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Technical considerations towards commercialization of porcine respiratory and reproductive syndrome (PRRS) virus resistant pigs

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Abstract

The selection and introduction of disease resistance genes in livestock not only provide health benefits to animals but opportunities for breeders and farmers to meet the growing demand for high-quality meat and milk while reducing agriculture's footprint on the environment. As traditional methods of classical breeding and selection for trait improvement are slow, recent progress in several areas of biology including (a) understanding host–pathogen interactions, (b) inexpensive and rapid DNA sequencing, and (c) robust gene editing like CRISPR-Cas provide geneticists tools to accelerate discovery and deployment of disease resistance alleles in livestock. Using these advances, the introduction of resistance genes into commercially relevant germplasm requires access to genetically superior livestock, an infrastructure for scalable allele deployment, freedom to operate, global regulatory approvals, and acceptance of gene edited livestock by producers and consumers. Importantly, academic researchers have recently discovered that modification of the CD163 gene in pigs can confer resistance to the virus that causes porcine reproductive and respiratory syndrome (PRRS). While this achievement represents a major step towards solving an important disease in livestock, to realize the positive impact on animal health while benefiting the pork industry and consumers, it is necessary to introduce this recessive disease resistance allele into commercial breeding populations. Rather than backcrossing the resistance gene from a few non-commercial founders, as a global supplier of high genetic merit livestock genetics, Genus plc and its pig division PIC (Pig Improvement Company) with Genus R&D have mobilized advances in reproductive biology, gene editing, DNA sequencing, and bioinformatics to simultaneously generate and introduce a single modified CD163 allele across four genetically diverse porcine lines of commercial importance that prevents PRRS virus (PRRSV) infection. This report focuses on technical aspects for a scaled gene editing program to consider for rapid and efficient generation and advancement of a small population of non-transgenic founder pigs for commercial breeding. This high genetic merit herd containing a PRRS disease resistance allele will provide important benefits to animal health and food chain value once approved for commercial sale and export.

Keywords: Pig, Disease resistance, PRRS, CD163, Gene editing, Commercialization

Introduction

Since the early domestication of plants and animals approximately 10,000 years ago, humans have incrementally increased food production, quality, and improved disease resistance, while simultaneously reducing labor input and food costs. Commonly these gains have been

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realized through a process called selective breeding, which relies on genetic variation caused by a combination of newly formed random mutations and the shuffling of existing variation through recombination. Significantly, in contrast to genetic gains observed through plant breeding, new allelic variation necessary to improve animal health traits has been slow mainly due to relatively small, highly-related populations and long reproductive cycles associated with livestock. Despite this difference, important positive cultural changes in society have benefited from the adoption of improved breeding techniques which continue to increase genetic performance in plants and animals to keep pace with population growth. Today, agriculture has realized a great step forward with the adoption of various technologies such as marker-assisted selection, high-throughput phenotyping and data analytics which enable the screening of large populations in different environments and under different stress conditions. Moreover, only with the incorporation of improved agricultural methodologies like high quality, inexpensive DNA sequencing, gene editing, comparative gene networks, advanced bioinformatic tools and genomics will genetics companies be able to keep pace with increased demand for high quality oils and proteins from plants, milk, meat and eggs.

Although slowed by livestock's long lifecycles and reduced genetic variability due to low numbers of recombinant progeny, animal traits have improved through classical breeding across many species including cattle and pigs. Selective breeding in dairy cows has more than doubled milk production (from 5000 kg to nearly 11,000 kg) over the past six decades, while the total number of dairy cows in the United States has been dramatically reduced (Capper et al. 2009; Capper and Cady 2007). Similarly, continued improvements in porcine genetics have led to improved feed conversion, faster growth rates and larger litter sizes which functionally translate to a reduction in animal numbers while increasing food production and driving down costs to the farmer and consumer (Tokach et al. 2016). However, despite these gains, intensive selection for traits yielding gains in milk production, feed conversion, and meat quality are often mirrored by reciprocal losses in fitness functions. For any livestock breeding program, ignoring the consequences of genetic selection programs that trade increased production performance for decreases in animal health can be detrimental in terms of societal acceptability and commercial credibility. A poorly understood biological system with possible internal trade-offs is disease resilience. Disease resilience in the context of livestock production has been described as "the ability of a host animal to maintain a reasonable level of productivity when challenged by infection" [after (Albers et al. 1987)]. Disease resilience captures two complementary host defense mechanisms against pathogens: resistance, i.e., the ability of a host animal to limit its within-host pathogen burden, either by preventing infection in the first place or by inhibiting within-host pathogen replication: and tolerance, i.e., the ability of an infected host to limit the damage caused by a given within-host pathogen load. Råberg et al. (Råberg et al. 2009) noted that "in the agricultural sector, attempts to select for increased yield in the face of parasite challenge may come to nothing (or even make things worse) if there is a trade-off between resistance and tolerance", i.e. if the genetic correlation between these traits is unfavorable. In that case, a breeding program that selects gradually for improved host resistance faces the risk of gradually decreasing host tolerance (and thus potentially also decreasing host resilience). These consequences are intensified if the pathogen co-evolves successfully to neutralize host resistance. One-sided gradual selection for increased resistance under unfavorable genetic correlations will then paradoxically lead to reduced tolerance, higher infection load and neutralized resistance, and therefore to reduced resilience. Similarly, one-sided selection for increased tolerance may achieve a similar reduction in disease resistance.

One way to avoid such scenarios would be to increase resistance or tolerance (both continuous traits) not gradually but completely, preferably in a few rapid modification steps. Completely resistant animals do not need any tolerance to achieve high performance levels under infectious challenge; and likewise, for completely tolerant animals the level of the pathogen load is irrelevant. In a gradual selection scenario, such rapid modification steps would require a host–pathogen mechanism with a high host heritability and a high prediction accuracy, so that results would be achieved quickly. This is most easily realized for traits that are controlled by a single gene, as exemplified by successful use of marker-assisted selection for resistance to (i) transmissible spongiform encephalopathy (scrapie) in sheep, (ii) *Escherichia coli* F4 and F18 in pigs, and (iii) infectious pancreatic necrosis (IPN) in Atlantic salmon. However, most resilience mechanisms are decidedly polygenic (Knap and Doeschl-Wilson 2020). An alternative route to achieve complete resistance or tolerance would be to move away from the gradual selection approach, and exploit (i) detailed biological knowledge of the relevant resilience mechanism and (ii) novel genomic technology to manipulate host response to pathogen challenges. Importantly, the adoption of new breeding practices that incorporate gene editing hold the potential to improve livestock health and solve current and emerging diseases through the discovery and

targeted modification of host genes capable of conferring resistance to viral and bacterial diseases.

To that end, the opportunity to solve an important livestock disease in pigs has recently presented itself through work by several academic groups. Porcine reproductive and respiratory syndrome (PRRS), is a panzootic infectious disease of pigs whose infectious agent is an enveloped, positive single-stranded RNA virus. PRRS emerged simultaneously in the late 1980s in the US and Germany, with a mortality rate of nearly 20%. While PRRS manifests in pigs of all ages, the disease primarily causes late-term abortions and stillbirths in sows and respiratory disease in piglets (Lunney et al. 2010). It cannot be overemphasized that PRRS virus-infected pigs suffer; weight loss, fever, respiratory distress, lethargy, depression, and reproductive failure are a few clinical manifestations in addition to mortality. PRRS infection results in a compromised respiratory immune system, which can lead to increased severity of any secondary infections and is commonly treated with antibiotics. While the disease is devastating to animals, pig producers also suffer emotionally and economically, with annual costs of > 650 million USD in the USA and 1,5 billion € in Europe (Holtkamp et al. 2013) where global producers generally assume that 60–80% of these production herds are infected (de Paz 2015). Until now, one strategy to treat PRRS infected herds has been through the administration of either modified live virus or killed virus vaccination. However, like other RNA viruses, PRRS viruses have a high rate of evolution primarily based on an error-prone viral RNA-dependent RNA polymerase and a significant rate of genetic recombination (Hanada et al. 2005; Kappes et al. 2013). Thus not surprisingly, vaccines have a limited range effectiveness (Chae 2021).

Through early reconstitution experiments in non-porcine cells, the porcine macrophage surface receptor, CD163, was shown to be capable of mediating infection of these otherwise PRRS virus non-permissive cell lines in culture (Calvert et al. 2007; Van Gorp et al. 2010). CD163 is a monocyte- and macrophage-expressed scavenger receptor found in mammalian cells. The CD163 gene (> 37 kb) in pigs encodes an approx. 1115 amino acid polypeptide (130 kDa) consisting of a signal peptide, 9 tandem cysteine-rich scavenger receptor superfamily (SRCR 1–9) domains, a C-terminal transmembrane domain and an intracellular cytoplasmic tail. CD163 not only exists as a membrane-bound protein, but can be found in a soluble form in plasma and other tissues (Etzerodt et al. 2017). Extensive studies in mice and humans have demonstrated that the CD163 receptor protein is responsible for endocytosis of hemoglobin:haptoglobin (Hb:Hp) complexes and, while serving as a viral entry point as described above, promotes the recognition and killing of bacterial pathogens like *Staphylococcus aureus* (Kneidl et al. 2012). Moreover, CD163 macrophage expression has been shown to be differentially regulated in response to inflammation (Etzerodt and Moestrup 2013).

As mentioned, cell studies demonstrated the requirement of the CD163 protein for PRRS virus infection. The importance of CD163 in animals for PRRS infection was verified by the use of gene editing technologies demonstrating that pigs containing a homozygous modified CD163 allele were resistant to infection with genotype 1 or genotype 2 (Type I and Type II) PRRSV (Whitworth et al. 2015; Burkard et al. 2017, 2018; Wells et al. 2017; Yang et al. 2018; Chen et al. 2019; Guo et al. 2019; Wang et al. 2019). These experiments yielded healthy, PRRSV resistant pigs through directing a variety of sequence changes into the CD163 gene, that either abolished or modified the function of the CD163 protein; no clinical signs (fever, cough, lethargy), no lung pathology and no viremia or PRRS coat protein antibody response were reported (Whitworth et al. 2015; Wells et al. 2017; Burkard et al. 2018; Yang et al. 2018). Importantly, Prather et al. (Prather et al. 2017) showed that dams with di-allelic CD163 knockout mutations were able to protect fetuses from PRRSV infection. Together these studies point to the important emergence of gene editing as a tool to discover and develop disease resistance traits in livestock, as DNA screens of 35,000 pigs of diverse genetic backgrounds did not identify potential knockout variants in the CD163 sequences examined (Johnsson et al. 2018). While developing positive selection screens for PRRSV resistant pigs through classical breeding may be an achievable target, such selection strategies are very inefficient for commercial applications as compared to alternatives like directed allele modification using gene editing tools. Further, real solutions to improve livestock health have been achieved through basic research and demonstrated by academic laboratories through the successful use of gene editing to overcome disease in animals (Whitworth et al. 2018; Luo et al. 2019; Xu et al. 2020). In addition to PRRS, other viral diseases have been successfully mitigated. Transmissible gastroenteritis virus (TGEV), caused by an alphacoronavirus, results in high morbidity and mortality in neonatal pigs by infecting and killing intestinal epithelial cells. However, pigs containing homozygous knockout mutations in porcine amino peptidase N (ANPEP) introduced by gene editing were shown to resist infection by TGEV and harbor no virus in enterocytes lining the ileum (Whitworth et al. 2018). The ability to rapidly introduce new allelic variants into cells provides a tremendous tool for mode of action discovery of other important livestock diseases like African Swine Fever, swine influenza, classical swine fever, and

Porcine Epidemic Diarrhea (PED) in pigs, and bovine respiratory disease (BRD) and mastitis in cattle, providing an opportunity to apply gene editing to eradicate disease and improve animal health.

Gene editing tools

In contrast to the generation and selection of random de novo mutations in the genome for improving disease resistance in livestock, the ability to specifically target candidate genes to introduce allelic variation for discovery and commercial breeding has the potential to revolutionize gains in animal health. Historically, targeted sequence variation has been accomplished through the use of molecular tools which introduce breaks in DNA and promote targeted mutagenesis including Zinc-finger nucleases (ZFNs), customized homing endonucleases (meganucleases), and transcription activator-like effector nucleases (TALENs) (Carroll 2014; Stella and Montoya 2016). However due to its simplicity, and its ease of programmability and use, the recently discovered CRISPR-Cas system has revolutionized gene editing across prokaryotes and eukaryotes (Jinek et al. 2012, 2013; Cong et al. 2013; Hsu et al. 2013, 2014; Doudna and Charpentier 2014). The backbone of this technology emerged from early studies using *Streptococcus thermophilus* bacteria for yogurt production (Barrangou et al. 2007; Deveau et al. 2008; Horvath and Barrangou 2010; Makarova et al. 2011; Sapranauskas et al. 2011; Gasiunas et al. 2012). While many CRISPR-Cas systems (Type I-VI) with different mechanisms have been described in bacteria and archea (Chylinski et al. 2014; Koonin et al. 2017; Makarova et al. 2020), functionally, to generate site-specific DSBs in DNA, *S.thermophilus* and *S.pyogenes Cas9* systems minimally require a Cas9 protein and a duplex of CRISPR RNA (crRNA), a trans-activating CRISPR RNA (tracrRNA), and the presence of a protospacer adjacent motif (PAM) which flanks the 3' end of the crRNA-targeted sequence (Gasiunas et al. 2012; Cong et al. 2013). PAM sequences are required and serve as the binding sites for the RNA-guided nucleases through protein-DNA contact (Nishimasu et al. 2014, 2015; Yamano et al. 2016). For the SpCas9 system, fusion of the crRNA and tracrRNA into a single guide RNA (gRNA) molecule has further simplified the system, consisting now of two instead of three components, but also significantly improved the frequency of genomic DNA cleavage (Jinek et al. 2013; Mali et al. 2013).

Since the discovery of *Spy and Stherm* Cas9s, other RNA-guided nucleases have emerged having PAM recognition sequences other than 5'-NGG-3' or 5'-NGGNG-3' (N = A, G, T or C), respectively. Distinct from these G-rich PAMs, the well characterized *Staphylococcus aureus* Cas9 (SaCas9) recognizes 5'-NNGRRT-3' (R = A or G) PAM sequence, (Ran et al. 2015), while the CPF1 class of nucleases from *Acidominococcus* and *Lachnospiraceae* recognizes a T-nucleotide rich PAM, 5'-TTTV-3'(Zetsche et al. 2015). Other unique features of Cpf1when compared to Cas9 include (i) the use of a single crRNA guideRNA and (ii) generation of staggered overhanging nucleotides at a PAM-distal target site whereas Cas9 generates a blunt end cut between the third and fourth bases 5' to the PAM site. Since 2012, other nucleases (Liu et al. 2019; Karvelis et al. 2020) and hybrid proteins, which fuse diverse functional domains to catalytically inactive Cas proteins, have expanded the scope and capabilities of genome editing tools (Anzalone et al. 2020).

Applications

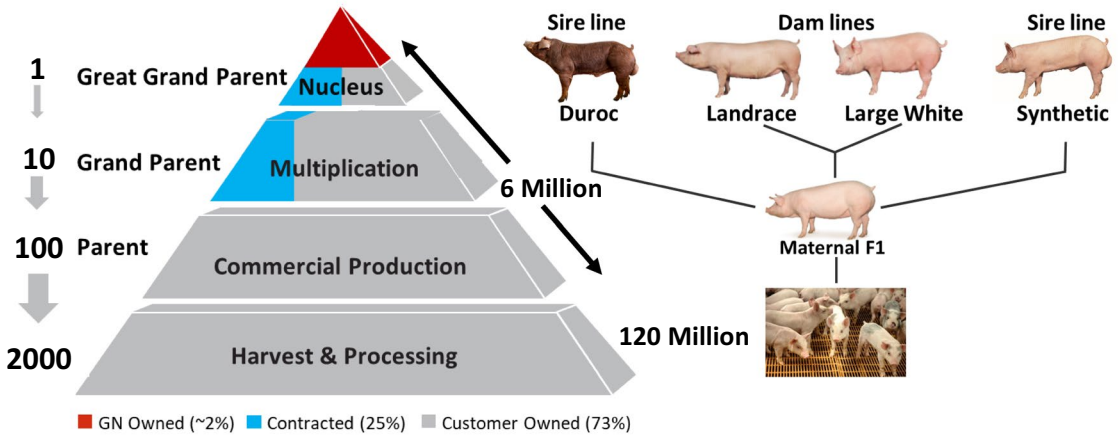
Thus, in contrast to ZFNs, meganucleases, and TALENs which require sophisticated protein engineering to establish specific target site recognition (Smith et al. 2000, 2006; Arnould et al. 2006; Maeder et al. 2008; Boch et al. 2009; Weber et al. 2011), the ease of programmability, simplicity and cost-effectiveness associated with this class of RNA-guided nucleases have significantly advanced medical and agriculture discovery and applications. To that end, since 2012, a spectrum of human clinical applications have emerged, including successful treatment of sickle cell disease (Frangoul et al. 2020) and the historic transplant of a modified pig heart into a human patient with terminal heart disease (Reardon 2022). Similarly, deployment of RNA-guided nucleases has positively improved disease resistance traits across a variety of plants (Wang et al. 2014; Nekrasov et al. 2017; Kim et al. 2019; Li et al. 2019) and led to production efficiency gains in germplasm conversion (Gao et al. 2020a, b), and trait stacking (Gao et al. 2020a, b) in maize. Together, programmable nucleases have clearly demonstrated their utility towards beginning to solve many human diseases and increase crop yields by reducing pathogen-mediated loss. When compared to crop improvement, adoption of this technology to mitigate disease loss at the commercial level in livestock has proved more difficult primarily due to the associated costs with animal agriculture, long generation intervals, small populations and lack of entrants willing to participate due in part to public acceptance and regulatory uncertainties. Setting those challenges aside, as mentioned earlier, several academic groups have demonstrated disease resistance proof-of-concept experiments in pigs, while gene-edited sea bream and pufferfish have recently been accepted in Japan for commercial production and public consumption (Kato-Unoki et al. 2018; Kishimoto et al. 2018).

In contrast to trait improvement in fish, translating a robust disease resistance trait in pigs has unique

challenges specifically related to the tiered pyramid structure of commercial pig production and pig breeding (Fig. 1). The conventional pig breeding and production process involves a series of four-tiered generations: nucleus, multiplier, commercial, and harvest, top to bottom, respectively. The generation interval of the pig is at least one year, so it takes at least 4 years to disseminate genetic progress from the nucleus to consumers. At the top of the pyramid is the "nucleus" level, where purebred great-grandparent (GGP) breeding stock is maintained, and within-line genetic improvement takes place via selection on breeding values estimated for any relevant recorded traits. The next tier downward (multiplier), is where purebred grandparent (GP) lines are produced and combined to produce a crossbred parent sow (maternal F1). At the commercial tier of the pyramid, crossbred parent sows are mated to purebred (sometimes crossbred) parent boars to produce three-way (and sometimes four-way) crossbred pigs for harvest and meat processing. The pyramidal structure, with its gene flow from top to bottom, reflects that the actual number of animals in each tier becomes progressively larger from top to bottom. Crossbreeding is practiced for two reasons: first, it invokes heterosis ("hybrid vigor") in the progeny exploiting dominance genetic effects that lead to increased performance mainly in traits with low heritability such as fertility and robustness; second, crossbreeding allows for line specialization: different purebred lines can be selected for different breeding goals so that combining them together leads to genetic synergy at the progeny level. Typically, the breeding goals of maternal lines are dominated by fertility traits, while paternal lines are focused on growth and carcass efficiency. For more detail on conventional pig breeding structures see Dekkers et al. (2011).

Scaled gene modification

Genetic suppliers function primarily at the nucleus or top tier of the production pyramid and provide multipliers and producers with superior genetics primarily through semen distribution, as this process is an efficient method to reduce cost while maximizing biosecurity. Reflecting



Landrace: Maternal line with large floppy ears, a multipurpose breed with low back fat but superior maternal genetics in the form of high milk production and a larger number of teats that can support large litters.
Large White: Yorkshire maternal line with short erect ears, moderate growth and back fat, a larger number of teats, and a high farrowing capability.
Maternal F1: The product of a Landrace-Large White cross. Used for crossing with terminal sires.
Synthetic terminal sire: Line consisting of a mixture of Pietrain, Large White, Hampshire, and Duroc breeds. Very fast-growing pig with high feed conversion and high meat quality.
Duroc: A terminal sire line characterized by its red color and slightly drooping ears. Durocs grow fast and have excellent meat quality.

Fig. 1 Pork production is a tiered multiplication and breeding pyramid. Diagram of the steps of pig production starting at the nucleus, multiplier, producer and final commercial pig for consumption. Each step of the pyramid is approximately 1 year and multiplication factor for each generation shown on the left. The red portion of the pyramid that is owned and operated directly by genetic nucleus (GN) providers; blue section represents independent producers under contract with genetic providers for sale to other producers; and gray portion represents customer operations for their internal use. According to the USDA, in March 2022, there were 72. 2 million pigs in production, producing approximately 120 million for processing annually (https://www.nass.usda.gov/Newsroom/2022/03-30-2022.php). Six million pigs make up the breeding population consisting of nucleus, multiplication and commercial levels. Maternal (dam lines) and paternal (sire lines) are shown on the right of the pyramid and describe at the bottom of the figure

on the process of genetic selection, multiplication and the production pyramid of pig breeding described, the deployment of PRRSV resistance traits using gene editing in a commercial environment must (i) take place within the backdrop of continuous genetic improvement and distribution through conventional production practices and, (ii) due to the recessive nature of this particular allele, requires that maternal and paternal lines maintain the modified CD163 allele in a homozygous state. Rather than backcrossing from a few (2–10) gene edited founders derived from non-elite porcine genetics, in an attempt to keep pace with genetic improvement at the nucleus level and accelerate commercialization of this new disease resistance allele, an alternative strategy would be to generate a single PRRSV resistance allele across the highest genetic merit maternal and paternal lines simultaneously. While a similar concept was proposed for porcine breeding more than 20 years ago (Visscher et al. 2000), maize breeding programs can now routinely introduce new traits directly into the most elite germplasm through gene editing (Gao et al. 2020a, b). This strategy accelerates time to market and importantly maintain high genetic merit typically lost due to introgression of these traits by backcrossing. In practice for commercial porcine gene editing applications, nucleus-derived lines would serve as the donor population to introduce the PRRSV resistance allele. For this new allele to be valuable to a porcine breeding program it would be necessary to maintain genetic diversity within and across lines during the process of generating a small population of gene edited founder animals. From a trait and genetic performance perspective, having the ability to establish and advance the single modified CD163 allele quickly into diverse lines has clear advantages over introgression by back-crossing because direct introduction enables early and rapid evaluation of disease resistance and of the conventional breeding values in the relevant commercial germplasm.

However, one challenge to the strategy outlined above, would be the ability to efficiently and reproducibly generate a single, commercially viable modified CD163 allele across the required 10–20 males per line minimally required to establish the initial gene edited nucleus breeding pool. As discussed above, several groups have shown that a variety of modified CD163 alleles, having insertions (1–7 bp) or deletions (1–>300 bp), result in the loss of a full-length CD163 protein in pigs yet conferred resistance to Type I and/or Type II PRRSV infection. These base deletions or insertions in the CD163 gene generated a translational reading frame in the CD163 mRNA harboring frameshifts and premature stop codons which likely lead to premature mRNA decay and absence of a full-length CD163 protein (Whitworth et al. 2015; Wells et al. 2017; Yang et al. 2018). Examination of the translation products generated by these frameshifts predicts that small stretches of new amino acids

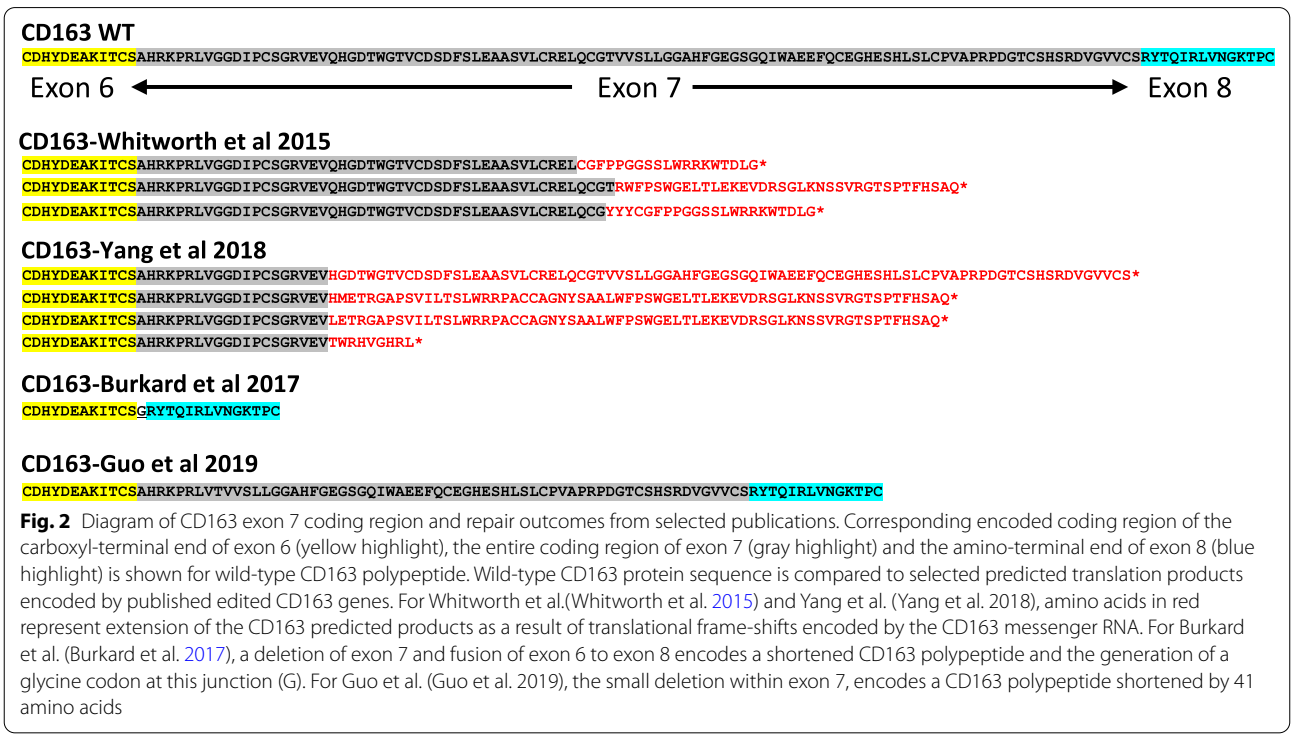


Fig. 2 Diagram of CD163 exon 7 coding region and repair outcomes from selected publications. Corresponding encoded coding region of the carboxyl-terminal end of exon 6 (yellow highlight), the entire coding region of exon 7 (gray highlight) and the amino-terminal end of exon 8 (blue highlight) is shown for wild-type CD163 polypeptide. Wild-type CD163 protein sequence is compared to selected predicted translation products encoded by published edited CD163 genes. For Whitworth et al.(Whitworth et al. 2015) and Yang et al. (Yang et al. 2018), amino acids in red represent extension of the CD163 predicted products as a result of translational frame-shifts encoded by the CD163 messenger RNA. For Burkard et al. (Burkard et al. 2017), a deletion of exon 7 and fusion of exon 6 to exon 8 encodes a shortened CD163 polypeptide and the generation of a glycine codon at this junction (G). For Guo et al. (Guo et al. 2019), the small deletion within exon 7, encodes a CD163 polypeptide shortened by 41 amino acids

are added to the carboxy-terminal end of the domain 5 truncated CD163 polypeptide (Fig. 2). Whether loss of CD163 function or the presence of these truncated versions of CD163 protein have a detectable impact on pig health, reproduction, production performance or meat quality has yet to be described using a large segregating pig population.

In contrast to the above reports that eliminate CD163 protein, two editing strategies remove small regions of the CD163 gene resulting in deletion of 105 or 41 amino acids corresponding to a complete or partial removal of domain 5 encoded by exon 7, respectively (Fig. 2) (Burkard et al. 2018; Guo et al. 2019). Both experiments used a dual-guide approach for modification of this region in CD163; for the deletion of the CD163 exon 7, two guideRNAs targeted DNA sequences in the flanking introns. The deletion of exon 7 and flanking DNA sequences (454 bp) results in fusion of intron 6 to intron 7. Upon transcription and with intron/exon splice junction sequences intact, 6:7 intron is processed resulting in an mRNA with exon 6 joined to exon 8. Fortuitously, splicing of exon 6 and 8 forms an in-frame glycine amino codon allowing translation of exon 7-less CD163 mRNA. Thus, the modified CD163 protein is a shorter version of the full-length protein with a single codon change (alanine to glycine) as a result of splicing exon 6 to exon 8 (Fig. 2).

Using the guideRNA pairs as described by Whitworth et al. (Whitworth et al. 2014, 2015), a 124 deletion was also recovered by Guo et al. (Guo et al. 2019) removing 41 amino acids in Domain 5 that corresponds to a proposed ligand-binding domain the CD163 polypeptide (Van Gorp et al. 2010). Although both deletions described result in a shorter mature CD163 protein, these experiments importantly provided data to support the hypothesis that modification or elimination of Domain 5 is sufficient to generate PRRSV resistant pigs while presenting data suggesting that other biological functions are maintained by the remaining eight encoded SRCR domains of near full-length CD163 protein. Similarly, while both studies have reported healthy pigs expressing these shortened versions of the CD163 polypeptide, additional studies would assist evaluating pig health and performance in a commercial setting.

In the studies described above, several methods were used to generate CD163 gene modifications, including delivery of either DNA vectors encoding Cas9 and guideRNAs or in vitro transcribed Cas9 mRNA and guideRNAs. DNA vectors expressing editing reagents were transfected into porcine somatic cells (fetal fibroblasts), with edited cells subsequently transferred to enucleated oocytes (somatic cell nuclear transfer: SCNT) and the resultant embryos implanted into synchronized recipient gilts. The piglets born would be genetically identical (both at the genome level and in terms of CD163 modification) to the edited somatic cells from which they arose. Alternative to these SCNT examples, microinjection of Cas9 mRNA and guideRNAs into 1-cell fertilized zygotes or in vitro fertilized (IVF) pig oocytes was also used to successfully generate pigs resistant to PRRSV. Several important differences exist when these methods are compared. First, SCNT requires a large number of embryos transferred (> 100 SCNT-derived embryos), whereas 30–50 embryos are transferred using injected fertilized zygotes. Further, slaughterhouse oocytes may also introduce an element of disease biosecurity risk. Second, SCNT methods often result in lower frequency of pregnancies of recipient females, smaller litter sizes, and piglets often displaying phenotypic differences, most likely due to epigenetic reprogramming associated with the transferred somatic cells which results in premature death (Kurome et al. 2013; Tanihara et al. 2021a, b). In contrast, higher pregnancy frequencies, larger litter sizes, and a large proportion of phenotypically normal piglets are observed when injected fertilized zygotes are transferred (reviewed in (Gil et al. 2017)). Third, because only a few fibroblast cell lines are the source for generating pigs by SCNT, genetic diversity is very limited and reduced as compared to the opportunity associated with zygote injection methods. For the latter, starting zygotes could be derived from nucleus males and females, thus maximizing genetic diversity required for continued performance improvement.

An issue with this strategy is that it has been well documented that microinjection of gene editing reagents into fish and mammalian zygotes results in progeny having more than two alleles (mosaicism), and often the frequency of these alleles is disproportionate and can vary across different tissues analyzed (Lamas-Toranzo et al. 2019; Mehravar et al. 2019; Hennig et al. 2020, 2022). Mosaicism is typically the result of one or more of the following variables: (i) timing of nuclease injection (premeiotic, one cell, two cell), (ii) nuclease activity at intended target site, (iii) class of nuclease (Zinc Finger, Meganuclease, TALEN, Cas9, CPF1), (iv) reagents delivered (DNA, mRNA, protein), concentration, and associated half-life, and (v) target site accessibility in different cell types. Moreover, depending on methods employed and tissues examined to detect intended target allele INDELs (insertion/deletion), mosaicism in the primary progeny is likely underrepresented. Large deletions may coincidentally remove PCR primer binding site(s) used for amplification and DNA sequencing, while a small number of cells may contain unmodified target alleles (wild-type) that may not have been detected. Given the potential presence of multiple, and often undetected alleles (both mutant and

wild-type) associated with mosaic animals, primary gene edited progeny derived from injected zygotes should be carefully evaluated through rigorous molecular analysis prior to phenotypic evaluation or source material for disease studies. This is particularly important for recessive traits where the presence of an undetected wild-type allele may confound experimental data interpretation.

Despite these challenges, injecting zygotes derived from parents with the most recent and most favorable genetic merit would be a preferred path for a scaled, commercial PRRSV resistance gene editing program. As routinely done in mouse gene editing applications, primary mosaic pigs (E0) would be bred to wild-type pigs to obtain a heterozygous generation of pigs (E1). Though adding a step to the breeding timeline and process, this cross is necessary as molecular characterization of sexually-transmitted gene edits is simplified when compared against the haploid genome descending from their wild-type mate.

One method during the early days of CRISPR-Cas9 was to use DNA vectors encoding Cas9 protein and guideRNAs to generate double-strand breaks (Petersen et al. 2016; Chuang et al. 2017). Because these exogenously added DNA vectors and repair templates can randomly integrate into the genome, other methods of delivering Cas9 and guideRNA are preferred for commercial scale PRRSV pig production. Cas9 and guideRNAs are now delivered routinely as either in vitro transcribed RNAs or a combination of Cas9 protein and guideRNA transcripts, with the latter commercially available. Not unexpectedly, delivering Cas9 protein over mRNA has been shown to increase resultant on-target INDEL frequencies while reducing observed mosaicism; like DNA-encoded Cas9, Cas9 mRNA requires time to be converted to protein and then complex with guideRNAs to form an active RNA-guided endonuclease. During this delay, the cell continues to divide and concurrently the concentration of both Cas9 mRNA and guideRNAs are likely reduced by cell-encoded RNA-degrading nucleases. This paradox may likely explain the reduced mutation frequencies and higher mosaicism for mRNA delivery when compared to Cas9 protein. Thus, from a timing perspective, delivering protein and guideRNA simultaneously allows for rapid formation of an active Cas9–gRNA ribonucleoprotein protein (RNP) complex able to generate INDELs more efficiently and prior to cell division and multicellular zygote formation. In addition, as demonstrated in mammals and plants (Kim et al. 2014; Lin et al. 2014; Liang et al. 2015; Svitashev et al. 2016; Hennig et al. 2020), RNP complex delivery reduces the degree of mosaicism and frequency of off-site cleavage as detected by the presence of INDELs at these predicted sites. Further, by adjusting RNP (protein:gRNA) concentrations, on-target editing can be optimized while reducing the generation of off-target INDELs (Kim et al. 2014; Rose et al. 2020). Although use of electroporation for RNP delivery to zygotes has been reported (Tanihara et al. 2016, 2020; Tanihara et al. 2021a, b), this method is not ready for commercial scale use due to the large number of donors needed to transfer electroporated zygotes per surrogate (> 200) and low pregnancy rates (approximately 1 piglet born per 50 zygotes transferred, of which 66–90% of the offspring contained modified alleles).

RNP injected zygotes

In order to maximize the genetic diversity of the founder population, zygote injection of RNPs is the current method of choice for scaled commercial production of PRRSV resistant pigs. However, nonhomologous end joining often produces random and numerous different repair outcomes. Thus, strategies to maximize the generation of one repair outcome that produces pigs with a unique and commercially relevant PRRSV resistant allele need to be considered. Factors that influence solutions to maximize efficiencies for scaled production of the CD163 allele include (i) whether the desired outcome produces a knockout or modified CD163 function, (ii) choice of endonuclease based on target site availability within CD163, (iii) DSB site and repair outcomes, (iv) activity of endonuclease at target site, and (v) off-target positions and frequencies. Eliminating the capacity of CD163 to bind PRRS virus by either complete or partial deletion of CD163 exon 7 would be a preferred strategy over a complete functional knockout of CD163. While this choice likely preserves accompanying functions of this scavenger receptor, generating precise, non-random deletions using an RNA-guided nuclease requires the placement of two guides which generate double-strand breaks are accurately repaired, removing DNA sequences between both cut sites.

Endonuclease-guideRNA prioritization screens

Regardless of nuclease choice, for commercial applications it would be beneficial to screen many guideRNA-protein combinations in order to maximize the efficiency of generating the desired CD163 allele in pigs. Given that a dual-guide strategy would be used for modification or removal of exon 7, each guideRNA would be tested individually and in combination for activity and frequency of cut-site to cut-site joining for precise sequence deletion. With the ultimate use of these editing reagents for zygote injections to produce a small founder population of genetically diverse PRRSV resistant pigs, a pipeline screening process is an important consideration for the identification of these guide-pair combinations. This pipeline would (i) identify pairs

to generate a high frequency of a desired commercial CD163 allele pig, and (ii) biochemically characterize whether guideRNA pairs have a potential to generate INDELs at non-target sites in porcine cells (off-target INDELs) (Fig. 3). Often it is necessary to balance these two considerations; whereas the best combinations for on-target frequencies and outcomes may need to be eliminated due to the associated off-target INDEL locations and frequencies generated by this pair.

Noting this dual-RNP approach would be used across 4 diverse porcine lines, building an accurate DNA sequence of the CD163 region is the first step in this screening process. This step allows the identification of line associated single nucleotide polymorphisms (SNPs) and elimination

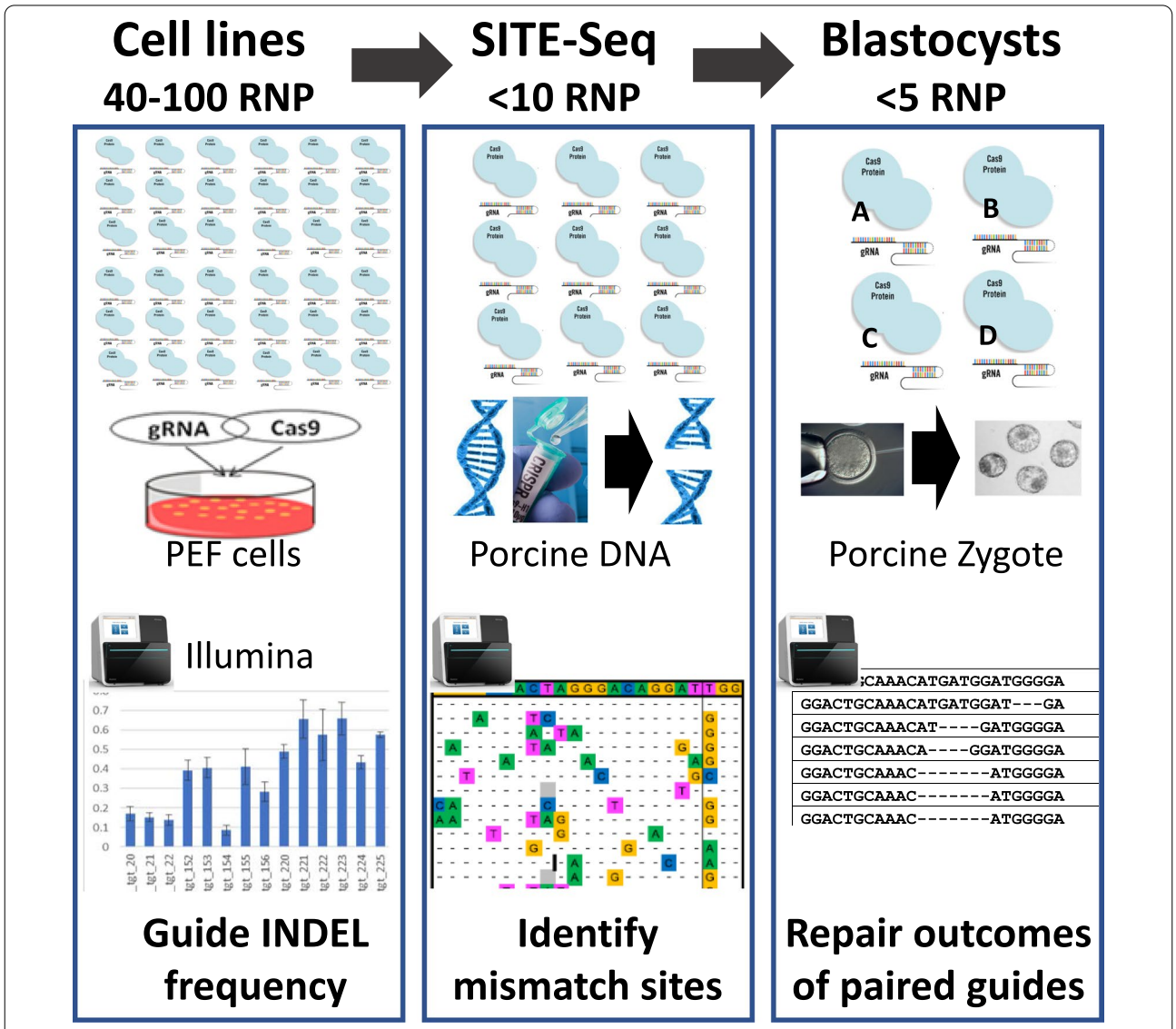


Fig.3 RNP screening process and prioritization steps for paired guide selection. The process for screening endonucleases to identify a Cas9, single dual-guide pair for CD163 allele modification is outlined. Left panel: Initially many guides are tested as RNPs in porcine fibroblast cell lines to measure on-target INDEL frequency short read Illumina DNA sequencing. Graph displays the frequency and range of on-target cutting by each RNP. This step eliminates no or low activity guideRNAs. Middle panel: Next a few high-activity RNPs (less than 10) are used to digest porcine genomic DNA in vitro to identify concentration dependent off-target sites by SITE-Seq biochemical screen. The sequences captured by SITE-Seq are aligned to the on-target sequence and mismatched nucleotides are highlighted in color. Right panel: A few RNP candidates having high frequency of desired repair outcomes and reduced mismatch off-target cutting sites are selected and injected as combinations (A + B, A + D, C + D, C + D) of dual-guide and screened in blastocysts to measure the on-target cut site to cut site repair and off-target INDELs at a few SITE-Seq identified mismatch sequences. Based on this criterion, one dual-guide pair is graduated to the commercial editing process (Fig. 4)

of those sequences for protein and guideRNA testing. This step is particularly important for intron containing DNA sequences, as non-coding introns tend to diverge more rapidly than coding exons because of the lack of selective pressure. With this CD163 reference sequence, the second step would be to select and test the activity of paired single guides to preferentially remove all or portions of CD163 exon 7. The DNA sequence of CD163 is unusual as removal of exon 7 and subsequent splicing of exon 6 to exon 8 does not alter the open-reading-frame of CD163 mRNA allowing translation of a near full length scavenger protein. Similarly, strategies which remove a small portion of exon 7, yet maintain CD163 function, would need to consider whether paired-guide removal of sequences and repair do not result in the formation of an in-frame translation termination codon.

A simple and rapid method to initially screen and select guideRNAs for downstream testing would be to transfect porcine embryonic fibroblasts cells (PEF cells) with individual RNP complexes and measure INDEL frequencies at the associated target site (Fig. 3). Given the reduction in DNA sequencing costs and the increased throughput by multiplexing, rather than sequencing using low throughput Sanger methods, Illumina short read technology would be the method of choice for this process screen. Genomic DNA extracted from individual pools of transfected PEFs is used for PCR amplification of the surrounding targeted sequences. By adding sample-specific index (barcodes) and Illumina sequencing adapters to the amplicons (typically 250- 350 nts) from each transfection pool, multiple amplicons can be sequenced simultaneously. Bioinformatic tools are used to deconvolute and analyze these DNA sequences and compare to wild-type reference sequences. DNA reads with the same INDEL are counted, collapsed into a single read, visually inspected to correspond to the expected site of cleavage and used to calculate the INDEL frequency of individual guides and associated repair outcomes relative to the total number of sequenced reads for that amplicon.

Using this approach, single guides that generate high INDEL frequencies would be advanced for secondary screening of paired guides. Individual selected guides would be paired and rescreened in PEFs to measure INDEL frequency and DNA repair outcomes associated with the dual-guide deletion approach. It should be noted that caveats to this cell pooling and screening strategy in porcine fibroblasts to advance single guide and guide pair deletions exist. First, depending on the size of region to be amplified by PCR, deletions can often be preferentially amplified and overrepresented in the sequence read pool; second, deletions that remove one or both primer binding sites used for amplification would not be represented in the sequencing pool and third, though the utility of screening in fibroblasts mirrors editing frequencies in sheep embryos for single guide screening (Wang et al. 2016), deletion frequencies directed by dual-guides in porcine fibroblasts may not reflect similar results in blastocysts.

However, despite these potential experimental limitations, as a first approximation, a limited number of paired guides ($n < 10$) that actively generate a high proportion of end-to-end deletions as determined by their repair outcomes are advanced for off-target analysis by a biochemical method called SITE-Seq. Given the potential that gene editing may be capable of introducing unintended, yet traceable and often reproducible, sequence changes to the genome (off-target INDELs), the process outlined in Fig. 3 incorporates a biochemical off-target screening analysis of RNA guides and endonucleases prior to scaled editing. This step identifies and advances a reduced number of guideRNA-pair combinations for subsequent screens in porcine zygotes.

In review, today there exists several bioinformatic and biochemical methods to assist prioritization of candidate guideRNAs which may have low or no off-target cutting in a cell. Publicly available bioinformatic tools use algorithms to search within a given reference genome for sites that have the potential for mismatch pairing of a Cas9 protein and guideRNA and ultimate INDEL production (Bae et al. 2014; Zhu et al. 2016; Clement et al. 2020; Patel et al. 2020). Determining the location and number of these sites could be considered for initial prioritization of guideRNA-protein. However, these tools are based on a reference genome and thus do not account for genetic variation within a species, nor fundamentally address the kinetic properties associated with each cutting event which is dependent on protein, guide, genomic site and accessibility. To that end, examining DNA sequence variation within these bioinformatically identified mismatch sites and across genetically different porcine lines would be a foundational step to prioritize guideRNA-endonucleases. Also, bioinformatic off-target tools may identify false-positive sites as they do not account for endonuclease accessibility to DNA for cutting as chromatin variation at different sites and tissues may occlude these locations to RNA-guided nuclease activity (Horlbeck et al. 2016; Meyenberg et al. 2021). While false-positive sites can be eliminated experimentally, use of bioinformatic tools risk missing kinetically active off-target sites and fall short for advancement of RNPs for commercial scale.

As mentioned, bioinformatic software identifies candidate off-target sites based on mismatch algorithms but does not address whether these sites are demonstrated substrates for cutting by gene editing reagents. A more direct method to enumerate these sites would be to incorporate biochemical screens which account for the

guideRNA-endonuclease ability to bind and cleave DNA in vitro. Since their development, methods for off-target detection have evolved and become important tools not only for off-target identification, but also for advancing the understanding and rapid improvement of nuclease activity and specificity in vivo (Clement et al. 2020; Rose et al. 2020; Atkins et al. 2021; Donohoue et al. 2021). An early example of an unbiased, biochemical off-target detection method was developed to improve meganuclease on-target specificity. Naked genomic maize DNA was subjected to digestion in vitro with meganuclease protein to which primers annealed to the exposed ends. Following amplification by PCR, the amplicons were sequenced and aligned to the maize reference genome. Analysis of these variant recognition sites (off-target cut sites) allowed researchers to modify meganuclease DNA-contacting amino acids and significantly improve on-target activity and eliminate off-target cutting (Deschamps et al. 2014). Other groups have since described a variety of methods to capture and sequence cleavage sites generated by guideRNA-endonuclease in vitro to examine the potential of these break site's ability to confer off-target INDELs in vivo (Kim et al. 2016; Cameron et al. 2017; Tsai et al. 2017). One method called SITE-Seq, and variants, has been described for unbiased biochemical identification of off-target site cutting by a number of groups (Cameron et al. 2017; Young et al. 2019; Patel et al. 2020; Donohoue et al. 2021). A feature of the SITE-Seq assay is that genomic DNA is digested with a range of RNP concentrations [RNP], from limiting to saturating (4–256 nM). This concentration range permits recovery of both high- and low-cleavage-sensitivity off-target sites being cleaved at low and high [RNP], respectively. The discovery of these concentration-dependent sites can then be used as a guide for comprehensive examination of possible off-target sites in cells by measuring INDEL frequencies in vivo. While this method may not be simple to implement into a gene editing program, inclusion of an in vitro method provides additional prioritization of high-activity guide-endonuclease combinations.

As shown in Fig. 3, SITE-Seq is the second screening step to advance dual-guideRNA pair for commercial production of PRRSV resistant pigs. To that end, biochemical identification of off-target sites by SITE-Seq serves to (i) map locations and determine whether these sequences can serve as substrates in vivo for off-target INDEL production during zygote injection, (ii) deprioritize guides which may induce INDELs genetically linked to CD163 on Chromosome 5, (iii) assist in the development of analytical methods to track these sites in vivo and (iv) direct experiments towards reducing off-target INDEL production in cells by modification of either guideRNA, protein or both. Paired with appropriate molecular screens, identification of in vivo off-target INDELs provides a means for a breeding organization to select animals that do not contain detected off-target INDELs for the generation of their nucleus herd. While sequence variation occurs in every generation through de novo mutations arising during the cell's DNA replication and repair process, this class of change is a driver of genetic gain or loss and dealt with by breeders evaluating performance. Like random de novo mutations, initially, it is not known whether gene editing-mediated sequence variation contributes positively, neutrally or negatively to performance. In contrast however, gene edited-induced INDELs are non-random, and if not identified, would be fixed in the breeding population and possibly negatively impact performance in certain biotic or abiotic environments. Off-target INDELs may be acceptable for proof-of-concept experiments. However, given the investment and commitment towards meeting customer objectives while introducing a new disease trait through new technologies, commercial entities may consider incorporating data-driven processes to remove tangible variation (off-target INDELs) early this process. This commitment is consistent with the use of molecular markers by commercial breeders to assure downstream customers that even very low frequency events which may be performance-negative in theory have been addressed.

Based on the activity and off-target selection steps presented in Fig. 3, a small subset of guideRNA pair/endonuclease combinations (2–5 pairs) that target sequences flanking CD163 exon 7 are next tested for their ability to generate similar high frequency cut-site to cut-site repair outcomes in blastocysts in preparation for identifying a single guide pair for scaled production (Fig. 3, right panel). This final screen would also examine whether high-cleavage-sensitive off-target sites (4–16 nM) detected by SITE-Seq are recovered in blastocysts cells after injection of editing reagents. In this last step, individual in vitro fertilized oocytes would be injected with candidate dual-guide RNP complexes and allowed to develop for 7–8 days whereupon on-target and selected high-cleavage sensitive off-target sites are amplified by PCR and sequenced. As done for fibroblast screening, short amplicons would be Illunima sequenced, counted, and inspected for the presence and frequency of the desired end-to-end CD163 deletion repair. Simultaneously, off-target regions would be similarly examined for presence or absence of INDELs and, if present, relative frequency determined. Together, these screening steps would identify a single, optimized pair of guideRNAs to maximize recovery of pigs that contain the desired CD163 allele from the fewest RNP-injected zygotes.

Scaled allele modification process and screens

Scaled production of a single modified CD163 allele across 4 different pig populations also requires coordination of a number of different steps and teams. As shown in Fig. 4, the first part of this scaled process involves (i) identification of the highest genetic merit donor females and males to contribute oocytes and semen, respectively, (ii) synchronization and mating of the nucleus-derived females for simultaneous production of fertilized zygotes, (iii) harvesting and injection of 1-cell zygotes with optimized dual-guide RNP complexes, and (iv) one day later, surgical transplant of < 40 injected and dividing zygotes into synchronized recipient surrogate females. Production of this population is highly dependent upon the skill and productivity of the surgery team, the efficiency of generating primary piglets containing the desired CD163 allele, litter size, and the age when animals become reproductive. Given these variables, to maximize efficiency of characterization and advancement of progeny, a commercial scaled program would focus each surgery round on a single donor line and anticipate generating a sufficient number of surrogates necessary to produce a target of 10–20 unique founders across each of the four lines (see below).

Approximately 4 months later, these surrogates would farrow an average of 6–12 live piglets per litter. In order to rapidly characterize, reduce and advance piglets to the breeding pool, a step-wise screening process, sequencing methods and rationale are described in Fig. 5. In this process different sequencing methods which have complementary approaches would be used to (i) initially screen all pigs by short amplicon sequencing to identify those containing the desired edit, (ii) long amplicons sequenced using an Oxford Nanopore platform to find large structural changes in CD163 not detected by Illumina and (iii) on a reduced set of pigs, hybridization-based capture for sequence resolution of the entire CD163 gene and off-target INDEL identification.

In the primary step, 250–350 bp amplicons from all pigs are barcoded, pooled, and sequenced on an Illumina platform (Fig. 4). Sequence reads are aligned to the porcine reference genome across CD163 exon 7 to identify piglets containing the cut-site to cut-site repaired CD163

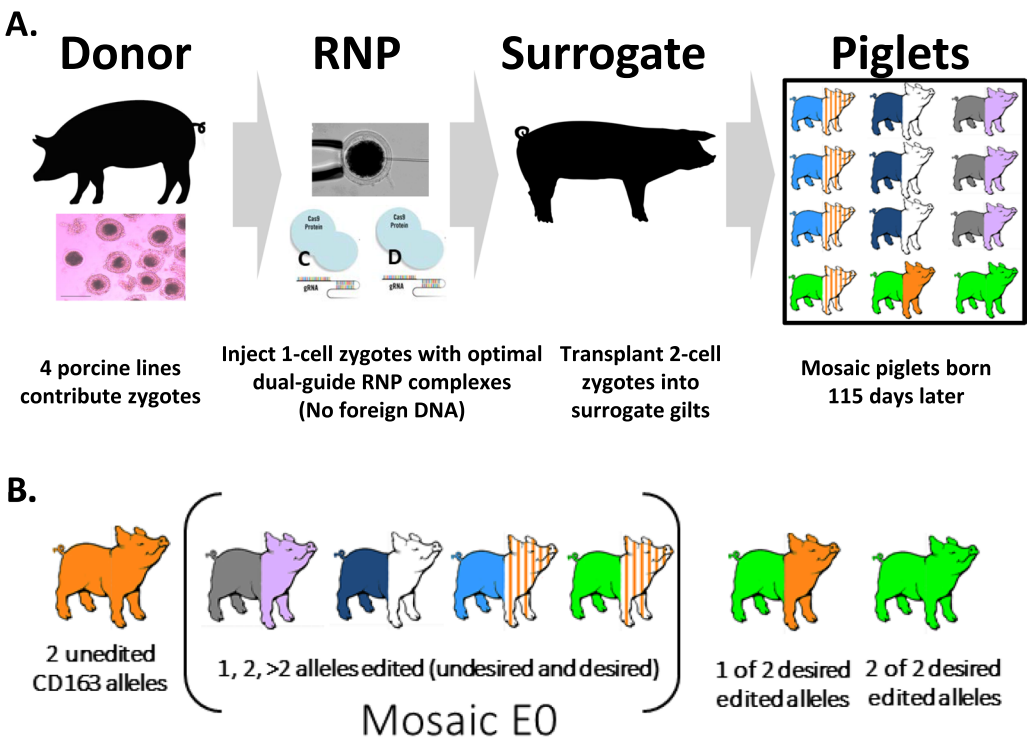


Fig. 4 Scaled production of pigs containing modified allele of CD163. A. High genetic merit gilts from nucleus are synchronized and artificially inseminated with semen from nucleus boars. Post fertilization, one-cell zygotes collected from donor gilts are microinjected with the dual-guide RNP complex (RNP C + D) identified in the process from Fig. 3 and then 25–35 two-cell stage zygotes transplanted into synchronized surrogate gilts. Approximately 115 days later these surrogates farrow litters ranging from 6 to 12 piglets. B. Cartoon depicting genetic makeup of pigs containing wild-type (tan), mosaic, heterozygous or homozygous modified CD163 allele (green)

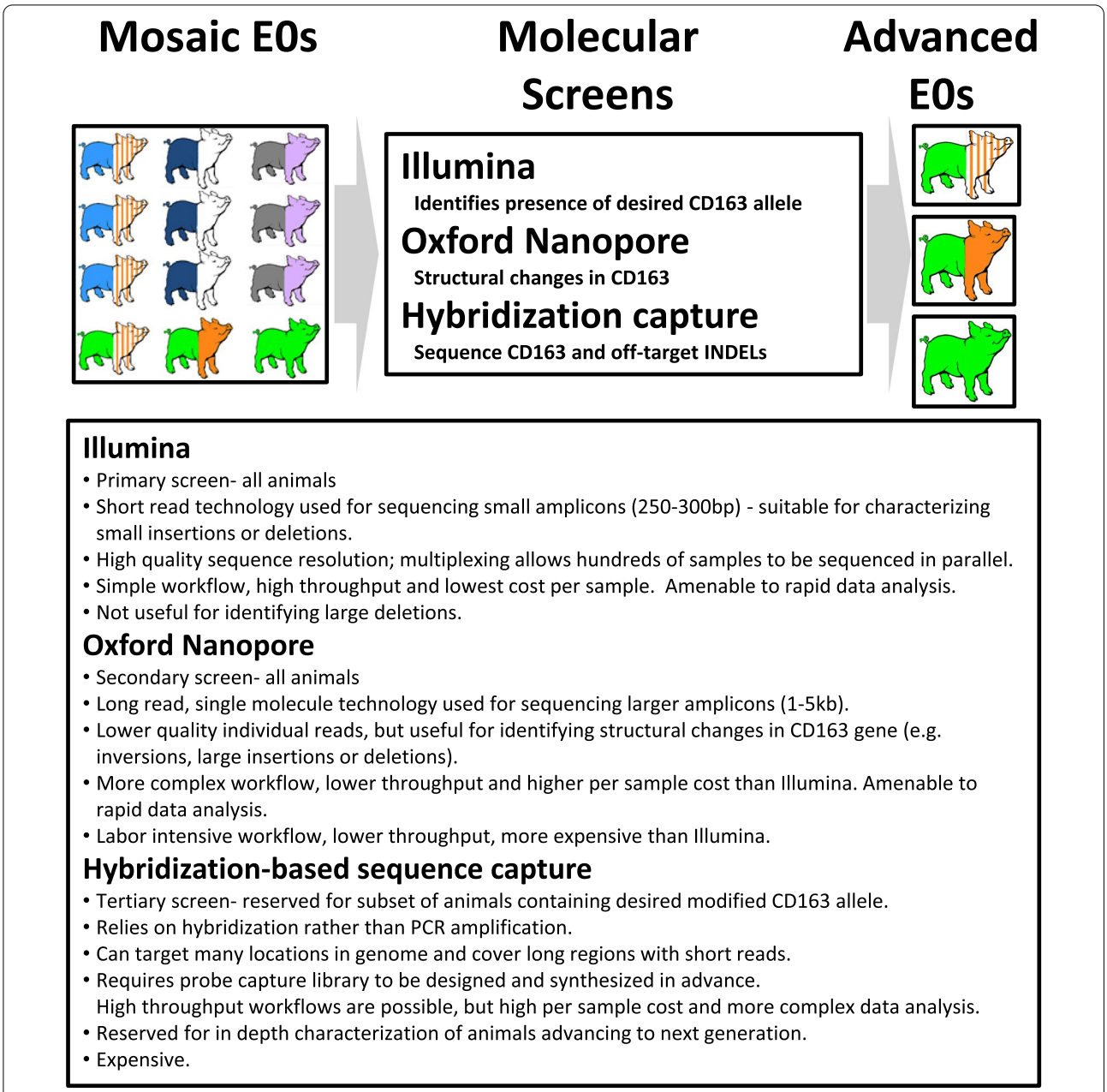


Fig. 5 Process and scaled screening steps of first-generation pigs (E0). Screening steps of primary generation of pigs farrowed from surrogate is described. DNA from tail samples of all live born E0 pigs is prepared and used for short (Illumina) and long (Oxford Nanopore) amplicon sequencing. A reduced number of pigs containing desired modified CD163 allele are then subsequently sequenced using hybridization capture to verify presence of on-target modified CD163 allele and identify gene editing reagent-mediated off-target INDELs. Selected E0 pigs are then crossed to wild-type, line identical, high genetic merit mates. A description of the DNA sequencing methods used in this process are outlined below

modified allele. As mentioned earlier, deep sequencing may uncover different CD163 alleles in this primary population of edited pigs generated by microinjection. In addition to identifying the desired commercial CD163 allele, understanding the diversity and frequency of these alleles is a consideration for advancement and breeding strategies. Further, commonly observed outcomes of NHEJ-break repair are small and large deletions (Carroll 2014). As reviewed by Ratner et al. (Ratner et al. 2021), often these deletions remove one or both primer binding sites used for sequence amplification, whereupon examination of this region may detect only one modified

CD163 allele. Amplification and sequencing strategies that do not account for this class of deletion often lead to misinterpretation of the modified CD163 allele being in the homozygous state, and complicate breeding outcomes. To address whether other sequence changes occur in the region surrounding the intended modified CD163 allele and uncover other CD163 alleles not detected by short amplicon sequencing, incorporation of a long amplicon sequencing step would provide further allele characterization. The Oxford Nanopore platform fits these criteria by determining the DNA sequence of a multi-kb region on a single read. Thus, PCR amplification of a large region of CD163 spanning exon 7 (1–5 kb) and sequenced by Nanopore would identify structural variation consisting of insertions, deletions, or rearrangements that are physically linked to the desired CD163 allele and likely uncover other variants of CD163 not revealed by Illumina.

Finally, a tertiary screen, targeted sequence capture is also introduced to identify RNA-endonuclease mediated sequence variations within CD163 and off-target INDELs. The advantage of hybridization-based sequence capture is that examination of specific genomic regions can be accomplished without the limitations associated with PCR-based amplification and sequencing. In brief, targeted sequence capture (Gnirke et al. 2009) uses a collection of short, tiled oligonucleotides (80–100 nucleotides) which span the 37 kb CD163 gene and 2 kb regions flanking SITE-Seq detected off-target sites. This capture probe pool is hybridized to a sheared whole-genome shotgun DNA library produced from a subset of the E0 edited pigs containing the desired CD163 allele. Enriched DNA fragments that have hybridized to the probes are sequenced on the Illumina platform at a depth of 1–2 million total reads and aligned to the latest *Sus scrofa* genome reference. Sequence comparison to DNA from the parents similarly captured (trio alignment) enables the identification of (i) parentally inherited variants across CD163 gene and changes not identified by Illumina or Nanopore and (ii) at the captured off-target regions, enabling the distinction of parental SNPs from endonuclease-mediated INDELs that may have arisen from the editing process.

Advancing and breeding strategies

Despite the potential of mosaic genetics in these E0 pigs, the described three-tier E0 molecular characterization process is important as it provides (i) data relating to the frequency of the desired commercial CD163 allele relative to other modified CD163 alleles and (ii) presence, frequency and location of off-target INDELs that may be inherited. This information has a direct impact on the strategy to accelerate and multiply a founder population of pigs containing homozygous modified CD163. To this end, a scaled editing program needs to contemplate that a given recipient female typically farrows 6–12 piglets (half male, half female) and assume that not all of these piglets will contain the unique modified CD163 allele. Support for this point was reported by Burkhard et al. (Burkard et al. 2017) which observed that of 32 live piglets born in those experiments, 4 pigs had a CD163 exon 7 deletion and only 1 of these 4 pigs had a precise clean deletion. In contrast to proof-of-concept experiments where first generation pigs containing multiple modified alleles might be considered for phenotypic testing and advancement, a commercial program should consider a sum of variables that influence the utility and breeding strategies for exon 7 deleted CD163 E0 animals. First, with rare exception, due to the recessive nature of this trait and mosaicism of E0 pigs generated by zygote injection, unmodified CD163 allele(s) may have gone undetected and as such may confound evaluation of PRRSV resistance using this primary generation. Second, Sanger or short-amplicon sequencing may not reveal complex modified CD163 alleles (e.g., large deletions or inversions) resulting in misinterpretation of the molecular makeup of the E0 animal. Third, molecular data relating to gene editing-mediated off-target INDELs, together with the above factors, negates considering a strategy that uses E0 males to be mated to a few E0 females. Together, litter size, low-frequency of a clean exon 7 deletion, detected and undetected multiple CD163 alleles, off-target INDELs, and mosaicism complicate founder generation. Rather, for a commercial program, the preferred path to speed the generation and molecular analysis this founder population would be to mate E0 females and males with line-identical wild-type males and females of high genetic merit, respectively (Fig. 6A). Especially for males, this would (i) fast-track the multiplication of this population, (ii) reintroduce latest high genetic merit from nucleus gilts and (iii) due to heterozygosity at these loci in next generation progeny (E1) greatly simplify molecular characterization of gamete-transmitted CD163 alleles and off-target INDELs. Moreover, outcrossing to a wild-type line identical mate also promotes the segregation of detected off-target INDELs from the desired, modified CD163 allele in the subsequent generation. E1 pigs multiplied by this mating would be screened by amplicon sequencing and targeted sequence capture across the modified and WT CD163 allele and at candidate off-target INDEL sites to identify pigs that maintain a CD163, cut-site to cut-site, deletion allele at a 50:50 frequency relative to WT CD163, absent additional CD163 sequence variation as well as nuclease induced off-target INDELs at other genome locations. Breeding values of these E1 progeny would also be estimated to select high

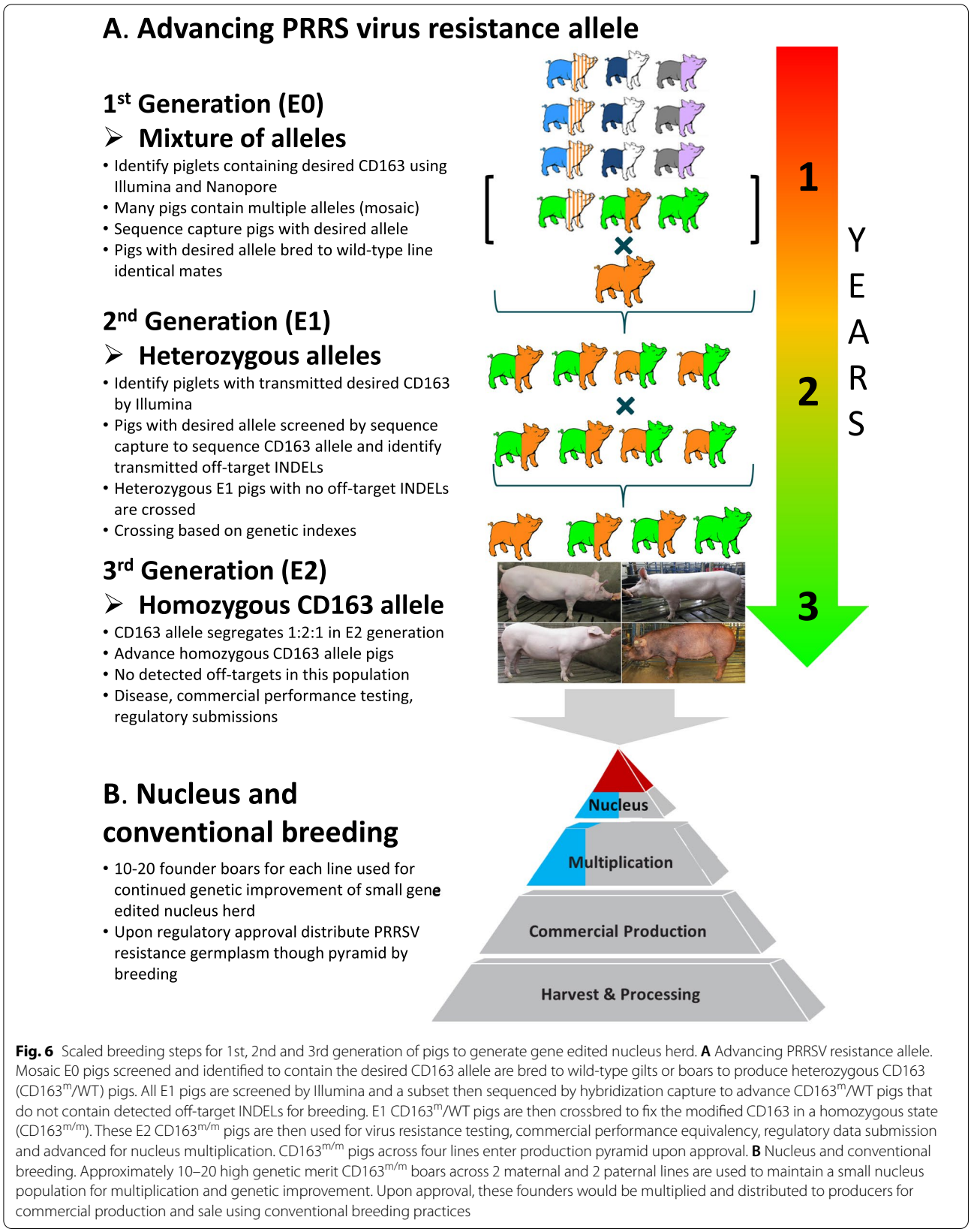


Fig. 6 Scaled breeding steps for 1st, 2nd and 3rd generation of pigs to generate gene edited nucleus herd. **A** Advancing PRRSV resistance allele. Mosaic E0 pigs screened and identified to contain the desired CD163 allele are bred to wild-type gilts or boars to produce heterozygous CD163 ($CD163^{m}$/WT) pigs. All E1 pigs are screened by Illumina and a subset then sequenced by hybridization capture to advance $CD163^{m}$/WT pigs that do not contain detected off-target INDELs for breeding. E1 $CD163^{m}$/WT pigs are then crossbred to fix the modified CD163 in a homozygous state ($CD163^{m/m}$). These E2 $CD163^{m/m}$ pigs are then used for virus resistance testing, commercial performance equivalency, regulatory data submission and advanced for nucleus multiplication. $CD163^{m/m}$ pigs across four lines enter production pyramid upon approval. **B** Nucleus and conventional breeding. Approximately 10–20 high genetic merit $CD163^{m/m}$ boars across 2 maternal and 2 paternal lines are used to maintain a small nucleus population for multiplication and genetic improvement. Upon approval, these founders would be multiplied and distributed to producers for commercial production and sale using conventional breeding practices

genetic merit males and females as parents of the E2 animals homozygous for the single PRRSV resistance allele. The E2 populations would be genotyped and expected to have an mendelian allele segregation pattern of 1:2:1 (wild-type: heterozygous: homozygous) at the CD163 locus. This population and subsequent generations would be used for phenotypic characterization of PRRSV disease resistance as well as equivalency of commercial production performance (growth, carcass composition, meat quality, and fertility as mentioned above).

Initiation of a breeding population via gene edited founders in the near-term needs to connect with wild type genetics for additional diversity and gain, thus initial targets of at least 10 founder males representing unique sire families provides a strong base for generation of initial generations and potential population expansion while attempting to optimize genetic merit and diversity. Thus prior to regulatory approval for sale, these edited founder males would be used to propagate a small gene edited nucleus herd with a goal of continued genetic improvement necessary to meet customer product standards. Upon approval, this pureline great-grandparent (GGP) nucleus population, homozygous for a unique CD163 PRRSV-resistant allele would be used to multiply the grandparent (GP) level exactly as described earlier for a traditional breeding pyramid (Fig. 1). Maternal and paternal GP lines are crossed to produce the parental generation, which in turn farrow pigs for harvest and meat processing (Fig. 6B). With current pig production practices, the timeline to distribute PRRSV resistance from the nucleus to producers, and finally consumers, spans at least a 4-year multiplication scenario. Despite this timeframe, strategic application of gene editing, paired with rigorous molecular screens and conventional breeding, a tangible opportunity to significantly improve animal health while demonstrating that these PRRSV resistant pigs are safe for consumption and safe for the environment, is close to realization.

Concluding remarks

With the advent and use of modern breeding tools, geneticists and breeders have a material opportunity to significantly and positively advance livestock health. Clearly demonstrated by several academic groups since its first report in 2015, and produced without the introduction of foreign DNA (i.e. non-transgenic), truncation or modification of a single gene can prevent PRRSV infection in pigs (Whitworth et al. 2015; Burkard et al. 2018). Importantly, these pigs appear normal and can protect fetuses from viral infection in utero. Like any technology, translating these proof-of-concept results into commercial practice requires investment in terms of people, infrastructure, germplasm, and time needed to produce this trait in relevant breeding populations. At the current time, cost estimates to generate these initial gene edited founders and their subsequent generations are almost impossible to calculate as dramatic variation can exist not only in editing efficiency and target numbers to generate, but also in the costs associated with dedicating an operation, e.g., available facilities of different size dedicated to the project, staffing costs and efficient utilization of both farm operations and technical teams. Moreover, commercialization often requires access to patents and licenses and the protection of private investment through the generation of intellectual property necessary to practice. Once achieved, trait value is dependent upon the persistence of the disease, durability of the PRRSV trait and commercial performance equivalency relative to the current elite genetics. In parallel to the development of the gene edited founders for breeding, commercialization also requires financing national and global regulatory approvals and market acceptance strategies. While the latter points are beyond the scope of this report, a science-driven, harmonized approach for regulatory approval of livestock is necessary to provide a clear path for industry and producers to bring gene edited products equitably and safely to market.

Conventional methods can be expected to continue to improve livestock performance only as long as (i) beneficial alleles exist, (ii) breeders have the ability to assemble the alleles in elite germplasm, and (iii) advanced breeding programs can maximize overall genetic progress based on these alleles. As exemplified above, there are cases where these beneficial alleles do not currently exist in breeding populations. In light of these new opportunities to introduce traits not attainable by random mutation and recombination, livestock genetic providers must develop new processes and navigate risks associated with bringing to market exceptionally beneficial health traits via gene editing. Thus, technical approaches and decisions should consider an end-to-end (bench to barn) process as a key ingredient towards building trust with regulatory agencies, producers, and consumers. This concept is particularly important as we consider that a PRRSV resistant pig represents a real opportunity to improve porcine health. Thus, being the early days of using gene editing technology to this end, having the ability to generate and introgress a single PRRSV resistant CD163 gene edit is a practical choice to consider in order to minimize unintended molecular or phenotypic differences across these genetically diverse, line-specific founders. While this has proved challenging at several levels, it may be possible to incorporate new gene modification platforms and deploy traits simultaneously, efficiently, and rapidly as reproductive and cell biology technologies improve in livestock. In summary, gene editing has already demonstrated a

profound positive impact on human health. For livestock applications, health and welfare traits would similarly benefit from responsible and transparent use of the technology, which is ultimately aimed towards continuing to improve sustainable agriculture practices to benefit animals, society and the environment.

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