Biallelic CRISPR-Cas9 editing of gene associated with coat color in microinjected bovine zygotes reaching the blastocyst stage

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Background

CRISPR studies in cattle have focused on cell editing and subsequent cloning (SCNT), attempting founder animal production. Further studies in cattle have been elucidating the role of cell differenciation genes in zygote using both micro-injection and SCNT [1,2]. Editing of these genes led to developmental arrest before reaching blastocyst stage as well as lower blastocyst rate. Additionnal efforts have been performed to reduce mosaicism by changing the injection time post-fertilization.

COPA is involved in ER-Golgi transport and its mutation is associated with coat color variation in cattle [3], but also in lung diseases and arthritis in humans [4] and increased proliferation, migration and invasion of cancer cells *in vitro* [5].

Aim

We investigated the feasibility of CRISPR-Cas9 gene editing via micro-injection and electroporation in a conventional IVF protocol to derive edited blastocysts.

Methods

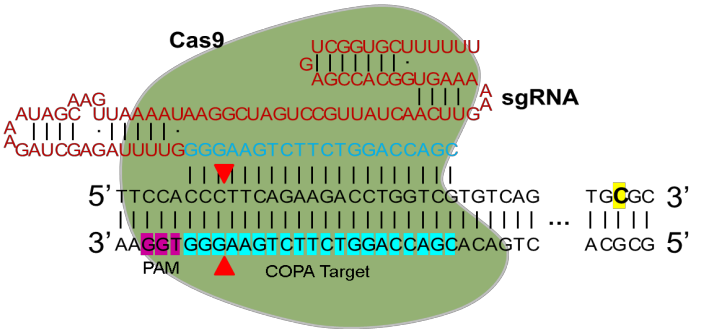

Figure 1 – Schematic representation of the COPA sgRNA target. The missense mutation associated with coat color in cattle is highlighted in yellow.

Micro-injection — *Selection*

Presumptive zygotes (24h post-fert.) → PX-458 plasmid → Day 8 GFP+

Presumptive zygotes (24h post-fert.) → Ribonucleoprotein (RNP) ATTO 550 → ~ 24h post-injection

Electroporation — *Pulse duration*

Presumptive zygotes (24h post-fert.) → Ribonucleoprotein (RNP) ATTO 550 → 1ms / 3ms

Editing validation

Nested PCR → T7E1 assay → Clonal sequencing

References

1. **Daigneault** B W, Rajput S, Smith G W, Ross P J. **Embryonic POU5F1 is required for expanded bovine blastocyst formation**. Scientific reports 8:7753 2018.
2. **Simmet** K, Zakhartchenko V, Philippo-Masser J, Blum H, Klymiuk N, Wolf E. **OCT4/POU5F1 is required for NANOG expression in bovine blastocysts.** PNAS 115(11) 2018.
3. **Dorshorst** B, Henegar C, Liao X, Almen M S, Rubin CJ, Ito S, Wakamatsu K, Stothard P, Van Doormaaf B, Plastow G, Barsh G S, Andersson L. **Dominant red coat color in holstein cattle is associated with a missense mutation in the coatomer protein complex, subunit alpha (COPA) gene**. PLoS ONE 10(6) 2015.
4. **Watkin** et al. **COPA mutation impair ER-Golgi transport and cause hereditary autoimmune-mediated lung disease and arthritis.** Nature genetics 47(6) 2015.
5. **Peng** et al. **A-tol RNA editing contributes to proteomic diversity in cancer.** Cancer Cell 33(5) 2018

Results

Fluorescence is not a good editing selection tool

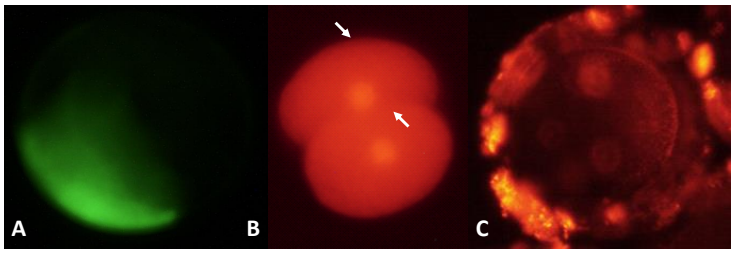

Figure 2 – Fluorescence selection of CRISPR injected presumptive zygotes(A, B) as well as electroporated zygotes (C). Arrows indicate the localization of the RNP effector complex in the nucleus.

Electroporation of RNP yields the highest editing rate

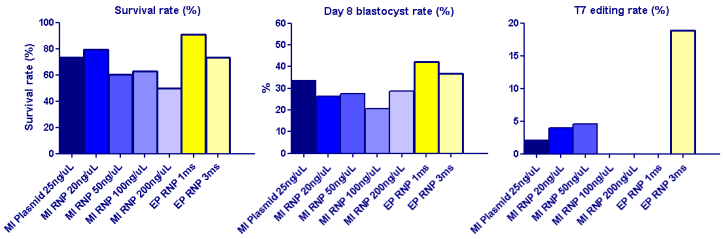

Figure 3 – Survival, blastocyst and T7 editing rate of both micro-injected (MI) and electroporated (EP) groups at various concentrations as well as pulse length.

COPA biallelic edited zygotes can reach blastocyst stage

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                1        10        * 20        30        40        48
                |--------+---------+---------+---------+---------|
      Reference TGCGCTCAGTTCCACCCT----------T-CAGAAGACCTGGTCGTG
Plasmid-BlastoGFP- TGCGCTCAGTTCCACCCT----------T-CAGAAGACCTGGTCGTG
  Plasmid-Morula1 TGCGCTCAGTTCCACCCT----------T-CAGAAGACCTGGTCGTG
  Plasmid-Morula2 TGCGCTCAGTTCCACCCT----------TTCAGAAGACCTGGTCGTG
  Plasmid-Morula3 TGCGCTCAGTTCCACCCAGTTCCACTCAGTTCAGAAGACCTGGTCGTG
   Plasmid-Morula TGCGCTCAGTTCCAC----------TCAGAAGACCTGGTCGTG
    Plasmid-8Cell TGCGCTCAGTTC----------AGAAGACCTGGTCGTG
   Plasmid-Blasto TGCGCTCAGTTC----------AGAAGACCTGGTCGTG
  Plasmid-8Cell2 TGCGCTCAG----------AAGACCTGGTCGTG
 Plasmid-Blasto2 TGCGCTCAG----------AAGACCTGGTCGTG
 Protein-Blasto TGCGCTCAGTT----------CCTGGTCGTG
      Consensus TGCGCTCAGtt.......  .....aagaCCTGGTCGTG
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Figure 4 – Characterization of editing events in plasmid and protein injected embryos. * represents the sgRNA guided restriction site.

Conclusion

Microinjection and electroporation of CRISPR plasmids and protein in presumptive zygotes is possible and mosaic editing as well as biallelic editing can occur from this procedure. Even if survival rates and developmental rates are similar, electroporation of RNP seems to yield a higher amount of editing in blastocysts.

Since coatomer protein complex subunit alpha (COPA) acts as a protein carrier, it is possible that homozygous knock-out causes developmental arrest in embryos. Homology-directed repair should ensure proper missense mutation of the gene in future founder animals.

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