Cryopreservation of Iranian Markhoz Goat Fibroblast Cells as an Endangered National Genetic Resource

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Abstract

The sustainable use of local animals is being eroded annually. Thus, a strategic vision for the conservation of biodiversity is of far-reaching emphasis to deal with unprecedented challenges in the local population growth process facing in the future. This study aimed to establish, characterize and cryopreserve endangered Markhoz goat (*Capra hircus*) fibroblast cell lines in vitro. These primary fibroblast cells were isolated from 58 Iranian Markhoz goats and individually cultured by explant technique in DMEM media supplemented with 10 % FBS. The cultured cell lines were morphologically consistent with fibroblast cells. The population doubling time for DMEM-cultured cells were 23±0.5 h. Chromosomal analysis indicated a total chromosome number of 2n = 60 with >95% frequency. Experimental assays for bacteria, fungi, yeast, and mycoplasma were reported negative. The efficiencies of VSV-G (pMDG) and lentiviral pCSGW vectors encoding fluorescent proteins showed an approximate value of 65%. Species identification for each sample was performed and confirmed correct goat cell banking without any miss- and cross-contamination. This study demonstrated the successful establishment of genetically stable fibroblast bank as a valuable genetic resource for endangered Iranian Markhoz goat breed.

Introduction

Biodiversity conservation aims to manage the human use of natural resources to get the greatest sustainable benefit of present and future generations. Preservation of genetic resources is an important issue in economic, social, and cultural categories; which lead to the establishment of productive ecosystems in the development of a sustainable community. The loss of livestock species in certain geographic areas causes to abolish the variability of the animal genetic resources and extinction (Niesenbaum 2019). Therefore, the conservation of genetic resources from native breeds, wild types, and domestic animals, is a key factor to protect biodiversity in a distinct ecosystem (Li et al. 2009a; Li et al. 2009b; Mehrabani et al. 2014). There are various complementary strategies for the conservation of animal resources, which include *in vitro* and *in vivo*. *In vitro* techniques include the preservation of sperm/ovule, embryos, genomic libraries, and somatic cell lines. One of the significant approaches is the establishment of fibroblast cell lines using cryopreservation technology (Guan et al. 2010; Li et al. 2009b; LIU et al. 2007). Fibroblast cells represent a rapid growth feature and early genetic screening that makes it a favorable cell type of conservation (Zhang et al. 1998). There are several reports which show that several fibroblast cell lines from various animals have been established until now, including the Caspian horse (Amoli et al. 2017), Sistani cattle (Gorji et al. 2017), Fars native goat fetal (Mehrabani et al. 2016), Simmental cattle (Ibrahim et al. 2009), Texel sheep (Li et al. 2009b), and Luxi cattle (Liu et al. 2008). These studies operated one of these two techniques to cultivate tissue, including enzymatic and direct explant techniques (Klingbeil et al. 2009). Markhoz goats are classified as domestic small ruminants, which can tolerate unpleasant conditions to survive. The main habitat for this breed is the Kurdistan province of Iran, which is the center of mohair product, obtained from this breed. Mohair has an important cultural role in the making local clothes in Kurdistan (Farshad et al. 2008). It is also noteworthy that this

breed is a major source of red meat (102,000 tons per year) especially in arid and semi-arid areas of Iran. So it is characterized as a multipurpose animal (Goodarzi and Hosseini 2013). An uncomfortable observation indicated that the population size of Markhoz goat is decreasing annually. In comparison to last decades, the distribution of this breed is limited in a few villages of Western Azerbaijan and a small part of Kurdistan. Based on the data obtained by Agricultural Jahad of Kurdistan (AJOK) there is a downward slope of Markhoz goat population size (22000 to 5000) from 1996 to 2005 (Bahmani et al. 2011), which this population size further decreased during the recent years. Due to the strategic value of Markhoz goat in the potential production of Mohair for textile industries to produce waterproof fabrics, biodiversity conservation of this livestock is crucial.

It was aimed at this research to cultivate the fibroblast cell lines derived from ear margin tissues of Markhoz goat (named CpHi). The cryopreserved cell lines were optimized in culture conditions and the established cell lines were characterized by the handling of cell viability, microbial quality control, chromosome analysis, species identification, and genes transfection of fluorescent proteins. Fibroblast cell cryopreservation is a fast and valuable strategy to develop a convenient and advantageous genetic pool for future genomic studies and the possible revival of this animal by genetic engineering (Ibrahim et al. 2009). Furthermore, the progression of novel technologies and introducing new achievements in future researches will increase the probability of utilization of these established cell lines in various categories, including tissue engineering, reprogramming, and producing extracellular matrix using these somatic cells (Franco-Barraza et al. 2016).

Materials And Methods

Primary fibroblast cell isolation and culture

In the present study, the ear samples from 58 specimens of Markhoz goat was obtained from the Ministry of Jahad Agriculture, Research Center, Sanandaj, Kurdistan province, Iran. The sample was moved to the laboratory using PBS containing Penicillin (200 U/ml) - Streptomycin (200 mg/ml). The fibroblasts were isolated from a small piece of animal ear sample using the explant technique described by Gorji et al. (Gorji et al. 2017). In brief, the samples were dissected out into small pieces of 1 mm^3 in size and 3-4 pieces were placed on the surface of small culture dishes, fixed by Lamell and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-Glutamine and Penicillin (200 U/ml) - Streptomycin (200 mg/ml) and then incubated at 37ºC with 5% CO_2 and 95% humidity for one week. In this period, the cell culture was checked routinely to investigate any probable contamination and replacement of the culture medium.

Cell proliferation and cryopreservation

After one week when the explant culture reached the 70-80% confluence, the cells were detached from culture plate using 0.025% trypsin-0.02% EDTA solution and sub-cultured into 25 mm^2 cell culture flasks.

In the culture process, the DMEM complete medium was added and the flasks were kept at 37ºC with 5% CO2 and 95% humidity. The medium was changed every two-three days and the cells were observed every day for density and morphology. For cryopreservation, the cells were again sub-cultured into a 75 mm^2 cell culture plate. When the cells reached the logarithmic growth phase, they were harvested and counted using a trypan blue solution. Around 1×10^6 cells/ml was harvested and resuspended in freezing medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO). The cell suspension was stored into sterile plastic cryovials labeled with the species name, cell ID number, cell count and the date of freezing. The cryovials were then kept at 4°C for 60 min, -80°C overnight and -196ºC nitrogen vapor phase for long-term storage. For cell reseeding, the cryovials were taken out from the nitrogen vapor phase after two weeks, six months and one year of storage. The cell viability before freezing and after thawing was measured by trypan blue staining method. For the growth rate estimation, the three independent cell lines were seeded into 24-well plates at a density of 5×10^4 cells /ml and cultured for 6 days in three repeats. After that, the cells were counted every day in triplicate. The mean cell numbers at each day were then plotted versus to time, and the population doubling time (PDT) was determined.

Chromosome analysis

The chromosome analysis was investigated by the protocol configured by Elyasi et al. (2015) but with some modifications. In brief, the cells were prepared in 50%–60% confluence and then colchicine was added into the medium at the concentration of 20 µg/ml. The medium was then removed after 1 hour and washed with PBS. After that, the cells were trypsinized, centrifuged at 300×g for 5 minutes and then the supernatant was removed. The hypotonic solution, 0.075 M KCl, was mixed gently with cell pellet and then incubated for 1 hour at 37ºC. After hypotonic treatment, 1 ml of cold fixative (3:1 methanol and acetic acid) was added and centrifuged for 10 minutes at 300×g. Finally, this step was repeated by 5 ml cold fixative. The remained suspension was dried on slides at 60ºC for 18 hours. For Giemsa staining, the slides were placed in a trypsin solution (0.025 %) for 35 seconds, and then slides were exposed to PBS and finally stained with Giemsa for 7 minutes. The slides were then analyzed at least 10 to 20 metaphases.

Microorganism detection

To detection of mycoplasma contamination, fluorescent DNA staining was investigated using by Hoechst 33258 (Sigma, USA) according to the protocol defined by Guan et al., (2005) (Guan et al. 2005). For this reason, the cells were cultured in antibiotic-free medium and stained with Hoechst 33258. To confirm the results of nucleus staining, the PCR method was also performed using the protocol released by Uphoff et al., (2002) (Uphoff and Drexler 2002). PCR method could detect the most common mycoplasma species, including Mycoplasma orale, Mycoplasma hyorhinis, Mycoplasma arginini, Mycoplasma fermentans, Mycoplasma hominis, Mycoplasma bovis and Acholeplasma laidlawii (Uphoff and Drexler 2002). In addition, the culture plates were followed microscopically for the presence of fungi, yeast and bacterial

contaminations every day. Furthermore, the cell supernatant (antibiotic-free) was cultured in microbial culture medium containing Thioglycollate Broth and Tryptone Soy Broth media for 14 days in 22ºC and 32ºC separately.

Species identification

Genomic DNA was extracted from primary cells via column-based DNA extraction kit (IBRC: MBK0021). The authentication of the primary cells was confirmed by the multiplex PCR method using the amplification of cytochrome C oxidase subunit I (COI) mitochondrial gene. The specific primers were used as reported by Cooper et al. (2007) (Cooper et al. 2007). Different animal species include mouse, rat, rabbit, camel, horse, cow, sheep, cat, dog, guinea pigs, pig, rhesus monkey, African green monkey, Chinese hamster, human, chicken, and goat could be detected by this method.

Transfection of fluorescent proteins

With the purpose of lentivirus production, 293LTV cells were seeded onto a culture plate and then transfected with 13.5 µg of transfer vector pCSGW, which carries PTP1B shRNA or cDNA, 9 µg of pCMVDR8.91, and 4.5 µg of pMDG using poly ethylenimine. Subsequently, the viruses were harvested after 48 hours by passing through a 0.45 µm pore-size filter and concentrated by ultracentrifugation at 100000 ×g for 90 min. Finally, to perform the infection, the Markhoz cells were transduced at multiplicities of infection (MOI) 6 for 16 hours. After that, the infected medium was removed and the cells were cultured in the absence of a virus for the next 72 hours. The relative fluorescence intensity of the induced fluorescent proteins was evaluated and recorded by a fluorescent microscope. The HIV packaging (pCMVDR8.91), VSV-G (pMDG) plasmids and the lentiviral pCSGW vector have been purchased from the Stem Cell Technology Center (Bonyakhteh).

Results

Establishment of Markhoz goat fibroblast cell line

It was observed that Markhoz cells emerged from tissue pieces into the culture flask around two-three days after being explanted. The migrated cells were including a combination of fibroblast-like cells and also epithelial-like cells, however, because of using fibroblast culture mediums, a relative pure fibroblast line was observed after passages three (figure 1,a). The cells were cryopreserved in freezing media (90% FBS and 10% DMSO) at passage number three (figure 1,b), in a density of 1×10^6 number/ml. The average viability before freezing and after thawing was estimated at 95-99% and 90-95% respectively. Furthermore, the cells could grow in several passage numbers after defreezing (figure 1, c), which represented the optimal cell culture condition and cryopreservation.

Growth curve of Markhoz cells

As shown in figure 1, d, the cell growth curves were plotted in ”s” shape and the population doubling time was calculated approximately 23±0.5 h. It was observed in this diagram that the lag phase lasted about two days, followed by three days exponential phase. Finally, the stationary phase was reached after about four days and took along for another two days.

Microbial analysis of primary cultured cells

Based on the routine investigations which including visible analysis by the naked eye, PCR analysis for mycoplasma (figure 2), microbial culture test, and Hoechst DNA staining (figure 3), the Markhoz goat cell culture medium did not display any contaminations by mycoplasma, bacteria, yeast, and fungi.

Species identification of Markhoz cell collection

As shown in figure 4 in this study, PCR analysis for species identification confirmed the origin of Markhoz goat cell lines and did not represent any cross-contamination, which was followed and examined by this method.

Chromosome analysis of Markhoz cells

Karyotyping analysis revealed that the chromosome number of the goat was 2n = 60 with >95% frequency, which included 29 pairs of autosomal chromosome showing the acrocentric pattern and 1 pair of sex chromosomes showing the sub-metacentric pattern. In this experiment, 50 representatives spread of metaphases were observed under the microscope at passage numbers 3-5, to be counted the chromosome distributions (figure 5,a) and numbers (figure 5,b).

Transfection of fluorescent proteins

According to the pictures obtained by the fluorescence inverted microscope (figure 5,c) the virus, which was carrying fluorescent protein genes, could be transfected successfully into the Markhoz cells with around 60-65% frequency after 72 hours of infection.

Discussion

Policy-makers in many countries are seldom aware of the diverse and significant contributions of animal genetic resources of food and agriculture. High-yielding domestic animals are one of the biodiversity challenges throughout the world, which can raise damage risk of the local animals. Native animals are a valuable resource of genetic pools for their potential resistance and adaptation to adverse biological

conditions. Therefore, a strategic vision for conservation and sustainable use of biodiversity is for the sustainable development of the economy and society (Li et al. 2009a). Native genes of endangered breeds should be conserved as much as possible for the increasing of the chance of somatic cell cloning to reproduce these breeds after extinction. Therefore, the conservation of endangered animals has become an urgent issue (Dohner 2001; Li et al. 2009c). The fibroblast-like cell culture from the Markhoz native goat was established using the explant culture method followed by storing at passage number three. In this experiment, the technique of primary explant culture was applied in small amounts of tissue with rather lower risks of losing cells than other methods (Li et al. 2009b). The analysis of this study indicated that established Markhoz goat fibroblast primary cells grew rapidly, which was genetically stable with correct species identification, and not any cross-contamination. Morphological observation showed that it was found both epithelial and fibroblast cells during the first passages of the explanted tissues. Since most epithelial cells were difficult to adhere to and solely attach in an unstable manner, a purified fibroblast line could be obtained after three passages (Guan et al. 2005; Zhou et al. 2005). To protect the potential biological and genetic alternation in vitro culture, cells were cryopreserved in a minimal number of passages (Hasoon et al. 2013). The survival rate after freezing decreased to some extent, possibly due to the cell injury that occurred during the process of freezing and recovery. The isolated cells were then examined for bacteria, yeast, fungi and mycoplasma contamination. Since the removal of the mycoplasma is difficult because they can coexist with cells for a long period, the contaminated cells with mycoplasma removed from the cell culture process exactly after the detection (Li et al. 2009b). The best methods for mycoplasma detection includes direct solid agar microbial culture, indirect DNA fluorescent staining, and PCR method (Ryan 2009). In this study, it was performed a combination of these methods as the best overall testing approach. The preserved goat fibroblasts were free of mycoplasma as determined by Hoechst 33258 staining methods and confirmed by PCR. According to the international standards, the karyotype analysis represented the 2n = 60 chromosomes, with the sex chromosomes X and Y (Iannuzzi et al. 1996). The diploid frequency of chromosome distribution was 70–75%, hence, it can be claimed that the Markhoz goat fibroblast cell culture was stably diploid. In this research, the expression of fluorescent proteins was around 65%, which different factors, including plasmid DNA concentration and incubation time, lipofectamine concentration and combination, and the utilization of serum in the medium can influence cell transfection efficiency (Escriou et al. 2001; Rui et al. 2006). However, the important issue was that under the standard conditions, the transfected cells had not shown any different growth rate, cell shape, and cell proliferation in comparison to untransfected cells. The Markhoz fibroblasts exogenous gene expression can be important in the future for the identification of specific genetic markers of this breed, and transgenic cloning research. Furthermore, in comparison to specialized cell lines, such as B-cells, foreign genes can be transfected and expressed in fibroblast cells in very facile conditions because of the reason that fibroblast cell lines are less differentiated (Bouchard et al. 1989).

Conclusions

In conclusion, this study demonstrated the establishment of the genetically stable Markhoz goat fibroblast cell lines, which are in the extinction danger and meet the criteria of cell line quality standards of the international cell collections. The established cell lines can be proposed as the valuable experimental materials for genomics, post-genomics, cloning, and other fields of life sciences in the future.

Declarations

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Conflict of interest

The authors declare no conflict of interest in the present study, and no significant financial support for this work could have influenced its outcome.

Ethics approval

This work was not submitted to any other journal in any form, and no animal experiments or human studies were used for this research.

Consent for participation

This manuscript has been read and approved by all named authors and that are no other persons who satisfied the criteria for authorship but are not listed. All the authors of this publication have a draft copy of this paper and are agree to participate in this manuscript with the proposed structure.

Consent for publication

The authors of this manuscript declare their consent to publish the results of study in this journal.

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Figures

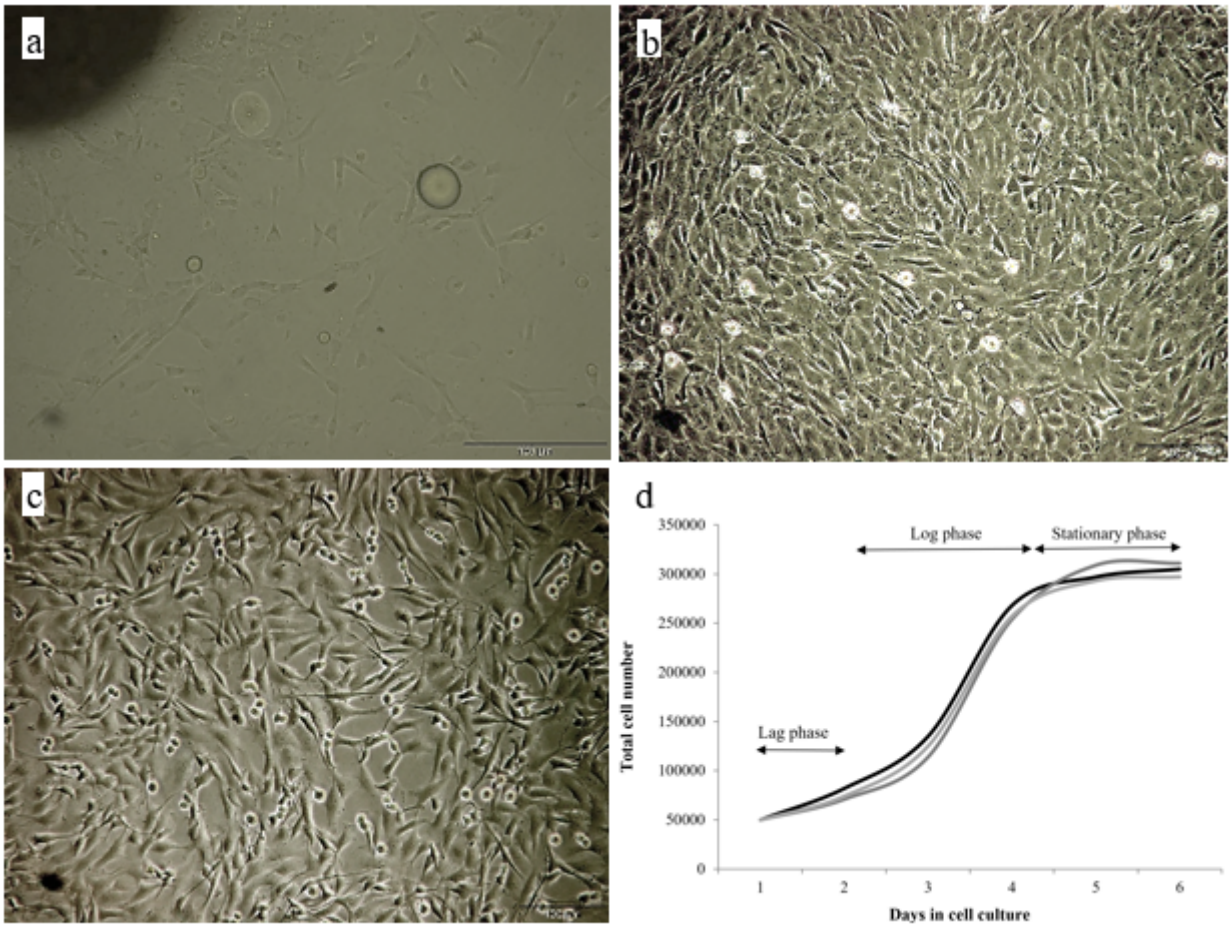

Figure 1

Growth of the goat fibroblast cells after the in vitro explant culture (a), before cryopreservation (b), 48 h after recovery (c) and Growth curve of the goat fibroblasts (d). The curve have a typical “S” shape, including lag phase, log growth phase, and stationary phase

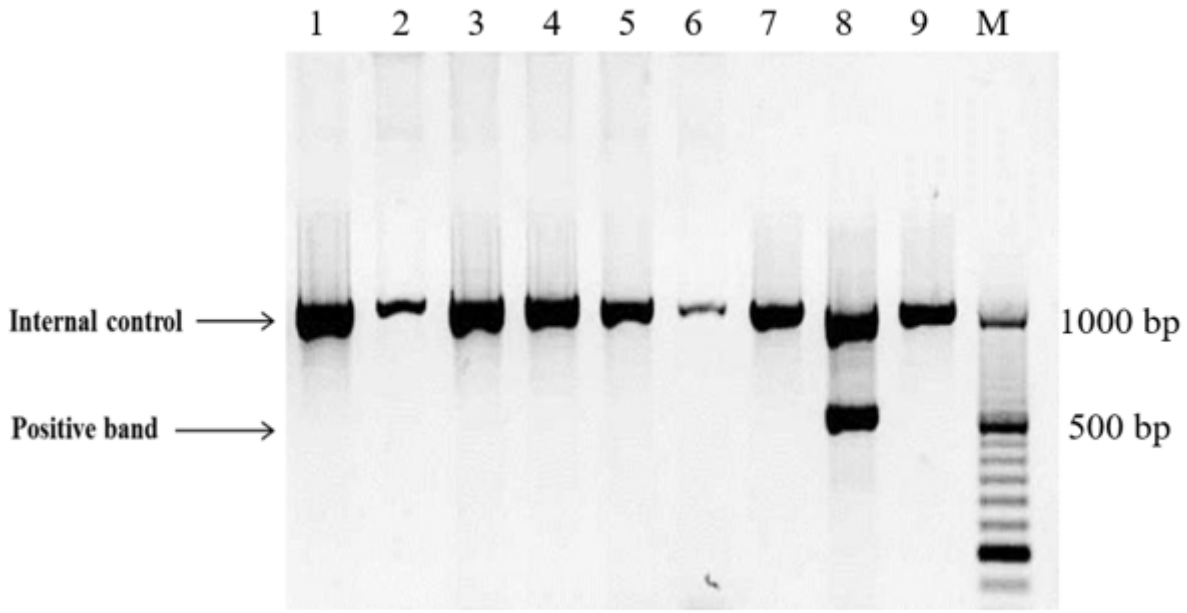

Figure 2

Gel electrophoresis of multiplex PCR for mycoplasma detection using specific primers. Lanes 1–7 demonstrated the CpHi cell lines without any mycoplasma contamination. Lane 8 showed PCR for mycoplasma positive control and lane 9 represented PCR for mycoplasma negative control. Identification of 1000 bp band was considered as multiplex PCR internal control.

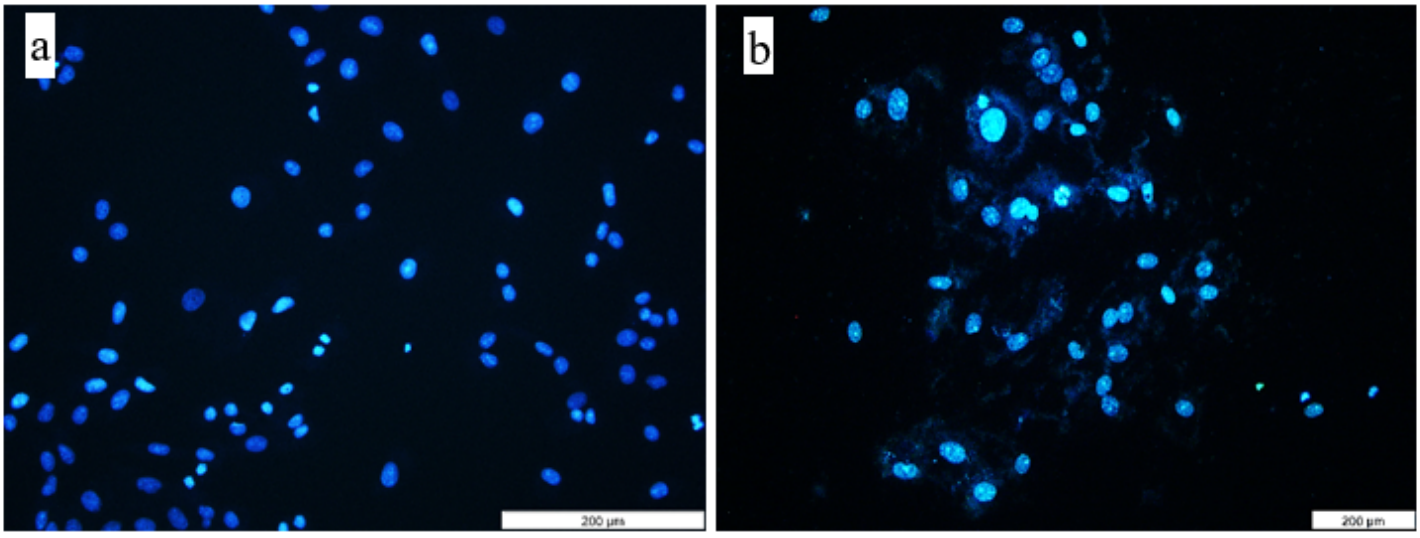

Figure 3

Analysis of the microbial quality control in Markhoz goat primary cell culture. DNA staining with Hoechst 33258 represented a clean cell culture condition without any microbial contamination as shown in the left side figure (a); furthermore, the figure in right side was used as the positive control (b)

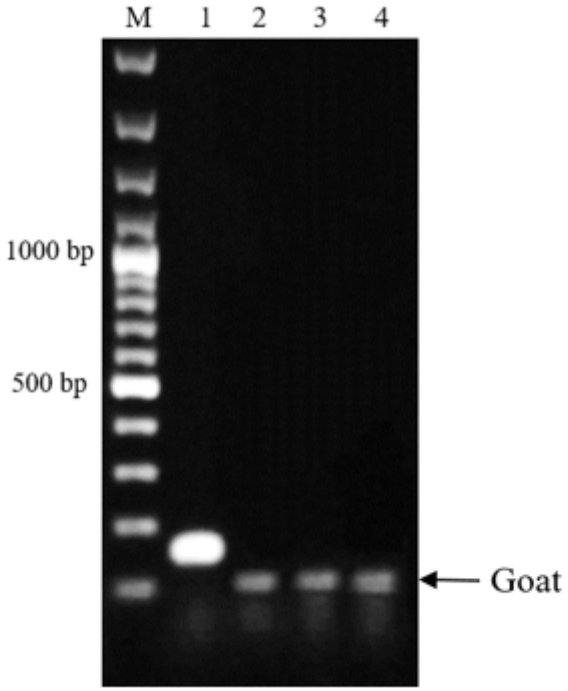

Figure 4

Gel electrophoresis of multiplex PCR for species identification with specific primers. Lanes 2-4 represented 117 bp specific band for the Markhoz goat with no cross-contamination. Lane 1 was used as a control group, which showed a cell line with the origin of the mouse.

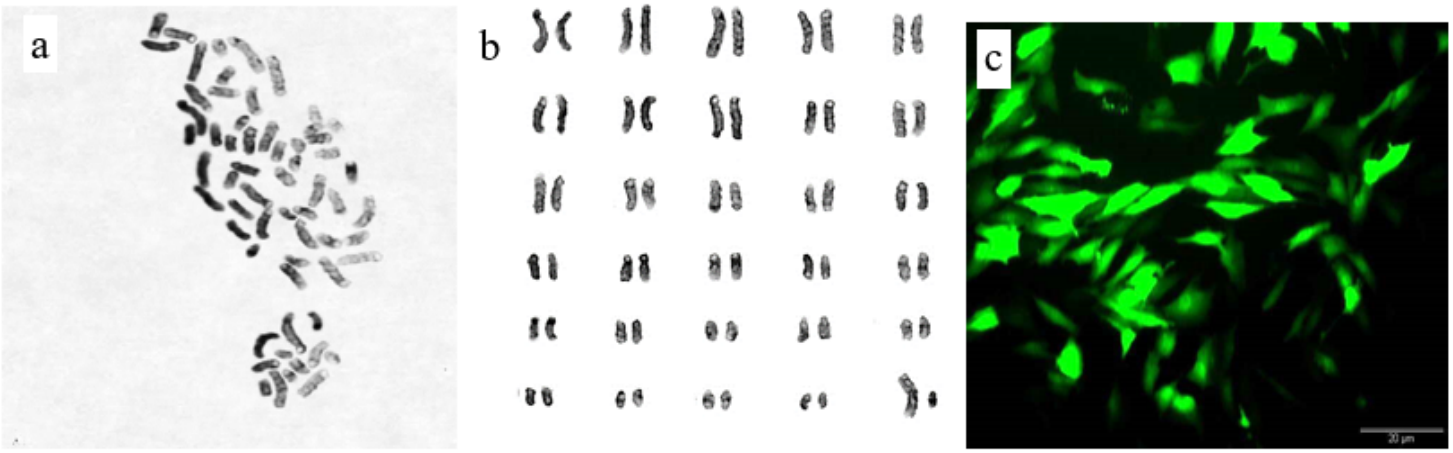

Figure 5

Metaphase spread (5, a) and karyotype analysis (5, b) of the Markhoz goat fibroblast cells. The chromosomes number was calculated 2n=60. The X chromosome was identified as the longest chromosome and the Y chromosome was categorized as the shortest chromosome in this species. Expression rate of the GFP in Markhoz goat fibroblast (5, c) after 72 h gene transduction. The gene expression was recorded using a fluorescent microscope.