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# CRISPR mediated BMP15 gene knockout in Caprine granulosa cells

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**Abstract**
BMP15, an oocyte derived protein, is a potent stimulator of granulosa cell mitosis. Site specific mutations of BMP 15 gene leads to increase in ovulation rate and litter size in sheep. Such mutations in BMP15 gene has not been observed in caprine species. Genetic control of fecundity is a key area needs to be addressed in caprine species. As a part of study of introduction of $FecX^{Gr}$ mutation in BMP15 gene in caprine zygotes to increase the fecundity, a prevalidation of knockout of BMP 15 gene was carried out in granulosa cells using CRISPR-Cas gene editing technology. In the present study CRISPR-Cas9 was used to knockout $FecX^{Gr}$ locus of BMP 15 gene in granulosa cells of goat. Granulosa cells were cultured, $FecX^{Gr}$ specific guide was designed. PCR amplification of the DNA template containing the sgRNA encoding sequence under the control of aT7 promotor was done which was followed by in vitro transcription to synthesize sgRNA. CRISPR components in the RNP (ribonucleoprotein) format was delivered via electroporation for knocking out of the desired gene in the cultured granulosa cells which was later confirmed by T7Endonuclease test.

**Keywords:** BMP15, CRISPR/Cas9, $FecX^{Gr}$, Granulosa cells

## 1. Introduction
Goat plays important role in generating income, employment and nutritional security for the poor farmers. With increasing human population and the demand supply gap for milk and meat production, the profitable goat rearing needs be encouraged. Increasing litter size by scientific manipulation is one way to increase the profitability in goat rearing.

The major genes responsible for prolificacy in sheep comprise of Booroola, BMP15 and GDF9 (Galloway *et al*., 2000; Souza *et al*., 2001) [8, 24] and certain mutations in these fecundity genes have been shown to regulate ovulation rate in the sheep. BMP15 an oocyte-derived protein, belongs to the transforming growth factor-beta superfamily. The BMP15 or FecX gene is expressed in the oocyte of many species including goat (Juengel *et al*., 2004) [11]. FecX gene is present in the X chromosome. BMP-15 strongly stimulates mitosis and proliferation of granulosa cell and interestingly its mitogenic effect is independent from follicle-stimulating hormone (Otsuka *et al*., 2000) [20].

There are several mutations in BMP15 gene discovered in various sheep breeds including $FecX^{H}$ (Hanna), $FecX^{B}$ (Belclare), $FecX^{I}$ (Inverdale), $FecX^{R}$ (Rasa Aragonesa), $FecX^{G}$ (Galway), $FecX^{L}$ (Lacaune), $FecX^{Bar}$ (Tunisian barbarine), $FecX^{Gr}$ (French Grivette), and $FecX^{O}$ (Polish Olkuska), (Galloway *et al*., 2000) [8] (Hanrahan *et al*., 2004) [9] (Lassoued *et al*., 2017) [15] (Demars *et al*., 2013) [6]. In fact, BMP15 gene is one of the most polymorphic loci among the genes affecting prolificacy in sheep.

Heterozygous ewes with $FecX^{H}$, $FecX^{bar}$ $FecX^{I}$, $FecX^{G}$, $FecX^{R}$, $FecX^{L,}$ and $FecX^{B}$ the mean ovulation rate increases in different breeds of sheep while in homozygous condition all phenotypes are sterile (Bodin *et al*., 2007; Juengel *et al*., 2013; Demars *et al*., 2013; Liu *et al*., 2015; Kaczor, 2017) [3, 12, 6, 16, 13]. $FecX^{Gr}$ and $FecX^{O}$ are the only two known FecX mutations known so far wherein both homozygous and heterozygous phenotypes causes increase in the ovulation rate and thus mean litter size without any sterility. $FecX^{Gr}$ mutations are found in the exon 2 of BMP-15. In case of $FecX^{Gr}$ mutation C to T change occurs at nucleotide 950 leading to conversion of threonine to isoleucine in exon 2 of BMP-15 in the French Grivette sheep. Substitutions of polar amino acid by nonpolar amino acid suspected to modify its three dimensional molecular structure and affecting the inherent properties of BMP15 protein.

FecX mutations *viz*. $FecX^{Gr}$ were absent in different goat breeds worldwide including India and had the wild type pattern only without any mutation (Chu *et al*., 2007; Hua *et al.*, 2008; Tejangookeh *et al*., 2009; Polley *et al*., 2009; Ahlawat *et al.*, 2013) [4, 10, 5, 21, 1]. Introduction of such mutation ($FecX^{Gr}$) in goat may lead to increased reproductive efficiency in goats also.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas (CRISPR associated) system is presently the excellent genome editing technology, having higher degree of specificity, precision, simplicity and economy over other similar tools. Harnessing the simplicity of CRISPR- Cas technology, FecX mutation can be introduced into the goat. The technology has already been utilised for the successful generation of ovine embryos consisting booroola mutation (Zhang *et al*., 2017) [27]. Lamb with Booroola gene ($FecB^{B}$) mutation has been produced by using CRISPR-Cas 9 (Zhou *et al.*, 2018) [28].

For enhancing the profitability of the farmers in goat sector, it necessary to improve the reproductive efficiency of the caprine species. As a part of study for generating BMP- 15 gene edited quality goat zygotes, the present study validate the BMP 15 gene knockout in granulosa cells.

## Materials and Methods

### Granulosa cell culture

The goat ovaries, collected from an abattoir, were transported to the laboratory at 37°C in sterile physiological saline containing antibiotic streptopencillin (Sigma, P4333) followed by washing twice with 1X PBS with streptopencillin. A primary granulosa cell culture was carried out (Fig1) as described by Babitha *et al*. (2014) [2]. In brief, follicular fluid was aspirated from large antral follicles using sterile needle and the contents were transferred into a petridish containing 1X PBS. The cell suspension was washed twice by centrifugation at 1500 rpm for 5 minutes. The obtained cell pellet were washed thrice in the culture media containing Dulbecco's modified Eagle's medium, 10% foetal bovine serum and 1% antibiotic-antimycotic solution at 1500 rpm for 5 minutes. Cell viability was checked with trypan blue exclusion dye technique. The 5 ml cell suspension in the culture media were transferred to T25 flask with an inoculation density of 5x $10^{5}$ cells and kept in 38.5 $^{0}$C at 5% $CO_2$.

### Production of $FecX^{Gr}$ knockout granulosa cells

CRISPR-Cas 9 technology was used for knocking out the granulosa cells. CRISPR-Cas9 machinery was electroporated as described below.

### Designing and synthesis of single guide RNA (SgRNA)

$FecX^{Gr}$ specific SgRNA were designed using available software. The SgRNA synthesis was performed according to an established protocol (Kumar *et al.,* 2020). The forward primer for SgRNA synthesis was designed consisting of T7 promotor sequence (17bp), $FecX^{Gr}$ specific guide sequence (20bp, Table 1), and scaffold template annealing sequence (15bp). The DNA template for $FecX^{Gr}$ SgRNA was produced by PCR amplification (denaturation at 98°C 1 min, reannealing at 60°C for 30 sec, elongation at 72°C for 30sec (Fig2). In vitro transcription (IVT) was performed to generate SgRNA followed by its purification (Takara Bio USA, Inc., 632636). The SgRNA was quantified using Nanodrop spectrophotometer (Eppendorf) and the quality was assessed by agarose gel (2%) electrophoresis (Fig3)

**Table 1:** Primers and Guide RNA sequence used in the experiment.

| | | |
|---|---|---|
| T7E1 Primers | Forward | AAAGCCTTCCCTGTTGCCCAA |
| | Reverse | CATTTGCCTCAATCAGAAGGATGC |
| Guide sequence | | CTATACCCCAAACTACTGTA |

### Electroporation of granulosa cells with CRISPR-Cas9 and *in vitro* culture

### Preparation of RNP complex

The Cas9 (TruecutCas9 protein $V_2$, Invitrogen, A36498) and SgRNA were delivered using Neon transfection system (Invitrogen). Electroporation of the CRISPR mix was done in the cultured granulosa cells (70-80% confluency) at 1200 volt with 20ms pulse width and 2 pulse frequency as described by established protocol (Kumar *et al.,* 2020). The transfected granulosa cells were cultured for 72 hours at 38.5°C and 5% $CO_2$.

### Mutation detection byT7E1 assay

The genomic DNA of the cultured granulosa cells were extracted using components from Invitrogen kit (A24372) following manufacture recommendations. T7E1 primers (listed in table-1) were designed in silico using FastPCR software. The target region was PCR amplified at 95 $^{0}$C 2 minutes followed by 40 cycles of 95 $^{0}$C 30s, 60 $^{0}$C 30s and 72$^{0}$C 45s followed by 72$^{0}$C for 5 minutes using mastermix (Promega). The T7 endonuclease assay of the edited amplicon was carried out by using T7 Endonuclease I (NEB, M0302S), and the product was analysed in agarose (2%) gel electrophoresis.

## Results and Discussion

The T7E1 assay results shown in the figure (4) clearly depict the knockout of the BMP15 gene. The heteroduplex formed as a result of denaturation followed by reanneling of the PCR amplicon obtained from the edited genomic DNA was cut by T7 endonuclease-1 which clearly showed the cleaved bands in the agarose gel electrophoresis.

In the mammalian cells, disruption of the DNA leads to activation of DNA damage response pathways to repair the damaged DNA that mainly comprises NHEJ (Non homologous end joining) and homology directed repair (HDR) pathways (Sansbury *et al.,* 2019; Yang *et al.,* 2020). NHEJ is the primary pathway employed by the mammalian cell to rejoin the broken DNA. This happens because NHEJ is faster, active throughout cell cycle and has the property of suppressing the HDR pathway (Yang *et al.,* 2020). However; this mechanism is error prone because of the introduction of insertions and deletions (INDELS) at site of repair (Sansbury *et al.,* 2019). This process has been explored (harnessed) by the scientists for knocking out of a gene by using CRISPR/Cas technology which causes the targeted mutagenesis resulting into formation of double stranded break (DSB) at the desired site (Sansbury *et al*., 2019) and subsequent repair by NHEJ. Conversely the HDR is high fidelity repair mechanism which occurs during S/G2 phases of the cell cycle in the presence of donor DNA or undamaged sister chromatid. Researcher explored this mechanism for insertion of interested mutation by using HDR template to repair the double stranded breaks caused by CRISPR/Cas9.

The various genome editing technology include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR. CRISPR/Cas9 genome

editing technology is highly efficient, accurate and easy in use for genome editing (Manghwar *et al.,* 2019) [17] and in the present study the tool has been utilised for successful knocking out the BMP15 gene.

The various factors that influence this genome editing efficiency by CRISPR/Cas9 technology include the proper designing of the guide sequence, quality of sgRNA and Cas9, the format used for delivery of CRISPR/Cas9, method of transfection etc. In our experiment we used RNP format delivered through electroporation which has been already optimised in our lab for granulosa cells (Kumar *et al.*, 2020).

The delivery methods of CRISPR/Cas9 include DNA, mRNA, and ribonucleoprotein (RNP) formats. The later format is a complex of pre-formed Cas9 protein and gRNA that does not require transcription or translation to occur in the cell. RNP format which was utilised in our study is the most efficient method of delivery, comprising high indel production and gene disruption and have the least chance of off-target effects owing to its speed and efficiency that can work within few seconds when coupled with transfection methods like electroporation (Liang *et al.,* 2015; Ringer *et al.*, 2018; Fajrail *et al.,* 2020) [22, 16].

Vehicles used to deliver CRISPR/Cas9 can be broadly classified into viral (Adeno-associated viruses, Lentiviruses etc.) or nonviral approaches. The nonviral approach includes various chemical (Lipid based delivery) and physical (microinjection and electroporation) delivery strategies and electroporation used in our experiment is an efficient delivery method in population of cells (Yip *et al*., 2020) [26].

FecX$^{Gr}$ is an important locus in the BMP 15 gene because this is responsible for increase in ovulation rate and litter size in sheep (Demars *et al.,* 2013) [6]. Substitutions of polar amino acid cytosine by nonpolar amino acid thymine is suspected to modify its three dimensional structure and affecting the intrinsic properties of BMP15 protein (Demars *et al.,* 2013) [6] which may be responsible for decreased BMP15 signalling. Reduced BMP-15 signaling leads to reduced granulosa cell mitosis and increase in the FSH sensitivity. Follicular sensitivity for FSH increases because BMP 15 tends to reduce FSHR expression (Otsuka *et al.,* 2001) [18, 19]. The increased follicular sensitivity of FSH promotes selection of additional follicles than normal which are smaller in size resulting in the enhancement of ovulation rate. We have disrupted the BMP 15 gene by using CRISPR/Cas technology and in future study there is always a possibility of insertion of desired mutation also. The present study will pave a way for knocking out of such gene in embryo and subsequent introduction of desired mutation to produce quality animals.

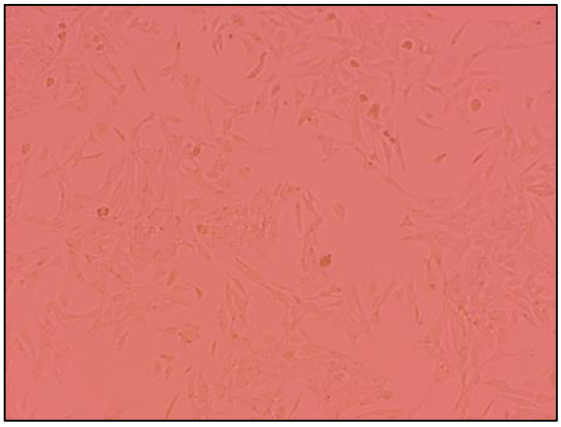

**Fig 1:** Caprine Granulosa cell culture

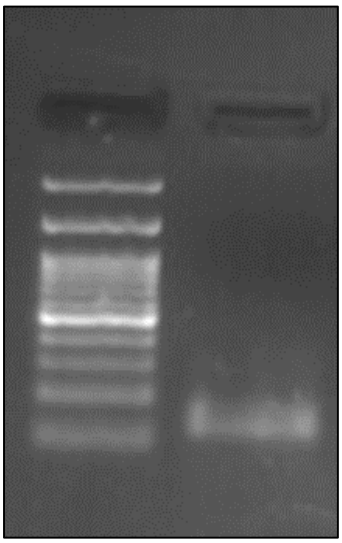

**Fig 2:** DNA template for FecX $^{Gr}$ SgRNA

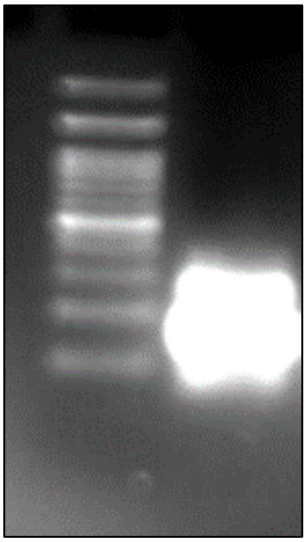

**Fig 3:** FecX $^{Gr}$ Specific SgRNA

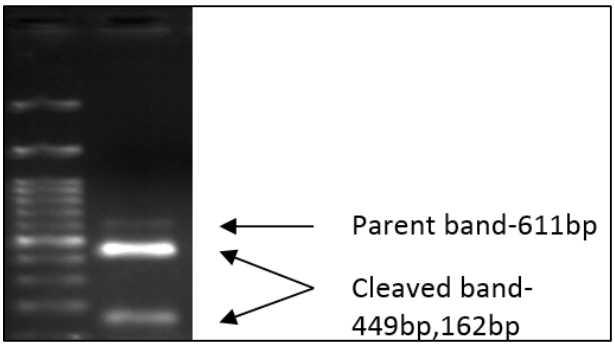


**Fig 4:** T7E1 assay products

## Conclusion

CRISPR-Cas 9 technology has been utilised for knocking out the BMP15 gene in the caprine granulosa cells and may be further used to unravel the functionality and expression studies of the gene. In addition the study leads a way to introduce the desired mutation in this fecundity gene to produce viable embryos and subsequently quality off-springs.

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