Microsatellite DNA analysis proves nucleus of interspecies reconstructed blastocyst coming from that of donor giant panda

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Abstract A method for DNA isolation from early development of blastocyst and further analysis of nuclear and mitochondrial DNA was developed in present study. Total DNA was prepared from interspecies reconstructed blastocyst and a giant panda specific microsatellite locus g^{010} was successfully amplified. DNA sequencing of the PCR product showed that two sequences of reconstructed blastocysts are the same as that of positive control giant panda. Our results prove that the nucleus of interspecies reconstructed blastocyst comes from somatic nucleus of donor giant panda.

Keywords: giant panda (*Ailuropoda melanoleuca*), interspecies cloning, microsatellite DNA, nucleus.

Recently, Chen et al.[1] got an interspecies reconstructed blastocyst from nucleus donor of giant panda. Their experiments showed that three somatic cells from skeletal muscle, uterus epithelial and mammary gland were able to reprogram in rabbit ooplasm and support the early embryo development. Mitochondrial DNA sequencing showed that the giant panda mitochondria occurred in the reconstructed blastocyst. More and more scientists pay attention to the work[2]. Animal cloning by somatic cell is of important significance for preservation and passage of genome in individual with special quality and in endangered and rare species[1]. Therefore, it is very important to test if the nucleus of the reconstructed blastocyst comes from that of donor. Chromosomal karotype analysis showed that the number of chromosome of reconstructed blastocyst cell is the same as that of donor giant panda and is different from that of rabbit[1]. However, it is the lack of more evidence at the DNA level.

The difficult problem for DNA analysis of reconstructed blastocyst is the method for trace DNA preparation from single blastocyst. Our previous studies showed that microsatellite DNA has the character of species specific and is an ideal genetic marker for species and individual identification[3,4]. This note is to answer the above question by approach of microsatellite DNA analysis.

1 Materials and methods

(i) Positive control. Uterus epithelium tissue was collected within 30 min after the death of a 12-year-old female giant panda. Total DNA isolation was performed according to standard method.

(ii) Negative control. DNA was prepared from recipient female white rabbit.

(iii) Primers for microsatellite DNA analysis. The giant panda specific primer sequence for microsatellite locus g^{010} was listed in our previous paper[4].

(iv) DNA preparation from reconstructed blastocyst. Single reconstructed blastocyst was put in a 0.5 mL of microcentrifuge tube containing 5 μL of ultra-pure water and then an equal amount of alkaline lysis solution (200 mmol/L KOH/50 mmol/L dithiothreitol) was added. After a 10-min water incubation at 65℃, 5 μL of neutralization solution (900 mmol/L Tris-HCL, pH 8.3/300 mmol/L KCL/200 mmol/L HCL) was added.

(v) Microsatellite DNA amplification. The first PCR conditions are as follows: a 3-min predenaturation at 95℃, followed by 25 cycles of a 40-s denaturation at 95℃, a 1-min annealing at 50℃ and a 2-min extension at 72℃. The second PCR conditions are as follows: a 3-min predenaturation at 95℃, followed by 40 cycles of a 50-s denaturation at 94℃, a 1-min annealing at 57℃ and a 30-s extension at 73℃. PCR reaction was 50 μL in volume. PCR cocktails contain 5 μL of 10×PCR buffer, 2 μL of dNTPs (each 2.5 mmol/L), 2 μL of BSA (10 mmol/L), 1 μL of each primer (10 μmol/L), 1 μL of *Taq* polymerase, 2 μL of template DNA, and brought to 50 μL with water.

(vi) DNA sequencing of microsatellite locus. The PCR product was directly sequenced in an ABI 377 automated DNA sequencer (Perkin-Elmer). Both strands were sequenced in order to improve the sequence reliability.

2 Results and analysis

After PCR condition optimization, the microsatellite locus g^{010} was successfully amplified from two reconstructed blastocysts. No amplified products were observed in negative controls with recipient rabbit and mock template. The PCR results are shown in fig. 1.

A total of 133 bp long DNA sequences were observed in both numbers 1 and 2 reconstructed blastocysts and positive control. All the three sequences are the same. The sequence results are shown in fig. 2.

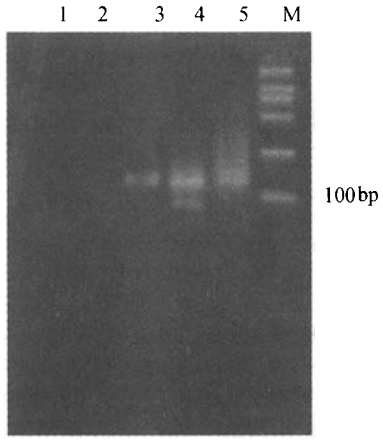

Fig. 1. Results of PCR using panda microsatellite locus g^{010}. 1, Negative control with mock template; 2, negative control with recipient rabbit template; 3, positive control with donor panda template; 4, reconstructed blastocyst 1; 5, reconstructed blatocyst 2; M, marker.

3 Discussion

Since 1997, the sheep "Dolly" was successfully cloned by using somatic cell, cloned mouse[5] and cattle[6] by using the same techniques were also reported recently. For endangered giant panda, due to small population size and the difficulty in acquiring recipient oocyte, it is more valuable to try interspecies cloning. However, when we get an early reconstructed blastocyst a question follows: Does the new nucleus come from donor giant panda or from recipient rabbit? In our previous studies, some microsatellite DNA loci were isolated and characterized by screening the library. The microsatellite DNA locus is a nuclear specific marker, the giant panda microsatellite DNA sequences we cloned have a little bit variability. The nucleotide substitution differences occurred even among different species within a family. The discrepancy of microsatellite locus among different individuals is focused on length difference. This DNA sequence has a characteristic of species identification and it is difficult to get PCR products in distant-related species by using these primers. Therefore, if we can get the PCR products and further confirm that they are the microsatellite locus, we can prove that the nucleus of reconstructed blastocyst is from donor nucleus of giant panda rather than from recipient rabbit. A giant

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          5′primer                                          50

GP   (GTTGCTCAAA GTTGACTTGA TGC)TATACAT GTACATGGAT ATATATACAT GCATATGTGT
RB1  (GTTGCTCAAA GTTGACTTGA TGC)TATACAT GTACATGGAT ATATATACAT GCATATGTGT
RB2  (GTTGCTCAAA GTTGACTTGA TGC)TATACAT GTACATGGAT ATATATACAT GCATATGTGT

                                                      100
GP   ATACACACAC ACACATACAT TATGGATATA TTTATCTGCA CAGACATAAA (GAAAAATTAA
RB1  ATACACACAC ACACATACAT TATGGATATA TTTATCTGCA CAGACATAAA (GAAAAATTAA
RB2  ATACACACAC ACACATACAT TATGGATATA TTTATCTGCA CAGACATAAA (GAAAAATTAA

          3′primer   133
GP   GCCTGGCAGC CTA)
RB1  GCCTGGCAGC CTA)
RB2  GCCTGGCAGC CTA)
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Fig. 2. Sequences for microsatellite locus g^{010}. Primer sequences are in parenthesis. GP, Giant panda positive control; RB, reconstructed blastocyst.

panda specific microsatellite locus g^{010} was successfully amplified from two reconstructed blastocysts in present study. Sequencing analysis showed that these sequences are the same as that of donor giant panda. Our results confirmed that the two nuclei of the reconstructed blastocysts come from that of donor giant panda.

In our last studies[1], a mitochondrial DNA fragment was amplified from the reconstructed blastocyst by using giant panda specific mtDNA primers. DNA sequence comparisons among donor giant panda, giant panda population and reconstructed blastocyst prove that the donor giant panda mitochondria occurred in the reconstructed blastocyst. However, we cannot exclude the co-existing possibility of donor somatic cell and recipient oocyte mitochondria during the development of embryos. Our present results plus previous chromosomal analysis results[1] confirmed that the nucleus of reconstructed blastocyst does come from that of donor giant panda. Our results show a clue for further studying the law and mechanism of mitochondria in reconstructed blastocyst.

It is a key technique to develop a method for DNA preparation from reconstructed blastocysts in molecular identification of reconstructed blastocysts. We had tried many reported methods, but the results were not satisfactory. At last we developed the above method for DNA preparation from early embryos and can further conduct nuclear and mitochondrial DNA analysis. This method operates simply and all reagents are commonly used in laboratories. Therefore, the method has a widely extended potentialities.

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