



Paternally and maternally imprinted gene perturbed expression in parthenogenetic diploid embryos in *Capra hircus*

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ABSTRACT

The present research work was undertaken to compare the expression of paternally imprinted (P57^{KIP2}, Grb10 and Xist) and maternally imprinted gene (Dlk1) among diploid parthenogenetic (DIP), female sexed *in vivo* and female sexed *in vitro* produced embryonic cell colony. The good quality caprine oocytes were matured in presence of cytochalasin B (CCB) and activated by ethanol (7%) for 5 min followed by incubation with 2 mM 6-dimethyl amino purine (DMAP) for 4 h for DIP embryos production. *In vivo* embryo was collected by surgical method from 10 super ovulated goats using Pluset®. *In vitro* embryos were produced by maturation of good quality oocyte followed by fertilization with superior male germplasm from the institute flock. Embryonic cell colony were developed from 8 to 16 and morula stage DIP, IVF and *in vivo* derived embryos. These colonies were used for studying the paternal and maternal imprinted genes expression. Karyotyping and sexing of embryonic cell colony was done to get only female sexed colony for this purpose. Paternally imprinted genes (P57^{KIP2}, Grb10 and Xist) showed more expression in DIP compared to *in vivo* and IVF derived embryonic cell colony. The expression of maternally imprinted genes (Dlk1) was low in DIP compared to *in vivo* and IVF derived embryonic cell colony. The present study demonstrated that the expression profile of both paternal and maternally imprinted genes were perturbed, which may be responsible for developmental failure of parthenogenetic embryos to full term following parthenogenetic embryo transfer in goat.

Key words: *Capra hircus*, Diploid parthenogenetic embryos, Dlk1, Grb10, P57^{KIP2}, Parthenogenesis, Xist

Parthenogenesis occurs as a natural process in the life history of many invertebrates and in sexually reproduced animals, the egg will be activated by artificial methods without fertilization called artificial or induced parthenogenesis. The stimulus for this may be physical, temperature, chemical, electrical etc. Preimplantation embryo development is characterized by the three distinct morphological steps, viz. compaction, cavitation and blastocoel expansion which require well-orchestrated expression of genes derived from the maternal and/or embryonic genome. *In vitro* produced embryos gene expression starts at the 2- to 4-cell stage. After fertilization, the early embryonic development is dependent on maternal mRNA that was transcribed during oocyte growth. It is only after a few cleavage divisions that the embryonic genome is activated and the embryo takes control of its own destiny.

Expression of development related genes is essential for survival of an embryo. Normal mammalian development requires both maternal (H-19, P57^{KIP2}, Igf2r, Grb10, Gnas, Mash/Hash, Xist etc.) and paternal (Igf2, Peg1, Peg3, Dlk1, Mest, Ndn, Nnat, Sgce etc.) genes contribution to the genome for complete gene expression (Barton *et al.* 1984). Further, the expression of Desmocollin Type I, II, III, Desmoglein 1, Plakophilin, Connexin 43, Hsp70.1, Oct4, Nanog, Glut-1, Glut-5, Igf-1-r, E-cad, Stat3, MnSOD, DNMT1, Bax, Bcl2, Fgf4, Chop 10, G6pd, Aquaporins 1,3,6,6,7,9 etc., genes is also required for normal early embryo development. Parthenogenetic embryo fails to develop to term and exhibit phenotypic abnormalities due either the lack of expression or to overexpression of several of these developmental related genes (Kaufman *et al.* 1977, Barton *et al.* 1984, Ranjan *et al.* 2014, Ranjan *et al.* 2015, Ranjan *et al.* 2016, Singh *et al.* 2013, Singh *et al.* 2014).

There is no live birth after parthenogenetic embryo transfer in higher mammal. Recently, mammalian parthenogenesis has been achieved with the birth of the mouse Kaguya (Kono 2004). This has opened up the hope of making parthenogenesis a successful method of cloning in domestic animal. So far no comparative gene expression between parthenogenetic, female sexed IVF and *in vivo*

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Table 1. Primers used for real-time PCR

Primer	Sequence (10 pmol/ μ l)	Annealing temp ($^{\circ}$ C)	Product size (bp)
β -actin	F 5'TCATCACCATCGGCAATG3' R 5'CCAATCCACACGGAGTAC3'	60	286
GAPDH	F 5'GGAGAA ACC TGCCAAGTATG3' R 5'TGAGTGTGCTGTTGAAGTC3'	60	126
P57 ^{KIP2}	F 5'GAGCAGCGTTCGGTTTCGTGTTTA3' R 5'CCAGTGTACTCCTTGGGCAGAATA3'	60	97
Grb10	F 5'AGTAGTTGTCCTGAGATTC3' R 5'CGTTCCTGACTCTGTTAG3'	60	253
Xist	F 5'TTTGCTGCAGGGAGAATATGGCTG3' R 5'GATGCAAAGCCCGCCAAGAATGTA3'	56	112
Dlk1	F 5'GGCATCGTCTTCTCAACAAG3' R 5'GCAGCAGCAGGTTCTTCTTC3'	60	88
Sry	F 5'TGAACGAAGACGAAAGGTGGCTCT3' R 5'ATCAGGGACTGTGAGCGGCATAAT3'	60	342

derived embryos has been done to identify the deficiency or molecular defects during normal parthenogenetic embryonic development and growth in goats. Scanty information is available in caprine (Singh *et al.* 2014, Ranjan *et al.* 2014, Kharche *et al.* 2015) and further the comparison has been made of unknown sexes of IVF or *in vivo* derived embryos. When parthenogenetic embryos are compared with female sexed IVF or female sexed *in vivo* embryos then only the actual status of gene expression pattern can be stated. Keeping in view above information and gaps in knowledge, the present research work was proposed to compare gene expression profile of P57^{KIP2}, Grb10, Xist and Dlk1 genes among DIP, *in vitro* produced and *in vivo* derived female sexed embryonic cell colony.

MATERIALS AND METHODS

All the experiments were approved by ethics committee of this institute.

Embryos production and culture of embryonic cells isolated from early stage embryos: *In vitro* embryo production was done as per Ranjan *et al.* (2013a). For parthenogenetic diploid (DIP) embryo development, isolated oocytes were cultured in maturation media supplemented with 15 μ g/ml of cytochalasin B (Ranjan *et al.* 2013a). Surgical embryo collection was done from 10 super ovulated goats as per standard protocol with Pluset® (Agrawal *et al.* 1982). The DIP, IVF and *in vivo* derived embryos of 8 to 16 cells and morula were zona lysed with proteinase-K (0.02%) and cultured in wells on mitomycin C inactivated goat fetal fibroblast monolayer. When the clumped blastomere of individual embryos were divided and make a colony, these cells were eluted by 0.25% trypsin EDTA solution (Puri *et al.* 2012).

Karyotyping of parthenogenetic embryo and sexing of embryonic cell colony: Karyotyping was done as per Ranjan *et al.* (2013a). The colonies of DIP embryos of cultures were used for gene expression studies. Colonies developed from individual IVF and *in vivo* derived embryos of 8–16 and morula stage embryos were sexed by SRY gene expression by real time PCR using genomic DNA (Ranjan

et al. 2013b). The female sexed colonies of IVF and *in vivo* derived embryos of cultures were used for gene expression studies.

RNA extraction and real time polymerase chain reaction: The total RNA was isolated from embryonic cell colony generated from 8 to 16 and morula stages of DIP (36 and 37), IVF (20 and 22) and *in vivo* (6 and 5) derived female sexed embryonic cell colonies by Quick-RNA™ MicroPrep. cDNA was synthesized using iScript select cDNA Synthesis kit. Relative quantification of different genes was performed using a real time PCR method by using Bio-Rad CFXManager™ software and EvaGreen supermix as per standard protocol developed in our laboratory (Ranjan *et al.* 2014, Singh *et al.* 2014). The detail of primers is given in Table 1.

Statistical analysis: Relative expression of gene in fold changes was calculated by $2^{-\Delta\Delta C_t}$ method (Pfaffl *et al.* 2002). The data was analyzed by one way ANOVA using SPSS 16 and a P-value <0.05 was considered to be significant.

RESULTS AND DISCUSSION

Three types of embryonic cell colonies (DIP, IVF and *in vivo*) and their 2 developmental stages (8–16 cell and morula) were used in the present study. The IVF and *in vivo* derived embryonic cell colony were sexed with PCR and female sexed colonies were separated. Parthenogenetic embryonic cell colonies were karyotyped and diploid parthenogenetic colonies were separated. *In vivo* derived 8–16 cell and morula stage of embryonic cell colony were used as control for relative expression analysis. The results indicated that CCB (15 μ g/ml) in maturation medium can be used for the production of optimum diploid parthenogenetic embryos in the caprine species (Ranjan *et al.* 2013a). The sex determination method of embryos can accurately and efficiently predict the sex of embryos. The overall sex ratio of male:female embryo was 1: 1.18 (Ranjan *et al.* 2013b).

In the present study, beta-actin and GAPDH were used as housekeeping genes to study the relative expression of P57^{KIP2}, Grb10, Xist and Dlk1. All developmental genes

were expressed in all the samples tested in present study. The expression of P57^{KIP2}, growth factor receptor-bound protein 10 (Grb10) and X-inactive specific transcript (Xist) was upregulated significantly ($P < 0.05$) in both stages of DIP as compared to IVF and *in vivo* one (Figs 1,2). There was no significant difference ($P < 0.05$) in P57^{KIP2} gene expression between 8–16 and morula of DIP, IVF and *in vivo* derived embryonic cell colony (Figs 1, 2). At 8–16 cell stage, there was no significant difference between DIP and IVF embryos, but in morula-stage, DIP embryos had significantly ($P < 0.05$) upregulated expression of Grb10 gene (Figs 1,2). Interestingly, at 8–16 cell stage, IVF embryos had significantly upregulated expression of this gene like DIP, but in morula stage, the difference was non significant albeit comparatively higher expression in IVF one. There was no significant difference between two stages of DIP and *in vivo* ($P < 0.05$) but morula of IVF showed significantly lower ($P < 0.05$) expression than 8–16 cell stage (Fig. 1). There was significantly higher ($P < 0.05$) Xist expression in morula stage of DIP and *in vivo* derived embryos as compared to 8–16 cell stage but no significant difference between two stages of IVF embryos (Figs 1, 2). The expression of delta-like1 (Dlk1) was downregulated significantly ($P < 0.05$) in 8–16 stage of DIP embryos as compared to *in vivo* and IVF derived one of similar stage (Figs 1, 2). However, there was no significant ($P < 0.05$) difference in both stages between IVF and *in vivo* derived embryos (Figs 1, 2). In morula stage, the expression of this gene was not significantly different among different types of embryos (Figs 1, 2). When compared between 8–16 and morula stage, there was no significant difference ($P < 0.05$) in expression of this gene in DIP, IVF and *in vivo* derived embryonic cell colony (Figs 1, 2).

P57^{KIP2} is a structurally distinct member of the p21Cip1 Cdk inhibitor family and is a candidate tumor suppressor gene. This gene lies –800 kb from Igf2 in both mouse and human and is a potent, tight-binding inhibitor of several G1 cyclin/Cdk complexes (Simon *et al.* 2011). This gene is expressed maternally in all mammalian species examined

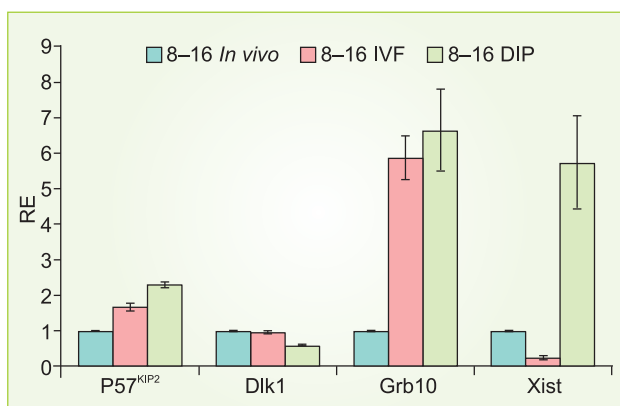


Fig.1. Relative expression of maternal and paternal imprinted genes in embryonic cell colony derived from 8–16 cell stages of DIP, *in vivo* and IVF derived embryos in caprine (Mean±SE). *Figure with different superscript differed significantly ($P < 0.05$). RE, Relative expression.

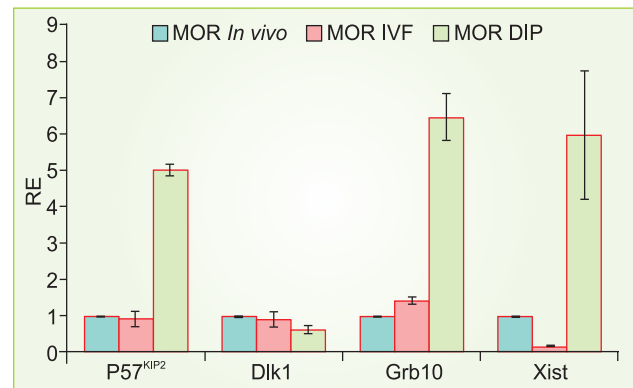


Fig. 2. Relative expression of maternal and paternal imprinted genes in embryonic cell colony derived from morula stages of DIP, *in vivo* and IVF derived embryos in caprine (Mean±SE). *Figure with different superscript differed significantly ($P < 0.05$) RE, Relative expression.

to date. The function of this gene is dependant with the expression of Igf2 gene and infact both the genes work in opposing manners to control cell proliferation during development (Zhang *et al.* 1997). It is down regulated in mice with high serum levels of Igf2 and its expression is reduced in Igf2 treatment of embryo fibroblast (Grandjean *et al.* 2000). P57^{KIP2} embryos were overgrown at E15.5 and at E18.5, just prior to birth but there was no difference in the weight of mutant and wild-type pups after birth (Simon *et al.* 2011). It is a maternally expressed gene and supported the prediction of the parental conflict hypothesis that the paternal genome silences genes that have an inhibitory role in embryonic growth (Andrews *et al.* 2007). Grb10 is maternally expressed in most tissues and known to bind the insulin and insulin like growth factor 1 (Igf-1) receptors. It was detected both in IVF and parthenogenetically activated (PA) blastocysts (Ruddock *et al.* 2004) as well as *in vivo* derived blastocysts in bovine (D’Cruz *et al.* 2008). It was observed that it is also expressed in human blastocyst, but so far no evidence could be provided for an allele specific expression (Adjaye *et al.* 2005). A study showed that this gene is expressed biallelically in ovine and porcine blastocyst (Thurston *et al.* 2008, Park *et al.* 2011). We also observed high expression of Grb10 in DIP as compared to *in vivo* and IVF samples. Xist transcripts were first detected at the 16–32-cell stage embryos, then detected in all morula and in 2 of 6 blastocysts (Ruddock *et al.* 2004) and in both male and female morulae and in female blastocysts in bovine (Peippo *et al.* 2002). In porcine it is expressed in preimplantation IVF and parthenogenetic embryos and are biallelically expressed (Park *et al.* 2011), while in mouse it shows monoallelic expression (Latham 2005). The expression of Xist RNA appeared to be elevated in haploid androgenetic blastocysts relative to diploid androgenetic and fertilized controls, but expression was quite variable and the difference was not statistically significant (Latham *et al.* 2002). In bovine, this gene displayed complete imprinting with expression in *in vivo* and parthenogenetic embryos in bovine (D’Cruz *et al.* 2008). The above results indicated

that this gene is expressed in preimplantation embryos in many species and have elevated expression as compared to *in vivo* derived embryos. However, no comparative expression profile has been studied among DIP, IVF and *in vivo* derived embryos. We also observed the expression of P57^{KIP2}, Grb10 and Xist in all stages of parthenogenetic, *in vivo* and IVF derived embryonic cell colony. The expression was upregulated significantly ($P < 0.05$) in DIP compared to their *in vivo* and IVF derived embryonic cell colony.

Dlk1 mRNA was expressed in mouse embryos during post implantation development, beginning between embryonic day 7 and day 11, as well as in the adult pancreas, bone marrow, adrenal, and pituitary glands (Schmidt *et al.* 2002). It has also been seen to express at high levels in the mouse embryo and placenta during development (Schmidt *et al.* 2002). However, the expression of this gene could not be detected in any of the oocyte or embryo samples of bovine (Ruddock *et al.* 2004). In this study, we observed a very low level of expression of this gene in DIP compared to IVF and *in vivo* embryos of caprine.

The results indicated that in caprine, the expressions were either low or enhanced in DIP as compared to IVF or *in vivo* derived embryos. The *in vitro* culture condition affects gene expression (Doherty *et al.* 2000, Khosla *et al.* 2001). Therefore, the changes of relative expression of genes in DIP and IVF embryos in the present study might be due to the effect of *in vitro* culture condition. Several *in vitro* developmental defects have been reported in parthenogenetic embryos compared with fertilized embryos (Loi *et al.* 1998). It was reported earlier that the fetus death may be due to lack of formation of placenta or perturbed developmental gene expression (Ranjan *et al.* 2013c, Renu *et al.* 2014, Ranjan *et al.* 2014, Kharche *et al.* 2015). The sequential expressions of developmental related genes are required for normal mammalian development *in utero* (Thomson *et al.* 1989, Nishita *et al.* 1996).

The expression profile of both paternal and maternally imprinted genes were perturbed, which may be responsible for developmental failure of parthenogenetic embryos in this species and warrants further modification of protocol for generation of DIP embryos in *Capra hircus*.

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