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# Expression profile of BMRPR1B gene in developing ovarian follicles of cross-bred Malabari goats

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**Abstract**
The study was performed to compare the expression of *BMPR1B* gene in whole follicles as well as granulosa cells from developing follicles of cross-bred Malabari goats. The study was conducted in about 120 ovaries of the cross-bred Malabari breed of goats retrieved from abattoir. The follicles were classified into large (>3 mm), small (1-3 mm) and very small (<1mm) based on the measurements taken using Stereo Zoom Microscope. The small and large follicles were isolated based on size for RNA isolation. Granulosa cells were also collected from the follicles of the same category by the aspiration method. The expression studies were carried out by real-time PCR. When the study was in whole follicles, *BMPR1B* gene was found to be 0.40 folds ($p<0.05$) down-regulated in large follicles when compared with the small antral follicle. However, the expression level of *BMPR1B* did not differ between the follicles of different sizes when granulosa cells were subjected to analysis. Therefore, it was concluded that all the compartments in the ovarian follicle contribute for *BMPR1B* expression and there was downregulation of the expression in this protein as the follicles develop to final stages.

**Keywords:** BMPR1B, follicles, granulosa cells, crossbred malabari breed, goats

## 1. Introduction

It has been proved that goats are the most adaptable of all domestic livestock. Among all the production traits, adaptability is most important. Adaptability is a trait with low heritability and, therefore, difficult to improve by selection (Baker and Gray, 2004) [1]. Goats get well acclimatized to a variety of environments; goat farming is relatively easy and economical. Henceforth, commercial farming becomes more profitable when the litter size is more. When there is multiple ovulation, litter size also increases considerably. Twinning occurs commonly in goats. However, quadruplet, quintuplet and sextuplet kids are very rare in goats (Palai *et al.,* 2013) [2]. In various reports, the estimated heritability of this trait is very low. Hence, selective breeding cannot be adopted. Molecular techniques emerged as a new concept to overcome this limitation. A non-prolific breed can be transformed into a prolific breed by introducing a prolificacy gene (Montgomery *et al*., 1995; Pardeshi *et al*., 2005) [3, 4].

Extensive studies were conducted in sheep and mutations in some genes were identified as responsible for prolificacy in certain breeds. Juengel *et al*. (2004) [5] identified TGF- β family as a key regulator of ovarian cell function in sheep. The members of the TGF-β superfamily have a major role in follicle development (Mcnatty, 2004; Gilchrist *et al*., 2004; Juengel and Mcnatty, 2005) [6, 7, 8]. The mutated genes, in brief, includes Booroola gene or Bone Morphogenetic Protein Receptor 1B (*BMPR1-B*), Growth Differentiation Factor 9 (*GDF9*) and Bone Morphogenetic Protein 15 (*BMP15*) (Davis, 2004) [9]. Among prolificacy genes, *BMPR1B* gene, also known as booroola or FecB or Activin-like Kinase located on chromosome 6 of sheep (Mulsant *et al*., 2001) [10] was identified first (Souza *et al*., 2001) [11], and the mutation at 746$^{th}$ base of coding region caused variation in ovulation rate in ovine species (Souza *et al*., 2001; Polley *et al*., 2010) [11, 12]. The role of BMPR1B in ovarian functions is now very clear, and mutation in *BMPR1B* is associated with increased ovulation rate in ewes (Mulsant *et al*., 2001; Souza *et al*., 2001; Fabre *et al*., 2003) [10, 11, 13] and impaired follicular development in mice (Yi *et al*., 2001) [14]. The studies on goat prolificacy are relatively less when compared to sheep as well as cattle (Evans, 2004) [15]. However, the genetic predisposition of twinning and triplet in goats follows a similar pattern to that of sheep (Hua *et al*., 2008) [16].

The Malabari breed of goat is a breed of Kerala, India otherwise called as Tellicherry goats. True-bred animals are found in the districts of Thalassery, Kasargod, Kannur, and Kozhikode in Kerala. They have no uniform colour. However, the majority are either white or black and

some with mixed colours and brown colour with patches also seen. They are of medium size. Twinning and triplets are common in this breed. Acharya (1982) [17] classified the goat breeds of India into different categories, and Malabari breed comes under high prolificacy breed with 42% twinning.
The present study is aimed to study and compare the pattern of expression of *BMPR1B* gene in whole follicles as well as granulosa cell from developing follicles of cross-bred Malabari goats.

## 2. Materials and Methods

### 2.1 Animals and sample collection

Around 120 ovaries of Cross- Bred (CB) Malabari goats were collected from a local slaughter house and brought to the laboratory within half an hour in chilled phosphate buffered saline (PBS) solution for further examination. The history of animals was unknown. The ovaries were washed thoroughly in PBS prior to further evaluation. The adipose tissue surrounding the ovaries was removed by dissection to clear the ovaries. The length and width of each ovary were measured with the help of Vernier Callipers, and their weights were also recorded using an electronic weighing machine.

### 2.2 Classification of ovarian follicles and isolation of whole follicles

The healthy follicles were measured and compared as per method described by Pramod *et al*., (2013) [18]. The total number of visible follicles on the surface of the ovary was counted and recorded. Then the follicles were classified (Silva *et al*., 2004; Pramod *et al*., 2013) [19, 18] into large (>3 mm), small (1-3 mm) and very small (<1mm) based on the measurements taken using Stereo Zoom Microscope (Vision Engineering, UK). The small and large follicles were isolated based on size and stored at -80$^0$C in RNAlater solution (Ambion, Austin) for RNA isolation.

### 2.3 Granulosa cell isolation

The follicular fluid from each healthy follicle was aspirated using sterilized 18-gauge hypodermic needles and syringes. Oocytes were removed from the follicular fluid by searching them under a stereozoom microscope. The follicular fluid collected from follicles were pooled according to their size in separate tubes and centrifuged at 8000 g for 3 min, after which the supernatant was separated. To the pelleted cells, RNAlater solution (Ambion, Austin) was added and stored at -80 $^0$C.

### 2.4 RNA isolation and cDNA synthesis

After thawing, the follicles and the granulosa cells were separated from RNAlater and immediately processed for RNA isolation. Three follicles were pooled and used for RNA isolation in the case of small follicles, and one follicle was used in the case of large follicles. The total RNA from ovarian follicles and granulosa cells was isolated using the RNeasy mini kit (Qiagen, Life technologies, USA) as per manufacturer's instruction with slight modifications. In brief, ovarian follicles were homogenized in lysis buffer (RLT buffer with β-mercaptoethanol) using tissue homogenizer (IKA). After thorough homogenization, an equal quantity of ethanol (70%) was added and mixed well by pipetting and then the mixture was transferred to spin column membrane and centrifuged for four min at 8000g. After discarding the flow-through, 700µL of wash buffer (RW1) was added and centrifuged for three minutes at 8000g followed by two times wash with 500µl wash buffer (RPE) and centrifuging each time for four min at 8000g. Then the spin column membrane was transferred to a collection tube and centrifuged for four min at 8000g. The membrane was washed, and 40µL nuclease-free water was added to the membrane and incubated at room temperature for 2 min and then centrifuged at 8000g for one min for elution of RNA.
The concentration and purity of RNA were determined spectrophotometrically (Thermo Scientific NanoDrop™1000 Spectrophotometer). The integrity of the RNA samples was ascertained using agarose gel (0.8%) electrophoresis. After analyzing the quantity and quality, the RNA samples were stored at -80$^0$C until cDNA synthesis. The total RNA was reverse transcribed into cDNA using the maxima first strand cDNA synthesis kit (Thermo Scientific, USA) as per manufacturer's protocol using oligo $(dT)_{18}$ primers and random hexamers. For cDNA synthesis, 500ng of RNA was reverse transcribed. The samples were thawed, and the reaction was set up as per the standard protocol of maxima first strand cDNA synthesis kit. The product of the first strand cDNA synthesis was stored at -80 $^0$C until use.
Exon-spanning primers (Table 1) were designed using online NCBI primer design software (Primer3, http://bioinfo.ut.ee/ primer3/) and specificity of the primer was checked using BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/). Beta-actin (*β-actin*) was used as a reference gene (Pramod *et al*., 2013) [18]. PCR reactions were carried out in 20 µL of a mixture. To 19 µL of master mix, 1 µL of template DNA was added. The tubes were spun briefly and placed in a thermal cycler. The details of primers used, reaction mix and PCR conditions used were given in Table 1, 2 and 3 respectively. After detecting the bands by electrophoresis, the product size of *BMPR1B* and *β-actin* was confirmed as 346bp and 54bp respectively (Fig 1). The PCR product was outsourced for sequencing and the sequence was verified using BLAST for the amplified product. Annealing temperature determined for both the genes was referred for further real-time PCR reaction.

### 2.5 Quantitative Real-Time PCR Analysis (qRT-PCR)

Expression of *BMPR1B* gene in large and small antral follicles and the isolated granulosa cell was quantified using real-time PCR (qRT-PCR). The qRT-PCR was carried out in a thermal cycler (Eco-Illumina Real-time Thermal cycler, USA) and was pre-programmed for temperature and cycling conditions specified (Table 5). All PCR reactions were performed in 12 µL volume in triplicates (technical replicates). In addition, one non-template control (NTC) for each gene and Reverse Transcription minus (RT minus) control for each sample and negative control (with only nuclease free water) were also included in the reaction (Table 4). A suitable plate setting was done prior to the start of the experiment. Master Mix with template DNA was loaded into the designated well of 48 well PCR plate, and the plate was sealed with an Eco-optical seal. The plate was centrifuged at 250xg for 30 Sec and was placed in Eco-Illumina Real-time Thermal cycler.
The expression of *BMPR1B* and *β- Actin* was studied using SYBR green chemistry (Maxima SYBR green qPCR master mix (Thermo Scientific, USA). qRT-PCR cycling conditions are given in Table 5. The expression of the target gene (*BMPR1B*) was compared with the reference gene (*β-actin*). For relative quantification by comparative method, the values were expressed relative to control sample called calibrator. β-

Actin was used as internal control. The Ct of the target gene and Ct of control gene were determined for each sample and calibrated (ΔΔCt method) (Livak and Schmittgen, 2001) [20]. The result is given in Table 6.

ΔCt = Ct (target gene) – Ct (reference gene)
ΔΔCt = ΔCt (test sample) – ΔCt (reference sample)
$RQ = 2^{-\Delta\Delta Ct}$

A melt curve analysis was performed after the reaction for checking the specificity of amplification.

## 3. Statistical analysis

Levels of expression of Caprine *BMPR1B* mRNA were analysed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) [20] and expressed as fold change relative to small follicles from the cross-bred Malabari breed of goat.

## 4. Result and Discussion

The current study evaluated the expression of *BMPR1B* gene in the ovaries of CB Malabari goats. Quantitative real-time PCR (RT-qPCR) analysis revealed differential expression of mRNAs encoding for *BMPR1B*. The gene was found to be 0.41 folds ($p<0.05$) down-regulated in large antral follicles compared to small antral follicle (Table 6). This downregulation in large antral follicles was found to be statistically significant when the whole follicle was isolated. The current result is in accordance with the studies of Gasperin *et al*. (2014) [21] in cattle, wherein they observed that *BMPR1B* expression was more in subordinate follicles and atretic follicles compared to the dominant follicle and they suggested that as a clear indication for the inhibitory role of *BMPR1B* in differentiation and steroidogenesis of follicle..

Silva *et al*. (2005) [22] and Lima *et al*. (2012) [23] reported that *BMPR1B* was expressed at all stages of follicular development in goats, and they proposed that BMP receptors long with other members of TGF β family might have a regulatory role in the development of goat ovarian follicle. Erickson and Shimasaki (2003) [24] also detected the presence of BMPR1B in antral follicles of rats. BMP receptors in granulosa cells regulate biological responses (Shimasaki *et al*., 1999) [25].

The expression of *BMPR1B* in ovaries of rats has been reported in granulosa cells as well. However, BMPR1B was absent in late preantral follicles and Graafian follicles (Shimasaki *et al*., 1999) [25] which support our findings of downregulation of *BMPR1B* expression in the large follicle. *BMPR1B* expression was reported in pigs (Quinn *et al*., 2004) [26], sheep (Souza *et al*., 2002) [27] and cattle (Glister *et al*., 2004) [28]. The Present study also demonstrated the presence of *BMPR1B* in whole follicles (small and large) and granulosa cells isolated from different stages of follicles. However, the present study was in disagreement with the observations depicting the upregulation of *BMPR1B* expression in granulosa cells of the large antral follicle of sheep (Chen *et al*., 2009) [29] and goats (Lima *et al*., 2012) [23]. However, the observations on the presence of *BMPR1B* in all antral follicular categories agree with the observations of Lima *et al*. (2012) [23]. Pramod *et al*. (2013) [18] also compared *BMPR1B* expression in small and large follicles in Black Bengal and Sirohi breeds of goats. However, they could not find any difference between *BMPR1B* expression in small and large follicles which is in contrary to the present observations. *BMPR1B* was expressed uniformly at high levels in all granulosa cells in all stages of follicles (Shimasaki *et al*., 1999) [25]. In the present study, there was no significant difference in *BMPR1B* expression between the granulosa cells of small and large follicles. Therefore, it was concluded that all the compartments in the ovarian follicle contribute for *BMPR1B* expression and there was downregulation of the expression in this protein as the follicles develop to final stages.

## 5. Tables

**Table 1:** Primers description

| Gene Name | Primer sequence (5”- 3”) | Primer Length (bp) | Product Size (bp) | Accession No |
|---|---|---|---|---|
| BMPR1B | GGAGCAGTGACGAGTGTCTC<br>TTCTCCCGCCTACAGACAGA | 20<br>20 | 346 | NM_001285575.1 |
| ACTB | AGCTCGCCATGGATGATGA<br>TGCCGGAGCCGTTGT | 19<br>15 | 54 | DQ661647 |

**Table 2:** Reaction mix for standard PCR reactions

| Components | Volume (µL) |
|---|---|
| Template (cDNA) | 1.00 |
| 10 X PCR buffer | 2.00 |
| MgCl2 (25 mM) | 1.20 |
| dNTP (2mM) | 2.00 |
| Forward primer (10 pM/µl) | 1.00 |
| Reverse primer (10 pM/µl) | 1.00 |
| Jumpstart Taq DNA polymerase (2.5 units/µl) | 0.10 |
| Nuclease-free water | 11.70 |
| Total | 20.00 |

**Table 3:** PCR conditions for amplification

| Steps | | Temperature | Time |
|---|---|---|---|
| Initial denaturation | | 95 $^0$C | 5 min |
| 35 cycles of | Denaturation | 95 $^0$C | 45 sec |
| | Annealing | 64 $^0$C | 45 sec |
| | Extension | 72 $^0$C | 1 min |

**Table 4:** Optimized concentrations of qRT-PCR Master Mix (12 µL) for *BMPR1B* and *β-Actin* gene

| Components | Volume (µL) |
|---|---|
| Template (cDNA) | 0.5() |
| Maxima SYBR Green qPCR Master Mix (2X) | 6.25 |
| Forward Primer (10 pM/ µl) | 1.0 |
| Reverse primer (10 pM/µl) | 1.0 |
| Nuclease free-water | 3.75 |

**Table 5:** qRT-PCR conditions for *BMPR1b* gene and *β-actin* gene

| Steps | | Temperature | Time |
|---|---|---|---|
| Initial denaturation | | 95 $^0$C | 3min |
| 40 cycles of | Denaturation | 94 $^0$C | 30sec |
| | Annealing | 64 $^0$C | 30sec |

**Table 6:** Expression of *BMPR1B* in small and large antral follicles

| Tissue Size | Tissue Type | Mean $C_T$ ± S.E | | $\Delta C_T$±S.E | $\Delta\Delta C_T$±S.E | Fold change from control ($2^{-\Delta\Delta CT}$) | p-Value |
|---|---|---|---|---|---|---|---|
| | | BMPR1B | β- Actin | | | | |
| GC | Small | 17.80 ±0.33 | 19.89 ±0.22 | -2.09 ±0.51 | 0.00 ±0.52 | $1^{ns}$ | 0.84 |
| | Large | 17.4 ± 0.22 | 19.40± 0.29 | -2.01± 0.36 | 0.08 ±0.28 | $0.95^{ns}$ | |
| WF | Small | 16.86 ±0.14 | 20.27 ± 0.16 | -3.41± 0.21 | 0.00 ± 0.21 | 1 | 0.007 |
| | Large | 19.15± 0.73 | 21.26 ±0.51 | -2.11± 0.89 | 1.29 ± 0.89 | $0.41^{**}$ | |

GC- Granulosa cells; WF- Whole follicle
**- *P*< 0.05

## 6. Figure

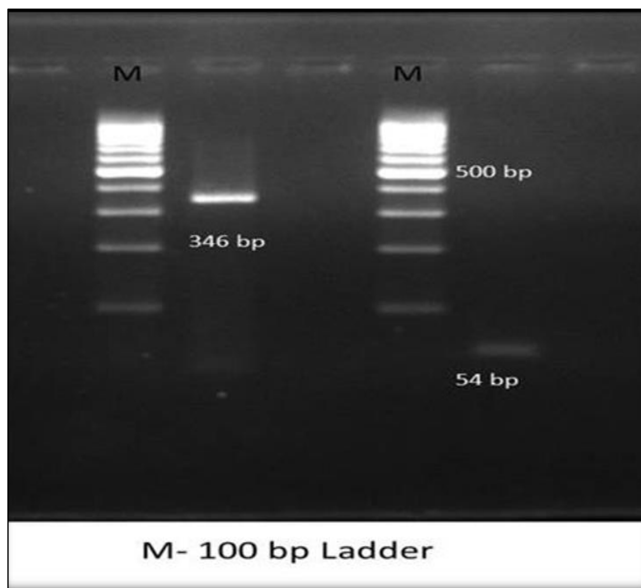


**Fig 1:** Agarose gel electrophoresis of PCR products of *BMPR1B* (346bp) and *β Actin* (54bp)

## 7. Conclusion

In the present study, *BMPR1B* expression was found to be down-regulated in large antral follicles compared to small ones, which suggests an inhibitory role for BMPR1B in dominant follicles. However, there was no significant difference in *BMPR1B* expression between the granulosa cells collected from small and large antral follicles. Therefore, it can be concluded that all compartments in the ovarian follicle contribute for *BMPR1B* expression and there was downregulation of the expression of this protein as the follicles develop to final stages.

## 8. Conflict of Interest Statement

The authors declare that there is no any conflict of interest in this manuscript.

## 9. Acknowledgments

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