Production of a Gene Knock-In Bull Calf by Embryo-Mediated Genome Editing

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Abstract

Genome editing offers an opportunity to introduce targeted gene insertions into livestock breeding programs. Molecular geneticists have typically employed a donor repair template and the homologous recombination (HR) pathway in somatic cells to introduce gene knock-ins into livestock genomes, followed by cloning. Editing embryos directly to achieve targeted gene knock-ins is inefficient, especially for introducing large DNA sequences. Here we report using a one-step method to produce a gene knock-in bull calf by cytoplasmic microinjection of CRISPR/Cas9 reagents into a bovine embryo. In vitro fertilized one-cell bovine zygotes were injected with a gRNA/Cas9 ribonucleoprotein complex and homology mediated end joining donor template containing the sex determining region Y (*SRY*) gene, the green fluorescent protein (*GFP*) reporter gene driven by the SV40 promoter, and one kilobase homology arms targeting the H11 safe harbor locus on bovine chromosome 17. Seven-day blastocysts were evaluated using fluorescent microscopy, and nine green fluorescent embryos were transferred to synchronized recipients. Ultrasound evaluation at 35 days revealed one pregnancy. In April 2020, a healthy 50 kg male calf was born. DNA was extracted from placenta, blood and a fibroblast line derived from the calf and analyzed for *SRY-GFP* knock-in, as well as genotypic sex. PCR and Sanger sequencing revealed the biallelic edit of the target location on chromosome 17, with the insertion of three or seven copies of the *SRY-GFP* construct in addition to donor plasmid backbone, or a 26 base pair insertion, and an XY genotype. Future analysis of the XX offspring inheriting the *SRY* gene on chromosome 17 from this knock-in bull will reveal whether inheritance of the bovine SRY gene is sufficient to trigger the male developmental pathway in cattle.

Objectives

The objectives of this project were to:

1) Produce a targeted *SRY-GFP* knock-in embryo by cytoplasmic microinjection of CRISPR/Cas9 reagents and homology-mediated end joining (HMEJ)[1] donor vector in presumptive zygotes six hours post insemination (6hpi)
2) Evaluate the presence of gene knock-in by fluorescent microscopy and non-surgically transfer high quality green fluorescent blastocysts
3) Evaluate the knock-in and level of mosaicism in resulting offspring

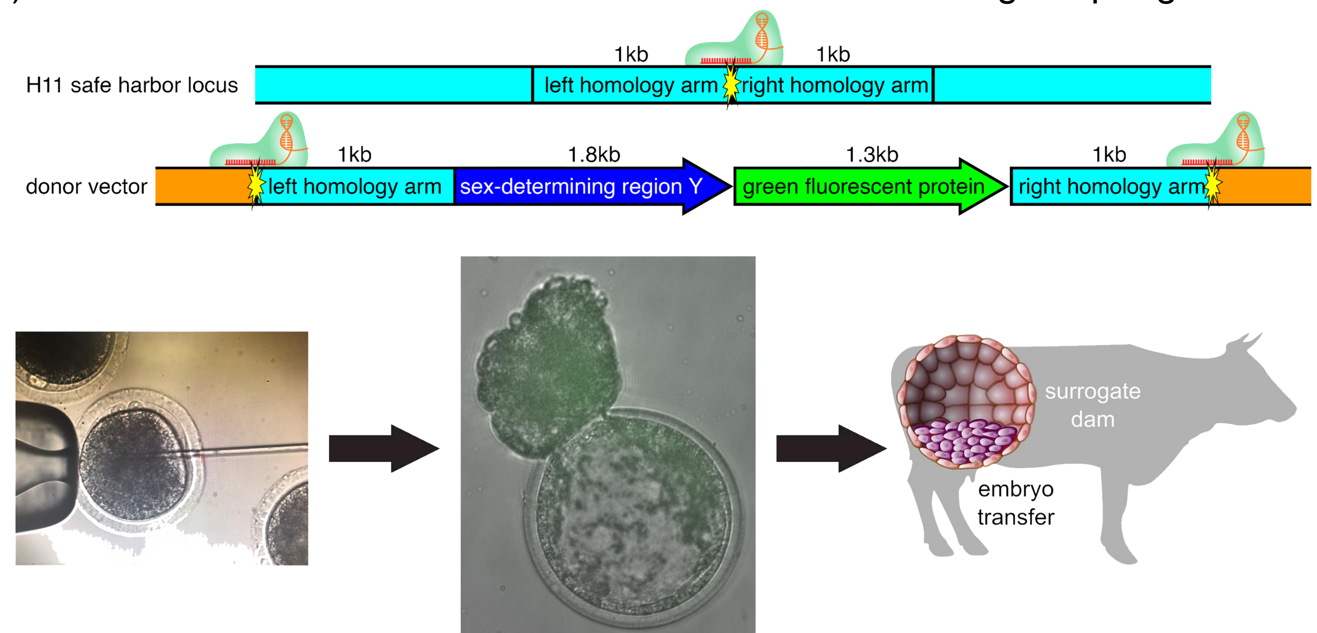

Figure 1. Schematic showing the methods used for producing a gene knock-in calf using the CRISPR/Cas9 system and an HMEJ donor vector in presumptive zygotes 6hpi. Yellow starburst = CRISPR target site at the H11 locus on chromosome 17 with gRNA/Cas9 ribonucleoprotein complex bound.

Results

Approximately 200 *in vitro* matured bovine zygotes were microinjected with the gRNA/Cas9 ribonucleoprotein complex and HMEJ-template at 6hpi as described[2]. Twenty two embryos reached the blastocyst stage, and nine (40%) showed green fluorescence indicating a successful knock-in (Fig. 2). These were non-surgically transferred at day seven to synchronized recipients as described[3].

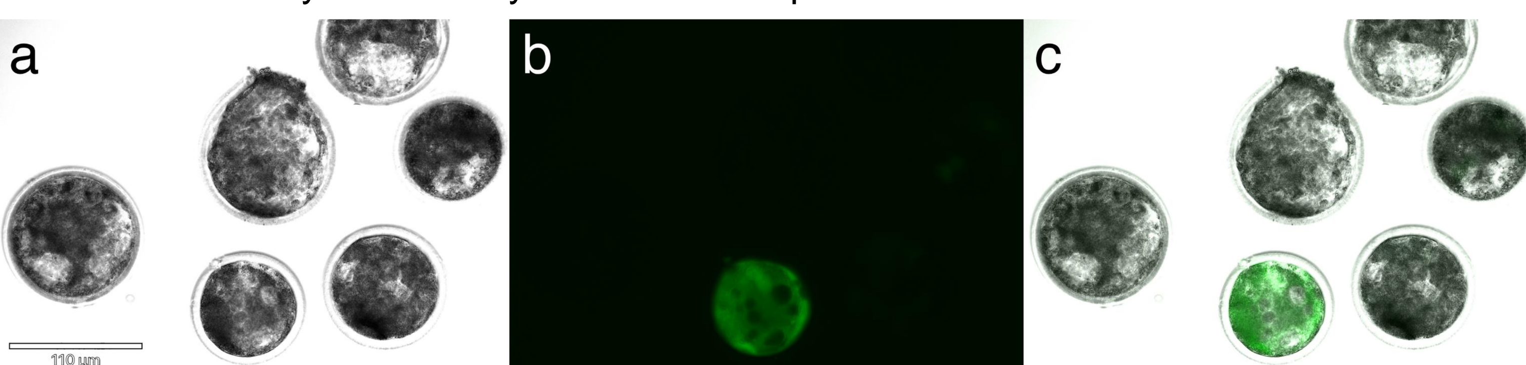

Figure 2. Non-invasive screening of bovine blastocysts to visualize knock-in embryos. (a) day seven microinjected bovine blastocysts under bright field (b) a filter specific for eGFP fluorescence showing knock-in of *SRY-GFP* into the H11 safe harbor locus, and (c) merge of bright field and fluorescent image.

One of the recipients (Tag 3113) was confirmed pregnant by transrectal ultrasonography at day 35 of development, and the phenotypic sex was likewise determined at day 68 with the fetus presenting a male gonad (Fig. 3). In April 2020, a healthy 50 kg bull calf named Cosmo was born (Fig 4a). DNA was extracted from placenta (trophectoderm), blood and a fibroblast cell line (mesoderm) derived from the calf and analyzed for the *SRY-GFP* knock-in, as well as genotypic sex. PCR and Sanger sequencing revealed a biallelic edit with the insertion of the *SRY-GFP* construct, and a 26 base pair insertion, in addition to an XY genotype. (Fig. 4b).

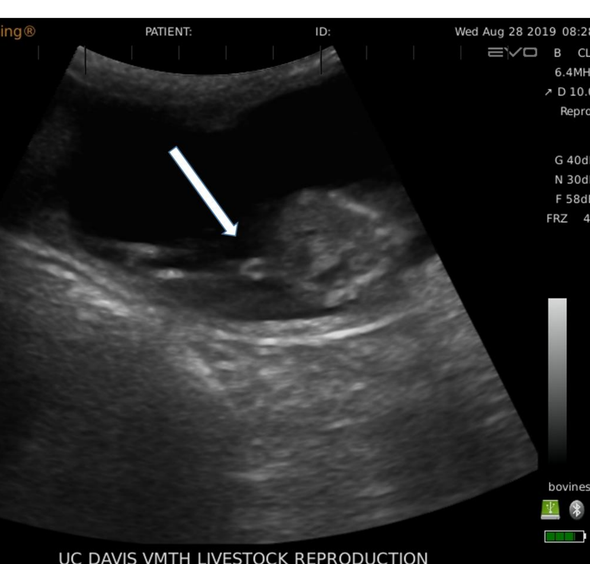

Figure 3. Ultrasound of the day 68 fetus revealing the male genital tubercle (arrow) caudal to the umbilicus indicating a male phenotype.

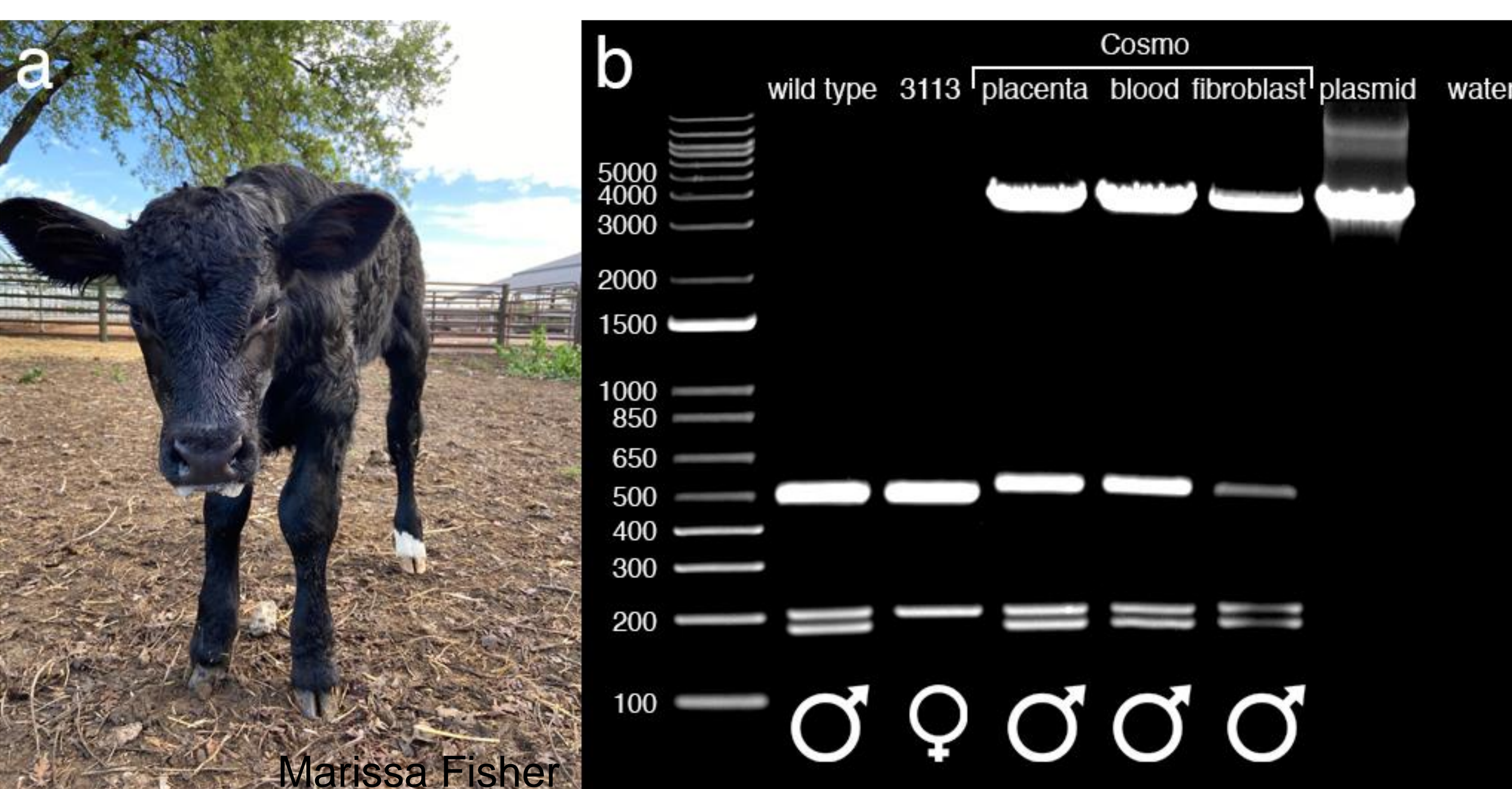

Figure 4. Development and analysis of the non-mosaic heterozygous *SRY-GFP* knock-in bull calf produced by homology mediated end joining and the CRISPR-Cas9 system in bovine zygotes. (a) the *SRY-GFP* knock-in bull calf Como at two days of age, (b) Analysis of *SRY-GFP* knock-in and genotypic sex by the polymerase chain reaction (PCR) of a wild type male, the recipient female (3113) and the *SRY-GFP* knock-in bull calf (Cosmo). DNA was extracted from three tissue types placental cotyledons (trophectodermal origin), blood and fibroblast (mesodermal origin). The donor plasmid was used as the positive control and water was used as the negative control. Upper bands (*SRY-GFP* knock-in): wild type 520bp, knock-in 3721bp. The wild type band from the calf can be seen to be slightly larger than wild type due to a 26 bp insertion confirmed by Sanger sequencing. Lower bands (sexing PCR): female 208bp, male 189bp & 208bp.

Results (cont.)

Genomic DNA from blood was used for Illumina whole-genome sequencing (paired-end, 150bp) on a NovaSeq 6000 sequencer (Novogene, USA) to 268X coverage. Raw reads were mapped to the complete *SRY-GFP* knock-in (Fig. 3a), the 26 base pair insertion allele (Fig. 3b), and the HMEJ-donor plasmid backbone (Fig. 3c). There was a 4X increase in reads that aligned to the knock-in compared to the 26bp insertion (Fig. 3b). In addition, reads were aligned to the HMEJ-donor plasmid backbone, showing the presence of pUC19 donor plasmid backbone in the bull calf's genome (Fig. 3c).

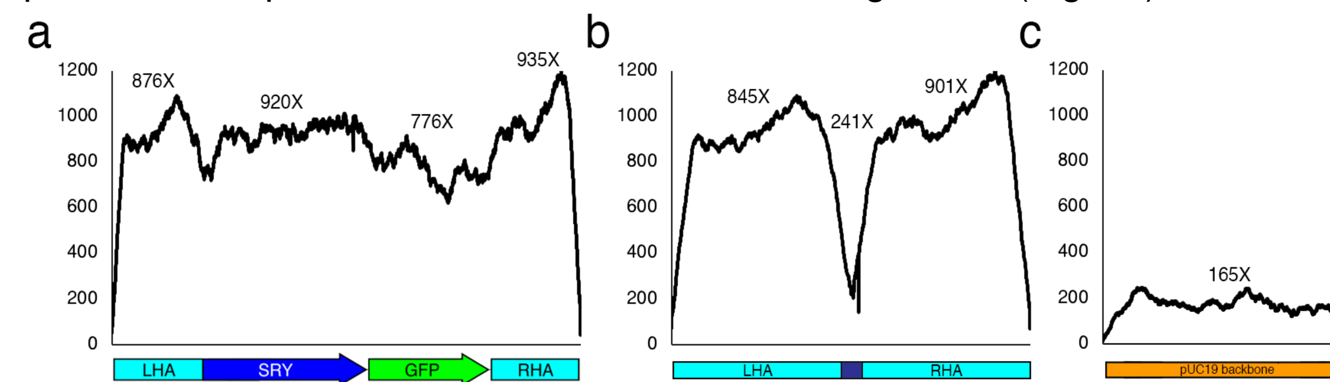

Figure 5. Coverage depth of mapped alignment of whole genome sequencing reads to the expected knock-in, the Sanger sequenced 26 bp knock-in allele, and the pUC19 donor plasmid backbone. (a) coverage depth of reads aligned to the complete 5.1 kb *SRY-GFP* knock-in, (b) coverage depth of reads aligned to the 26 bp insertion, (c) coverage depth of reads aligned to the 2.7 kb pUC19 donor plasmid backbone.

Genomic DNA was used for PacBio long read sequencing in a Sequel II SMRTcell. Reads were aligned against the bovine reference ARS-UCD1.21, the pUC19 plasmid, and the complete knock-in sequence. Three individual insertion alleles were identified: one containing the 26bp insertion (Fig. 5b), a second with seven copies of the knock-in with one copy of the backbone, and a third with three copies of the knock-in with one copy of the backbone (Fig. 6). The 18 kb sequence is identical to the 38 kb allele but missing 4 copies of the knock-in, suggesting it is a contracted deletion allele rather than a separate editing event.

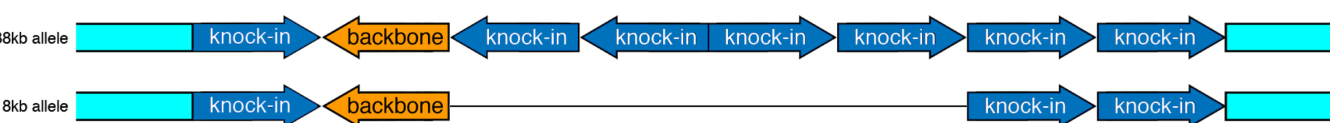

Figure 6. Two complex insertion alleles containing multiple copies of the *SRY-GFP* knock-in as determined by long read sequencing. "knock-in" = 5.1 kb (1 kb left homology arm + 3.1 kb *SRY-GFP* + 1 kb right homology arm complete donor template); "backbone" = 2.7 kb pUC19 donor plasmid backbone.

Next Steps

Fluorescent *in* situ hybridization of chromosomal spreads will be used to confirm the chromosomal location and zygosity of the knock-in insertions. Hi-C, Illumina short read and PacBio long read data will be jointly used to investigate both the haplotype phase of chromosome 17, and evaluate SNPs and alterations in the bull's genome. Future analysis of the XX offspring inheriting the *SRY* gene on chromosome 17 from this knock-in bull will reveal whether inheritance of the bovine *SRY* gene is sufficient to trigger the male developmental pathway in cattle.

References

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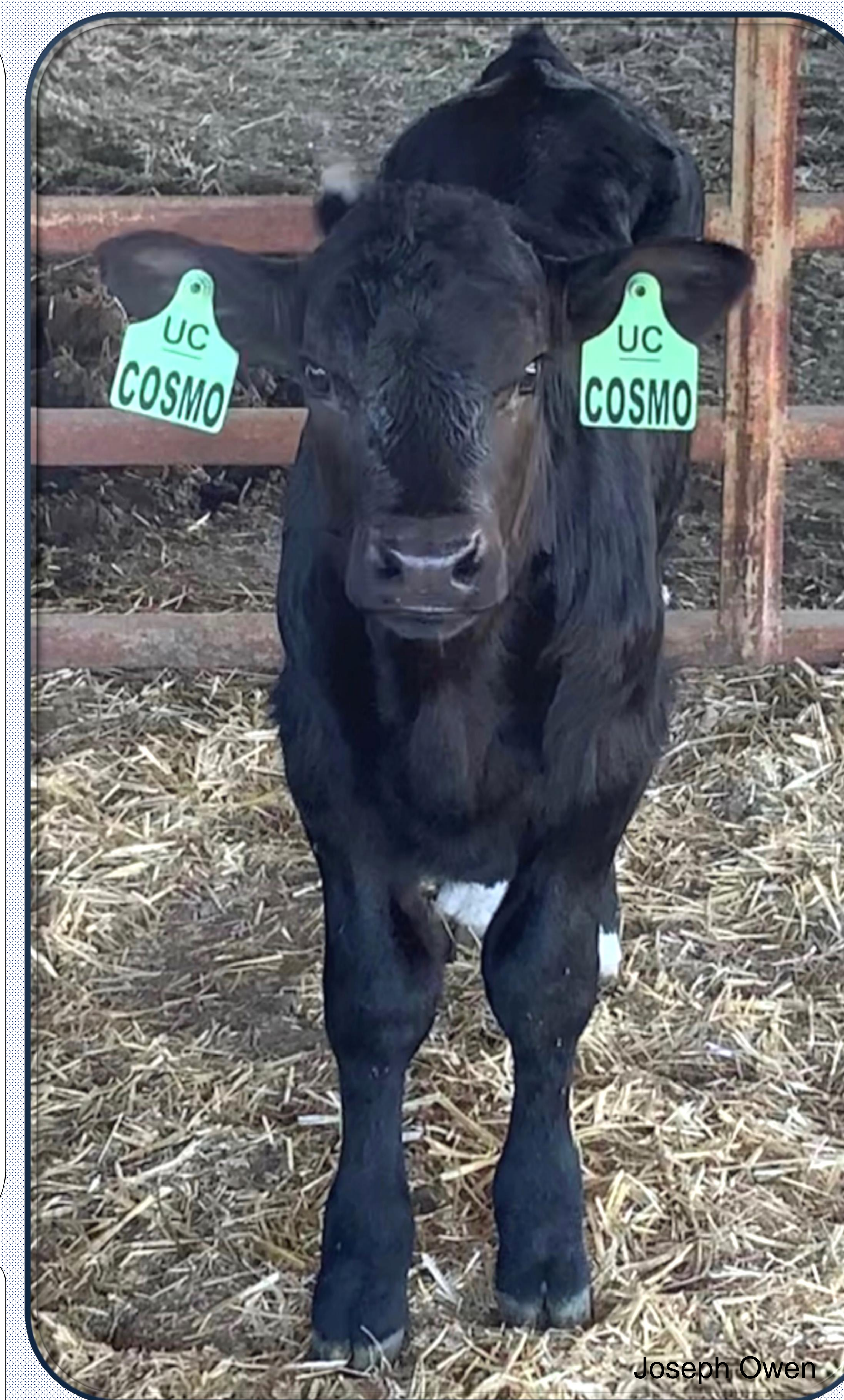

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