



# Cryopreservation of Iranian Markhoz goat fibroblast cells as an endangered national genetic resource

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## Abstract

**Background** The continuous accessibility of local animals for sustainable use 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 extension facing in the future. This study aimed to establish and cryopreserve endangered Markhoz goat (*Capra hircus*) fibroblast cell lines in vitro.

**Methods and results** These primary fibroblast cells were isolated from 58 Iranian Markhoz goats and individually cultured by explant technique in DMEM medium supplemented with 10% FBS and 2 mM L-Glutamine, in the presence of Penicillin (200 U/ml)—Streptomycin (200 mg/ml) during the first passage number. The extracted cell lines were confirmed morphologically as fibroblast cells. The population doubling time for DMEM-cultured cells was  $23 \pm 0.5$  h. Chromosomal analysis indicated a total chromosome number of  $2n = 60$  with  $> 95\%$  frequency. The cultured cells were checked for bacteria, fungi, yeast, and mycoplasma contaminations and the results were reported negative. The efficiencies of the fluorescent protein encoded by VSV-G (pMDG) and lentiviral pCSGW vectors reported in a range of 65% value. According to the species identification analysis, the goat cell lines were banked and confirmed without any miss- and cross-contamination.

**Conclusions** The significant issue in this paper can be concluded about the first report of the establishment of endangered Markhoz goat cell banking inside the country. This study demonstrated the successful establishment of a genetically stable fibroblast bank as a valuable genetic resource for the endangered Iranian Markhoz goat breed.

**Keywords** Markhoz cell line · Explant technique · Primary cell preservation · Molecular characterization

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## Introduction

Biodiversity conservation aims to manage the human use of natural resources to get the sustainable benefit of present and future generations. Preservation of genetic resources implies the different aspects of economic, social, and cultural issues, which lead to the establishment of productive ecosystems to develop a sustainable community [1]. The loss of the livestock species inside certain geographic areas causes the abolishment of the variability of the animal genetic resources and extinction [2]. Therefore, the conservation of genetic resources from native breeds, wild types, and domestic animals is the key factor to protect biodiversity in a distinct ecosystem [3–5].

There are various complementary strategies for the conservation of animal resources which including in vitro and in vivo. In vitro techniques include the cryopreservation of

sperm/ovule, embryos, genomic libraries, and somatic cell lines [6]. One of the significant *in vitro* approaches in cryopreservation technology is the establishment of the fibroblast cell lines [3, 7, 8]. Fibroblast cells represent a rapid growth feature and early genetic screening that makes it a favorable cell type of conservation [9]. Several reports indicated the establishment of the fibroblast cell lines from various animals which including the Caspian horse [10], Sistani cattle [11], Fars native goat fetal [12], Simmental cattle [13], Texel sheep [3], and Luxi cattle [14]. These studies operated one of these two techniques to cultivate tissue, including enzymatic and direct explant techniques [15].

Markhoz goats, a class of small domestic ruminants, can tolerate unpleasant conditions to survive. The favorable habitat for this breed is the Kurdistan province of Iran, known as the center of mohair by-products obtained from this breed. Mohair plays the basic cultural role in making local clothes in Kurdistan [16]. It is also noteworthy that this breed is a valuable source of red meat (102,000 tons per year) in arid and semi-arid areas of Iran [17]. So it can be characterized as a multipurpose animal [18].

An uncomfortable observation indicated that the population size of the Markhoz goat is decreasing annually. In comparison to the 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 Jihad of Kurdistan (AJOK), there is a downward slope of Markhoz goat population size (22,000 to 5000) from 1996 to 2005 [19], with a further decrease during the recent years [18]. Therefore, the Markhoz goat is an endangered animal threatened for extinction [20]. According to the strategic role of Markhoz goat in making the waterproof fabrics by Mohair in textile industries, biodiversity conservation of this breed is crucial. Furthermore, due to its impressive adaptability to the environment [18, 20], the establishment of fibroblast cell bank, as an unlimited source of genetic pool, leads to further studies for genetic improvement and variability to enhance its future productive performances. This work can employ future exhaustive studies, including characterization of molecular markers for the management strategies and improvement of specific traits in endangered populations [18].

The essential aim of this research was the conservation of fibroblast cell lines derived from the ear margin tissues of Markhoz goat (named CpHi). The derived cell lines were cultured by explant technique and the established cell lines were cryopreserved followed by assessment of the 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 [13]. 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 different branches, including tissue engineering, reprogramming, and producing extracellular matrix using these somatic cells [21].

## Materials and methods

### Primary fibroblast cell isolation and culture

In the present study, the ear samples from 58 specimens of Markhoz goat were obtained from the Ministry of Jihad Agriculture, Research Center, Sanandaj, Kurdistan province, Iran. The ethical approval of sampling protocols arranged and confirmed by the human and animal cell bank of IBRC.

The small pieces of animal ear samples were moved to the laboratory using PBS containing Penicillin (200 U/ml)—Streptomycin (200 mg/ml). The fibroblast cells were isolated from the received sample using the explant technique described by Gorji et al. (2017) [11]. In brief, each sample was dissected out into small pieces of 1 mm<sup>3</sup> in size, placed on the surface of the culture plate (32 mm diameter), fixed by pre-cleaned 18 × 18 mm coverslip glass, 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). After that, the culture plates were kept and incubated at 37 °C with 5% CO<sub>2</sub> and 95% humidity during one week. In this period, the cell culture was checked routinely to investigate the probable contaminations and to refresh the culture medium.

### Cell proliferation and cryopreservation

The cells were proliferated and cryopreserved according to Stacey et al. (2008) protocol, but with a few modifications [22]. After a week, the non-contaminated cultured cells reached 70–80% confluency and were detached from the culture plate using 0.025% trypsin-0.02% EDTA solution and sub-cultured into 25 mm<sup>2</sup> cells culture flasks. In continue, the cells were cultured using the free-antibiotic DMEM medium containing 10% FBS, and 2 mM L-Glutamine followed by kept at 37 °C with 5% CO<sub>2</sub> and 95% humidity. The medium was changed every two or three days, and the cells were checked routinely for their density and morphology. Finally, the cells were cultured into 75 mm<sup>2</sup> cell culture plates, harvested, and counted using a trypan blue solution. Around 1 × 10<sup>6</sup> cells/ml was resuspended in a freezing medium containing 90% FBS and 10% dimethyl sulfoxide (DMSO). The cell suspension was stored in 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

– 196°C nitrogen vapor phase for long-term storage. For cell reseeded, 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 the trypan blue staining method. For the growth rate estimation, the three registered cell lines were seeded into 24-well plates at a density of  $5 \times 10^4$  cells/ml and cultured for 6 days in three repeats. After that, the cells were counted every day triplicate, and the mean cell numbers at each day were plotted versus time, followed by the determination of the population doubling time (PDT).

### Chromosome analysis

The chromosome analysis was investigated by the protocol configured by Gorji et al. (2017) but with some modifications [11]. In brief, the cells were prepared in 50–60% confluency, and then colchicine was added into the medium at the concentration of 20 µg/ml. The medium was then removed after 1 h and washed with PBS. After that, the cells were trypsinized, centrifuged at  $300 \times g$  for 5 min, and then the supernatant was removed. The hypotonic solution, 0.075 M KCl, was mixed gently with cell pellet and then incubated for 1 h at 37 °C. After hypotonic treatment, 1 ml of cold fixative (3:1 methanol and acetic acid) was added and centrifuged for 10 min at  $300 \times g$ . Finally, this step was repeated by the addition of a 5 ml cold fixative solution. The remained suspension was dried on glassy slides at 60 °C for 18 h. For Giemsa staining, the slides were placed in a trypsin solution (0.025%) for 35 s, and then the slides were exposed to PBS solution followed by stained with Giemsa for 7 min. Finally, the slides were then investigated for at least 10–20 metaphases.

### Microorganism detection

In order to recognition of mycoplasma contamination, fluorescent DNA staining was investigated according to the protocol defined by Guan et al. (2005) [23]. For this reason, the cells were cultured in an antibiotic-free medium and stained with Hoechst 33,258 (Sigma, USA). To confirm the results of nucleus staining, the PCR method was also performed using the protocol released by Uphoff et al. (2002) [24]. The primers used in 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* [24]. For microbial contaminations, the culture plates were investigated microscopically for the presence of fungi, yeast, and bacterial contaminations every day, and the cell supernatant (antibiotic-free) was cultured in microbial culture medium containing Thioglycollate Broth and Tryptone Soy Broth medium for 14 days in 22 °C and 32 °C separately.

### Species identification

Genomic DNA was extracted from primary cells via a column-based DNA extraction kit (IBRC, Iran). The authentication of the primary cells was confirmed by the multiplex PCR method assembled using the amplification of cytochrome C oxidase subunit I (COI) mitochondrial gene. The specific primers were used according to the study reported by Cooper et al. (2007) [25]. 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.

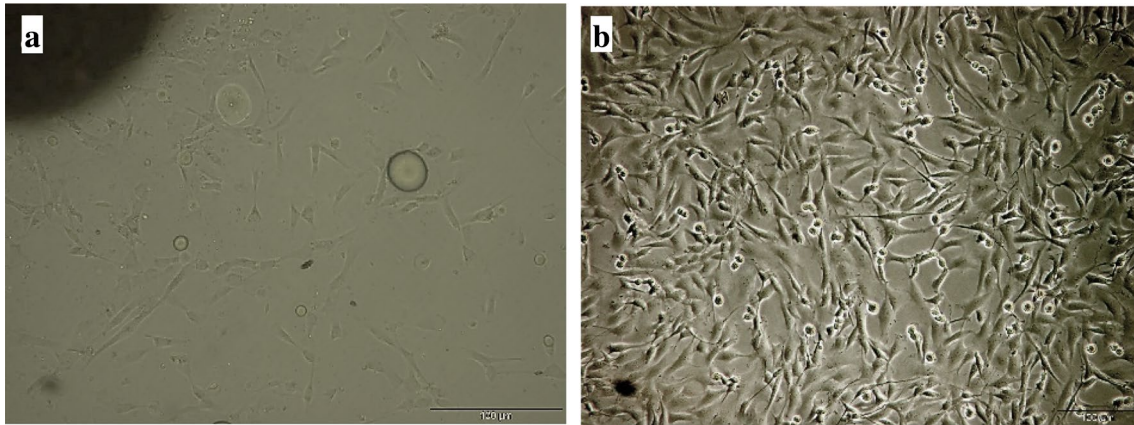
### Transfection of fluorescent proteins

According to the 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 h by passing through a 0.45 µm pore-sized filter and concentrated by ultracentrifugation at  $100,000 \times g$  for 90 min. Finally, to complete the infection, the Markhoz cells were transduced at multiplicities of infection (MOI) 6 for 16 h. After that, the infected medium was removed and the cells were cultured in the absence of a virus for the next 72 h. 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 to three days after being explanted. In the first days, the migrated cells included 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 number three (Fig. 1a). The cells were cryopreserved in freezing media (90% FBS and 10% DMSO) at passage number three, in a density of  $1 \times 10^6$  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 (Fig. 1b), which represented the optimal cell culture condition and cryopreservation process.



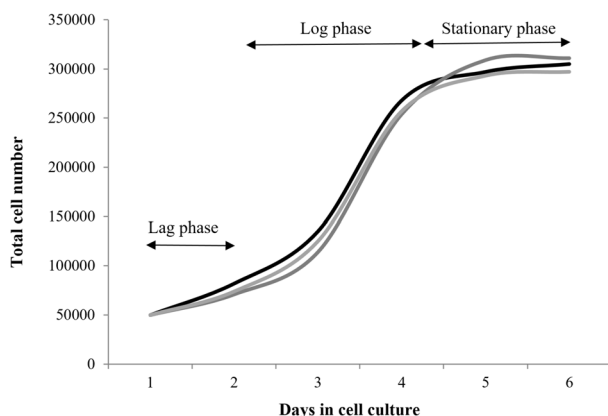
**Fig. 1** Growth of the goat fibroblast cells after the in vitro explant culture (a), 48 h after recovery (b)

### Growth curve of Markhoz cells

As shown in Fig. 2, the cell growth curves were plotted in the style of “s” shape and the population doubling time was calculated approximately  $23 \pm 0.5$  h. As shown in this diagram, 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 two days.

### Microbial analysis of primary cultured cells

Based on the comprehensive investigations in microorganisms detections, the visible analysis by the naked eye, PCR analysis for mycoplasma (Fig. 3a), microbial culture assay, and Hoechst DNA staining (Fig. 3b, c) represented that the Markhoz goat cell culture medium did not display any contaminations by mycoplasma, bacteria, yeast, and fungi.



**Fig. 2** Growth curve of the goat fibroblasts. The curve has a typical “S” shape including lag phase, log growth phase, and stationary phase

### Species identification of Markhoz cell collection

As shown in Fig. 4, the PCR analysis for species identification confirmed the pure originality of Markhoz goat cell lines and did not represent any cross-contamination according to this method.

### Chromosome analysis of Markhoz cells

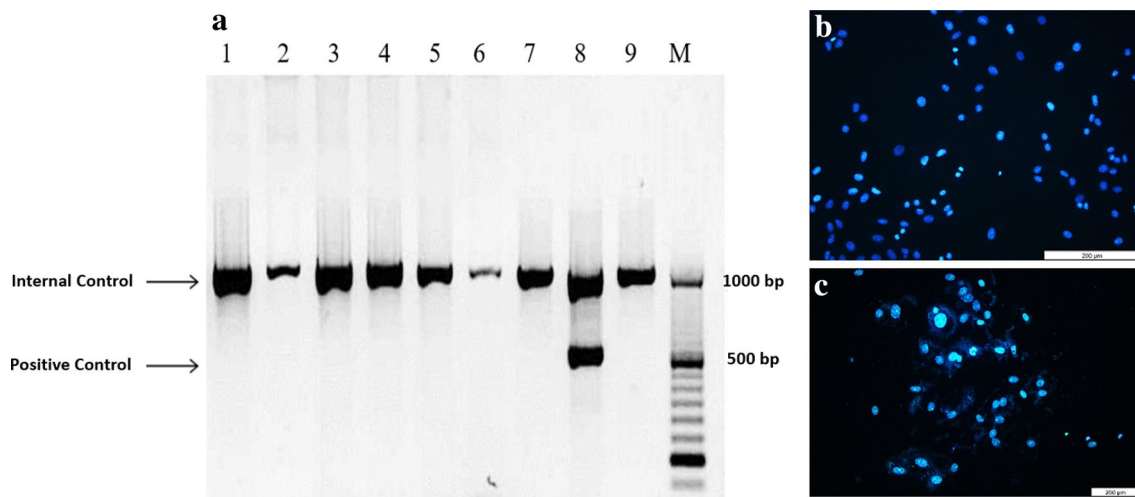
Karyotyping analysis revealed that the chromosome number of the Markhoz goat was  $2n = 60$  with  $> 95\%$  frequency in distribution which carrying the 29 pairs of autosomal chromosome showing the acrocentric pattern and one pair of sex chromosomes showing the sub-metacentric pattern. In this experiment, 50 independent spreads of metaphase step were observed under the microscope using by the cells at passage numbers 3–5, and the chromosome distribution patterns (Fig. 5a) and numbers (Fig. 5b) was figure out.

### Transfection of fluorescent proteins

According to the pictures obtained by the fluorescence inverted microscope (Fig. 6) the virus, which was carrying fluorescent protein genes, could be transfected successfully into the Markhoz cells with around 60–65% efficiency rate after 72 h of infection.

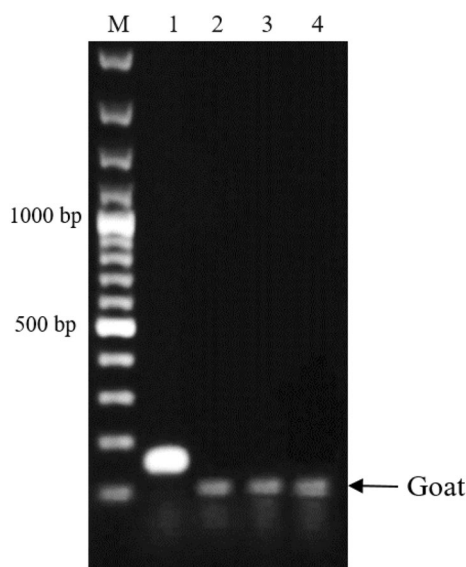
### Discussion

The contemporary biodiversity conservation problem is determined by the reduction and extinction of various animal populations and species all over the world [26]. 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



**Fig. 3** Gel electrophoresis of multiplex PCR for mycoplasma detection using specific primers (**a**) and analysis of microbial quality control in Markhoz goat primary cell cultures (**b–c**). Lanes 1–7a demonstrated the CpHi cell lines without any mycoplasma contamination. Lane 8a showed mycoplasma positive control and lane 9a represented

mycoplasma negative control. Identification of 1000 bp band was considered as multiplex PCR internal control. DNA staining with Hoechst 33,258 represented a clean cell culture condition without any microbial contamination (**b**) and positive control (**c**)



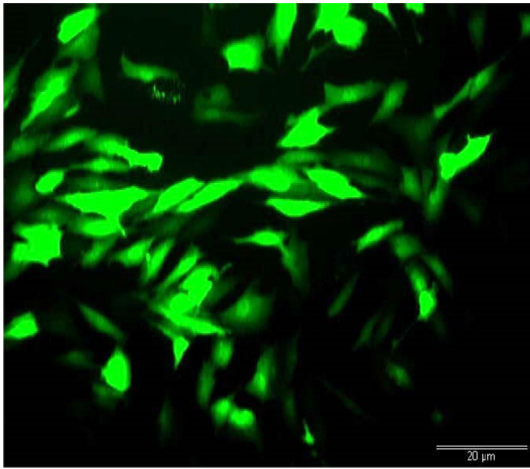
**Fig. 4** Gel electrophoresis of multiplex PCR for species identification with specific primers. Lanes 2–4 represented 117 bp specific band for the Markhoz goat with no cross-contamination. Lane 1 was used as a control group, which showed a cell line with the origin of the mouse

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 the sustainable development of the economy and society [4].



**Fig. 5** Metaphase spread (**a**) and karyotype analysis (**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

Reduction of animal specified native area, population size and herd numbers are the main indicators of obligatory danger of native animal extinction in the close future [27]. 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 [28, 29]. The ideal priority to conserve the animal breeds is the live population preservation; however, this approach is expensive and unless the breed can be used for production, it is not almost to be economic. Therefore, the in vitro conservation strategies emerged to cryopreserve animal genetic resources according to the genome/gene banks with the hope to manage



**Fig. 6** Expression rate of the GFP in Markhoz goat fibroblast after 72 h gene transduction. The gene expression was recorded using a fluorescent microscope

the exchange of genetic diversity or regenerate a particular population in the future [30, 31]. The main *in vitro* conservation approaches of livestock genetic resources have been focused on cryopreservation of gametes, embryos, and somatic cells as well as testis and ovarian tissues [31, 32]. However, according to the comprehensive studies, the above-mentioned conservation programs lack of a standardized protocol to apply for any kind of embryos [33], oocytes [34], and sperms [34] which seems to be a difficult procedure.

Fibroblast cells, considered as a source of disease modeling after cell reprogramming, are characterized as a group of cell culture with easily accessible in the body as a transient primary cell culture or permanent transformed cell line [35]. The fibroblast-like cell culture from the Markhoz native goat was established using the explant culture method followed by storing the cells at passage number three. In this experiment, the primary explant culture technique was applied in small pieces of tissue with rather lower risks of losing cells than other methods [3]. 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 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 [23, 36]. To protect the potential biological and genetic alternation *in vitro* culture, cells were cryopreserved in a minimal number of passages [37]. The survival rate after freezing decreased to some extent, possibly due to the cell injury that occurred during the process of freezing and recovery. However Stacey

et al. (2008) believed for most of cell types at least 80% cell viability can be accepted [22]. The isolated cells were then examined for bacteria, yeast, fungi and mycoplasma contamination. Since the removal of the mycoplasma is difficult due to the coexistence of the cells for a long period, the contaminated cells with mycoplasma removed from the cell culture process exactly after the detection [3]. The best methods for mycoplasma detection includes direct solid agar microbial culture, indirect DNA fluorescent staining, and PCR method [38]. In this study, a combination of the above-mentioned methods was performed as the best overall testing approaches. The free of mycoplasma goat fibroblasts were preserved according to the experimental tests determined by Hoechst 33,258 staining followed by confirmation of PCR methods.

Similar to the previous research the karyotype analysis represented the  $2n = 60$  chromosomes, with the sex chromosomes X and Y [39]. 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 [40, 41]. 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 fibroblast 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 fibroblast cell lines are less differentiated [42].

## Conclusions

This study demonstrated the establishment of the genetically stable Markhoz goat fibroblast cell lines which are classified as the endangered extinction breed, followed by meet the criteria of the quality standards of the international cell banking collections. The established cell lines can be proposed as valuable experimental materials for genomics, post-genomics, cloning, and molecular fields of future studies.

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## Declarations

**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.

**Ethical approval** This work was not submitted to any other journal in any form, and the results of this study were not used in any animal experiments or human studies.

**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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