GENETIC IDENTITY OF DONNOR SOMATIC CELL AND CLONED BOVINE EMBRYOS USING SINGLE BLASTOMERE BIOPSY FROM THE 8-CELL STAGE EMBRYO

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Abstract: Animal cloning is the process of producing an entire organism that theoretically has the same genome as its donor cell line. To validate the success of SCNT, one must verify that the genetic source of the cloned organism is identical to the donor cell. However, none of the studies have performed to identify the genetic characteristics in the stage of preimplantation development of cloned embryo. This study will propose 2 methods for the validation of genetic source of cloned embryos at the preimplantation development, including molecular sexing and sequence-based typing (SBT). The result of molecular sexing revealed that all samples showed positive signals except for the recipient oocyte. The result of sequence-based typing had shown that all samples were successfully amplified exon 2 of BoLA-DRB3 gene, even from a single embryonic cell. In addition, these were able to read its sequence by direct sequencing method and be able to identify the allele of samples. Validation of SCNT embryos using molecular sexing is quick and simple, but it can be only applied if the donor cell is male. Method applying SBT of MHC is more difficult compared to molecular sexing due to extreme polymorphism and heterozygosity. However, this is more accurate than the previous method and can be applied in most situations. SBT used in this study is dependent on the sequence of the locus, which can be applied for other species if the sequence of MHC locus is known. In conclusion, these two methods are effective to validate the genetic identity of donor cell and cloned embryonic cell at the early development.

Keywords: Bovine leukocyte antigen DRB3, cloned bovine embryo, molecular sexing, preimplantation genetic diagnosis, sequence-based typing.

1. INTRODUCTION

Animal cloning is defined as the procedure for the production of genetically identical individuals. Somatic Cell Nuclear Transfer is the technique that transfers the nucleus from the somatic donor cell into the recipient oocyte which lacks the nucleus by the process of enucleation (Campbell et al., 2007). To verify that the sample is cloned embryo/species derived from donor cell, one must validate that the genomic information is identical to each

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Molecular sexing is the process to determine the sex of the preimplantation embryo by using reliable molecular methods. This has become a crucial technique for livestock management and reproductive biotechnology (Chen et al., 1999). Currently, the common approach to determine the sex of cattle using PCR is to amplify sequences of Y chromosome (Sachan et al., 2020). PCR-SBT is the combination of PCR and direct sequencing from the resulting PCR product. In this method, the target sequence is directly sequenced without the process of gene cloning. It has been the most accurate method for MHC typing because it provides exact allele assignment (Erlich, 2012). The objectives of this study were to establish the procedure to verify the success of SCNT by validate the genetic identity between donor cell and cloned embryonic cell by applying (1) molecular sexing by direct single-cell PCR, and (2) SBT by using BoLA-DRB3.

2. MATERIALS AND METHOD

All chemicals were purchased from Sigma-Aldrich Chemical Company, unless otherwise indicated.

2.1. Bovine fibroblast cell preparation and collection

In this study, bovine fibroblast cells of male Hanwoo with known BoLA-DRB3 Allele (DRB3 *0902/*4301) were used. To subculture fibroblast cells, the original cell culture must reach a confluency of at least 70%. Cell culture was diluted with PBS and incubated for 5 minutes, then washed for 2 times. Fibroblast cell clusters were then trypsinized for 1 to 2 minutes to dissociate adherent cells. After trypsinization, DMEM supplemented with 40% FBS was added to stop the activity of Trypsin. Dissociated cells were centrifuged at 1000 rpm for 5 minutes and washed with 900 µL of DMEM wash. After third centrifugation, DMEM supplemented with 10% FBS was added to the pellet. Donor cells with passage 3 to passage 10 are appropriate for SCNT.

2.2. Preparation of recipient bovine oocytes

Bovine ovaries were collected from Ut Hao Slaughterhouse in Binh Duong Province. Oocyte cumulus granulosa cell complexes (OCGCs) were aspirated from large antral follicles (4-7 mm in diameter) using an 18-gauge needle. OCGCs were transferred into HEPES medium, TCM 199, and IVM medium. After In Vitro Maturation for 18-20 hours, cumulus cells were removed by repeated pipetting in HEPES medium supplemented with 0.1% hyaluronidase. Oocytes that have extruded the first polar body with good morphology consist of homogenous cytoplasm, were chosen for SCNT.

2.3. Somatic cell nuclear transfer in bovine

Matured oocytes were placed in HEPES medium supplemented with 10 µg/mL cytochalasin B. and the culture dish was covered with mineral oil. For enucleation, XYClone laser (Hamilton Throne) was used to open a hole on the zona pellucida right at the MII site, then slowly aspirating the cytoplasm containing spindle and MII chromosome into enucleation needle and take it out. Enucleated metaphase plate was stored in an Eppendorf tube for Direct PCR. During somatic cell injection, somatic cell was gently aspirated using injection pipette, to disrupt cell membrane and collect nucleus. Nucleus
was inserted to the enucleated oocyte using Piezo-pulses to open inner cell membrane. After SCNT, embryos were cultured in the modified synthetic oviduct fluid (mSOF) supplemented with 0.3 % BSA and incubated at 38.5 °C with 5 % CO₂ for In Vitro Development (IVD).

2.4. Collection of embryonic cells from the 8-cell stage embryos by biopsy

Nikon Eclipse Ti-U (Nikon) and XYClone laser (Hamilton Throne) were used for blastomere biopsy. This process was applied after the cloned embryo has reached to the 8-cell stage. 8-cell embryos were incubated for 2-5 min in Ca²⁺/Mg²⁺ free – bicarbonate – buffered medium to loosen cell-adhesion. During biopsy, the embryo was held in a fixed position by moderate suction of the holding pipette. XYClone laser was set up at a wavelength of 250 nm to open a 30 μm hole in zona pellucida. Single blastomere was taken out by aspiration using biopsy pipette (40 μm in diameter). The biopsied embryonic cells were then prepared for cell lysis.

2.5. Molecular sexing

DNA segment of Y chromosome was amplified for molecular sexing of a single embryonic cell from cloned bovine embryo. The reactions were carried out in a 20 μL reaction mixture containing 5 μL of lysed sample, 10 pmol of BY-F and BY-R primers, 1:200 of Fu14 DNA polymerase, 5X PCR reaction buffer, and 0.125 mM dNTPs. The thermocycling condition consisted of initial denaturation at 95 °C for 5 min, followed by 45 cycles of 1 min at 95 °C, 20 sec at 58 °C, 20 sec at 72 °C, and 5 min final extension at 72 °C with the addition of 1 min at 4 °C for holding.

2.6. PCR of BoLa-DRB3 exon 2 and SANGER sequencing

Exon 2 of BoLA-DRB3 gene was amplified for sequence-based typing of blastomere. In this method, semi-nested PCR was applied using primers DRB3FRW and DRB3REV as the first PCR primer set, which both were designed by Dr. Miltiadou (Miltiadou et al., 2003). The component of the reaction was the same as reaction of molecular sexing, except that primer is changed to 10 pmol of DRB3FRW and DRB3REV. At the second PCR, inner forward primer was changed to DRB3-seq-F. 0.5 μL of first PCR product was used for the template of the second PCR. The result of PCR product was analyzed by 1.2 % agarose gel electrophoresis. Direct sequencing of PCR product was performed to identify the allele of each sample. Exo-SAP-IT (Invitrogen) was used to clean up the PCR product. Sequencing reactions were performed using the ABI PRISM BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) followed by the manufacturer’s recommendations. The resulting sequencing products were further purified by ABI BigDye XTerminator™ Purification Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Capillary electrophoresis was performed on ABI 3500xl Genetic Analyzer (Applied Biosystems).

2.7. Sequence analysis and allele determination

Base-calling from the raw sequence output was performed using Sequencing Analysis Software. The forward and reverse sequence readings for each sequencing
sample were assembled using the Sanger Reads Editor program from Unipro UGENE Software. Local BLAST (Basic Local Alignment Sequence) was applied to identify alleles of each sample.

2.8. Statistical analysis

Each experiment was replicated at least three times to confirm the validity of this procedure.

3. RESULT AND DISCUSSION

3.1. Characterization by molecular sexing

According to the gel electrophoresis in Figure 1, all samples had successfully amplified a single band of 300 bp, except for recipient oocyte and non-template control. This suggested that the cloned blastomere was derived from a male donor cell.

![Figure 1](image)

**Figure 1.** Gel electrophoresis for molecular sexing using Y-specific primer. 1: molecular size marker, 2: Genomic DNA extracted from donor cells, 3: Donor cell, 4: Blastomere from 8-cell of cloned bovine, 5: Cloned Blastocyst, 6: Recipient oocyte, 7: non-template control

In molecular sexing, the desired amplicon could be produced in single round PCR from single cell, because PCR efficiency of BY primer set was extremely high compared to BoLA-DRB3. This method requires only single run of PCR, thus provides an easier and quicker method. However, the drawback is that it cannot be applied if the donor cell was female which lacks Y chromosome. Overall, this method is easy and quick, but lacks the versatility.

3.2. Characterization by sequence-based typing using BoLA-DRB3 exon 3

From the agarose gel electrophoresis, a single band of PCR product with a size of 319 bp was observed in each sample (Fig. 2). This confirmed that exon 2 of DRB3 gene was successfully amplified in all samples. Although the presence of smear occurs above the target band, it is still acceptable for direct sequencing. However, the result of this gel electrophoresis cannot be concluded that the cloned blastomere had same genetic material as donor cell. In order to clarify the genetic characteristics of each sample, these must be sequenced by Sanger sequencing using capillary electrophoresis.

The results of the sequencing chromatogram of donor cell, recipient oocyte and single cell derived from 8-cell embryos are showed in Figure 3. The results showed that the sequence of the chromatogram is similar except for the recipient oocyte. All sequences of BoLA-DRB3 gene matched among four samples including genomic Hanwoo, donor cell, cloned blastomere (single cell), and cloned blastocyst.

In this study, semi-nested PCR was used to amplify BoLA-DRB3 gene from a single cell. Semi-nested PCR is modification of nested PCR in which only one primer is changed for the second PCR run, instead of changing both two forward and reverse primers. Although semi-nested PCR provides high sensitivity for the amplification from the limited amount of genetic material, disadvantages of this technique including time-consuming process, potential for cross-contamination/carryover contamination, and overuse of reagent may limit the usage (Ogawa et al., 2004). Therefore, this needs to be further optimized to overcome these disadvantages. Direct Sanger sequencing of PCR is usually more challenging compared to cloning- sequencing because the low resolution is caused by high background noise due to prematurely truncated amplified products and excess unpurified components of PCR reaction (Linder et al., 2015). However, advantage of direct sequencing of PCR products are significant for diagnosis of the preimplantation embryo. The first advantage is the speed. Along with speed, direct sequencing has an advantage to sequence heterozygous in one reaction. Cloning sequencing can only sequence one allele in a reaction. This typing method is dependent on the locus-specific primer, which means this method can be performed not only for the examination of BoLA-DRB3 but can be applied to other loci.
Figure 3. Developmental competence of porcine embryo in difference energy substrate combination. (Direct Sequencing chromatogram of BoLA-DRB3 exon2 in samples of: A. Genomic DNA of Hanwoo, B. Hanwoo Donor cell used for SCNT, C. Blastomere collected from 8-cell cloned Hanwoo embryo, D. Cloned Hanwoo blastocyst, and E. recipient oocyte. Red: thymine, blue: cytosine, green: adenine, black: guanine. *: indicate the peaks of different bases.

The procedure is applicable to other species, if the most polymorphic MHC locus is known for specific species, the primer to amplify specific locus in MHC is known, and there is an adequate database to identify the allele of the individual. Therefore, this method can be applied to examine the genetic characteristics of the cloned embryonic cell from other livestock such as porcine, sheep, and goats.

4. CONCLUSION

In this study, cloned Hanwoo blastocysts were successfully produced, and both methods including molecular sexing and SBT using polymorphic marker were able to validate the success of SCNT during preimplantation development. Molecular sexing is simple and quick to proceed, yet it has limitation that it cannot be applied if the donor cell is female. Although high knowledge and techniques are needed to perform SBT using DRB3, it is extremely accurate and able to produce reliable result. This is the first study to examine the genetic identity between cloned embryonic cell and donor cell. It is
suggested that these methods can be applied effectively in both basic research of animal cloning and livestock industry.

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PHÔI BÒ NHÂN BẢN 8 TẾ BÀO

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Tóm tắt. Nhân bản với tính đồng vật là quá trình sinh sản từ một nhân tế bào chuyển vào một trường chín đã được lấy nhân và đồng vật nhân bản sẽ có cùng bộ gene với nguồn tế bào cho. Để xác nhận sự thành công của chuyển nhân tế bào sinh dưỡng (CNTBSD), chúng ta phải xác minh rằng nguồn gene của đồng vật nhân bản phải giống hoàn toàn với đồng tế bào cho nhân. Tuy nhiên, không có nghiên cứu nào được thực hiện để xác định các đặc điểm di truyền của phi hồi bò nhân bản với tính trong giai đoạn tiến làm tổ. Nghiên cứu này sẽ đề xuất 2 phương pháp xác nhận nguồn gene của phi hồi bò nhân bản với tính, bao gồm phương pháp xác định giới tính phân từ (XDGPT) và phương pháp xác định trình tự genotype (XDDTG). Kết quả của XDGPT cho thấy tất cả các mẫu đều có tính hiệu dương tính ngoại trừ tế bào trứng nhân. Kết quả của XDDTG đã cho thấy rằng tất cả các mẫu đã được khuyến đề đi đoạn exon 2 của gene BoLA-DRB3, ngày càng từ một tế bào nguyên bào phi hồi. Ngoài ra, chúng được xác định từ bằng phương pháp giải trình tự trực tiếp và có thể xác định alen của các mẫu. Việc xác thực CNTBSD bằng cách sử dụng phương pháp XDGPT rất nhanh chóng và đơn giản, nhưng phương pháp này chỉ có thể được áp dụng nếu nguồn đồng tế bào cho là còn đặc. Phương pháp XDDTG áp dụng cho phức hợp hòa hợp mờ chịu yếu (MHC) khó hơn so với XDGPT do tính đa dạng và giả hợp tự. Tuy nhiên, cách này có kết quả chính xác hơn so với XDGPT và có thể áp dụng trong hầu hết các tính huống. XDDTG được sử dụng trong nghiên cứu này phù hợp vào trình tự của lỗ-cut gen, có thể áp dụng cho các loại khác nếu biết trình tự lỗ-cut MHC. Kết luận, hai phương pháp này có hiệu quả cao để xác nhận danh tính di truyền của tế bào cho nhân và tế bào phối nhân bản.

Từ khóa: Chân đoạn di truyền tiến làm tổ, gene kháng nguyên đặc cụ thể con bò (BoLA) - DRB3, phi hồi bò nhân bản, xác định giới tính phân tử, xác định trình tự genotype.

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