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Commentary

CRISPR Bacon: A Sizzling Technique to Generate Genetically Engineered Pigs

Franco J. DeMayo[1] and Thomas E. Spencer,[2] *Editors-in-Chief, Biology of Reproduction*

[1]*Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas*
[2]*Department of Animal Sciences and Center for Reproductive Biology, Washington State University, Pullman, Washington*

The ability to manipulate the genome by adding, removing, or modifying DNA sequences in a sequence-specific fashion is essential to studies that investigate the genetic underpinning of physiology. Technology based on the prokaryotic CRISPR (clustered regularly interspersed short palindromic repeats)/Cas9 system is completely revolutionizing genome engineering. During the past year, CRISPR/Cas9 editing has been implemented in a multitude of model organisms and cell types and has already started to supplant incumbent genome-editing technologies, such as TALENs (transcription activatorlike effector nucleases) and ZFNs (zinc finger nucleases) [1]. In this issue of *Biology of Reproduction*, sizzling work by Whitworth and colleagues [2] illustrates the efficient and easy use of CRISPR/Cas9 technology to generate genetically engineered pigs. Historically, genetic engineering has been an inefficient process in many animals that lack embryonic stem (ES) cells, such as large animals (pigs, cattle, sheep, chickens, and nonhuman primates) and smaller animals (rats and guinea pigs). Those animals are important for human health as they provide high-quality protein for the world's population and/or serve as important biomedical research models. Genetically engineered animals were produced first by pronuclear injection [3]. Subsequent technologies involved genetically engineering somatic cells for use as nuclear donors in somatic cell nuclear transfer (SCNT) to produce targeted genome modifications [4]. Although improvements are being made with SCNT, the efficiency of this procedure remains low in most animals. Embryonic stem cells have made the mouse the model organism for many studies, but even this approach in mice is relatively inefficient and costly with respect to time and animal resources. The process of genetically engineering ES cells in vitro using homologous recombination (HR) and subsequent creation of mouse models after injecting them into blastocysts and germline transmission via chimeras and breeding is inefficient, requires a minimum of a year, and is expensive. In addition to these limitations, this approach is restricted to two strains of mice, the 129SV and C57BL6N strain for which germ line-competent ES cells are available. These problems led researchers to seek and discover new methods to conduct genetic engineering.

During the past decade, genome engineering in animals without ES cells has been made possible with meganucleases, such as ZFNs, TALENs, and now CRISPR/Cas9. As illustrated in Figure 1, targeted meganucleases can induce double-strand breaks (DSBs) at specific locations in the genome and cause either random mutations through nonhomologous end joining (NHEJ) or stimulation of HR if donor DNA is provided [5–8]. Targeted modification of the genome through HR can be achieved with meganucleases if donor DNA is provided along with the targeted nuclease. After introducing specific modifications in somatic cells, these cells were used to produce genetically engineered animals via SCNT. Indeed, TALENs and ZFNs have been used to engineer the genome of several different animals. Both TALENs and ZFNs depend on making custom proteins for each DNA target, which is cumbersome. Additionally, TALENs are expensive and do not easily target some areas of the genome. The problems with those meganucleases are abrogated in large part with CRISPR/Cas9, which is supported by the article by Whitworth and colleagues [2]. Indeed, the CRISPR/Cas9 system has been successfully used to generate genetically engineered animals in various vertebrates, including zebrafish [9], monkeys [10], mice [11], rats [12], and pigs [13].

In nature, the Cas9 system requires three components: 1) an RNA (~20 bases) that contains a region complementary to the target sequence (cis-repressed RNA [crRNA]), 2) an RNA that contains a region complementary to the crRNA (trans-activating crRNA [tracrRNA]), and 3) Cas9, the enzymatic protein component in this complex. A single guide RNA (gRNA) can be constructed to serve the roles of the base-paired crRNA and tracrRNA. The gRNA/protein complex can scan the genome and catalyze a DSB at regions that are complementary to the crRNA/gRNA [14] (Figure 1). Unlike other designed nucleases, only a short oligomer needs to be designed to construct the reagents required to target a gene of interest, whereas a series of laborious cloning steps are required to assemble ZFNs and TALENs. Indeed, reagents for the CRISPR/Cas9 system can be acquired for minimal cost through commercial sources, synthetic homologous recombination targeting vectors can be acquired from vendors at reasonable prices, and bioinformatics tools for the design of gRNAs are freely available. Further, CRISPRs can add and delete base pairs at specifically targeted DNA loci and have been used to modify five genes at once [1].

In addition to gene editing, CRISPR/Cas applications in cells and zygotes include gene knock-in, reversible knock-down, gene activation, and gene repression [14]. In terms of gene editing, CRISPRs can be used to add or delete base pairs at one or more specifically targeted DNA loci. CRISPR interference, like RNA interference, turns off genes in a reversible fashion by targeting but not cutting a site. In bacteria, the presence of Cas9 alone is enough to block

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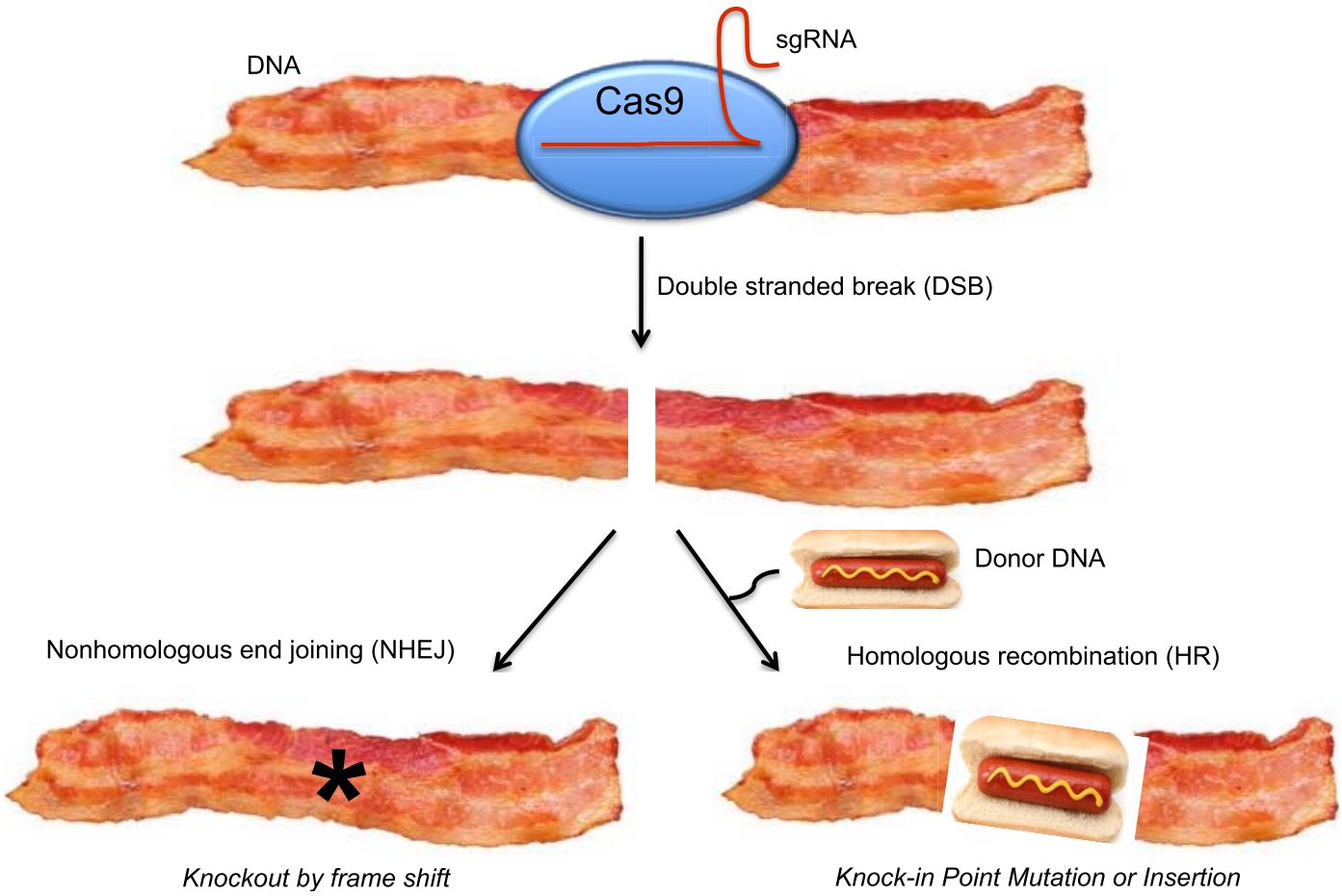

FIG. 1. Overview of the CRISPR/Cas9 system for genomic engineering. The enzyme Cas9 is a DNA endonuclease found in many bacteria in which it functions as part of a defense system against invading DNA molecules such as viruses. Cas9 has two active sites that each cleave one strand of a double-stranded DNA molecule. The enzyme is guided to the target DNA by an RNA molecule that contains a sequence that matches the sequence to be cleaved. RNA-guided Cas9 activity creates site-specific double-stranded DNA breaks (DSBs), which are then repaired by either nonhomologous end joining (NHEJ) or homologous recombination (HR). The process of NHEJ can cause small deletions (*), resulting in a frame shift and knockout. During homologous recombination, the addition of donor DNA enables new sequence information to be inserted at the break site.

transcription, but for mammalian applications, a section of protein is added. Its gRNA targets regulatory DNA, called promoters, that immediately precede the gene target. Cas9 can be used to carry synthetic transcription factor activation or repression domains that activate or repress specific genes. The technique can be refined by targeting multiple CRISPRs to slightly different areas of the promoter region.

The cisgenic and transgenic capabilities of the CRISPR/Cas9 system provides important applications to create animal models and in agriculture and human health. The gene editing can be used to introduce specific base pair changes to recreate important single nucleotide polymorphisms (SNPs). The transgenic approach can be used to create small insertions for introduction of *flox* sites for downstream conditional deletion strategies using Cre recombinase. Further, it can be used to create large insertions for the knock-in of reporter alleles (*LacZ, eGFP,* etc.), Cre recombinase, or entire genes to replicate natural copy number variation (CNV). Thus, the CRISPR/Cas9 system should be useful to create designer animals that contain natural SNP and CNV associated with a specific trait or disease [15]. This approach is critical to develop new animal biomedical research models and also agricultural animals with specific desired traits without having to use a complicated breeding scheme for introgression of the SNP or CNV. Introgression for species that have long generation intervals is untenable. Additionally, therapeutics to correct human and animal diseases are another application. In summary, we expect this technology to revolutionize all aspects of science and to provide more sizzling manuscripts in *Biology of Reproduction*.

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