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# Insulin like growth factor 2 (Igf2) and its receptor gene (Igf2r) showed opposite expression in diploid parthenogenetic embryos in *Capra hircus*

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Parthenogenesis is a reproductive process where a single egg can develop to term in the absence of male genetic counterpart and without meiotic chromosome reduction. The normal mammalian development requires balanced expression of both maternal (H-19, $P57^{kip2}$, Igf2r, Grb10, Gnas, Mash/Hash, Xist etc.) and paternal (Igf2, Peg1/Mest, Peg3, Dlk1, Ndn, Nnat and Sgce etc.) as well as other developmental related genes (Barton *et al.* 1984). Therefore, the fundamental reason of failure of parthenogenetic embryos to live birth in mammals is associated with lack of or perturbed expression of these genes (Singh *et al.* 2013, Ranjan *et al.* 2014, Singh *et al.* 2014).

The expression of early embryonic developmental as well as paternal and maternally imprinted genes was reported in pre-implantation embryos in mice, cattle, sheep, pig etc. (D'Cruz *et al.* 2008). There are also sporadic studies on mice (Perecin *et al.* 2009), but scanty information is available on caprine (Singh *et al.* 2014, Ranjan *et al.* 2014). 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 *in vivo* embryos then only the exact status of gene expression can be stated.

Therefore, we studied the comparative gene expression profile of Igf2 and Igf2r gene among DIP, *in vitro* produced and *in vivo* derived female sexed embryonic cell colony. All the experiments were approved by ethics committee of the Indian Veterinary Research Institute, Izatnagar, India.

*Embryos production and culture of embryonic cells isolated from early stage embryos: In vitro* embryo production was done as per standard protocol developed in our laboratory (Ranjan *et al.* 2013a). For parthenogenetic diploid (DIP) embryo development, isolated oocytes were cultured in maturation media supplemented with 15µg/ml of cytochalasin B (Ranjan *et al.* 2013a). Surgical embryo collection was done from 10 goats as per standard protocol (Agrawal *et al.* 1982). The DIP, IVF and *in vivo* derived embryos of 8–16 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.

*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).

*RNA extraction and real time polymerase chain reaction:* The total RNA was isolated from embryonic cell colony generated from 8–16 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 kit. cDNA was synthesized using iScript select cDNA synthesis kit. Relative quantification of different genes was performed using a real time PCR method by using software and Eva Green supermix as per standard protocol (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 $2^{-\Delta\Delta Ct}$ method (Pfaffl *et al.* 2002) and analysed by one-way ANOVA using SPSS 16 and a P-value <0.05 was considered to be significant.

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 percentage of embryos showed higher diploid values in 15 µg/ml cytochalasin B (CCB) (83.66±1.13), followed by 20 µg/ml (72.22±1.22), 10 µg/ml (68.57±1.17), and 5 µg/ml (62.00±2.48). These results indicated that CCB (15 µg/ml) in maturation medium can be used for the production of diploid parthenogenetic embryos in the caprine species (Ranjan *et al.* 2013a). There was consistently fixed banding patterns in male DNA samples extracted from blood of known phenotypic sex. The negative control resulted in no amplification, thus foreign DNA contamination was

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Table 1. Primers used for Real-time PCR

| Primer | Sequence (10pmol/µl | Annealing temp(°C) | Product size (bp) |
|---|---|---|---|
| β-actin | F 5′TCATCACCATCGGC AATG3′<br>R 5′CCAATCCACACGGA GTAC3′ | 60 | 286 |
| Gapdh | F 5′GGAGAA ACC TGCC AAGTATG3′<br>R 5′TGAGTGTCGCTGTT GAAGTC3′ | 60 | 126 |
| Igf2r | F 5′GAACTGTAAGCAGC AGAATCAC3′<br>R 5′GAGTCGTCCACCA AGTAAGC3′ | 60 | 252 |
| Igf2 | F 5′TGCTGCTATGCTGC TTAC3′<br>R 5′AGATGTCATATTGG AAGAACTTG3′ | 60 | 295 |
| SRY | F5′TGAACGAAGACGAA AGGTGGCTCT3′<br>R 5′ATCAGGGACTGTGA GCGGCATAAT3′ | 60 | 342 |

excluded. The method of sex determination of goat embryos that we used can accurately and efficiently predict the sex of embryos. The sex ratio of male: female embryos were 1: 1.5 and 1: 1 in 8–16 cell and morula stage *in vivo* embryo derived embryonic cell colony respectively. For IVF embryos, the sex ratio of male: female was 1: 1.25 and 1.1: 1 in 8–16 cell and morula stage of derived embryonic cell colony respectively. The overall sex ratio of male: A female embryo was 1: 1.18 (Ranjan *et al.* 2013b).

The expression of Igf2r gene was detected in all the samples tested in the present study (Figs 1–2). The expression of Igf2r gene was up regulated significantly (P<0.05) in DIP and IVF derived 8–16 and morula as compared to *in vivo* one (Figs 1–2). Igf2r encodes a

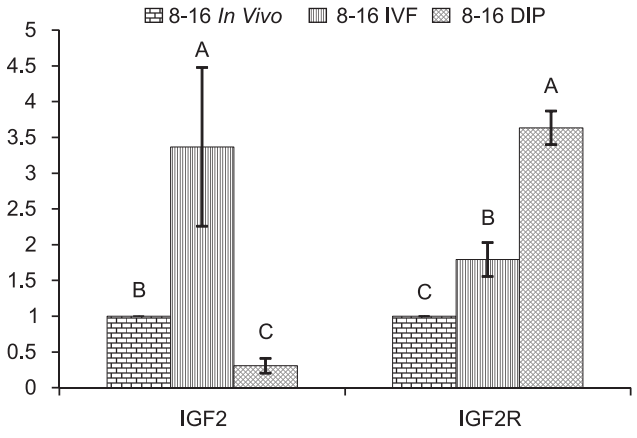


Fig. 1. Relative expression of Igf2 and Igf2r in embryonic cell colony derived from 8–16 cell stages of DIP, *In Vivo* and IVF derived embryos in caprine (mean±SE). *Figure with different superscript differed significantly (P<0.05).

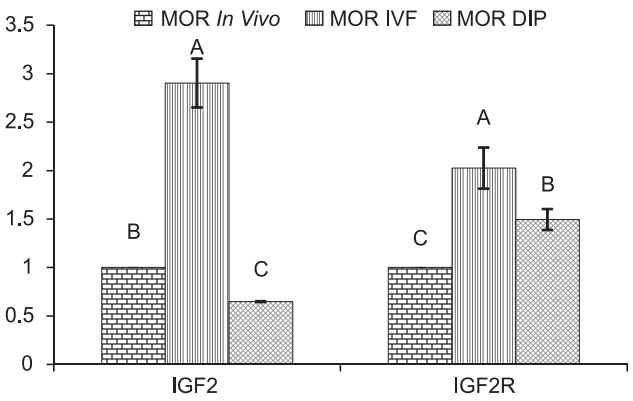


Fig. 2. Relative expression of Igf2 and Igf2r in embryonic cell colony derived from morula stages of DIP, *In Vivo* and IVF derived embryos in caprine (mean±SE). *Figure with different superscript differed significantly (P<0.05).

multifunctional receptor that is involved in the regulation of cell growth and differentiation. Knockout experiments demonstrated that Igf2r null mice exhibit fetal out growth or late gestational lethality (Bartolomei *et al.* 1993). A three-fold increase expression of this gene was observed in porcine parthenogenetic blastocyst as compared to androgenic and IVF blastocyst (Park *et al.* 2011). Another report indicated a higher expression of this gene in parthenogenetic fetus as compared to control fetus in mouse (Sotomaru *et al.* 2002). In sheep also, there was increased levels of expression of this gene in parthenogenetic embryo as compared to the control embryo suggesting that in this species Igf2r is expressed from the maternal allele predominantly (Yang *et al.* 2003).

The expression of Igf2, maps to sheep chromosome 21q21–qter (Ansari *et al.* 1994) and encodes a major fetal growth factor. In mice and humans, Igf2 is expressed from the paternal chromosome exclusively (Ohlsson *et al.* 1993), but does not express in parthenogenetic embryos in mouse (Walsh *et al.* 1994). We have also observed very low expression in DIP embryos in contrast to high level of expression in IVF and *in vivo* derived embryos. This expression pattern strongly suggested that Igf2 may be imprinted and paternally expressed in caprine also. In analogy with the studies in the mouse, where genetic ablation of the Igf2 gene leads to reduced fetal growth (De Chiara *et al.* 1991), it seems likely that the absence of Igf2 expression in parthenogenetic caprine fetuses would also involve in their growth retardation. Feil *et al.* (1998) also found same type of observation in sheep fetus and membranes. Because parthenogenones contain only maternally derived chromosomes, these results indicated that maternal Igf2 genes can be expressed during pre implantation mouse development and therefore are not inactivated as a consequence of the maternal imprint (Latham *et al.* 1994). In cattle, there was a lower Igf2 expression rate in the somatic cell nuclear transfer (SCNT) (0.19) and parthenogenetic (0.02) groups, when compared to *in vivo* and IVF embryos (2.01; $P < 0.05$). This low expression of Igf2 in parthenogenetic embryos and chorio-

allantoic membranes confirms its imprinted status in cattle (Perecein *et al.* 2009). In sheep, parthenogenetic embryos express low levels of Igf2 and high levels of Igf2*r* and have retarded growth when compared to control embryos (Feil *et al.* 1998; Yang *et al.* 2003).

The results indicated that in caprine, the DIP embryos expressed paternally as well as maternally imprinted genes during preimplantation development as was in IVF and *in vivo* derived ones. However, the expressions were either low or enhanced in DIP as compared to IVF or *in vivo* derived embryos. The reasons for this somewhat different expression in DIP embryos could not be ascertained, however, it was observed that in IVF derived embryos, the expression was also quite up regulated as compared to *in vivo* derived one. There have been reports which indicated that *in vitro* culture condition affects gene expression (Doherty *et al.* 2000, Khosla *et al.* 2001). Therefore, the changes of relative expression of Igf2 and Igf2r 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 identified in parthenogenetic embryos viz. delayed development, reduced total cell number, fewer cells in the inner cell mass of blastocyst compared with fertilized embryos (Loi *et al.* 1998). In our earlier experiment, we also observed early fetus death due to lack of formation of placenta or perturbed developmental gene expression (Ranjan *et al.* 2013c, Ranjan *et al.* 2014). The sequential expressions of development related maternal and paternal genes are required for normal mammalian development *in utero* (Thomson *et al.* 1989, Nishita *et al.* 1996). There are several reports that the death of parthenogenetic mammalian embryos is determined by the absence of expression of the genes of imprinted loci of the maternal or paternal genome, which leads to significant defects in development of tissues and organs (Platonov 2005).

In conclusion, the present study demonstrated the comparative expression profile of Igf2 and Igf2r genes, which seems responsible for proper growth of fetus among DIP, IVF and *in vivo* derived pre implantation embryos. 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 having normal gene expression as compared to *in vivo* and IVF one.

## SUMMARY

The present research work was proposed to compare the expression of development related genes (Igf2 and Igf2*r*) 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 and embryo development was recorded. We have explored comparative expression profile of paternally (Igf2*r*) and maternally (Igf2) imprinted genes among DIP, female sexed IVF and female sexed *in vivo* derived embryos. Embryonic cell colony were developed from 8–16 and morula stage DIP, IVF and *in vivo* derived embryos and these colonies were used for studying the paternal and maternal imprinted genes. Overall, we observed higher expression of maternally expressed genes (Igf2*r*) in DIP compared to *in vivo* and IVF derived embryonic cell colony. The expression of paternal genes (Igf2) was low in DIP compared to *in vivo* and IVF derived embryonic cell colony.

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