Establishment and characterization of a fibroblast cell line derived from Gansu alpine fine-wool sheep

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ABSTRACT: A fibroblast cell line was successfully established from ear marginal tissue of Gansu alpine fine-wool sheep by using a primary explant technique and cell cryogenic preservation technology. Cryopreserved cells were checked for quality and biological characteristics and showed typical fibroblast morphology when cultured again in vitro. The growth curve was typically S-shaped as the cell population passed through a lag phase, a logarithmic phase and a plateau phase. Biological analysis showed that the population doubling time (PDT) for reviving cells was approximately 24 h. The cells were also free from bacterial, fungal, viral and mycoplasma contamination. Karyotyping analysis indicated that the chromosome number of a normal cell was of \(2n = 54\) and 95.4% of the entire population was diploid. The transfection efficiencies of three fluorescent proteins (pEGFP-N3, pDsRed-N1, pEYFP-N1) optimal at 48 h were from 18.5% to 30.1%. The corresponding fluorescence was distributed throughout the cytoplasm and nucleus 24 h after transfection. This newly established cell line meets the quality control standards established by the American Type Culture Collection. It not only preserves the genetic resources of the Gansu alpine fine-wool sheep at the cellular level but also provides valuable materials for genomic, postgenomic, and somatic cloning research in these animals. Moreover, the establishment of these methods may provide both technical and theoretical support for preserving the genetic resources of other livestock and poultry at the cellular level.

Introduction

Livestock and poultry breed resources were an indispensable part of overall biological breed resources and formed a basis for human survival and sustainable development of the society (Wu, 1999). The development of animal husbandry worldwide has emphasized high-yielding breeds, resulting in the extinction of many local ones with poor economic performances (Woelders et al., 2006). Unless these genomic resources are conserved in some form before they are lost, we will not only lose the genes peculiar to rare breeds but will also find it impossible to explore the cytological and molecular biological mechanisms that are required to reproduce these breeds by somatic cell cloning. It is therefore urgent to commence conservation of Chinese threatened breeds. Preservation of individual species, semen samples, embryos, genomic libraries and cDNA libraries are all practical methods. In addition to these methods, modern somatic cell cloning techniques have made somatic cells an attractive resource in the conservation of animal genetic materials (Changxin, 1999).

Gansu alpine fine-wool sheep is the only ovine breed in West Gansu, China. These sheep are rustic and particularly able to climb and to withstand long walks. This paper presents an original approach for the conservation of threatened domestic breeds which are economically, socially and/or ecologically important. A fibroblast cell line was developed from ear marginal tissue for long-term preservation of genetic material of this valuable breed. This cell line may also provide a useful experimental material for research in biodiversity.
Materials and methods

Isolation, primary culture and subculture
A marginal tissue sample was obtained from each ear, rinsed and then cut into ~1 mm³ pieces, which were subsequently seeded in a tissue culture flask containing DMEM + 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO₂. The culture was observed every 24 h for the occurrence of a substantial outgrowth of cells from the tissue pieces. The medium was refreshed when the cells reached 80–90% confluence, and one day later they were treated with 0.25% trypsin and separated into prepared culture flasks at 1:2 or 1:3 ratios (Guan et al., 2005; Zhou et al., 2004). The cell suspension was divided into new culture flasks and incubated at 37 °C in a 5% CO₂ atmosphere.

Cryogenic preservation and recovery
Cells were supplemented with fresh medium 24 h prior to freezing. The monoplastic cell suspension was obtained by digesting cells in 0.25% trypsin. The cells were centrifuged at 1000 rpm for 8 min, and the cell pellet was resuspended in sufficient media for freezing (10% DMSO plus 50% fetal bovine serum plus 40% DMEM) to reach a final cell density of 3-5×10⁶ viable cells/ml. Cell suspensions were aliquoted into sterile plastic cryovials and then kept at 4 °C for 30 min, before putting them into a programmed cryopreservation system for 12 h, and finally transferred to liquid nitrogen for long-term storage.

To recover and reseed the cells, the frozen tubes were removed from liquid nitrogen and quickly 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 renewed 24 h later.

Estimation of cell viability
Viabilities were determined before freezing and after recovery using the Trypan blue exclusion test (Weingartl et al., 2002). The number of dead cells was determined for 1000 cells.

Growth kinetics
Cells were seeded in 24-well microplates (~2.5×10⁴ cells per well, Gu et al., 2006) and cultured for 7 d, and three wells were monitored on a daily basis for cell concentration until a plateau phase was reached. The mean cell counts at each time point were then used to plot the growth curve, based on which the PDT was calculated (Qi et al., 2007; Hirofumi et al., 2006; Weingartl et al., 2002).

Chromosome analysis
Cells were harvested at 80-90% confluence, and chromosomes preparations were fixed, and stained following standard methods (Suemori et al. 2006). Chromosome numbers were counted for 50-100 individual chromosome spreads, and relative chromosome length, arm ratio, and centromere index were determined.

Microorganism detection
For such purpose, the cells were cultured in DMEM supplemented with 10% fetal bovine serum with no antibiotics and observed for the presence of bacteria and fungi at 3d after subculture according to the method of Doyle et al. (1990). Hay’s hemadsorption protocol was used to examine the samples for cytopathogenesis using phase-contrast microscopy (Hay, 1992; Wu et al., 2008). Mycoplasm detection was made using the American Type Culture Collection protocol, the cells were cultured in antibiotic-free medium for at least 1 week, and then fixed and stained with Hoechst 33258 (Masover et al., 1998).

Expression of fluorescent protein gene
To obtain the highest transfection efficiency and low cytotoxicity, optimized transfection conditions by varying cell density as well as plasmid DNA (BD Biosciences Clontech product) and Lipofectamine 2000 (Invitrogen) concentra-
ations were found, according to lipofectamine media methods of Escriou et al. (2001) and Tsuchiya et al. (2002). The plasmid DNA (lg) to Lipofectamine 2000 (II) ratio was 1:3. The medium was renewed 6 h after transfection, and cells were observed at 24, 48 and 72 h, and at 2 weeks and 1 month after transfection, using a Nikon TE-2000-E inverted confocal microscope with excitation wavelengths of 405, 488 and 543 nm to determine the transfection efficiency and the morphology of positive cells. For each experimental group, images were captured from 10 visual fields to determine the total and positive cell counts in each field for the calculation of transfection efficiencies (Kain et al., 1995; Guan et al., 2006).

Results

Morphology of fibroblast cells
A few epithelial-like and fibroblast-like cells grew from the different tissue samples (Fig. 1A), after the cells had been attached in culture for approximately 2 weeks. As time passed, cells continued to proliferate and were subcultured when they reached 90% confluences (Fig. 1B). Fibroblasts grew rapidly and gradually replaced the epithelial cells in subcultures (Barile et al., 1993). After 2–3 passages, a relatively purified fibroblast line was obtained. The viabilities of fibroblasts as measured by Trypan Blue staining were 98.6% and 93.7%, before freezing and after recovery, respectively.

Growth kinetics
The growth curve was S-shaped and the PDT was 42.8 h. There was a lag time or latency phase of about 72h after which the cells proliferated rapidly and entered the exponential phase. As cell density increased, proliferation was retarded and by the sixth day, the cells entered a plateau phase (Fig. 2).

Microbial analysis
Tests for contamination with mycoplasma and other bacteria and fungi were negative, and there was no cytopathogenic evidence in the hemadsorption test.

Chromosome and karyotype analysis
The chromosome number observed was 2n = 54, consisting of 26 pairs of euchromosomes and two sex chromosomes (Fig. 3). The X chromosome was the longest acrocentric chromosome and the Y chromosome was the shortest submetacentric one. Chromosomes were counted at first and third passage and 95.4% of the cells analyzed were diploid (2n = 54) in 100 cell spreads.

Expression of fluorescent protein genes
Three fluorescent proteins, pEGFP-N3, pDsRed-N1, pEYFP-N1, were highly expressed in Gansu alpine fine-wool sheep fibroblasts (Fig. 4A, B, C). Positive cells were observed
at 24, 48, 72 h, and at 2 weeks and 1 month after transfection. Both green and cyan fluorescences could be observed throughout the cytoplasm, while red fluorescence showed a granular pattern (Fig. 5A-C).

Most positive cells shrunk and shed 72 h after transfection. The number of cells expressing fluorescent proteins decreased and the fluorescence intensity gradually faded, and disappeared 7-14 days after transfection. These results showed that the expression of exogenous genes has no obvious effect on fibroblast growth and proliferation.

Discussion

We have established a fibroblast cell line from ear marginal tissue of Gansu alpine fine-wool sheep using an adherent culture method. Morphologically, the cultured cells were typical fibroblasts. All of our analyses of these cells indicate that this newly established fibroblast line is genetically stable and grows rapidly. We are now able to conserve the genomic resource of Gansu alpine fine-wool sheep in the long term by freezing fibroblasts in liquid nitrogen. After freezing and thawing, the cell survival rate decreased to some extent, possibly owing to cell injury that occurred during the process of refrigeration and recovery.

Our morphological observations indicated that there were both epithelial and fibroblast cells present during the primary and early passages of the explanted tissue. Fibroblasts adhered more easily and could be trypsinized more readily, whereas the epithelial cells weren't adherent or stably adherent and detached with only mechanical agitation, according to which distinction the fibroblasts could be purified through serial passage.

Karyotypic data can confirm the origin of a cell line and identify possible cross-contamination. Normal cells possess stable chromosome numbers, shape and structure and therefore, karyotype analysis can be an effective method for distinguishing normal cells from variants. Fluorescent proteins were rapidly becoming important reporter molecules for monitoring gene expression and protein localization in vivo, in situ and in real time. DNA concentration, lipofectine concentration, DNA incubation time and lipofectine combination, and the presence of serum can all affect cell transfection efficiency.
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References


