Gene Editing in Cattle: Recent developments and regulations

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Gene editing allows the introduction of double-stranded breaks at a specific sequence in the genome.

Zinc Finger Nucleases

TALENs

Guide RNA

CRISPR/Cas9

"Knock-out"

Nuclease-induced double-strand break

NHEJ

Deletions

Insertions

Variable length indels

Donor template

HDR

Precise insertion or modification

To get an homology-directed repair (HDR) knock-in donor template is often provided in a plasmid.

“Knock-in”
# How might gene editing be used in cattle breeding programs?

<table>
<thead>
<tr>
<th>Target</th>
<th>Targeted Trait/Goal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraspecies <em>POLLED</em> allele substitution</td>
<td>No horns/welfare trait</td>
<td>Carlson et al., 2016</td>
</tr>
<tr>
<td>Intraspecies <em>SLICK</em> allele substitution</td>
<td>Heat tolerance</td>
<td>Sonstegard et al., 2017</td>
</tr>
<tr>
<td>Myostatin (MSTN) gene knockout</td>
<td>Increased lean muscle yield</td>
<td>Proudfoot et al., 2014</td>
</tr>
<tr>
<td>Beta-lactoglobulin gene knockout</td>
<td>Elimination of milk allergen</td>
<td>Yu et al., 2011</td>
</tr>
<tr>
<td>Prion protein (PRNP) knockout</td>
<td>Elimination of prion protein</td>
<td>Bevacqua et al., 2016</td>
</tr>
<tr>
<td>Intraspecies <em>CALPAIN &amp; CAPASTATIN</em> allele substitution</td>
<td>Improved meat tenderness</td>
<td>Casas et al., 2006 (not reduced to practice)</td>
</tr>
<tr>
<td>Insertion of lysostaphin/lysozyme transgene</td>
<td>Resistance to mastitis</td>
<td>Liu et al., 2013 &amp; 2014</td>
</tr>
<tr>
<td>CD18 gene edit</td>
<td>Resistance to bovine respiratory disease</td>
<td>Shanthalingam et al., 2016</td>
</tr>
<tr>
<td>Insertion of SP110, NRAMP1</td>
<td>Resistance to tuberculosis</td>
<td>Wu et al., 2015; Gao et al., 2017</td>
</tr>
<tr>
<td>Intraspecies SRY translocation onto X chromosome</td>
<td>All male offspring</td>
<td>Owen et al., 2018</td>
</tr>
<tr>
<td>NANOS gene knockout</td>
<td>Infertile males (for surrogate sire and gonial cell transfer)</td>
<td>Ideta et al., 2016</td>
</tr>
</tbody>
</table>

Gene editing to produce Tuberculosis resistant cattle

Genetic improvement (permanent, cumulative) as a solution to animal disease rather than antibiotics/chemicals
Gene Edited Polled Calves
Naturally-occurring bovine allele at polled locus

Production of hornless dairy cattle from genome-edited cell lines

To the Editor:
Physical dehorning of dairy cattle is practiced to protect animals and their handlers. Genetic analyses have identified variants that are associated with hornlessness (referred to as ‘polled’) in cattle, a trait that is common in beef but rare in dairy breeds. We have introgressed a candidate POLLED allele into dairy cattle by genome editing and reproductive cloning, providing both evidence for genetic causation and a means to introduce POLLED into livestock with the potential to improve the welfare of millions of cattle annually.

In the United States, an estimated 80%\(^1\) of all dairy calves (4.8 million per year) and 25% (8.75 million animals) of beef cattle are dehorned every year. A lower proportion of beef cattle than dairy cattle need to be dehorned because the dominant POLLED locus is nearly fixed in beef cattle such as Angus, whereas dairy breeds such as Holstein have a much lower frequency of POLLED because of the small number of sires (6%) producing commercially available POLLED semen\(^2\). Physical dehorning of cattle, which is done to protect animals and producers from accidental injury is not only

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\(^1\) Van Eenennaam 10/23/2019

\(^2\) Van Eenennaam 10/23/2019
Precision breeding offers a new alternative to dehorning
YouTube: https://youtu.be/-Qks_LMmodw
Current polled dairy sires have inferior genetic merit

- Daughters of polled Holstein sires will earn less over their lifetimes
- Polled allele frequency is 0.0071
- Adding polled to selection indices is not effective
- If used exclusively polled sires would increase inbreeding & slow genetic gain

Simulation of introgression of the POLLED allele via conventional breeding versus gene editing.
Gene Edited Polled Calves
Naturally-occuring bovine allele at polled gene

10 base pairs (p)

212 base pairs (P)
A

RC1002

RC.dams1-6 (L to R)

RC.calves1-6 (L to R)

B

HH.sire1

HH.dams1-3 (L to R)

HH.calves1-3 (L to R)

C

HO1

HO1.dams1-3 (L to R)

HO1.calves1-3 (L to R)
We analyzed these six polled calves and horned controls for two years.
You may recognize heifer #1 (Princess) from her magazine cover shot.
Crispr could give us a more humane world. Will humans let that happen?

Genomic and phenotypic analyses of six offspring of a genome-edited hornless bull

Amy E. Young¹, Tamer A. Mansour²,³, Bret R. McNabb²,³, Joseph R. Owen¹, Josephine F. Trott¹, C. Titus Brown³ and Alison L. Van Eenennaam¹*

Genome editing followed by reproductive cloning was previously used to produce two hornless dairy bulls. We crossed one genome-edited dairy bull, homozygous for the dominant P_c Celtic POLLED allele, with horned cows (pp) and obtained six heterozygous (P_c/p) polled calves. The calves had no horns and were otherwise healthy and phenotypically unremarkable. We conducted whole-genome sequencing of all animals using an Illumina HiSeq4000 to achieve -20x coverage. Bioinformatics analyses revealed the bull was a compound heterozygote, carrying one naturally occurring P_c Celtic POLLED allele and an allele containing an additional introgression of the homology-directed repair donor plasmid along with the P_c Celtic allele. These alleles segregated in the offspring of this bull, and inheritance of either allele produced polled calves. No other unintended genomic alterations were observed. These data can be used to inform conversations in the scientific community, with regulatory authorities and with the public around ‘intentional genomic alterations’ and future regulatory actions regarding genome-edited animals.

Received: 4 February 2019; Accepted: 28 August 2019; Published online: 07 October 2019

The (A) wild type HORNED allele, and (B) wild type P_c POLLED allele.

The 212 bp repeat sequence (purple) is duplicated in the wild type P_c POLLED allele and replaces the 10 bp (CTGGTATTCT) orange sequence (*) in the HORNED allele.

The 1.6 kb homology directed repair (HDR) template sequence (red) is identical in sequence to the wild type P_c POLLED allele.

HORNED allele

POLLED allele

POLLED allele - edited

The genome-edited bull carried allele c) the exact same sequence as the wild type P\(_c\) POLLED allele, and allele d) which included both the pCR2.1 plasmid sequence (yellow) and a duplication of the Pc HDR template (red). There was an additional 3.9 + 1.6 = 5.5 kb inserted on one of the two POLLED alleles.

Just for perspective the bovine genome is 3 billion bp

\[
\frac{5,500 \text{ bp}}{3,000,000,000 \text{ bp}} = 0.0002\%
\]

4 offspring (1, 4, 5, 6) inherited the plasmid-bearing allele
2 offspring (2, 3) inherited the naturally-occurring allele
> 86.5 million genomic alterations (SNPs; Indels) between different breeds of cattle

1000 Bull Genomes Project: International consortium sequenced 2703 cattle to 11x fold coverage

The number of SNP variants relative to the ARS-UCD1.2 bovine reference genome


Van Eenennaam 10/23/2019
Gene-edited cattle have a major screwup in their DNA

6th August, 2019

FDA Finds Unexpected Antibiotic Resistance Genes in ‘Gene-Edited’ Dehorned Cattle

12th August, 2019

Genetically engineered hornless cattle: flaws in the genome overloo
e

10th August, 2019
Template plasmid integration in germline genome-edited cattle. 7/28/2019

Alexis L. Norris, Stella S. Lee, Kevin J. Greenlees, Daniel A. Tadesse, Mayumi F. Miller, Heather Lombardi
https://www.biorxiv.org/content/10.1101/715482v1.full; doi: https://doi.org/10.1101/715482
Picnic Day
Surveyed public audience on gene editing
What percentage of animal products like milk, meat, and eggs currently come from animals that have been produced using genetic engineering?

<table>
<thead>
<tr>
<th>Percentage</th>
<th>Before Presentation</th>
<th>After Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;75%</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>51-75%</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>25-50%</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>&lt;25%</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>0%</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>No Idea</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Decline to Answer</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N/A</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
How do you feel about the use of gene editing to address an animal welfare concern?

88% of respondents at this event were strongly or moderately supportive of using gene editing to address an animal welfare concern (i.e. polled).
In conclusion, many participants reported positive attitudes towards GM polled cattle; we suggest that people may be more likely to support GM technologies when these are perceived to benefit the animal.

Editing as a Cherry on Top of the Breeding Sundae

It will be able to introduce useful alleles without linkage drag, and potentially bring in useful novel genetic variation from other species.

Genome Editing

- In vitro embryo fertilization (IVF)
- Genomic Selection
- Embryo Transfer
- Artificial insemination
- Progeny testing
- Performance recording
- Development of breeding goals
- Association of like minded breeders
March 28th, 2018 USDA statement
No additional regulatory requirements if plants could otherwise have been developed through traditional breeding
January 18th, 2017 FDA draft guidance 187 considers the “intentional alterations” in animals whose genomes have been edited to be unapproved animal drugs.

<table>
<thead>
<tr>
<th>DATE</th>
<th>EVENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/2014</td>
<td>Cell line edited &amp; Embryo Transfer</td>
</tr>
<tr>
<td>5/2015</td>
<td>Two edited bulls born in IA</td>
</tr>
<tr>
<td>11/2015</td>
<td>Bulls move to Davis</td>
</tr>
<tr>
<td>9/2017</td>
<td>Six edited bull’s calves born</td>
</tr>
<tr>
<td>2017</td>
<td>Meat collection from bulls</td>
</tr>
<tr>
<td>12/2018</td>
<td>Sequence &amp; phenotype data shared with FDA</td>
</tr>
<tr>
<td>3/2019</td>
<td>Plasmid detected</td>
</tr>
<tr>
<td>9/2019</td>
<td>Meat collection from male offspring (and controls)</td>
</tr>
<tr>
<td>10/2019</td>
<td>Female heifer offspring bred</td>
</tr>
<tr>
<td>6/2020</td>
<td>Milk collection from female offspring (and controls)</td>
</tr>
</tbody>
</table>
2019
Australian Office Gene Technology Regulator

10 base pairs (p)

POLLED GENE

212 base pairs (P)

Template-guided knock-ins regulated, not knock-outs

- not gene technology, not GMOs, not regulated
- gene technology, GMOs, regulated

Natural mutations
mutagenesis
SDN-1

no template

+ template

SDN-2 and ODM
oligonucleotide
long template

Inserting transgenes

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European High Court rules all genome edits are “GMOs”

“Organisms obtained by mutagenesis are GMOs within the meaning of the GMO Directive, in so far as the techniques and methods of mutagenesis alter the genetic material of an organism in a way that does not occur naturally. It follows that those organisms come, in principle, within the scope of the GMO Directive and are subject to the obligations laid down by that directive.

The Court states, however, that it is apparent from the GMO Directive that it does not apply to organisms obtained by means of certain mutagenesis techniques, namely those which have conventionally been used in a number of applications and have a long safety record.” (defined as before 2001)
Would gene-edited polled Holsteins be subject to additional regulations in this country?

<table>
<thead>
<tr>
<th>Country</th>
<th>Additional Regulations?</th>
<th>Basis of trigger/regulation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>No</td>
<td>Novel DNA sequence/transgene</td>
</tr>
<tr>
<td>Australia</td>
<td>Yes</td>
<td>Use of repair template</td>
</tr>
<tr>
<td>Brazil</td>
<td>No</td>
<td>Novel DNA sequence/transgene</td>
</tr>
<tr>
<td>Canada</td>
<td>No</td>
<td>Trait novelty (i.e. novel product risk)</td>
</tr>
<tr>
<td>European Union</td>
<td>Yes</td>
<td>Is a GMO if used a mutagenesis technique not in existence before 2001</td>
</tr>
<tr>
<td>Japan</td>
<td>No</td>
<td>No exogenous genes</td>
</tr>
<tr>
<td>New Zealand</td>
<td>Yes</td>
<td>Using of in vitro technique that modifies the genes/genetic material</td>
</tr>
<tr>
<td>United States</td>
<td>Yes</td>
<td>New Animal Drug</td>
</tr>
</tbody>
</table>
Executive Order on Modernizing the Regulatory Framework for Agricultural Biotechnology Products

The Secretary of Agriculture (Secretary), the Administrator of the Environmental Protection Agency (Administrator), and the Commissioner of Food and Drugs (Commissioner), to the extent consistent with law and the principles set forth in section 3 of this order, shall......

“use existing statutory authority, as appropriate, to exempt low-risk products of agricultural biotechnology from undue regulation.”
Conclusions

- Gene Editing offers an approach to **precisely knock out** undesirable traits and **precisely knock-in** desirable traits in food animal breeding programs.

- It opens up new opportunities for animal breeders to address critical problems such as disease resistance, animal welfare and resilience, and product quality traits.

- Currently there are a patchwork of proposed regulatory approaches for the use of gene editing of food animal species which will potentially result in trade disruptions.

- Harmonizing the regulations associated with gene editing in food species is imperative to allow both plant and animal breeders access to gene editing tools to introduce useful sustainability traits like disease resistance, climate adaptability, and food quality attributes into global agricultural breeding programs.
Thanks for inviting me!

My laboratory receives public funding support from the National Institute of Food and Agriculture and the Biotechnology Risk Assessment Grant (BRAG) program, U.S. Department of Agriculture, under award numbers 2015-67015-23316, 2015-33522-24106, and 2017-33522-27097 (Comparative Evaluation Of The Phenotype, Genomic Stability, And Animal Products Derived From Offspring Of Genome Edited And Control Bulls).