

Association between *BMP4* gene polymorphism and *in vitro* embryo production traits in Gyr cows[□]

*Asociación entre polimorfismo en el gen **BMP4** y características de producción in vitro de embriones en vacas Gyr*

*Associação entre o polimorfismo no gene **BMP4** e características de produção in vitro de embriões em vacas Gyr*

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Summary

Background: candidate genes and their polymorphisms have been associated with traits of economic interest in cattle, representing an important strategy for genetic improvement of complex traits. Bone morphogenetic protein 4 (*BMP4*) is a member of the transforming growth factor beta (TGF β) superfamily, which is involved with several events of embryonic, fetal, and adult development in vertebrates. **Objective:** the aim of this study was to evaluate the degree of association between polymorphism in the *BMP4* gene (guanine for thymine-G>T, SNP *rs109778173*) and the performance of Gyr oocyte donors, including the rate of *cumulus-oophorus* complex obtained in each session of follicular aspiration (OPU), embryo development, and pregnancy rates. **Methods:** DNA was extracted from hair follicles from 50 oocyte donors and genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Data from 212 OPU-IVP sessions was collected, and the following traits were associated with *BMP4* polymorphism (SNP *rs109778173*): number and ratio of viable *cumulus-oophorus* complexes; number of cleaved embryos at day 4 of culture; number of transferable embryos at day 7 of culture, and pregnancies on days 30 and 60 after embryo transfer. **Results:** the studied *BMP4* polymorphism was significantly associated ($p<0.01$) with the number and ratio of viable cumulus-oocyte complexes, and the ratio of pregnancies at 30 days. **Conclusion:** the GT genotype (SNP *rs109778173*) was associated with inferior performance regarding OPU-IVP traits. This finding suggests

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possible genetic effects of *BMP4* on performance of Gyr oocyte donors. Studies are needed on the *BMP4* gene regarding subsequent changes in embryonic development of said mutation.

Keywords: *genetic variability, molecular markers, mutation, OPU-IVP, SNPs.*

Resumen

Antecedentes: genes candidatos y sus polimorfismos han sido asociados a características de interés económico en ganado, representando una excelente estrategia para el mejoramiento genético de características complejas. La proteína morfogénica ósea 4 (*BMP4*) es un miembro de la superfamilia del factor de crecimiento transformante beta (TGF β); que controla innumerables eventos del desarrollo embrionario, fetal y de adultos en vertebrados. **Objetivo:** el objetivo de este estudio fue evaluar el grado de asociación entre un polimorfismo en el gen *BMP4* (guanina por timina-G>T-, SNP *rs109778173*) y el desempeño de donantes de ovocitos de la raza Gyr, incluyendo la tasa de complejos *cummulus-oophorus* obtenidos en cada sesión de aspiración folicular (OPU), el desarrollo embrionario y la tasa de preñez. **Método:** el ADN fue extraído del foliculo piloso de 50 vacas Gyr donantes de ovocitos, el genotipado fue realizado por la técnica de reacción en cadena de polimerasa-polimorfismos de longitud de fragmentos de restricción (PCR-RFLP). Datos de 212 sesiones de OPU-IVP fueron colectados y las características a seguir fueron asociadas con el polimorfismo en el gen *BMP4* (SNP *rs109778173*): número y tasa de complejos de *cummulus-oophorus* viables, de embriones clivados al día 4 de cultivo, de embriones transferibles al día 7 de cultivo, y de preñeces a los días 30 y 60 posterior a la transferencia de los embriones. **Resultados:** el polimorfismo fue significativamente asociado ($p<0,01$) con número y tasa de complejos *cummulus-oophorus* viables y tasa de preñez al día 30. **Conclusión:** el genotipo GT (SNP *rs109778173*) fue asociado a resultados inferiores relacionados con características de OPU-IVP. Este resultado sugiere posibles efectos genéticos del gen *BMP4* sobre el desempeño de donadoras de oocitos de la raza Gyr, siendo necesarios estudios del gen *BMP4* en relación a las alteraciones en el desarrollo embrionario subsecuentes a la mutación citada.

Palabras clave: *marcadores genéticos, mutación, OPU-IVP, SNPs, variación genética.*

Resumo

Antecedentes: genes candidatos e seus polimorfismos têm sido associados a características de interesse econômico no gado bovino, representando uma importante estratégia para o melhoramento genético de características complexas. A proteína morfogênica óssea 4 (*BMP4*) é um membro da superfamilia do fator de crescimento transformante beta (TGF β); que controla diversos eventos do desenvolvimento embrionário, tanto fetal quanto adulto em vertebrados. **Objetivo:** avaliar o grau de associação entre um polimorfismo no gene *BMP4* (guanina por timina-G>T-, SNP *rs109778173*) e o desempenho de doadoras de oócitos da raça Gyr, incluindo a taxa de complexos *cummulus-oophorus* obtidos em cada sessão de aspiração folicular (OPU), o desenvolvimento embrionário e a taxa de prenhez dos embriões. **Método:** o DNA foi extraído do foliculo piloso de 50 vacas Gyr doadoras de oócitos, e a genotipagem foi realizada pela técnica de reação em cadeia da polimerase-polimorfismo no comprimento de fragmentos de restrição (PCR-RFLP). Dados de 212 sessões de OPU-IVP foram coletados, e as características a seguir foram associadas com o polimorfismo no gene *BMP4* (SNP *rs109778173*): número e taxa de complexos *cummulus-oophorus* viáveis dos embriões clivados no dia 4 de cultivo, dos embriões transferíveis no dia 7 de cultivo e de prenhez no dia 30 e 60 após a transferência dos embriões. **Resultados:** o polimorfismo estudado foi significativamente associado ($p<0,01$) com o número e a taxa dos complexos *cummulus-oophorus* viáveis e a taxa de prenhez ao dia 30. **Conclusão:** o genótipo GT (SNP *rs109778173*) foi associado a resultados inferiores relacionados a características OPU-IVP. Este resultado sugere possíveis efeitos genéticos do gene *BMP4* sobre o desempenho de doadoras de oócitos da raça Gyr, fazendo-se necessários estudos no gene *BMP4* em relação às alterações no desenvolvimento embrionário subsecuentes ao polimorfismo citado.

Palavras chave: *marcador molecular, mutação sinônima, OPU-IVP, SNP, variabilidade genética.*

Introduction

Bone morphogenetic protein 4 (*BMP4*) is a member of the transforming growth factor beta

(TGF β) superfamily, which controls numerous events of embryonic, fetal and even adult development in vertebrates (Chen *et al.*, 2004).

Several BMPs have been suggested as autocrine/paracrine regulators of bovine ovarian follicular development, demonstrated by the expression of *BMP4* and *BMP7* in granulosa and theca cells (Glister et al., 2011).

During the follicular development, the *BMP4* gene is expressed first in the stromal cells and later in the theca cells. Its receptors are found in granulosa cells as well as in the oocyte itself (Glister et al., 2004; Fatehi et al., 2005). Those receptors participate in primordial to primary follicular transition, stimulate granulosa cells proliferation, pre-antral follicular growth, and follicular survival, and regulate steroidogenesis in granulosa cells (Knight and Glister, 2006).

There is new evidence to support the hypothesis that *BMP4* and *BMP7* originated from theca and stromal cells to promote the initial transition of primordial follicles to enhance follicle survival (Phil and Claire, 2006). Moreover, in later developmental stages, *BMP4* induces embryonic and extra embryonic mesoderm formation (Degrelle et al., 2011) and is also related to vasculogenesis (Astorga and Carlsson, 2007) in the embryo and in the developing placenta as well. As a result, *BPMs* are of major importance for pregnancy success (La Rosa et al., 2011). These authors also concluded that *BMP4* is implicated in oocyte maturation and embryonic development in bovines, affecting the cleavage rates and the pluripotent state of embryonic cells.

Since the introduction of ovum pick-up/*in vitro* production (OPU-IVP), many efforts to improve embryo production efficiency have been undertaken. These were all focused on non-genetic factors. The genetic component to IVP was approached by Machado et al., (2006), suggesting its influence on the characteristics associated with IVP and, therefore, their heritability.

Genome-wide association studies on economic aspects have shown a clear genetic divergence between *Bos taurus* and *Bos indicus* (Fortes et al., 2010; Canavez et al., 2012; Höglund et al., 2012). Previous studies have reported an association between single nucleotide polymorphism (SNP) in *BMP4* gene and the blastocyst rates in Holstein cows evaluated

in vitro (Li et al., 2012), suggesting a major effect of *BMP4* on the performance of oocyte donors. In this study we evaluated the association between G>T, SNP *rs109778173* polymorphism in *BMP4* gene and *in vitro* production traits and pregnancy rate of Gyr cows.

Material and methods

Ethical considerations

This study was approved by the Ethics Committee for Animal Experimentation of Universidade Estadual do Norte Fluminense-UENF (Protocol number 243, March 11, 2014).

DNA samples and data collection

DNA was extracted from hair follicles via alkaline method, according to standard procedure (adapted from Coelho, 2001). DNA concentrations were measured using a NanoDrop[™] 2000 (Thermo Science, New York, NY, USA) spectrophotometer. Data from 212 OPU-IVP sessions of 50 Gyr oocyte donors were collected in 2012 and 2013 (Rio de Janeiro, Brazil). The OPU-IVP procedures were performed by two companies with extensive experience in the field.

The OPU-IVP traits associated with the SNP marker were: number and ratio of viable *cumulus-oophorus* complexes (Nvcoc and Pvcoc) classes I, II, and III that were successfully matured, fertilized, and *in vitro* cultured. Number and ratio of cleaved embryos at day 4 of culture (Ncleavd4 and Pcleavd4). Number and ratio of transferable embryos at day 7 of culture (Ntembd7 and Ptembd7) as a ratio of Nvcoc, according to the international embryo transfer society (IETS) classification as stage 4 (morula), grade 1 (excellent or good); and stages 5 to 7 (early blastocyst, blastocyst, and expanded blastocyst), grade 1 and 2 (excellent and regular).

Besides the characteristics mentioned above, SNP marker was also associated with number and ratio of pregnancies at days 30 and 60 after transfer (NPrD30, PPrD30 and NPrD60, PPrD60). All characteristics are described considering each OPU-IVP session. Data for all characteristics were recorded and ceded for research purposes.

Single nucleotide polymorphism analysis

Detection of *BMP4* polymorphism was carried out according to Li *et al.* (2012). A segment of the *BMP4* gene (exon 2, SNP *rs109778173*) was amplified by PCR with a specific primer (Table 1). The PCR reaction was performed in a final volume of 20 μ l, using 1x

PCR buffer [10 mM Tris-HCl (including Mg^{+2}), 0.5 mM dNTP mix (Promega, Madison, WI, USA), 1U of Taq DNA polymerase (Promega, Madison, WI, USA), 0.5 mM of each primer (Invitrogen, Sao Paulo, SP, Brazil), 50 ng of DNA extracted, and deionized water. A negative control was included for each replicate of the PCR reaction.

Table 1. Conditions for the primer used in the amplification of SNP *rs109778173* in *BMP4* gene.

Gene	Primer sequence 5'-3'	Annealing temperature (°C)	PCR product size (bp)	Reference
<i>BMP4</i>	F 5' TAGAACATCTGGAGAACATC R 5' GGCTTCATAACCTCATAAATG	55	190	Li <i>et al.</i> , 2012

The cycling conditions consisted of an initial state of 95 °C for 1 min, followed by 40 amplification cycles with denaturation at 95 °C for 30 s, annealing of primers at 55 °C for 1 min, and extension at 72 °C for 1 min. After the last cycle, reactions were led to a final step of 7 min at 72 °C for the final extension of the tapes. PCR was conducted using a thermocycler (Applied Veriti® 96-Well, Applied Biosystems, Foster City, CA, USA). The product was separated by polyacrylamide gel (8%) stained with silver nitrate to confirm the amplification (Sigma-Aldrich, Sao Paulo, SP, Brazil).

Polymerase chain reaction-restriction-fragment length polymorphism (PCR-RFLP) was used for genotyping the amplified samples. The restriction enzyme *HinfI* used (Life technologies, Sao Paulo, SP, Brazil) recognizes and cleaves the 5' G⁺ANTC 3' sequence (Table 2). This enzyme recognizes only one cleavage site in the amplified fragment, with a reaction product of two fragments sized 80 bp and 110 bp. The expected result was cleavage of the samples that did not display the G>T mutation. The cleavage reaction was carried out in a final volume of 20 μ l, including *HinfI* specific buffer, 5 IU restriction enzyme, 4 μ l PCR product, and deionized water.

Table 2. *BMP4* SNP and restriction endonuclease information.

Gene	SNP number NCBI	SNP sequence	Restriction enzyme	T _m -RFLP (°C)
<i>BMP4</i>	<i>rs109778173</i>	GGGCCCTGGTCCACCTGCTCCCGGAA[G/T] AGTCGAAGCTCGGCAGACGAGATCA	<i>HinfI</i>	37

T_m = melting temperature.

To confirm the digestion reaction, the product was separated by polyacrylamide gel (8%) and stained with silver nitrate (Sigma-Aldrich). After this visualization, the samples were submitted to capillary electrophoresis using the equipment for allelic discrimination by size and polymorphism identification (Fragment Analyzer™, Advanced Analytical, Ames, IA, USA).

Statistical analysis

Genotypic and allele frequency, Hardy-Weinberg equilibrium probability (P-HW), gene homozygosity and heterozygosity (H_o and H_e), effective allele number (N_e) and polymorphism information content (PIC) were statistically analyzed according to the approaches by Nei and Roychoudhury (1974) and

Nei and Li (1979). These tests were conducted using PowerMarker v.3.25 software.

The association between SNP marker and OPU-IVP traits and pregnancy rates was determined by analysis of variance of repeated data using the following model:

$$Y_{ij} = \mu + E_i + \varepsilon_{ij}$$

Where:

Y_{ij} is the observation for the OPU-IVP traits.

μ is the mean for each trait.

E_j is the genotype effect.

ε_{ij} is the random error.

This model was analyzed using the PROC GLIMMIX procedure of SAS 9.2 program (SAS Institute Inc., Cary, NC, 1999).

Results

The protein coding domain of *BMP4* gene is located into exon 2. We amplified a fragment of DNA sequence in exon 2, where the SNP mutation number *rs109778173* was identified in Holstein cows (Li et al., 2012). We found the mutation G>T in Gyr cows using PCR-RFLP and capillary electrophoresis methods. Three genotypes were identified (GG, TT and GT) with genotypic frequencies of 0.64, 0.32, and 0.04, respectively. Allelic frequency and Hardy-Weinberg equilibrium are described in Table 3.

Table 3. Genotypic and allelic frequency of *BMP4* gene in Gyr cows oocyte donors.

Polymorphism	Genotypic frequency		Allele frequency	P-HW* (Chi-squared)
	%	(n)		
Exon 2 SNP <i>rs109778173</i>	GG (0.64)	(32)	0.8 ± 0.03 G	0.53205
	TT (0.32)	(16)	0.2 ± 0.04 T	
	GT (0.04)	(2)		

*The Hardy-Weinberg equilibrium probability was tested at 5% (Chi-squared values >0.05 means that the SNP are in disequilibrium).

The results displayed in Table 4 show heterozygosity, polymorphism information content, and effective number of alleles with medium values. This data points out that polymorphism levels and genetic variation were medium, according to the classification of PIC (low polymorphism if PIC value <0.25, medium polymorphism if 0.25<PIC value <0.5, and high

polymorphism if PIC value >0.5). The Chi-squared test showed that genotypic distributions in the population studied agreed with Hardy-Weinberg equilibrium ($p>0.05$).

Medium values of SNP polymorphism for the characteristics above mentioned indicate its potential use as a molecular marker (Fu et al., 2013).

Table 4. Population genetic indexes at the *BMP4* exon 2 (187 bp) region.

Location	Gene homozygosity (Ho)	Gene heterozygosity (He)	Effective allele number (Ne)	Polymorphism information content (PIC)
rs109778173	0.30	0.36	1.56	0.26

Electropherogram and capillary electrophoresis gel for G>T mutation in *BMP4* gene are shown in Figure 1. Alleles of 75bp and 105bp were obtained after cleavage with *HinfI* restriction enzyme. The

mutant allele was not cleaved because the recognizing site was absent, and the allele size was 187 bp, leading us to conclude that the mutation was present in heterozygous.

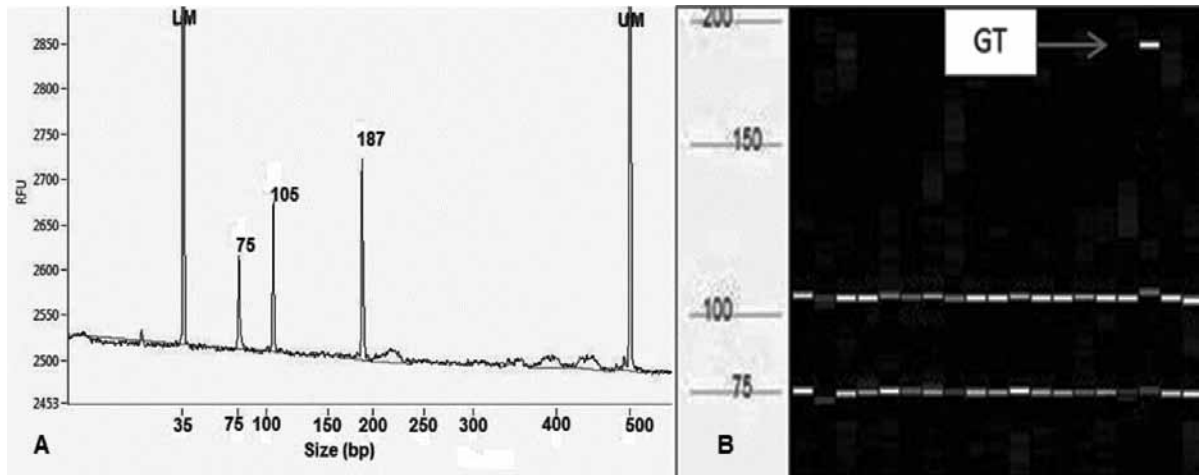


Figure 1. Electropherogram (A) of *HinfI* PCR-RFLP. The GT genotype shows two fragments for the cleaved allele (75bp and 105bp) and for the 187bp allele not cleaved (mutation in heterozygous). And capillary electrophoresis gel (B) shows one sample with G>T mutation (arrow).

Polymorphism variance analysis for OPU-IVP traits and pregnancy rates at *rs109778173* -*BMP4* gene exon 2 (187bp) locus showed significant effect of genotype GT and characteristics of number and ratio of viable *cumulus-oophorus* complexes (Nvcoc and Pvcoc) and ratio of pregnancies at 30 days (PPrD30). Means of GT genotype showed lower performance for the characteristics listed above at probability level of 1% (Table 5). The GT genotype might be an unfavorable genotype.

The mean values for the other characteristics evaluated showed no significant effect depending on genotype, although all of them were numerically lower in mutant genotype when compared with other genotypes (GT vs. GG and TT). Genotypes GG and TT showed no significant effect on OPU-IVP traits and pregnancy rates.

Table 5. Association between the SNP (*rs109778173*) genotype of the *BMP4* gene and embryo production for ovum pick-up in Gyr cattle.

Traits*	Genotypes (number/OPU-IVP sessions) (mean ± SE)		
	GG (32/157)	TT (16/44)	GT (2/11)
NVcoc	10.01 ± 6.95 ^a	9.25 ± 6.03 ^a	5.72 ± 4.51 ^b
PVcoc	75.82 ± 20.46 ^a	76.73 ± 18.26 ^a	60.50 ± 28.69 ^b
NcleavD4	6.25 ± 4.72	7.2 ± 5.24	4.27 ± 4.73
PcleavD4	64.59 ± 34.68	74.13 ± 30.12	67.17 ± 37.27
NTembd7	2.56 ± 2.78	2.90 ± 3.65	1.45 ± 2.38
PTembD7	36.91 ± 35.40	32.84 ± 33.26	26.90 ± 35.27
NPrD30	0.68 ± 1.17	0.59 ± 0.87	0.18 ± 0.60
PPrD30	29.27 ± 39.15 ^a	32.65 ± 43.25 ^a	6.09 ± 64.20 ^b
NPrD60	0.64 ± 1.12	0.50 ± 0.82	0.18 ± 0.60
PPrD60	27.07 ± 37.97	25.45 ± 39.61	6.09 ± 20.20

*NVcoc and PVcoc, number and ratio of viable *cumulus-oophorus* complexes; Ncleavd4 and Pcleavd4, number and ratio of cleaved embryos at day 4 of culture; Ntembd7 and Ptembd7, number and ratio of transferable embryos at day 7 of culture; NPrD30 and PPrD30 number and ratio of pregnancies at day 30 after transfer and NPrD60 and PPrD60, number and ratio of pregnancies at day 60 after transfer.

^{ab}Mean between genotypes for the SNP with different superscript letters are significantly different (p<0.01).

Discussion

The *BMP4* gene, located at chromosome 10 can be found in humans, chimpanzees, rhesus monkeys, dogs, mice, rats, and zebra fish. *B. taurus* is one of 65 orthologous species with the human *BMP4* gene (Waterhouse et al., 2011).

Involvement of bone morphogenetic proteins in development processes of embryonic tissues has been documented starting from the gastrulation process. Gastrulation is a highly dynamic process. When gastrulation evolves properly, it allows two major events of early development of vertebrates: I) the establishment of morphologically visible antero-posterior (head or tail) and dorsal-ventral axis of the cord (or mouth-spinal), and II) the specification and standardization of early germ layers, especially the mesoderm (Downs and Davies, 1993).

Mutants obtained during the gastrulation process recently revealed that the axis of the embryo proper specification consists of a series of reciprocal interactions between the embryonic and extraembryonic tissues, so genes involved in these interactions can then contribute to the morphogenic development of the embryo (Pfister et al., 2007; Tam and Loebel, 2007).

Animals displaying G>T mutation in *BMP4* gene in this study presented 4.29 less viable oocytes per OPU session compared to the GG genotype, which represents a 16.23% loss to the TT genotype. Also, pregnancy rate at 30 days from transferred embryos obtained from the GT cows was 26.5% lower compared to the TT genotype.

Li et al. (2012), using an *in vitro* model in Holstein cows, showed similar results for the G>T mutation in *BMP4* gene. They concluded that embryos produced from genotype TT cows showed 10.5 and 16.1% higher blastocyst rates than GG and GT cows, respectively. The same authors showed that blastocyst rate was significantly associated with SNP *rs109778173* of *BMP4* ($p = 0.006$), whereas the association with fertilization rate was not statistically significant ($p = 0.095$).

La Rosa et al. (2011) demonstrated that *BMP4* slows down the development of only *in vitro* fertilized

embryos, and concluded that it influences bovine *in vitro* oocyte maturation and affects cleavage rates and the pluripotent state of embryonic cells, suggesting that a balance of *BMP* signaling is needed for a proper development during pre-implantation of bovine embryos.

The genotypic frequency in the Gyr cows population studied was 0.64, 0.32 and 0.04 for the GG, TT and GT genotypes, respectively, concluding that the GT genotype is less present, in contrast with the genotypic frequencies observed by Li et al. (2012) (approximately 0.56, 0.05, and 0.37, respectively). Therefore, the GT genotype has a higher ratio for the *BMP4* gene (SNP *rs109778173*) in Holstein cows, compared with the same genotype in the Gyr population studied in this work.

Probability values for the Hardy-Weinberg equilibrium ($p > 0.05$) in both populations show disequilibrium with the evaluated SNP, and therefore, with their ability to segregate (Ardlie et al., 2002).

We hypothesized that the difference between genotypic frequencies for GT in two populations could be associated with selection processes aimed at increasing milk production. However, studies in both populations (Holstein and Gyr) involving SNPs related to milk production and fertility must be conducted to test the hypothesis of joint segregation.

The widespread use of artificial insemination in Nordic Red cattle has resulted in increased selection intensity, leading to increased productivity. However, cow fertility has concurrently severely declined. Kadri et al. (2014) identified a 660-kb deletion encompassing four genes as the causative variant. They showed that the deletion is a recessive embryonically lethal mutation. This study demonstrates that embryonic lethal mutations account for a non-negligible fraction of the decline in fertility of domestic cattle, and that associated positive effects on milk yield may account for part of the negative genetic correlation.

The G>T mutation at SNP *rs109778173* is classified as silent or synonymous, given that the replacement of guanine by thymine nucleotide in codon (CTG by CTT) does not alter the amino acid sequence in the protein (in the case of the protein

coding of *BMP4* gene it is leucine). This phenomenon is often referred to as redundancy of the genetic code.

Despite this redundancy, synonymous codons are not used with equal frequency due to a phenomenon known as codon bias (Ikemura, 1981). If codon bias is the result of natural selection, a change from a preferred to an undesirable codon should lead to reduced protein expression, caused by a decrease in the efficiency or fidelity of translation or a combination of both (Bulmer, 1991). Adoligbe *et al.* (2012) showed a significant effect for synonymous mutation in *GDF10* gene with body traits in indigenous Chinese cattle.

When a synonymous or silent mutation occurs, the change is often assumed to be neutral, meaning that it does not affect the fitness of the individual carrying the new gene to survive and reproduce. Synonymous changes may not be neutral because certain codons are translated more efficiently (faster and/or more accurately) than others (Carlini and Stephan, 2003).

The association between *BMP4* gene polymorphism and OPU-IVP traits in Gyr cattle confirmed the influence of this gene in embryonic development and fertilization rates after embryo transfer. The GT genotype negatively affects characteristics such as number and ratio of viable *cumulus-oophorus* complexes and ratio of pregnancies at day 30 after transfer. This finding could be an indication of a genetic effect of *BMP4* on these characteristics.

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Conflicts of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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