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The Culture of Embryonic Stem Cells Derived from *in vivo* Fertilized Embryos of Arbas Cashmere Goats

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Abstract: Research on the embryonic stem cells of large livestock has recently attracted the attention of scholars. However, it is difficult to keep goat embryonic stem cells in an undifferentiated state during cell passaging in culture. In this study, fertilized embryos of Arbas cashmere goats were obtained by superovulation *in vivo*. The key issues in the culture of Arbas cashmere Goat Embryonic Stem Cells (AgESCs) were explored: the addition of differentiation-inhibiting factors, the selection of medium as well as the passaging, cryopreservation and thawing methods used. This study found that high-quality *in vivo* fertilized embryos could be cultured in either serum-containing medium or serum-free medium and that AgESCs could be passaged in either medium for 30 generations. The mechanical method was superior to the trypsin digestion method for passaging AgESCs. There was no change before and after cryopreservation and thawing with regard to AgESC morphology, alkaline phosphatase staining, the formation of embryoid bodies, immunofluorescence staining or the PCR detection of pluripotency factors. Finally this study provides the experimental basis for the establishment of goat embryonic stem cell lines.

Key words: Arbas Cashmere goat embryonic stem cells, passage, freezing, N2, B27

INTRODUCTION

Embryonic stem cell lines established in mouse initiated a new field for stem cell study (Evans and Kaufman, 1981; Martin, 1981) which initiated the new field of embryonic stem cell research. In 1998, Thomson *et al.* (1998) derivated human embryonic stem cell lines. Since, then the research team of Sham Blott derivated human embryonic germ cell lines (Shamblott *et al.*, 1998). Yangm *et al.* (2012) successfully derivated mouse parthenogenetic embryos stem cell lines. The successful derivation of human and mouse embryonic stem cell lines opened a new chapter in biological research. The successful establishment of embryonic stem cell lines of large livestock would promote livestock-related research, the application of biotechnology and accelerate the pace of livestock breeding technology. Tian *et al.* (2006) established goat embryonic stem cell lines and passaged them beyond 8 generations by adding a factor from cultured mouse embryonic stem cells to the culture medium. Pawar *et al.* (2009) derivated goat embryonic stem cell lines via *in vitro* fertilization which were then passaged more than 6 generations. Behboodi *et al.* (2011) cultured goat embryonic stem cell lines that could be passaged for many generations using goat fibroblasts as the feeder layer. Due to limited resources and high economic value, the embryonic stem cell lines of livestock such as goats have not yet been established. Although, Arbas cashmere goats, known as the fiber gem, play an important role in economic development, Arbas cashmere Goat Embryonic Stem Cell (AgESC) lines have not yet been derivated. Successful derivation of AgESC lines could provide excellent seed cells to engage in modern biotechnological breeding of the cashmere goat.

During the cultivation of embryonic stem cells, fetal fibroblasts are usually selected as the feeder layer for cultured Embryonic Stem (ES) cells. MEF can secrete factors which inhibit ES cell differentiate and promote ES cells proliferate. Therefore, it can effectively promote the ES cells proliferate while maintaining their undifferentiated state and pluripotency (Evans and Kaufman, 1981). LIF is expressed in the trophectoderm but not in the inner cell mass. LIF Receptor (LIFR) and gp130 are expressed in the inner cell mass. This expression pattern suggests that the inner cell mass is mediated by paracrine LIF from trophoblast cells. Undifferentiated human embryonic stem cells cannot be cultured in serum-free medium when only LIF and Bone Morphogenic Proteins (BMPs) are added.

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The inhibition of BMP signals improves the process of culturing human embryonic stem cells. Basic Fibroblast Growth Factor (bFGF) can prevent the nuclear translocation of phosphorylated Smad I or inhibit the nuclear activity of Smad I thereby inhibiting downstream BMP signaling events and allowing human embryonic stem cells both to proliferate in the absence of fibroblasts and to maintain an undifferentiated state (Mathieu *et al*., 2012). In recent years, BMP growth factors have been identified as the active ingredient of serum. In N2 and B27 culture media, some inhibitors block the FGF signaling pathway and can eliminate the dependence of embryonic stem cells on BMP4 but LIF retains sensitivity to embryonic stem cells (Kunath *et al*., 2007; Wilder *et al*., 1997). When culturing embryonic stem cells in a serum-free, chemically defined medium (such as N2 or B27), only relevant factors are needed to co-maintain the self-renewal and pluripotency of ESCs without the need of serum. It has been reported that using this method, the cultured embryonic stem cells can produce chimeras therefore meeting the pluripotency standard (Ying *et al*., 2003). In this study, high-quality fertilized embryos were obtained via superovulation from Arbas cashmere goats. The fertilized embryos were then directly cultured and cell adhesion was simulated to obtain an inner cell mass. A comparative analysis was conducted to compare the impact of various culture media and passaging methods, cryopreservation and thawing on the culture of AgESCs to explore the culture process of AgESCs and provide an experimental basis to eventually establish an AgESC line.

MATERIALS AND METHODS

Preparation of the feeder layer: Kunming white strain mice (SCXK (Mongolia) 2002-0001) were provided by the National Laboratory of Animal Breeding, Inner Mongolia University. The animals were 12.5-13.5 days pregnant and were sacrificed (Roach *et al*., 2006) to obtain fetal mice. The fetal mice were rinsed twice in a Ca^{2+} free, Mg^{2+} free PBS (Gibco, 14190) solution. After removing the heads, limbs and internal organs, the fetal bodies were cut into pieces with sterile ophthalmic surgical scissors. Next, 0.05% trypsin-EDTA (Gibco, 25300) was added, followed by digestion in an incubator (37°C, 5% CO_2, saturated humidity) for 4-6 min. Fetal mouse fibroblast cell culture medium (high glucose DMEM (Gibco), 20% FBS (Tbd), 100 IU mL^{-1} penicillin, 0.05 mg mL^{-1} streptomycin) was added to stop digestion. A single cell suspension was seeded onto a cell culture dish (Corning, USA) and then cultured in an incubator (37°C, 5% CO_2, saturated humidity). After 24 h, the medium was replaced to remove the 0.05% trypsin-EDTA. In the later stage of the experiment, the medium was replaced every other day. The cells were passaged when they reached 90% confluency. Confluent fetal mouse fibroblast cells were treated with 10 μg mL^{-1} Mitomycin-C (MMC) (Zhejiang Hisun Pharmaceutical Co., Ltd. China, H33020786) for 2-5 h. Next, the cells were flushed three times with Ca^{2+} free, Mg^{2+} free PBS, followed by another 0.05% trypsin-EDTA digestion for 0.5-1 min.

The fetal mouse fibroblast cell culture medium was used to stop the digestion. The cells were suspended and centrifuged at 1500 rpm for 5 min. After centrifugation, the cell density was adjusted to 2×10^4-2.5×10^4/cm^2. The cells were then seeded onto 4 well plates (Corning) coated with 0.1% gelatin (Sigma, G9382) and cultured in an incubator (37°C, 5% CO_2, saturated humidity). The feeder layer was usually prepared the day before embryo transplantation (Roach *et al*., 2006; Munoz *et al*., 2008; Bryja *et al*., 2006; Li *et al*., 2004).

Obtaining *in vivo* fertilized embryos: The estrus cycle for Arbas cashmere ewes is 20 days. Arbas cashmere ewes with excellent physiques were used for these experiments. Two methods were adopted for estrus: synchronized estrus and natural estrus. The synchronized estrus was performed by placing a progesterone vaginal suppository deep into the vagina of a ewe on any day in the estrous cycle; 10-12 days later, the suppository was removed. After removing the suppository, each ewe was injected with 200-500 IU Pregnant Mare's Serum Gonadotropin (PMSG) (Ningbo Hormone Products Factory). During the following 2-3 days, all of the treated ewes were in estrus and were mated with high-quality Arbas goats. For natural estrus, a high-quality Arbas goat was used to test ewes twice daily and the estrus time of ewes was recorded (the day of estrus was day 0). On the 15-17th days of the estrus cycle of the donor ewes an intramuscular injection of 30 IU mL^{-1} Follicle Stimulating Hormone (FSH) was administered to the donor ewes twice daily November 23, 2013 (Ningbo Hormone Products Factory). An intravenous injection of 60 IU mL^{-1} Luteinizing Hormone (LH) was administered to the ewes when they re-entered estrus (Ningbo Hormone Products Factory). The ewes were then mated with high-quality Arbas goats. Between 48-80 h after mating, 2~8 cell embryos were collected. Egg flushing agent (Ca^{2+} free, Mg^{2+} free PBS, 3 mg mL^{-1} BSA [Sigma]) was injected into the fallopian tubes at the junction of the fallopian tubes and the uterus and a hand-made glass tube was used to collect the egg-flushing agent from the tubal fimbria. The collected embryos were cultured in SOFaa (Tervit *et al*., 1972) with essential amino acids (Sigma) and non-essential amino acids (Sigma) to obtain blastocysts 3-4 days later.

Table 1: AgESC culture medium

Medium type	Medium components
Medium I	DMEM F12 with 10% FBS, 0.1 mM β-Mercaptoethanol, 0.1 mM non-essential amino acids, 0.1 mM L-Glutamine, 100 IU mL^{-1} Penicillin, 0.05 mg mL^{-1} Streptomycin, 20 ng mL^{-1} LIF, 50 ng mL^{-1}b FGF
Medium II	DMEM F12 with BSA, N2, B27, 0.1 mM β-Mercaptoethanol, 0.1 mM non-essential amino acids, 0.1 mM L-Glutamine, 100 IU mL^{-1} Penicillin, 0.05 mg mL^{-1} Streptomycin, 20 ng mL^{-1} LIF, 50 ng mL^{-1} bFGF

DMEM F12 (Gibco), FBS (Gibco), non-essential amino acids (Gibco), Penicillin and Streptomycin (Gibco), β-Mercaptoethanol (Millipore), N2 (Gibco), B27 (Gibco), LIF (Millipore), bFGF (Promega), L-Glutamine (Gibco), BSA (Sigma)

Obtaining and culturing the inner cell mass: The embryonic stem cell culture medium was replaced with the prepared feeder layer at 1-2 h prior to embryo transplantation (Table 1). After the medium was in equilibrium, the selected hatching blastocysts were seeded onto a 4 well plate with one embryo for each well. The plate was then placed into an incubator (37°C, 5% CO_2, saturated humidity). The embryos were evaluated 3 days later for attachment. The growth of the embryos was then observed every day.

Passage of the AgESCs: Mechanical passaging and the trypsin-digestion method were used to passage the AgESCs. For the mechanical passaging method, the Arbas cashmere goat embryos were transplanted onto the feeder layer 5-6 days after hatching and the inner cell mass grew significantly. Under the microscope, the inner cell mass was mechanically separated and the AgESC-mass was cut into small pieces with a pipette. After repeated pipetting, the small pieces became small clumps and were transplanted onto a new feeder layer that had been prepared on the previous day. The 2 days later, the AgESCs were adhesive and the medium was then replaced every day. After the AgESCs were passaged, the embryonic stem cells had grown significantly. At this point, a 200 μL pipette could be used to draw a cross-shape at the bottom of the petri dish. The embryos were then detached and repeatedly pipetted to disperse the clone clumps mechanically which were then passaged onto a new feeder layer for continued culture.

For the Trypsin-Digestion Method, after the AgESCs had grown significantly, the medium was removed and embryos were rinsed once with Ca^{2+} free, Mg^{2+} free PBS. After 0.25% trypsin-EDTA was added, the plate was placed into an incubator (37°C, 5% CO_2, saturated humidity) to digest for 3-5 min. The plate was taken out after digestion and medium was added to stop the digestion. The suspended AgESCs were placed into a 15 mL centrifuge tube and centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was discarded and the cells were re-suspended. The cell suspension was passaged onto a newly prepared fetal mouse fibroblast feeder layer for continued culture.

The cryopreservation and thawing of AgESCs: To passage the AgESCs, the medium was first discarded and the cells were rinsed with Ca^{2+} free, Mg^{2+} free PBS. Fresh embryonic stem cell culture medium was added and a pipette was used to gently detach the cells from the bottom of the petri dish and thoroughly disperse the cells. The cell suspension was suctioned and placed into 15 mL centrifuge tubes to centrifuge at 1500 rpm for 5 min. Then, the supernatant was discarded and 1 mL of cryoprotectant solution was added (90% FBS, 10 DMSO) to adjust the cell density to 1×10^6 mL^{-1}. Each cryovial held 1 mL cryoprotectant solution with a cell suspension and was placed into an isopropanol cell freezing box and stored at -80°C for over 17 h, followed by long-term storage in liquid nitrogen. While thawing, the cryovial was taken out of the liquid nitrogen tank, placed into a 37°C water bath for 1-2 min (or placed in an incubator at 37°C, 5% CO_2, saturated humidity). After the cells were completely thawed they were centrifuged and the cryoprotectant solution was discarded. The cell pellet was resuspended with fresh embryonic stem cell culture medium and the cells were seeded onto a prepared feeder layer for further culture.

Alkaline phosphatase staining: AgESCs were rinsed with Ca^{2+}free, Mg^{2+}free PBS three times and fixed with 4% paraformaldehyde (Sigma) at 4°C overnight. The fixed cells were then rinsed twice with Ca^{2+} free, Mg^{2+} free PBS. Alkaline phosphatase staining solution (Westang, c1601) was added and the cells were incubated at room temperature for 30 min. Next, the cells were observed under the microscope.

Embryoid body formation assay: The mechanical method was used to separate the AgESCs from the fetal mouse fibroblast feeder layer. The AgESCs were then rinsed twice with Ca^{2+} free, Mg^{2+} free PBS. Next, 0.25% trypsin-EDTA was added the AgESCs to digest for 3-4 min and a medium of DMEM F12 (Gibco) with 20% FBS (Tbd), 100 IU mL^{-1} penicillin and 0.05 mg mL^{-1} streptomycin was added to stop the digestion. The single cell suspension was placed into 15 mL centrifuge tubes for centrifugation at 1500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in a medium of DMEM F12 with 20% FBS, 100 IU mL^{-1} penicillin and 0.05 mg mL^{-1} streptomycin. The cell suspension was made into suspension drops on the cover of a 90 mm petri dish. Ca^{2+} free, Mg^{2+} free PBS was added

to the petri dish. After 7-10 days of culture in suspension, spherical bodies became visible (Yao *et al*., 2006).

Immunofluorescence staining: The AgESCs that attached to the glass slides were rinsed with Ca^{2+} free and Mg^{2+} free PBS three times and fixed in 4% paraformaldehyde at 4°C overnight. Then, 0.1% Triton-X100/PBS was added to the cells and they were allowed to incubate at room temperature for 10 min. Next, the cells were rinsed with 0.4% BSA/PBS for 5-10 min twice. The cells were incubated with goat serum (1:10 dilution) for 30 min at room temperature to block non-specific antibodies. After the blocking solution was discarded, the diluted primary antibody (Chemicon ES Cell Marker Sample kit, diluted at 1:50 with PBS and 4% BSA; Nanog, Abcam, diluted at 1:150 with PBS and 4% BSA; and Sox-2 and Therm, diluted at 1:150 with PBS and 4% BSA) was added and incubated at 4°C overnight. The following day, the cells were rinsed with PBS with 0.4% BSA three times; each rinse was 5-10 min. Biotin-labeled goat-anti-rat IgM, goat-anti-rabbit IgG or goat-anti-mouse IgG (Abcam, diluted at 1:300 in PBS) that corresponded to each of the above antibodies were added and the cells were incubated at room temperature for 1 h. The cells were then rinsed with 0.4% BSA in PBS three times and DAPI staining agent was added during the last rinse. The cells were sealed onto slides and observed under the microscope.

PCR detection and determination: The *in vivo* Arbas Cashmere goat embryos and AgESC specimens were placed in enzyme-free tubes. Total Ribonucleic Acid (RNA) was extracted according to the instructions of the RNeasy plus Micro kit (QIAGEN) and was reverse transcribed into cDNA using the Sensiscript RT kit (QIAGEN). Polymerase Chain Reaction (PCR) primers were designed according to the bovine octamer-binding transcription factor 4 (OCT-4) and homeobox protein (NANOG) cDNA sequence reported in the NCBI database. OCT-4 forward primer: 5'-CGCCCTATG ACTTGTGTGG-3', reverse primer: 5'-AAGGGTCTC TGCCTTGCATA-3' (synthesized by Invitrogen). The size of the target fragment was 505 base pairs (bp). NANOG forward primer: 5'-CACCCATGCCTGAAGAAAGT-3', reverse primer: 5'-GGCAGGTTTCCAGGAGAGTT-3' (synthesized by Invitrogen). The size of the target fragment was 516 bp. The PCR reaction contained 1 μL reverse transcription product and 1 μL (2 μmoL L^{-1}) of each primer. The following reaction conditions were used: 94°C denaturing for 3 min; followed by 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, for a total of 35 cycles and a final extension at 72°C for 10 min. Agarose gel (1%) electrophoresis was used to detect the amplified products.

RESULTS AND DISCUSSION

Comparison of the two methods to obtain *in vivo* fertilized embryos: Two methods were adopted to obtain *in vivo* embryos during the experiment. One method was to synchronize the estrus of Arbas Cashmere ewes and then obtain embryos through superovulation. The second method was to induce superovulation in ewes in natural estrus to obtain embryos. The results showed a difference between the two methods in the number of embryos obtained. For synchronized estrus, the number of ovulations in each ovary was 6.1±3.1 after superovulation (Fig. 1A). However, for natural estrus, the number of ovulations in each ovary was 10.6±3.8 after superovulation (Fig. 1B). The difference between these two methods in the number of ovulations in each ovary of Arbas cashmere ewe was highly significant ($p<0.01$) (Table 2). When the *in vivo* embryos were compared with the *in vitro* fertilized embryos (obtained with either of the two methods), the *in vivo* embryos were denser and more high-quality (Fig. 1C). The quality of the all of the embryos met the requirements of the experiment.

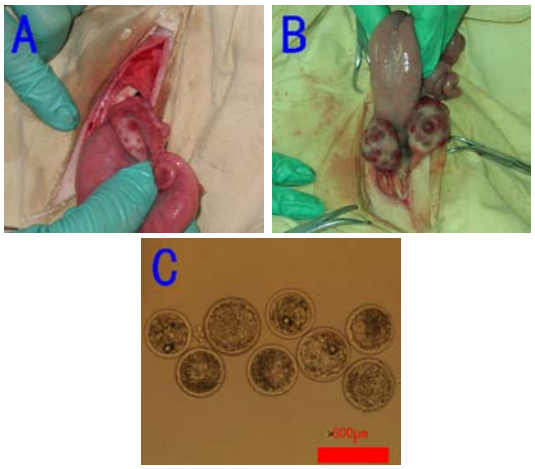

Fig. 1: Images of the ovaries of ewes after estrus synchronization and superovulation. The number of ovulations in each ovary of the ewes was significantly different between the two methods; A) the ovaries of ewes after estrus synchronization and superovulation there were few ovulations; B) the ovaries of ewes after natural estrus and superovulation there were more ovulations than in a and C) the *in vivo* embryos

Table 2: The effect of the different methods to obtain the ovulation of Arbas cashmere ewes

Estrus methods	No. of ewes	No. of ovulations per ovary
Natural Estrus	78	10.6±3.8^{A}
Synchronized Estrus	67	6.1±3.1^{B}

$^{A, B}$Indicate a highly significant difference ($p<0.01$)

Therefore, the method to obtain embryos through superovulation in ewes in natural estrus was superior to that of synchronized estrus and there was no difference in the quality of the obtained embryos between the two methods when the type of fertilization was held constant.

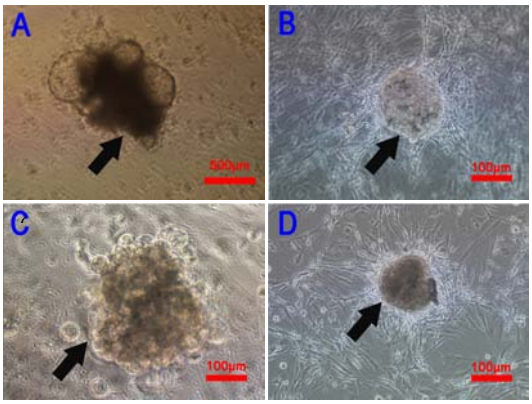

Fig. 2: Morphology of AgESCs passaged by microdissection and trypsinization. AgESCs were cultured in medium I and II. Both the mechanical and trypsin digestion methods were used for passaging. The AgESC clones that formed after mechanical passaging had clear edges and normal morphology. A and B are the morphologies of AgESCs with mechanical passaging and cultured in medium I and II, respectively. B and D are the morphologies of AgESCs with the trypsin digestion method of passaging and cultured in medium I and II, respectively

Comparison of the two methods of passaging: This experiment used fetal mouse fibroblast as the feeder layer and evaluated the mechanical separation and trypsin digestion methods to passage AgESCs. When the mechanical separation method was used to passage AgESCs, the cells had normal cell growth, strong growth activity, faster increase of cell clones and vacuoles in the process of passage (Fig. 2A and C). When the trypsin digestion method was used to passage AgESCs, the edges of the clones were not clear, the growth rate of clones was slower, fibrous cells appeared and the number of clones was relatively small (Fig. 2B and D). Therefore, mechanical separation was superior to the trypsin digestion method in passaging embryonic stem cells.

The impact of cryopreservation and thawing on AgESCs: During the experiment, the cultured AgESCs were cryopreserved, thawed and recovered. All of the cells survived after thawing and could be passaged normally. The results of alkaline phosphatase staining (Fig. 3) and immunofluorescence staining (Fig. 4 and 5) of the AgESCs before and after thawing were all positive.

Comparative analysis of two types of media: The impact on obtaining the inner cell mass of the embryos. In this experiment, 103 *in vivo* Arbas cashmere goat embryos

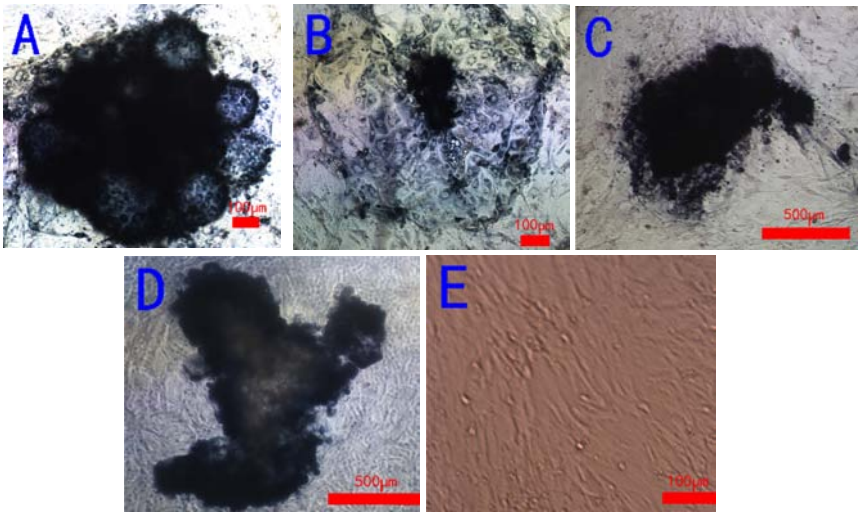

Fig. 3: Alkaline phosphatase staining of AgESCs before and after thawing. AgESCs cultured in medium I and II showed positive alkaline phosphatase staining before and after cryopreservation and thawing; A) and B) are the morphologies of primary AgESCs cultured in medium I and II, respectively; C) and D) are the morphologies of the 10th generation of AgESCs cultured in medium I and II, respectively and E) is the alkaline phosphatase staining of fetal mouse fibroblasts (negative control)

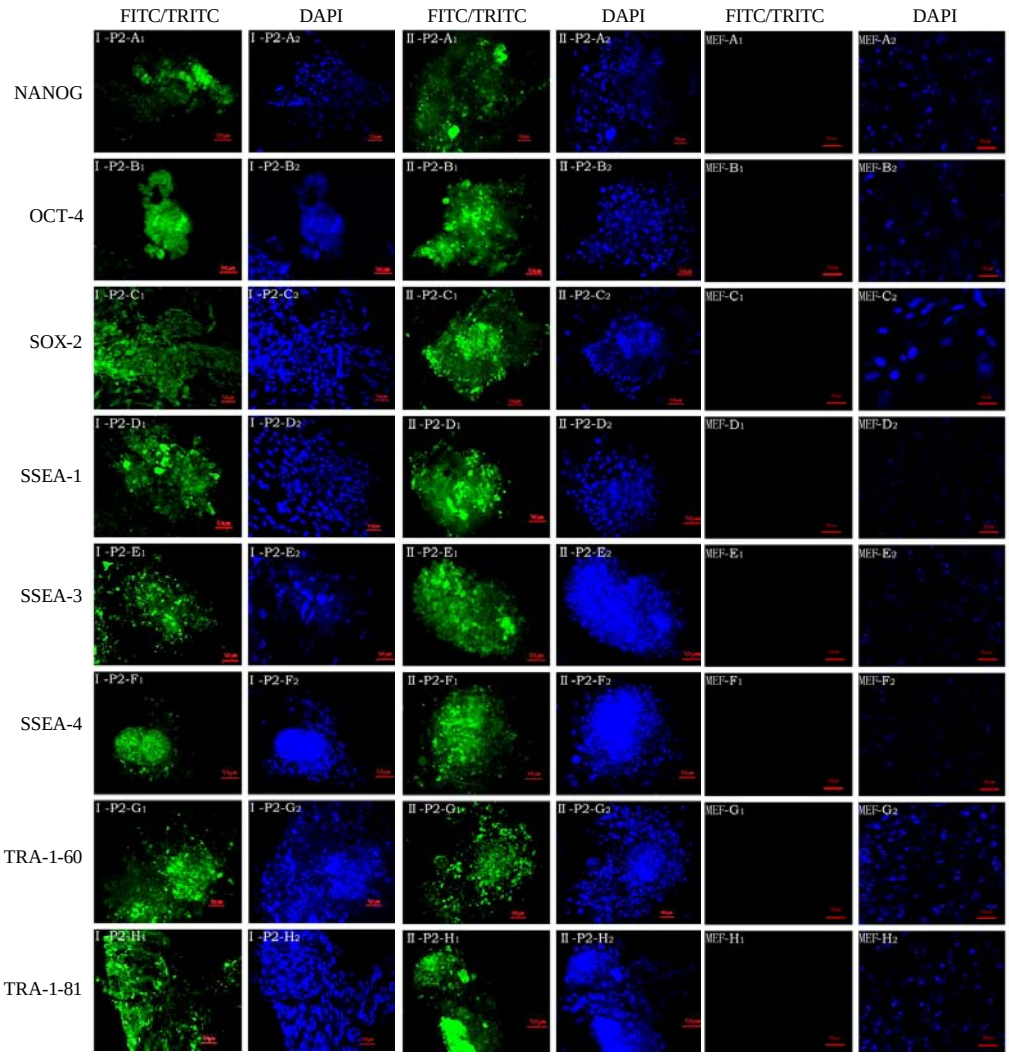

Fig. 4: Immunofluorescence staining of the 2nd generation of AgESCs. The 2nd generation of AgESCs cultured in medium I and II. AgESCs were stained positive using the following antibodies: NANOG, OCT-4, SOX-2, SSEA-1, SSEA-3, SSE2A-4, TRA-1-60 and TRA-1-81. 1: Positive staining; 2: Control

were obtained and 59 and 44 hatching blastocysts were selected to transplant into culture systems using medium I and II, respectively. The results are shown in Table 3. The growth time of the formation of the primary inner cell mass from hatching blastocyst was 2-3 days. The attachment times were 5-6 and 4-5 days for hatching the blastocysts cultured in medium I and II, respectively. The attachment time was shorter in medium II.

Morphological identification of embryonic stem cells: As shown in Fig. 5, the growth of the inner cell mass of primary AgESCs cultured in medium II (Fig. 6E) was significantly greater compared with that of the AgESCs cultured in medium I. Trophoblast cells and cells of the inner cell mass cultured in medium II grew faster than those cultured in medium I after 3 days, among cells that attached to the wall at the same time, the clone diameter of the cultured cells was approximately 100 μm (Fig. 6A) in medium I and approximately 500 μm in medium II (Fig. 6E). The inner cell mass of the primary AgESCs cultured in medium 2 grew faster with tighter intercellular connections and a clear boundary from the trophoblast, relative to the AgESCs cultured in medium I. As shown in Fig. 6, the morphology of the AgESCs that were derived from hatching blastocysts and then cultured in either of the two media was in line with the morphology of undifferentiated embryonic stem cells: the cells were large and compact; the intercellular connections were tight; it

Table 3: The effect of AgESC culture in two types of media

Mediums	Type of seeded embryos	Attachment time (days)	The growth time of the primary generation (days)	No. of embryos	Adhesion rate (%)
Medium I	Hatching blastocyst	5-6	2-3	59	100
Medium II	Hatching blastocyst	4-5	2-3	44	100

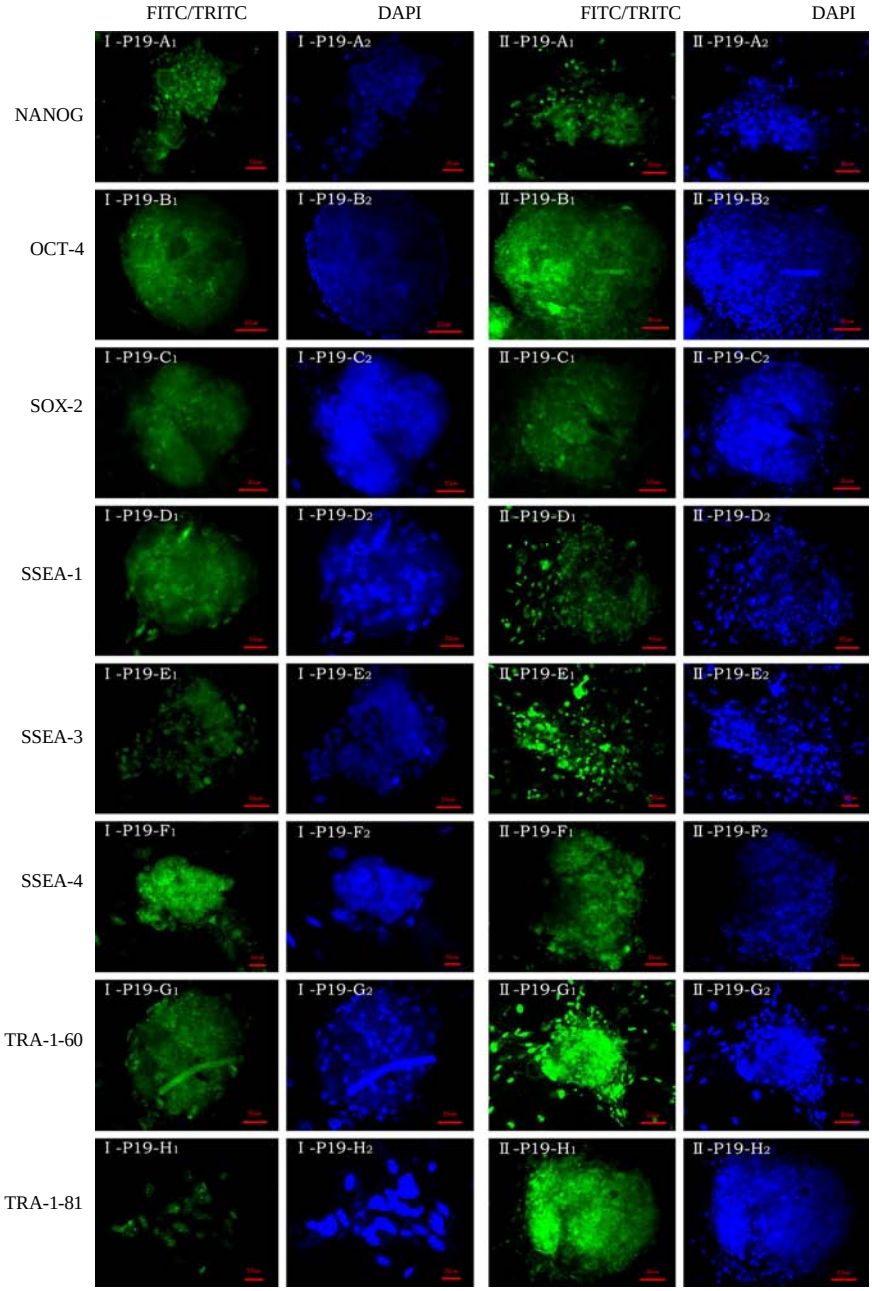

Fig. 5: Immunofluorescence staining of the 19th generation of AgESCs. The 19th generation (cryopreserved and thawed) of AgESCs cultured in medium I and medium II. AgESCs were stained positive using the following antibodies: NANOG, OCT-4, SOX-2, SSEA-1, SSEA-3, SSE2A-4, TRA-1-60 and TRA-1-81. 1: Positive staining; 2: Control

was difficult to distinguish the cell to cell connections between the cells and the surface of the stem cell clones was smooth with a clear boundary from the feeder layer (Roach *et al*., 2006). This study found that AgESCs

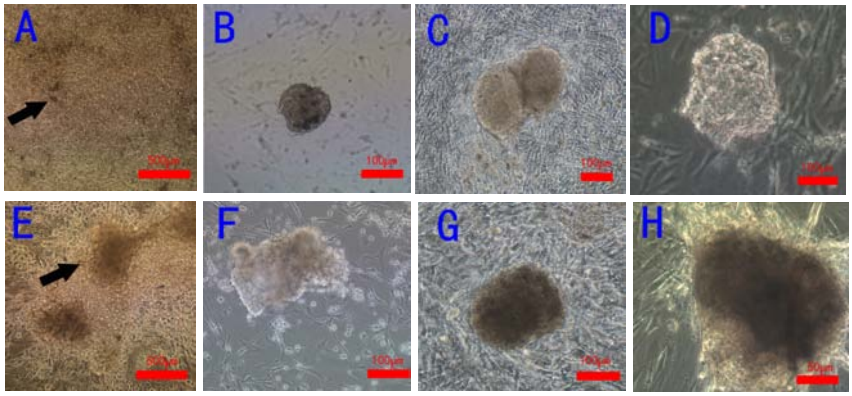

Fig. 6: Morphology of AgESCs cultures. AgESCs cultured in medium I and II; A) primary AgESCs cultured in medium I; E) primary AgESCs cultured in medium II; B, C and D) the 5th, 10th and 30th generation of AgESCs cultured in medium I; G, H and I) the 5th, 10th and 30th generation of AgESCs cultured in medium II

cultured in either medium could grow normally. For AgESCs cultured in medium I, trophoblast growth was significant and there were relatively more vacuoles in the process of passage and more dead cells in the medium. For AgESCs cultured in medium II, the stem cells grew faster, trophoblasts gradually disappeared during the process of passage and there were fewer dead cells in the medium.

***In vitro* differentiation of AgESCs:** *In vitro* differentiation experiments were conducted with AgESCs cultured in each of the two types of media. The results indicated that cells cultured in either type of media could form *in vitro* embryoid bodies (Fig. 7). In this regard, there was no significant difference between medium I and II.

PCR detection and determination of embryonic stem cells: The results of PCR detection and determination indicated that both *OCT-4* and *NANOG* genes were expressed in the 10th generation of AgESCs cultured in either medium (Fig. 8).

In this study, *in vivo* Arbas Cashmere goat embryos were obtained through two methods: Synchronized Estrus and Natural Estrus. A comparison between these two methods showed that there were fewer ovulations in ewes in synchronized estrus and that the difference was highly significant. An explanation for this phenomenon is that when a large amount of exogenous hormone drugs are used at the beginning of the estrus synchronization on ewes at different phases of the estrus cycle these drugs are antagonistic to the normal hormones in the bodies of the ewes therefore, fewer follicles formed than occurs during natural estrus.

The number and quality of the *in vivo* embryos obtained through superovulation of either natural or

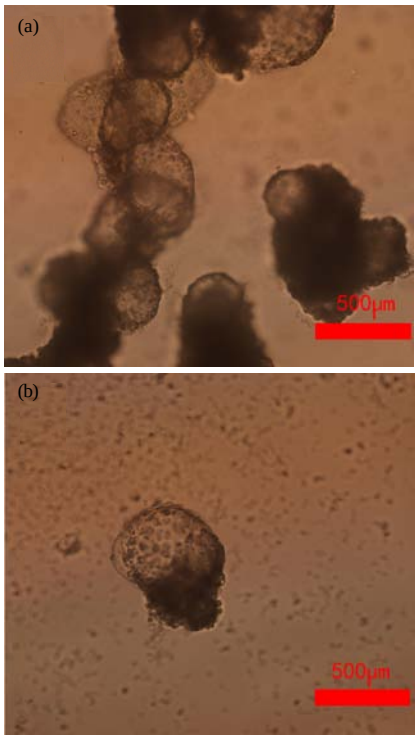

Fig. 7: *In vitro* differentiation of AgESCs. Embryoid bodies were formed with no factors and no feeder layer. The 8th generation of AgESCs cultured in either medium I; a) or medium II and b) could form embryoid bodies

synchronized estrus was superior to those of the *in vitro* embryos. In this study, the lowest number of *in vivo* embryos obtained through superovulation was 6.1±3.1 from each ovary, whereas for *in vitro* fertilization, approximately 3.2 oocytes were obtained from each ovary.

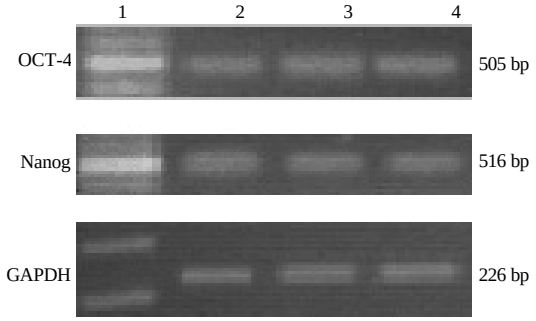

Fig. 8: RT-PCR analysis of gene expressions related to the nondifferentiation of AgESCs OCT-4 and Nanog-mRNA was used as markers of the undifferentiated state in Embryonic Stem (ES) cells. Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as an internal standard. Lane 1: 100 bp DNA ladder; Lane 2: *in vivo* embryos; Lane 3: AgESCs cultured in medium I (passage 10); Lane 4: AgESCs cultured in medium II (passage 10)

After *in vitro* maturation and development, a low number of blastocysts were formed (Pawar *et al*., 2009). When the quality of the *in vivo* and *in vitro* embryos was compared, the results showed that the connections between the *in vivo* embryonic trophoblast cells were tight and that the cells exhibited strong growth. When the inner cell mass formed, the attachment time was longer for *in vivo* embryos which may be because the connection between the *in vivo* embryonic cells was tight and cells were less susceptible and more immune to the impact of the external environment.

The time required for the *in vivo* embryos to reach 100% adhesion rate was significantly different from that of the *in vitro* fertilized embryos (Pawar *et al*., 2009). After the embryonic stem cell clones were formed, the intercellular connections between embryonic stem cells derived from *in vivo* embryos were tighter and had more integrity. This study found that high-quality *in vivo* embryos were conducive to the cultivation of goat embryonic stem cells.

This study used 2nd generation fetal mouse fibroblasts as the feeder layer to culture AgESCs. For the culture process of embryonic stem cells, the use of homologous and heterologous fibroblasts as a feeder layer has grown in popularity among scholars. Gjorret and Maddox-Hyttel (2005) succeeded in using heterologous fibroblasts to culture bovine embryonic stem cells. Previously, researchers also found that the growth effect of using fetal mouse fibroblasts as the feeder layer was superior to that of bovine fetal fibroblasts in culturing bovine embryonic stem cells (Jin *et al*., 2012). Familari and Selwood (2006) succeeded in using homologous fibroblasts as a feeder layer to culture mink, marsupial, human and pig embryonic stem cells but they failed to culture sheep, bovine and chicken embryonic stem cells. After evaluating several previous studies, heterologous fetal mouse fibroblasts were selected as the feeder layer for AgESC culture.

Compared with the homologous Arbas Cashmere fetal goat fibroblasts, the fetal mouse fibroblasts had the following characteristics: the morphology was homogeneous, the growth time was short, the vitality was better, the growth of cells was vigorous and the cells were able to secrete large amounts of embryonic stem cell growth factors such as LIF. Moreover, as a feeder layer, the cell metabolism was low, the purification time was short and they were easy to obtain.

At the beginning of the experimental design, homology analysis established that large animals and humans shared a high level of homology therefore the selection of medium was mostly based on human embryonic stem cell culture medium. The main difference between medium I and II was that in medium II, N2, B27 and BSA were used to replace the role of serum in medium I and II therefore became a medium with defined components. During the process of passage, no significant differences were found in the attachment time, adhesion rate or passage time of the embryos whereas the inner cell mass grew significantly faster in medium II than in medium I. During passage, the growth of trophoblasts was significantly slower than for embryonic stem cells cultured in medium I and there were more dead cells. The reason for this phenomenon may be that the serum components of medium I were complex and contained a variety of proteins and factors which inhibited the growth of stem cells.

To date, the AgESCs have been passaged for 30 generations using the mechanical method. Using the trypsin digestion method, the number of clones gradually decreased during the process of passage, the edges became unclear and many fibroblast-like cells started to form clusters, suggesting that trypsin caused the cells at the edge of the stem cell clones to begin to slowly differentiate which was not conducive to the passage and culture of embryonic stem cells.

Huang *et al*. (2010) found that the mechanical method was more conducive to the growth of embryonic stem cells (Gjorret and Maddox-Hyttel, 2005; Huang *et al*., 2010) which is in line with the conclusion of this study. In this experiment, AgESCs were cryopreserved and recovered. Alkaline phosphatase staining and immunohistochemical staining were used to identify the

embryonic stem cells before and after freezing. The growth of the embryonic stem cells before and after freezing was also compared. This study found that alkaline phosphatase staining of the embryonic stem cells before and after freezing was positive and moreover, the staining results of the following antibodies were also all positive: OCT-4, Stage-Specific Embryonic Antigen-1 (SSEA-1), SSEA-4, SSEA-3, Nanog, sry-related HMG box-2 (Sox-2), TRA-1-60 and TRA-1-81. These results suggest that cryopreservation and thawing would not result in the differentiation of the embryonic stem cells and that AgESCs cultured in both types of media retained the characteristics of stem cells.

CONCLUSION

Results showed that the serum-containing medium and the serum-free medium with clear ingredients can both be used to culture AgESCs and that the serum-free medium with clear ingredients is more conducive to cell growth. The mechanical method of passaging is more suitable for the passaging of AgESCs. The common cryopreservation and thawing method can effectively store AgESCs.

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