Indian Journal of Animal Sciences **92** (6): 729–733, June 2022/Article
https://doi.org/10.56093/ijans.v92i6.105874

Establishment and characterization of fetal fibroblast cells of Black Bengal breed

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Received: 12 October 2020; Accepted: 25 March 2022

ABSTRACT

The somatic cells are as valuable as germ cells and an attractive resource for conserving animal genetic materials. The present study was undertaken to establish and characterize the caprine fetal fibroblasts of Back Bengal goat's genetic resources. The fetal fibroblast cells of Black Bengal goat breed at the age of 1-2 month was cultured and evaluated for its growth kinetics, cryogenic preservation ability, transgenic protein expression ability including the identification of cell specific marker identification. The cells were highly prolific, identified by molecular markers, with a population doubling time (PDT) of approximately 73.6 h and could be useful for gene expression study. These cells will be valuable assets for other genomics, post genomics, somatic cloning, and other fields of biological sciences in the future.

Keywords: Black Bengal, Characterization, Fetal Fibroblast Cells, Growth kinetics, Marker

Animal cells are valuable genetic resources and are an important component of biodiversity. The culture of animal cells has become a major tool for routine investigation of endocrinology (Bols and Lee 1991), virology (Wolf 1988), biotechnology and aquaculture (Bols and Lee 1991), and resources protection (Zhou *et al.* 2008, Chen and Qin 2011), immunology (Bols *et al.* 2001), and pharmacology including the regenerative medicine. Modern biotechnology has emerged because of the in depth and extensive study of cell culture and its associated techniques. The culture of animal cells is one of the major connectors between fundamental research and industrial exploitation. Cell culture provides the basis for studying the regulation of cell proliferation, differentiation, and product formation in carefully controlled conditions. Especially the cloning aspects have made the somatic cells as valuable as germ cells and the establishment of the somatic cell bank has made a revolution as an attractive resource for conserving animal genetic materials.

Fibroblast is a type of cell that synthesizes the extracellular matrix and collagen, has a critical role in wound healing and maintains the structural integrity of connective tissues (Annonymous 2014*)*. Fibroblasts are morphologically heterogeneous and derived from primitive mesenchyme. During tissue injury, fibroblasts differentiate into contractile and secretory myofibroblasts that helps in tissue repair during wound healing (Anonymous 2014). The fetal origin of fibroblasts is highly prolific cells (Unpublished) as compared to other groups of cells in culture cells. To reduce the time and specification of requirements in the experiment, the fibroblast culture is more suitable.

The present study was planned to establish and characterize the caprine fetal fibroblast cell culture of Black Bengal goat. This breed is one of the highly prolific breeds among Indian breeds of goat and gives multiple births with short generation intervals, etc. The meat of 6-8 months' kid of Black Bengal goat is world-famous for its tenderness and produces fine skin with excellent quality for high-class shoe-making. The fetal fibroblast cells of Black Bengal goat breed at the age of 1-2 months will provide valuable experimental materials for genomics, post-genomics, somatic cloning, and other fields of biological sciences in the future.

MATERIALS AND METHODS

Primary cell culture: Slaughterhouse fetuses/aborted caprine fetuses (about 45 days) were collected following the guideline of the Institutional Ethics Committee. The following protocol was adopted for the establishment of primary transplant culture.

The gravid uterus/aborted caprine fetus (Fig. 1) were collected in Dulbecco's phosphate-buffered saline (DPBS) containing antibiotics and transported to the laboratory. All procedure were carried out under strict hygienic condition. In the laboratory, the uterus was washed thrice by normal saline solution in a clean tray. Thereafter, the uterus along with the tray was taken to the Biosafety cabinet (Class II).

In the laminar air flow, the uterus was cut by Bard-Parker (BP) blade and a fluid-filled balloon-like structure containing a fetus was observed and transferred to a 90 cm petri dish containing gentamicin and antimycotic

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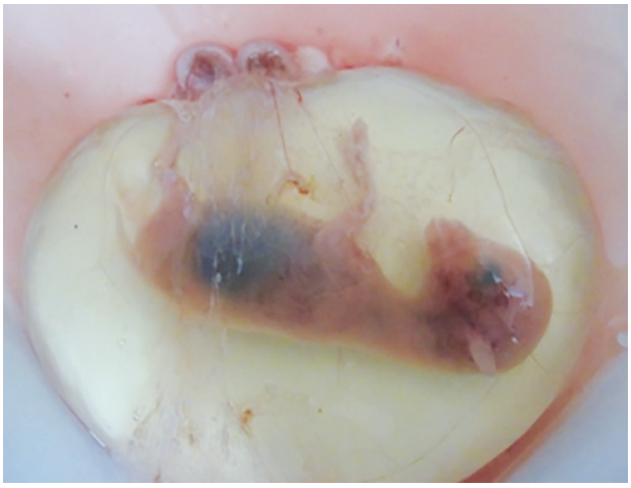

Fig. 1. Fluid-filled balloon-like structure containing the fetus.

(Amphotericin B) solution. The fetus was washed thrice and with the help of fine forceps, the uppermost layer of the skin was removed and transferred to another petriplate containing complete medium, i.e. Dulbecco's Modified Eagle Medium (DMEM) and 15% Fetal Bovine Serum (FBS) with Gentamicin. The skin pieces were cut into small pieces of 1 mm^2 and transferred into a 25 cm^2 tissue culture flask (Falcon), in few drops of medium and kept in a CO_2 incubator at 38.5˚C maintaining 5% CO_2 with 98% humidified environment for 2 to 4 h. When tissue pieces adhered to the surface of flasks, 4 ml of complete medium (15% FBS) was added slowly from the side and incubated for 24 h. Next day, the flasks were checked for contamination or floating tissue. Floating tissues were reseeded again in new tissue culture flasks. The culture was allowed to grow until 60 to 70% confluence before reseeding for better growth.

Sub-culture and maintenance: Fibroblast cultures that reached 60 to 70% confluence were digested with 0.25% trypsin – Ethylenediaminetetraacetic acid (EDTA) solution at a rate of 300 µl for 25 cm^2 cell culture flask for 1.5- 2 min and kept in a CO_2 incubator at 38.5˚C maintaining 5% CO_2 and 98% humidity. Every 1 min, the cells were observed under a microscope for rounding. After rounding, the action of trypsin was neutralized by 1 ml DMEM containing 10% FBS. The cell suspension was taken into the 1.5 ml multicentifuge tube and centrifuged at 3000 rpm for 2 min. The medium was decanted and pellet resuspended in 1 ml of media (10% FBS). Cells were counted in a hemocytometer and subcultured.

Growth kinetics of cells: Cells were placed at an initial density of 1×10^5 cells before seeding in each well of a 6-well plate (Falcon, USA) and cultured in fresh media (10% FBS), without antibiotic at 38.4˚C. Cells were collected and counted daily for 9 days using a hemocytometer (three wells each time). This process was repeated three times.

Cryogenic preservation and reseeding of cells: Cells were supplemented with fresh medium 24 h before freezing to make sure the nutrition was sufficiently absorbed by the cells. The cell suspension was acquired by digesting cells in 0.25% trypsin–EDTA solution. The suspension was centrifuged at 3000 rpm for 3 min and the supernatant was discarded. The cells were collected to make sure cell density approaches 10^6-10^7/ml. The cells were preserved in a freezing medium containing 10% Dimethylsulphoxide (DMSO)+90% fetal bovine serum. The tubes were tightly packed and immersed in aqueous methylene blue (0.05%) at 4ºC for 20–30 min. Further, they were placed at -20ºC for 2 h and then at -80ºC freezer for overnight. Finally, tubes were put in liquid nitrogen for long term storage.

For reseeding, the tubes were thawed at 37˚C and the cells suspension placed in DMEM and centrifuged at 1000 rpm for 10 min to remove the DMSO. The cells were then resuspended in fresh DMEM and seeded into petridishes, and cultured under 5% CO_2 at 38.5ºC. Medium was changed after 24 h.

Cell viability: Cell viability was determined using trypan blue staining as described previously (Weingartl *et al.* 2002). The number of dead cells was determined from a field of 1000 cells.

Microbial analysis for detection of bacteria, fungi, and virus: The cells were cultured in DMEM containing 10% fetal bovine serum without antibiotics and tested for the presence of microbes and viruses 3 days after subculture. The explanted fibroblasts were cultured and analyzed 3 days after subculture.

Expression of fluorescent protein gene in the caprine fetal fibroblastic cell: Transient transfection of the caprine fetal fibroblastic cell with pGFP-V-RS expression plasmid containing green fluorescence protein (GFP) and U6 promoter was performed with TurboFectin™ 8.0 transfection reagent in 1:3 ratio (2 µg Plasmid DNA: 6 µl TurboFectin). The wells showing 70-80% confluence were selected for setting up the reaction. To view the transfection, the cells containing the complete medium with transfection complex were visualized under an inverted fluorescence microscope (DM IL LEDTM, Leica, Germany) after 48 h of transfection in different magnifications and photographs of each field were taken under visible light as well as UV light simultaneously.

Cell origin identification by fibroblast-specific protein-1 marker: To establish the origin of the cell caprine, fibroblast, species-specific primers Fp-5'TACAGGAGACTTCCAGGGATT 3', Rp-5' CATGACAGAAGGACTGGGTAAG 3' were designed using the sequence XM_005677499.1, from NCBI that specifically amplify the sequence Fibroblast-specific protein 1(FSP1) gene sequence of fibroblast of caprine origin only. The primer could amplify the product of 143 bp in the designed RT-PCR reaction.

The cultured cells after the fourth passage were harvested and used for total RNA extraction. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The quality of RNA was checked and treated with DNaseI (Fermentas) for the removal of possible genomic DNA contamination. The

cDNA was synthesized using random hexamer primers of RevertAidTM MMLV RT enzyme of the first strand cDNA synthesis kit (Fermentas). Final reaction volume of 25 µl containing 1 µl of each gene-specific forward and reverse primer (10 p.mol), 3 µl of cDNA template was used. Thermal cycling condition comprised of initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 45 sec, primer annealing 60°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C for 5 min. Finally, the RT-PCR product was observed on 2% agarose gel.

RESULTS AND DISCUSSION

Morphological study (Primary cell culture and passaging of cell fetal fibroblast): The prominent growth of cells, i.e. splitting was noticed under phase-contrast microscopy on the next day after seeding of small tissue pieces into tissue culture flask having complete media and incubated. The cells had adhered tightly to the cell culture flask and the clear rapid growth of caprine fetal fibroblast cells (Figs 2 and 3) was observed. When the cells had reached 80-90% confluence on 3rd day, sub-culturing was carried out by adding a 0.25% trypsin-EDTA solution. The first 2-3 passages the cells were heterogeneous in population and fourth passage onward it became the complete fibroblast type. Morphologically, all cells showed clear typical fibrous and fusiform morphologies with centered oval-shaped nuclei. The cell morphology did not change during long-term cell culture (up to 30 passages). After the cryopreservation, the recovery was found to be 60–70% and cells grew well in the culture.

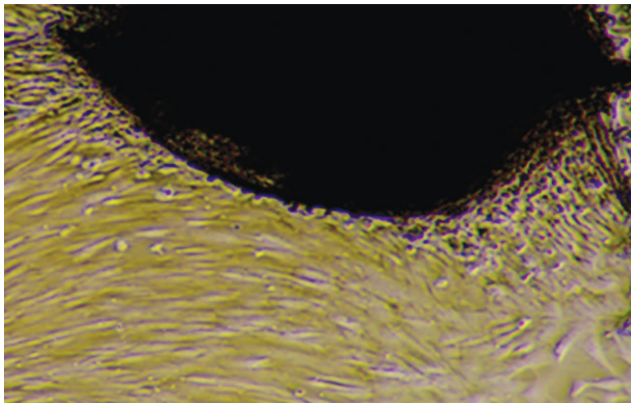

Fig. 2. Growth of caprine fetal fibroblast cells (10×).

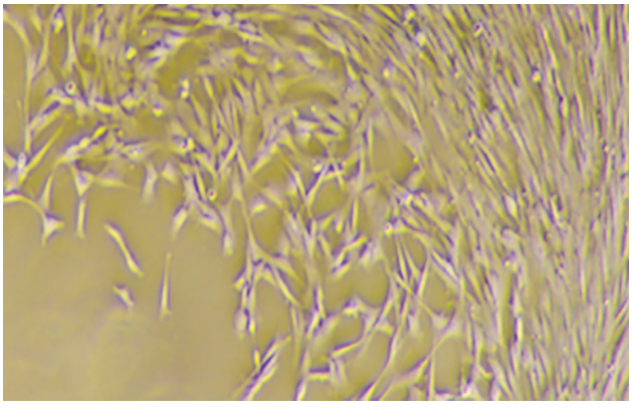

Fig. 3. Growth of caprine fetal fibroblast cells occupying spaces of the culture flask (10×).

Growth curve analysis: The cells of the fifth passage were seeded at an initial density 1×10^5 cells in each well of a 6-well plate and cells were collected and counted daily for 9 days using a hemocytometer. The population doubling time (PDT) was determined based on the average cell counts at each time point and the curve was plotted.

The growth curve of Black Bengal goat fetal fibroblasts had an "S" shape (Fig. 4) and the PDT (Roth 2006) was approximately 73.6 h. When the cell density increased, proliferation was reduced by contact inhibition and the cells entered the plateau phase after the 7th day. After the plateau phase, the population began to collapse and entered the apoptotic phase after the 9th culture day.

Expression of fluorescent protein gene in black goat fetal fibroblastic cell: For expression, the fluorescent protein gene in Black Bengal goat fibroblastic cells, transfection of pGFP-V-RS expression plasmid containing shRNA cassette (Origene, USA) with TurboFectin™ 8.0 transfection reagent in 1:3 ratio (2 µg Plasmid DNA: 6 µl TurboFectin) was used. The cells were visualized under an inverted fluorescence microscope (DM IL LEDTM, Leica, Germany) after 24, 48, 72 h transfection in different magnifications, and photographs of each field were taken

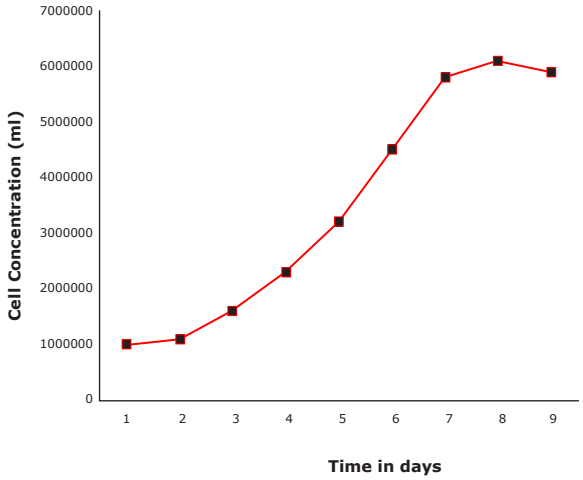

Fig. 4. Growth curve of Black Bengal goat fetal fibroblast cells.

under visible light as well as UV light simultaneously (Fig. 5). In each microscopic field, the transfected (Presence of GFP) and non-transfected cell (absence of GFP fluorescence) were counted for calculation of transfection efficiency. The black goat fetal fibroblastic cells showed good (40-60%) transfection ability and can be useful for subsequent studies.

Detection of bacteria, fungi, and Mycoplasma contamination: Generally, Fungi propagation can be observed by the medium turbidity or the culture medium turning flavor-green in colour or the presence of colony

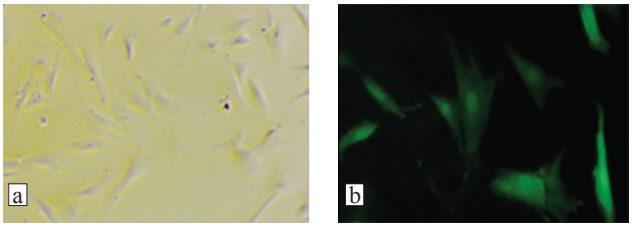

Fig. 5. The caprine fetal fibroblast cells expressing the Green fluorescent protein (20×): a) Transfected cells in Normal filter; b) Under GFP filter of Transfected cells.

or hypha that could be easily observed under an inverted microscope as well as by naked eyes.

In bacterial contamination, there is a sudden change in *p*H, usually, a decrease with most infections, cloudiness in the medium under a low-power microscope (~100×), spaces between cells appear granular and may shimmer with bacterial contamination, high-power microscopy (~400×), the individual bacteria can be distinguished into rods and cocci (Freshney 2010).

Generally, it is difficult to detect mycoplasma contamination in the early phase of cell culture. But in later phase, cells collapse, cell membrane fragments visible in the medium, and the cell edge becomes vague and out of shape, indicative of mycoplasma contamination.

During our experimentation, the culture medium was clear at all times and no abnormal changes were observed under the microscope that indicated that our Black Bengal goat fibroblasts were free of bacteria, fungi, viruses, and mycoplasma contamination.

Marker identification by RT-PCR: RT-PCR experiments showed that the fibroblasts expressed the relevant cell marker FSP1 gene with a product size of 143 bp on 2% agarose gel (Fig. 6). This was indicative of the origin of the cell to caprine fibroblast only.

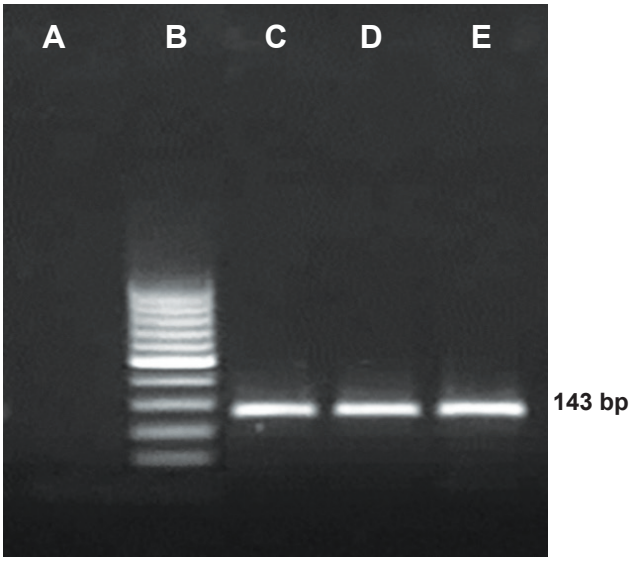

Fig. 6. Agarose gel (2%) showing the RT-PCR product of fibroblast-specific protein 1(FSP1) gene. Note: Lane A, NTC; B, 50 bp ladder; C, D, and E, RT-PCR product.

Animal genetic resources are an integral part of biodiversity. These resources have an important potential to improve the social-economic life of human beings and are considered as the insurance of the future. The somatic cells are as valuable as germ cells and the establishment of the somatic cell bank has made a revolution as an attractive resource for conserving animal genetic materials.

The Black Bengal goat breed is found in West Bengal, Bihar, Assam, Odisha, and Bangladesh. The breed is very popular because of small size, low demand for feed, high kid production rate, high disease resistance and produces high-quality meat and skin.

The present procedure of establishing the fibroblast cell culture of Black Bengal goat with the addition of 10% of serum was found to be successful. We observed that the addition of extra serum (15-20% of FBS) increases cell proliferation in the early days of culture (Lab observation). Morphologically, the cell population was heterogeneous in early passages (2-3) and contained both epithelial and fibroblast cells. From the fourth passage onwards, the cells were homogenous and completely fibroblast type. The fetal fibroblast of Black Bengal goat showed good freezing-thawed recovery of 60-70% and subsequent cultures did not show any abnormalities. The cells were found to be highly prolific with the population doubling time of approximately 73.6 h indicative of the usefulness of carrying the research activity in a faster way. The GFP can be successfully expressed in the growing cells and this provides valuable experimental materials for genomics, post genomics, somatic cloning, and other fields of biological research in the future.

Recently, the full genome sequence of the Black Bengal Goat of Bangladesh was decoded by a group of scientists (Prothom Alo 2019). The biological characterization with molecular characterization of the breed will fulfill the future course of research in the field of modern biotechnology and more particularly in genomic and proteomic research.

ACKNOWLEDGEMENTS

Authors are thankful to Department of Biotechnology, Government of India for providing fellowship and other support to carry out the research work.

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