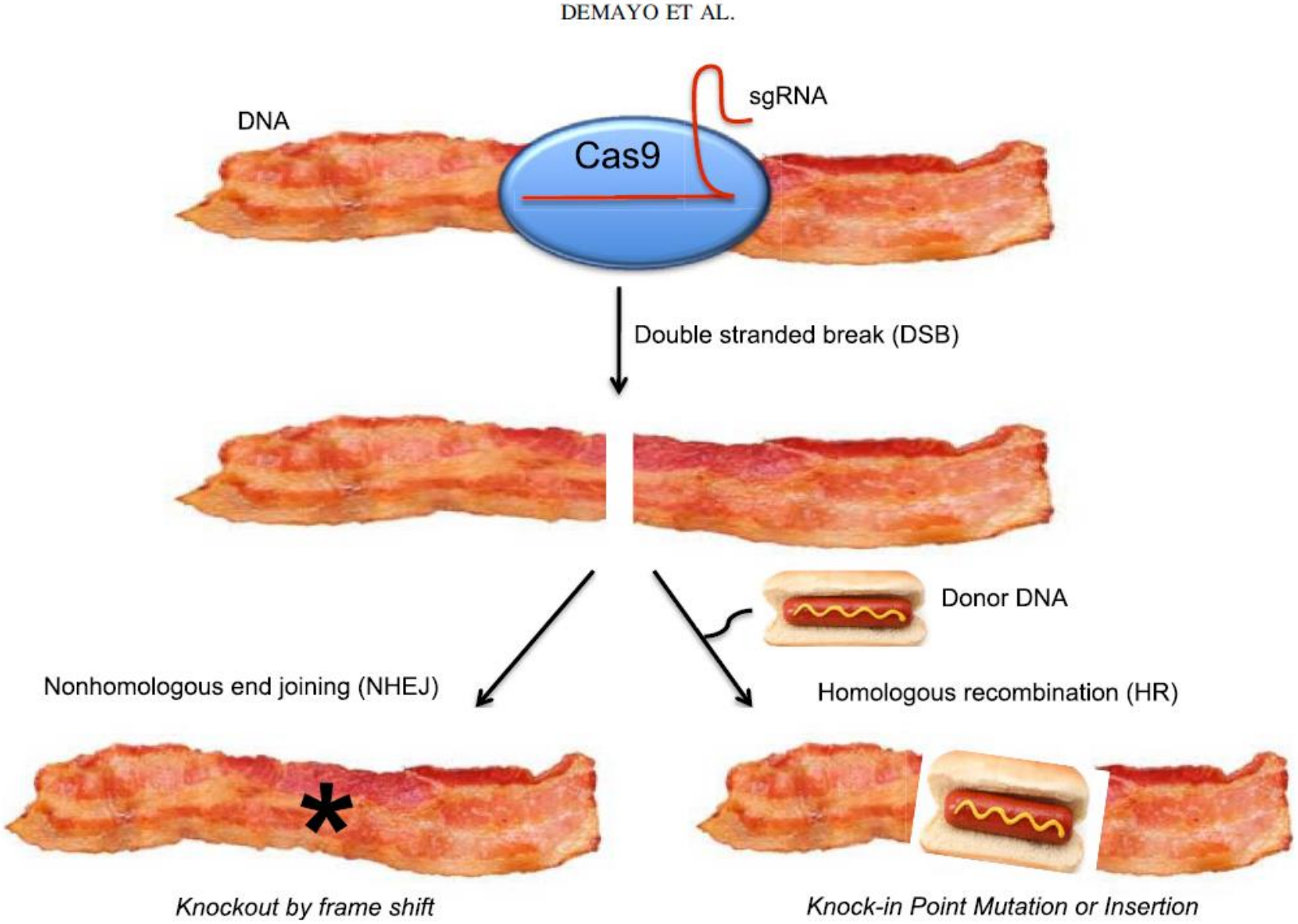


- CRISPR-Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave invading foreign genetic elements (Sorek et al., 2008; Mkarova et al., 2011)

- This system has been repurposed for mammalian genome engineering using SpCas9 along with a fusion of the tracrRNA and mature crRNA to create a chimeric single guide RNA (sgRNA)

Ran et al, 2013

(DeMayo and Spencer Commentary BOR 2014)



CD163 knock-out

CD163 Domain Swap

Project Spotlight
CRISPR

http://www.broadinstitute.org/what-broad/areas-focus/project-spotlight/crispr

The ability to precisely edit the genome of a living cell holds enormous potential to accelerate life science research, improve biotechnology, and even treat human disease.

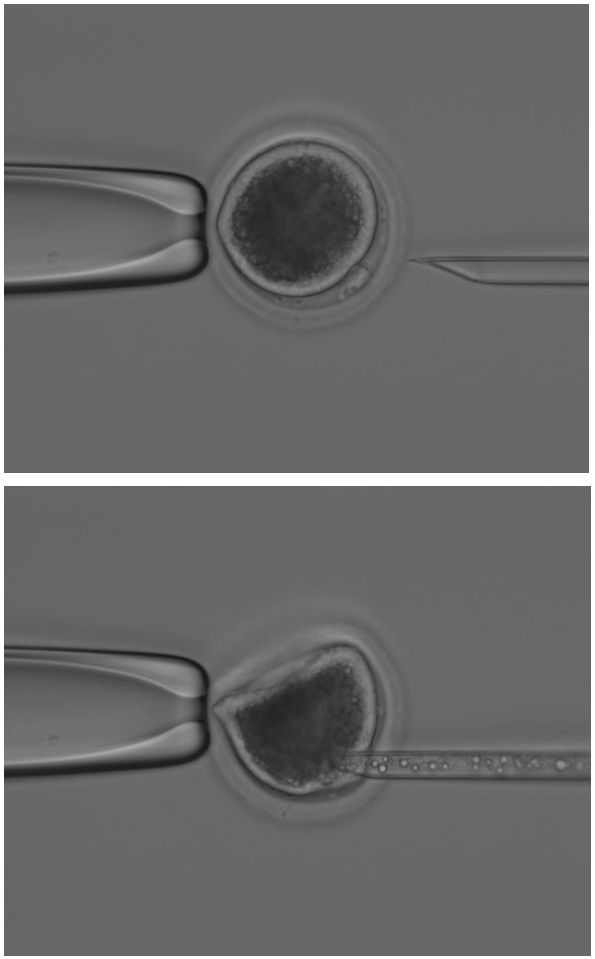
Methods for genome editing — primarily zinc finger nucleases and Transcription Activator-Like Effector (TALE) Nucleases — have existed for several years, but in 2013 these were quickly eclipsed by the efficiency, effectiveness and precision of the engineered CRISPR-Cas9 system that was first harnessed for mammalian

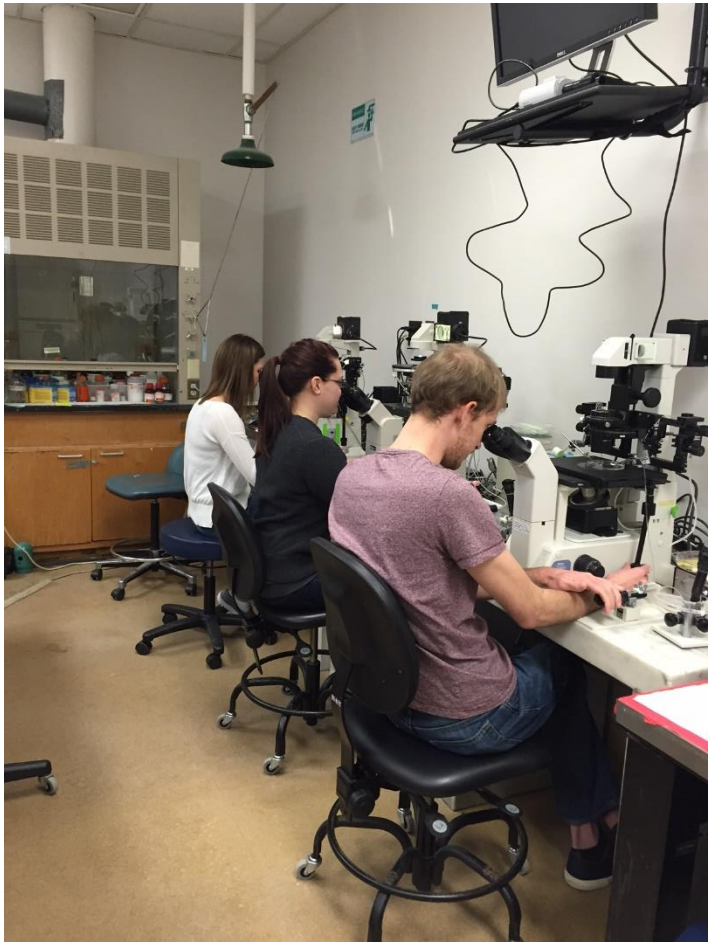
Spotlight: CRISPR

- CRISPR >
- Questions and Answers about CRISPR >
- CRISPR Timeline >
- Zhang Lab Website >
- Zhang Lab CRISPR Genome Engineering Resources >
- Feng Zhang Bio >
- Patents & Licensing >
- News & Press Releases >
- Press Kit >
- Obtaining Materials >
- Office of Strategic Alliances and Partnering >

Somatic Cell Nuclear Transfer

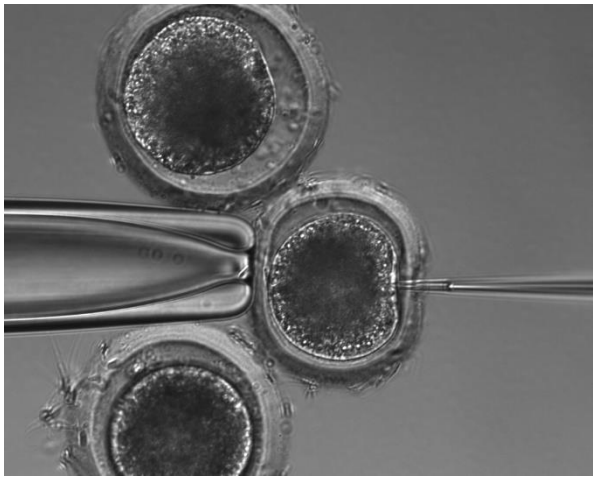
Using the CRISPR/Cas9 system to edit specific genes in fetal fibroblast cell line and then clone the pigs

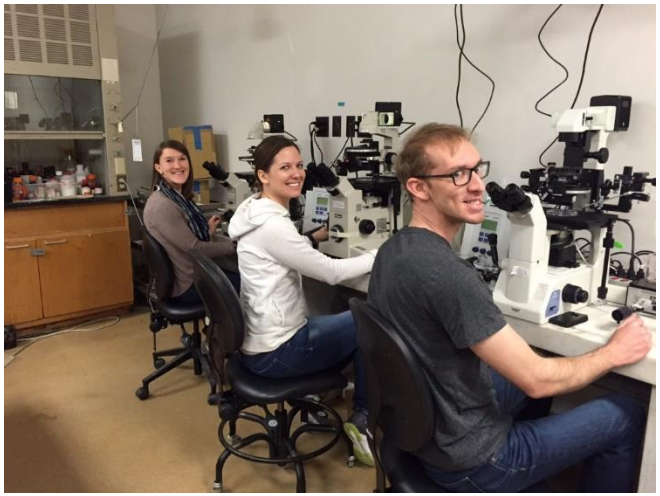




Zygote Injection

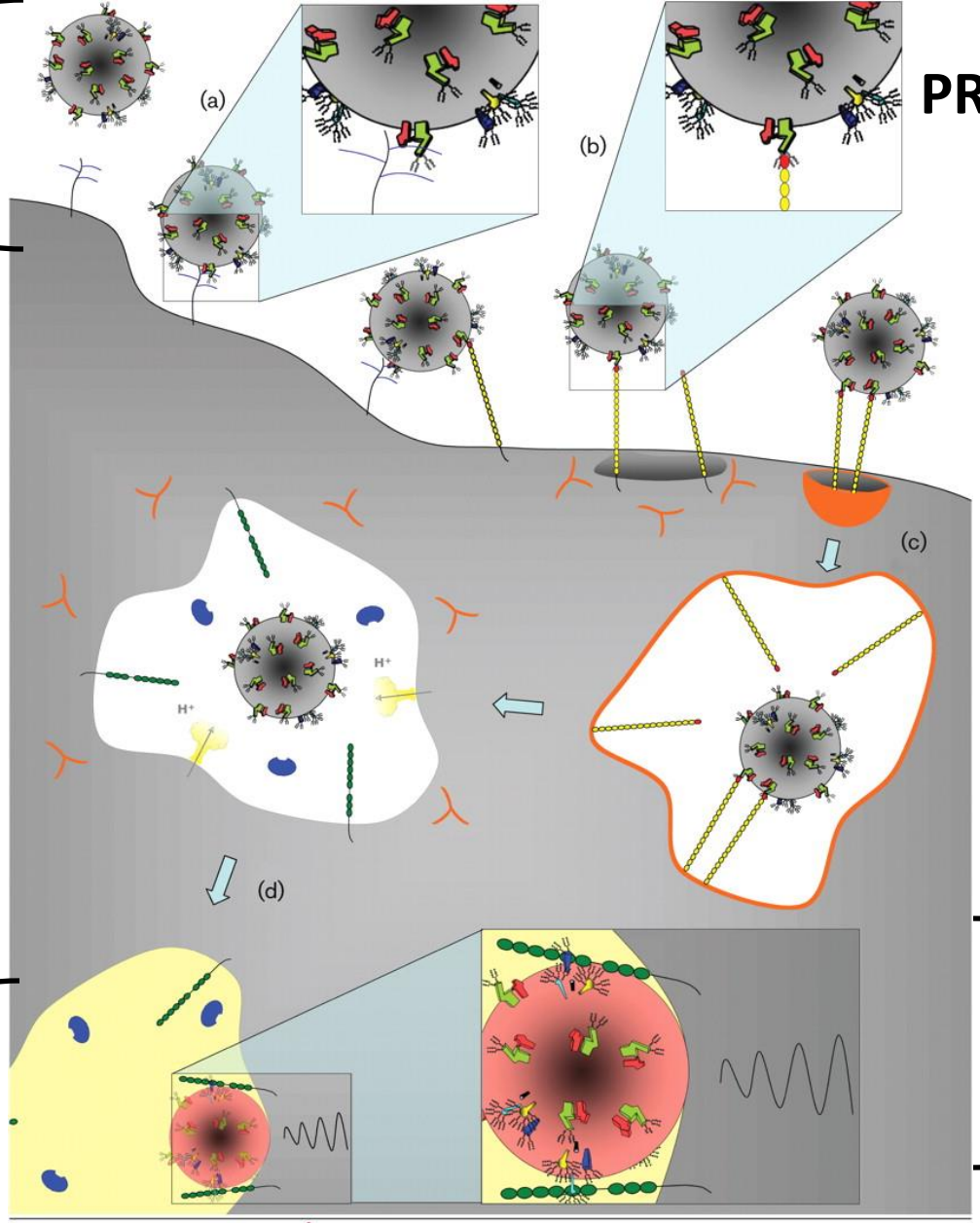
Using the CRISPR/Cas9 system to edit specific genes in pig zygotes





PRSSv Infectivity

Heparan Sulphate Binding



Binding and Internalization by *SIGLEC1*

Uncoating of the virus by *CD163 (SRCR domain 5 specifically)*

Genome Release in endosomes dependent on low pH

Van Breedam et al., 2010

JVI
Journals.ASM.org

An Intact Sialoadhesin (Sn/SIGLEC1/CD169) Is Not Required for Attachment/Internalization of the Porcine Reproductive and Respiratory Syndrome Virus

Randall S. Prather,[a] Raymond R. R. Rowland,[b] Catherine Ewen,[b] Benjamin Trible,[b] Maureen Kerrigan,[b] Bhupinder Bawa,[b] Jennifer M. Teson,[a] Jiude Mao,[a] Kiho Lee,[a] Melissa S. Samuel,[a] Kristin M. Whitworth,[a] Clifton N. Murphy,[a] Tina Egen,[a] Jonathan A. Green[a]

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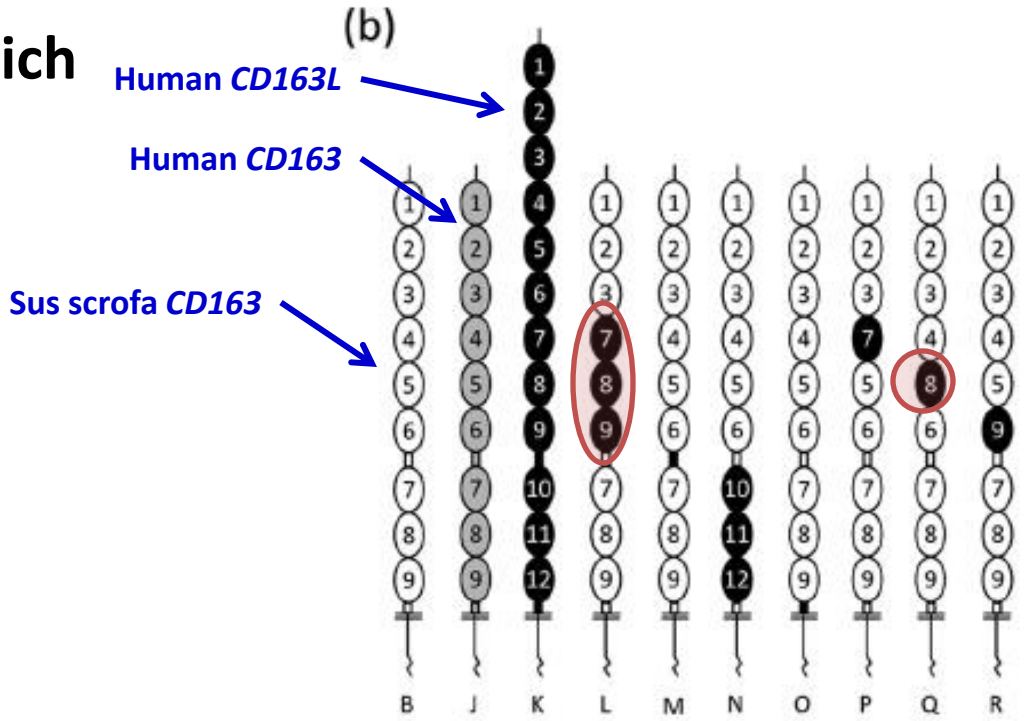
Surface expression of SIGLEC1, also known as sialoadhesin or CD169, is considered a primary determinant of the permissiveness of porcine alveolar macrophages for infection by porcine reproductive and respiratory syndrome virus (PRRSV). *In vitro*, the attachment and internalization of PRRSV are dependent on the interaction between sialic acid on the virion surface and the sialic acid binding domain of the *SIGLEC1* gene. To test the role of SIGLEC1 in PRRSV infection, a *SIGLEC1* gene knockout pig was created by removing part of exon 1 and all of exons 2 and 3 of the *SIGLEC1* gene. The resulting knockout ablated SIGLEC1 expression on the surface of alveolar macrophages but had no effect on the expression of CD163, a coreceptor for PRRSV. After infection, PRRSV viremia in *SIGLEC1*$^{-/-}$ pigs followed the same course as in *SIGLEC1*$^{-/+}$ and *SIGLEC1*$^{+/+}$ littermates. The absence of SIGLEC1 had no measurable effect on other aspects of PRRSV infection, including clinical disease course and histopathology. The results demonstrate that the expression of the *SIGLEC1* gene is not required for infection of pigs with PRRSV and that the absence of SIGLEC1 does not contribute to the pathogenesis of acute disease.

CD163?

CD163

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily

CD163 Deletion constructs showed that extracellular domain 5 encoded by exon 7 was important for PRSSRv infectivity



Van Gorp et al., 2010

Two Proposed Targeting Approaches

- Traditional knockout by NHEJ
  - INDEL changes reading frame
  - Premature stop codon
- Domain Swap
  - Remove extracellular domain SRCR5 from *CD163*
  - Replace with extracellular domain 8 from h*CD163L* (exon 11) mimic
  - Maintain CD163 function

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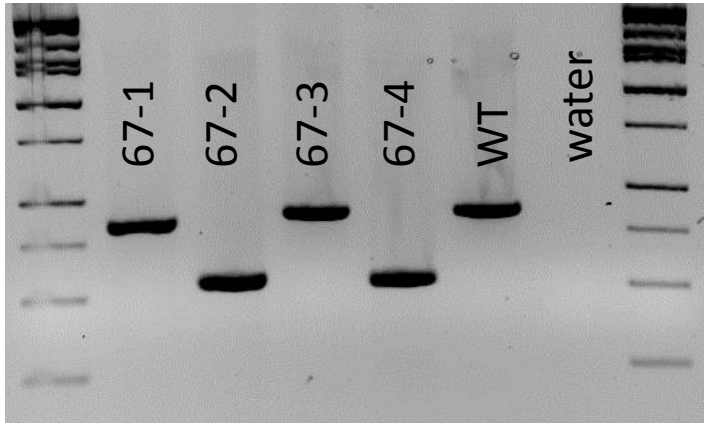
A



B

C CD163 exon 7 modifications

WT	TGCTGTGCAGGGAACTACAGTGCGGCACTGTGGTTTCCCTCCTGGGGGG
#67-1	TGCTGTGCAGGGAACT----------CTGTGGTTTCCCTCCTGGGGGG ★
#67-2	-(Δ124 bp)----------CTGTGGTTTCCCTCCTGGGGGG -(Δ123 bp)----------ACTGTGGTTTCCCTCCTGGGGGG
#67-3	TGCTGTGCAGGGAACTACAGTGCGGC**A**ACTGTGGTTTCCCTCCTGGGGGG
#67-4	-(Δ130 bp)----------TCCTGGGGGG -(Δ132 bp)----------CTGGGGGG

100% of piglets born had an edit in exon 7 of the *CD163* gene

★ **This boar 67-1 is almost four years old**

Whitworth et al., 2014

Are CRISPR/Cas9 *CD163* edited pigs resistant to PRRSv?

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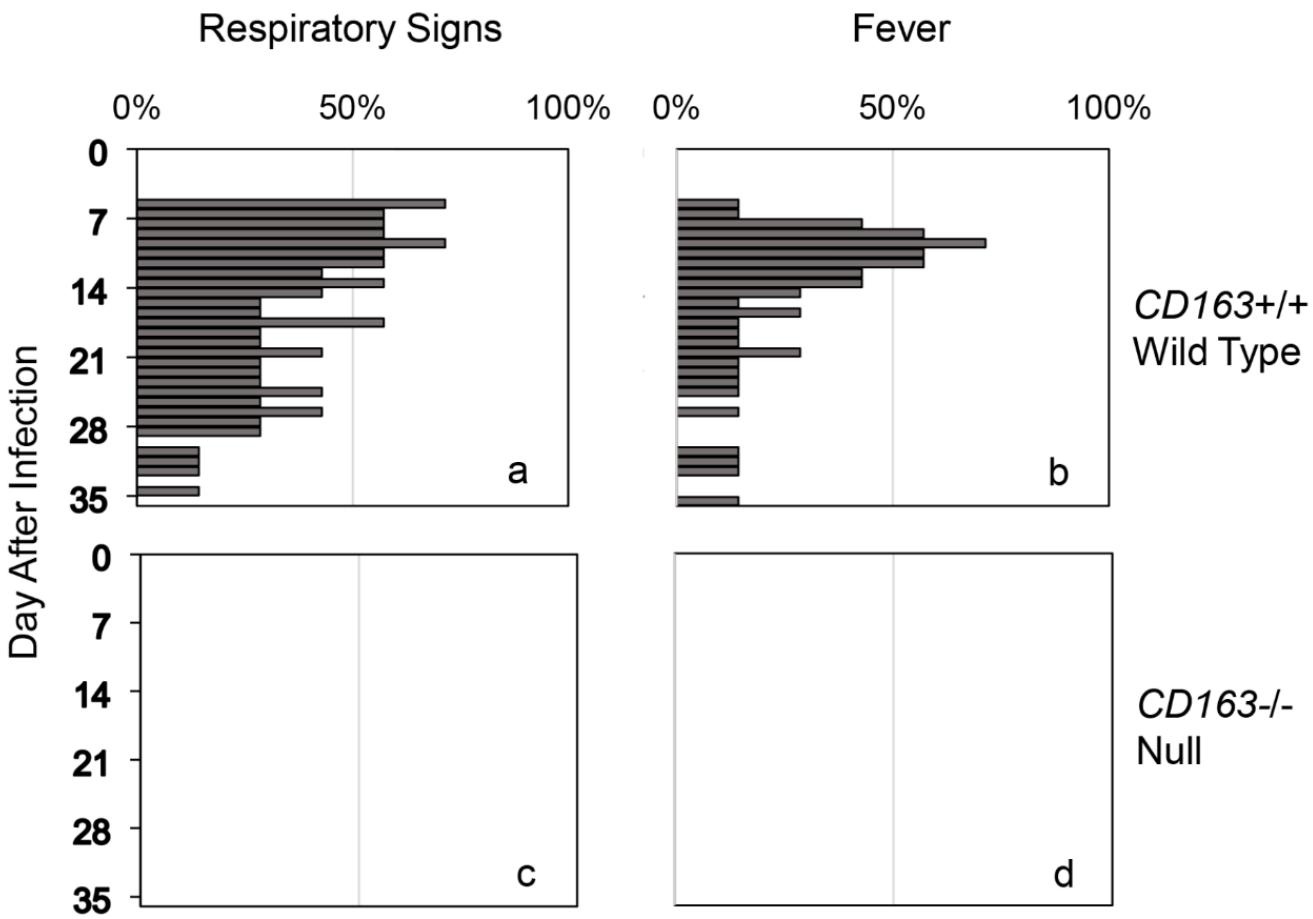
Weaned wildtype and *CD163* edited piglets prior to transport to Kansas State University.

NVSL 97-7985

Whitworth et al, ‘16
Nature Biotechnology

College of Agriculture Food and Natural Resources

Clinical Signs



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Whitworth et al, '16
Nature Biotechnology

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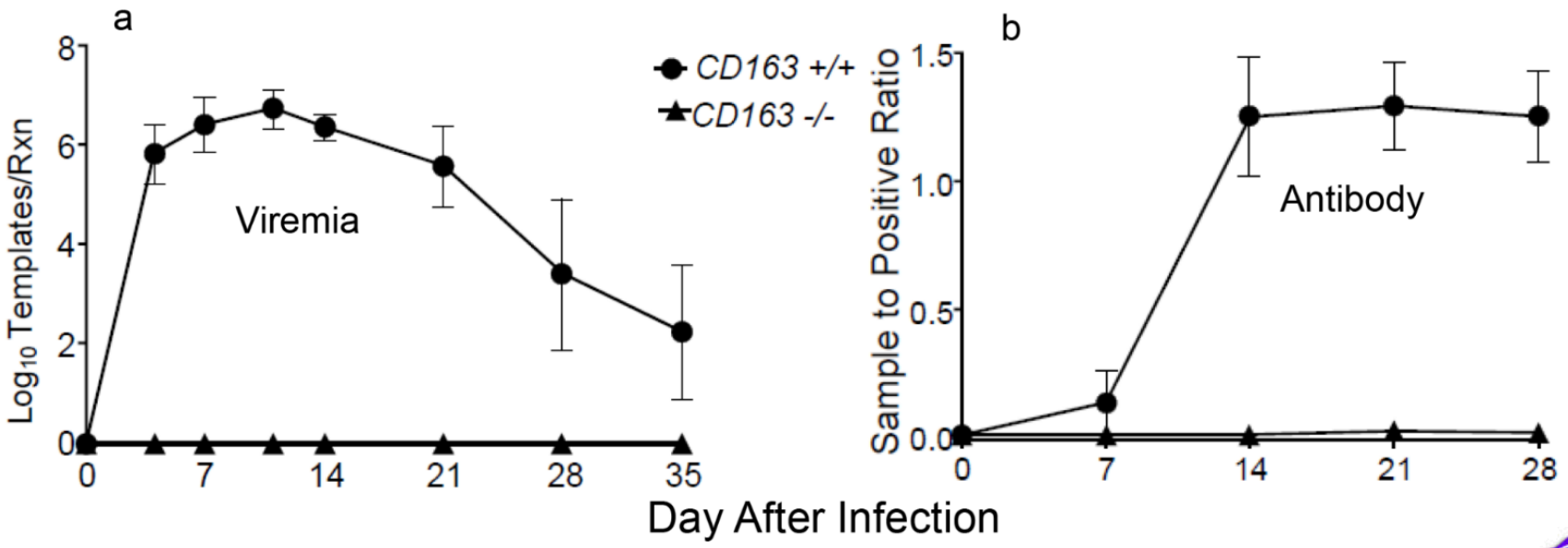
Viremia and Antibody



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Whitworth et al, '16
Nature Biotechnology

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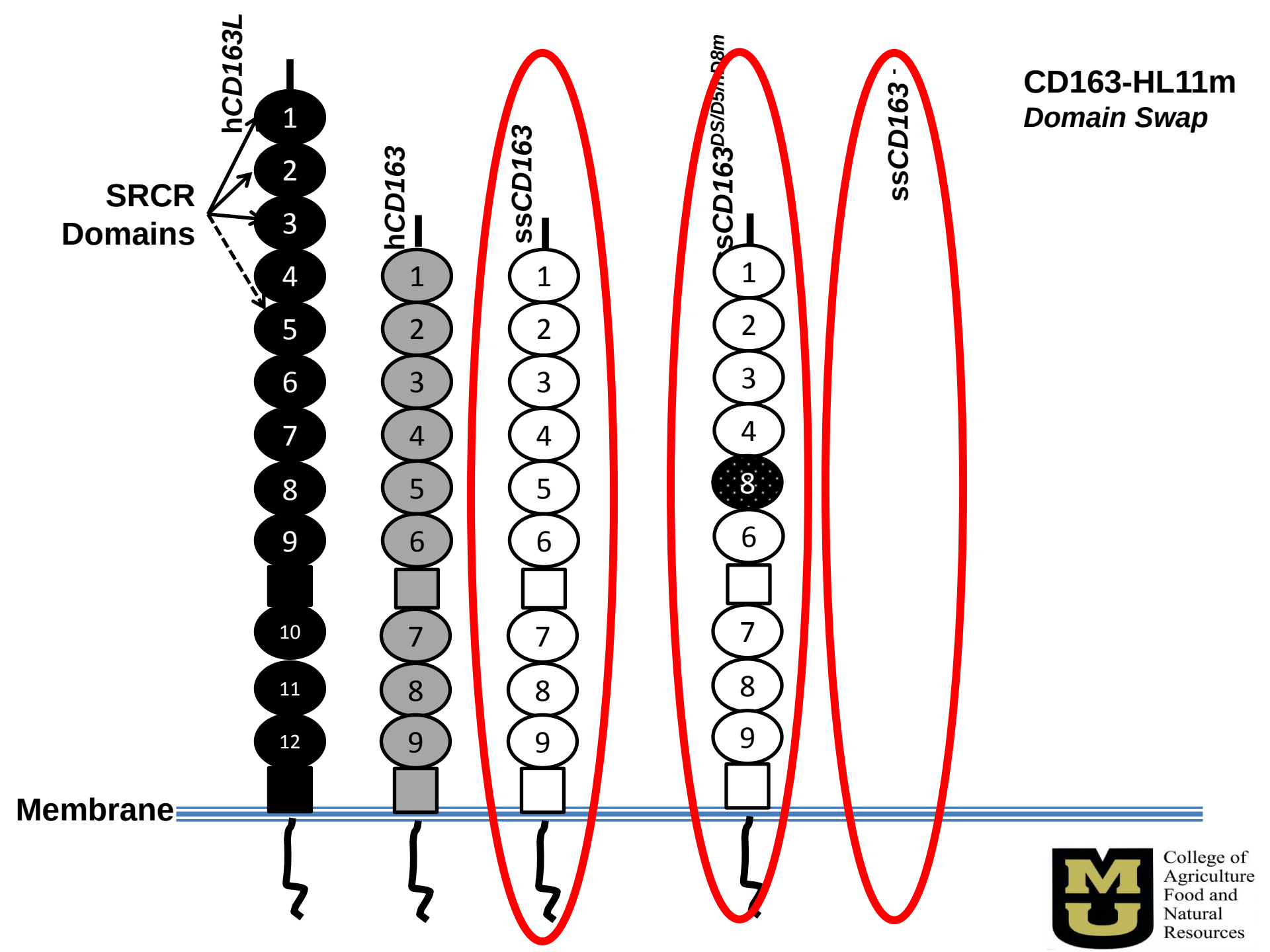
What about other PRRSv isolates?

- Initial CD163 knock-out study challenged with a North American (Type 2) (NVSL 97-7985)
- What about other Type 2 viruses?
- What about European (Type 1) viruses?

Wells et al., 2016



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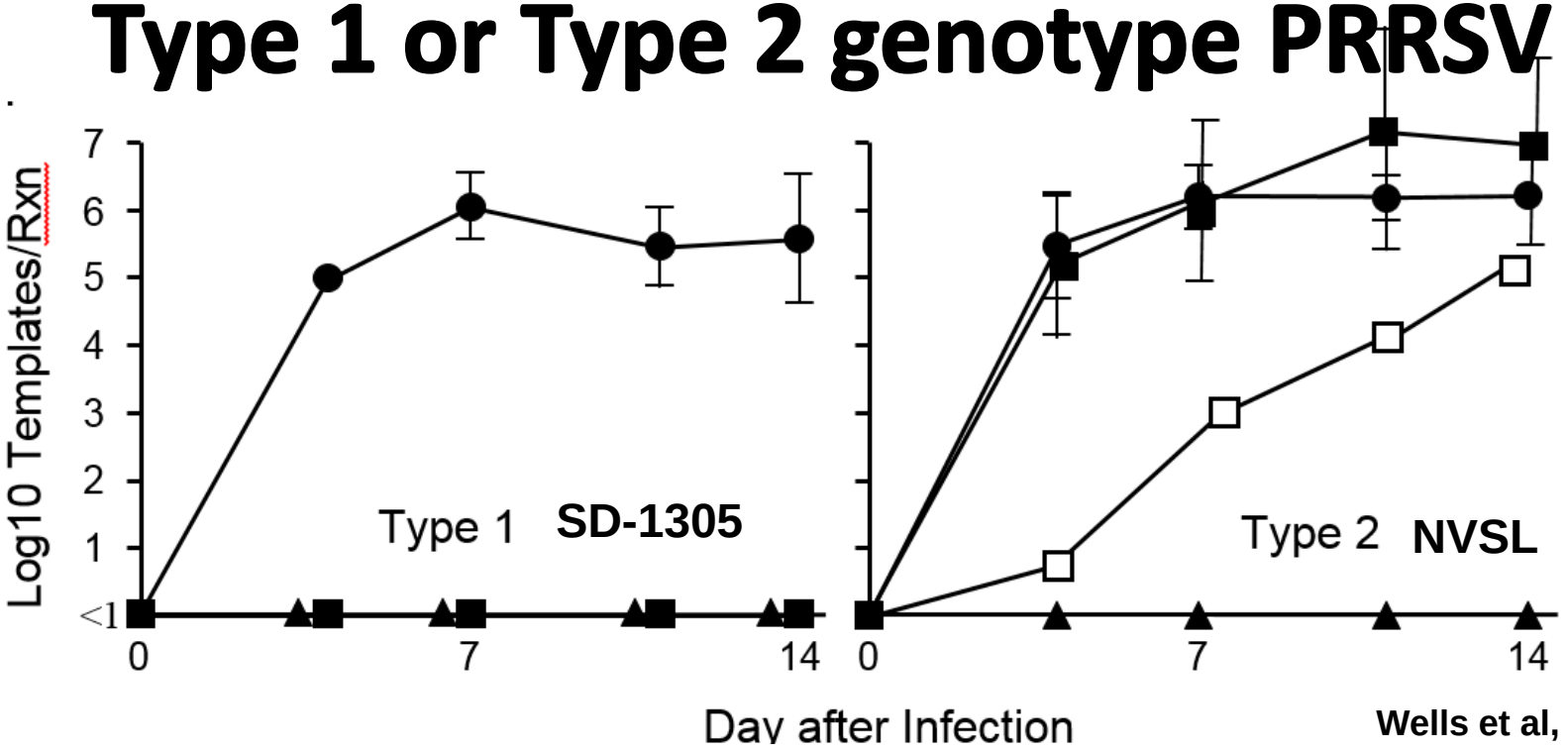
		Source of Macrophages				
Type 1		**WT**		**Domain Swap**		**Null**
13-15		56 +/-9		0		0
Lelystad		62 +/-15		0		0
03-1059		50 +/-18		0		0
03-1060		61 +/-12		0		0
01-08		64 +/-20		0		0
4353-PZ		62 +/-15		0		0
Type 2						
NVSL 97		59 +/-15		8 +/-8		0
KS-06		56 +/-20		12 +/-9		0
P129		64 +/-11		8 +/-6		0
VR2332		54 +/-5		6 +/-3		0
CO 10-90		43 +/-18		8 +/- 8		0
CO 10-84		51 +/-22		7 +/-4		0
MLV-ResP		55 +/-12		3 +/-1		0
KS62		49 +/-3		10 +/-11		0
KS483		55 +/-23		6 +/-3		0

***Results are presented as percent infected PAMs (n=3: mean±S.D.)**

Infection of CD163 modified pigs with Type 1 or Type 2 genotype PRRSV



Wells et al, Submitted

WT (circles), HL8 (squares) and Null (triangles) CD163 modified pigs were infected with a contemporary Type 1 isolate, SD-1305 or a Type 2 isolate, NVSL.

The open box shows viremia for the HL11m pig#101. The number pigs in each group for the Type 1: WT n=4, HL11 n=5 and null n=3; and for the Type 2: n=4 for WT, n=4 for HL11 and n=3 for the null pigs.

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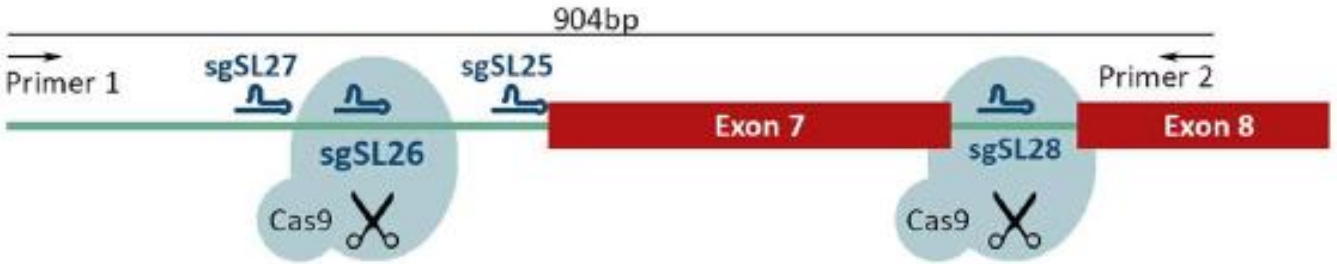
PLOS | PATHOGENS

RESEARCH ARTICLE

Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function

Christine Burkard[1], Simon G. Lillico[1], Elizabeth Reid[2], Ben Jackson[2], Alan J. Mileham[3], Tahar Ait-Ali[1], C. Bruce A. Whitelaw[1], Alan L. Archibald[1]*

1 The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, United Kingdom, 2 The Pirbright Institute, Ash Road, Pirbright, Woking, United Kingdom, 3 Genus plc, DeForest, Wisconsin, United States of America



Clean deletion of domain 5 (exon 7) also confers resistance to both Type 1 and Type 2 PRRSV (Burkard et al., 2017)

CRISPR/Cas9 Editing of CD163

- CRISPR/Cas9 can be used to efficiently create biallelic edits in CD163
- Protects young growing pigs from PRRSV
  - Both type 1 and type 2 isolates
- Protects both pregnant pigs and the fetuses from PRRSV

African Swine Fever

SCIENTIFIC REPORTS

OPEN

Mammalian interspecies substitution of immune modulatory alleles by genome editing

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Published: 22 February 2016

Simon G. Lillico[1], Chris Proudfoot[1], Tim J. King[1], Wenfang Tan[1], Lei Zhang[2], Rachel Mardjuki[2], David E. Paschon[2], Edward J. Rebar[2], Fyodor D. Urnov[2], Alan J. Mileham[3], David G. McLaren[3] & C. Bruce A. Whitelaw[1]

We describe a fundamentally novel feat of animal genetic engineering: the precise and efficient substitution of an agronomic haplotype into a domesticated species. Zinc finger nuclease in-embryo editing of the RELA locus generated live born domestic pigs with the warthog RELA orthologue, associated with resilience to African Swine Fever. The ability to efficiently achieve interspecies allele introgression in one generation opens unprecedented opportunities for agriculture and basic research.

Edited the RELA locus in domestic pig embryos with ZFNs to convert to the ASF resistant warthog RELA
Not challenged yet

Lillico et al., 2016

Virology 501 (2017) 102–106

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Genetically edited pigs lacking CD163 show no resistance following infection with the African swine fever virus isolate, Georgia 2007/1

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ARTICLE INFO

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African swine fever
Virus
Receptor
CD163

ABSTRACT

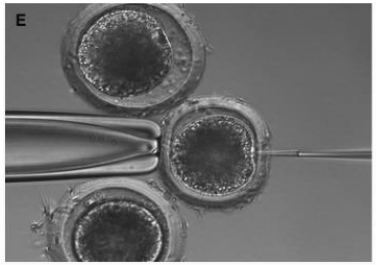
African swine fever is a highly contagious, often fatal disease of swine for which there is no vaccine or other curative treatment. The macrophage marker, CD163, is a putative receptor for African swine fever virus (ASFV). Pigs possessing a complete knockout of CD163 on macrophages were inoculated with Georgia 2007/1, a genotype 2 isolate. Knockout and wild type pen mates became infected and showed no differences in clinical signs, mortality, pathology or viremia. There was also no difference following in vitro infection of macrophages. The results do not rule out the possibility that other ASFV strains utilize CD163, but demonstrate that CD163 is not necessary for infection with the Georgia 2007/1 isolate. This work rules out a significant role for CD163 in ASFV infection and creates opportunities to focus on alternative receptors and entry mechanisms.

CD163 pigs are not resistant to African Swine Fever

Popescu et al. 2017

College of Agriculture Food and Natural Resources

Swine Flu



Transgenic Research
February 2017, Volume 26, Issue 1, pp 97–107

Zygote injection of CRISPR/Cas9 RNA successfully modifies the target gene without delaying blastocyst development or altering the sex ratio in pigs

Authors | Authors and affiliations

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TMPRSS2- transmembrane protease, serine 2

Host cell protease that cleaves a glycoprotein on the virus surface that activates the influenza virus

Whitworth et al., 2017

Pig Management
Melissa Samuel
Jason Dowell
Sabrina Hammond
Elizabeth Queathem
Teagan

Genotyping
Monica Witzke
Mykel Anderson

Zygote Injections, Somatic Cell Nuclear Transfer and IVF
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Joshua Benne
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Kansas State University
Bob Rowland
Maureen Kerrigan

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Kevin Wells

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