Genetics and Molecular Research

Association of growth factor receptor-bound protein 10 gene polymorphism with superovulation traits in Changbaishan black cattle

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ABSTRACT. The application of assisted reproductive technology in animal production benefits the economy and conservation of biological resources. Single nucleotide polymorphism (SNPs) was used as predictive markers for breeding and reproduction. In the present study, we examined the association between a SNP of the *grb10* gene and superovulation traits in cattle. Sequencing results indicated a point mutation and statistical analysis showed a significant association of the mutation with superovulation traits. The high number of embryos collected from the heterozygotes suggested that the mutation in the

grb10 gene exerted a significant effect on the number of embryos recovered although the quality was not affected. The *grb10* gene may serve as a useful biomarker for donor selection.

Key words: *grb10*; Polymorphism; Superovulation traits; Cattle

INTRODUCTION

Female infertility and low birth rate are significant public health issues with profound social, psychological, and economic consequences (Hung et al., 2016). Assisted reproductive technologies (ART), including superovulation, artificial insemination, and embryo transfer have been used worldwide to contribute to the dissemination of breeds that are at a risk of extinction, and to an accelerated genetic improvement in domestic animals (Meuwissen, 1998). However, these technologies are reaching a plateau; no remarkable progress was made in the last twenty years. Researchers continually optimize techniques and processes to improve fertilization rates and live births. Many factors affect the efficiency of ART, including the variability in hormone response (Perez Mayorga et al., 2000), developmental synchrony, and genetics (Spearow and Barkley, 1999; Spearow et al., 1999).

As a major component of ART, superovulation has been used for bovine embryo production for more than 60 years. Improvement of herds by traditional selection is difficult because of the low heritability and long reproductive cycle. There is increasing preference for single nucleotide polymorphisms (SNPs) as predictive markers for ovarian responses that promote ovulation (Fauser et al., 2008). This candidate gene approach provides an early breeding tool to accelerate improvements in reproduction.

Several candidate genes, including follicle stimulating hormone receptor (*FSHR*) (Perez Mayorga et al., 2000; Simoni et al., 2002; de Castro et al., 2003), have been reported to participate in the superovulation, and it was suggested that ovarian response to hormone stimulation is a polygenic trait (de Castro et al., 2004). Growth factor receptor-bound protein 10 (*grb10*) is a highly isoform-specific and tissue-specific imprinted gene, and was reported to play an important role in mammalian feto-placental growth and development (Garfield et al., 2011). Although GRB10 is a critical regulator of many physiological and pathological processes, the impact of this gene on female reproduction has not been elucidated.

Consequently, in the current study, *grb10* was selected as a candidate gene for the association with superovulation traits. Here, SNPs of *grb10* were studied by PCR-restriction fragment length polymorphism (PCR-RFLP) and sequencing. The present study demonstrated a strong relationship between *grb10* gene polymorphism and superovulation traits in Changbaishan black cattle. Real-time PCR data showed *grb10* mRNA expression at various developmental stages of the embryo. This study suggested an important role of *grb10*in bovine oocyte maturation and early embryonic development.

MATERIAL AND METHODS

Chemicals and reagents

Unless otherwise specified, all chemicals and reagents were purchased from Sigma-Aldrich (Shanghai, China).

Animals and superovulation treatment

Changbaishan black beef cattle originate from the hybridization of local Chinese and Japanese black cattle lines. We conducted experiments from June to September 2011 in Jilin Province, China (127.33°E, 43.67°N). All cattle subjects received trans-rectal palpation to exclude gynecologically abnormal individuals before the superovulation treatment.

Superovulation was induced in the subjects by a 16-day FSH-CIDR [EAZI Breed™ CIDR (progesterone), cattle insert]-Prostaglandin-Luteinizing hormone-releasing hormone protocol, provided by the Beijing AnBo Embryo Biotech Center (Deng et al., 2015a,b). Each subject in estrus received two doses of frozen semen, artificially collected from one fertile Wagyu bull at 12-h intervals. Six days after artificial insemination, the embryos and ova were recovered by a standard, nonsurgical uterine flushing technique. The recovered embryos were evaluated at 70X magnification at a light microscope based on compactness and homogeneity of cell mass and the cells were graded according to Lindner and Wright (1983).

Briefly, available cells were classified as M1 (morulae, grade 1: embryos with single or small extruded blastomeres comprising less than 15% of total cellular material), M2 (morulae, grade 2: large cells or individual blastomeres extruded from the embryonic mass, constituting more than 15% but less than 50% of the total cellular material), or the blastocoel. Despite containing nucleated blastomeres, embryos that were severely underdeveloped to be considered viable, were classified as degenerate. An ovum was designated as unfertilized in the absence of cleavage or if all cytoplasmic fragments lacked nuclei. Experiments herein were performed in accordance with "Guiding Principles in the Use of Animals", adopted by the Chinese Association for Laboratory Animal Sciences. All animal treatments and protocols applied here were approved by the Animal Experiment Ethical Management Committee at Jilin University.

Genomic DNA extraction

Immediately after the recovery of embryos, blood samples were collected from the jugular vein in sterile tubes containing ethylene diamine tetra acetic acid. From these samples, genomic DNA was extracted using the Blood Genomic DNA miniprep kit (Axygen Co., Hangzhou, China), and detected using 1% agarose gel electrophoresis. Dilute DNA samples were stored at -80°C until further use.

Primer synthesis and PCR amplification

Primers were designed using the Oligo 6.0 software (Molecular Biology Insights, Inc., CO, USA) (http://www.oligo.net/). The primers were 5'-CTCGGAGACAGTGCCCCTC-3' upstream and 5'-GGTGGTTCAGGGACCCCATCACAC-3' downstream (Shanghai Sangon Biological Engineering Technology & Services Co., China). The PCR mixture contained 50 ng DNA template, 10 pM upstream and downstream primers, 2.5 mM dNTP mixture, 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase in a 20-μL reaction volume. Amplification conditions were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 30s, 64°C for 30 s, 72°C for 30 s, and a final step at 72°C for 10 min. The amplification products were run on a 1% agarose gel and were visualized using a UV transilluminator.

Identification of genetic variation and sequencing

To detect *grb10* gene polymorphism, we used the restriction endonuclease, *Hin*1II (MBI Fermentas Life Science, Ontario, Canada) for the digestion of PCR products according to the manufacturer protocol. The digestion mixture composed of 10 U enzyme, 5 µL PCR product, 10X digestion buffer, and was maintained at 37°C for 8 h. The digested fragments were run on a 2% agarose gel and their genotypes were classified based on band patterns. The resultant PCR products were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Bovine embryo culture and RT-PCR

Fresh Changbaishan black cattle ovaries were acquired from slaughterhouses and the cumulus-oocyte complexes (COCs) were transferred to droplets of maturation medium and incubated at 39°C in 5% CO_2 for 22-24 h. Mature COCs were washed with D-PBS and *in vitro* fertilization (IVF) stock solution before being transferred to IVF droplets containing the sperm cells. Oocytes were removed after 24 h, washed in D-PBS, shaken for 1.5 min to remove cumulus cells, and transferred to *in vitro* culture droplets at a concentration of 10-15 cells per droplet.

The total RNA was extracted using RNeasy Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer protocol. PrimeScript RT reagent kit (TaKaRa, Dalian, China) was used for reverse transcription to obtain cDNAs. SYBR Green Real-time PCR Master Mix (TaKaRa) dye and MasterCycler RealPlex (Eppendorf, Hamburg, Germany) were used to detect relative levels of the expression of *grb10* related to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). The primer sequences were shown in Table 1.

Table 1. Sequence of qRT-PCR primers used in this study.

Gene	Primer sequence (5'-3')	Product length (bp)
gapdh	F-GCC ATC AAT GAC CCC TTC AT R-TGC CGT GGG TGG AAT CA	70
grb10	F-CAG AAG TCA CTG TGT GGA CGA R-CCT GGA CTA CCA GCT CAT GG	101

Statistical analysis

Data are reported as means ± SE. The statistical significance of differences between groups was determined by independent *t*-test or one/two-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference, post-hoc test, unless otherwise indicated. Statistical significance was set at $P< 0.05$.

RESULTS

Genotypes

Gene fragments of g*rb10* were amplified from genomic DNA of all samples (Figure 1A). According to two-band patterns observed after the digestion reaction, subject cattle were divided into two subtypes: AA and AB. In the coding sequence, the 492nd base change from

C to T is a substitution of the non-polar aliphatic valine by the polar neutral methionine, a polymorphism that forms the restriction site of the *Hin*1II endonuclease. Figure 1B shows DNA restriction fragments at locus C492T generated by *grb10*-*Hin*1II polymorphisms: 293 bp for the AA genotype (296 cattle), and 293, 177, and 116 bp for the AB genotype (4 cattle). Polymorphic sequencing results are detailed in Figure 1C and D.

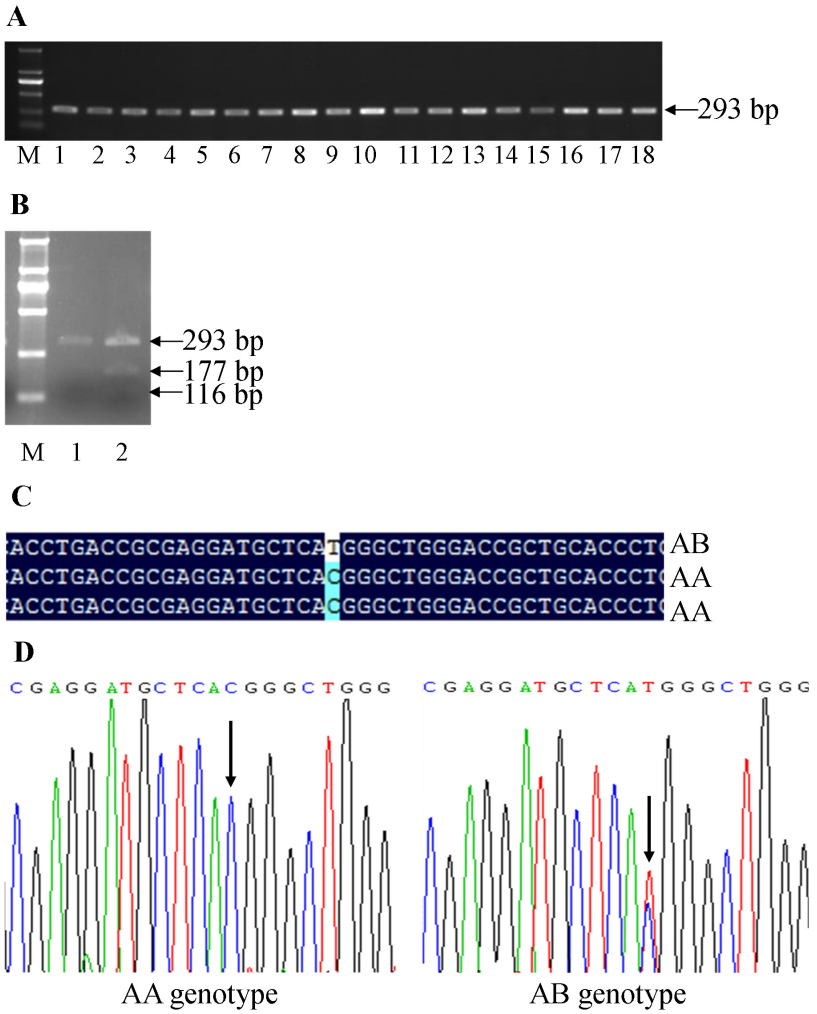

Figure 1. RFLP and sequencing results of *Grb10* gene in Changbaishan black cattle. **A.** PCR products of *grb10* gene fragments analyzed using 1% agarose gel electrophoresis. *Lanes 1-18* refer to 18 PCR products selected from random samples. The arrow shows the expected 293bp band of *grb10* gene fragments amplified from genomic DNA of all samples. **B.** Representative RFLP genotypes of the *grb10* gene fragment analysis using 2% agarose gel electrophoresis. *Lane 1*, with one band at 293 bp, refers to the AA genotype. *Lane 2*, with three bands at 293, 177 and 116 bp, refers to the AB genotype. *Lane M* refers to the DL2000 marker. **C.** Sequence alignment of AA and AB genotypes. Sequence alignment shows a C/T transition in *grb10* gene fragments. **D.** Sequencing of *grb10* gene fragments. Representative sequencing results of different genotypes. Arrows indicate the mutation site and double peaks are observed in the heterozygous AB genotype.

Association of genotypes with superovulation traits

Association analysis between superovulation traits and two *grb10* genotypes are presented in Table 2. Cattle with the AB genotype at the *grb10* locus showed increased numbers of unfertilized embryo (NUE, 1.652 ± 0.147 versus 5.750 ± 2.175, P = 0.002), available embryos (NAE, 8.236 ± 0.383 versus 30.250 ± 3.544, P < 0.001), total embryos (NTE, 12.770 ± 0.447 versus 42.000 ± 5.354, P < 0.001), M2 embryos (NM2, 1.593 ± 0.122 versus 3.750 ± 1.109, P = 0.043), and M1 embryos (NM1, 6.171 ± 0.364 versus 26.250 ± 2.394, P < 0.01). No significant difference was detected according to the ratios, as shown in Table 2.

Table 2. Association of genotypes with superovulation traits.

Superovulation traits	Genotype AA	Genotype AB	P value
NUE	**1.652 ± 0.147**	**5.750 ± 2.175**	**0.002**
NDE	2.909 ± 0.192	6.000 ± 1.225	0.063
NAE	**8.236 ± 0.383**	**30.250 ± 3.544**	**<0.001**
NTE	**12.770 ± 0.447**	**42.000 ± 5.354**	**<0.001**
NM2	**1.593 ± 0.122**	**3.750 ± 1.109**	**0.043**
NM1	**6.171 ± 0.364**	**26.250 ± 2.394**	**<0.001**
NBE	0.435 ± 0.080	0.250 ± 0.250	0.788
PUE	14.1 ± 1.21%	13.0 ± 4.59%	0.918
PDE	22.6 ± 1.24%	14.1 ± 1.93%	0.425
PAE	63.8 ± 1.80%	72.9 ± 6.40%	0.558
PM2	11.8 ± 0.83%	8.73 ± 2.33%	0.664
PM1	48.9 ± 1.79%	63.7 ± 5.41%	0.338
PBE	2.99 ± 0.52%	0.43 ± 0.43%	0.569

Superovulation traits correspond to stereomicroscope observation: NUE = number of unfertilized embryos, NDE = number of degenerated embryos, NAE = number of available embryos, NTE = number of total embryos, NM2 = number of M2 embryos, NM1 = number of M1 embryos, NBE= number of blastulas, PUE = percentage of unfertilized embryos = NUE / NTE x 100%, PDE = percentage of degenerated embryos = NDE / NTE x 100%, PAE = percentage of available embryos = (NM1 + NM2 + NBE) / NTE x 100%, PM1 = percentage of M1 embryos = NM1 / NTE x 100%, PM2 = percentage of M2 embryos = NM2/NTE×100%, PBE = percentage of blastulas = NBE / NTE x 100%. Bold numbers indicate that the cattle from two different genotypes share significant differences.

Gene expression of *grb10* in bovine embryos at different developmental stages

Upon further investigation, we detected *grb10* mRNA in all early bovine embryonic stages (Figure 2) and its expression was the highest in 4-cell embryos, approximately 4.4-fold greater than metaphase II (MII) oocytes (P < 0.001). Expression levels were similar in MII oocytes, 2-cell embryos, and morulae whereas those of 8-16 cell embryos were moderate at approximately 2.3-fold greater than those of MII oocytes (P < 0.05).

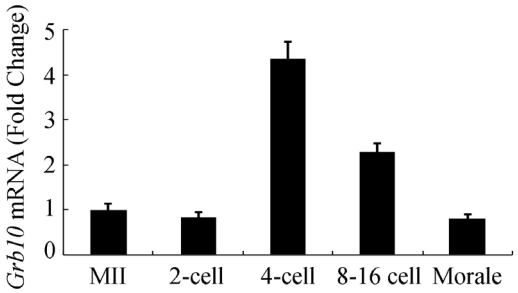

Figure 2. Expression of *grb10* gene in bovine embryos at different developmental stages. Bars represent the means ± SE of pool data from three experiments.

DISCUSSION

Gene sequences and variations among them are entry points for studying gene expression and function (Liu et al., 2009). The transcriptional regulatory function may be changed by point mutations in the regulatory regions of genes that control protein configuration (Mayo et al., 2006). Using genetic screening, mutations involving defects in transcriptional regulation were identified in important developmental genes (Gibson and Honeycutt, 2002). Mutations provide novel options for animal breeding and improved production because of alternations in transcriptional regulation. However, few mutations have been discovered compared to that of the number of genes.

Mutation of C to T at the 492nd base represents a substitution of the non-polar, aliphatic amino acid, valine by the neutral amino acid, methionine. BLAST analysis of bovine GRB10 sequence confirms a high homology with other species: 94% with *Homo sapiens sapiens* and 88% with *Mus musculus*. GRB10 shares identical sequences across species, including the conserved region of interest containing valine (Figure 3). Location of the target valine in a conserved region implies an important maintenance role of the interaction with chaperone proteins. Further investigation is required to elucidate resulting changes in protein properties.

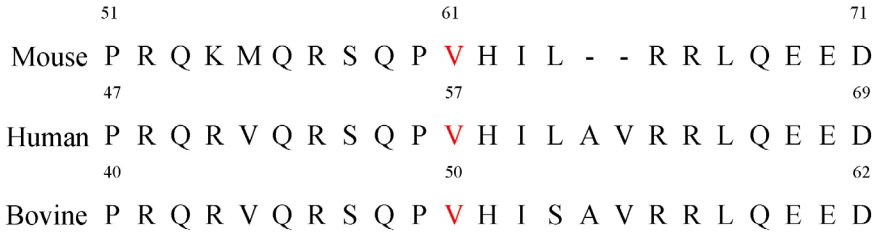

Figure 3. Amino acid sequence of the bovine GRB10 with other species. The valine is shown in bold red. In a number of species, these amino acids, including valine, were highly conserved. GenBank accession code: mouse (AAH16111.1), human (AAH24285.1), bovine (NP_001179515).

GRB10 is an intracellular adapter protein that is known to modulate diverse intracellular signaling pathways by interacting with various tyrosine kinase receptors, such as the insulin growth hormone receptor and insulin-like growth factor I receptors (Hu et al., 2010; Mroue et al., 2015; Yang et al., 2016). However, functions of *grb10* in female reproduction and early embryonic developmental remain unclear. An interaction was reported between human insulin receptor and GRB10 in the placenta that is closely related to human feto-placental growth. Overexpression of certain isoforms of *grb10* inhibits the activity of relevant tyrosine kinases and leads to growth suppression (Davies et al., 2005; Isles et al., 2006; Mukhopadhyay et al., 2015). Deletion of the paternally methylated region on *Meg1/grb10* leads to severe pre- and post-natal growth retardation, which might be the etiology of the Silver-Russell syndrome (Lim et al., 2004).

In the present study, the statistical analysis showed a strong relationship between *grb10* polymorphism and the number of embryos recovered after superovulation and AI. The heterozygote cattle with AB genotype at *grb10* locus showed increased number of NUE, NTE, NAE, NM1, and NM2. In this case, heterozygotes show a significant desirable trait advantage although breeding yielded 296 AA and only 4 AB genotype progeny. Although

the heterozygote showed a higher number of NDE, as well as higher ratios of percentage of available embryos and percentage of M1 embryos, no significant difference was detected by the data analysis. This is likely because of the limited number of heterozygotes detected by the RFLP and sequencing in the herds.

Upon further investigation, *grb10* mRNA was detected by real-time PCR in early bovine embryonic stages. The expression of grb10 mRNA was unchanged in MII oocytes and 2-cell embryos, and the expression reached the highest point in 4-cell embryos. Then the expression level fall down, and the morulae yielded similar expression levels as MII oocytes. This constitutes the first report on *grb10* mRNA expression patterns in bovine oocytes/embryos. Heightened expression in 4- to 16-cell embryos suggests that GRB10 might participate during maternal-to-zygote transition. However, lower expression in 8- to 16-cell embryos relative to the 4-cell stage might be because of developmental heterogeneity as a result of the blockade in embryos at that point.

In the present study, we discovered a strong correlation of the detected *grb10* polymorphism with superovulation traits. Heterozygous AB genotype at *grb10* locus exhibited a superior ovulation performance. The exon mutation in *grb10* has a significant effect on a number of superovulation traits. Based on the data, the AB genotype is better suited for embryo production. In summary, the collective expression data of *grb10* suggested that this gene might play an important role in bovine oocyte maturation and early embryonic development. For improved embryo production, *grb10* could serve as a useful molecular marker for donor selection.

Conflicts of interest

The authors declare no conflict of interest.

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