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Single nucleotide polymorphisms in the bovine genome are associated with the number of oocytes collected during ovum pick up

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ABSTRACT

The number of follicles recruited in each estrous cycle has gained practical importance in artificial reproductive technology, as it determines the oocyte yield from ultrasound-guided ovum pickup for *in vitro* embryo production. We aimed to identify single nucleotide polymorphisms (SNPs) in bovine genes related to reproductive physiology and evaluate the association between the candidate SNPs and the number of oocytes collected from ultrasound-guided ovum pickup. We sequenced genomic segments of GDF9, FGF8, FGF10 and *BMPR2* and identified seventeen SNPs in the *Bos taurus* and *Bos indicus* breeds. Two SNPs cause amino acid changes in the proteins GDF9 and FGF8. Three SNPs in *GDF9*, *FGF8* and *BMPR2* were genotyped in 217 Nelore cows (*B. indicus*), while two previously identified mutations in *LHCGR* and mitochondrial DNA (mtDNA) were genotyped in the same group. The polymorphisms in *GDF9*, *FGF8*, *BMRP2* and *LHCGR* were significantly associated ($P < 0.01$) with the number of oocytes collected by ovum pickup, whereas the SNP in the mtDNA was not. In addition, we estimated an allelic substitution effect of 1.13 ± 0.01 ($P < 0.01$) oocytes for the SNP in the *FGF8* gene. The results we report herein provide further evidence to support the hypothesis that genetic variability is an important component of the number of antral follicles in the bovine ovary.

1. Introduction

Oocytes have been recovered from cows using non-surgical procedures for many years, but only recently have studies investigated the control mechanisms affecting the number of follicles recruited (reviewed by Binelli and Murphy, 2010). In cows, the number of follicles recruited per follicle wave is highly consistent between consecutive estrous cycles but varies largely between individuals (Ireland et al., 2007). This observation can also be extended to the number of oocytes collected in each ultrasound-guided ovum pick up (OPU) session, as the oocyte yield is highly correlated with the number of follicles recruited (Tamassia et al., 2003). The estimated within-animal consistency in the number of follicles per wave is high (0.89) in heifers (Ireland et al., 2007), and some heritability in the number of oocytes that are recovered by OPU and successfully fertilized (0.25 ± 0.06) has been reported (Merton et al., 2009). In addition, a high correlation between the number of antral follicles and the number of healthy follicles (0.89) and atretic follicles (0.90) has been reported (Ireland et al., 2008), indicating that the

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variation in the ratio of healthy and atretic follicles in follicular waves is small. Taken together, these reports suggest that a genetic component may regulate folliculogenesis and that allelic variation in the genes involved in such regulation may lead to different ovarian phenotypes.

Several genes are regulated in a very dynamic manner during folliculogenesis (Hasegawa et al., 2009; Yoon et al., 2006). The expression of key regulatory genes varies across the pre-antral stages of development (from primordial to secondary stages), with this variation extending to the recruitment, growth, ovulation and atresia of antral follicles (Aerts and Bols, 2010a,b). In addition, knockout studies in mice have demonstrated that the disruption of 114 genes can compromise folliculogenesis (Mouse Genome Informatics, http://www.informatics.jax.org). While a number of studies have described the expression patterns of several regulatory genes throughout folliculogenesis (*i.e.*, *Akl3*, *Akl5*, *Akl6*, *Amh*, *Bmp15*, *Bmpr2*, *Cnx37*, *Cnx43*, *Figα*, *Figlα*, *Fgf2*, *Fgf7*, *Gdf9*, *Kit*, *Kitl*, *Tgfb1* and *Tgfb2* (Dean, 2002; Epifano and Dean, 2002; Choi and Rajkovic, 2006; McNatty et al., 2005)), the variability in the nucleotide sequences of these genes and its impact on reproductive physiology is not yet fully understood.

Fibroblast growth factor (FGF) genes are also important regulators of folliculogenesis. *FGF8* mRNA exclusively localizes to the oocyte in rodents (Valve et al., 1997), and localizes to preantral follicles and both germ (oocyte) and somatic (theca and granulosa) cells of antral follicles in cattle (Buratini et al., 2005a,b). FGF8 family members (FGF17 and FGF18) share the same FGF receptors with FGF8 and are expressed in bovine antral follicles, where these proteins have been shown to inhibit steroidogenesis and alter the progression of the cell cycle, inducing follicle atresia (Machado et al., 2009; Portela et al., 2010). Moreover, FGF8 acts synergistically with BMP15 to stimulate glycolysis in mouse cumulus cells, which is fundamental for supplying energy to the oocyte (Sugiura et al., 2009). In cattle, *FGF10* is expressed in preantral follicles and theca cells and oocytes from antral follicles. FGF10 inhibits the secretion of estradiol from granulosa cells, and the expression of FGF10 in theca cells is negatively associated with the growth of dominant follicles (Buratini et al., 2007). Therefore, variations in FGF nucleotide sequences can potentially interfere with different phases of folliculogenesis.

The early stages of follicle development are independent of gonadotropins (Knight and Glister, 2001); however, theca cells produce mRNA coding for the luteinizing hormone receptor (luteinizing hormone/choriogonadotropin receptor – LHCGR) in preantral follicles, and the expression of *LHCGR* increases in the cells of non-atretic follicles measuring 2–4 mm in diameter (Braw-Tal and Roth, 2005). The expression of *LHCGR* decreases in the theca cells of follicles that are larger than 5 mm, whereas the transcription of *LHCGR* is activated in granulosa cells on the 2nd day of wave emergence (Mihm et al., 2006) and increases as the follicles grow (Nogueira et al., 2007). The action of the luteinizing hormone during the early stages of folliculogenesis is uncertain, but should not be disregarded, as theca cells express mRNA for the corresponding receptor. In addition, polymorphisms in the *LHCGR* gene have been associated with both male and female reproductive disorders in humans (reviewed by Themmen, 2005) and reproductive traits in cattle (Hastings et al., 2006).

Despite the expanding knowledge of the genes controlling the physiology of the folliculogenesis, no studies have addressed the genomic variability of this trait at the nucleotide level in cattle. We hypothesize that genetic variability is associated with the recruitment and growth of antral follicles during the estrous cycle. The goals of this study were to identify polymorphisms in the bovine *GDF*9, *FGF*8, *FGF*10 and *BMPR*2 genes and to analyze the effects of new and previously described polymorphisms in the bovine *LHCGR* gene and the mitochondrial genome on the number of oocytes collected during ovum pick up in Nelore cows (*Bos indicus*).

2. Materials and methods

2.1. Samples

DNA was extracted from blood samples collected from Nelore cows (*B. indicus*) during OPU procedures with a standard protocol (Sambrook et al., 1987). The OPUs were performed by two private companies in Brazil, and the number of viable oocytes that qualified for *in vitro* fertilization procedures (NVO) and the number of blastocysts produced per *in vitro* fertilization procedure were recorded and ceded for research purposes. The oocytes were collected from follicles larger than 3 mm and used for *in vitro* fertilization (IVF) if the cytoplasm was homogeneous and contained at least 5 layers of compact cumulus cells. All the cows selected for this study had records for more than one OPU with at least 20% of the collected oocytes resulting in blastocysts in each IVF session [(number of blastocysts produced per IVF cycle × number of oocytes fertilized^{-1}) × 100]. Thirty cows were selected for single nucleotide polymorphism (SNP) discovery analysis, and 217 cows were genotyped for allele frequency and genotype–phenotype association analysis.

In addition, blood samples from 20 purebred Holstein cows (*Bos taurus*) from a local herd were processed for DNA extraction and used in the SNP experiments. These samples were collected to compare the *B. indicus* and *B. taurus* genomes.

2.2. Single nucleotide polymorphism analysis

Segments of the *GDF*9, *FGF*8, *FGF*10 and *BMPR*2 genes were amplified by PCR with specific primers (Table 1) that were chosen based on the *B. taurus* genome reference sequence (Btau4.0). The reactions consisted of 1× enzyme buffer, 1.5 mM $MgCl_2$, 400 μM of each dNTP, 5 μM each for the forward and reverse primers, 1 U of Taq DNA polymerase (Invitrogen, Grand Island, NY) and 50 ng of DNA as template in a final volume of 25 μL. The cycling conditions consisted of an initial 94 °C step for 2 min, 35× (94 °C for 30 s, annealing temperature (Table 1) for 30 s and 72 °C for 45 s), and a 72 °C step for 5 min. An aliquot of each reaction was subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide (0.5 mg/mL). The gels were exposed to ultraviolet light, digitalized with the FujiFilm 3000 laser scanner (Fuji, Japan) and analyzed using Image

Table 1
Primers used for PCR and sequencing of bovine GDF9, BMPR2, FGF8 and FGF10 fragments.

Gene	BTA[a]	Exons	Code	Primers (5′–3′)	Annealing temperature	GenBank accession
*GDF*9	7	1	GDF9-A	TTGCTAATTCTTCCAAGCCATG TGGAAGCTCTAAACCCACTGTAAC	53 °C	194719537
		1	GDF9-B	GCCCACCCACACACCTAAAGTTTA GCACACCAACAGCTGAAAGAGGTA	65 °C	
		2	GDF9-C	TGTATGAGAGAGATGGGAGCAGTGC TCCCGTGCTGAAGGATGCTG	65 °C	
		2	GDF9-D	AAGACTCTCCCTAGAGCTCCATACTC TAGAACTGCAATTCCACCCAAG	55 °C	
		2	GDF9-E	AAGAGAGGGCTGTCTGCCTGTCC ACCGCACACAGAAAATTTATGCCAC	63 °C	
*FGF*8	26	1, 2	FGF8-A	ATGTGCATCCCTGGCCCCATGA TCAACCGAACACGTGCCGAGGAC	63 °C	194719403
		4, 5, 6	FGF8-B	CAGCCAGGTGGGGGTGGTTG GGTCCCTTCCCCAGCCCTCTC	64 °C	
FGF10	20	2	FGF10-A	TGGAATTGAGACATAGGCAGTTGAG CTTGGTGACTAGCCCTTTGTGC	63 °C	194719397
		2	FGF10-B	GGTCTCTTTACCTAAGCTAGGGTGGC CCTTGCCCGCCCTTGAAAC	61 °C	
		3	FGF10-C	ACACAGGAATTCACAAAGCAAGG CTATGAGTGTACCACCATCGGAAG	53 °C	
*BMPR*2	2	6	BMPR2-A	ATACCAATTTCAGAGCCATGC GCAGGTATGCTTTACCATTTGAG	63 °C	194719396
		10	BMPR2-B	CTTAAACAATTCAGTGGGCCAG TTTACACAATCCCTCCACTTACAC	63 °C	
		11	BMPR2-C	TCTCCTGTCAGTACCCCCTCCTAC AAGTTACTAGGCCTCGTTTGGGAA	62 °C	

[a] Bovine chromosome.

Gauge software (Fuji, Japan) to determine the presence of non-specific PCR products.

The amplicons were purified using the ExoSAP-IT kit (GE Healthcare - Amersham, Piscataway, NJ) as recommended by manufacturer. A total of 10 ng of purified PCR product was used as a template in the sequencing reactions, which also contained the respective pair of primers and the BigDye® Terminator v3.1 reagent (Applied Biosystems, Foster City, CA).

The raw sequences were automatically processed and assembled using Prhed/Phrap (Ewing et al., 1998) and aligned with the *B. taurus* reference genome sequence (BGSAC, 2009). The contigs were visually inspected with Consed (Gordon et al., 1998), and the SNPs were automatically identified with Polyphred (Bhangale et al., 2006). This step was followed by a visual inspection of the sequencing chromatograms.

2.3. Genotyping

Two of the SNPs discovered through sequencing (*GDF*9 and *FGF*8) and two of the SNPs previously described in the literature (*LHCGR* (Marson et al., 2008) and mtDNA-*Nd5* (Meirelles et al., 1999)) were analyzed by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) using specific primers and restriction enzymes (Table 2).

The reaction conditions for the amplification of the *GDF*9 and *FGF*8 gene segments were the same as those described in the previous section. The PCR amplification of the *LHCGR* and mtDNA-*Nd5* gene segments was conducted using the following reaction mixture: 1× enzyme buffer, 3.5 mM $MgCl_2$, 100 μM of each dNTP, 5 μM of each primer, 1 U Taq DNA polymerase (Invitrogen, Grand Island, NY), and 100 ng DNA template in a final volume of 25 μL. The thermocycling protocol was as follows: 94 °C for 2 min, 35× (94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s), and 72 °C for 5 min. A 15 μL aliquot of each reaction was subjected to enzyme digestion as recommended by the manufacturer, and the digested products were subjected to electrophoresis in 2% agarose gels containing ethidium bromide (0.5 mg/mL). The gels were exposed to ultraviolet light, digitalized in the Fuji-Film 3000 laser scanner (Fuji, Japan) and analyzed in Image Gauge software (Fuji, Japan).

One SNP in the *BMPR*2 gene was genotyped using a TaqMan® assay with the ABI Prism 7500 Sequence Detection System (Applied Biosystems) and the following primers: 5′-GCT AAT ATT TGA CTT GCT CTT TGT TCT GT-3′ and 5′-TCC TTC CGG GCA TTT TAA TAA ACA TAC T-3′. The probe sequences were as follows: 5′VIC-CTC TTA CGA ACT TCC and 5′FAM-CTC TTA CAA ACT TCC. The TaqMan® assays were carried out with Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ) according to the manufacturer's instructions.

2.4. Statistical analysis

The genotypic frequencies ($F_{(ii)}, F_{(ij)}, F_{(jj)}, i \neq j$) were calculated by directly counting the corresponding genotypes and dividing by the total number of genotyped individuals (N). The standard deviations of the genotypic frequencies were estimated using the formula $((F_{(ii)}(F_{(ii)} - 1))/N)^{-0.5}$ as described elsewhere (Evett and Weir, 1995). The allele frequencies ($f_{(i)}$ and $f_{(j)}$, $i \neq j$) were calculated as follows:

Table 2
Primers and restriction enzymes used for PCR product generation and digestion.

Gene-fragment	Primers	Restriction enzyme
GDF9-B	GCCCACCCACACACCTAAAGTTTA GCACACCAACAGCTGAAAGAGGTA	Nde I
FGF8-A	ATGTGCATCCCTGGCCCCATGA TCAACCGAACACGTGCCGAGGAC	Msp I
LHCGR	CAA ACT GAC AGT CCC CCG CTT T CCT CCG AGC ATG ACT GGA ATG GC	Hha I
mtDNA-*Nd5*	CCC AAC GAG GAA AAT ATA CC AAC CGC AAA CAA CCT CTT C	Hind III

$f_{(i)} = F_{(ii)} + (F_{(ij)}/2)$ and $f_{(j)} = 1 - f_{(i)}$, and the standard deviations were estimated using the formula $((f_{(i)}(1 - f_{(i)}))/2N)^{-5}$ (Evett and Weir, 1995).

The Fisher exact test was used to test for Hardy–Weinberg equilibrium (Hardy, 1908) and independence between the mitochondrial genotype and each nuclear gene (H_0) using GenePop v4.0 software (Raymond and Rousset, 1995).

The data set followed a Poisson distribution (not shown), as expected for most counted data. Therefore, we transformed the data using Log_2 to attain normality within the dataset (not shown). The association between each DNA marker and the NVO was determined by analysis of variance (ANOVA) of repeated data using the following model: $Log_2(Y_{ijklm}) = \mu + A_{ij} + SNP_k + F_l + Y_m + e_{ijklmn}$; where Y_{ijklmn} is the observation for animal *ijklm*; μ is the overall phenotypic mean; A_{ij} is the random effect of cow *i* at OPU *j*; SNP_k is the fixed effect of a SNP; F_l is the fixed effect of the place of OPU *j*; Y_m is the fixed effect for year of j_{th} OPU; e_{ijklmn} is the associated error for observation *ijklm*, assuming mean $\cong 0$ and variance $\cong 1$. The differences ($\overline{D}$) between the genotypic values were estimated by comparing the least square means and the gene/marker effect of significances with a Student's *t*-test (H_0: $\overline{D} = 0$; H_1: $\overline{D} \neq 0$).

The animals heterozygous for the *FGF8* marker exhibited intermediate values compared to the homozygous animals; therefore, we estimated the allele substitution effect with a multiple regression analysis that used the above model with numeric values for the genotypes (SNP_k, $k = 0, 1, 2$). The slope of the regression line (β) estimated the average substitution effect for each allele, and the significance was tested with a Student's *t*-test (H_0: $\beta = 0$; H_1: $\beta \neq 0$).

The probability of significance (*P*) values were corrected for multiple tests with the Bonferroni procedure (Bland and Altman, 1995), and the alternative hypotheses (H_1) were assumed when the adjusted *P* value was <0.01. The association analysis tests and probability adjustments were performed in SAS/STAT® software v8, and the NVO results are presented in the natural scale of the data.

Table 3
SNPs identified in Nelore cattle.

Gene	NCBI_ss#	Localization	Position[b]	Bovine Ref. Seq.[c]	Nelore sequence[d]	Allele frequency in Nelore sample	P (HW)[e]	AA substitution[f]
GDF9	104806867	Exon 1	Chr7:44093437[a]	C	C/A	A- 0.19 ± 0.08	0.6918	Ala/Glu
	104806874	Intron 1	Chr7:44092967	C	T	T- 1.00		
	104806881	Exon 2 (3′UTR)	Chr7:44090844	T	G	G- 1.00		
FGF8	104806875	Intron 1	Chr26:22818083	A	G	G- 1.00		
	104806876	Intron 1	Chr26:22817910		ACAA	ACAA- 1.00		
	104806866	Exon 2	Chr26:22817344[a]	G	G/C	C- 0.25 ± 0.15	0.8007	Arg/Pro
	104806877	Exon 2	Chr26:22817318	T	C	C- 1.00		Ser/Pro
	104806878	Intron 2	Chr26:22817235	C	G	G- 1.00		
FGF10	104806864	Intron 2	Chr20:32501605	C	G/C	C- 0.35 ± 0.07	0.3208	
	104806879	Exon 3	Chr20:32502010	T	C	C- 1.00		Lys/Glu
	104806865	Exon 3 (3′UTR)	Chr20: 32502286	C	T	T- 1.00		
BMPR2	104806868	Intron 5	Chr2:95614590	A	A/C	C- 0.69 ± 0.12	0.4376	
	104806869	Intron 5	Chr2:95614807	C	C/T	T- 0.61 ± 0.07	1.0000	
	104806870	Exon 11 (3′UTR)	Chr2:95635857	T	T/A	A- 0.50 ± 0.16	0.3208	
	104806871	Exon 11 (3′UTR)	Chr2:95636182	A	A/G	G- 0.56 ± 0.12	0.8604	
	104806872	Exon 11 (3′UTR)	Chr2:95636437[a]	G	G/A	A- 0.45 ± 0.11	0.9465	
	104806873	Exon 11 (3′UTR)	Chr2:96636632	C	T/C	C- 0.19 ± 0.10	0.2932	

[a] SNPs confirmed.
[b] SNP position on the bovine reference sequence.
[c] Nucleotide observed on *Bostaurus* (Btau 4.0).
[d] Nucleotide observed in this study from Nelore samples.
[e] Fisher exact probability for Hardy Weinberg equilibrium.
[f] Amino acid substitution observed from *B. taurus* reference sequence to Nelore (*B. indicus*) sequence.

Table 4
Genotype and allele frequencies for markers in the Nelore sample.

Gene	SNP position	*N*	Genotype frequency			Alelle frequency		P (HW)
GDF9	Chr7:44093437	217	AA 0.02 ± 0.01	CA 0.16 ± 0.02	CC 0.82 ± 0.03	A 0.10 ± 0.01	C 0.90 ± 0.01	0.1195
FGF8	Chr26:22817344	203	CC 0.20 ± 0.03	CG 0.51 ± 0.04	GG 0.29 ± 0.03	C 0.46 ± 0.02	G 0.54 ± 0.02	0.7790
BMPR2	Chr2:95636437	217	AA 0.16 ± 0.02	AG 0.45 ± 0.03	GG 0.40 ± 0.03	A 0.38 ± 0.02	G 0.62 ± 0.02	0.4718
LHCGR	Chr11:32195548	218	CC 0.02 ± 0.01	CT 0.25 ± 0.03	TT 0.73 ± 0.03	C 0.14 ± 0.02	T 0.86 ± 0.02	1.0
mtDNA	nt:12178	218	Indicine 0.24	na	Taurine 0.76	na	na	na

na: not applicable.

3. Results

3.1. SNP identification and genotyping

Seventeen SNPs were identified when the genome sequences for Nelore cows (*B. indicus*) were compared with the taurine reference genome sequence (Table 3). Eight SNPs were putative breed or sub-species specific nucleotide variations, with 2 of the SNPs resulting in amino acid changes in the respective protein (*FGF*8 – Chr26:22817318 T/C; *FGF*10 – Chr20:32502010 T/C) and the remaining six SNPs located in intronic or untranslated regions. The nine remaining SNPs were found only within the Nelore samples. Two of these SNPs result in amino acid substitutions (*GDF*9 – Chr7:44093437 C/A; *FGF*8 – Chr26:22817344 C/G). The allelic frequencies of these 9 SNPs were in Hardy–Weinberg equilibrium.

Table 4 shows the allelic and genotypic frequencies estimated for the SNPs in *GDF*9 (Chr7:44093437), *FGF*8 (Chr26:22817344), *BMPR*2 (Chr2:95636437), *LHCGR* (Chr11:32195548) and mtDNA (nt:12178), which were genotyped in a larger number of animals and were in Hardy–Weinberg equilibrium. The genotypes of the Holstein cows were identical to those found in the cattle reference genome.

3.2. Association analysis

The average number of viable oocytes (NVO) retrieved from a total of 1426 OPUs performed on 193 cows was 29.6 ± 25.2. The descriptive statistics for the dataset used in the association analysis are presented in Table 5. The SNPs in the *GDF9*, *FGF8*, *BMPR2* and *LHCGR* genes exerted significant effects on the NVO. The NVO retrieved from the genotypes with higher and lower averages ranged from 4.87 (*FGF8*) to 9.67 (*LHCGR*) (Table 6).

Table 5
Descriptive summary of viable oocytes collected per OPU.

	Data original format	Transformed data (Log_2)
Number of animals	193	193
Number of OPUs	1426	1426
Mean	29.6	4.4
Standard deviation	25.2	1.2
Range	1–194	0–7.6
Median	22	4.4
Mode	10	3.3
Coefficient of variation (%)	85.15	1.82

4. Discussion

We identified genetic variants in four genes involved in oocyte maturation process that are specific to Nelore cattle. The presence of fixed alleles in each breed was expected, as the average genetic distance (F_{ST}) between the zebuine and taurine genomes has been estimated at 22.8% (Chan et al., 2010), and the diversity ratio between the Angus (*B. taurus*) and Brahman (*B. indicus*) genomes is estimated to be 0.3–0.55 when the calculations are based on mutation rates and 0.4–0.7 when the calculations are based on pairwise nucleotide heterozygosity (Bovine HapMap Consortium, 2009). The percentage of Nelore animals showing taurine mitochondrial DNA (76%) was in agreement with previously published results (79%, Meirelles et al., 1999).

The average NVO observed in our study (29.6) was in agreement with previously reported estimates of the total number of follicles recruited in each follicular growth wave in Nelore cows between 20 and 24 months old (33.4 ± 3.2, Carvalho et al., 2008). Moreover, the observed average NVO was also similar to the number of oocytes recovered from 60 to 90-day-old Nelore calves (24.5 ± 93.5, Malard et al., 2001) and the number of oocytes recovered from Aberdeen Angus cows by OPU (25.4 ± 2.5, Carvalho et al., 2008).

The GDF9 protein functions as a dimer, forming either homodimers or heterodimerizing with BMP15 (Liao et al., 2003). The malfunction of this protein can impair the development of secondary follicles (Epifano and Dean, 2002; Dong et al., 1996), and immunization against GDF9 decreases the number and size of antral follicles in cattle (Juengel et al., 2009). The *GDF9* SNP causes an amino acid substitution (Ala/Glu) at protein residue 95, which is highly conserved across mammalian species (Fig. 1A). We observed a significant difference in the number of viable oocytes (6 oocytes) retrieved from the heterozygous (CA) and CC homozygous cows. It is possible that the signaling from the GDF9 dimer may be partially impaired by the amino acid substitution in one of the GDF9 proteins.

We do not yet understand the role of FGF8 in the regulation of folliculogenesis, and this lack of understanding

Table 6
Averages of viable oocytes collected by OPU per genotype. Data is presented as least square means (LSM) ± standard error (SE) of viable oocytes collected for each genotype. *N*, number of cows.

Gene	SNP position	*F* values	Genotypes	*N*	LSM ± SE
GDF9	Chr7:44093437	18.37*	AA	04	20.80 ± 0.44[a]
			CA	34	14.00 ± 0.02[b]
			CC	180	19.99 ± 0.01[a]
FGF8	Chr26:22817344	6.66*	GG	59	22.07 ± 0.01[a]
			CG	103	19.48 ± 0.01[a,b]
			CC	41	17.20 ± 0.02[b]
BMPR2	Chr2:95636437	24.29*	AA	34	22.83 ± 0.01[a]
			AG	97	16.76 ± 0.01[b]
			GG	86	21.23 ± 0.01[a]
LHCGR	Chr11:32195548	17.78*	CC	04	26.06 ± 0.03[a]
			CT	54	16.39 ± 0.01[b]
			TT	160	21.17 ± 0.01[a]
mtDNA	nt:12178	0.04	Taurine	53	19.61 ± 0.01[a]
			Indicine	165	19.41 ± 0.01[a]

* Significant ($P < 0.01$).
[a] Different letters between genotypes within marker represent significant statistical differences ($P < 0.01$).
[b] Different letters between genotypes within marker represent significant statistical differences ($P < 0.01$).

is complicated by the unfeasibility of knockout models, as the null mutant does not develop beyond gastrulation (Itoh and Ornitz, 2008). In cattle, the *FGF*8 gene is transcribed in primordial, primary and secondary preantral follicles (Buratini et al., 2005a,b), in addition to the oocytes, granulosa cells and theca cells of antral follicles (Buratini et al., 2005a,b). FGF8 most efficiently activates the *FGFR*3*C* and *FGFR*4 receptors, both of which are transcribed in

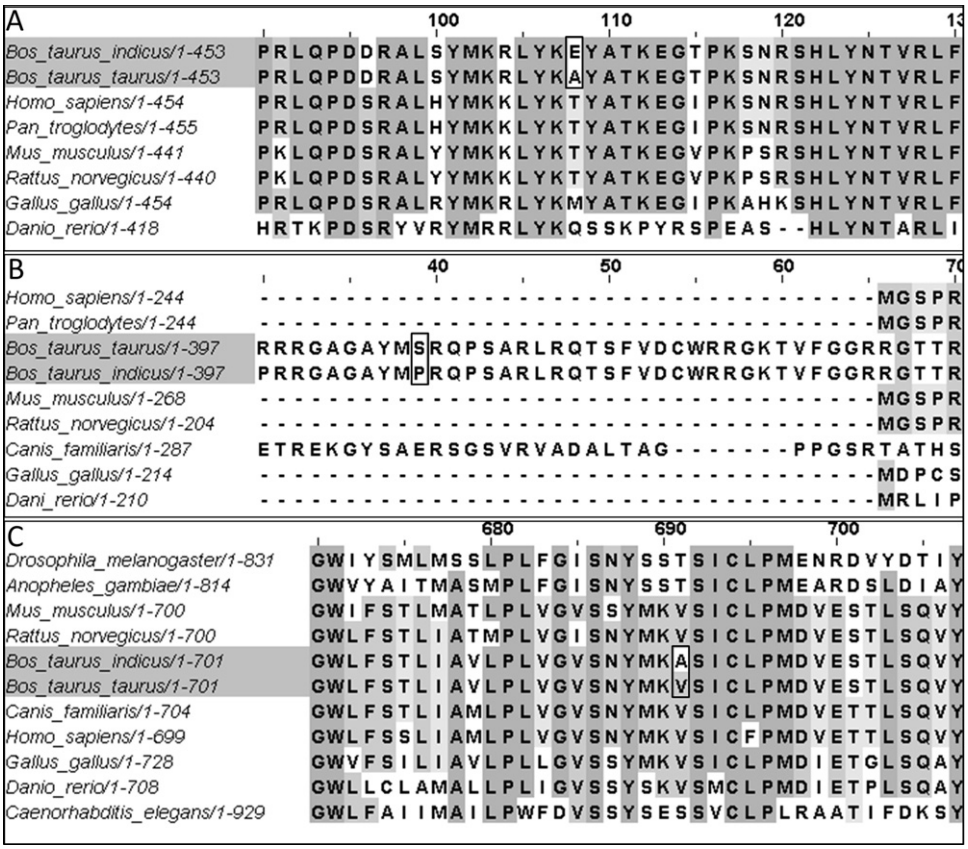

Fig. 1. The alignment of the *Bos taurus* and *Bos indicus* GDF9 (A), FGF8 (B) and LHCGR (C) proteins is shown. The amino acid differences between *B. taurus* and *B. indicus* that were investigated in this study are marked by a vertical rectangle. The proteins are ordered by similarity. The dashed lines represent gaps in the alignments, while the shades of gray represent the degree of similarity (100% similarity is assumed for the positions in the alignment where the amino acids are the same across species). The numbers following the species name represent the number of amino acids in that protein for a given species.

theca cells (with FGFR3C also being transcribed in granulosa cells), suggesting that FGF8 signaling occurs within the bovine antral follicle (Buratini et al., 2005a,b; Itoh and Ornitz, 2008). FGF8 seems to interact with other proteins to regulate folliculogenesis, as it was shown to function together with BMP15 to stimulate glycolysis in cumulus cells (Sugiura et al., 2009). While the observed alteration in the 30th amino acid of the protein is in a non-conserved region (Fig. 1B), this alteration could still lead to a mutant protein with a slightly altered function, which would have consequences for FGF8 signaling and lead to changes in the number of follicles recruited in each wave. The homozygous wild-type females (GG) produced on average a total of 4.87 more viable oocytes than the homozygous mutant females (CC). The heterozygous females showed intermediate values, suggesting an additive relationship between the alleles (Falconer and Mackay, 1996). The estimated allele substitution effect for the C allele was 1.13 ± 0.01 ($P = 0.0003$) oocytes.

BMPR2 functions as part of a protein complex that also contains Activin receptors 2 and 2B and BMPRI and BMPRIB. BMPR2 also has a key role in the TGF beta pathway and the stimulation of cell differentiation through the Smad intracellular signaling pathway (Miyazono et al., 2010). While scientists are uncertain as to how BMPR2 is assembled as a protein complex, BMPR2 was shown to interact with GDF9 to stimulate granulosa cell proliferation in the small antral follicles of mice (Vitt et al., 2002). The polymorphism present in the 3′UTR of the BMPR2 gene affected the number of viable oocytes collected by OPU. The homozygous females produced 5–6 more oocytes than the heterozygous females. The 3′UTR of BMPR2 has several sites that are predicted targets for many micro RNAs (Friedman et al., 2009), and miR-21 has been shown to down-regulate the level of BMPR2 protein (Qin et al., 2009). Despite the previous connection between the BMPR2 protein and follicle recruitment and growth, further studies are required to evaluate the relationship between the polymorphisms in the 3′UTR of the gene and follicle recruitment and growth in cattle.

The functional LHCGR may only occur after follicular deviation, as previously suggested (Nogueira et al., 2007; Barros et al., 2010). The down-regulation of LHCG:LHCGR signaling around the time of dominant follicle selection has a significant effect on the gene expression of theca cells (STAR and CYP17A) and granulosa cells (PAPPA) (Luo et al., 2011) in cows. While the importance of LHCG/LHCGR signaling during the follicle selection is well accepted, the role of the LHCG/LHCGR in the early stages of bovine folliculogenesis has yet to be studied. In mouse models, the *Lhcgr* gene is expressed in granulosa cells by the 4th day of the estrous cycle (Xu et al., 1995). The importance of the *Lhcgr* gene in follicular development is emphasized by the fact that knockout mice (*Lhcgr*−/−) do not mature follicles past the early antral stage (Lei et al., 2001). In addition, these follicles do not respond to gonadotropins (Pakarainen et al., 2005).

The *LHCGR* polymorphism investigated in this study significantly affected the NVO. The OPU procedure collected 4–10 more oocytes from the homozygous females than from the heterozygous females. Polymorphisms in the *LHCGR* gene have been associated with the estimated breeding values for calving interval, days to first service and condition score (Hastings et al., 2006), but the SNPs linked to these values were different from the SNP investigated in the present study.

The effect that the *LHCGR* polymorphism exerts on the NVO can be caused by an abnormal interaction between the LH protein and the LHCGR protein. The C/T SNP at position 62478 of the bovine LHCGR gene causes an amino acid substitution (aa: 443 alanine/valine) in a transmembrane domain that resides in a highly conserved region of the protein (Fig. 1C).

The comparison of the least square means for the NVOs of the genotypes with each polymorphism revealed different patterns of allele interactions. The analysis for the *FGF8* SNP indicated that C and G have an additive interaction. In comparison, the results for the *GDF9*, *BMRP2* and *LHCGR* SNPs suggest underdominance between the two alleles for each SNP. The allelic distribution for the three SNPs might follow the expected population equilibrium (Table 4) because the number of antral follicles present in a given follicular wave may not be under selective pressure in a species that normally ovulates one oocyte. The biochemical mechanisms that control the interactions between GDF9 and LHCGR are yet not fully elucidated, and all the interactions for each of these proteins have yet to be identified. The formation of homodimers or heterodimers is possible for both GDF9 and LHCGR to perform their functions (Liao et al., 2003; Urizar et al., 2005). Thus, we hypothesize that the formation of GDF9 and LHCGR homodimers is impaired by the amino acid change. However, further experiments are necessary to clarify why the heterozygous cows averaged fewer NOV during the OPU. The *BMPR2* polymorphism is positioned in the 3′UTR and thus has no effect on protein conformation. Therefore, it is important to consider the fact that one or all these SNPs may not be causative mutations and might rather be linked to a haplotype that effectively influences the phenotype. Follow up studies using high throughput sequencing will help elucidate the genetic basis of the quantitative phenotypic variation on a case-by-case basis (Mackay et al., 2009).

In contrast to the nuclear markers, the two mitochondrial genomes (taurine and indicine) showed no significant differences in the analyzed characteristic. However, the average follicle number recruited in each follicular wave is reported to be different for the *B. indicus* and *B. taurus* breeds (Nelore: 33.4; Aberdeen Angus: 25.4 (Carvalho et al., 2008)). Altogether, these results could indicate that the divergence between the taurine and indicine mitochondrial genomes has little or no effect on the number of follicles recruited in each follicular wave and that this phenotype is controlled by nuclear genes.

In summary, we identified new polymorphisms in the bovine *GDF9*, *FGF8*, *FGF10* and *BMPR2* genes, some of which are associated with the oocyte OPU yield and may be correlated with the number of antral follicles recruited during follicle maturation in purebred Nelore cattle. This finding is an indication of a genetic effect on either the number of primordial follicles recruited to grow in each follicular wave or a genetic effect on the growth of healthy follicles during the wave. Despite the limited number of molecular markers tested, our findings may have a significant

impact on the selection of cows as oocyte donors for *in vitro* embryo production. Further studies using high throughput technologies (Seidel, 2010) are needed to confirm the relationship between genetic background and oocyte yield.

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