Original Article

Development of PCR based approach to detect potential mosaicism in porcine embryos

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ABSTRACT Direct injection of genome editing tools such as CRISPR/Cas9 system into developing embryos has been widely used to generate genetically engineered pigs. The approach allows us to produce pigs carrying targeted modifications at high efficiency without having to apply somatic cell nuclear transfer. However, the targeted modifications during embryogenesis often result in mosaicism, which causes issues in phenotyping founder animals and establishing a group of pigs carrying intended modifications. This study was aimed to establish a genomic PCR and sequencing system of a single blastomere in the four-cell embryos to detect potential mosaicism. We performed genomic PCR in four individual blastomeres from four-cell embryos. We successfully amplified target genomic region from single blastomeres of 4-cell stage embryo by PCR. Sanger sequencing of the PCR amplicons obtained from the blastomeres suggested that PCR-based genotyping of single blastomere was a feasible method to determine mutation type generated by genome editing technology such as CRISPR/Cas9 in early stage embryos. In conclusion, we successfully genotyped single blastomeres in a single 4-cell stage embryo to detect potential mosaicism in porcine embryos. Our approach offers a simple platform that can be used to screen the prevalence of mosaicism from designed CRISPR/Cas9 systems.

Keywords: blastomere, CRISPR/Cas9, gPCR, mosaicism, pig

INTRODUCTION

With the recent development of genetic scissors such as Zing Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9, scientists are increasingly turning to these methods for generating transgenic organisms (Hauschild et al., 2011; Lutz et al., 2013; Sander and Joung, 2014). In particular, CRISPR/Cas9 system has been successful in introducing

targeted modifications to the genome of different species with relatively low manufacturing cost and greater efficiency (Albadri et al., 2017; Ma et al., 2017; Cho et al., 2018a; Tian et al., 2018; García-Tuñón et al., 2020).

Pigs are physiologically and anatomically similar to humans, making them suitable for a candidate species for xenotransplantation. Other advantages of pigs as an animal model include relatively short gestation periods, high litter size, highly developed embryo micromanipulation system, and the possibility of artificial insemination

(Ji et al., 2017; Lee et al., 2017; Cho et al., 2018b; Choi and Lee, 2019). During the last 20 years, genetically engineered (GE) pigs have been mainly produced through manipulating genes in the somatic cells, followed by somatic cell nuclear transfer (SCNT) (Dai et al., 2002; Lai et al., 2002; Choi et al., 2017; Jeon and Rho, 2020). Although successful, GE pigs produced via SCNT often present health complications (Carter et al., 2002; Piedrahita et al., 2004; Lee et al., 2005). Alternatively, direct injection of genome editing tools such as CRISPR/Cas9 system into developing embryos can introduce targeted modifications. GE pigs produced via direct injection of CRISPR/Cas9 system do not possess health complications at birth; however, the pigs may carry more than two alleles if targeted modifications occur after cell division. The mosaicism is considered to be a shortcoming of the approach because the phenotype of mosaic pigs often does not represent targeted modifications and the genotype of progeny derived from the mosaic pigs is difficult to predict.

The frequency of mosaicism is difficult to assess because capturing all alleles from developing embryos is extremely challenging. Establishing an effective system to detect the frequency of mosaicism will assist us to design CRISPR/Cas9 systems with minimal mosaicism. Typically, genomic PCR followed by sequencing is performed to detect potential mosaicism using blastocysts (Hirata et al., 2019). It is easier to conduct the PCR analysis using blastocysts because sufficient amount of genetic content can be obtained. However, PCR amplification of genomic DNA from blastocysts will not be able to calculate the degree of mosaicism and detect all possible alleles as PCR amplifications can be biased from a pool of genomic DNA. Single cell sequencing approach may be used to detect the level of mosaicism in blastocysts; however, cost and labor associated with the analysis are extensive.

In this study, we attempted to develop a simplified method to identify genomic information of 4-cell stage embryos by genotyping single blastomeres in the embryos. Specifically, we amplified and sequenced genomic region of *TET1* gene from single blastomeres of 4-cell stage embryos to verify the feasibility of detecting mosaicism in early stage embryos. Our procedure will greatly benefit efforts to prevent mosaicism when engineering the genome using CRISPR/Cas9.

MATERIALS AND METHODS

Experiments of this study including *in vitro* embryo production and genotyping, were conducted based on our previous reports (Lei et al., 2016; Ryu and Lee, 2017; Uh et al., 2020). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

In vitro maturation of pig oocytes

Sow ovaries obtained from a local abattoir were transported to the laboratory and washed with DPBS. Oocytes were aspirated from the ovaries using an 18-gauge needle attached to syringes. Aspirates were washed with TL-Hepes medium three times and placed in petri dishes. Cumulus-oocyte complexes (COCs) with evenly granulated cytoplasm and intact surrounding cumulus cells were collected using a finely drawn glass capillary pipet under a microscope. Approximately 50 COCs were placed in a single well of 4-well dishes containing maturation medium (TCM-199 supplemented with 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/mL epidermal growth factor (EGF), 0.5 µg/mL luteinizing hormone (LH), 0.5 µg/mL follicle stimulating hormone (FSH), 10 ng/mL gentamicin, and 0.1% polyvinyl alcohol (PVA); pH 7.4), and incubated for 42–44 h at 38.5°C, 5% CO₂, and 100% humidity.

In vitro fertilization of pig oocytes

The COCs incubated in maturation medium were placed in a 1.5 mL centrifuge tube containing denuding medium (0.3 M mannitol, 0.001% BSA, 0.03% hyaluronidase, 5% TL-Hepes medium in distilled water; pH 7.4), and then the cumulus cells were removed by vortexing. Oocytes with a polar body were collected in manipulation medium (TCM-199 supplemented with 0.6 mM NaHCO₃, 2.9 mM Hepes, 30 mM NaCl, 10 ng/mL gentamicin, and 3 mg/mL bovine serum albumin [BSA]) and placed in 50 µL droplets of fertilization medium (modified Tris-buffered medium with 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂, 11 mM glucose, 20 mM Tris, 2 mM caffeine, 5 mM sodium pyruvate, and 2 mg/mL BSA) in a group of 25–30 oocytes. Fresh semen obtained from boars were mixed with semen extender and stored at 17°C up to one week. After placing the oocytes in fertilization medium, 1 mL semen was diluted by adding 9 mL DPBS supplemented with 0.1% BSA, and then washed at 720 × g for 3 min by centrifugation.

After 3 washing steps, the sperm pellet was resuspended with fertilization medium, and 50 μ L of the resuspension was added to the droplet containing oocytes. The final concentration of sperm in the droplet was approximately $0.25 \times 10^6/\text{mL}$. Oocytes and sperms were coincubated in the fertilization medium at 38.5°C, 5% CO₂ for 5 h, then moved to culture droplets containing PZM3 medium (Yoshioka et al., 2002) and incubated at 38.5°C, 5% CO₂, 5% O₂.

Genotyping of single blastomeres

At day 2 after *in vitro* fertilization, 4-cell stage embryos were collected for genotyping. Embryos were placed in DPBS with 1% BSA, adjusted to pH 1.98, and gently pipetted a few times to completely remove the zona pellucida and attached sperm. Then, blastomeres were separated into individual cells through repetitive pipetting in DPBS with 1% BSA. To isolate gDNA, individual blastomere was placed in a PCR tube with 12 μ L embryo lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.5, 0.5% Nonidet P40, 0.5% Tween-20, and 200 μ g/mL proteinase K) and incubated at 65°C for 30 min, followed by 95°C for 10 min. Target region of *TET1* gene was amplified using Dream Tag DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Primers used for PCR amplification are forward: 5'-CTGAGAGA-ATGCATAGGAAAAAGACTGACTTCCAG-3' and reverse: 5'-GTCCTATCCAAATTCATTCTGCTGCTCC-3'. Thermocycling conditions were as follows: 95°C for 2 min; followed by 39 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min, and storage at 4°C. Amplicons were visualized using 2% gel electrophoresis, then purified using a

PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA) and used for Sanger sequencing. Schematic summary of the genotyping approach is depicted in Fig. 1.

RESULTS

Successful PCR amplification of a target gene from a single blastomere

We examined whether PCR amplification from a small amount gDNA is feasible. Similar to our previous study (Uh et al., 2020), a region of *TET1* gene was successfully amplified from a single blastocyst (Fig. 2). Sensitivity of the PCR amplification was high enough to amplify the *TET1* region from a single 4-cell stage embryo, i.e. four copies of genomic DNA. To examine the feasibility of amplifying the target region from a single blastomere, 4 blastomeres were separated from a 4-cell stage embryo. Genomic DNAs derived from the individual blastomeres were used for PCR, and target region was successfully amplified from the blastomeres. This result indicates that a single copy DNA in a single blastomere of a 4-cell stage embryo gDNA is sufficient to amplify a target genomic region through conventional PCR approach.

Genotyping of single blastomeres from a 4-cell stage embryo

The PCR amplicons obtained from the whole 4-cell stage embryo and the single blastomeres of the 4-cell embryo were purified and sequenced to verify whether the quantity and quality of the amplified DNA are appropriate for genotyping. Sequencing result of the whole 4-cell embryo showed clear single peaks in the all nucleotide sequences of the target region in chromatogram (Fig. 3A and 3B). Clear single peaks matched to target sequence

Fig. 1. Schematic diagram of the approach for genotyping of single blastomere in 4-cell embryos.

Fig. 2. Gel-electrophoresis image of *TET1* gene that PCR amplified from embryonic DNA. The size of amplified region of wild-type *TET1* gene is 431 bp. The first lane is a size marker. The target regions were successfully amplified from single blastomeres (lane 2-5) of a 4-cell embryo. The four blastomeres were isolated from an identical 4-cell embryo. The target region was also amplified in a whole 4-cell embryo (lane 6) and blastocyst (lane 7). BM, blastomere; BL, blastocyst.

Fig. 3. Sequencing reading of *TET1* gene from a whole 4-cell embryo and single blastomeres of a 4-cell embryo. (A) and (B): Clear sequence reading from two individual embryos at 4-cell stage. (C) and (D): Clear sequence reading from two individual blastomeres of a 4-cell stage embryo. (E) and (F): Major chromatogram peaks were completely matched to *TET1* gene sequence although there were some baseline noises.

of *TET1* gene were also observed in Sanger sequencing of two blastomeres of the 4-cell embryo (Fig. 3C and 3D). In sequencing of other two blastomeres, there were some baseline noise, but major peaks were completely matched to the target sequence (Fig. 3E and 3F). This result suggests that mutation types generated by introducing CRISPR/Cas9 into zygotes can be determined in early stage embryos. The approach of this study could promote the efficiency of producing genetically engineered animals by detecting mosaicism in relatively early stage of preimplantation development.

DISCUSSION

Prevalence of mosaic genotype is one of the major disadvantages to utilize direct injection of CRISPR/Cas9 system into developing embryos because animals carrying mosaic genotypes often interfere with phenotyping and breeding the founder animals (Lei et al., 2016; Park et al., 2017). Developing an effective approach to detect the frequency of mosaicism assist us to design and optimize CRISPR/Cas9 systems that introduce mosaicism at minimal level. In this study, we successfully genotyped a single blastomere from porcine 4-cell stage embryos, demon-

strating that it is possible to perform genomic PCR on a single copy of genomic DNA. We propose that PCR-based genotyping of single blastomere is an efficient approach to identify genomic-modifications in early stage embryos after direct injection of CRISPR/Cas9 system into presumable zygotes.

Previous reports suggest that microinjection timing (Sato et al., 2018) and CRISPR/Cas9 concentration (Tanihara et al., 2019) can influence the frequency of mosaicism in CRISPR/Cas9 injected embryos. For example, microinjection of CRISPR/Cas9 into germinal vesicle-stage oocytes appears to produce non-mosaic transgenic embryos (monoallelic knockouts) (Su et al., 2019). Another study identified optimal microinjection conditions in terms of CRISPR/Cas9 concentration and type, injection time, and embryos (Liu et al., 2016). Rapidly detecting the frequency of mosaicism at a low cost can assist in developing conditions that result in low mosaicism in porcine embryos. For instance, we can select an optimal microinjection timing that minimizes mosaicism. Normally, *in vitro* matured oocytes are incubated with washed sperm in microdroplets for 6 h to induce *in vitro* fertilization. The time point at which sperm enters the oocyte varies for each zygote, with a potential difference of up to 6 h. Comparing the

frequency of mosaicism after injecting CRISPR/Cas9 system at various time points during/after IVF system should help us identify a timing that can minimize the prevalence of mosaicism. Similarly, different source of Cas9 mRNA/protein can be examined for their capability to introduce mosaicism.

Unlike whole genome sequencing approach, our PCR-based detection of mosaicism is simple and can deliver the frequency of mosaicism in just a few days. Although detecting mosaicism using whole genome sequencing offers an extensive snapshot of possible alleles in embryos or animals, the method requires library preparation and bioinformatic analysis of sequencing information, which can take months. Alternatively, our PCR-based approach does not require specific equipment or expertise to detect mosaicism and ability to identify mosaicism after CRISPR/Cas9 injection can be comparable to the whole genome sequencing. Due to the lack of knockout embryos at the time of this study, no targeted event has been detected using the approach. However, our system should be instantly applied to embryos carrying targeted modifications.

In conclusion, we demonstrated that Sanger sequencing of PCR amplicons obtained from single blastomere DNA in 4-cell stage embryo is a feasible method for genotyping of mammalian embryos. Our technique opens the doors to future research that can determine optimal microinjection conditions for minimizing or eliminating mosaicism, therefore, improving the use of CRISPR/Cas9-mediated genome modification in mammalian embryos.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

JC and KL designed experiments and wrote the manuscript. JC, KU, JR, XF, and SB performed experiment and data analysis. KL supervised the work. All authors read and approved the manuscript.

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