*Indian Journal of Animal Sciences* **83** (5): 502–505, May 2013/Article

# Comparative expression profile of developmental related genes in haploid and diploid parthenogenetic embryos in caprine

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Received: 22 October 2011; Accepted: 26 November 2012

ABSTRACT

Several reports suggested that haploid parthenogenetic embryos have less developmental ability as compared to diploid parthenogenetic or IVF derived embryos, may be due to lesser expression of developmental related genes. The present study was, therefore, carried out to compare expression of early embryonic developmental related genes in different stages of haploid and diploid parthenogenetic embryos in goat. Good quality oocytes were aspirated from the ovary and matured in maturation medium without (haploid embryo generation) or with cytochalasin B (for diploid embryo generation). After maturation, oocytes were activated using ionomycin followed by incubation with 6-DMAP in mSOF. The activated oocytes were further cultured in mSOF for generation of parthenogenetic embryos of different stages. Total RNA was isolated from pool of, viz 2–4, 8–16 and morula cell stages embryos of haploid and diploid, respectively, and cDNA was synthesized. The relative expression of *Nanog*, *Sox-2*, *Klf4* and *Foxd3* genes were analyzed by real time PCR. The result indicated that the relative expression of all 4 genes could not be detected in 2–4 and 8–16 cell stage of haploid embryos, whereas 2–4 and 8–16 cell stage diploid embryos expressed all these genes. When both type of embryos reached at morula stage, the expression of all the genes were present and no significant changes were observed between haploid and diploid parthenogenetic embryos. From the results, it can be concluded that haploid parthenogenetic embryos showed undetectable level of embryonic develpomenta lrelated genes like *Nanog, Sox-2*, *Klf4* and *Foxd3* at early embryonic develpomental stage in comparison to diploid parthenogenetic embryos. This may be one of the major reasons for poor developmental competence of parthenogenetic haploid embryos.

**Key words**: Caprine, Embryos, Gene expression, Parthenogenesis

Haploid parthenogenetic embryonic development is a normal part of the life cycle for some lower animals (Rougier and werb 2001), but it was not observed in mammals (Rasch*et al*. 1977). Mammalian parthenotes do not survive to long term, because the maternal and paternal chromosomes of mammals are not equivalent (McGrath and Solter 1984). Normal mammalian development requires genomic contributions from both the mother and father. Several studies have been conducted in mammals like mice, bovine and rabbit, which indicate development of haploid parthenogenones is significantly weakened (Tarkowski and Rossant 1976, Modlinski 1980). Development of haploid rabbit parthenogenones is inefficient (Escriba and Garcia-Ximinez 2000). Presumably, gametic imprinting is the main cause of unipaternal failure in development. Although nonimprinted genes such as insulin-like growth factor-1 receptor may be misregulated in parthenogenetic embryos (Rappolee *et al*. 1992).

Embryos cultured in the laboratory can display epigenetic changes, aberrant gene expression and perturbed patterns of fetal growth. In *in-vitro* produced embryos, gene expression starts at the 2 to 4 cell stage. *Oct4, Nanog, Sox-2, Desmocolin I, II, III, desmogelin 1, plakophilin, connexin 43 Hsp70.1, Glut-1, trophoblast protein* gene (TP), polymerase etc. are some of the important genes related to development of embryo (Wrenzycki*et al*. 1997) and *Oct–4, Nanog, Sox-2*, *Klf4* and *Foxd3* gene maintain pluripotency related genes. Three transcription factors that exert a dramatic amount of genetic control over pluripotency are *Oct-4, Nanog and Sox-2* (Magnani and Cobat 2008). Klfs were implicated in the regulation of an autoregulatory network, known as the core pluripotency network, that plays a key role in ESC self-renewal. Klfs and the Oct4/Sox2/Nanog network are strongly

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interconnected since Klf2, Klf4 and Klf5 activate the expression of *Nanog, Sox2*, and *Oct4* (Jiang *et al*. 2008). Another pluripotency gene *Foxd3* is required for epiblast formation in blastocysts and stem cell pluripotency in mouse (Hanna*et al*. 2002). Parthenogenetic embryo fails to develop to full term and exhibit somewhat complementary phenotypic abnormalities (Surani*et al*. 1986,Solter 1988). The reason of limited developmental potential of haploid embryos may be imbalance expression of embryonic developmental related genes during different cell stages of embryos. Over expression of nang in mouse ES cells bypasses the requirement of leukemia inhibitory factor (LIF) and promotes self renewal (Chambers *et al*. 2003) and the over expression of a dominant negative form of Sox-2 leads to differentiation of ES cells into cells with a trophectoderm phenotype (Li *et al*. 2007, Masui *et al*. 2007). Several interesting possibilities were observed critically but none has provided credible explanation to develop the parthenogenetic embryos. Therefore, the present study was designed to compare the expression of embryonic developmental related genes in different stages of haploid and diploid parthenogenetic embryos in goat to improve the development of parthenogenetically produced embryos.

## MATERIALS AND METHODS

*Recovery and* in vitro *maturation of oocytes:* Goat ovaries collected from goats of unknown reproductive status slaughtered at local abattoir were carried to the laboratory in normal saline solution (NSS) (0.85%) fortified with antibiotic in a thermos flask at 35–37°C within 2 h of slaughter. In laboratory, the extra ovarian tissues were trimmed off and ovaries were washed several times with NSS (35–37°C). Cumulus oocyte complex (COCs) were aspirated from all visible non atretic follicles by an 18 gauge needle attached to 5 ml syringe containing oocyte collection media, (OCM). The COCs along with follicular fluid were pooled into 50 ml sterile plastic tube and were allowed to settle for 10 min. Sediments were taken in large petri-dish (90 mm) and the COCs were searched under zoom stereomicroscope. Only excellent (i.e., more than 5 layers of cumulus cells and evenly granulated cytoplasm) and good (more than 3 layers of cumulus cells and evenly granulated cytoplasm) COCs were selected and drop washed several times in OCM. The COCs were further drop-washed 5–6 times with *in vitro* maturation (IVM) medium. Groups of 15–20 selected oocytes were placed in 60 µl droplets of IVM medium. IVM medium was TCM 199, supplemented with 10% fetal bovine serum (FBS), bovine serum albumin (BSA) (3 mg/ml), FSH (0.5 µg/ml); LH (10 IU/ml); goat follicular fluid (5%); L-glutamine (0.1mg/ml) and gentamycin (50 µg/ml). For diploid embryo generation oocytes were matured in maturation medium supplemented with cytochalasin B (20 µg/ml). The droplets were covered with warm non-toxic mineral oil and cultured at 37°C, in an atmosphere of 5% $CO_2$ and 20% oxygen under humidified air (95%) for 27 h in $CO_2$ incubator. The evaluation of maturation were based on the visual assessment of degree of cumulus expansion under inverted microscope described by Kobayashi *et al.* (1994).

*Oocyte activation:* After maturation, the oocytes were treated with 0.1% hyaluronidase in TCM-199 followed by pipetting to remove the cumulus cells. Finally cumulus free oocytes were activated using 5µ mionomycin in mSOF for 5 min followed by 4 h incubation with 2 mM 6-DMAP in mSOF.

In vitro *development culture:* After activation treatment, oocytes were taken out of 6-DMAP drop, washed several times in modified synthetic oviductal fluid (mSOF) supplemented with 0.8% BSA and essential and non-essential amino acids and cultured in 100 µl of same media in $CO_2$ incubator at 37°C, 5% $CO_2$, 20% $O_2$ and 95% relative humidity until assessment to determine cleavage. For embryo development, cleaved oocytes were subsequently transferred in fresh drop of mSOF and were further cultured.

*RNA extraction and reverse transcription:* For gene expression analysis, embryos of different cell stages were picked up from the culture media separately and washed with 1× PBS. Then, transfer the embryos in 2 ml centrifuge tube for RNA isolation. The total RNA was isolated. cDNA was synthesized using synthesis kit. After preparing cDNA,O.D of cDNA was maintainedat 500 ng/µl and used for real time PCR study.

*Real time PCR:* Relative quantification was performed using a real time polymerase chain reaction method. The mtDNA copy number was determined. For amplification of genes, primers were designed in beacon software. Primers for real time PCR were: ß-actin F5'-CGCAC CACTGGCATTGTCAT-3' and R5'-TCCAAGGCGACGTA GCAGAG-3' NanogF5'-GTCCCGGTCAAGAAACAAAA-3' andR5'-TGCATTTGCTGGAGACTGAG-3' Sox-2 F5'-CACAACTCGGAGATCAGCAA-3' and R5'-CACAACT CGGAGATCAGCAA-3' Klf4 F5'-GGAAGTTTGCCCGC TCAGAT-3' and R5'-GCCTCTTCATGTGTAAGGCG-3' Foxd3 F5'-GCTGACTCTGAGCGGCAT-3' and R5'-TCCTCGGACTGAGGGTCCA-3'. The real time PCR thermocycling conditions were: an initial denaturation step at 95°C for 30 min followed by 40 cycles of 95°C for 5 sec, annealing for 15 sec. Transcript level of all 4 genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene â-actin.

*Analysis of data:* The relative expression changes were determined using the $2^{-\ddot{A}\ddot{A}Ct}$ method (Pfaffl 2001).

## RESULTS AND DISCUSSION

In the present study, the relative expression of embryonic developmental related genes, viz. *Nanog, Sox-2, Klf4* and *Foxd3* was done in diploid embryos in relation to haploid,

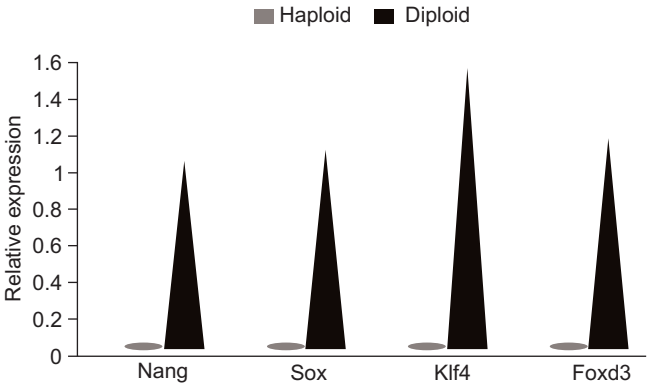


Fig. 1. Relative expression of *Nang, Sox-2, Klf4* and *Foxd3* genes between 2–4 cell stage of haploid and diploid parthenogenetic embryos.

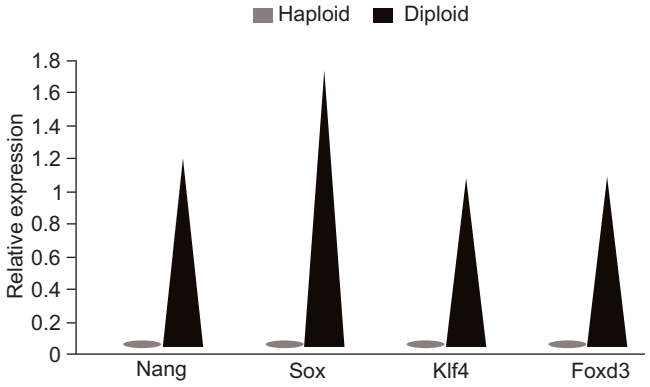


Fig. 2. Relative expression of *Nang, Sox-2*, *Klf4* and *Foxd3* genes between 8–16 cell stage of haploid and diploid parthenogenetic embryos.

and results are presented in Figs 1–3. The expression of all 4 genes was not observed in 2–4 and 8–16 cell stage of haploid embryos whereas 2–4 and 8–16 cell stage of diploid embryos expressed all these genes (Figs 1, 2). The morula stage embryos of haploid and diploid expressed all the 4 genes and interestingly there was no significant difference on relative expression (Fig. 3). This could be because haploid embryo has only X chromosome and diploid embryo has XX chromosome that has somewhat similar gene imprinting, X inactivation and gene expression phenomenon of XX and XY chromosome of *in vitro* fertilizated embryos. *In vitro* fertilized embryos have both maternal and paternal genomes that is why they survive easily due to balance gene expression, methylation, gene imprinting and X inactivation. Genomic imprinting offers possible explanation, as haploid embryo possess exclusively maternal or paternal chromosome (Latham *et al*. 2002).

Since haploid embryo possess only X chromosome that is why haploid embryo do not survive long time due to reduced or over expression of some genes (Chambers *et al*. 2003, Li *et al*. 2007, Masui *et al*. 2007). For example, Latham *et al*. (2002) observed that haploid parthenogenones over

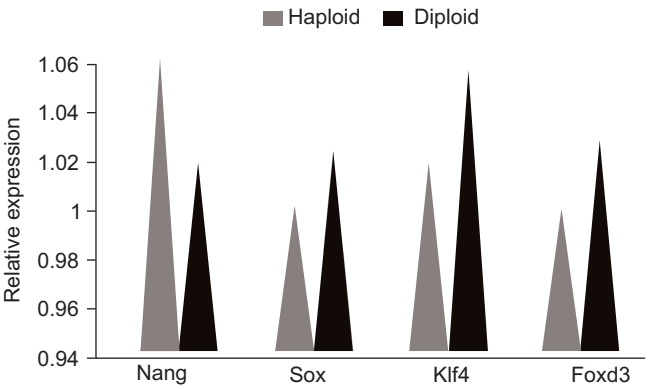


Fig. 3. Relative expression of *Nang, Sox-2, Klf4* and *Foxd3* genes between morula cell stage of haploid and diploid parthenogenetic embryos.

expressed bex 1 mRNA relative to fertilized control embryos at both morula and blastocyst stages. Another study also revealed that mouse embryos with a loss of function for *Oct-4, Nanog* and *Sox-2* genes showed altered development with embryonic lethality between days 4.5 and 6.5 due to premature differentiation of the ICM cells (Boyer *et al*. 2006). In the present study, the disturbance in the gene pattern observed in haploid embryos may have some effects on the control of pluripotency, especially during 2– to 16-cell stage where haploid embryos were shown the highest gene expression difference as compared to diploid embryos. In conclusion, the study indicated that the reduced developmental competence of haploid parthenogenetic embryos may be due to reduce expression of developmental related genes.

## ACKNOWLEDGEMENT

The authors are thankful to NAIP, ICAR for providing fund for the present work. Further, the authors are also thankful to the Director, IVRI for providing all the facilities for conducting the present research work.

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