Genetic variants related to gap junctions and hormone secretion influence conception rates in cows

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The recent decline in fertility is a serious problem in the dairy industry. To overcome this problem, we performed a genome-wide association study using 384 Holsteins and identified four loci associated with conception rates. Two of them contained gap junction-related genes: *PKP2* and *CTTNBP2NL*. Further analysis confirmed that PKP2 increased connexin 43, a gap junction protein, whereas CTTNBP2NL dephosphorylated connexin 43. Knockdown of *PKP2* or overexpression of *CTTNBP2NL* inhibited embryo implantation in mice. The other two loci contained neuroendocrine-related genes: *SETD6* and *CACNB2*. Additional experiments indicated that SETD6 is involved in the transcriptional regulation of gonadotropin-releasing hormone, whereas CACNB2 controlled the secretion of follicle-stimulating hormone in cattle. The total allele substitution effect of these genes on conception rate was 3.5%. Our findings reveal important roles for gap junction communication and the neuroendocrine system in conception and suggest unique selection methods to improve reproductive performance in the livestock industry.

Conception rate has decreased dramatically over recent decades in the dairy industry (1). Dairy cattle have been selected primarily for milk yield, not for reproductive performance, which has led to an improvement of the genetic component for production and a decline in fertility (2). To improve genetics for conception rate, a candidate gene approach, which focuses on associations between genetic variation within prespecified genes of interest and infertility, has been conducted (3, 4). However, conception is a complex process consisting of follicle development, ovulation, fertilization, implantation, and placental differentiation (5). In addition, paracrine, autocrine, and endocrine signals modulate these events (5). Therefore, the list of prespecified genes is very long.

Instead of checking the long list, we conducted a genome-wide association study (GWAS) to find genes responsible for fertility. GWAS comparing many cases with controls provides valuable insights into the genetic architecture of complex processes (6). We compared dairy cows with low conception rates with cows with high conception rates and discovered four loci correlated with fertility that harbored two gap junction-related genes and two neuroendocrine-related genes.

Gap junctions are membrane-bound structures composed of connexins (Cxs) that mediate communication between neighboring cells (7). Such cell-to-cell communication is critical for cell growth and differentiation. The spatial and temporal expression patterns of Cx26 and Cx43 proteins are strictly regulated in the rat uterus from preimplantation to placental differentiation (8). Expression of Cx26, Cx32, and Cx43 proteins is observed in placental tissues during pregnancy in cows (9). Conditional deletion of Cx43 in mice resulted in severe fertility defects (10). Although such studies suggest that gap junctions are indispensible for mammalian implantation, it remains unclear how these structures modulate conception.

The neuroendocrine system is another modulator of reproduction. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) in a pulsatile pattern, which leads to the release of FSH and luteinizing hormone (LH) from the anterior pituitary gland (5). Disruption of GnRH, FSH, or LH causes infertility in mice (11–14). However, it is largely unknown how the pulsatile secretion of GnRH is controlled.

We used GWAS to identify four loci associated with conception rate in Holsteins, a breed known as the world's most productive dairy cattle. These loci harbored two gap junction-related genes, *plakophilin 2* (*PKP2*) and *cortactin-binding protein 2 N-terminal like* (*CTTNBP2NL*), and two neuroendocrine-related genes, *SET domain containing 6* (*SETD6*) and *calcium channel, voltage-dependent, beta 2 subunit* (*CACNB2*). Our GWAS uncovered unexpected roles for these genes and provides a potential solution for the problem of declining conception rates in the livestock industry.

Results

Loci Correlate with Conception Rates of Holsteins. To identify genes associated with fertility, we collected DNA from 4,362 Holstein cows (females) and evaluated the estimated breeding value (EBV) for conception rates (15, 16). EBV is a genetic component obtained by subtracting an environmental component from a phenotype. The mean EBV for conception rates ± SD of these collected samples was 45.3 ± 3.5% (Fig. S1). We selected 192 low samples (conception rates lower than 41.1%) and 192 high samples (conception rates higher than 51%) among the collected samples. Based on typing a total of 384 samples for 54,001 SNPs, we identified six loci associated with conception rates on chromosomes 3, 5, 13, 18, and 28 (Fig. 1*A*). Because the associated regions surrounding two significant SNPs, ARS-BFGL-NGS-72055 (chromosome 3) and BTB-01171634 (chromosome 28), did not include any known genes, the other four loci were selected for further analysis as described below.

Significance

A genome-wide association study has uncovered four variants, located at *PKP2*, *CTTNBP2NL*, *SETD6*, and *CACNB2*, that modulate conception rates in cattle. *PKP2* and *CTTNBP2NL* function in gap junction communication and influence embryo implantation, whereas *SETD6* and *CACNB2* are involved in the release of follicle-stimulating hormone from the anterior pituitary gland. The discovery of such unexpected roles for these genes could help to combat the declining conception rates reported by the dairy industry and potentially contribute to understanding human reproductive events at the molecular level.

Author contributions: M.S., S.S., and Y.S. designed research; M.S. and S.S. performed research; Y.N. and Y.A. contributed new reagents/analytic tools; M.S., S.S., Y.G., and T.K. analyzed data; and M.S., S.S., and Y.S. wrote the paper.

Conflict of interest statement: M.S. and Y.S. are named on Japan patent application number 2013-111480, which is for the method of determining conception rate in cattle. This application does not alter adherence to policies on sharing data and materials.

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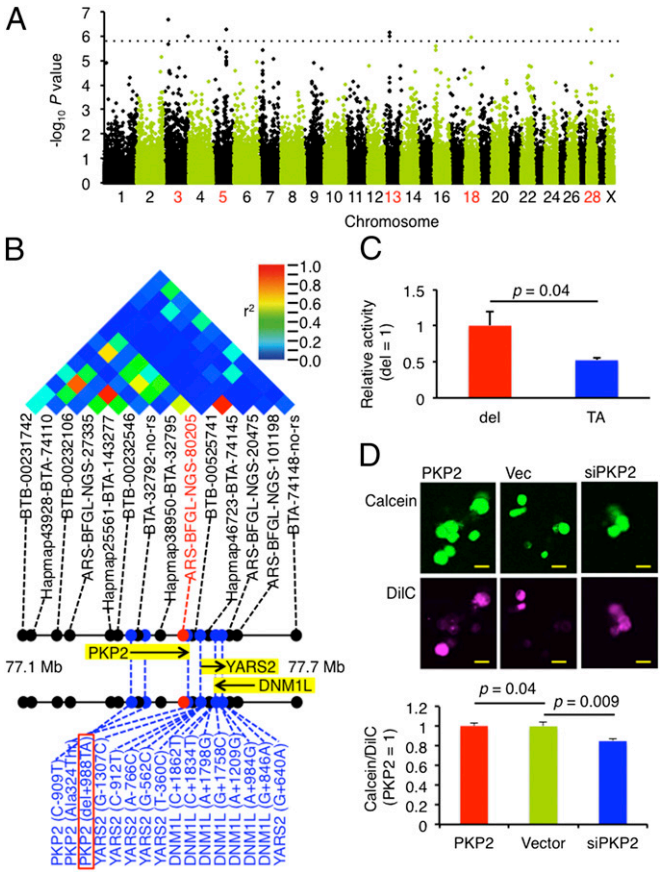

Fig. 1. PKP2 enhances gap junction communication. (*A*) Manhattan plots for the genome-wide screen for loci associated with conception rate. The dotted line represents the threshold for genome-wide significance of $P < 1.2 \times 10^{-6}$ based on the Bonferroni correction for multiple comparisons. (*B*) A pairwise linkage disequilibrium diagram showing a schematic representation of the genes (black arrow) located in the associated region on chromosome 5. Red, black, and blue dots represent the genome-wide significant SNPs, the original SNPs, and the newly developed SNPs, respectively. (*C*) The average luciferase activity ± SEM of the 3′ UTR of *PKP2* in uterus-derived BEnEpCs ($n = 6$). (*D*) The average ratio of calcein to DiIC transfer ± SEM in BEnEpCs transfected with PKP2, vector (Vec), or siPKP2 relative to PKP2 ($n = 6$ each). Representative images are shown. (Scale bars: 20 μm.)

PKP2 Enhances Gap Junction Communication. The associated region harboring the significant SNP, ARS-BRGL-NGS-80205, on chromosome 5 includes *PKP2*, *tyrosyl-tRNA synthetase 2, mitochondrial* (*YARS2*), and *dynamin 1-like* (*DNM1L*; Fig. 1*B*). To detect possible causative polymorphisms in this region, we sequenced all exons and the 5′ and 3′ UTRs of these genes and found 16 unique SNPs (Fig. 1*B*). Reanalysis with the newly developed SNPs demonstrated that PKP2 (del+988TA) was the most significant SNP (Dataset S1, Table S1). We also confirmed that the association signal for ARS-BRGL-NGS-80205 disappeared when the PKP2 (del+988TA) genotype was included as a covariate in a logistic regression analysis ($P = 0.49$). Moreover, we genotyped PKP2 (del+988TA) in an additional 1,034 cows and 2,528 sires and found that cattle harboring the del/del genotype exhibited a higher conception rate than cattle harboring the TA/TA genotype (Dataset S1, Table S2). Thus, PKP2 (del+988TA) was the most promising causative SNP on chromosome 5.

PKP2 (del+988TA) is located in the 3′UTR of *PKP2* and may influence the expression level of this gene. Because *PKP2* is expressed in several bovine tissues, including the uterus (Fig. S2), we used uterus-derived bovine endometrial epithelial cells (BEnEpCs) and compared the luciferase activity. Reporters carrying the del allele exhibited higher luciferase activity than those carrying the TA allele (Fig. 1*C*). Consistent with the results of the luciferase assay, the level of messenger RNA (mRNA) generated from the del allele was higher than for the TA allele, based on determining the allelic mRNA ratio (Fig. S3*A*). Consequently, the expression level of *PKP2* might affect the conception rate in cattle.

PKP2 is a desmosomal plaque protein (17), and inhibition of its expression decreases Cx43 levels in the rat heart (18). To examine whether the expression level of PKP2 affects the Cx43 content in the bovine uterus, we transfected BEnEpCs with siPKP2 (a construct that represses the expression of endogenous *PKP2*), empty vector, or PKP2 (a *PKP2* expression plasmid). Both immunostaining and immunoblotting of transfected BEnEpCs indicated that reduced expression of PKP2 decreased Cx43 expression (Fig. S4). Cattle carrying the *PKP2* del allele might have exhibited higher expression of Cx43 in their uterus than cattle carrying the TA allele.

Because Cx43 is a gap junction protein (7), the level of PKP2 expression might influence transfer efficiency through gap junctions in the bovine uterus. To explore this possibility, we performed a dual-label cell coupling assay using a gap-junction-permeable dye (calcein) and a gap-junction-impermeable dye (DilC) in BEnEpCs transfected with siPKP2, vector, or PKP2. We found that the lower the PKP2 expression level, the less calcein diffused (Fig. 1*D*). Reduced expression of PKP2 could impair gap junction communication through decreasing Cx43 expression.

CTTNBP2NL Inhibits Gap Junction Communication. The associated region surrounding INRA-641 on chromosome 3 includes *suppression of tumorigenicity 7 like* (*ST7L*), *wingless-type MMTV integration site family, member 2B* (*WNT2B*), and *CTTNBP2NL* (Fig. 2*A*). We sequenced all of the exons and the UTRs of these genes but found only one SNP (Fig. 2*A*). Reanalysis with this newly developed SNP indicated that INRA-641 was still the most significant SNP (Dataset S1, Table S1). Cattle harboring the G/G genotype presented a higher conception rate than cattle harboring the A/A genotype (Dataset S1, Table S2). Accordingly, INRA-641 was the most promising causative SNP in this region.

Because INRA-641 is located in the 3′UTR of *CTTNBP2NL* and *CTTNBP2NL* is expressed in the bovine uterus (Fig. S2), we examined the luciferase activity in BEnEpCs. Reporters carrying the G allele showed lower luciferase activity than those carrying the A allele (Fig. 2*B*). The level of mRNA generated for the G allele was lower than for the A allele (Fig. S3*B*), suggesting that the expression level of *CTTNBP2L* might affect the conception rate in cattle.

The function of CTTNBP2NL is currently unknown. However, CTTNBP2NL interacted with the protein phosphatase 2A catalytic subunit (PP2Ac) in a human embryonic kidney cell line (19). To examine whether CTTNBP2NL interacts with PP2Ac in the bovine uterus, we conducted an immunoprecipitation assay using BEnEpCs. Immunoblotting of the immunoprecipitated proteins revealed that CTTNBP2NL interacted with PP2Ac in BEnEpCs (Fig. 2*C*). Moreover, knockdown of CTTNBP2NL decreased phosphatase activity, whereas overexpression of CTTNBP2NL increased phosphatase activity (Fig. 2*D*). Treatment with an inhibitor of PP2A, okadaic acid (20), decreased phosphatase activity (Fig. 2*D*). Differences in the level of CTTNBP2NL expression might influence phosphatase activity through interacting with PP2Ac.

One of the known targets of PP2A is Cx43 (21), and the phosphorylation status of Cx43 affected cell-to-cell communication in murine anterior pituitary cells (22). The level of CTTNBP2NL expression might alter the phosphorylation status of Cx43 and the gap junction transfer efficiency in the bovine uterus. To test this hypothesis, we investigated the ratio of phosphorylated Cx43 to total Cx43 in BEnEpCs. The level of phosphorylated Cx43 was

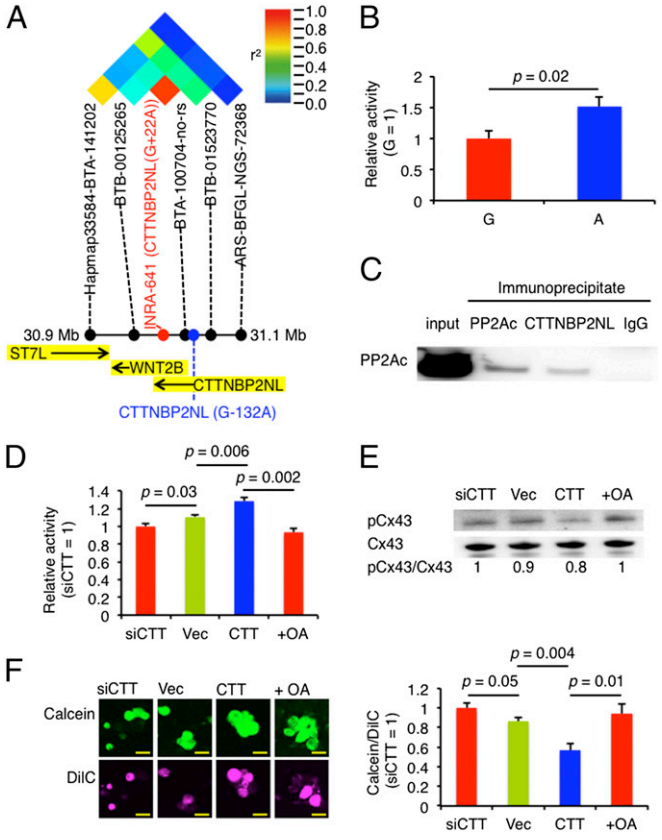

Fig. 2. CTTNBP2NL inhibits gap junction communication. (*A*) A pairwise linkage disequilibrium diagram with a schematic representation of the genes (black arrow) located in the associated region on chromosome 3. Red, black, and blue dots represent the genome-wide significant SNPs, the original SNPs, and the newly developed SNPs, respectively. (*B*) The average luciferase activity ± SEM of the 3′ UTR of *CTTNBP2NL* in uterus-derived BEnEpCs (n = 6). (*C*) Representative immunoblots with an anti-PP2Ac antibody for the input for immunoprecipitation, immunoprecipitated with PP2Ac, CTTNBP2NL, or IgG as a negative control. (*D*) The average phosphatase activity ± SEM of BEnEpCs transfected with siCTTNBP2NL (siCTT), vector (Vec), CTTNBP2NL (CTT), or CTTNBP2NL following treatment with okadaic acid (+OA) relative to siCTT (n = 3 each). (*E*) Representative immunoblots with anti-phosphorylated Cx43 (pCx43) and anti-total Cx43 antibodies. (*F*) The average ratio of calcein transfer to DilC transfer ± SEM in BEnEpCs transfected with siCTT, Vec, CTT, or +OA relative to siCTT (n = 3 each). Representative images are shown. (Scale bars: 20 μm.)

clearly decreased in cells in which CTTNBP2NL was overexpressed and increased under either knockdown of CTTNBP2NL or treatment with okadaic acid (Fig. 2*E*). Consistent with the phosphorylation status of Cx43, a dual-label cell coupling assay showed that overexpression of CTTNBP2NL decreased calcein transfer, whereas either knockdown of CTTNBP2NL or treatment with okadaic acid increased it (Fig. 2*F*). An increased level of CTTNBP2NL expression might decrease the transfer efficiency through dephosphorylation of Cx43.

Either siPKP2 or CTTNBP2NL Inhibits Implantation. To further evaluate the effects of PKP2 and CTTNBP2NL on conception, we examined the murine uterus after transferring murine siPKP2, which represses the expression of endogenous *pkp2* in mice, or CTTNBP2NL using electroporation. Our experiments using BEnEpCs indicated that PKP2 improved cell-to-cell communication through Cx43, whereas CTTNBP2NL inhibited it through Cx43 (Figs. 1*D* and 2*F*). Induced expression of Cx43 has been observed in the uterine stromal cells of mice on day 5 of pregnancy, within 12 h of the initiation of implantation (10). We therefore introduced control to the left uteri and siPKP2 or CTTNBP2NL to the right uteri of female ICR mice on day 3 of pregnancy, before the initiation of implantation and induction of Cx43 expression. When pregnancy had progressed to day 7, 4 d after electroporation with either siPKP2 or CTTNBP2NL, the left and right uteri were morphologically different and the right uteri were shorter and weighed less than the left uteri (Fig. 3*A*). In agreement with the results from our cell-based assays (Fig. S4 and Fig. 2*E*), we confirmed that electroporation of siPKP2 decreased the expression of Cx43 in the uterus, whereas introducing CTTNBP2NL decreased the level of phosphorylated Cx43 (Fig. S5). Further immunohistochemical analysis demonstrated that electroporation of siPKP2 or CTTNBP2NL in the right uteri inhibited embryo implantation and development of endothelial network expressed platelet/endothelial cell adhesion molecule 1 (ref. 23; Pecam1, Fig. 3*B*), although rates of implantation were previously demonstrated to be higher in the right than in the left cornu of the uterus in laboratory mice (24). Because the mouse fetuses had undergone sufficient growth on day 17 of pregnancy, we counted them and confirmed that electroporation of siPKP2 or CTTNBP2NL reduced the litter size (Fig. 3*C*). Consequently, the SNPs in bovine *PKP2* and *CTTNBP2NL* might affect conception rates through influencing implantation efficiency.

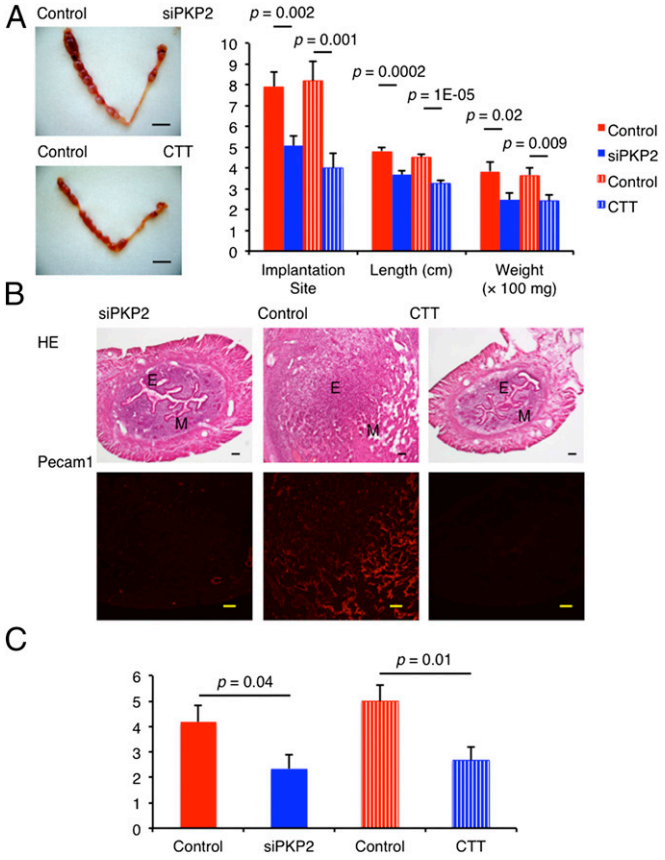

Fig. 3. Either siPKP2 or CTTNBP2NL inhibits implantation. (*A*) The average number of implantation sites and the lengths and weights ± SEM for uteri electroporated with the control, murine siPKP2, or CTTNBP2NL (CTT), with a representative image showing a uterus on day 7 of pregnancy. The data for siPKP2 came from 12 mice, and those for CTTNBP2NL came from 11 mice. (Scale bars: 1 cm.) (*B*) Representative images of uterine sections obtained from electroporated mice stained with either H&E (HE) or immunostained to detect Pecam1. E, embryo; M, mesometrial region. (Scale bars: 100 μm.) (*C*) The average number of fetuses ± SEM in the electroporated uteri counted from six mice each.

SETD6 Affects the Release of GnRH. The associated region harboring ARS-BFGL-NGS-20779 on chromosome 18 includes *NDRG family member 4* (*NDRG4*), *SETD6*, and *CCR4-NOT transcription complex, subunit 1* (*CNOT1*; Fig. 4*A*). We sequenced all of the exons and the UTRs of these genes and found 23 SNPs (Fig. 4*A*). Reanalysis with the newly developed SNPs indicated that SETD6 (Ala360Glu) was the most significant SNP (Dataset S1, Table S1). The association signal for ARS-BFGL-NGS-20779 disappeared when the SETD6 (Ala360Glu) genotype was included as a covariate in a logistic regression analysis ($P = 0.01$). Cattle harboring the Ala/Ala genotype exhibited a higher conception rate than cattle harboring the Glu/Glu genotype (Dataset S1, Table S2). Hence, SETD6 (Ala360Glu) was the most promising causative SNP on chromosome 18.

SETD6 catalyzes methylation of lysine residues in the RelA subunit of NF-κB (25), which is involved in the transcriptional regulation of GnRH (26). We compared the lysine methyltransferase activity of bovine SETD6^{Ala} and SETD6^{Glu} using immortalized murine hypothalamic neurons synthesizing GnRH, referred to as GT1-7 cells (27), when repressing endogenous *setd6* by cotransfecting murine siSETD6. Unexpectedly, SETD6^{Ala} exhibited lower activity than did SETD6^{Glu} (Fig. 4*B*). Ala360Glu is located in one of the α-helices of the C-terminal domain of the SETD6 protein (25, 28), and the Glu residue is highly conserved from chickens to humans (Fig. 4*C*). Likewise, the number of cattle harboring the Ala/Ala genotype was much lower compared with those harboring Glu/Glu (Dataset S1, Table S2). A recent mutation in the amino acid at position 360 of bovine SETD6 might reduce its methyltransferase activity, which happens to increase conception rate.

Methylation of RelA by SETD6 inhibits the transactivation activity of RelA (25). We detected RelA bound to the promoter of GnRH via ChIP in GT1-7 cells transfected with SETD6^{Glu}, but not SETD6^{Ala} (Fig. S6*A*). Cotransfection of GT1-7 cells with an NF-κB–driven reporter gene and either SETD6^{Ala} or SETD6^{Glu} demonstrated that only SETD6^{Glu} repressed the activity of this reporter gene (Fig. S6*B*). Consistent with a negative role for SETD6 in NF-κB signaling, the secretion of GnRH in GT1-7 cells transfected with SETD6^{Glu} was lower than in those transfected with SETD6^{Ala} (Fig. 4*D*). A mutation in amino acid 360 of bovine SETD6 might influence GnRH release through NF-κB signaling.

The pulsatile release of GnRH leads to the production of FSH and LH in the anterior pituitary gland (5). Because SETD6 variants might affect the secretion of these hormones through regulating GnRH release from the hypothalamus, we examined the concentrations of FSH and LH in the serum of Ala/− and Glu/Glu cows at approximately day 9 of the estrus cycle and found that Ala/− cows presented higher concentrations of FSH, but not LH, than Glu/Glu cows (Fig. 4*E*). SETD6 might, therefore, control FSH secretion through the transcriptional regulation of GnRH.

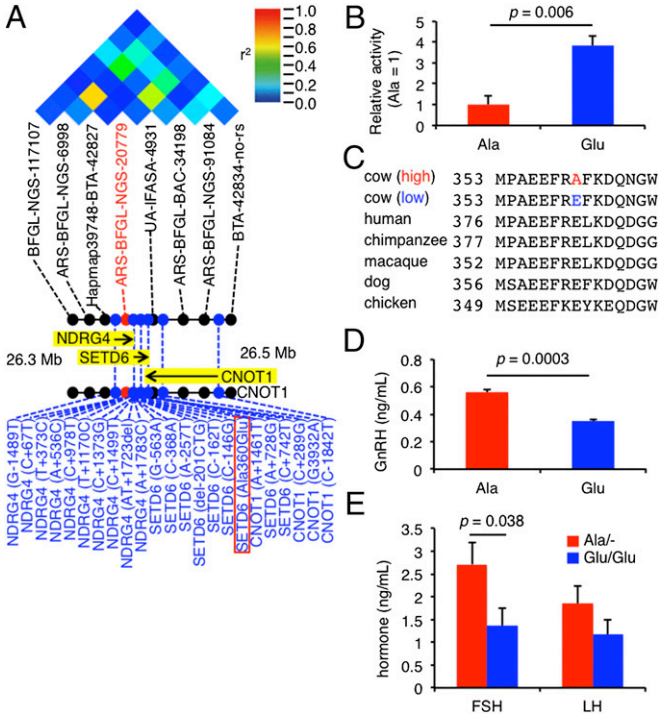

Fig. 4. SETD6 regulates the release of GnRH. (*A*) A pairwise linkage disequilibrium diagram with a schematic representation of the genes (black arrow) located in the associated region in chromosome 18. Red, black, and blue dots represent the genome-wide significant SNPs, the original SNPs, and the newly developed SNPs, respectively. (*B*) The average methyltransferase activity ± SEM of GT1-7 cells transfected with SETD6^{Ala} (Ala) or SETD6^{Glu} (Glu) ($n = 3$ each). (*C*) Alignment of the partial amino acid sequence of SETD6. (*D*) The average concentration ± SEM of GnRH released from GT1-7 cells transfected with Ala or Glu ($n = 3$ each). (*E*) The average concentrations ± SEM of FSH and LH in the serum of Ala/- ($n = 28$) or Glu/Glu cows ($n = 22$).

CACNB2 Controls the Secretion of FSH. The associated region surrounding BTA-32318-no-rs and its adjacent UA-IFASA-6479 on chromosome 13 includes *CACNB2*, *NOP2/Sun domain family, member 6* (*NSUN6*), *enhancer of polycomb homolog 1 (Drosophila)* (*EPC1*), and *kinesin family member 5B* (*KIF5B*; Fig. 5*A*). We sequenced all of the exons and the UTRs of these genes and found 12 SNPs (Fig. 5*A*). Reanalysis with the newly developed SNPs indicated that CACNB2 (del+613AT) was the most significant SNP (Dataset S1, Table S1). The association signals for BTA-32318-no-rs and its adjacent UA-IFASA-6479 disappeared when the CACNB2 (del+613AT) was included as a covariate in a logistic regression analysis ($P = 1E-05$ and 6E-05, respectively). Cattle harboring the del/del genotype displayed a higher conception rate than cattle harboring the AT/AT genotype (Dataset S1, Table S2). Thus, CACNB2 (del+613AT) was the most promising causative SNP on chromosome 13.

GnRH mediates the influx of calcium through L-type voltage-gated calcium channels (29). We compared the luciferase activity using the murine gonadotroph-derived LβT2 cell line (30), which expresses the GnRH receptor. Reporters carrying the del allele exhibited higher luciferase activity than those carrying the AT allele (Fig. 5*B*). Similar results were observed in the allelic mRNA ratio (Fig. S2*C*), suggesting that the expression level of *CACNB2* might affect conception rate in cattle.

Calcium influx through L-type voltage-gated channels is required for the GnRH-induced activation of extracellular signal-regulated kinase (29), which in turn leads to secretion of FSH (31, 32). Thus, a high expression level of CACNB2 might increase FSH secretion. To explore this possibility, we transfected LβT2 cells with siCACNB2, vector, or CACNB2 and stimulated them with GnRH and activin A (33). Knockdown of CACNB2 reduced FSH secretion, whereas overexpression of CACNB2 increased its release (Fig. 5*C*). Moreover, cows harboring both the del allele in *CACNB2* and SETD6^{Ala} exhibited a higher concentration of FSH than AT homozygous cows at approximately day 9 of the estrus cycle, whereas there was no difference in SETD6^{Glu} homozygotes (Fig. 5*D*). CACNB2 might control FSH secretion in the SETD6 pathway.

Impact of Genetic Variants Related to Gap Junctions and Neuroendocrinology. Taking all of our results together, four genes were found to be associated with conception rates in cattle. To estimate the impact of these genes on the phenotype, we evaluated allele

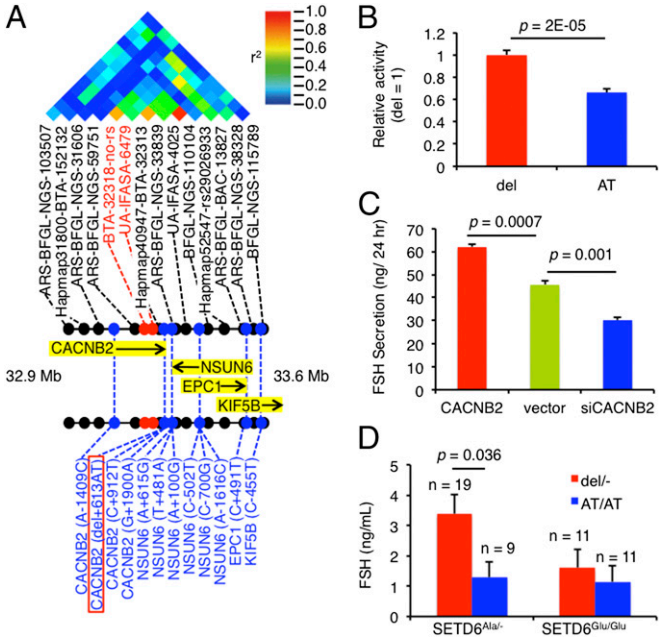

Fig. 5. CACNB2 controls the secretion of FSH. (*A*) A pairwise linkage disequilibrium diagram with a schematic representation of the genes (black arrow) located in the associated region in chromosome 13. Red, black, and blue dots represent the genome-wide significant SNPs, the original SNPs, and the newly developed SNPs, respectively. (*B*) The average luciferase activity ± SEM of the 3′ UTR of *CACNB2* in LβT2 cells ($n = 8$). (*C*) The average secretion ± SEM of FSH by LβT2 cells transfected with CACNB2, vector, or siCACNB2 after treatment with GnRH and activin A ($n = 3$ each). (*D*) The average concentrations ± SEM of FSH in the serum of del/- ($n = 30$) or AT/AT cows ($n = 20$).

substitution effects based on a fixed or random model. Both models indicated that each gene showed an effect ranging from 0.62 to 1.05% per allele, and the total allele substitution effect was 3.5% (Dataset S1, Table S3). Moreover, these genes had favorable effects on the length of the days open period, which was shortened by 8.5 d as the total allele substitution effect (Dataset S1, Table S3). The days open, which is the length of the interval between delivery and conception, is an important fertility trait among cattle, and its additive genetic variance is 90.5 d (34). We obtained similar results both for the conception rate and days open in terms of the deregressed EBV (35) and daughter yield deviation (36), which are more reliable genomic predictors than the traditional EBV (Dataset S1, Table S3). Therefore, genetic variants related to gap junctions and neuroendocrinology influence conception rates as well as days open.

Discussion

We identified naturally occurring genetic variants in four genes—*PKP2*, *CTTNBP2NL*, *SETD6*, and *CACNB2*—that were associated with conception rates in cattle. The total allele substitution effect of these four genes is quite large, given that the additive genetic variance for the conception rate was 3.5–3.9% (16). Selection of cattle on the basis of the SNPs identified in the present study should be beneficial for the livestock industry.

PKP2 and CTTNBP2NL influence implantation through gap junction communication. Gap junction communication in the cumulus–oocyte complex of the ovary is also important for oocyte maturation and pregnancy outcome (37). The molecules entering the oocyte from the cumulus include cAMP and glucose (38, 39). Identifying the molecules that traverse gap junctions in the uterus should be studied. Moreover, it might be worthwhile to investigate whether PKP2 and CTTNBP2NL influence oocyte maturation.

SETD6 controls release of GnRH. We previously demonstrated an association between *glutamate receptor, ionotropic, AMPA 1* (*GRIA1*), and ovulation rate in Japanese Black cattle (40). Like SETD6, GRIA1 regulates release of GnRH; however, GRIA1 affects only the timing of the LH surge and does not alter FSH secretion in cattle (40). Interestingly, GnRH differentially regulates the synthesis of LH and FSH through changes in the pulsatile pattern of GnRH secretion (41): A fast GnRH pulse preferentially increases LH synthesis, whereas a slow GnRH pulse increases FSH synthesis (42). GRIA1 and SETD6 might, therefore, be involved in the differential pulsatile release of GnRH.

CACNB2 increases FSH secretion and is a member of the voltage-gated calcium channel superfamily (43). Numbers of rat anterior pituitary cells secreting FSH are increased by activin A through Ca^{2+}-dependent mechanisms (44). CACNB2, along with other members of the calcium channel superfamily, might participate in the development of gonadotroph-producing FSH.

Peñagaricano et al. (45) reported that the functional categories such as calcium ion binding [gene ontology (GO): 0005509] and plasma membrane part (GO:0044459) showed significant enrichment of gene statistically associated with bull fertility. Our candidate genes such as *CACNB2* and *PKP2* might also be involved in bull fertility as well as female conception rate.

This study found genes that play important roles in fertility of mammals. As a proof of principle, a naturally occurring mutation has been found in *bone morphogenetic protein 15* (*BMP15*) in sheep that increases ovulation rate (46). This discovery led to the prediction of an overresponse to recombinant FSH based on BMP15 alleles in humans (47) and to the identification of an associated mutation related to hypergonadotrophic ovarian failure in humans (48). Similarly, our results in cattle could potentially further the understanding of reproduction events among humans.

Materials and Methods

Ethics Statement. All animal experimentation was undertaken with the approval of the National Livestock Breeding Center Committee on Animal Research (H25-2).

Whole-Genome Scan. Samples were genotyped using the BovineSNP50 v1 DNA analysis kit (Illumina) and adjusted population stratification by principal component analysis (49; $\lambda = 1.02$). A logistic regression analysis was conducted using PLINK software (50). A conditional logistic regression analysis was performed by including PKP2 (del+988TA), SETD6 (Ala360Glu), or CACNB2 (del+613AT) as a covariate.

Development of Unique SNPs. Each of the exons, 2 kb of the 5′ UTR and 2 kb of the 3′UTR of genes located in the associated regions based on the November 2009 *Bos taurus* draft assembly (51), were amplified by PCR and sequenced. Regions including the genome-wide significant SNPs and their neighboring SNPs with an r^2 of more than 0.2 were defined as the associated regions. The primers for each gene and the samples used for comparing sequences are shown in Dataset S1, Tables S4 and S5, respectively. We selected the samples with homozygous high- or low-specific haplotypes comprising the genome-wide significant SNPs and their neighboring SNPs.

Genotyping of Additional Samples. DNA was extracted from blood samples drawn from the 1,034 cows with EBV for conception rates of 41–51% and semen from 2,528 sires whose EBV for conception rates were calculated as described (16). Genotyping was performed after PCR amplification.

Real-Time PCR. RNA was extracted from bovine tissue samples using TRIzol reagent (Life Technologies). Real-time PCR was conducted with the ABI 7900HT sequence detection system using the comparative Ct method and GAPDH as an internal control (Life Technologies). The primers used in these assays are shown in Dataset S1, Table S6.

Luciferase Assay. Fragments of the 3′ UTRs of *PKP2*, *CTTNBP2NL*, and *CACNB2* were generated using PCR with the primers forward and reverse (Dataset S1, Table S7). These PCR products were further amplified via PCR with the primers forward2 and reverse2 (Dataset S1, Table S7) to generate a restriction site. The fragments produced were then cloned into the pMIR-REPORT

miRNA expression reporter vector (Life Technologies). The luciferase assays were performed using a dual-luciferase reporter assay system (Promega).

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