CONSERVATION OF QINGHAI FINE-WOOL SHEEP AT SOMATIC CELL LEVEL IN STERILE CULTURE

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ABSTRACT

This study aimed to establish the fibroblast cell bank of Qinghai fine-wool sheep in the form of somatic cells, and to study its biological characteristics, to help for long-term conservation and sustainable use of this genetic resource. Ear marginal tissue of the Qinghai Fine-wool sheep was used to establish a fibroblast cell line by direct culture of explants and cryopreservation techniques, and the quality of the cell line was assessed. This study not only shows the effective preservation of the Qinghai Fine-wool sheep germplasm at the cellular level, but also provides a available experimental resource for fields including cell biology, genomics, post-genomics, genetic engineering and embryo engineering.

Key words: Biological characteristics, Fibroblast cell line, Qinghai fine-wool sheep.

INTRODUCTION

Animal genetic resources are playing important role in the biological and genetic diversity (Kohler-Rollefson, 1997). Livestock and poultry genetic resource conservation is an important filed of research, as it is essential for the sustainable development of animal husbandry and biodiversity.

The Qinghai fine-wool sheep originated in the in Gang cha County, Qinghai province and is one of the China-famous breeds. However, the number of Qinghai fine-wool sheep gradually declined in recent years due to changes in the market orientation. Nowadays it is very necessary to carry out research on preservation of germplasm resources of Qinghai fine wool sheep. Compared with the traditional in vivo preservation, somatic cell preservation has obvious advantages such as small occupied space, low investment, without the group and individual physiological restrictions (Corley-Smith and Brandhorst, 1999). In addition, the establishment of Qinghai fine-wool sheep somatic cell lines has an important significance for the development of the biotechnology research, such as somatic cloning and transgenic animal production.

At present, no report is available on Qinghai fine-wool sheep fibroblast cell line. This experiment established Qinghai fine-wool sheep fibroblast cell line by using the tissue culture and cryopreservation technique, and then we conducted a systematic analysis of culture conditions and cell biological characteristics. The research provided the necessary resources and technical support for the development of Qinghai fine-wool sheep genetic resources preservation and related biological technology.

MATERIALS AND METHODS

Cell culture: Ear marginal tissue samples were taken from 40 Qinghai Fine-wool sheep that were provided by the Sheep Breeding Farm of The Chinese Academy of Agricultural Sciences, Beijing, PR China. The samples were collected into separate tubes which contained DMEM (Gibco, Carlsbad, CA, USA) supplemented with ampicillin (100 U/ml) and streptomycin (100 µg/ml) (Solarbio, China, Beijing).

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The samples were then rinsed and chopped into 1-mm³ pieces, which were seeded onto the surface of a tissue culture flask containing 90% DMEM and 10% fetal bovine serum (FBS) in an incubator at 37°C with 5% CO₂. When the cells reached 80–90% confluency, they were harvested and divided into prepared culture dishes at 1:2 or 1:3 ratios.

**Cryogenic preservation and recovery:** Cells of passages 3-5 were selected. The culture medium was changed 24 h before harvesting and the cultures were maintained in an actively growing state to ensure optimum health and good cell recovery. After 24 h the cells were centrifuged at 1200 rpm for 8 min. The supernatant was removed and the cell pellet was re-suspended in a freezing medium containing 10% dimethylsulfoxide (DMSO; Gibco, Carlsbad, CA, USA), 50% FBS and 40% DMEM to reach a final cell density of 3×10⁶ to 5×10⁶ viable cells/ml which was confirmed by trypan blue exclusion test. Subsequently, 1 ml of the cell suspension from each sample was allocated into sterile plastic cryogenic vials and vials were sealed and stored in boxes filled with the proper amount of isopropyl alcohol at –80°C for overnight. After overnight storage the cells were transferred to a liquid nitrogen storage system (Werners et al., 2004). At recovery, the frozen vials were taken from the liquid nitrogen and thawed in a 42°C water bath, and then the cells were transferred into a flask with complete DMEM. The cells were cultured at 37°C in a 5% CO₂ atmosphere and the medium was refreshed every 24 h (Speirs et al., 1991).

**Estimation of cell viability:** Assays of cell viability before cryopreservation and after recovery were detected using Trypan blue exclusion test. The cells were seeded in a six-well plate and 1000 cells were counted for calculation (Strobe, 2001).

**Growth curve analysis:** Cells were plated on 24-well microplates with a density of 4×10⁴ cells/well and cultured for 7 days. cell number was counted daily until a plateau phase was reached. Mean values were used to plot a growth curve and to calculate the PDT (Kozak, 1992).

**Microorganism detection:** The cells were cultured in media without antibiotics for 3 days and tested for the presence of microbes under high microscope. The cultured cells were detected by the EZ-PCR (Williamson et al., 1992) mycoplasma test kit using PCR technology to detect mycoplasma contamination in cell cultures. The primer set is specific to the highly conserved 16S rRNA coding region in the mycoplasma genome and allows various mycoplasma species as well as Acholeplasma and Spiroplasma species to be detected with high sensitivity and specificity. Bands of the amplified DNA fragments are identified after gel electrophoresis. (primer sequences F: 5' GAA CGG GTG AGTAACACGT 3' R: 5' GGTGTTCTTCCATATATCTACGC 3')

**Chromosome and karyotype analysis:** Cells were harvested at 80–90% confluence. The cells were grown in a media containing colcemid for 6h and then harvested. Microslide preparation and chromosome staining were performed as described by Muller et al. (1997). Chromosome numbers were counted for 50–100 individual chromosome as previously described (Kawarai et al., 2006). This method focuses on three important parameters: relative chromosome length, arm ratio, and centromere index.

The microphotographs of metaphase chromosomes were used to measure the long and short arms of 27 pairs of chromosomes. The chromosomal parameters of relative length, centromere index and kinetochore type were calculated according to the method of Lavania and Sharma (1981).

**Isoenzyme analysis:** Isoenzyme patterns of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were identified by non-continuous native polyacrylamide (Sigma-Aldrich, USA) gel electrophoresis (PAGE). The stacking gels were at an acrylamide concentration of 5% w/v and the separating gel was at an acrylamide concentration of 13% w/v. The cells were harvested and protein extraction solution was added to cells at a density of 5×10⁷ cells/ml. The suspension was centrifuged and stored in aliquots at = 70°C. Equal volumes of native gel sample loading buffer (Solarbio) were added to the samples. The gel buffer was prepared by using Tris-citric acid buffer at two different buffer: 0.078 mol/l Tris-citric acid (stacking gels; pH 6.8) and 0.017 mol/L Tris-citric acid (separating gel; pH 8.9). The electrophoretic buffer was Tris-glycin (pH 8.7).

The buffered staining mixture was 0.1 M Tris-HCL (pH 8.0) and 100 mM DL-lactate lithium salt
FIG 1: Morphology of Qinghai Fine-wool sheep fibroblasts cultured in vitro: (A) 5–12 days after explanting; (B) near confluence; before cryopreservation; (C) 24 h after recovery from cryostorage. (D) Growth curve of Qinghai Fine-wool sheep fibroblasts.

Results and Discussion

Cell morphological observation and viability: A few fusiform-like and oval-like cells migrated from tissue pieces 5–12 day after explanting (Fig. 1A). When observed over time, fibroblasts were seen to move out of the vicinity of the tissue and then multiplied quickly. The cells were having a typical long spindle shape with slow primary cell growth. The cells were sub cultured when they reached 85% confluence. After passage, growth accelerated and plateaued after 3–4 d. The average viability before freezing was 98.2% and after thawing was 96.5%, which demonstrated that the cells were healthy and the culture conditions were optimal (Fig. 1B–1C).

Growth curve (cell dynamics): The growth curve of Qinghai Fine-wool sheep fibroblast cells appeared as a typical ‘‘S’’ shape (Fig. 1-D). There was a lag time or latency phase of about 48 h after the cells were seeded, corresponding to the adaptation of primary cells in recovery that were repairing protease damage.

Microorganism detection: The culture media being surveyed did not become turbid or display other visible changes, whereas the positive test control was visibly turbid with precipitation. (Data not show). These results demonstrated that the newly established Qinghai Fine-wool sheep fibroblast line was not contaminated by bacteria or fungi. EZ-PCR analysis indicated no microbial contamination in the fibroblast line (Fig.2).

Chromosome and karyotype analysis: In Qinghai Fine-wool sheep the diploid number of chromosomes was 54, consisting of 26 pairs of euchromosomes and XX sex chromosomes (Fig.4). The chromosomal properties of relative length, centromere index, and kinetochore type were shown in Table 1.

Isoenzyme analysis: LDH and MDH isoenzyme patterns were obtained from Qinghai Fine-wood sheep fibroblasts five bands were apparent representing LDH1, LDH2, LDH3, LDH4 and LDH5 from anode to cathode (Fig. 3A). The patterns of MDH showed two bands (Fig. 3B). The results indicated that there was no cross-
contamination with cells from other species.

**Exogenous expression of fluorescent proteins**

The expression of pEGFP-N3, pEYFP-N1 and pDsRed1-N1 at 24 h, 48 h and 72 h was recorded by laser confocal microscopy with a specific excitation wavelength. The results indicated that the three fluorescent protein genes were expressed in most positive cells, and the strongest fluorescence intensity and the highest transfection efficiency of the exogenous genes appeared at 48 h after transfection. The expression efficiencies of the three fluorescent proteins at 24 h, 48 h and 72 h after transfection were between 13.6% and 44.3% (Table 2). All positive cells appeared shrunken and disintegrated at 24 h after transfection (Fig. 5). The number of positive cells increased at 48 h and had many non-fluorescent vacuoles in the cytoplasm at 48 h and 72 h after transfection.

**Cell isolation and culture:** Enzymatic digestion and tissue culture are methods to obtain the single cell for primary cells (Outani et al., 2013). Trypsin and collagenase digestion culture methods could make the tissue block dispersed into cell clusters or single cells. The latent cell growth period was shortened and the working efficiency and the cell yield was high with this method (Eslaminejad et al., 2010). The method of enzymatic digestion culture was suitable to train a large number of organizations, but this protocol was more cumbersome and easy to avoid contamination (Pylayeva-Gupta et al., 2013). However, using tissue culture method, cell growth rate was relatively slow, especially ear organization contained collagen that was unfavorable to the spread and growth of cells. The advantage of the method was simple and able to avoid contamination (Uphoff and Drexler, 2013). The experiment used tissue culture method that the surrounding overflowed fibroblast and epithelial cells after 5 days, which was consistent with previous reported (Bai et al., 2011).

In the early days of cell lines, epithelial cells and fibroblasts was mixed. Epithelial cells and fibroblast cells had different sensitivity to trypsin. When cells were digested and subcultured typically fibroblast were shedding prior and adherent faster; in a short period of time, epithelial cells were difficult to attach or attached unstable vibration slightly floats. Therefore, we could use the difference getting
TABLE 1: Chromosome parameters of Qinghai Fine-wool sheep fibroblasts (±)

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>Relative Length</th>
<th>Centromere morphology</th>
<th>Chromosome number</th>
<th>Relative length</th>
<th>Centromere morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.4 ± 0.36</td>
<td>M</td>
<td>15</td>
<td>5.1 ± 0.15</td>
<td>T</td>
</tr>
<tr>
<td>2</td>
<td>19.0 ± 0.12</td>
<td>M</td>
<td>16</td>
<td>4.9 ± 0.39</td>
<td>T</td>
</tr>
<tr>
<td>3</td>
<td>18.3 ± 0.12</td>
<td>M</td>
<td>17</td>
<td>4.3 ± 0.37</td>
<td>T</td>
</tr>
<tr>
<td>4</td>
<td>9.3 ± 0.28</td>
<td>T</td>
<td>18</td>
<td>4.5 ± 0.33</td>
<td>T</td>
</tr>
<tr>
<td>5</td>
<td>9.0 ± 0.03</td>
<td>T</td>
<td>19</td>
<td>4.1 ± 0.11</td>
<td>T</td>
</tr>
<tr>
<td>6</td>
<td>8.8 ± 0.16</td>
<td>T</td>
<td>20</td>
<td>3.9 ± 0.9</td>
<td>T</td>
</tr>
<tr>
<td>7</td>
<td>8.2 ± 0.03</td>
<td>T</td>
<td>21</td>
<td>3.6 ± 0.15</td>
<td>T</td>
</tr>
<tr>
<td>8</td>
<td>8.1 ± 0.22</td>
<td>T</td>
<td>22</td>
<td>3.3 ± 0.12</td>
<td>T</td>
</tr>
<tr>
<td>9</td>
<td>7.8 ± 0.16</td>
<td>T</td>
<td>23</td>
<td>3.3 ± 0.26</td>
<td>T</td>
</tr>
<tr>
<td>10</td>
<td>7.2 ± 0.21</td>
<td>T</td>
<td>24</td>
<td>3.4 ± 0.17</td>
<td>T</td>
</tr>
<tr>
<td>11</td>
<td>7.3 ± 0.16</td>
<td>T</td>
<td>25</td>
<td>3.1 ± 0.11</td>
<td>T</td>
</tr>
<tr>
<td>12</td>
<td>6.2 ± 0.18</td>
<td>T</td>
<td>26</td>
<td>3.2 ± 0.27</td>
<td>T</td>
</tr>
<tr>
<td>13</td>
<td>5.3 ± 0.17</td>
<td>T</td>
<td>X</td>
<td>9.7 ± 0.22</td>
<td>T</td>
</tr>
<tr>
<td>14</td>
<td>5.0 ± 0.22</td>
<td>T</td>
<td>X</td>
<td>9.4 ± 0.19</td>
<td>T</td>
</tr>
</tbody>
</table>

Abbreviate (M represent metacentric chromosome, T represent telocentric chromosome)

TABLE 2: Transfection efficiency for three fluorescent proteins

<table>
<thead>
<tr>
<th>Transfection time (h)</th>
<th>pEGFP-N3 (%)</th>
<th>pDsRed1-N1 (%)</th>
<th>pEYFP-N1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>30.3</td>
<td>13.6</td>
<td>32.5</td>
</tr>
<tr>
<td>48</td>
<td>42.7</td>
<td>20.9</td>
<td>41.5</td>
</tr>
<tr>
<td>72</td>
<td>38.5</td>
<td>18.9</td>
<td>42.5</td>
</tr>
</tbody>
</table>

FIG 5: Efficient transfection of fibroblast cells for 48 hours with pEGFP-N3 (A), pDsRed1-N1 (B), and pEYFP-N1 (C) using Lipofectamine 2000. (Scar bar 100ìm) The picture (a-c) was magnified, respectively. (Scar bar 10ìm and 20ìm)

purified fibroblasts by trypsin digestion and adherence repeatedly by 2 to 3 passages (Tong et al., 2012).

Growth curve: The growth curve of ear marginal tissue fibroblasts of the Qinghai Fine-wood sheep had an obvious “S” shape and the PDT was about 48 h. There was a lag time or latency phase of about 48 h after seeding, corresponding to the adaptation and recovery of the cells from protease damage, after which the cells proliferated rapidly and entered exponential phase. As the cell density increased, cell proliferation was inhibited by contact inhibition. After the sixth day, the cells no longer proliferated and entered into plateau phase followed by
subsequent degeneration and decline phase (Kafri et al., 2013).

**Cell viability:** Studies have shown freezing affected the cell viability (James et al., 2011), while in our work, the cell viability before freezing (98.2%) and after thawing (96.5%) showed no significant differences suggesting that the freezing had little effect on the viability of the cells. So we could preserve Qinghai fine-wool sheep fibroblasts by liquid nitrogen preservation way, in order to achieve the purpose of preservation.

**Chromosome and karyotype analysis:** The chromosome number of Qinghai fine-wool sheep is 2n=54 cells (Liu et al., 2014) and majority of chromosomes were diploid (> 98.2%) indicating that the building of the cell bank was stable diploid cell bank. With increasing passage number, only few cell chromosome number (< 2%) had some changes viz. hyperdiploid chromosomes, hypodiploid chromosomes or polyploidy chromosomes was increased suggesting that the cell chromosome was relatively stable and the cells were stable diploid cells. The influencing factor of transfection of exogenous gene included DNA-liposome ratio, dosage and transfection time (Xiong et al., 2011). Better transfection efficiency largely depends on the optimization conditions. The transfection rate also can be used as a measure of cell viability index. In this experiment, 3 kinds of extrinsic fluorescent protein were transfected on Qinghai fine-wool sheep fibroblast cells and achieved high transfection rate (20.8~44.3%) suggesting that the cells had the ability of exogenous gene expression and could be used as donor cells in transgenic research.

An ear marginal fibroblast cell bank was established from the Qinghai fine-wool sheep using tissue culture and repeated digestion passage method, as the same method as described by De Falco et al., 2013). Cell biology test had reached the standard of ATCC(2010). The cell viability before freezing and after thawing had high viability, respectively 98.2% and 96.5%. Chromosome karyotype was stability. Isozyme showed no cross contamination phenomenon with other species cells. In addition, cells could be transfected and expressed, and the transfection rate was 13.6~42.7%. The above results showed that the research of Qinghai fine wool sheep ear marginal tissue fibroblasts cell lines can be used as a form of the species resource conservation, as well as can be provide valuable experimental materials for cell biology, medical science, genomics, gene engineering, embryo engineering and life science research.

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