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Effects of genetic background on responses to superovulation in Japanese Black cattle

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ABSTRACT. We investigated the effects of genetic background on the responses to superovulation in Japanese Black cattle. The genotype frequencies of *GRIA1* and *FSHR* relating to ovulation and follicular development in each of the major bloodlines—Tajiri, Fujiyoshi, and Kedaka—were analyzed. The Tajiri line had the lowest frequency of G allele homozygosity of c.710A>G in *GRIA1* among the three bloodlines, and deviation from Hardy–Weinberg equilibrium was detected. Genotype frequencies of c.337C>G, c.871A>G, and c.1973C>G in *FSHR* were in Hardy–Weinberg equilibrium in all bloodlines. The results of generalized linear mixed-model analyses showed that farm, levels of plasma anti-Müllerian hormone (AMH) concentration, age in months, repeated superovulation, c.337C>G in *FSHR*, and bloodlines had significant effects on the responses to superovulation. The number of transferable embryos in the group heterozygous for c.337C>G in *FSHR* was significantly higher than that in the group homozygous for the C allele. The Kedaka line showed a significantly higher number of ova/embryos, fertilized embryos, and transferable embryos than the Tajiri and Fujiyoshi lines. The concentration of circulating AMH is a useful endocrine marker for antral follicle counts. This study revealed the effects of genetic background on the responses to superovulation using levels of plasma AMH concentration as a covariate. The prominent effect of genetic background on superovulation in the Kedaka line requires additional studies to confirm the genomic regions and polymorphisms that are involved in the trait.

KEY WORDS: bloodline, *FSHR*, *GRIA1*, Japanese Black cattle, superovulation

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Embryo transfer is an important technique that is used to improve genetic capacity in cattle. Recently, oocytes collected from slaughterhouse ovaries or live animals using ovum pick-up have been frequently used for the production of *in vitro*–fertilized embryos; however, the production of *in vivo*-fertilized embryos by superovulating donor animals is also important. In general, conception rate and resistance to cryopreservation of the *in vivo* embryos is better than *in vitro* embryos [7]. On the contrary, considerable individual variability in the responsiveness to superovulation has been a limiting factor that affects the efficiency of *in vivo* embryo production [1].

The impact of genetic background on the responsiveness to superovulation in cattle has yet to be fully elucidated. Sugimoto *et al*. [13] demonstrated that a single nucleotide polymorphism (SNP) of glutamate ionotropic receptor AMPA type subunit 1 (*GRIA1*) in Japanese Black cattle correlated with an increased number of ovulations. Cory *et al*. [2] reported that SNPs in follicle-stimulating hormone receptor (*FSHR*) in Holstein cows were related to the responsiveness to superovulation; however, trials of superovulation are scant. Recently, Parker Gaddis *et al*. [9] conducted a large-scale study to estimate the genetic parameters involved in the responsiveness to superovulation. Their study in Holstein cows revealed several genomic regions related to superovulation and suggested that, with this information, the selection of specific traits in offspring should be possible. The effects of candidate genes within these genomic regions remain to be investigated.

Antral follicle counts (AFCs) in the ovary vary greatly among individuals, and the number of small antral follicles that are

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responsible for exogenous administration of follicle-stimulating hormone (FSH) has been found to significantly correlate with the number of embryos after superovulation [6, 11]. The concentration of circulating anti-Müllerian hormone (AMH) produced by the granulosa cells of healthy growing follicles is strongly associated with AFCs and useful as an endocrine marker for ovarian reserves in cattle. We have reported that the level of plasma AMH concentration and the *GRIA1* genotype have a synergistic effect on the responsiveness to superovulation, which is helpful in the accuracy of predictions of those responses [4]; however, differences in plasma AMH concentrations has a larger effect on superovulation than the *GRIA1* genotype; therefore, the study of the animals' genetic background on superovulation must take into account the potential factors in the responses, such as AFCs and levels of plasma AMH concentration.

Japanese Black cattle are derived from native Asian cattle and have been specifically selected to produce densely marbled beef. There are three basic bloodlines in Japanese Black cattle—Tajiri, Fujiyoshi, and Kedaka—that evolved from areas of regional geographic isolation in Japan. The bloodlines have characteristic genetic traits for meat quality, growth rate, frame type, and maternal ability. In addition, although experience shows that embryo production efficiency in response to superovulation varies among the bloodlines, any detailed studies of the specific characteristics of these bloodlines with regard to their response to superovulation are unreported.

In this study, we investigated the effects of the *GRIA1* genotype, *FSHR* genotypes, and the bloodlines on the responsiveness to superovulation in Japanese Black cattle using a generalized linear mixed model (GLMM) with levels of plasma AMH concentration as a covariate.

MATERIALS AND METHODS

All procedures for animal experiments were carried out in accordance with guidelines and ethics approved by the Animal Experiment Committee of the Animal Research Center.

Superovulation and embryo collection

Between 2010 and 2013 at the Animal Research Center (farm A) and between 2007 and 2013 at a commercial farm (farm B) located in the eastern area of the Hokkaido Prefecture, Japan, superovulation was induced in Japanese Black cattle, and the embryos were collected. Superovulation was induced in the animals using FSH (20 IU/cow, Antrin R-10, Kyoritsu Seiyaku Corp., Tokyo, Japan) administered twice daily in decreasing doses over 3 days. An injection of prostaglandin F2α (cloprostenol 0.5 or 0.75 mg/cow, Resipron-C, ASKA Animal Health Co., Ltd., Tokyo, Japan) was administered on the third day of the superovulation treatment. A controlled internal drug release (CIDR) device (Eazi-Breed, Pfizer Japan Inc., Tokyo, Japan) was inserted into the cows for 7–10 days before the prostaglandin F2α was administered. An injection of 1 mg of cow estradiol benzoate (Kyoritsu Seiyaku Corp.) was administered 4 days before the initiation of FSH injections at farm A. An injection of 2 mg of cow estradiol benzoate was administered 5 days before the initiation of FSH injections at farm B. The donor cows were inseminated 12 and 24 hr after detection of estrus by visual observation, and the embryos were recovered 7–8 days after insemination. A highly skilled technician specific to each farm collected the embryos, which were then classified according to the criteria set by the International Embryo Technology Society [14]. For this study, code 1 and code 2 embryos were defined as transferable embryos.

Genotyping GRIA1 and FSHR

DNA was extracted from venous blood samples using the DNeasy Blood and Tissue Kit (QIAGEN GmbH, Hilden, Germany). Polymerase chain reaction (PCR) primers and restriction enzymes are listed in Table 1. The genetic polymorphism of *GRIA1* was determined according to Sugimoto *et al*. [13]. Direct DNA sequencing of PCR products or an analysis of PCR-restriction fragment length polymorphism (PCR-RFLP) was conducted to determine the genotype of c.710A>G. The genetic polymorphisms of *FSHR* were determined according to Cory *et al*. [2]. PCR-RFLP analysis was conducted to determine the polymorphisms of c.337C>G, c.871A>G, and c.1973C>G.

Table 1. Primer sequences and restriction enzymes for PCR-RFLP

Gene	SNP	Primer	Restriction enzyme	PCR products (bp) [restriction fragments]
GRIA1	c.710A>G	AGCCTCCCTACCAGCTCTCT CGTTGTTGCCAGCCTCAC	BfuAI	241 [120, 87, 26]
FSHR	c.337C>G	GGACAAAGGGTGAATAACTG CCCCACATCTTTGATTACAA	HgaI	286 [150, 136]
	c.871A>G	AGGGCAGACAGACTGTTAGA GTGATGGCCAGGATGCTAAT	BsrI	409 [265, 144]
	c.1973C>G	CATCTTCACCAAGAACTTCC TGCCAGGGAGATTAAATTAG	MnlI	329 [226, 103]

Table 2. Basic statistical data on superovulation

Farm	Animals	Total of SOV	Age in months at SOV	Repetition of SOV	No. of ova/embryos	No. of fertilized embryos	No. of transferable embryos	AMH (*ng*/m*l*)
A	41	162	92 ± 39	4.0 ± 3.5	12.3 ± 10.4	10.0 ± 9.5	5.9 ± 6.1	0.274 ± 0.297
B	102	835	50 ± 16	8.2 ± 4.7	22.6 ± 12.6	17.6 ± 11.0	12.6 ± 8.2	0.465 ± 0.382
Total	143	997	56 ± 26	6.9 ± 4.8	21.0 ± 12.8	16.3 ± 11.2	11.5 ± 8.3	0.434 ± 0.376

Data presented are the mean ± standard deviation. SOV: superovulation.

Plasma AMH concentration

Venus blood samples were collected in heparinized tubes and centrifuged at 3,000 × g for 10 min at 4°C to recover the plasma, which was stored at −20°C until the AMH assays were conducted. At farm A, blood was collected before each superovulation, and the interval between blood collection and embryo collection was 38 ± 66 days (mean ± standard deviation). At farm B, blood was collected from all animals only once in March 2013; therefore, the interval between blood collection and embryo collection was −275 ± 432 days. AMH concentrations were measured as previously reported using an AMH Gen II ELISA kit (Beckman Coulter, Brea, CA, U.S.A.). Undiluted plasma (20 μl) was used for the analysis [4].

Statistical analyses

GLMM was used to analyze the effects of the farm, levels of plasma AMH concentration, age in months, repeated superovulation, genetic polymorphisms in *GRIA1* and *FSHR*, and the bloodlines of donor animals on the response to superovulation. We considered individual animals as random effects. The probability distribution of trait values was assumed as the poisson distribution, and the natural logarithm was used as the link function. Bloodline of donor animals was defined as the lineage of the sire and was divided into three major groups—Tajiri, Fujiyoshi, and Kedaka—of Japanese Black cattle based on the definition provided by the Livestock Improvement Association of Japan, Inc. The minor bloodlines, Shigekane and Eiko, were grouped into the Tajiri and Kedaka lines, respectively, which were closest to them in lineage. The numbers of donor animals belong in the Tajiri, Fujiyoshi, and Kedaka lines were 11 (27%), 5 (12%), and 25 (61%) in farm A and 40 (39%), 22 (22%), and 40 (39%) in farm B. The numbers of animal and basic statistical information on the outcome of superovulation (e.g., number of embryos and AMH levels) are presented in Table 2. Some of these data were used in a previous study that investigated the correlation between the levels of plasma AMH concentration and the responsiveness to superovulation [5]. The effects of the independent variable on the numbers of ova/embryos (NOEs), fertilized embryos (NFEs), and transferable embryos (NTEs) were evaluated using the Type II Wald χ^2 test. The least squared means of NOEs, NFEs, and NTEs were calculated using models. The effects of genetic polymorphism in *GRIA1*, *FSHR,* and bloodlines on NOEs, NFEs, and NTEs were evaluated using Tukey multiple comparison test. Deviation from Hardy–Weinberg equilibrium was tested using the χ^2 test. The experimental data were analyzed using the R statistical software implementing the lme4 package and the lsmeans package.

RESULTS

Donor Japanese Black cows had the lowest frequency of homozygosity for A allele of c.710A>G in *GRIA1* (Table 3). A different genotype frequency in the SNP was observed in the Tajiri bloodline compared with that in the Fujiyoshi and Kedaka lines. The Tajiri line had the lowest frequency of being homozygous for G allele among the three bloodlines, and a deviation from Hardy–Weinberg equilibrium was detected.

In the polymorphisms of *FSHR*, the frequency of homozygosity for G allele at c.337C>G was the lowest regardless of bloodlines. The highest frequency of homozygosity for C allele was observed in the Tajiri and Kedaka lines, whereas heterozygosity was highest in the Fujiyoshi line. The frequencies of heterozygosity for c.871A>G and c.1973C>G were high in all bloodlines. Genotype frequencies of these SNPs in *FSHR* exhibited Hardy–Weinberg equilibrium.

Mean ± standard deviation of plasma AMH concentration (*ng*/m*l*) in the Tajiri, Fujiyoshi, and Kedaka lines were 0.359 ± 0.319, 0.302 ± 0.205, and 0.341 ± 0.340, respectively. There was no significant difference in plasma AMH concentrations among bloodlines.

Environmental factors that may affect the superovulatory response were incorporated into GLMMs as covariates (Table 4). The results of the Type II Wald χ^2 test showed that farm, AMH concentration, and repeated superovulation had significant effects (P<0.001) in GLMMs on NOEs, NFEs, and NTEs. Age in months had significant effects in GLMMs on NOEs (P<0.001), NFEs (P<0.001), and NTEs (P<0.01).

The least square means of NOEs, NFEs, and NTEs were estimated using GLMMs according to each genotypes and bloodlines (Table 5). Significant effects of genetic polymorphisms in *GRIA1* were not detected. Significant effects on NTEs were detected only in C.337C>G of *FSHR*. NTEs in those cattle heterozygous for c.337C>G were significantly higher than those homozygous for C allele. NOEs, NFEs, and NTEs in the Kedaka bloodline were significantly higher than those in the Tajiri and Fujiyoshi lines.

Table 3. Genotype frequency in major bloodlines of Japanese Black cattle donor

Gene	Genotype and HWE	Bloodline			Total (n=143)
		Tajiri (n=51)	Fujiyoshi (n=27)	Kedaka (n=65)	
GRIA1	AA	0.157	0.111	0.031	0.091
c.710A>G	AG	0.686	0.370	0.415	0.503
	GG	0.157	0.519	0.554	0.406
	HWE	*P*=0.0078	n.s.	n.s.	n.s.
FSHR	CC	0.627	0.296	0.600	0.552
c.337C>G	CG	0.353	0.630	0.385	0.420
	GG	0.020	0.074	0.015	0.028
	HWE	n.s.	n.s.	n.s.	n.s.
FSHR	AA	0.078	0.111	0.215	0.147
c.871A>G	AG	0.549	0.481	0.615	0.566
	GG	0.373	0.407	0.169	0.287
	HWE	n.s.	n.s.	n.s.	n.s.
FSHR	CC	0.431	0.444	0.185	0.322
c.1973C>G	CG	0.510	0.481	0.600	0.545
	GG	0.059	0.074	0.215	0.133
	HWE	n.s.	n.s.	n.s.	n.s.

HWE: Hardy–Weinberg equilibrium.

Table 4. Effects of environmental factors in generalized linear mixed models for superovulatory response

Covariate	No. of ova/embryos		No. of fertilized embryos		No. of transferable embryos	
	Estimate	*P* value	Estimate	*P* value	Estimate	*P* value
Farm	1.614	<0.001	1.674	<0.001	1.581	<0.001
Plasma AMH concentration	0.732	<0.001	0.592	<0.001	0.413	<0.001
Age in months	1.227	<0.001	1.282	<0.001	0.755	<0.01
Repetition of superovulation	−0.042	<0.001	−0.041	<0.001	−0.030	<0.001

Table 5. Least square means of superovulatory response estimated using generalized linear mixed models

Genotype and bloodline		n	Least square mean ± standard error		
			No. of ova/embryos	No. of fertilized embryos	No. of transferable embryos
GRIA1 c.710A>G	AA	87	2.26 ± 0.23	1.80 ± 0.29	1.35 ± 0.25
	AG	490	2.25 ± 0.16	1.85 ± 0.20	1.42 ± 0.18
	GG	420	2.43 ± 0.16	2.06 ± 0.19	1.60 ± 0.17
FSHR c.337C>G	CC	490	2.14 ± 0.12	1.74 ± 0.15	1.41 ± 0.13$^{b)}$
	CG	490	2.35 ± 0.13	2.05 ± 0.16	1.74 ± 0.14$^{a)}$
	GG	17	2.45 ± 0.36	1.91 ± 0.44	1.22 ± 0.40
FSHR c.871A>G	AA	142	2.40 ± 0.35	2.14 ± 0.43	1.84 ± 0.38
	AG	555	2.16 ± 0.26	1.63 ± 0.31	1.17 ± 0.28
	GG	300	2.39 ± 0.32	1.93 ± 0.39	1.37 ± 0.35
FSHR c.1973C>G	CC	321	2.04 ± 0.31	1.64 ± 0.38	1.43 ± 0.34
	CG	545	2.46 ± 0.23	2.19 ± 0.28	1.69 ± 0.25
	GG	131	2.44 ± 0.39	1.88 ± 0.48	1.25 ± 0.42
Bloodline	Tajiri	335	2.32 ± 0.17$^{b)}$	1.86 ± 0.21$^{b)}$	1.40 ± 0.19$^{b)}$
	Fujiyoshi	162	1.97 ± 0.20$^{B)}$	1.55 ± 0.25$^{B)}$	1.16 ± 0.22$^{B)}$
	Kedaka	500	2.65 ± 0.16$^{A,a)}$	2.30 ± 0.20$^{A,a)}$	1.81 ± 0.18$^{A,a)}$

Lower cases (a and b) letters indicate $P<0.05$; upper cases (A and B) indicate $P<0.001$.

DISCUSSION

The Japanese Black is the most popular breed of Wagyu and has been bred in each region of Japan for many years. The Tajiri bloodline originates from a sire born in the Hyogo Prefecture and its outstanding genetics produces finely marbled beef. The Kedaka and Fujiyoshi bloodlines were formed in the Tottori and Shimane Prefectures, respectively. These closed breedings not only contributed to the improvement of beef marbling standard number but also led to specific characteristics within the lines, such as growth rates, frame size, and temperament; therefore, comparisons among bloodlines might provide valuable information about hereditary factors that are responsible for reproductive traits. In the present study, we investigated the effects of genetic background on the response to superovulation in Japanese Black cattle.

First, we analyzed the genotype frequencies of the genes related to ovulation and follicular growth. The genotype frequency of c.710A>G in *GRIA1* was similar to that in the report on donor Japanese Black cows by Sugimoto *et al*. [13], in which homozygosity for the A allele of c.710A>G corresponding to an asparagine at amino acid residue 306 had the lowest response to superovulation treatment and genotype frequency. We analyzed the genotype frequencies in the bloodlines, and deviation from Hardy–Weinberg equilibrium was observed only in the Tajiri line. The genotype frequency in the Tajiri line of heterozygosity for c.710A>G was the highest among the three bloodlines, and the genotype frequency of homozygosity for G allele was the lowest. Cory *et al*. [2] compared genotype frequencies of three SNPs—c.337C>G, c.871A>G, and c.1973C>G—in *FSHR* among Holstein, Jersey, Angus, and Charolais cows. The present results indicate that genotype frequencies of the three SNPs of *FSHR* in Japanese Black cows were similar to those in Holstein cows. No deviation from Hardy–Weinberg equilibrium was observed in any SNPs or bloodlines. These results suggest that genetic selection during the process of establishing the Tajiri line affected the genotype frequency of *GRIA1* polymorphism. The Tajiri line produces finely marbled beef and in general exhibits a smaller frame, lower birth weight, and lower growth rate than those of other lines; therefore, breeding that focused on marbling might have had adverse effects on the propensity for superovulation.

Many factors, such as breed, age, nutrition, and other management factors, affect ovarian responses [3, 8]. In this study, significant effects on the superovulatory response were found in farm, levels of plasma AMH concentration, age in months, and repeated superovulation. The difference between the results from the two farms might be attributed to differences in the management conditions. In concert with previous studies, levels of plasma AMH concentration were positively correlated with embryo production efficiency [5, 10, 12]. We previously reported that the levels of plasma AMH concentration in donors gradually decreased with repeated superovulation [5]. Although these levels in cows from farm B might not be accurate because blood was collected only once, and the intervals between embryo recovery and blood collection varied widely with individual cows, an obvious correlation was found between levels of plasma AMH concentration and responses to superovulation. The superovulatory response was positively affected by age in months. Prolonged use of donors that respond better to superovulation might cause the positive effect. Although previous studies suggest that embryo production is not decreased when superovulation is repeatedly induced in donors by progestin and prostaglandin F2α [3, 8], reduced responsiveness to repeated superovulation is commonly understood among practitioners. In this study, the above four environmental factors were incorporated into GLMMs as covariates to evaluate effects of genetic factors.

The NOE arithmetic means according to the c.710A>G genotype of *GRIA1* were 19.3, 19.7, and 22.8 in those homozygous for A allele (n=87), those heterozygous (n=490), and those homozygous for G allele (n=420), respectively, and the highest NOEs were observed in those homozygous for G allele, as observed in previous studies [4, 13]; however, the statistically significant effect of *GRIA1* genotype using GLMM analysis was not observed. The difference of statistical results may be due to the use of GLMM analysis with consideration of environmental factors. A significant effect of genetic polymorphism was detected only in c.337C>G of *FSHR*. Although homozygosity for G allele of c.337C>G was less frequently (17 out of 997 superovulation) used to evaluate the effects on superovulation, NTEs in those heterozygous for SNP was significantly higher than in those homozygous for C allele. Cory *et al*. [2] reported that the percentage of viable embryos in those homozygous for G allele in c.337C>G was significantly higher than that in those homozygous for C allele, but that there were only two animals homozygous for G allele. The present study suggests that the G allele of c.337C>G might have a positive effect on the viability of embryos produced by superovulation. Further investigation is needed to elucidate the effect of SNP on FSH signaling in granulosa cells.

Variation of embryo production efficiencies among Japanese Black cattle bloodlines has been empirically recognized. For the first time, we analyzed the effects of the bloodlines with regard to the levels of plasma AMH concentration that varied greatly among individuals and had a strong association with the responsiveness to superovulation. The Kedaka line showed significantly higher embryo production efficiency than did the Fujiyoshi and Tajiri lines. The responsiveness to superovulation is a quantitative trait, and the recent study using genome-wide association analyses showed that some genomic regions in BTA5, BTA8, BTA13, BTA14, and BTA21 are involved in that trait [9]; therefore, genetic polymorphisms in *GRIA1* and *FSHR* that are located on BTA7 and BTA11, respectively, might have a minor function in superovulation. The prominent effect of genetic background in the Kedaka line on superovulation is interesting and requires further studies to confirm the association with candidate genomic regions suggested by Parker Gaddis *et al*. [9].

In summary, GLMM analyses on the responses to superovulation in Japanese Black cattle suggest that c.337C>G in *FSHR* had a significant effect on NTEs. Distinct differences in this response were found among the three bloodlines. The Kedaka line showed significantly higher embryo production efficiency than did the Tajiri and Fujiyoshi lines. These findings provide important information for improving the efficiency of *in vivo* embryo production by genetic selection.

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