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Friend Leukaemia Integration 1 is Associated with Conception Rate in Holsteins

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Abstract

Background: Conception rate is an economically important trait in the dairy industry; however, it has decreased dramatically over recent decades. Conception is a complex process including follicle development, ovulation, fertilization, implantation, and placental differentiation and numerous factors contribute to this event. The present study aims to explore the genetics of conception rate in Holsteins using a genome-wide association study (GWAS).

Methods and Findings: Our GWAS for conception rate based on 2,559 Holstein sires found that the conception rate is influenced by a single nucleotide polymorphism GA-266del in the 5' untranslated region of *friend leukaemia integration 1 (FLI1)*. Cows with higher conception rates carried the GA polymorphism in the FLI1 5' untranslated region. Luciferase assays and quantitative analysis of allele ratios revealed that FLI1 transcripts with the GA polymorphism were expressed at higher levels than those carrying the deletion polymorphism. FLI1 is a member of the ETS gene family of transcription factors and disruption of FLI1 increased natural killer cell population. High levels of natural killer cells were correlated with spontaneous abortion in human. Cows with the deletion polymorphism released higher levels of perforin, a product of natural killer cells, than did cows with the GA polymorphism. Moreover, cows with the deletion polymorphism have lower successful rate for pregnancy after embryo transfer than cows with the GA polymorphism.

Conclusions: These observations suggest that cows carrying the deletion polymorphism in FLI1 might have lower conception rates because of the enhanced perforin production. Thus, this study provides novel insights into the role of FLI1 during reproduction process.

Keywords: Conception; GWAS; FLI1; NK cells; Perforin

Introduction

Dairy production depends on the frequency at which cows conceive; thus, conception rate (CR) is essential for this industry. Studying the genetics of CR using a genome-wide association study (GWAS) may be helpful for understanding the underlying biological mechanisms and beneficial for the industry. Here we report a new gene, *friend leukaemia integration 1 (FLI1)*, which is associated with CR in the Holstein cattle population.

FLI1 is a proto-oncogene and belongs to the ETS gene family of transcription factors [1]. The targeted disruption of Fli1 in mice increased numbers of natural killer (NK) cell [2]. High levels of NK cells in the peripheral blood of patients were correlated with spontaneous abortion in human [3]. Activation of NK cells by polynucleotides can cause abortion in pregnant mice [4]. Moreover, the decidual Va14 NKT cells, a subset of NK cells, were involved in abortion through perforin-dependent pathway [5]. Perforin, a pore-forming protein, acts as a host defence molecule protecting both the mother and the fetus from a wide spectrum of pathogens and also acts as an effector molecule causing the apoptosis of trophoblast cells leading to various pregnancy disorders [6]. Therefore, FLI1 might be correlated with CR through perforin-dependent pathway in ruminants.

In this work, we performed a GWAS on 2,559 Holstein sires and identified a single nucleotide polymorphism (SNP) in the 5' untranslated region (UTR) of FLI1 that influences the CR of cows. Cows with the deletion polymorphism were less fertile than cows with the GA polymorphism, and this SNP decreased the expression of FLI1. We demonstrated that cows with the deletion polymorphism release more perforin compared with cows with the GA polymorphism. In addition, cows with the deletion polymorphism have lower success rate for pregnancy after embryo transfer than cows with the GA polymorphism. Thus, we propose that FLI1 keeps a low level of perforin that maintains pregnancy.

Methods

Samples

We collected DNA from 2,559 Holstein sires and evaluated the estimated breeding values (EBVs) for the CRs [7, 8]. The EBVs for the CRs of the sires were evaluated based on their daughters' CRs. The EBVs for CRs of daughters were evaluated by threshold linear models using insemination event data after first calving. The threshold-linear repeatability animal model to estimate EBV of CR can be written as:

where is a vector of unobserved liabilities converted from insemination events data, which collected as a longitudinal binary response of either a success or a failure. ; is the fixed vector of systematic effects (age at insemination, month of insemination, days from calving to insemination, regression coefficients on inbreeding); is the random vector of herd-year-season at insemination; is the random vector for interaction of service sires and years ; is the random vector of additive breeding values; is the random vector of permanent environmental effects; is the random vector of residual terms; and , , , , and are known incidence matrices with the appropriate dimensions. The EBV for the CRs of the population was distributed as shown in Figure 1A.

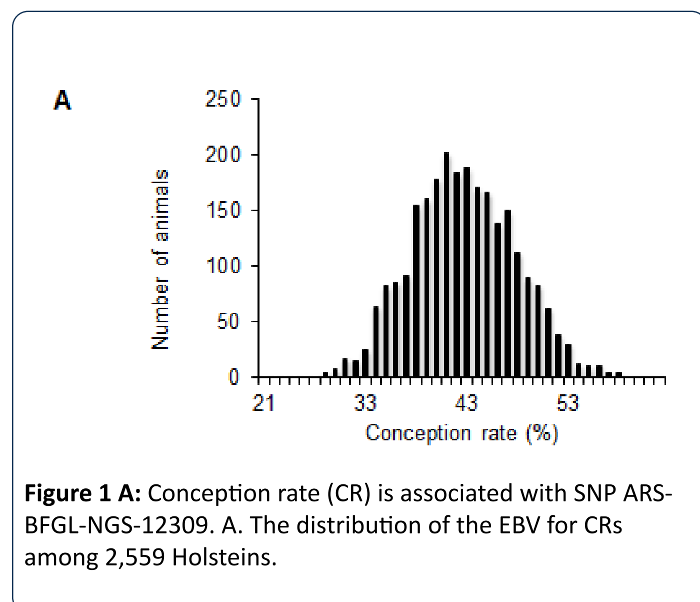

Figure 1 A: Conception rate (CR) is associated with SNP ARS-BFGL-NGS-12309. A. The distribution of the EBV for CRs among 2,559 Holsteins.

Whole-genome scan

We genotyped 2,559 samples using a Bovine SNP 50 v1 DNA Analysis Kit (Illumina, San Diego, CA, USA) for a total of 54,035 SNPs and conducted an association analysis using EMMAX software [9].

Identification of novel SNPs

Based on the Nov. 2009 Bos taurus draft assembly [10] (UMD_3.1), each of the exons, 2 kb of the 5'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. The genome-wide regions that included significant SNPs as well as their neighbouring SNPs with r^2 values greater than 0.2 were defined as the associated regions. The r^2 values were calculated by a linkage disequilibrium analysis using PLINK software [11]. The primers for each gene and the samples used to compare the sequences are shown in Tables S1 and S2, respectively.

Allelic substitution effects

We genotyped 2,682 cows and 4,165 bulls for *FLI1, PKP2, CTTNBP2NL, SETD6, CACNB2, UNC5C, and FAM213A. FLI1* was identified as a gene that was associated with the CR in the present study. *PKP2, CTTNBP2NL, SETD6, CACNB2* and *UNC5C* have previously been identified as genes associated with the CR, whereas FAM213A has previously been identified as a gene that was associated with the fertility selection index (SI) [12–14]. The SI consists of the 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 delivery [15]. The EBVs of cows and bulls with these six traits included in the SI were estimated by a animal model using 1,881,898 records. The EBVs of cows and bulls in relation to the CR were estimated by a threshold-linear 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:

where = the deregressed EBV [16] of animal *i* (= 1, 2, ..., n) for the CR, DO, NI, SFI, P70, P90, P110 or SI; = the general average value of the population; = the genotype covariate (coded as 0 or 2 for the two homozygotes and 1 for heterozygotes) of gene j in animal i; = the random regression coefficient representing the allelic substitution effect for gene j; and = 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, heart, kidney, liver, lung, ovary, pancreas, skeletal muscle, spleen, stomach, and uterine tissue 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 S3.

Luciferase assay

Fragments of the 5'UTR of FLI1 were generated using PCR with the respective forward and reverse primers (Table S4). These PCR products were further amplified via PCR using the forward2 and reverse2 primers (Table S4) to be cloned into a pGL3 (R2.2)-basic vector (Promega, Madison, MI, USA) using an In-Fusion Advantage PCR Cloning Kit (Takara Bio Inc., Shiga,

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Japan). Luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega).

SNaPshot and quantitative analysis of allele ratios

The allelic messenger RNA (mRNA) ratio was determined using a SNaPshot Multiplex Kit (Life Technologies), and the primers used are shown in Table S5. For cDNA preparations, each mRNA was converted to cDNA in three separate experiments Table 1-5.

Table 1 (S3-S5): Primers used to search for SNPs.

Gene	Position	Primer	Sequence
ETS1	5'UTR-1	Forward	GTGGTTAGCAGTGTTTAGGCT
		Reverse	ACACACCTGCTTACCTCATCT
	5'UTR-2	Forward	TTCTCTTCCTGGCTCCTTCC
		Reverse	TGTCACCACTGGCCAAAATT
	5'UTR-3	Forward	CAGAGCTGTGCATCATGTTTT
		Reverse	TCCACGCATTCTTGAGGACT
	5'UTR-4	Forward	AGCAGCCCAAGTCCAGTATT
		Reverse	GGGGAATCGGACCTTCTTCT
	exon 1	Forward	AGAGATCCTGAGGGTGGGG
		Reverse	GGGGAGAAGTGGAGGGGA
	exon 2	Forward	GCAGAACGATCACCACCATC
		Reverse	GGTCCATCCTCTCTCCTTCC
	exon 3	Forward	TTTCGTGTAGTCTCCGAGGC
		Reverse	CACCCTGTCCTCATGCATTT
	exon 4	Forward	TGAGATCACTGTGGTCCTCG
		Reverse	GGAAGAGAGAAGAGGAGCCA
	exon 5	Forward	TCTCCTCTCTCCAATCGCAC
		Reverse	TGGCTAAGAGTGAGGGAGGA
	exon 6	Forward	GTGTCTCTTCCCATCCCTCC
		Reverse	CAGAAGTGTCCAGGGAGCC
	exon 7	Forward	TTCACCATGGCTTGTGTCTC
		Reverse	ACACCATCAAGCCCCATACA
	exon 8	Forward	CACCAATGAGTGCAGGCATA
		Reverse	TCAGAATCCTCAGTCGGCAA
	3'UTR-1	Forward	GATGGACTTCAGTGGGGAGG
		Reverse	ATAAATGTGGGGTGCTGGGA
	3'UTR-2	Forward	GGAAAGAGGGAGTGAAGGGA
		Reverse	TTGACAATGGCCTCGGTTTG
	3'UTR-3	Forward	AAGGAAGGAAGCTTGAAGGC
		Reverse	CTCCCTGAGCAGCTCCTAAA
	3'UTR-4	Forward	GCCCAGCTGTGTATTGTGAT
		Reverse	TCTTCCTGGGATGGTCTCTG

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FLI1	5'UTR-1	Forward	GAGGAAAGGGTTAAGCCTGATT
		Reverse	CTTCTTTCTCCCCGACTTCC
	5'UTR-2	Forward	AAAGTCCAAAGCGTGGTCTG
		Reverse	TGCATCCAATGGGAAGTTTT
	5'UTR-3	Forward	GAGCTCTCCAGTAGCCCAGA
		Reverse	TTGTTCCCGGGAGATAAGG
	5'UTR-4	Forward	TGCAGACTTTGGGAATCAGG
		Reverse	GCGGAAGGAAGGGAGAGT
	exon 1	Forward	CTTTTTCGCTCCGCTACAAC
		Reverse	GCGGAAGGAAGGGAGAGT
	exon 2	Forward	GGGCTCTGTGTCCTTCTCTG
		Reverse	CGTCTGCCACAGACACACTT
	exon 3	Forward	CCTTCCCCTGAGCTTTGTCT
		Reverse	AGTGAAAGGGTTCCCGAAGT
	exon 4	Forward	TTGCTAACAGCCTGTCTCTCC
		Reverse	TAGGGACCGGGCACTTAC
	exon 5	Forward	GTTTTTGCTTCGCCTTTCAG
		Reverse	CCCAGTCTTCCCATCACAGT
	exon 6	Forward	CTGCCACTCCATGAGCTGTA
		Reverse	CGCTTATGACCCTGTTCTCC
	exon 7	Forward	GGGAGTGAGTGAATGGGAAA
		Reverse	AGGGTTCGAACATCATGGAC
	exon 8	Forward	TCAGGCTTTCCTATGATCTCAA
		Reverse	ACACAACCTCTCAGGCCAAA
	exon 9	Forward	TCTCAGGTGGAGCCTGTTTT
		Reverse	CCACCGATGAGGAAGCAT
	3'UTR-1	Forward	CATCTACCCCAACCCCAAC
		Reverse	GTTCCAGTTGCCCTCCACT
	3'UTR-2	Forward	GGCAGGAAGCTTATCATCTTATC
		Reverse	AACGTACAAGCAGCCCAAAT
	3'UTR-3	Forward	GAGTTGACCTCGGTCACAGAT
		Reverse	CTGGGAAAACCCTTGGACCT
	3'UTR-4	Forward	TTGTGCCTTCTTCTCTCAGAAC
		Reverse	CTACACCATCAGCCGGTTTC
KCNJ1	5'UTR-1	Forward	AGGCTGGTCTGAGGGACAAT
		Reverse	CCTTTCTCCCTGGCTTTACC
	5'UTR-2	Forward	AAAGAAAAGCCTTCCATGAGC
		Reverse	CTCCAGTCAGTGCAGAACCA
	5'UTR-3	Forward	TGCAAATGAATGAGGCACTT

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		Reverse	TGGCATTGAGTGACTGTTCC
	5'UTR-4	Forward	TGTGTGAGCCAGAGATGACC
		Reverse	TCAGACCAGCTGCCAACTC
	exon 1	Forward	TGTATCCTGCCCACTTACCC
		Reverse	TATGGCATTTCTCCGCTTAC
	exon 2	Forward	CTTCTCTTAGTGACTTTCTGTTCTGA
		Reverse	CCCCTGTCCTGTGATGAATG
	exon 3	Forward	TCTGTTTTGTCTTTCTCTGATGTGT
		Reverse	ACTCGTGTGGAAAACTCAGC
	3'UTR-1	Forward	TGAAACAGACGACACCAAAA
		Reverse	GAGCCAATGTTCAAATAAAAGTGA
	3'UTR-2	Forward	ATGGACAGGCCAAATGAGAT
		Reverse	TGCCTTCTTGGAAGATCAGC
	3'UTR-3	Forward	CGCTGGCTTCAAATCTGTTA
		Reverse	GGACGTCACAACGTCAGAGA
	3'UTR-4	Forward	GTATTCTGGAGCGGACGGTA
		Reverse	TCATAGCAATGGGTCAGCAG
ARHGAP32	5'UTR-1	Forward	CTGTGCCTCTCATTGTGCTG
		Reverse	TGAGTTGTATGAGCTACTTGTGT
	5'UTR-2	Forward	ACAGAATGGGAGGAAATATTTGC
		Reverse	TCATCTCTAGCTCCATCCATGT
	5'UTR-3	Forward	ATCCTGTAGTGCCACTCCTG
		Reverse	GACTGTGAACCAATCCAACATAA
	5'UTR-4	Forward	ACTTCAACTTAAGGGGAACGTG
		Reverse	CGAATCCAACCAGAACACG
	exon 1	Forward	GAACAACCTGGACACTGCTG
		Reverse	AGATGAGGGAGGTGGAGAGA
	exon 2	Forward	TGGATTGTAGTCATTGGAAGGC
		Reverse	TGCTTCCCCTGTTCCTTTTC
	exon 3	Forward	TCTTTTGGTATGGAGTTAGGACC
		Reverse	TGTATGGACAGTAAGAGCTCATT
	exon 4	Forward	TGTTCCAGGATCTTGTCTCTCA
		Reverse	ACGCCTCGCTTCAGTATGTA
	exon 5	Forward	ACCGTGACTTTCTTTCCCTCT
		Reverse	TGCAGAAATGCCAATGTGACT
	exon 6	Forward	TCGCTAGAGGGTTTTGGAGT
		Reverse	CCCATGAATCTTCCTGAGTGC
	exon 7	Forward	TCTGTGAAGAACCTCTGTGACT
		Reverse	CACATGGGATTTCTTTCGTAGGT

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	exon 8	Forward	CTTGCAAGTCCCGTGTCTTT
		Reverse	GCTAGAAAGGCAGCACAGAC
	exon 9	Forward	GACCAGTCTTTGTGCTGCTT
		Reverse	CACACCGAATTCTTTGTTGCG
	exon 10	Forward	GCTCACACACTGGTCTGTTC
		Reverse	TGACAACGAACACAACAGCT
	exon 11	Forward	TGCACTTCATTTCCTCTTGCT
		Reverse	GGAGATCAACAGGGAGAGGT
	exon 12	Forward	TTAATCTCCACCCCTTACAGC
		Reverse	GAGGCCAAGGTTTTCTGATACT
	exon 13-14	Forward	CTCACTGAGCTGGAGGTTAAT
		Reverse	TGGCATTTTACAGAGCGTGG
	exon 15-16	Forward	ACTCCCTGATGTTTCTTTGTGT
		Reverse	TTAAAGATACGTTCCCAGGGG
	exon 17	Forward	AGTTTGAGTCTTGTCGTTGCT
		Reverse	CCACAGTCGAATGAACAGGT
	exon 18	Forward	TTGGATGCCTTAATGCGGAC
		Reverse	AAGGGAAGGCGTGAACACT
	exon 19	Forward	ACGTTGTTTGGTTTTGATTCTGC
		Reverse	CGTAGTGACTGAACAACAACAAC
	exon 20	Forward	AGCTGACTCATAGGGCACTG
		Reverse	AAGCAAGAATGGAGGAAAACAAA
	exon 21-1	Forward	ACACAAGCTACCTTTTCACTTTC
		Reverse	GGCACACTGGTCTTCACAGA
	exon 21-2	Forward	CAGAGTCACTTCCGTTCCCT
		Reverse	GGCACACACTGATGGAGAGT
	exon 22-1	Forward	ATTCCGCCTGCAGTCCAT
		Reverse	GGTCTGGAAGCCCTTTGG
	exon 22-2	Forward	GGGCAGAGTATGTGTCCTCA
		Reverse	GGAGTCCAGTTTTCCCAGGA
	exon 22-3	Forward	GAGAAATACCGCCTGCAGTC
		Reverse	CCTCCAGGTTATCGTACTGC
	exon 22-4	Forward	GGGTCACCTGTTTTCTTTGTCA
		Reverse	ACTGAAGTGTGTGGAGCAAC
	3'UTR-1	Forward	GACCCATTAGATCCAGGCTGA
		Reverse	GCTGGTGTGGATGGCAATTA
	3'UTR-2	Forward	GGAATTACCGTGTTGTGTCTTC
		Reverse	ACACCTAACAGAGTATTTCCACA
	3'UTR-3	Forward	TCATGTGATTGCATTTTAAGGGT

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		Reverse	ACGTTTCACACTTTCACCAGG
	3'UTR-4	Forward	GGTCACACACACTGTTTACTCC
		Reverse	TACTGAAAGGAGGCGGCATC
JAM3	5'UTR-1	Forward	GCATAGACTCCACAGCCCTA
		Reverse	CAGCCTCTGTCCCATAAACA
	5'UTR-2	Forward	AAGTCAGCGGGCCTAAGTAG
		Reverse	TGCTCCAGGAAACAACAAACT
	5'UTR-3	Forward	TGACTGTGTGAAAAGTGACGT
		Reverse	GAACCCGGGTCTCCTTCAT
	5'UTR-4	Forward	TTCAGCACTTTCCCCTCTCA
		Reverse	CCTCAGCGCCATGTCGAG
	exon 1	Forward	TCCATAGCAACCAGACTCGG
		Reverse	CGAGACCCTTCCCTGACG
	exon 2-3	Forward	CGTCTCTGACTTGGCTTTTCT
		Reverse	GGGCCTTCTGTACAAAGAGG
	exon 4-5	Forward	TCCTTTAACGGGGAAGCCTT
		Reverse	GGTCTGTAGCTCTGGTCTCC
	exon 6	Forward	ACATTGTTGGTGTGTTCGGG
		Reverse	GAGGCTCAGCAGACACTCA
	exon 7-8	Forward	ACAGCATCTTCTCACCCCTC
		Reverse	CGTCTCCAGGCTCCCTTAC
	exon 9-1	Forward	ATCGAGGGAAGTGGTGTCAG
		Reverse	GGTTTCCTAAGCCACCAGTG
	exon 9-2	Forward	TGTTCTGCTTTTCTATGGGTGT
		Reverse	TGTCTTCATGGCAGAGGGAC
	exon 9-3	Forward	AGGAAAAGGCTACCCACTCC
		Reverse	GAAAGAAACTGGGCTGGCTC
	exon 9-4	Forward	AAAAGGCTACCCACTCCAGT
		Reverse	AAAGAAAGGTCAACACAGTCTTG
	exon 9-5	Forward	ATGGTCCAGGGCCAAAGG
		Reverse	AATGAAGAGGCTGAGCTGCT
	exon 9-6	Forward	TGCCATGAGAACTGGTAGCA
		Reverse	GACCCACACTCACTCCTCTC
	3'UTR-1	Forward	GCTACTAACACACCTGCACG
		Reverse	GGGACCCAAGCTTTGTTTCA
	3'UTR-2	Forward	CTCCCGTTGCTCTGGTAAAA
		Reverse	TGGTGCTCAGAAAGTGGTCA
	3'UTR-3	Forward	AGGGAGAGAAGCTGGGAGTA
		Reverse	GCGGTTTCCAAGGTACATCC

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	3'UTR-4	Forward	GGGATGATTGTACATGTGCAGT
		Reverse	CTCTGCTACCCGACTGAAGG
SPATA19	5'UTR-1	Forward	TGTGCAGATTTAGCGCTTATG
		Reverse	CTGTTTCCATCCTTACTGGTG
	5'UTR-2	Forward	CAAGGTTGAAGAGACAGGGAAG
		Reverse	CTCCCCAGCTTCCCTAAAAT
	5'UTR-3	Forward	GCCTAGCATATTCTGATCAATAGAGA
		Reverse	CACAAGTGATTCAGATCACAAAAA
	5'UTR-4	Forward	TCCTTCACAAGAATTGGCACT
		Reverse	TCCATTGAAGCAGCCTGAG
	exon 1	Forward	CCAATCAGGTAGGCACCAC
		Reverse	CTCTCCACCTAGCCATCACC
	exon 2	Forward	TGGATGTGGATAGTGGAGCA
		Reverse	TGATGAATCAGACGCAGCTC
	exon 3	Forward	GGACCAGACGAGTGAAGGAA
		Reverse	CGGCTAACAGGCTCCATTAC
	exon 4	Forward	CTTGCTGGGCAGTAACCACT
		Reverse	GGTTTGTGTCAGCCAGGAAT
	exon 5	Forward	ATCCCAATGCTTGACGATGT
		Reverse	GACCCACAGAGACCAGAAGC
	exon 6	Forward	CTGCTGTGGTCTGTGCTCTG
		Reverse	CCCCTTGCTGGTATTCTCAA
	exon 7	Forward	GCATTTCCCTCTTTCCATGT
		Reverse	TAGCAAGGCACTCACACCAG
	3'UTR-1	Forward	AGTGGCAGCACTGGAATCTT
		Reverse	TGTGTAAGGAAAGGACGCACT
	3'UTR-2	Forward	GTGACATGTGCGCTTCACTC
		Reverse	AAGATCTGGGGGACAATTCC
	3'UTR-3	Forward	TTGTTCCTCTCCTGGTTTCC
		Reverse	ACAGCTGCCGGAAAGAGTAA
	3'UTR-4	Forward	GGCCTTCATCGTGAGGTTTA
		Reverse	CTGCAGGAATCCTTCCAGAC

Table 2: Samples used for developing new SNPs.

ID	34	55	109	152
Paternal ID	2182318	2183007	2265005	2290977
Maternal ID	769202561	15937840	123597843	128824973
EBV	0.521	0.4807	0.2988	0.2937
Hapmap51018-BTA-65434	G	G	G	G

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	G	G	G	G
Hapmap34976-BES2_Contig422_801	G	A	G	G
	A	A	A	G
BFGL-NGS-112928	G	G	G	G
	G	G	A	G
ARS-BFGL-NGS-70760	C	C	A	A
	C	C	A	A
BTA-65424-no-rs	A	A	A	A
	A	A	A	A
BFGL-NGS-109714	A	A	A	A
	G	G	G	A
ARS-BFGL-NGS-62183	C	A	A	A
	C	A	A	A
BTB-01020010	G	A	A	G
	G	G	G	G
BFGL-NGS-110219	A	A	G	G
	G	G	G	G
Hapmap40017-BTA-65421	C	C	A	A
	C	C	C	A
ARS-BFGL-NGS-36127	A	A	A	A
	A	A	A	A
UA-IFASA-7281	G	G	A	A
	G	G	A	A
ARS-BFGL-NGS-87575	G	G	A	A
	A	G	A	A
ARS-BFGL-NGS-12309	G	G	A	A
	G	G	A	A
BTA-65417-no-rs	G	G	A	G
	G	G	G	G
UA-IFASA-7219	A	G	A	A
	A	A	A	A
ARS-BFGL-NGS-1333	A	A	G	G
	A	A	G	G
BTA-65410-no-rs	A	G	G	G
	G	G	G	G
UA-IFASA-8767	G	A	A	A
	G	A	A	A
UA-IFASA-9430	A	G	A	A
	A	A	A	A

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Hapmap48423-BTA-65404	A	G	A	A
	A	A	A	A
BFGL-NGS-119359	G	G	G	G
	G	G	G	G
ARS-BFGL-NGS-16031	A	A	G	G
	A	A	G	A
Hapmap58618-rs29012371	G	G	A	G
	G	A	A	A
BTA-65537-no-rs	A	A	A	A
	G	G	G	A
BFGL-NGS-117839		G	G	G
		A	A	A
BTB-01023253	A	G	A	A
	G	G	A	G
ARS-BFGL-NGS-102550	A	G	A	A
	A	A	A	A
ARS-BFGL-NGS-24769	G	G	G	G
	G	G	G	G
ARS-BFGL-NGS-41631	A	A	A	A
	A	A	A	A
BFGL-NGS-115193	A	A	A	A
	G	G	G	A
Hapmap52938-rs29027128	A	A	A	A
	A	A	A	A
ARS-BFGL-NGS-40178	G	G	G	G
	G	A	G	G
BTA-65587-no-rs	A	A	A	A
	A	A	A	A
UA-IFASA-8226	G	A	A	A
	A	A	A	A

Table 3: Primers used for real-time PCR.

Gene	Primer	Sequence
FLI1	Forward Reverse Probe	CGTCAAGCGCGAGTACGA TGCCGCATTTGCTGACACT TGGGTCCAGGGAGTCTCCGGTG
GAPD	Forward Reverse Probe	GCCCTCAACGACCACTTTGT CCTGTTGCTGTAGCCAAATTCA AAGCTCATTTCCTGGTACGA

Table 4: Primers used for generating reporter constructs.

Primer	Sequence
Forward	TATATAGTGTGTGTGATGCG
Reverse	TTGGCCAAGTCTGCAGCCGA
Forward2	CCGAGCTCTTACGCGTTATATAGTGTGTGTGA
Reverse2	CTTAGATCGCAGATCTTTGGCCAATCTGCAG

Table 5: Primers used for SNaPshot.

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Primer	Sequence
Forward	TATATAGTGTGTGTGATGCG
Reverse	CTTTGCGAATGGGGAGGAAG
Extension	GCGCGAGACAGAGAGAGAGAGAGAGAGAGAGA

Enzyme immunoassays

The concentration of perforin released from the bovine serum was assayed using a Perforin Human ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Embryo transfer to recipient female

The embryos were transferred non-surgically into Holstein heifers to the uterine horn ipsilateral to the existing corpus luteum using an embryo transfer device (Misawa Medical Industry Co., Ltd, Tokyo, Japan) on days 6–8 of the estrous cycle. Pregnancy was determined by real-time B-mode ultrasonography (Honda electronics Co. Ltd., Toyohashi, Japan) on days 30 and 60 of gestation. Calves were delivered spontaneously without induction of parturition.

Results

Our GWAS identified ARS-BFGL-NGS-12309 as the most significantly associated SNP on chromosome 29 (Figure 1B), and the region associated with this SNP included 6 genes (Figure 2A). To detect possible causative polymorphisms in this region, we sequenced all exons and the 5' and 3' UTRs of these 6 genes and found 35 novel SNPs (Figure 2A). A reanalysis of the newly sequenced SNPs demonstrated that FLI1 (GA-266del) was the most significant (Figure 2A).

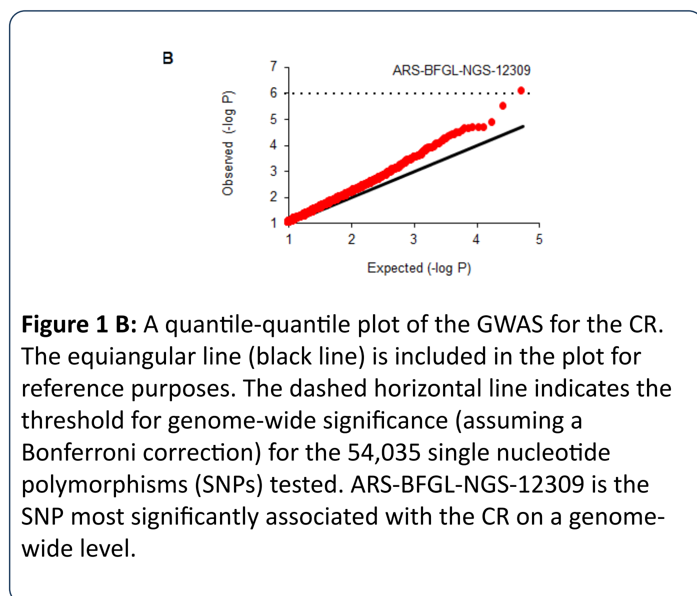
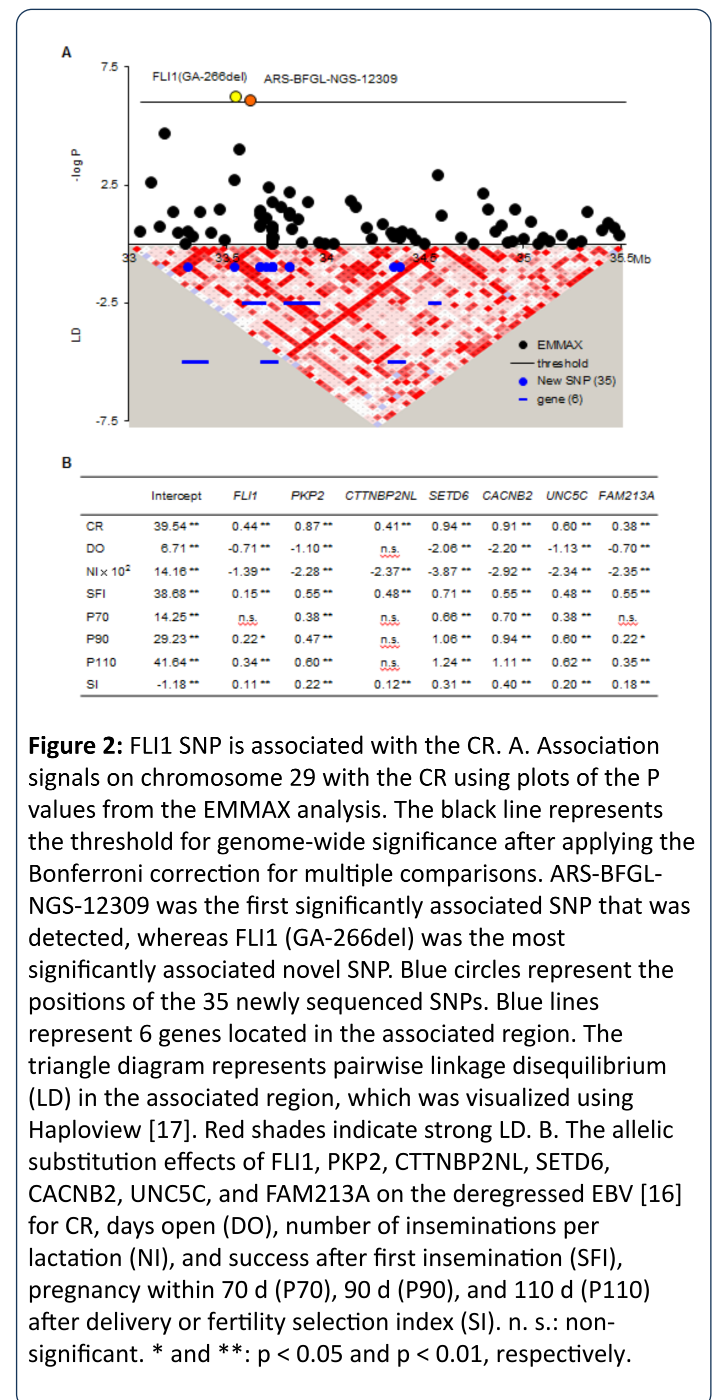


Figure 1 B: A quantile-quantile plot of the GWAS for the CR. The equiangular line (black line) is included in the plot for reference purposes. The dashed horizontal line indicates the threshold for genome-wide significance (assuming a Bonferroni correction) for the 54,035 single nucleotide polymorphisms (SNPs) tested. ARS-BFGL-NGS-12309 is the SNP most significantly associated with the CR on a genome-wide level.

Moreover, we genotyped FLI1 (GA-266del) in 2,682 cows and 4,165 bulls and found that the allele substitution effect of FLI1 on the dEBV for the CR was 0.44 (Figure 2B). Cows with the GA/GA genotype exhibited a 0.44 higher CR than those with the GA/del genotype. FLI1 also had favourable effects on the dEBV for the traits that compose the SI (DO, NI, SFI, P90, and P110) and the SI itself. The effects of FLI1 were similar to those of *PKP2, SETD6, CACNB2, UNC5C, or FAM213A*, which have previously been identified to be associated with the CR or SI in the Japanese dairy cow population [12–14] (Figure 2B). Therefore, FLI1 (GA-266del) was the most promising causative SNP on chromosome 29 and had a similar impact on the CR as the five genes previously identified to be associated with CR or SI.



	Intercept	*FLI1*	*PKP2*	*CTTNBP2NL*	*SETD6*	*CACNB2*	*UNC5C*	*FAM213A*
CR	39.54 **	0.44 **	0.87 **	0.41 **	0.94 **	0.91 **	0.60 **	0.38 **
DO	6.71 **	-0.71 **	-1.10 **	n.s.	-2.06 **	-2.20 **	-1.13 **	-0.70 **
NI × 10^2	14.16 **	-1.39 **	-2.28 **	-2.37 **	-3.87 **	-2.92 **	-2.34 **	-2.35 **
SFI	38.68 **	0.15 **	0.55 **	0.48 **	0.71 **	0.55 **	0.48 **	0.55 **
P70	14.25 **	n.s.	0.38 **	n.s.	0.66 **	0.70 **	0.38 **	n.s.
P90	29.23 **	0.22 *	0.47 **	n.s.	1.06 **	0.94 **	0.60 **	0.22 *
P110	41.64 **	0.34 **	0.60 **	n.s.	1.24 **	1.11 **	0.62 **	0.35 **
SI	-1.18 **	0.11 **	0.22 **	0.12 **	0.31 **	0.40 **	0.20 **	0.18 **

Figure 2: FLI1 SNP is associated with the CR. A. Association signals on chromosome 29 with the CR 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. ARS-BFGL-NGS-12309 was the first significantly associated SNP that was detected, whereas FLI1 (GA-266del) was the most significantly associated novel SNP. Blue circles represent the positions of the 35 newly sequenced SNPs. Blue lines represent 6 genes located in the associated region. The triangle diagram represents pairwise linkage disequilibrium (LD) in the associated region, which was visualized using Haploview [17]. Red shades indicate strong LD. B. The allelic substitution effects of FLI1, PKP2, CTTNBP2NL, SETD6, CACNB2, UNC5C, and FAM213A on the deregressed EBV [16] for CR, days open (DO), number of inseminations per lactation (NI), and success after first insemination (SFI), pregnancy within 70 d (P70), 90 d (P90), and 110 d (P110) after delivery or fertility selection index (SI). n. s.: non-significant. * and **: $p < 0.05$ and $p < 0.01$, respectively.

FLI1 (GA-266del) is located in the 5'UTR of *FLI1* and may influence the expression level of this gene. Because *FLI1* is expressed in several bovine tissues, including the uterus (Figure 3A), we used BEnEpC derived from bovine uterine tissue to assess luciferase activity. Reporters carrying the GA allele exhibited higher activity than those carrying the *del* allele

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(Figure 3B). Consistent with the results of the luciferase assay, the level of mRNA generated in the presence of the GA allele was higher than that produced in the presence of the *del* allele according to the allelic mRNA ratio measured in the bovine uteruses (Figure 3C). Consequently, the *FLI1* expression level might affect CR in cattle.

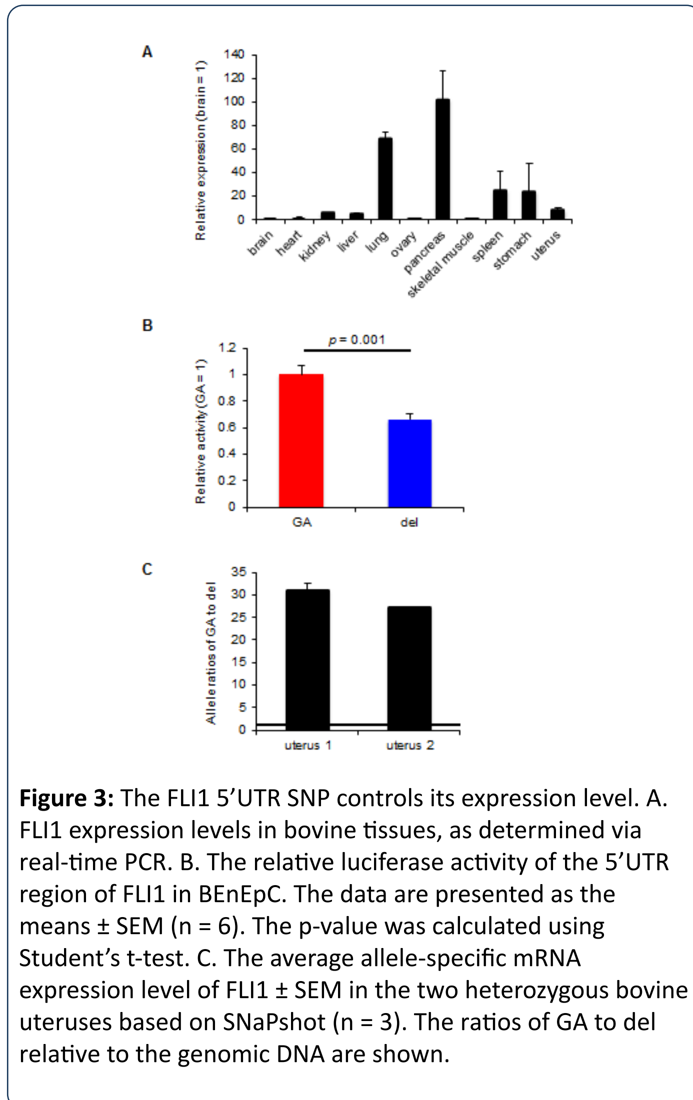
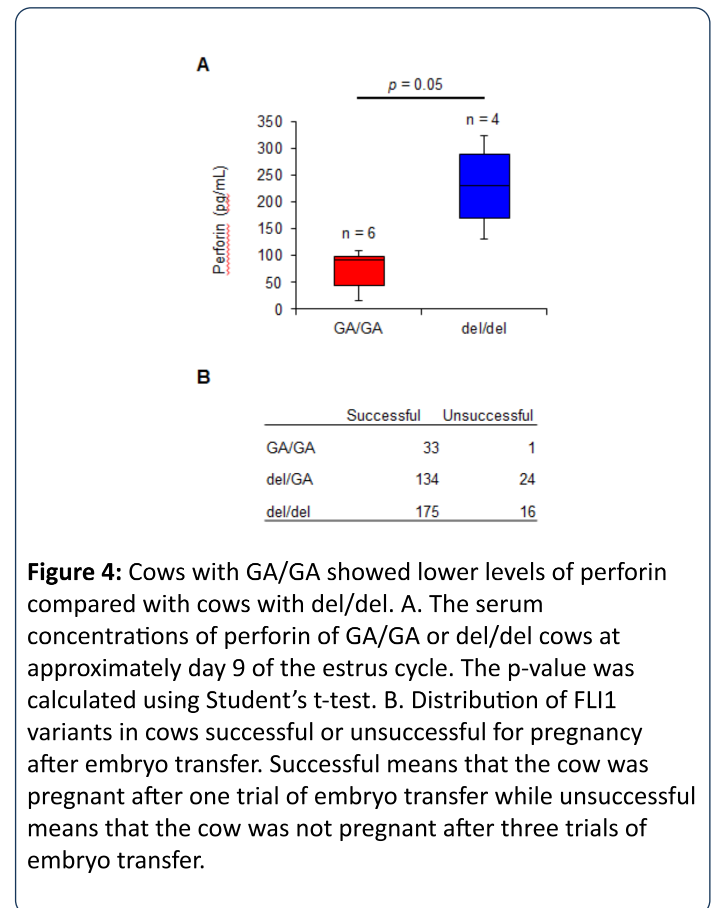


Figure 3: The FLI1 5'UTR SNP controls its expression level. A. FLI1 expression levels in bovine tissues, as determined via real-time PCR. B. The relative luciferase activity of the 5'UTR region of FLI1 in BEnEpC. The data are presented as the means ± SEM (n = 6). The p-value was calculated using Student's t-test. C. The average allele-specific mRNA expression level of FLI1 ± SEM in the two heterozygous bovine uteruses based on SNaPshot (n = 3). The ratios of GA to del relative to the genomic DNA are shown.

The reduced expression of *Fli1* in mice increased numbers of NK cell [2] and NK cells were involved in the acceptance of the fetus to the mother through perforin-dependent pathway [5]. Thus cows with the reduced expression of FLI1 might show high concentration of perforin and low CR. To explore the possibility, we examined the FLI1 genotypes and their perforin concentration in cows. As expected, the serum concentrations of perforin of cows carrying del/del were higher than those of cows carrying GA/GA (Figure 4A). Moreover, we found a relationship between the FLI1 genotype and outcome of embryo transfer (Figure 4B). 33 cows carrying GA/GA were pregnant after one trial of embryo transfer (Successful) while only 1 cow carrying GA/GA was not pregnant after three trials of embryo transfer (Unsuccessful). On the other hand, 16 out of 191 cows carrying del/del were unsuccessful. The chi-square statistic for the distribution of *FLI1* variants gives a p-value of 0.038. Therefore, the reduced expression of *FLI1* increased perforin production, which might decrease CR through inhibiting to accept the embryo to the recipient.



	Successful	Unsuccessful
GA/GA	33	1
del/GA	134	24
del/del	175	16

Figure 4: Cows with GA/GA showed lower levels of perforin compared with cows with del/del. A. The serum concentrations of perforin of GA/GA or del/del cows at approximately day 9 of the estrus cycle. The p-value was calculated using Student's t-test. B. Distribution of FLI1 variants in cows successful or unsuccessful for pregnancy after embryo transfer. Successful means that the cow was pregnant after one trial of embryo transfer while unsuccessful means that the cow was not pregnant after three trials of embryo transfer.

Discussion

The present study is the first to demonstrate that FLI1 modulates CR. Analyzing the whole genome of 2,559 Holstein sires identified a novel mutation associated with the CR. Although we have previously identified several genes associated with the CR in the Japanese Holstein female population [12, 13], *FLI1* is a novel gene associated with CR, which has previously been known as a proto-oncogene and belongs to the ETS gene family of transcription factors [1]. One of the reasons for this GWAS result might be that we scanned the whole genome of sires whose EBVs for traits are more precise than females because of their large number of offspring. A second reason might be that we analyzed all of the 2,559 samples rather than comparing two groups of samples carrying the extremes of CR.

We found that the SNP in the 5'UTR of FLI1 is correlated with CR. The region including the SNP identified is not predicted to be a binding site of transcription factor (TRANSFAC 7.0, http://www.gene-regulation.com/pub/datab ases.html), however, it might affect interaction with an unknown nuclear protein [18]. *cis*-Acting polymorphisms would affect transcription, mRNA processing, mRNA stability, and protein translation [19]. Several GWAS reported that SNPs located in 5'UTR of the genes were associated with a broad range of phenotypes [20–22]. Since the polymorphism in the 5'UTR of FLI1 influences its expression level

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(Figures 3B & C), the associated genetic variant should harbor the functional effect and affect the phenotype.

Several studies implicate that oncogenes play an important role in fertility [23, 24]. One example is pleomorphic adenoma gene 1 (PLAG1) known as an oncogene associated with pleomorphic adenomas of the salivary gland, which belongs to the PLAG family of zinc finger transcription factors [25]. The study of Plag1 knockout mice suggests that PLAG1 deficiency causes growth retardation as well as reduced fertility [26]. GWAS in humans and domestic animals indicated that polymorphisms in the PLAG1 genomic region were associated with body growth and reproductive traits [27, 28]. Possible mechanisms linking PLAG1 to reproductive physiology could be related to growth hormone (GH) and insulin-like growth factor (IGF) 1 or 2 signalling [24]. Interestingly, IGF1 is a target gene of FLI1 [29]. Moreover, administering of bovine somatotropin to dairy cows increased plasma concentrations of GH and IGF1 and enhanced conceptus size and fertility [30]. FLI1 might influence CR through GH and IGF1/2 signalling as well as PLAG1.

We demonstrated here that the polymorphism in *FLI1* affected the serum concentration of perforin (Figure 4A). In cattle, perforin was highly expressed in the peri-implantation endometrium, suggesting that it may play important roles in the establishment and maintenance of gestation during normal pregnancy in ruminants [31]. By producing perforin, uterine NK cells act as a double-edged sword at the maternal-fetal interface to protect the host from the pathogens and along with apoptosis of fetus [6]. In dairy cattle, infection of the mammary gland is associated with a reduction in pregnancy rate and an increase in the number of inseminations required to establish pregnancy [32], suggesting that activation of the immune system by infection inhibits pregnancy. Keeping the appropriate level of perforin would be critical for normal pregnancy in ruminants as well as other mammals.

Several reports inferred the link between immunity and female fertility. The long pentraxin 3 is produced by innate-immunity cells in response to proinflammatory signals and acts as a predecessor of antibodies, but it is also essential in female fertility by acting as a nodal point for the assembly of the cumulus expansion [33]. Peroxisome proliferator-activated receptor gamma regulates immune cell activation and facilitates the release of oocytes each estrous cycle [34]. FLI1 might have dual roles for controlling NK cell population and CR.

In conclusion, the present study investigated the whole genome in 2,559 samples, and a significant association was observed between CR and FLI1 with a polymorphism in 5’UTR. Further functional studies revealed that this SNP was correlated with the expression of FLI1, the concentration of perforin, and the outcome of embryo transfer. These results indicate that FLI1 plays a role in female fertility in cows.

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