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Timing of CRISPR/Cas9-related mRNA microinjection after activation as an important factor affecting genome editing efficiency in porcine oocytes

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ABSTRACT

Recently, successful one-step genome editing by microinjection of CRISPR/Cas9-related mRNA components into the porcine zygote has been described. Given the relatively long gestational period and the high cost of housing swine, the establishment of an effective microinjection-based porcine genome editing method is urgently required. Previously, we have attempted to disrupt a gene encoding α-1,3-galactosyltransferase (*GGTA1*), which synthesizes the α-Gal epitope, by microinjecting CRISPR/Cas9-related nucleic acids and enhanced green fluorescent protein (*EGFP*) mRNA into porcine oocytes immediately after electrical activation. We found that genome editing was indeed induced, although the resulting blastocysts were mosaic and the frequency of modified cells appeared to be low (50%). To improve genome editing efficiency in porcine oocytes, cytoplasmic injection was performed 6 h after electrical activation, a stage wherein the pronucleus is formed. The developing blastocysts exhibited higher levels of EGFP. Furthermore, the T7 endonuclease 1 assay and subsequent sequencing demonstrated that these embryos exhibited increased genome editing efficiencies (69%), although a high degree of mosaicism for the induced mutation was still observed. Single blastocyst-based cytochemical staining with fluorescently labeled isolectin BS-I-B_4 also confirmed this mosaicism. Thus, the development of a technique that avoids or reduces such mosaicism would be a key factor for efficient knock out piglet production *via* microinjection.

1. Introduction

Recently, a series of nuclease-based genome editing technologies, including zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)9 (CRISPR/Cas9), have been developed. They enable targeted and efficient modification of a variety of eukaryotic species, particularly mammalian [1,2]. In the case of CRISPR/Cas9-based genome editing, it requires a guide RNA (gRNA) which can bind to the specific chromosomal DNA site together with the Cas9

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endonuclease [3–6]. Once bound, two independent nuclease domains in Cas9 will each cleave one of the DNA strands 3 bases upstream of the protospacer adjacent motif (PAM), introducing double-strand breaks (DSBs) at the host chromosome target site, which are repaired by non-homologous end-joining (NHEJ). The NHEJ-based repair process generates nucleotide insertion or deletion (indel mutations), or causes a frame-shift, which disables encoded proteins or forms premature stop codons, ultimately leading to the generation of a loss-of-function allele. Due to the difficulties in design and assembly, and the limited availability of target sites [7], CRISPR/Cas9 is becoming the most frequently used tool for producing genetically modified organisms [1,2]. To perform CRISPR/Cas9-mediated genome editing efficiently, the selection of appropriate gRNA is important, since targeting efficiency can vary between loci, and even between target sites within the same locus [8–10].

Direct zygotic injection of CRISPR/Cas9 components is becoming a major tool for the production of biallelic knock out (KO) animals, including mice, rats, rabbits, and monkeys [11–18]. In the case of KO pig production, Hai et al. [19] first demonstrated that cytoplasmic injection of mRNAs as CRISPR/Cas9 components into zygotes isolated from oviducts, and subsequent return of the treated zygotes to the source oviducts, led to the production of biallelic KO piglets. Whitworth et al. [20] also achieved similar results using *in vitro*-produced zygotes. These findings encouraged us to produce KO piglets through one-step injection of CRISPR/Cas9-related components (mRNA) into zygotes. On this occasion, the most important issue was determining the appropriate gRNA to enable efficient genome editing. Most porcine studies have focused on molecular analysis of resulting fetuses or newborns to confirm the presence of genome editing-induced mutations; determining whether the constructed gRNA is optimal for efficient target gene KO is both time-consuming and labor-intensive. Sakurai et al. [21] proposed a single blastocyst-based assay in mice, which is centered on the T7 endonuclease 1 (T7E1)-based cleavage assay for CRISPR/Cas9-induced indel detection. They used repeated PCR of genomic DNA isolated from a single blastocyst for target region amplification and successfully identified mutant alleles. In mice and swines, it takes 3 and 7 days, respectively, to reach the blastocyst stage after fertilization. Therefore, rapid evaluation is possible if blastocysts are used for molecular analyses. The other advantage to using a single blastocyst as an experimental target is that localization of the protein generated from a target locus can be simply assessed by cytological methods using lectin or antibody probes. In other words, assessing the consequence of mutations at a target locus should be possible by single embryo staining in the presence of such probes.

α-1,3-galactosyltransferase (α-GalT) synthesizes the α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R), which is expressed on the cell surface in almost all mammals, except for humans and Old World monkeys [22,23]. The α-Gal epitope is specifically recognizable by staining with the isolectin, BS-I-B_4 (IB4) [24,25]. We have previously described the expression of α-Gal epitope in porcine embryos using quantitative reverse transcription (RT)-PCR and cytochemical staining with fluorophore-conjugated lectin, which showed that its expression was already observable in oocytes and their zona pellucida (ZP), likely because of maternally accumulated products [26]. α-GalT mRNA (generated from the embryonic genome) is detectable throughout the embryonic cleavage stage and reaches peak expression during the blastocyst stage [26]. However, our previous experiment using α-Gal epitope negative cells as a somatic cell nuclear transfer (SCNT) donor demonstrated that α-Gal epitope expression detected by staining with a fluorophore-conjugated IB4 was almost diminished in both ZP and embryo itself in the SCNT-derived blastocysts [27], which suggested the disappearance of the accumulation of the maternally inherited α-Gal epitope in the cytoplasm of embryo and ZP, during embryogenesis and up to the blastocyst stage. This unique detection system using a fluorophore-conjugated IB4 is beneficial to researchers, since the consequence of CRISPR/Cas9-mediated genome editing can be visualized as a loss of the α-Gal epitope from the blastocyst surface. In our previous study, we injected mRNA CRISPR/Cas9 components targeted to the porcine α-GalT gene (*GGTA1*) into parthenogenetic oocytes immediately after electric activation, and found that genome editing was indeed successful, although its mode was mosaic and its frequency appeared to be low (50%) [28].

There are several factors affecting the genome editing efficiency of zygote injection experiments, apart from appropriate gRNA selection. For example, there may be a particular mRNA concentration that confers the greatest target gene KO efficiency. Furthermore, there may be a particular zygotic injection time that permits the greatest KO efficiency. In the previous successful KO piglet studies [19,20], mRNA was injected into *in vivo*-fertilized oocytes collected from females the day after insemination or *in vitro*-fertilized (IVF) oocytes at 14 h post-fertilization, suggesting that injection during the pronuclear stage may be suitable. Therefore, in this study, we injected mRNA into porcine oocytes 6 h after electric activation, when most oocytes should contain pronuclei [29], to assess whether this improved the rate of successful genome editing.

2. Materials & methods

The experiments described were performed in accordance with the guidelines of *Kagoshima University Committee on Recombinant DNA Security* and approved by the *Animal Care and Experimentation Committee of Kagoshima University* (no. S28003; 16th May 2016).

2.1. Experimental outline

An outline of this study is shown in Fig. 1A. We chose parthenogenetically activated (PA) porcine oocytes that had been electrically activated because they develop normally *in vitro*, like the IVF porcine oocytes [30]. Immediately after electrical activation, CRISPR/Cas9-related mRNA + enhanced green fluorescent protein (*EGFP*) mRNA was injected into the cytoplasm of oocytes (control group; Cont), which were then cultured *in vitro* for 7 d to reach the blastocyst stage. In the experimental (Exp) group, the PA oocytes were cultured for 6 h after electrical activation, and then injected with the same mRNAs described above. The injected oocytes were treated as shown for the Cont group. The development rates of the injected oocytes to the 2-cell and blastocyst stages were recorded for each group. All resulting blastocysts were fixed and stained red with Alexa Fluor 594-labeled IB4 (AF594-IB4), which can specifically bind to the α-Gal epitope [25]. Thus, it is likely that blastocysts with EGFP-derived fluorescence may also have exhibited decreased α-Gal epitope expression due to CRISPR/Cas9-mediated disruption of the α-GalT gene. However, blastocysts without any EGFP-derived fluorescence would exhibit normal AF594-IB4 staining, since they may have lost the injected mRNA and therefore had no target gene mutations. After fluorescence photography, each blastocyst was transferred to a small tube and lysed to isolate genomic DNA, which was subjected to whole genome amplification (WGA) for a T7E1-based assay and sequencing, as detailed below.

2.2. Preparation of mRNAs for microinjection

All CRISPR/Cas9 components were the same as reported in our previous paper [27]. For Cas9 expression in mammalian cells, we used a plasmid encoding humanized Cas9, a gift from George Church (Addgene plasmid # 41815) [31]. The plasmid for gRNA#3