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Assessment of different stages of parthenogenetic embryos for production of embryonic stem cell like colonies

JUHI PATHAK, S D KHARCHE*, ANJANA GOEL, A K S SIKARWAR, SONIA SARASWAT, RAVI RANJAN, CHETNA GANGWAR, S P SINGH, A K GOEL and M S CHAUHAN

ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura, Uttar Pradesh 281 122 India

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Unlike other vertebrates, mammals are not capable of parthenogenetic reproduction. Mammalian parthenogenetic embryos undergo early developmental demise due to imbalanced expression of imprinted genes and exhibit cell proliferation defects and restricted contribution to mesodermal and endodermal tissues when combined with normal embryos as chimeras. Frequent homozygosis at the major histocompatibility locus, permitting efficient immune matching, has raised interest in parthenogenetic ESCs as a cell source for transplantation (Revazova *et al.* 2008). Establishment of ESC lines in domestic animals, especially from cow, buffalo, sheep, and goat, would be useful for production of pharmacological products for treatment of human and animal diseases (Zakrzewski *et al.* 2019) and also conservation system of endangered animals (Hou *et al.* 2018). Also, goat pluripotent stem cells might be a better material for developing new strategies to improve the yields of transgenic and cloned embryos or animals for developing nations. However, progress in derivation of ESC from domestic animals has been slow. To definitively determine the therapeutic potential of parthenognetic ESC derivatives, we investigated the production of ESC like cells from different stages of partheogenetic embryos.

Collection of ovaries, recovery of oocytes and *in vitro* maturation (IVM) was done as per the method of Kharche *et al.* (2016). Briefly, after 27 h of *in vitro* maturation, denuded oocytes were then chemically activated with 5 µM calcium ionophore for 5 to 7 min followed by 4 h culture in 2 mM DMAP. Embryos were then cultured in RVCL at 38.5°C in humidified atmosphere with 5% CO_2 in CO_2 incubator.

For the preparation of feeder layer, fibroblast cells were isolated from abdominal skin of goat foetus (40–60 days) and cultured. Embryonic fibroblast cells in log phase (80–90% confluent) were inactivated by adding 20 µg/ml Mitomycin-C for 4 h followed by washing with culturemedium.

For stem cell production, embryos produced by parthenogenetic activation were divided in to four groups based on their stages of development (Fig. 1).

Group 1 (*8–16 cell*): Zona of embryos (n=328) was dissolved with 0.25% pronase and were cultured on inactivated goat fetal fibroblast monolayer in stem cell culture media.

Group 2 (*Morula*): Zona of embryos (n=786) was dissolved with 0.25% pronase and were cultured on

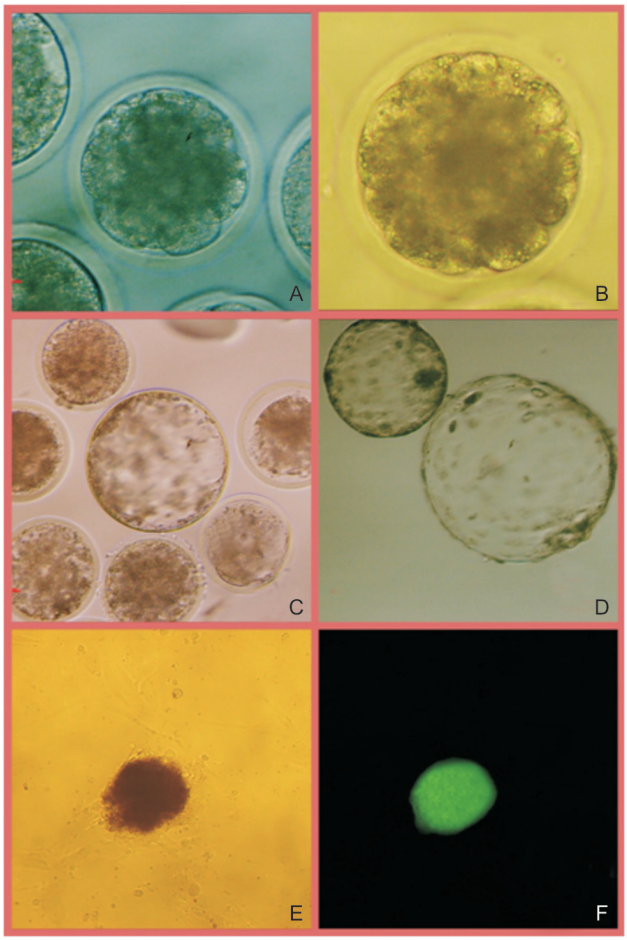

Fig. 1. Parthenogenetic stem cell production from different stages of embryos. **A**. 8–16 cell stage, **B**. Morula, **C**. Blastocyst, **D**. Hatched Blastocysts, **E**. pES cell colony and **F**. Immuno-fluorescence with OCT-4.

*Corresponding author e-mail: kharche62@gmail.com

inactivated goat fetal fibroblast monolayer in stem cell culture media.

Group 3 (*Blastocyst*): Zona of embryos (n=196) was dissolved with 0.25% pronase were cultured on inactivated goat fetal fibroblast monolayer in stem cell culture media.

Group 4 (*Hatched blastocyst*): ICM (Inner cell mass) from hatched blastocysts (n=122) produced from activation was isolated and were cultured on inactivated goat fetal fibroblast monolayer in stem cell culture media.

These were cultured on Mitomycin-C inactivated goat fetal fibroblasts feeder layer in stem cell culture medium [(DMEM supplemented with FBS (20%), 1% L-glutamine, 1% non-essential amino acids, 50 µg/ml gentamycin, LIF (40 ng/ml), SCF (20 ng/ml), IGF-1 (10 ng/ml) and β-FGF (4 ng/ml)]. Subsequent colonies were passaged mechanically using a microblade and media was replaced after every 24 h. Small clumps of colonies were reseeded on fresh mitomycin-C treated feeder layers and cultured for 4–5 days.

Undifferentiated ES like colonies were also seeded on fresh mitomycin-C treated feeder layers in culture medium without leukemia inhibitory factor (LIF) for random differentiation.

Characterization of goat ES-like cells colonies was performed with alkaline phosphatase staining kit (Sigma Chemical Co., USA). The expression of surface marker SSEA-3 and intracellular marker (OCT4) was examined by carrying out immunofluorescence staining with ES cells marker sample kit (Merck Life Science Pvt Ltd., India).

ES cells derived from embryos produced by parthenogenetic activation represent a potential solution to the problem of rejection, as any replacement cells would be genetically identical to the recipient's cells. Progress in derivation of ESC from domestic animals has been slow. In most farm animals, including cattle, pig, and small ruminants (Renard *et al.* 2007, Zakrzewski *et al.* 2019), the derivation of ES cell lines has been attempted from the ICM; however, the data are inconsistent.

In this study, a total of 7,325 chemically activated oocytes with Calcium Ionophore and DMAP were cultured *in vitro* for development of embryos and the cleavage rate recorded was 76.36%. The percentages of 2 cell, 4 cell, 8–16 cell, morula, blastocyst and hatched blastocyst were 32.59%, 24.29%, 21.18%, 15.77%, 3.629% and 2.538%, respectively.

A total of 887 ES like colonies were obtained. Out of these, 137 pESC like colonies were obtained from 8–16 cells, 488 from morula, 157 by culturing blastocysts and 105 were formed by culturing ICM from hatched blastocyst (Table 1).

Undifferentiated ES like cells formed round colonies with clear margins. Cells were tightly packed with each other with close cell membrane contact and were rounded. ES like colonies when exposed to ALP staining stained red indicating a high expression of alkaline phosphatase whereas the feeder cells were lightly coloured. The expression of ES like colonies against the SSEA-3 and Oct 4 was also positive.

Table 1. Efficiency of different stages of parthenogenetic embryos for production of embryonic stem cell colonies

Stages of embryo	No. of embryos used	No. of embryonic stem cell colony formation (%)
8–16 cell	328	137[a] (41.76)
Morula	786	488[a] (62.08)
Blastocysts	196	157[b] (80.10)
Hatched blastocysts	122	105[b] (86.06%)

The goat ES like cells described here exhibited the hallmarks of pluripotency: Long-term proliferation in culture in an undifferentiated state and expression of known markers of pluripotent cells. In our study, ESC like colony formation with ICM of hatched blastocyst was comparatively higher as compared to blastocysts while ESC like colony formation with blastocysts and ICM of hatched blastocyst was significantly higher as compared 8–16 cells and morula. It is observed that inner cell mass source is an important criterion for the ES like cells derivation in goats. Also, in comparison to the expanded blastocysts (80.10%), hatched blastocysts showed higher (86.06%) attachment rate and primary colony formation rate. This observation encourages for the presence of more viable inner cell mass or higher cell number in ICM in turn showing increased efficiency of hatched blastocysts and expanded blastocysts in comparison to morula. Further superiority of HBs and ExBs over morula can be of two reasons, firstly as no proteolytic enzyme was used on hatched blastocysts and expanded blastocysts and they were seeded like this on the goat fetal fibroblast monolayer, the damage to the inner cell mass can be expected to be minimal. Secondly, hatched blastocysts and expanded blastocysts are more developed stages than morula that could have given them an edge over the morula. In some studies it is showed that inner cell mass from from morula and early stages of blastocyst gave unsatisfactory results as compared to hatched blastocysts (Verma *et al.* 2007).

Morphologically, the goat pES like cells reported here formed large colonies with distinct boundaries similar to those generated from mouse, monkey and other animals but also share some similarity with those reported in previous studies on ES-like cells from large domestic animals that formed mostly monolayers of cells (Mitalipova *et al.* 2001). Goat pES like cells possessed alkaline phosphatase activity and were characterized by expression of OCT 4 as well as SSEA3. This is similar to expression patterns reported for undifferentiated primate ESC and mouse stem cells.

Furthermore, the presence of LIF and inactivated goat fetal fibroblast feeder cells had positive influence. We observed frequent, spontaneous differentiation of ES-like cells in the absence of LIF that resembled epithelial-like or neuron-like cells. The presence of LIF inhibited the differentiation of ES like cells with support of feeder cells (Mulas *et al.* 2019). Another factor promoting the successful

derivation of goat pES cells reported here may have been the availability of species-specific feeder cells. Several different cell types have been used as feeder layers for the culture of ESCs. However, in farm animals none of these cell types could sustain prolonged ES cell proliferation and prevent spontaneous differentiation. In other farm animals, studies regarding the utility of LIF and feeder cells have yielded conflicting results (Saito *et al.* 2003, Wang *et al.* 2005). Our results support the use of human LIF in addition to goat fetal fibroblast.

The morphology of cells did not change during culture in the presence of LIF, but removing LIF from the culture allowed the cells to differentiate into epithelial-like cells and neuron like cells. This spontaneous differentiation has also been reported in other ruminants (Verma *et al.* 2007). Because of the potential limitations of *in vitro* experiments, it is necessary to examine cell differentiation *in vivo* after cell transplantation.

However, during later passages, the cells colonies lost their integrity, compactness and floated in culture medium indicating sign of degeneration. Continuous proliferation of ES cells requires optimal *in vitro* conditions to maintain self-renewal and feeder layer secretes important growth factor for preventing differentiation of ES cells.

The results indicated that the goat pES like cells from different stages of parthenogenetic goat embryos that expressed known ES cell markers, could be maintained *in vitro* in undifferentiated state.

SUMMARY

Parthenogenetic activation of oocytes has gained new interest in recent years as an alternative approach to create embryos with no reproductive purpose for research in areas such as assisted reproduction technologies itself and for derivation of clinical grade pluripotent embryonic stem cells for regenerative medicine. In this study, we described the production of goat parthenogenetic ES like cells from different stages of parthenogenetic embryos. We compared the source material on the pESCs outgrowth and culture. 8–16 cell stage embryos, morula, blastocysts and ICM (inner cell mass) from hatched blastocysts produced from chemical activation were cultured on goat fetal fibroblast monolayer in stem cell culture media. Colonies were passaged when signs of differentiation were visible. ESC like colonies were cultured on feeder cells in the presence of hLIF however, some ESC like colonies were also cultured in absence of hLIF for random differentiation. In our study, ESC like colony formation with ICM of hatched blastocyst was comparatively higher as compared to blastocysts while ESC like colony formation with blastocysts and ICM of hatched blastocyst was significantly higher as compared 8–16 cells and morula. It is observed that inner cell mass source is an important criterion for the ES like cells derivation in goats. Also, in comparison to the expanded blastocysts (80.10%), hatched blastocysts showed higher (86.06%) attachment rate and primary colony formation rate. ESC like colonies in absence of hLIF differentiated *in vitro* into epithelial like and neuronal like cells. Undifferentiated ESC like colonies stained positive for alkaline phosphatase, SSEA-3 and OCT-4.

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