Potential for Selection of Beneficial Traits in Swine with Site-Specific Nucleases

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I am located in the Animal Biosciences and Biotechnology Laboratory of the Agricultural Research Service (ARS) in Beltsville, Maryland. Our lab falls under the purview of the national program or NP 101, which is food animal production, as well as NP 103, which is animal health. Within the lab we have five projects in three broad categories:

- Growth and reproduction,
- Health and alternatives to antibiotics, and
- Development of genome-editing tools.

A partner on campus, Dr. Bhanu Telugu with the University of Maryland, works closely with us and is a major contributor to this presentation.

As we prepare projects, it's important to recognize the interdependence of the health of all species and, furthermore, being part of the ARS, it's important that whatever knowledge that we acquire and intervention strategies that we develop should have global application when and wherever possible.

Our research falls under the USDA's priority *Global Food Supply and Security* and the focus is to maintain the efficient production of nutritious, affordable and safe food for human consumption. This will continue to be a priority, and, as we go forward, we anticipate challenges. For example, it is anticipated that, by 2050, there will be about a 70 percent increase in the demand for animal protein, not only due to an increase in the global population but also due to increased individual wealth in less-developed countries, particularly as those people adopt a more western lifestyle, they will demand more meat in their diets. Also, it is predicted that, by 2050, a large increase in urbanization will have

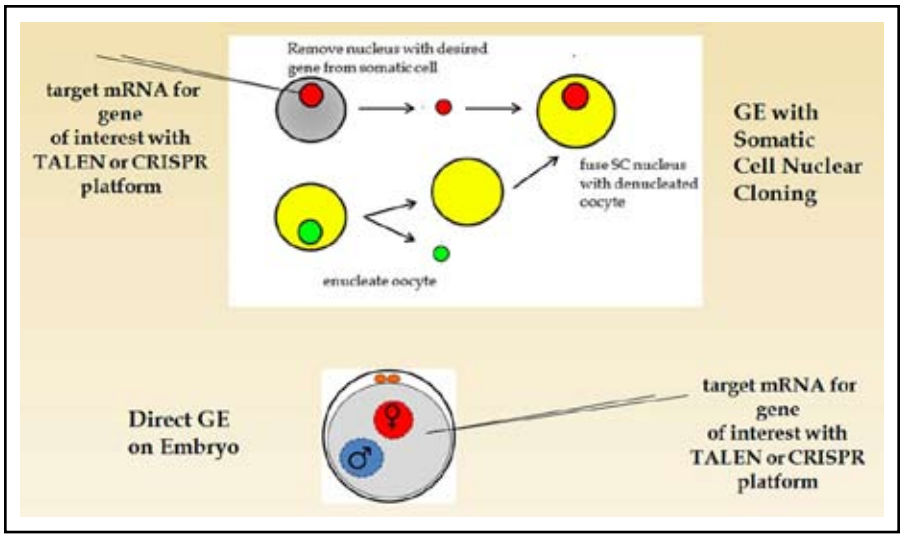

Figure 1. Genetic engineering.

occurred with decreases in rural populations, including the number of farmers. And it is expected that crop-farmland and animal-pasture "footprints" will be reduced in the next few decades.

Aside from challenges within the livestock-production system, another issue is animal disease as global travel increases. How do we mitigate current diseases as well as minimize the appearance of new diseases? We need to develop alternatives to antibiotics while fostering growth promotion. Feed efficiency is another salient issue; we have very efficient animals now, but can we boost their efficiency further? Other issues are animal welfare and the interactions of animals with the environment and how these affect production.

Breeding and Selection

Selective breeding has been beneficial for the agricultural community with the production of superior animals with desirable production traits including increased growth rate, increased feed efficiency, increased meat yield per animal as well as resistance to disease. On the other hand, frequently along with desirable traits, undesirable traits also segregate, such as the susceptibility to other diseases and, in litter-bearing species—particularly referring to swine—we've seen increased weight variability, and with attempts to increase muscle mass we may alter body structure, which has been known to affect reproductive capacity as well as raise animal-welfare concerns.

Another caveat for selective breeding is the length of time that it takes to achieve genotypic improvement; for cattle it can be about a quarter century. Selective breeding will continue, particularly considering increased genome information on animals, but the bigger question is whether there will be value in genetic engineering of livestock, particularly if we can guarantee precise targeting of an allele or quantitative trait nucleotides, improve traits and, therefore, produce healthy and safe animals.

Swine

1. **LDL receptor:** Daniel Carlson et al., Oct. 2012 – UMinn, Roslin Instit., TX A&M. TALENs in SCNT and *in vitro* embryo & biallelic KO pigs.

2. **v-rel avian reticuloendotheliosis:** Lillico et al., Jul. 2013 – Roslin Instit., UMinn., Recombinetics. TALENs in IVF derived embryos/mono- & biallelic KO pigs.

3. **α-1,2,galactosyltransferase:** Xin et al., Dec. 2013 – multiple Chinese Instit. TALENS in somatic cells/biallelic KO pigs.

Cattle and Sheep

1. **Myostatin:** Proudfoot et al., Sept. 2014 – Roslin Instit., Recombinetics, TX A&M, Genus PLC. TALENs in bovine and sheep IVF embryos/mosaic bull & heterozygous lamb.

Figure 2. Reported gene targeting of livestock.

Gene Targeting

Primarily, the ARS's role in genetic engineering is development and refinement of tools. We are investigating technologies that utilize natural cellular mechanisms for genome repair that do not leave behind foreign DNA and precisely target genes of interest. And beyond development of tools, we seek opportunities for their implementation either through collaborative efforts or by passing on technologies to academia and industry. Like many present at this conference, our interests include TALENs and CRISPRs, investigating their utility primarily in swine and for addressing agricultural concerns such as animal well-being and zoonotic diseases.

We consider that there are two approaches for genetic engineering in farm animals (Figure 1). One is to modify the gene of interest in the genome of a somatic cell with either TALENs or CRISPRs and establish a cell line, and then use those cells for somatic nuclear cloning where you actually remove the genetically engineered (GE) nucleus fuse with an enucleated oocyte. Once the nucleus is in the oocyte, it is reprogrammed and a GE embryo generated. The second way is by direct modification of the gene of interest in the embryo genome again using TALENs and CRISPRs that are introduced directly into the embryo itself.

TALENs

Figure 2 shows some recent reports of success in genetically engineering swine, cattle, and sheep by *somatic cell nuclear transfer (*SCNT) as well as direct embryo modification.

Initially, we tried to use the TALEN platform to target a gene that was in a safe-harbor site for knock-out; for our proof-of-concept experiment, we selected the prion gene. Again we were interested in trying to use direct swine-embryo modification using *in vitro* matured oocytes that were *in vitro* fertilized to generate embryos. We chose direct embryo modification over SCNT primarily because, with SCNT, the efficiency had been low and, in addition, quite often there are developmental issues with offspring.

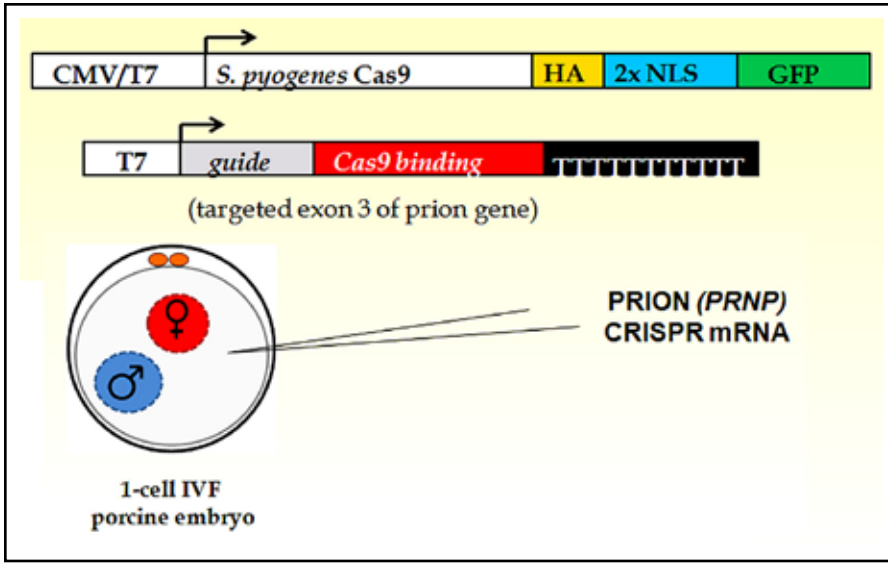

Figure 3. Gene knockouts in swine using the CRISPR/Cas NHEJ system.

Briefly wrapping up our experiments with TALENs, at least in our hands only a low percentage of the embryos developed to the blastocyst stage. Additionally, when we evaluated the sequences, many of our embryos had only a one-codon deletion. Accordingly, we moved on to CRISPRs because we had that technology operational at the same time, and, it seemed to work better in our hands.

CRISPRs

We chose the CRISPR-Cas9 system with which two approaches are possible, either through non-homologous end-joining, where you create an indel-mutation or, if you are interested in more-precise gene editing, you can adopt the homology-directed recombination method.

In our first approach, as stated, we wanted to target the prion gene and, particularly, modify exon 3 in the prion gene, so we used the Cas9 nuclease with a T7 promoter for *in vitro* transcription (Figure 3). The construct also had the nuclear localization signal as well as green fluorescent protein (GFP) as a marker; the guide RNA had the T7 promoter as well. These constructs were *in vitro* transcribed and then the RNA was injected directly into one cell each of *in vitro* fertilized porcine embryos. Those embryos were then cultured *in vitro* and, at 24 hours post-injection, we found that about 80 percent were GFP-positive. Also, we were able to identify their localization—within the cells of the embryo—which was good for us. Furthermore, we were able to mature 30 percent of those embryos to the blastocyst stage, superior to our experience with the TALEN technique.

Figure 4 shows sequences of clones obtained from the embryos that were analyzed to determine whether we had bi-allelic or mono-allelic or mosaic modifications. The bottom line is that we were successful in getting bi-allelic knock-outs or modifications in

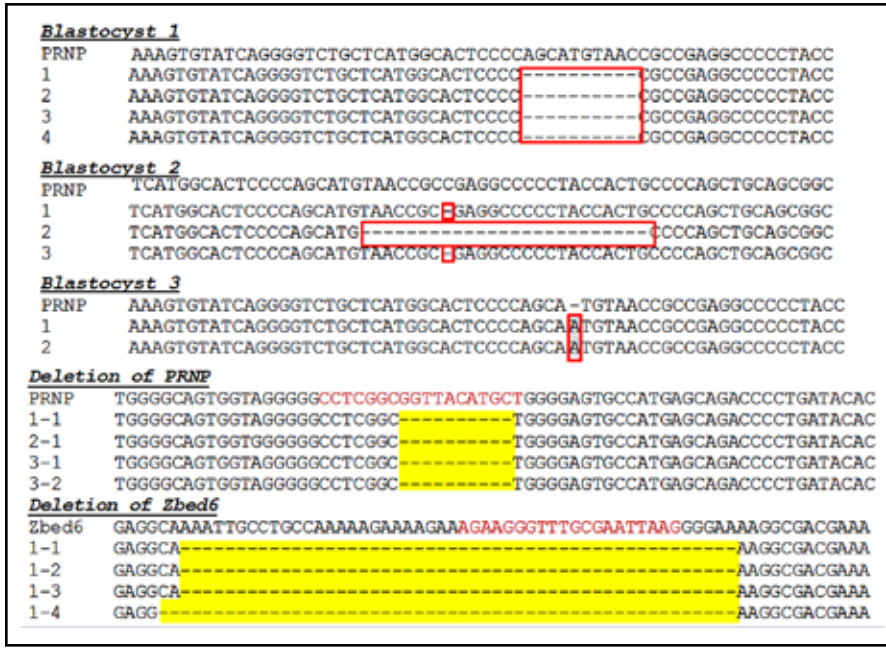

Figure 4. Successful single and double KOs.

about 80 percent of the embryos. Both deletions and insertions were observed between embryos, and, in some cases, we saw embryos that were mosaic. Because one of the targets that we were eventually going after is actually a bigenic disease, we were also interested in seeing if we could knock out two genes at one time. In addition to the prion gene, we chose the zinc-finger bed containing six transcription factors; the take-home message here is that we were able to create deletions in both genes simultaneously in the same embryo. That was good proof-of-concept, indicating that we could go ahead and target our genome of interest.

The next approach was to see if we could actually insert into or directly edit a particular sequence within a gene. In this case, we decided to insert a short sequence into the Zbed6 gene using the Cas nuclease with a guide tRNA, and a single-stranded DNA oligo that contained a loxP—*i.e.*, the sequence that we wanted to insert—the construct was flanked on the 5' end by an EcoR1 site (Figure 5). Again, the RNA constructs (nuclease and guide) plus the single-stranded DNA were injected into embryos, and, after we collected the embryos, a PCR was performed to see if we had actually been able to insert our short sequence. Figure 5 shows the banding patterns. The lower band is the wild-type allele that we expected. The upper band was of a size indicative of a 34-base-pair insertion. In addition, some embryos had the wild type and also had a lower band indicative of deletions in those embryos. The third pattern had a band of the same size as the wild type, but, because of its diffuse nature, we deduced that some other event had occurred in these embryos.

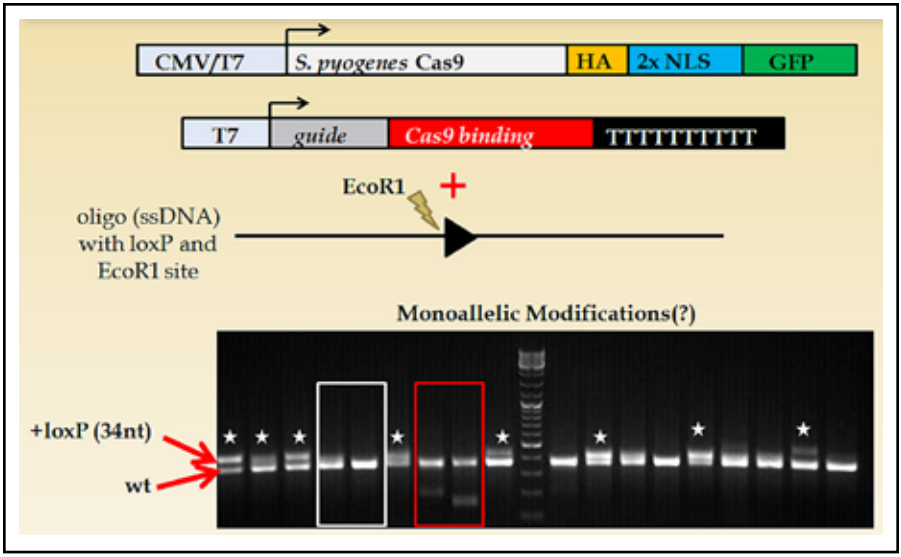

Figure 5. Gene-editing potential with HDR.

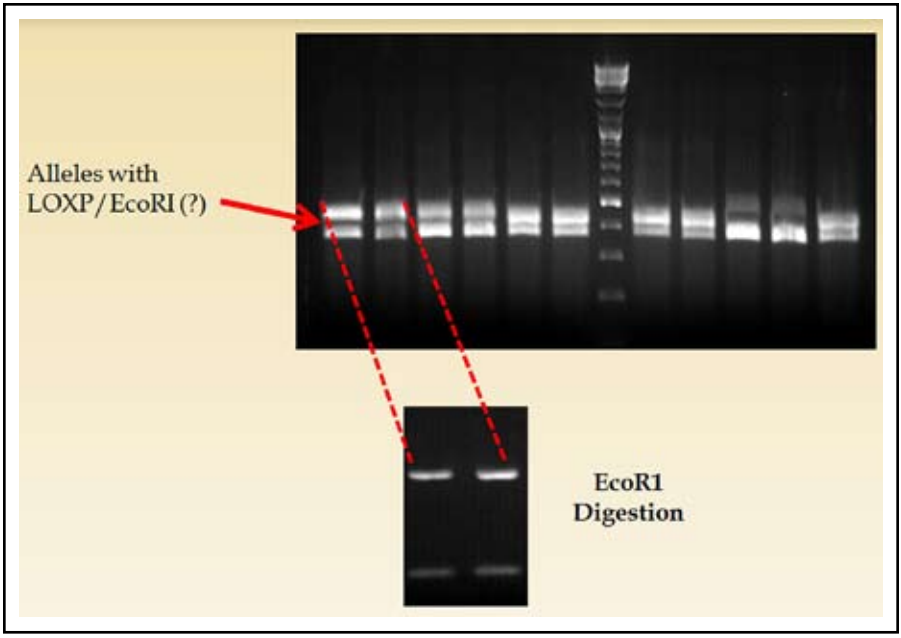

Figure 6. Confirmation of an EcoR1 site.

With the embryos that contained, what we thought was the loxP insertion, we did an EcoR1 digestion on the upper band; the allele that we thought contained loxP/EcoR1, and, sure enough, we were able to show that the EcoR1 site was inserted (Figure 6).

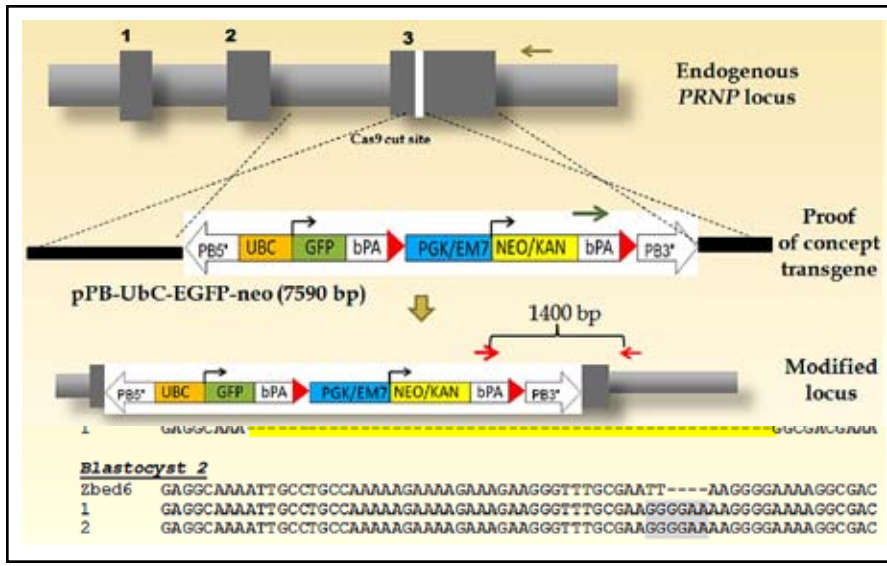

Figure 7. Mono-allelic modifications with precise gene editing.

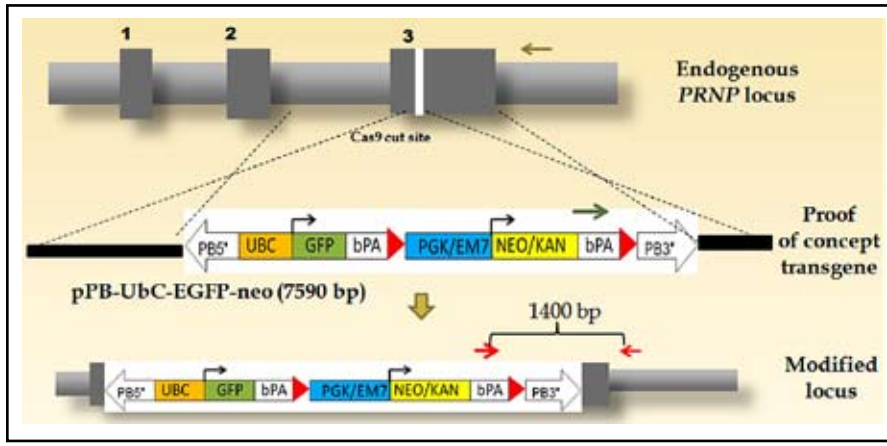

Figure 8. Gene insertion/replacement with Cas9 by homologous recombination.

Sequencing provided confirmation (Figure 7). We showed that we had the EcoR1 site plus the loxP gene inserted in a portion of the embryos, suggesting the ability to precisely target and modify a gene of interest; however, the sequence data also showed some random deletions and insertions. Although we need to work on this platform further, overall we were content that we were able to produce mono-allelic modifications.

Lastly, we were interested in determining if we could insert a transgene into a particular locus. In Figure 8, the upper schematic again shows the prion gene that we targeted for the introduction of a transgene into exon3. The middle schematic shows our proof-of-

concept transgene, which consisted of a ubiquitin promoter, GFP as a marker and then the phosphoglycerate kinase and EM7 promoters that flanked the gene providing neomycin and kanamycin resistance. The entire transgene construct was about 7.6 kb. The bottom schematic shows the modified locus subsequent to the insertion of the transgene; opposing arrows denote where we had designed PCR primers in the 3' end of the transgene construct and in the intron of the prion gene in order to amplify a 1,400-base-pair section to verify actual insertion of the transgene into the genome.

The construct along with the Cas9 guide RNA were injected into embryos, which we cultured to the blastocyst stage at day 7 (Figure 9) and, indeed, we were able to see GFP being expressed, indicating that we had successfully inserted our transgene. Furthermore, a PCR band appeared at the expected size subsequent to insertion of the transgene. Sequencing confirmed that many of the distinct embryos and the clones derived from those embryos also contained the transgene. On the other hand, we saw deletions in some of the embryos.

In Summary

We feel that we developed and successfully tested several CRISPR-based approaches for gene targeting in swine. We produced embryos and we were able to perform putative KOs using non-homologous end-joining and we plan to use this technology to address animal-welfare issues. We also feel that we can use the oligo-based insertion approaches to modify genes with homology-directed recombination, and we were also able to show that we could direct the targeting of expression cassettes into the genome of embryos as well.

On Going

We are probably a few years behind, but we are performing embryo transfers with our *in vitro* produced embryos in collaboration with the University of Maryland. We are trying to establish a dependable system to produce *in vivo* oocytes, so that we may actually go back and repeat this work, and then again try to refine the system by examining off-targeting and increasing efficiency.

Even though we have not produced animals, pigs in particular have been produced with the CRISPR/Cas 9 system using the SCNT for CD163 or direct embryo modification CD1D (Figure 9).

As an adjunct to our agricultural interests, when we focus on disease or welfare there is opportunity to study gene function particularly as more information is derived about the genome itself and as annotation is improved. There are also possibilities of dual benefit to agriculture and biomedicine where developmental issues and diseases are similar in swine and humans. Additionally, the pig has use as a model; for certain human diseases it is a better model than lab animals and it can also be applied to human transplantation.

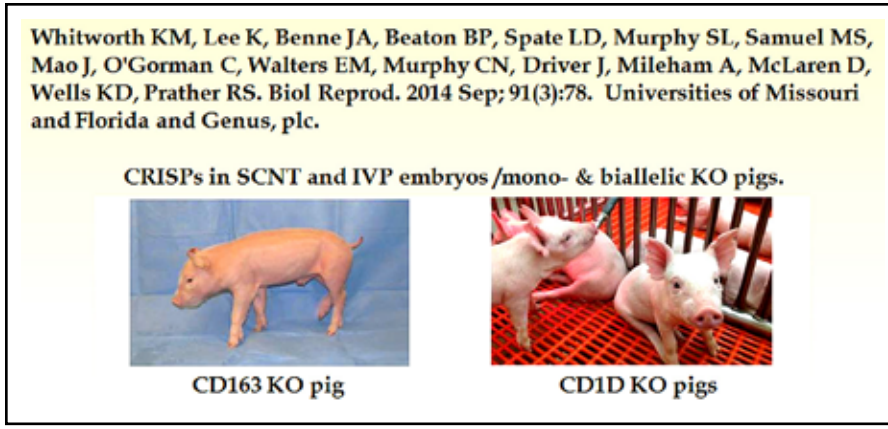

Figure 9. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from *in vitro*-derived oocytes and embryos.

LE ANN Blomberg is the research leader for the Animal Biosciences and Biotechnology Laboratory, Animal and Natural Resources Institute, US Department of Agriculture-Agricultural Research Service in Beltsville, MD. She received a BS in biology at John Brown University and subsequently received an MS and PhD from Georgetown University for her work in defining molecular cues regulating spontaneous postnatal alveoli formation in altricial animals and retinoic acid-induced alveolar regeneration in a rodent emphysema model. After joining the USDA as a postdoctoral fellow in 2002, she became a scientist and subsequent research leader for the unit.

DR. BLOMBERG'S work has focused on reproduction in swine with interests in understanding physiological aspects regulating embryo competence and the impact of uterine stress on fetal/neonatal development.