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Original Research Article

Expression of transcription factor genes (*Oct*-4, *Nanog and Sox 2*) by putative amniotic fluid stem (AFS) cells of sheep (*Ovis aries*)

Anil Khuttan[1], Kapil Dev[1], Birbal Singh[2], Dheeraj Mohania[3], Manoj Kumar [4], Vinod Verma[5], Anal Kant Jha[6] and Sanjeev K. Gautam[1*]

[1]Department of Biotechnology, Kurukshetra University, Kurukshetra -136 119 Haryana India.
[2]Regional Station, Indian Veterinary Research Institute, Palampur-176 061 H.P. India.
[3]Department of Research, Sir Ganga Ram Hospital, New Delhi.
[4]National Institute of Nutrition, Hyderabad
[5]Department of Biotechnology, University of Allahabad, Allahabad
[6]Aryabhatta Centre For Nanoscience and Nanotechnology Aryabhatta Knowledge University, CNLU Campus, Patna-800001

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Abstract

The current study was carried out to isolate, culture, characterize and cryopreserve the amniotic fluid stem (AFS) cells in sheep. Putative AFS cells were cultured without feeder cells, in DMEM containing 10% FBS, 1% penicillin/ streptomycin and 1% L-glutamine in 5% CO2 in humidified air at 37±0.5°C. After 6 days of culture different morphologies of cells were observed. Most of the cells started anchorage-dependent growth after day 6 of the culture. In order to check their pluripotency properties when the cells were subjected to RT-PCR a strong positive expression of Oct-4, Nanog and Sox2 was observed. Using species-specific primers, a PCR amplicon of 290, 501 and 362 bp was observed for Oct-4, Nanog and Sox-2 respectively. The cells were found to have a normal karyotype at different passages. These results may contribute towards establishing non-embryonic pluripotent stem cells for various therapeutic and reproductive biotechnological applications in this species.

Keywords: *Sheep, amniotic fluid, stem cells, characterization and RT-PCR.*

1. Introduction

Sheep an important species of livestock for India represents sheep biodiversity in the form of breeds and strain. The domestication of animals is being carried out from Neolithic times along with the cultivation of cereals. First sheep and goats, second cattle and pigs, and finally draft animals such as horses and asses were domesticated. They contribute greatly to the agrarian economy, especially in areas where crop and dairy farming are not economical, and play an important role in the livelihood of a large proportion of small and marginal farmers and landless laborers. Among the domesticated livestock species, there are over 30 breeds of cattle, 10 breeds of river buffaloes, 42 breeds of sheep, 20 breeds of goats, 8 breeds of camel, 7 breeds of hoarse and 18 of the indigenous poultry. India's vast genetics resources in the sheep is reflected by the availability of 42 breeds of sheep. Small ruminants especially sheep contribute to the livelihoods of millions of rural poor in most of the developing countries of the Asia and Africa, However, introduction of exotic breeds, natural calamities and change in farming system have been resulted decline in pure breed population and in dilution of the genetics merit.

The productivity of Indian sheep is low, yet considering the nutritional and physical environmental conditions under which they reared it cannot be considered inefficient. Major reasons for this low productivity are inadequate grazing resources, disease problems and serious lack of organized efforts for genetic improvement. There is little selection of rams and bucks used for breeding, and much inter–mating among neighboring breeds takes place. The traditional breeding program involving selections based on production performance have contributed significantly to improvement of production performance of these goats. However, these techniques require long duration to improve to a particular trait. Therefore, researchers are attempting to improve sheep reproduction through innovative approaches using various reproductive technologies. Coordinated systems of reproductive management have been developed based on a thorough understanding of the endocrine, cellular and molecular factors controlling ovarian and uterine functions. Reproductive endocrinological interventions have contributed to improvements in sheep productivity, particularly through increased embryo production and the birth of the sheep through embryo transfer technology. Notable reports have presented a comprehensive account of the information on reproductive endocrinological advances, including ovarian follicular dynamics, knowledge of which may lead to better synchronization (as well as embryo production and transfer) in ewes.

The productive and reproductive efficiency of sheep is poor due to lower genetic potential which has not been studied in relation to functional genomics. Stem cells represent an ideal tool to study embryogenesis under in vitro conditions, particularly the genes involved in the functional development. Moreover, availability of sheep

*Corresponding author: **Sanjeev K. Gautam**

Table 1 Primer sequences and their fragment size of Oct-4, Nanog ans Sox-2 genes in sheep

Gene	Primer	Primer sequence	Primer position	Cycle Number	Fragment size	Reference
Oct4	Oct4 (F)	5’-CAATTTGCCAAGCTCCTAAA-3’	37–56	52x15	290	Daniela Sanna Cell Biol. Int. (2010) 34, 53–60
	Oct4 (R)	5’-TTGCCTCTCACTTGGTTCTC-3’	307–326			
Nanog	Nanog (F)	5’-TTCCCTCCTCCATGGATCTG-3’	327–346	53x15	501	Daniela Sanna Cell Biol. Int. (2010) 34, 53–60
	Nanog (R)	5’-AGGAGTGGTTGCTCCAAGAC-3’	805–824			
Sox2	Sox2 (F)	5’-TGATACGGTAGGAGCTTTGC-3’	1571–1590	53X15	362	Daniela Sanna Cell Biol. Int. (2010) 34, 53–60
	Sox2 (R)	5’-GGTCTCTAAAGGGGCAAAAG-3’	1913–1932			

The reverse transcriptase PCR (RT–PCR) was carried out at 42°C for 60 min. followed by denaturation at 95°C for 8 min. After RT-PCR the gel was run on 1.8% agarose.

stem cells could facilitate development of efficient methods for somatic cell cloning, transgenics, and gene regulation in this species. As for as the sheep amniotic fluid stem cells are concerned, there is very limited information available on these lines. To the best of our knowledge, no work has been carried out on scanning electron microscopy for morphologocals studies of these cells. Looking at scarcity of the information and usefulness of the these cells the current study was designed.

2. Materials and Methods

2.1 Chemicals and media

All chemicals i.e. reagents, culture media and antibiotics used during the study were of cell culture grade, obtained from Hi Media Laboratories (Mumbai, India) unless otherwise indicated. Trizol was from Invitrogen (USA). Disposable 35 mm x10 mm cell culture Petri dishes, 6 well tissue culture plates, and centrifuge tubes were procured from Tarsons Products Pvt. Ltd. (Kolkata, India). Membrane filters were from Advanced Micro-devices (Ambala, India). The primers were got synthesized from GenxBio (India). The culture media were reconstituted freshly as per manufacturers’ instructions and filter-sterilized (0.22µm) prior to use.

2.2 Transportation and collection of sample

Sheep amnion was obtained from a nearby abattoir, washed 2-3 times with isotonic saline fortified with 1% of penicillin/streptomycin and transported to the laboratory in a thermally insulated ice box within 5 hour. Uterine cut, fetus and membranes were located and AF was aspirated aseptically with the help of 20 ml syringe fitted with 16 gauge hypodermic needle. Twenty milliliters of AF was collected in Oakridge tubes. The appearance (fluid without cells, blood-cell-free, blood-cell, bloody or brown-coloured fluid), of fluid volume which was collected were observed and carefully documented.

2.3 Isolation and culture of putative AFS cells

The putative AFS cells were separated by centrifugation. Amniotic fluid was centrifuged at 3000g for 10 min and washed twice with phosphate buffered saline (PBS) and this procedure was repeated two to three times. During centrifugation a layer was formed at bottom of the tube and a buffycoat formed in which blood cells were seen.

2.4 Culturing of putative AFS cells

The cells were ready to culture. The cells were seeded at density of 103 cells / cm2 in a 6 well culture plates containing cell culture medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1% non-essential amino acids, 1% Penicillin / Streptomycin / Ampicillin, 1% Vitamin solution. Cultured plates were incubated in humidified CO2 Incubator (Lark, China) at 38.5 ± 0.5ºC in presence of 5% CO2 in humidified air (Dev *et al*.,

2012). The cells were allowed to grow and were subcultured by passaging after achieving >80% confluency. All chemicals were added from the side wall of the plate so that no bubbles are formed. No “feeder layer” was used for culturing of putative AFS cells.

2.5 Expression of Oct-4, Nanog and Sox 2 by Reverse Transcriptase PCR

2.5.1 mRNA Formation

The method proposed by Hummon *et al*; 2007 with minor modifications was followed for extraction of total cellular RNA. RNA was extracted from approximately 0.6×107 cells using the Trizol agent. Procedure adopted was as follows: 300 µl sample of putative amniotic fluid stem cells was taken and 700 µl of Trizol reagent was added in an eppendorf tube and mixed properly. 200 µl chloroform was added, gently mixed for 5 minutes. Centrifugation was carried out at 12000g for 15 min. Two separate layers i.e. aqueous and organic were formed. Aqueous layer was carefully taken and transferred to a fresh eppendorf tube. To it added 198 µl chloroform and 8 µl isoamyl alcohol (24:1), mixed and centrifuged at 12000g for 15 min. The supernatant obtained was collected. To it 500 µl isopropyl alcohol was added then again centrifuged at 12000g for 5 min. Upper layer was carefully removed and 700 µl absolute alcohol was added and centrifuged at 12000g for 10 min.

Supernatant was discarded and 70 µl RNAase free DEPC–treated water was added for removing RNAases. RNA was prepared then run on 0.8% agarose gel using agarose gel electrophoresis.

2.5.2 cDNA Formation

The cDNA was synthesized by reverse transcription of mRNA purified from the putative AFS cells. The reaction mixture comprised of total cellular 5 ng RNA, 0.2 µg / µl random hexamer, 7 µg / µl cDNA direct RT, 10 µM / µl AMV reverse transcriptase and 40 U / µl RNase inhibitor in a total volume of 20 µl (one step RT–PCR kit, Novagen). The primer sequences used for Oct-4, Nanog and Sox-2 is given below:-

2.6 Cryopreservation and thawing of putative AFS cells

The putative AFS cells were cryopreserved using slow freezing method. The cells were collected and washed twice with PBS. Total two cryovials were used for freezing. Cryopreservation medium was prepared which contained 10% DMSO and 50% FBS and was named CP-M . CP-M was added drop wise in both the cryovials containing cells. The cryovials were put in minicooler and incubated at -8ºC for 12 hrs. After 12 hours the mincoolers were shifted to -20ºC and kept for 5 hour. Afterword, the vials were transferred in LN2 (-196ºC). The cryovials were kept in LN2 for 38 hrs. For thawing, the vials were taken out from LN2 container and put in water bath (37ºC) for 30 sec to 1 min. The cells were transferred to 6 well plates having fresh medium containing DMEM+10% FBS. The cells were washed 3-4 times in fresh medium.

2.7 Viability assay of putative AFS cells

For measuring the viability of the cells trypan blue assay was performed. The preparation of 1:1 dilution of the suspension using a 0.4% trypan blue solution was followed. Loaded the counting chambers of a hematocytometer (ROHEM, India) with the dilution and observed the cells under compound microscope.

3. Results

3.1 Culturing of putative AFS cells

After collection, all the cells were spherical and variable in sizes. No anchorage was observed before 48–72 h (Fig.1) of culturing the cells. After day 6, morphologically different cells viz., star shaped (62.7%), spherical with–out nucleus (1.9%), spherical with nucleus (26.4%), pentagonal (0.4%), and free floating and rounded cells (8.3%) were observed (Fig. 2). At day 7 of culturing, most of the cells converted into star shaped cells which subsequently started anchoring to surface (Fig. 3). The anchorage–dependent cells subsequently gained typical fibroblast like shape and formed a confluent monolayer (Fig.4) A few spherical and freely floating cells were also visible. Instead of forming uniform cell monolayer, certain cell clumps were also observed. Initially, the cells reached 70–80% confluence after two weeks. However, the passaged cells exhibited higher growth rate, reaching a 90–100% confluence after day 6 of culturing.

3.2 Cryopreservation and viability of putative AFS cells

After cryopreservation and thawing, the viability assay was performed to check the percentage of viable and dead cells. It was observed that 70% of the cells were viable after tryopan blue staining (Fig. 5).

3.3 Characterization of putative AFS cells by Oct-4, Nanog and Sox-2

Agarose gel electrophoresis for analysis of RT-PCR product revealed amplicons of 290, 501 and 362 bp of Oct-4, Nanog and Sox-2 (Fig. 6).

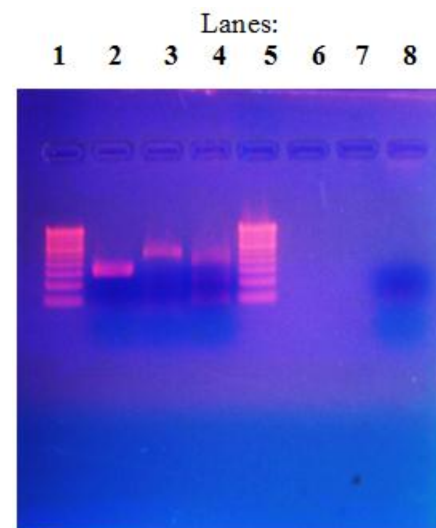

Fig.6 Lane1: 100bp ladder; Lane 2: Oc4-4 (290 bp); Lane 3: Nanog (501 bp); Lane 4: Sox-2 (362 bp)

4. Discussion

Mammalian AF contains diverse cell types representative of three germ layers (Gosden, 1989; Fauza, 2004). Amniotic membrane and AF–derived cells have therefore, attracted a deal of attention globally as an alternative cell sources for transplantation and tissue engineering, and as a possible reserve of pluripotent stem cells that may be useful for clinical application in regenerative medicine (Delo *et al*, 2006; Parolini *et al*., 2009; Dobreva *et al*., 2010) and reproductive biotechnological applications (Zhao and Zheng, 2010). However, the potential of AF stem cells in livestock assisted reproduction and health applications are yet to be exploited. The establishment of pluripotent stem cell lines in domestic species could have great impact in the agricultural as well as in the bio–medical field (Yadav *et al* ., 2011). Accordingly, the study of the AF stem cells in live–stock species has become a new focus recently (Zhang and Chen, 2008). Efforts are being made to study various types of stem cells (Verma *et al*., 2007; Dev *et al*., 2012; Sritanaudomchai *et al*., 2007) in domestic animals. The

present study is a preliminary effort to investigate whether the AFS cells in goat AF can be cultured and exhibit stem cell–like attributes. It has been observed that goat AF cells were able to grow without feeder cells. The choice of the culture medium and conditions chosen to grow goat AF cells are based on reports already established for human AF stem cells (De Coppi *et al*., 2007). However, the final selection was based on our preliminary observations on the growth of the cells in various combinations of culture media and supplements in buffalo species (Dev *et al*., 2012). After 3 to 5 days of incubation, the sheep AF cells had five different types of morphologically different cells (Fig. 1). The polygonal or star shaped cells were cultured for prolonged periods (at >10th passage). It was found that these cells transformed into fibroblast–like cells (Fig. 3). The cells had similar morphology to the AFS cells of buffalo (Dev *et al*., 2012) and as also reported by Mihu *et al*., 2009 where the authors observed the AF stem cells to show morphological features similar to fibroblasts. The sheep AF cells were found to have enlarged nuclei compared to adult skin fibroblasts, cumulus cells and granulose cells (data not shown). The AF cells, which were initially round in structure, started anchorage–dependent growth after day 3 to 5 of culture in vitro. After cryopreservation, the cells had around 70 % viability rate. Almost same viability rate has been observed in a study in our own lab by Sumita *et al* (Verma *et al*, 2012) which indicates that AFS cells of sheep can be cryopreserved successfully and can be used later on.

Conclusion

In summary, the present study is a preliminary attempt on isolation, culturing and cryopreservation of putative amniotic fluid stem cells in sheep. These putative AFS cells can be used in various purposes in future for various investigations. However, use of AFS cells in sheep therapeutic and assisted reproductive biotechnology needs further studies.

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