# Gene Editing in Equine Embryos: A Model for Gene Therapy to Reduce Burden of Genetic Diseases

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## Background

- OCT4 is a core transcriptional factor that plays a role in maintaining cellular pluripotency in the developing embryo and in culture.[1]
- OCT4 is well conserved across mammals, suggesting conserved function.[2] Three regulatory elements were identified with four conserved regions (CR) across species.[3]
- One equine study showed spatial and temporal differences in OCT4 expression (**Fig. 1**) and that the uterine environment may play a role in OCT4 expression in horses (**Fig. 2**).[4]
- OCT4 knockout bovine embryos were created using CRISPR/Cas9 technology.[5]
- One study produced CRISPR modified equine embryos through somatic cell nuclear transfer [6] (**Fig. 3**), but no direct CRISPR modified equine embryos have been created.

## Hypothesis and Objectives

- Hypothesis: The equine CRs, gene and protein will have comparable sequence similarity to the other species and *OCT4* expression in adult tissues will be low except in ovarian tissue.
- Objective: Compare DNA and protein sequences of across species and investigate RNA-sequencing data to assess gene expression in adult tissues.

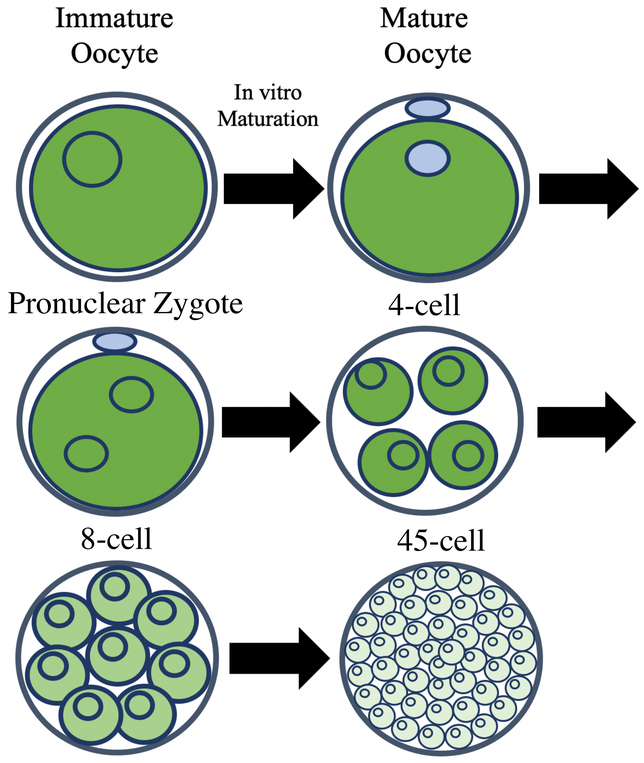


**Figure 1. OCT4 Expression During *In Vitro* Equine Embryo Development.** OCT4 expression is designated in green and shade demonstrates the amount of protein expressed. Blue coloring indicated weak or no OCT4 staining. OCT4 is expressed in the immature oocyte, but after maturation, expression restricts to the oocyte cytoplasm.[4] OCT4 expression continues after fertilization, but decreased until about the 40-cell stage.[4]

## Materials and Methods

**Conserved Region Analysis:**

- Bovine, murine and human sequences were taken from Nordhoff *et al*. 2001.
- NCBI nucleotide BLAST used to identify equine CRs.
- Sequences aligned using MUSCLE (EMBL-EBI).

**Gene Sequence Comparison**

- mRNA sequences were pulled from NCBI references.
- Aligned sequences using NCBI nucleotide BLAST.

**Protein Sequence Comparison:**

- Protein sequences were pulled from NCBI references.
- Sequences were aligned using NCBI protein BLAST.

**RNA-seq Analysis:**

- Data collected for the Functional Annotation of Animal Genomes (FAANG) initiative assessed for expression in the annotated location of *OCT4* (ECA20:30,998,000-31,014,300).[7]
- 46 adult tissues assessed from two replicates.
- Data was visualized with the Integrative Genomics Viewer (IGV, The Broad Institute, San Diego, CA).[8]

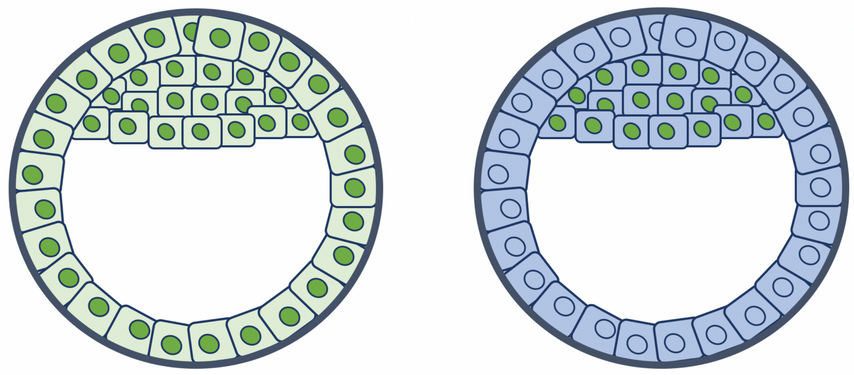


**Figure 2. Comparison of OCT4 Expression in *In Vitro* and *In Vivo* Produced Equine Blastocysts.** As the embryo develops *in vitro*, OCT4 expression increases in the nuclei of all blastomeres.[4] This is in contrast with *in vivo* produced embryos as those embryos only express OCT4 in the inner cell mass.[9] This trend is not seen in other species, suggesting that an extrinsic factor in the equine uterine environment plays a role in regulating equine OCT4 expression.

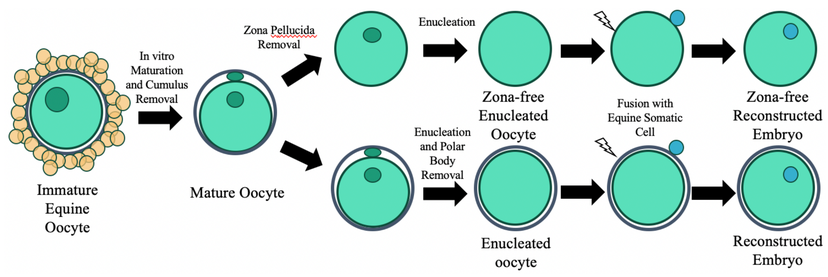


**Figure 3. Schematic Representation of Equine Somatic Cell Nuclear Transfer.** Oocytes are collected via transvaginal aspiration or from abattoir equine ovaries. Oocytes that successfully mature in culture are enucleated and electrofused with the somatic cell donor nucleus. Adapted from Gambini *et al*. 2016.

**Table 1. Comparison of Equine Sequence with Bovine, Murine and Human Conserved Regions.** The percent sequence identity is presented in the table with the number of equal base pairs out of the total base pairs presented in parenthesis.

| | Cattle | Mouse | Human |
|---|---|---|---|
| CR1 | 92.31% (120/130) | 85.38% (111/130) | 94.62% (123/130) |
| CR2 | 96.43% (189/197) | 93.50% (187/200) | 95.41% (187/196) |
| CR3 | 85.85% (91/106) | 80.00% (84/105) | 85.71% (90/105) |
| CR4 | 93.28% (125/134) | 70.15% (94/134) | 91.79% (123/134) |

**Table 2. Gene Sequence Comparison Between Equine, Bovine, Murine and Human *OCT4*.** The percent sequence identity determined from BLAST alignments is presented.

| | Horse | Cattle | Mouse | Human |
|---|---|---|---|---|
| Horse | 100% | - | - | - |
| Cattle | 91.32% | 100% | - | - |
| Mouse | 81.60% | 80.09% | 100% | - |
| Human | 92.47% | 89.30% | 82.12% | 100% |

**Table 3. Protein Sequence Comparison Between Equine, Bovine, Murine and Human OCT4.** The percent sequence identity determined from BLAST alignments is presented.

| | Horse | Cattle | Mouse | Human |
|---|---|---|---|---|
| Horse | 100% | - | - | - |
| Cattle | 94.44% | 100% | - | - |
| Mouse | 83.06% | 81.63% | 100% | - |
| Human | 95.00% | 90.83% | 84.04% | 100% |

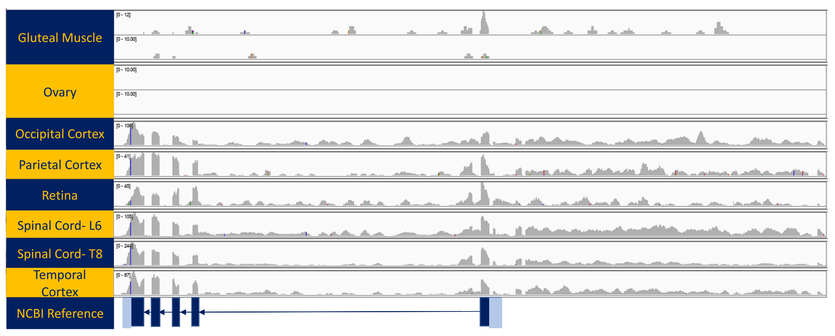


**Figure 4. RNA-seq Comparison Across Adult Equine Tissues.** IGV was utilized to visualize the genomic region containing the OCT4 gene. This figure contains a subset of the tissues assessed showing examples with no expression (gluteal muscle and ovary, two replicates shown) and with expression (remaining tissues, one replicate shown). An adaptation of the NCBI annotation for one isoform of *OCT4* (XM001490108.6) is presented for comparison. The exons are in dark blue while the untranslated regions are in light blue.

**Table 4. Adult Equine Tissues with no OCT4 mRNA expression.** Analysis of the available equine RNA-seq data identified that eight tissues did not have OCT4 mRNA expression whereas the remaining 34 tissues did show mRNA expression in this region. The tissues with no expression are listed here.

| | | |
|---|---|---|
| Gluteal Muscle | Larynx | Longissimus muscle |
| Metacarpal 3 | Ovary | Right Ventricle |
| Superficial digital flexor tendon | Sesamoid bone | |

## Results

- The four CRs were identified in the equine sequence to be located at ECA20:31003077-31003206, ECA20:31004007-31004202, ECA20:31004534-31004638 and ECA20:31005099-31005232.
- Sequence comparison identified high sequence similarity between the proposed equine CRs and the bovine and human CRs (**Table 1**). There was less similarity between the equine and murine sequences, but homologous regions were still identified.
- The *OCT4* mRNA comparison identified the highest sequence similarity between the equine and the human sequences (**Table 2**). The murine sequence was the least similar to the equine sequence, but the sequence identity was similar to the findings between the murine sequence and the cattle and human sequences.
- The protein comparison identified the highest similarity between the equine and human proteins (**Table 3**). The murine protein consistently had the lowest identity with other species.
- Analysis of the RNA-seq data in the genomic region of the *OCT4* gene identified *OCT4* mRNA expression in 34 of the 46 assessed tissues. Sequencing reads coincided with the locations of the exons as annotated in the NCBI equine reference genome for one isoform (XM001490108.6, **Fig. 4**).
- Only 8 tissues assessed did not express *OCT4* mRNA, including ovary (**Table 4**).

## Conclusions

- Analyses of CRs, mRNA, and protein sequences identified high sequence similarity between horses, cattle, humans and mice. Despite the high sequence similarity, previous work suggests that there may be a different functional role of OCT4 in the equine embryo.[4,9]
- RNA-seq analysis identified OCT4 expression in 34 of 46 tissues. Our hypothesis that ovary would have the highest *OCT4* expression was not supported as this tissue had no evidence of expression at this locus.

**Future Directions:**

- Functional work in equine embryos, such as a CRISPR mediated knockout, is needed to ascertain the role of OCT4 in the equine embryo.

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