20 Generation of myostatin knockout horse embryos using clustered regularly interspaced short palindromic repeats/CRISPR-associated gene 9 and somatic cell nuclear transfer

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Abstract

Historically, livestock improvement by selective breeding was the principal selection force in animal production and welfare, but the desired phenotype may involve more than 1 generation. Nowadays, new technologies such as CRISPR/Cas9 could overpass these limits and improve animal quality by insertion or modification of the desired genotype. In this work, we aim to knock out the myostatin (MSTN) gene, a negative regulator of muscle mass development, in equine cells and to generate equine cloned embryos with this modified genotype. To achieve this, 1×10^5 equine fibroblasts were nucleofected with 5, 2, or 1 μg of the plasmid hspCas9-2A-Puro V2.0 that codified for the Cas 9 nuclease and a single guide RNA (gRNA). Two different gRNA (gRNA 1 and gRNA 2) complementary to the first exon of the MSTN gene were designed and evaluated. Cells were also nucleofected with the enhanced green fluorescent protein-N1 plasmid in order to determine the transfection efficiency, obtaining more than 90% of enhanced green fluorescent protein+ cells in the 3 conditions. Forty-eight hours after nucleofection, cells were treated with 2.5 μg mL^{-1} of puromycin for 48 h to isolate cells that incorporated the plasmid. After that, clonal culture was achieved by plating individual cells in 96-well plates. The clones were then expanded individually and genomic DNA was isolated from each one, genotyped for the MSTN exon 1 locus by PCR amplification, and Sanger sequenced. Both gRNA had mutational activity, with 96% efficiency (24/25 clones) for gRNA 1 and 55.5% mutation activity (10/18 clones) for gRNA2. We obtained different genotypes depending on the gRNA and the dose that was used-gRNA 1: 1 μg = 57% wt/wt, 14% wt/mutX, and 29% mutX/mutX; 2 μg = 33.3% wt/wt, 33.3% mutX/mutY, and 33.3% mutX/mutX; 5 μg = 40% wt/wt and 60% mutX/mutY; gRNA 2: 1 μg = 17% wt/wt, 17% wt/mutX, 50% mutX/mutY, and 17% mutX/mutX; 2 μg = 67% mutX/mutY and 34% mutX/mutX; 5 μg = 54% mutX/mutY and 46% mutX/mutX. Two of the gRNA2 mutated cell lines were used for embryo generation by NT, 1 wt/mutX line (gRNA2-1ug-C2, heterozygote clone) and 1 mutX/mutX line (gRNA2-5ug-C13, homozygote clone). Before that, to assess specificity, the first 2 highly ranked off-target sites of gRNA2 were checked by Sanger sequencing in the selected clones, not observing modifications in their sequences. In both cases, we could generate edited MSTN equine cloned blastocysts: 3/153 (2%), 3/155 (2%), 8/140 (6%), and 9/73 (12%) for gRNA2-1μg-C2, gRNA2-5μg-C13, control fibroblasts, and control mesenchymal cells, respectively. In conclusion, genome edition by CRISPR/Cas9 is an efficient method to edit the genome of horse fibroblasts in a dose-dependent manner with apparent high specificity. Moreover, equine embryos can be generated with these cells with lower blastocyst rates than control fibroblasts of the same cell line or mesenchymal cells, probably due to higher cell passages needed for cell clone isolation and expansion. To the best of our knowledge, this is the first report of genome edited horse embryos.

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