Family with Sequence Similarity 213, Member A is Associated with the Fertility Selection Index in Holsteins

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Abstract:

Female fertility is an economically important trait in the dairy industry, and the fertility selection index has been developed as a method of including female fertility in the breeding goals of this industry. This index considers a combination of factors, including days open, number of inseminations per lactation, success after first insemination, and pregnancy within 70 d, 90 d, and 110 d after parity. Based on a genome-wide association study of the fertility selection index using 442 Holsteins, we found that the index is influenced by a variation in the thioredoxin fold region of the family with the sequence similarity 213, member A (FAM213A) protein. FAM213A is a CXXC motif-containing peroxiredoxin 2-like protein that regulates cellular redox status. A replacement of isoleucine with valine in FAM213A was associated with poor fertility in cows. The overexpression of $FAM213A^{Val}$ in bovine endometrial epithelial cells reduced reactive oxygen species to a lesser extent relative to the overexpression of $FAM213A^{Ile}$ and caused a decrease in cyclooxygenase-2 expression. Downregulation of cyclooxygenase-2 led to a decline in prostaglandin E2, which is critical for implantation because it protects the conceptus from the maternal immune system. Cows with $FAM213A^{Val}$ showed lower levels of prostaglandin E2 than did cows with $FAM213A^{Ile}$, suggesting that cows with $FAM213A^{Val}$ are less fertile than cows with $FAM213A^{Ile}$ because of their reduced uterine environment. Thus, the present study found that FAM213A unexpectedly modulates female fertility in cattle.

Introduction

Dairy production depends on the frequency at which cows conceive; thus, female fertility is essential for this industry. To improve overall female fertility, the dairy industries in certain countries have combined several reproductive components into one overall index: the fertility selection index (SI) [1–4]. In Japan, the SI consists of the estimated breeding values (EBVs) for days open (DO), the number of inseminations per lactation (NI), success after first insemination (SFI), and pregnancy within 70 d (P70), 90 d (P90), and 110 d (P110) after parity [5]. The first two traits are continuous traits, whereas the other four traits are binary traits that show either success or failure after insemination. The genetic correlations among these reproductive traits are not necessarily ± 1.0 [1, 3]. Therefore, a principal components analysis is used to estimate weights for each trait and combine them into the SI to allow for the selection of these six fertility traits simultaneously.

Several groups have already conducted genome-wide association studies (GWASs) for SI using Danish Jersey [4], Nordic Red [6], and Italian Holstein [7]. These studies revealed some single nucleotide polymorphisms (SNPs) associated with both SI and individual traits included SI, suggesting that important fertility traits for selection in the population would generate the signal. Thus, studying the genetics of the SI using a GWAS may be helpful for understanding the underlying biological mechanisms and improving the general fertility level of the population.

Materials and Methods

Samples

We collected blood from 7,034 Holstein cows and evaluated their SI [5]. The mean SI was 0, and cows with a SI lower than -1.35 were in the 5th percentile, whereas cows with a SI higher than 1.46 were in the 95th percentile of the study population (Fig. 1A). We selected samples from 212 cows with an SI

lower than -1.35 as well as samples from 230 cows with an SI higher than 1.46.

Whole-Genome Scan

We genotyped 176 low SI samples and 174 high SI samples using the BovineHD BeadChip (Illumina, San Diego, CA, USA) for a total of 782,696 SNPs. SNPs were excluded if they deviated significantly from Hardy-Weinberg equilibrium ($p < 0.001$), if they had low minor allele frequency (< 0.01), or if they had a call rate < 95 %. We have previously genotyped 36 low and 56 high SI samples using the BovineSNP50 BeadChip (Illumina) [8] and combined these genotypes into the BovineHD data by treating these 92 samples missed genotype for HD-specific SNPs. We conducted an association analysis using EMMAX software [9].

Identification of Novel SNPs

Based on the Oct. 2011 *Bos taurus* draft assembly [10] (Baylor Btau_4.6.1), each of the exons, 2 kb of the 5' untranslated regions (UTRs) and 2 kb of the 3' UTRs of the genes located in the associated regions were amplified by polymerase chain reaction (PCR) and sequenced. We defined the genome-wide regions that included significant SNPs as well as their neighboring SNPs with r^2 values greater than 0.2 as the associated region because a linkage disequilibrium decayed rapidly until r^2 reached 0.2 in cattle [11]. The r^2 values were calculated by a linkage disequilibrium analysis using PLINK software [12]. The primers for each gene and the samples used to compare the sequences are shown in Tables A and B in the Supplemental data, respectively.

Allelic Substitution Effects

We genotyped 2,682 cows and 4,165 bulls for *the family with sequence similarity 213, member A (FAM213A), plakophilin 2 (PKP2), cortactin-binding protein 2 N-terminal like (CTTNBP2NL), SET domain containing 6 (SETD6), calcium channel, voltage-dependent, beta 2 subunit (CACNB2),* and *unc-5 homolog C (UNC5C). FAM213A* was identified as a gene that was associated with the SI in the present study,
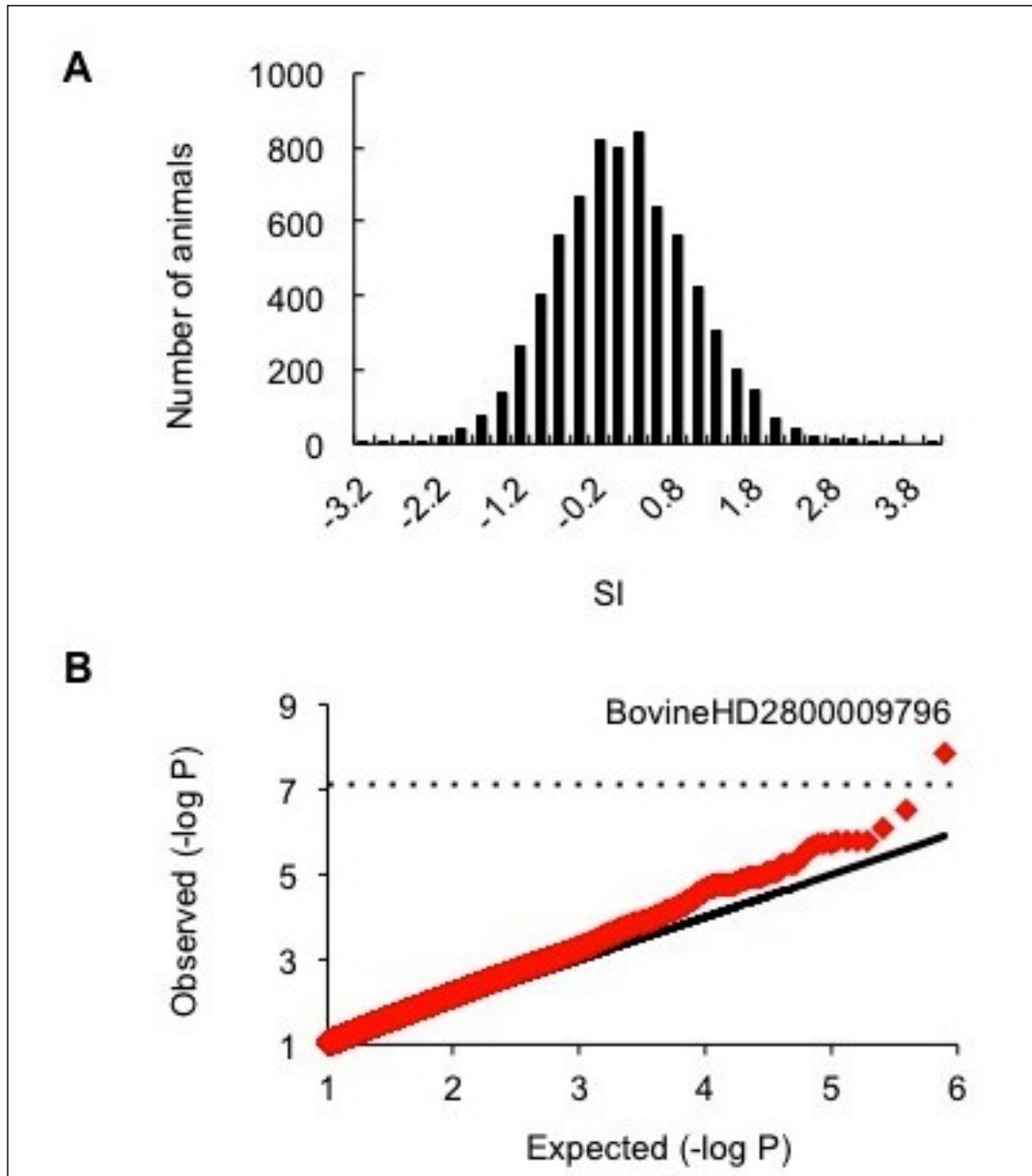
Figure 1. Fertility selection index (SI) is associated with SNP BovineHD2800009796. A. The distribution of SI values among 7,034 cows. B. A quantile-quantile plot of the GWAS for the SI. The equiangular line (black line) is included in the plot for reference purposes. The dashed horizontal line indicates the threshold for genome-wide significance (assuming a Bonferroni correction) for the 782,696 single nucleotide polymorphisms (SNPs) tested. BovineHD2800009796 is the SNP most significantly associated with the SI on a genome-wide level.

whereas *PKP2*, *CTTNBP2NL*, *SETD6*, *CACNB2* and *UNC5C* have previously been identified as genes associated with the conception rate (CR) [8, 12]. The EBVs of cows and bulls with these six traits included in the SI were estimated by a multiple-trait animal model using 1,881,898 records. The EBVs of cows and bulls in relation to the CR were estimated by a threshold repeatability animal model using 3,428,666 values after first parturition. The data were collected between January 1990 and September 2015 by the dairy herd improvement program of Hokkaido, Japan. The allelic substitution effects of these genes were determined using the following equation:

$$y_i = \mu + \sum_{j=1}^{6} x_{ij}g_j + e_i$$

where y_i = the deregressed estimated breeding value (dEBV) [13] of animal i (= 1, 2, …, n) for the SI, DO, NI, SFI, P70, P90, P110, or CR; μ = the general average value of the population; x_{ij} = the genotype covariate (coded as 0 or 2 for the two homozygotes and 1 for heterozygotes) of gene j in animal i; g_j = the random regression coefficient representing the allelic substitution effect for gene j; and e_i = the random residual effect for the value of animal i. We performed the analyses with the SAS MIXED procedure.

Real-time PCR

RNA was extracted from individual samples of bovine brain, kidney, liver, lung, mammary gland, muscle, ovaries, pancreas, stomach, and uterine tissue or from transfected cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Real-time PCR was conducted with an ABI 7900HT Sequence Detection System using the comparative Ct method and glyceraldehyde-3-phosphate dehydrogenase (GAPD) as an internal control (Life Technologies). The primers used

in these assays are shown in Table C in the Supplemental data.

Generation of Overexpression Constructs

We generated bovine *FAM213A* by PCR with the gene's respective forward and reverse primers (Table D in Supplemental data. These PCR products were further amplified via PCR with the forward-2 and reverse-2 primers (Table D in the Supplemental data) for cloning into a pCAGGS (R-N) vector [14] using an In-Fusion Advantage PCR Cloning Kit (Takara Bio Inc., Shiga, Japan).

Cell-viability Assay

To check the redox activity in cells transfected with bovine *FAM213A*, we performed cell-viability assays. Bovine endometrial epithelial cells (BEnEpCs; Cell Applications, San Diego, CA, USA) were plated and transfected. H_2O_2 was diluted in PBS and further diluted in culture medium to the indicated concentrations. Cells were incubated in medium containing H_2O_2 for 24 h, and cell viability was evaluated with the MTT-based Cell Growth Determination Kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. The amount of MTT formazan produced was determined by measuring absorbance with a microplate reader (Bio-Rad, Hercules, CA, USA) at a test wavelength of 570 nm and a reference wavelength of 655 nm. The *p*-value was calculated using Student's t-test.

Measurement of the GSH/GSSG Ratio

We also measured the GSH/GSSG ratio to compare the cellular redox status in cells transfected with bovine *FAM213A* using the glutathione detection kit (ENZO Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. In brief, total glutathione (GSHt) and GSSG concentrations were derived from GSH and GSSG standard curves and converted into nanomoles per milligram of protein. The concentrations of reduced GSH were found by subtracting GSSG from GSHt. Finally, the GSH/GSSG

ratio was calculated by dividing the difference between GSHt and GSSG concentrations by the GSSG concentration [ratio = GSHt – 2 (GSSG)/GSSG]. The *p*-value was calculated using Student's t-test.

Immunoblotting Analysis

To investigate cyclooxygenase-2 (COX-2) expression in cells transfected with bovine *FAM213A*, we analyzed immunoblotting. Protein was extracted from transfected cells using NucleoSpin RNA/Protein (Machrey -Nagel, Düren, Germany). The extracted proteins were separated, transferred to a membrane, and blocked. The blots were incubated with an anti-COX-2 (ab15191, Abcam, Cambridge, UK) or anti-actin (A2066, Sigma-Aldrich) antibody and detected with ECL Prime (GE Healthcare, Buckinghamshire, UK). To estimate the ratio of COX-2 to actin, the obtained images were quantified using ImageQuant LAS 4000 (GE Healthcare).

Enzyme Immunoassays

To assess prostaglandin E2 (PGE2) secreted by cells transfected with bovine *FAM213A*, we conducted immunoblotting analysis. Pyrrolidine dithiocarbamate (PDTC) and N-(2-cyclohexyloxy-4-nitrophenyl)-methane sulfonamide (NS-398) were purchased from Sigma-Aldrich. Before transfection, BEnEpCs were pretreated with 10 µM PDTC or 10 µM NS-398 for 30 min. The concentration of PGE2 released from the transfected BEnEpCs or the bovine serum were assayed using a PGE2 ELISA kit (ENZO Life Sciences) according to the manufacturer's instructions. The *p*-value was calculated using Student's t-test.

Results

Our GWAS identified BovineHD2800009796 as the most significantly associated SNP on chromosome 28 (Fig. 1B), and there were no more SNP that may be impacting SI as shown in Graph A in the Supplemental data. The region associated with this SNP included 13 genes (Fig. 2A). To detect possible causative polymorphisms in this region, we sequenced all exons

and the 5' and 3' UTRs of these 13 genes and found 42 novel SNPs (Fig. 2A). The positions, the minor allele frequencies, and the genotype counts in the GWAS sample for the novel 42 SNPs in Table E in the Supplemental data. A reanalysis of the newly sequenced SNPs demonstrated that FAM213A (A31G: I11V) was the most significant (Fig. 2A). Moreover, we genotyped FAM213A (A31G: I11V) in 2,682 cows and 4,165 bulls and found that the allele substitution effect of *FAM213A* on the dEBV for the SI was 0.18 (Fig. 2B). Cows with the A/A genotype, which codes for Ile/Ile, exhibited a 0.18 higher SI than those with the A/G genotype, which codes for Ile/Val, whereas cows with the G/G genotype, which codes for Val/Val, exhibited a 0.18 lower SI than those with the A/G genotype. *FAM213A* also had favorable effects on the dEBV for the traits that compose the SI (DO, NI, SFI, P90, and P110). The effects of FAM213A were similar to those of *PKP2* (chromosome 5: 82,452,366 bp), *CTTNBP2NL* (chromosome 3: 33,171,753 bp), *SETD6* (chromosome 18: 26,204,767 bp), *CACNB2* (chromosome 13: 32,851,153 bp), and *UNC5C* (chromosome 6: 31,142,900 bp), which have previously been identified to be associated with the CR in the Japanese dairy cow population [8, 12] (Fig. 2B). Therefore, FAM213A (A31G: I11V) was the most promising causative SNP on chromosome 28 and had a similar impact on the SI as the five genes previously identified to be associated with CR.

FAM213A protein is a CXXC motif-containing peroxiredoxin (PRX) 2-like protein [16]. PRXs and thioredoxins (TRXs) are redox regulatory proteins. PRXs play a protective antioxidant role in cells through their peroxidase activity, whereas TRXs alter the redox state of target proteins by catalyzing the reduction of their disulfide bonds through a CXXC motif. FAM213A has a similar sequence to PRXs and contains a CXXC motif that is shared by many enzymes with redox regulatory functions. Thus our identified two types of FAM213A might modulate reactive oxygen species (ROS) differently. To assess its redox activity, we used
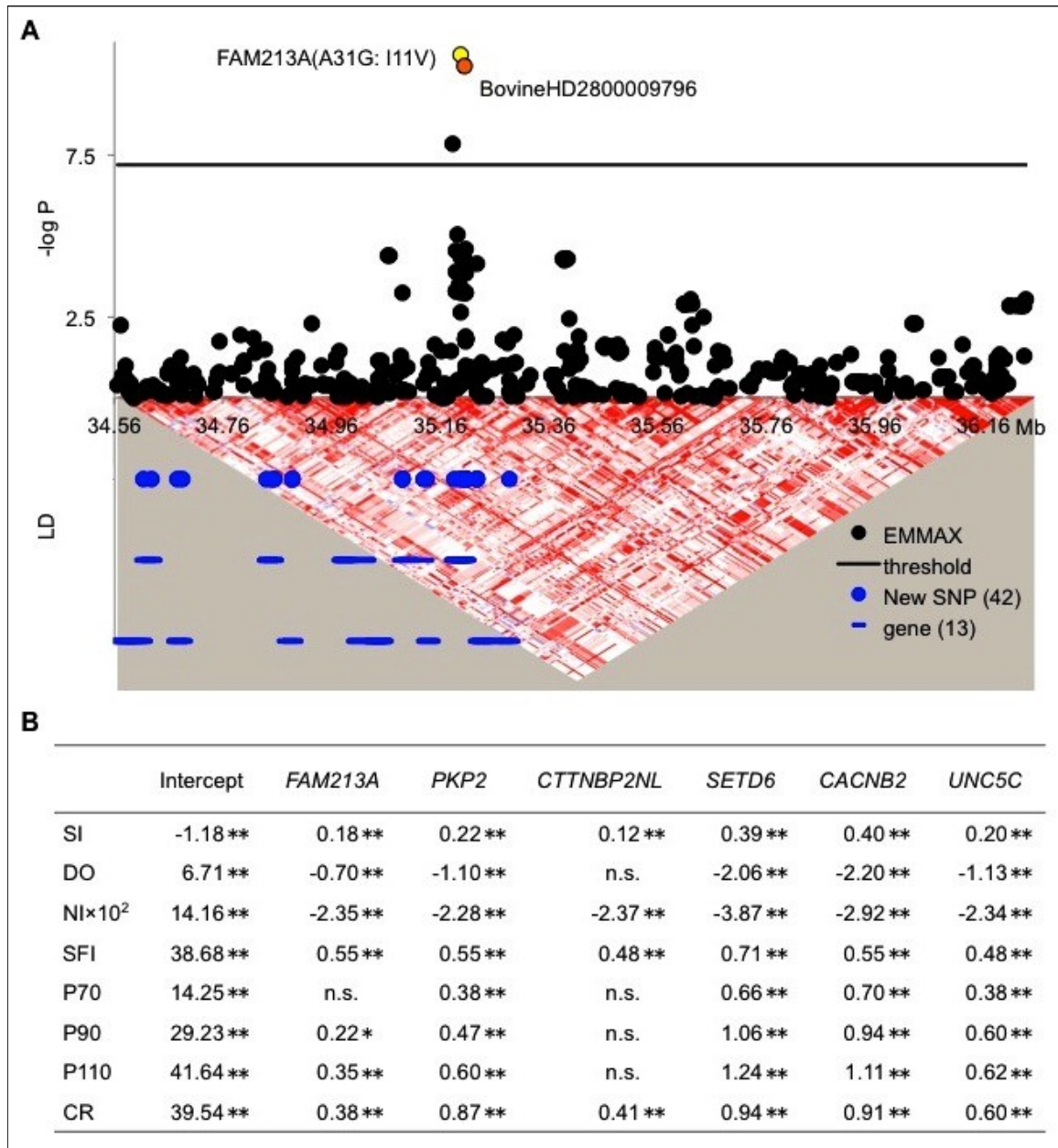
Figure 2. *FAM213A* SNP is associated with the SI. A. Association signals on chromosome 28 with the SI using plots of the *P* values from the EMMAX analysis. The black line represents the threshold for genome-wide significance after applying the Bonferroni correction for multiple comparisons. BovineHD2800009796 was the first significantly associated SNP that was detected, whereas FAM213A (A31G: I11V) was the only significant novel SNP. Blue circles represent the positions of the 42 newly sequenced SNPs. Blue lines represent 13 genes located in the associated region. The triangle diagram represents a pairwise linkage disequilibrium (LD) in the associated region, which was visualized using Haploview [35]. Red shades indicate strong LD. B. The allelic substitution effects of *FAM213A*, *PKP2*, *CTTNBP2NL*, *SETD6*, *CACNB2*, and *UNC5C* on the deregressed estimated breeding value (dEBV) [14] for the SI traits days open (DO), number of inseminations per lactation (NI), success after first insemination (SFI), pregnancy within 70 d (P70), 90 d (P90), and 110 d (P110) after parity or for the conception rate (CR). n. s.: non-significant. * and **: $p < 0.05$ and $p < 0.01$, respectively.

BEnEpCs derived from bovine uterine tissue because FAM213A is expressed in several bovine tissues including the uterus (Fig. 3A). Cell-viability assays using BEnEpCs transfected with FAM213A revealed that oxidative stress induced by H_2O_2 treatment decreased cell viability in a dose-dependent manner and indicated that cells with FAM213A^{Val} had increased viability relative to that of FAM213A^{Ile} in the presence of 0.2 mM H_2O_2 (Fig. 3B). Consistent with the results of the cell-viability assay, the GSH/GSSG ratio, a proxy for the cellular redox status, was increased in BEnEpCs transfected with FAM213A^{Val} to a greater extent than in BEnEpCs transfected with FAM213A^{Ile} (Fig. 3C). Furthermore, the GOR prediction for a protein's secondary structure [17] revealed that FAM213A^{Val} would likely have a shorter b-sheet than FAM213A^{Ile} (Fig. 3D). These results suggest that the activity of FAM213A^{Val} shifts conditions towards a more reduced environment than does FAM213A^{Ile} and indicate that this single amino acid substitution has an impact on the function of this protein.

ROS are important factors for fertility. In the uterus, the establishment of a maternal-fetal connection is associated with an increase in ROS [18]. Although ruminants have different types of placentation relative to humans or mice, ROS fluxes occur before implantation in all of these mammals [19]. Increases of ROS production by peripheral bovine lymphocytes have been reported as pregnancy progresses [20]. ROS triggers the NF-κB pathway, which enhances COX-2 expression [21, 22], suggesting that the more reduced environment created by FAM213A^{Val} might decrease COX-2 expression. To explore this possibility, we conducted real-time PCR using RNA extracted from BEnEpCs transfected with FAM213A. As expected, BEnEpCs transfected with FAM213A^{Val} expressed less *COX-2* than cells transfected with FAM213A^{Ile} (Fig. 4A). Moreover, pretreatment of BEnEpCs with a selective inhibitor of NF-κB, 10 μM PDTC, significantly repressed *COX-2* expression (Fig. 4A). We found the same result in the level of protein expression (Fig. 4B). Thus, the more reduced environment created by FAM213A^{Val} appears to decrease COX-2 expression through effects on the NF-κB pathway.

A COX-2 deficiency in mice results in implantation defects [23], and one of the products of COX-2, PGE2, can correct these implantation defects in COX-2-deficient mice [24]. Therefore, decreased COX-2 expression might reduce the production of PGE2 to levels that are inadequate for implantation. Our enzyme immunoassays revealed that BEnEpCs transfected with FAM213A^{Val} released less PGE2 compared with cells transfected with FAM213A^{Ile} (Fig. 5A). Furthermore, pretreatment of BEnEpCs with a selective inhibitor of COX-2, 10 μM NS-398, or a selective inhibitor of NF-κB, 10 μM PDTC, significantly repressed PGE2 production (Fig. 5A). Additionally, the serum concentrations of PGE2 of cows carrying FAM213A$^{Ile/Ile}$ were higher than those of cows carrying FAM213A$^{Val/Ile}$ or FAM213A$^{Val/Val}$ (Fig. 5B). Therefore, the reduced uterine environment created by FAM213A^{Val} decreased PGE2 production through effects on the NF-κB/COX-2 pathway, which might decrease the SI of cows carrying FAM213A^{Val}.

Discussion

The present study is the first to demonstrate that FAM213A modulates fertility. Scanning the whole genome of 442 Holsteins and analyzing the results with EMMAX identified a novel mutation associated with the SI. Although we have previously identified several genes associated with the CR in the Japanese Holstein population using a principal component analysis approach [8, 13], *FAM213A* is a novel gene associated with fertility, and it has only previously been reported to modulate osteoclast differentiation *in vitro* [16]. One of the reasons for this GWAS result might be that the SI follows a normal distribution, which makes it possible to compare samples belonging to the 5th percentile with samples belonging to the 95th percentile of the population (Fig. 1A). A second reason might be that the EMMAX method could correct for severe stratification
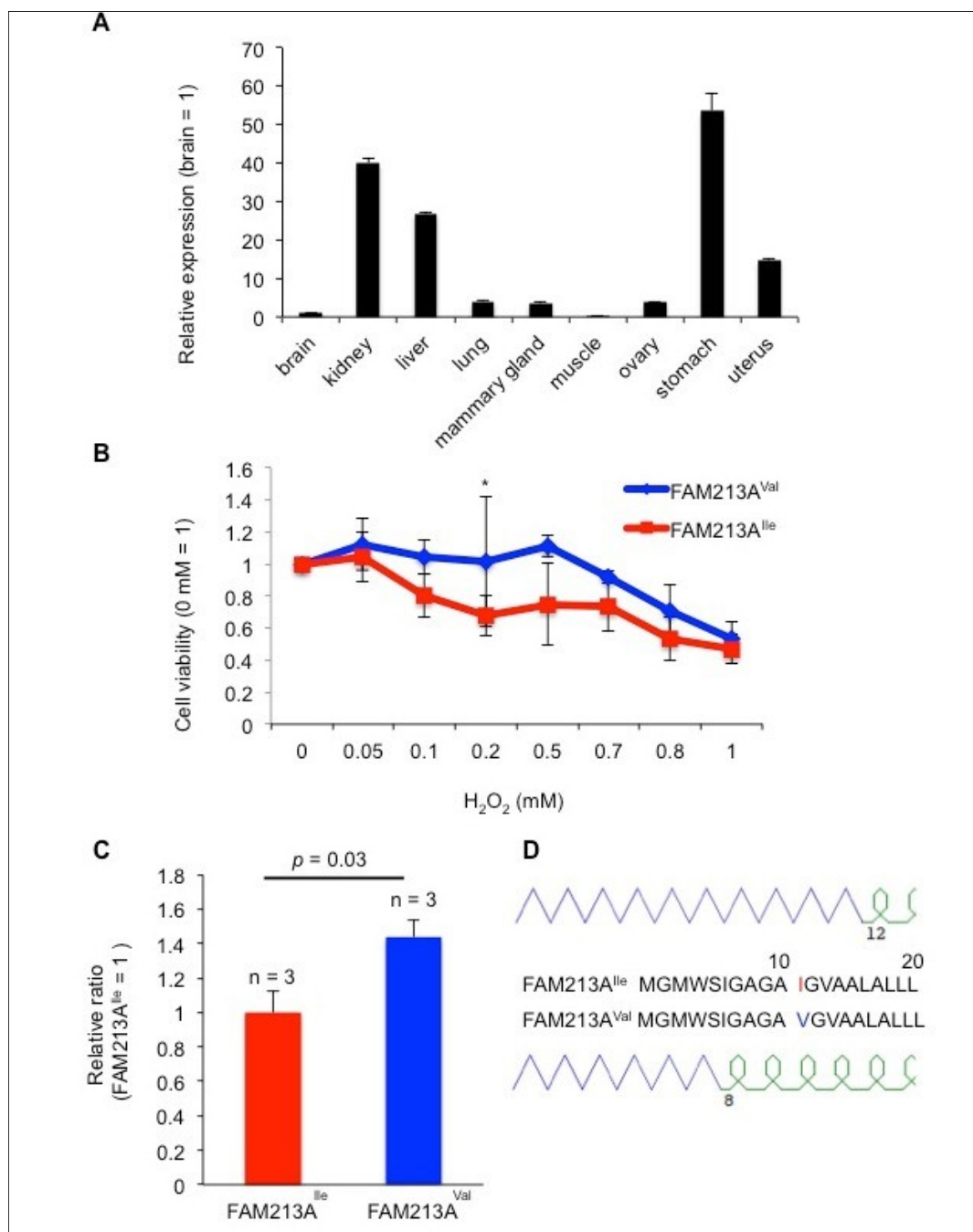
Figure 3. Overexpression of FAM213A^{Val} reduced reactive oxygen species (ROS) to a lesser extent relative to the overexpression of FAM213A^{Ile} in bovine endometrial epithelial cells (BEnEpCs). A. *FAM213A* expression levels in bovine tissues as determined via real-time PCR. B. Relative cell viability of FAM213A^{Ile} or FAM213A^{Val} transfected in BEnEpCs in the presence of H_2O_2. The data are presented as the mean ± SEM (n = 3). The *p*-value was calculated using Student's t-test. *: $p < 0.05$. C. The relative GSH/ GSSG ratio in BEnEpCs transfected with FAM213A^{Ile} or FAM213A^{Val} in the presence of 0.2 mM H_2O_2. D. Schematic illustration of the GOR-predicted protein secondary structure for the partial amino acid sequences of FAM213A. Purple lines represent the b-sheet and green curves represent a-helices.
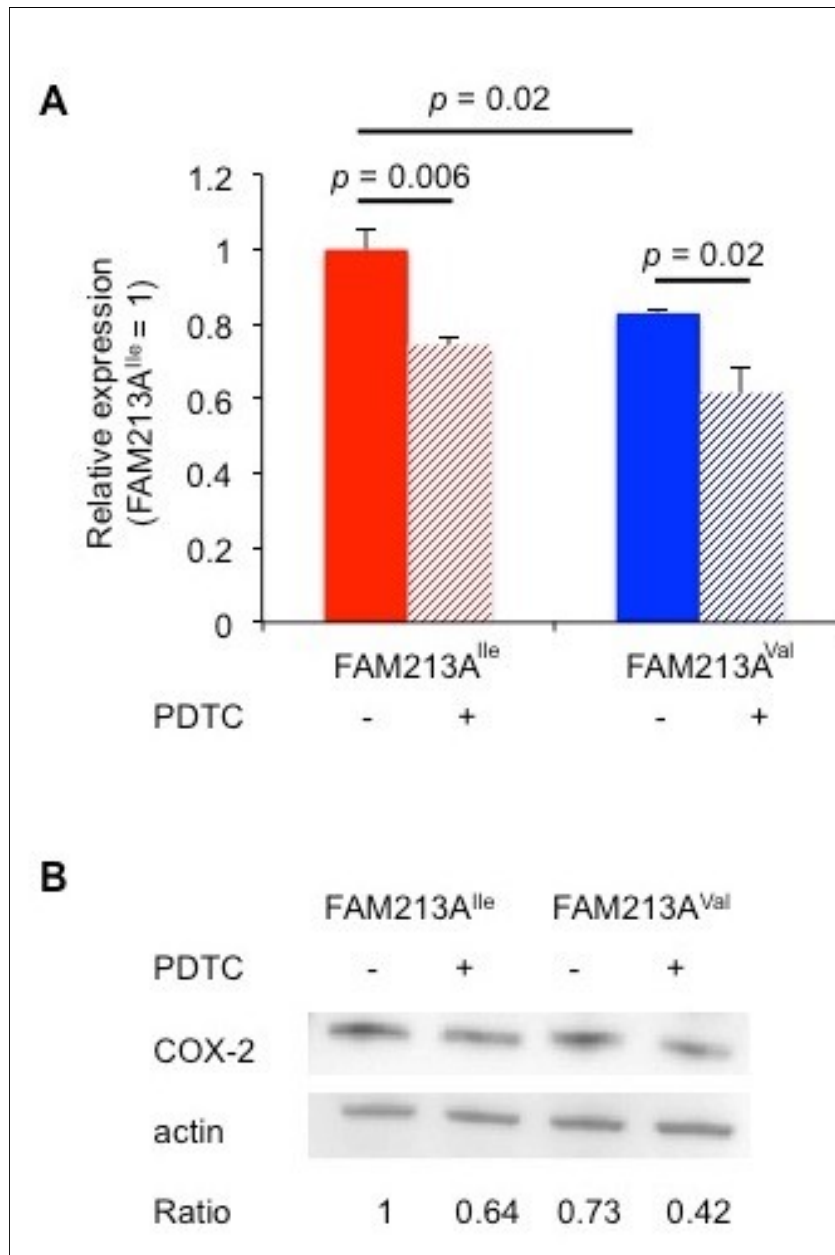
Figure 4. Overexpression of FAM213A^{Val} in BEnEpCs resulted in less cyclooxygenase-2 (COX-2) expression compared with cells overexpressing FAM213A^{Ile}. A. The relative *COX-2* expression of BEnEpCs transfected with FAM213A^{Ile} or FAM213A^{Val} in the absence or presence of pretreatment with 10 μM pyrrolidine dithiocarbamate (PDTC) before treatment with 0.2 mM H$_2$O$_2$. The data are presented as the means of 3 experiments ± SEM. The *p*-value was calculated using Student's t-test. B. Representative immunoblots with an anti-COX-2 or anti-actin antibody from BEnEpCs transfected with FAM213A^{Ile} or FAM213A^{Val} in the absence or presence of pretreat-ment with 10 μM PDTC before treatment with 0.2 mM H$_2$O$_2$. Relative ratios of COX-2 to actin (FAM213A^{Ile} = 1) are shown.
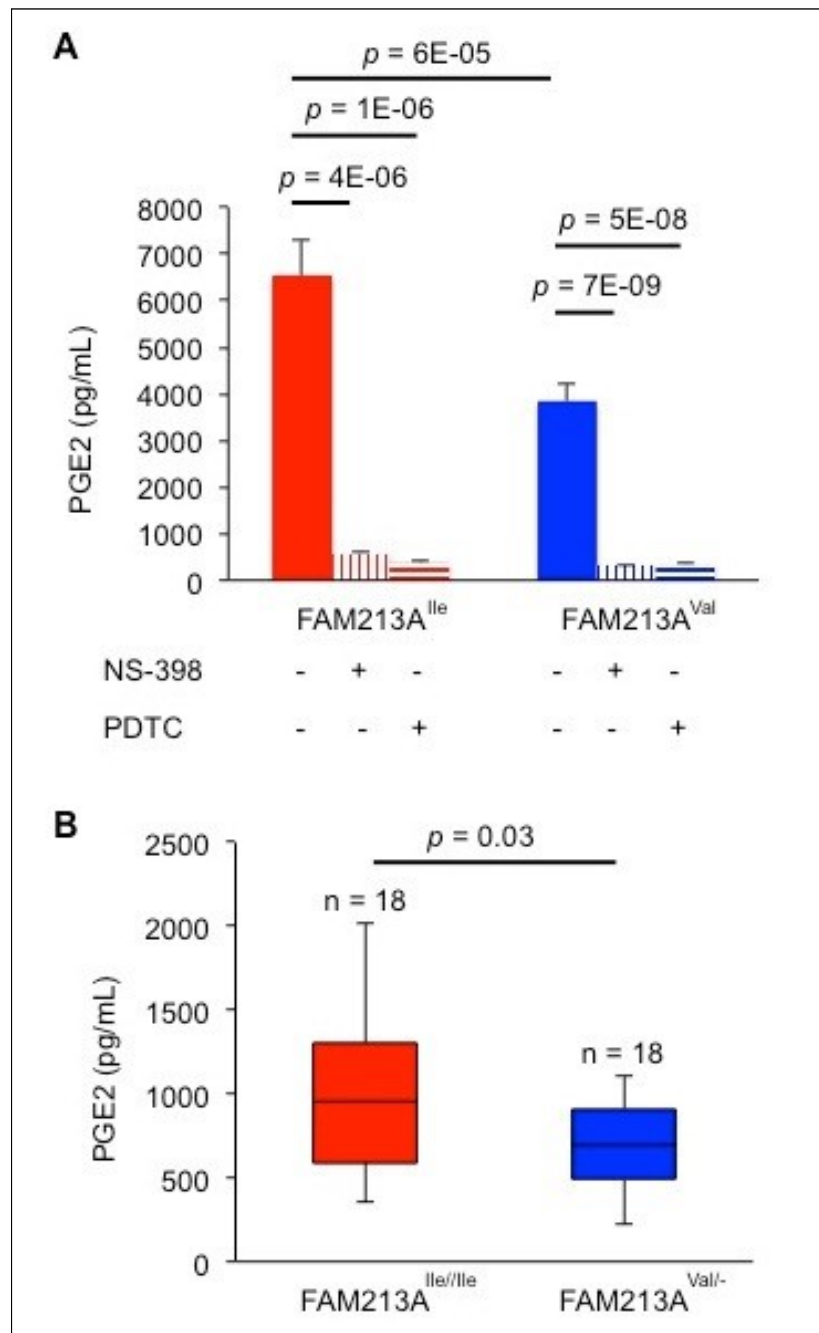
Figure 5. Cows with FAM213A^{Val} showed lower levels of prostaglandin E2 (PGE2) compared with cows with FAM213A^{Ile}. A. The concentration of PGE2 released from BEnEpCs transfected with FAM213A^{Ile} or FAM213A^{Val} in the absence or presence of pretreatment with 10 µM N-(2-cyclohexyloxy-4-nitrophenyl)-methane sulfonamide (NS-398) or 10 µM PDTC before treatment with 0.2 mM H$_2$O$_2$. The data are presented as the means of 3 experiments ± SEM. The p-value was calculated using Student's t-test. B. The serum concentrations of PGE2 of FAM213A$^{Ile/Ile}$ or FAM213A$^{Val/-}$ cows at approximately day 9 of the estrus cycle.

and reduce the effects of unusual clusters of cows that are significantly larger because of intensive genetic selection and the extensive use of artificial insemination in the dairy industry [25].

However, in the present GWAS, significant SNPs were not observed near the genes previously identified as associated with CR, although the allelic substitution effects of these genes on the SI were significant among the 2,682 cows and 4,165 bulls (Fig. 2B) that were analyzed. The SI consists of reproductive traits that have low heritabilities ranging from 0.05 to 0.14 [5]. In dairy cattle, only *DGAT1* and *ABCG2* have been identified and verified by multiple studies to have significant effects on fat and protein concentrations, which have the highest heritabilities of all of the traits that are routinely analyzed [26]. The heritabilities of these traits range from 0.20 to 0.61 [27]. Thus, using multiple GWASs to identify and verify the genes responsible for reproductive traits that have low heritabilities might be more difficult than for milk production traits that have high heritabilities.

We found that cows carrying FAM213A^{Val} have a lower SI than cows carrying FAM213A^{Ile}. This single amino acid substitution is located in the thioredoxin fold region and predicted to change the length of the b-sheet (Fig. 3D) in this region. The thioredoxin fold region contains a b-a-b-a-b-a-b-b-a arrangement [28], and the length of the first b-sheet of the region might be critical for the redox regulatory function of this protein.

The identified single amino acid substitution decreases ROS, which reduces COX-2 expression through the NF-κB pathway (Fig. 4) and reduces PGE2 production (Fig. 5). It is worth noting that COX-2 expression and PGE2 production are also induced by interferon-tau [29], the pregnancy recognition signal produced by the trophoblast prior to implantation in ruminants, which has been shown to enhance fetal survival when administered to mice [30, 31]. Along with the interferon-tau pathway, enhancing COX-2 expression and PGE2 production via

the ROS/NF-κB pathway might be important for implantation.

Although we demonstrated effects using BEnEpCs in the present study, FAM213A might also have impacts on different tissues in cows because the SI consists of several reproductive components [5]. In the ovary, oocyte maturation requires ROS [32], and FAM213A was found to be expressed in the ovary (Fig. 3A). Ovulation is initiated by a surge in luteinizing hormone, which generates ROS [33]. After ovulation, the corpus luteum is produced in the ovary, which also generates ROS [34]. Moreover, COX-2-deficient mice have demonstrated multiple failures in female reproductive processes, including fertilization, ovulation, and implantation [23]. Thus FAM213A might affect multiple reproductive processes through the ROS/NF-κB/COX-2/PGE2 pathway.

Conclusion

The present study investigated the whole genome in samples from 212 cows with a low SI and samples from 230 cows with a high SI, and a significant association was observed between the SI and FAM213A with a single amino acid polymorphism. Further functional studies revealed that this single amino acid polymorphism in FAM213A reduced ROS, thus leading to decreased COX-2 expression and lower PGE2 production. These results indicate that FAM213A plays a role in female fertility in cows.

Acknowledgments

Supplemental data:

Primers used to search for SNPs (Table A). Samples used for developing new SNPs (Table B). Primers used for real-time PCR (Table C). Primers used

for generating the overexpression constructs (Table D). A quantile-quantile plot of the GWAS for the fertility selection index without BovineHD2800009796 (Graph A). The minor allele frequencies and the genotype counts in the GWAS sample for 42 novel SNPs in chromosome 28 (Table E). The standard error for the allelic substitution effects of *FAM213A*, *PKP2*, *CTTNBP2NL*, *SETD6*, *CACNB2*, and *UNC5C* on the deregressed estimated breeding value for the SI traits days open (DO), number of inseminations per lactation (NI), success after first insemination (SFI), pregnancy within 70 d (P70), 90 d (P90), and 110 d (P110) after parity or for the conception rate (CR) (Table F).

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