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Genetic Polymorphism and mRNA Expression Studies Reveal *IL6R* and *LEPR* Gene Associations with Reproductive Traits in Chinese Holsteins

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Abstract: Genetic selection of milk yield traits alters the energy distribution of high producing cows, resulting in gene-induced negative energy balance, and consequently, poor body condition scores and reduced reproductive performances. Here, we investigated two metabolic-syndrome pathway genes, *IL6R* (Interleukin 6 receptor) and *LEPR* (Leptin receptor), for their polymorphism effects on reproductive performance in dairy cows, by applying polymorphism association analyses in 1588 Chinese Holstein cows (at population level) and gene expression analyses in granulosa cells isolated from eight cows (at cell level). Among the six single nucleotide polymorphisms we examined (two SNPs for *IL6R* and four SNPs for *LEPR*), five were significantly associated with at least one reproductive trait, including female fertility traits covering both the ability to recycle after calving and the ability to conceive and keep pregnancy when inseminated properly, as well as calving traits. Notably, the identified variant SNP g.80143337A/C in *LEPR* is a missense variant. The role of *IL6R* and *LEPR* in cattle reproduction were further confirmed by observed differences in relative gene expression levels amongst granulosa cells with different developmental stages. Collectively, the functional validation of *IL6R* and *LEPR* performed in this study improved our understanding of cattle reproduction while providing important molecular markers for genetic selection of reproductive traits in high-yielding dairy cattle.

Keywords: Holstein cattle; fertility; gene expression; ovarian follicle; haplotype

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1. Introduction

Over the past few decades, the decline of reproductive performance has been widely observed in dairy cattle, partially resulting from unfavorable genetic correlations with intensively selected milk production traits [1,2]. Poor reproductive performance and increasing involuntary culling rate increase the cost of herd management and veterinary care, placing a significant burden on the dairy industry [3]. It has been noticed by breeders globally that genomic selection of female fertility, such as integrating potential causative mutations into prediction models [4], needs to be carefully strategized [5]. One foreseen challenge is that, given the complexity and the highly polygenic characteristic of cattle reproduction, candidate genes identified for reproduction traits might also exert pleiotropic effects for other economically important traits.

Genetic selection of milk yield traits alters the energy distribution of high producing cows, resulting in gene-induced negative energy balance, and consequently, lower body condition scores [6,7]. Coupled with challenging environmental restraints, some of the lactating cows may suffer from reduced reproductive performance [8,9]. We hypothesized that some of the genes highly involved in energy balance procedure might exert shared effects on female reproductive traits as well. Here, we used genes *IL6R* (interleukin 6 receptor, in BTA3) and *LEPR* (Leptin receptor, in BTA3) as examples, both of which are involved in metabolic syndrome pathways [10] and showed evidences of associations with reproductive traits from large scale association studies [11]. The gene *IL6R* encodes a protein containing antibody folding [12], which is a specific binding receptor of Interleukin 6 (IL6). IL6 binds with IL6R to form complexes, and then with trans-membrane protein gp130 (signal transducer of IL6 family cytokines), the formed high-affinity complexes can lead to signal transduction [13]. The gene *LEPR* is the controller of leptin, one type of hormone mainly produced by fat cells, playing an important role in controlling body weight, fat deposition, food intake, immune function, and reproduction. It has been reported that LEPR exists in the ovaries, at which leptin binds to LEPR to modulate steroid production of the ovaries [14].

Previous studies in human have shown that the high expression of IL-6 can change the characteristic of cytokines pattern [15], which can further induce infertility [16,17], habitual abortion [18], and preeclampsia [19] in women. Additionally, multiple polymorphisms in *IL6R* are reported to be associated with either function or pathophysiology of reproduction [20,21]. However, there was a lack of reporting in the literature regarding its role in reproduction in cattle. For *LEPR*, a few studies reported the associations between the polymorphism of *LEPR* and fertility consequences [22,23] and reproductive traits [24]. There is a lack of focused analyses on reproduction traits covering different perspectives of female reproduction. Furthermore, validation in expression profiles remains important for understanding the physiological and molecular process of *LEPR* gene regulating reproduction.

Bovine ovarian follicle, consisting of an oocyte that ovulates, fertilizes, and forms an embryo, is surrounded by granulosa and theca cells, which can produce molecular signals and hormones to cause the oocyte's developmental competence [25]. During follicle growth, GCs replicate, secrete various hormones, and ensure an essential micro-environment for follicle growth [26]. The proliferating and differentiating GCs are critical for normal follicle growth and are involved in oocyte development, ovulation, and the process of luteinization [27,28]. Due to its indispensable roles in reproduction process, ovarian follicles can be more suitable tissues for studying reproductive traits. Gene expressions in GCs may change at different stages of follicular development, which in turn regulate follicular development and, hence, the reproductive outcomes.

Collectively, the objectives of this study were to (1) examine the effects of genes related to metabolic syndrome pathways, including *IL6R* and *LEPR* on dairy cattle reproduction, employing both SNP- and haplotype-based association analyses; (2) investigate their roles in reproduction process by employing expression analyses in GCs. The findings from this study could improve our understanding of genes with pleiotropic effects for complex traits and positively contribute to the genetic improvement of reproduction performances in dairy cattle.

2. Materials and Methods

2.1. Genotype and Haplotype-Based Association Analyses

2.1.1. SNP Identification in 68 Bulls

Premier 5.0 and Oligo 7.0 (Sangon Biotech Co., Ltd., Shanghai, China) were used to design primes of all exons and flanking regions (within 2 kb distance of 5′ and 3′ end) in two candidate genes. In total, 38 pairs of primers (Table S1) were designed, including 14 for *IL6R* and 24 for *LEPR*. These primers were then synthesized at Sangon Biotech (Shanghai, China).

The DNA samples were extracted from frozen semen samples of 68 Holstein bulls (Beijing Dairy Cattle Center, Beijing, China) using a standard phenol–chloroform method, and then, DNA concentrations were diluted to 200 ng/μL with TB buffer. We randomly mixed DNA samples into three pools (one pool contained samples from 28 bulls and two pools contained 20 samples each) and then used pooled samples for the subsequent PCR amplification (ABI3730XL DNA analyzer (Applied Biosystems, Foster, CA, USA)). Sequencing data were aligned to the reference genome (UMD 3.1) using Ensemble (https://asia.ensembl.org/, accessed on 1 March 2017) and examined for potential polymorphisms using Chromas (http://technelysium.com.au/wp/chromas/, accessed on 1 March 2017) and DNAman (https://www.lynnon.com/dnaman.html/, accessed on 1 March 2017). In total, 35 variants were detected for gene *IL6R* and *LEPR*.

2.1.2. Genotyping and Phenotyping of 1588 Cows

A total of 1588 Holstein cows from eight dairy farms (Beijing Sunlon Livestock Development Co., Ltd., Beijing, China) were genotyped, of which complete pedigree and reproductive information have been recorded. For each cow, DNA of whole blood samples were extracted using TIANamp Blood DNA Kit (TIANgen, Beijing, China). To shortlist variants included for KASP (Kompetitive allele-specific PCR) genotyping [29], a pilot analysis was performed in 46 (out of 1588) randomly selected samples. Only one variant was kept if the distance between two variants was less than 200 bp. Ultimately, six variants (Table 1) remained and used for genotyping in all 1588 cows with the KASP method [29]. A chi-square test was used to determine whether allelic frequencies of any variant deviated from the Hardy-Weinberg equilibrium. For candidate gene *IL6R* and *LEPR*, haplotype blocks were constructed based on LD structures of identified SNPs by using the Haploview4.0 software [30,31], and the default parameters of Haploview4.0 software are used in haplotype block construction.

Table 1. The detail information of six SNPs within the gene *IL6R* and *LEPR* in 1588 Holstein cows.

Gene	SNP	Genotype	Number of Individuals	Genotypic Frequency	Allele	Allelic Frequency	H-W-E Test, χ^2 Value (df = 1)
IL6R	g.16210680C/A	G:G	155	0.125	G	0.373	0.034
		T:G	614	0.496	T	0.627	
		T:T	469	0.379			
IL6R	g.16177843C/T	A:A	455	0.325	A	0.570	0.945
		A:G	684	0.489	G	0.430	
		G:G	259	0.185			
LEPR	g.80143337A/C	G:G	439	0.299	G	0.550	0.581
		G:T	736	0.502	T	0.450	
		T:T	291	0.198			
LEPR	g.80101398A/-	-:-	125	0.085	-	0.297	0.600
		A:-	621	0.423	A	0.703	
		A:A	722	0.492			
LEPR	g.80071722T/G	A:A	53	0.037	A	0.177	0.145
		C:A	403	0.280	C	0.823	
		C:C	985	0.684			
LEPR	g.80101081A/T	A:A	721	0.494	A	0.706	0.387
		A:T	619	0.424	T	0.294	
		T:T	119	0.082			

In this study, we detected the associations of six variants with nine reproductive traits covering the broad mechanisms of cattle reproduction [32,33], including age at the first service (AFS), age at the first calving (AFC), the interval from calving to the first insemination (ICF), the interval from first to last insemination in heifers (IFL_H) and cows (IFL_C), stillbirth in heifers (SB_H) and cows (SB_C), and calving ease score in heifers (CE_H) and cows (CE_C). Estimated breeding values (EBVs) for above nine reproduction

traits were estimated using single-trait animal models [34] in routine genetic evaluation (Independent Innovation League of Dairy Breeding, Beijing, China), which were used as the response variable of association analyses. The effects in evaluation models for various reproductive traits included fixed effects (age at first insemination, herd-year of calving or herd-year of first insemination within parity, year-month of calving or year-month of first insemination within parity, AI technician, sexed semen, and parity) and random effects (additive genetic effect, permanent environmental effect, and random residual effect). Furthermore, a full pedigree dataset was provided by the Dairy Association of China (Beijing, China), and each animal was traced back as many generations as possible. In total, the pedigree included 109,676 females and 3609 males, with birth years from 1930 to 2020. Furthermore, the sub-pedigree linked to the genotyped population (1558 cows) included 7757 females and 1478 males. Descriptive statistics of EBVs for each reproductive trait were presented in Table S2.

2.1.3. Association Analysis

The SNP- and haplotype block-based association analyses for each reproductive trait were performed by employing the GLM procedure of SAS 9.2 (www.sas.com, accessed on 1 March 2017). False positives resulting from multiple testing in association analyses was controlled using Bonferroni *t*-test. The model used for association analyses of each reproductive trait is as follows:

$$Y_{ij} = \mu + G_i + e_{ij}$$

where Y_{ij} was an individual's EBV; μ was the overall mean; G_i was the fixed effect corresponding to the genotype or haplotype combination; and e_{ij} was the random residual effect. The proportion of the phenotypic variance (σ_P^2) explained by each identified SNP was calculated as (σ_g^2)/(σ_P^2), where (σ_g^2) was the variance explained by SNP, and it was calculated as the following formula: $\sigma_g^2 = 2pq[a + d(q - p)]^2$ [35]; and (σ_P^2) was the phenotypic variance (Table S3).

2.2. Gene Expression Assay of IL6R and LEPR

2.2.1. Ovary Collection, Follicle Selection, and Granulosa Cell Isolation

Bovine ovaries of eight dairy cows were collected at an abattoir and put into thermally insulated bottles (28–30 °C) containing sterile physiological saline (with 100 U/mL Penicillin and 0.1 mg/mL Streptomycin) immediately. Within the time of two hours, ovaries were transported to laboratory. After washing three times with warm (37.5 °C) 0.9% NaCl solution and rinsing in 70% warm ethanol for 30 s, ovaries were washed with Dulbecco's phosphate-buffered saline (DPBS) three times. Healthy ovarian follicles with amber-colored fluid were kept for the further operation. According to their diameters (d), ovarian follicles were classified into four developmental stages, including stage 1 (d $\leq$ 3 mm), 2 (3 mm < d $\leq$ 7 mm), 3 (7 mm < d $\leq$ 10 mm), and 4 (d > 10 mm). The follicular fluid contained both cumulus–oocyte complexes (COCs) and GCs. A filter (diameter = 70 μm) was used to filter out COCs and then was used to centrifuge the filtrates (contained GCs) at 1500× *g* for 5 min. The supernatant of the follicular fluid was discarded by aspiration. The GC cells were washed three times in phosphate-buffered saline (pH 7.4) and placed in DMEM/F-12 (Gibco, Life Technologies Inc., Grand Island, NY, USA), which contained 1% penicillin-streptomycin and 10% fetal bovine serum (FBS, Gibco, Life Technologies Inc., Grand Island, NY, USA).

2.2.2. Quantitative Reverse Transcription PCR (RT-qPCR)

The RT-qPCR was conducted to detect the relative expression level of *IL6R* and *LEPR* in GCs collected from ovarian follicles at different developmental stages. Total RNA was extracted from three biological replicates of GCs. Reverse transcription was carried out through first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA) with oligo (dT) 18 primers according to the manufacturer protocols. The gene-specific primers

spanning the exons were designed using Primer 3 (version 4.0, https://bioinfo.ut.ee/primer3-0.4.0/, accessed on 1 March 2017) and Primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 1 March 2017) (Table S4).

The RT-qPCR was carried out through iTaq™ Universal SYBR® Green Super-mix (Bio-Rad Laboratories GmbH, Munich, Germany) in Applied Biosystem® Step-OnePlus™ (Ap-plied Biosystems, CA, USA). A reaction volume of 20 µL was used, including 7.4 µL of double-distilled H_2O, 0.3 µL of forwarding primer, 0.3 µL of reverse primer, 10 µL of 1× SYBR Green master mix (Bio-Rad Laboratories GmbH, Munich, Germany), and 2 µL of cDNA template. Light Cycler 480 instrument (Roche, Mannheim, Germany) was used to perform RT-qPCR. The second derivative maximum method was employed for data acquiring. In this study, the gene *GAPDH* was used as the reference gene, and the $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels of gene [36].

3. Results

3.1. Screening of Polymorphisms in Association Popualtion

In this study, a total of 35 variants (Table S5) were identified from polymorphisms screening using mixed pooled DNA of 68 bulls, including eight in *IL6R* and 27 in *LEPR*. The detailed information of the 35 variants are presented in Table S5. By the pilot analysis using 46 randomly selected samples, a total of six SNPs were confirmed using initial Kompetitive allele-specific PCR (KASP) genotyping. As shown in Figure 1, the clustering distributions of three genotypes for each SNP were obviously separated, suggesting that the reliability of KASP genotyping was guaranteed.

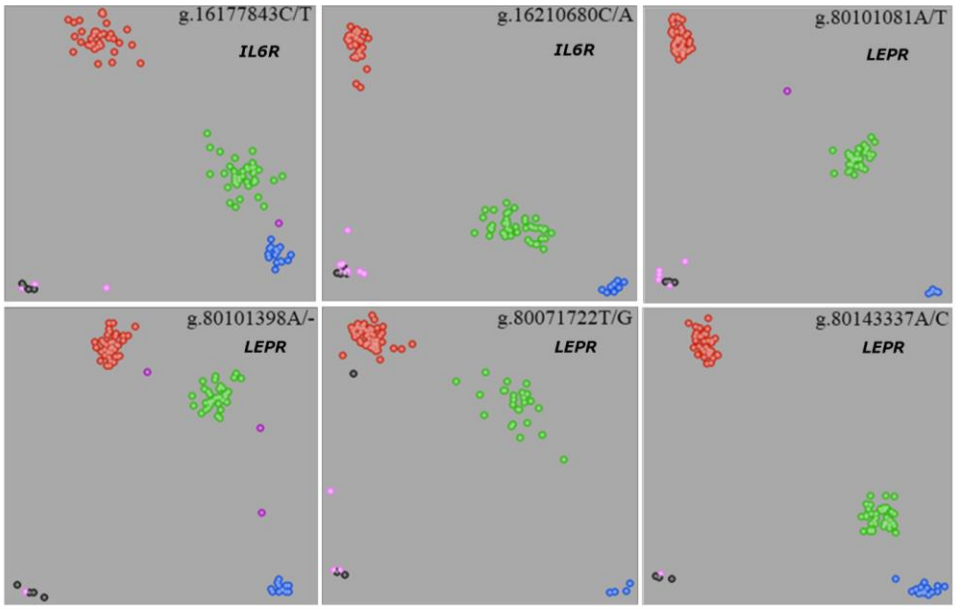

Figure 1. Distributions of SNP clustering for the identified six SNPs *IL6R* and *LEPR* using KASP genotyping method. The red and blue represents two homozygous genotypes; the green represents heterozygous genotype; the pink represents no or weak signal; the purple represents signal for no genotyping; and the black represents blank control.

Subsequently, these six SNPs in gene *IL6R* (two) and *LEPR* (four) were genotyped by KASP genotyping in 1588 cows, and the allele frequencies and the corresponding *p*-value derived from Hardy-Weinberg equilibrium test for six SNPs are presented in Table 1. We detected three SNPs (g.16210680C/A, gene *IL6R*; g.80071722T/G and g.80071722T/G, gene *LEPR*) in the exon region, one in the downstream regulatory region (g.16177843C/T, gene *IL6R*), and two in the intronic region (g.80101398A/- and g.80101081A/T, gene *LEPR*).

3.2. Association Analyses of SNPs with Reproductive Traits

In total, nine significant associations ($p < 0.05$ in F-test) for gene *IL6R* and *LEPR* were observed with AFC, CE_C, CE_H and ICF. For gene *LEPR*, all SNPs had the significant association with ICF. Furthermore, the SNP g.80143337A/C had the significant association with AFC, CE_H and CE_C. For gene *IL6R*, only one significant association was found between SNP g.16177843C/T and CE_H. The least square means of various genotypes for each SNP are presented in Table 2.

The proportion of phenotypic variance explained by each SNP for every reproductive trait are presented in Table S6. Among all associations between six SNPs and nine reproductive traits, the SNP g.80143337A/C in gene *LERP* explained the largest phenotypic variance for ICF (0.009%). For gene *IL6R*, the SNP g.16177843C/T and g.16210680C/A explained a large phenotypic variance for AFC (0.003%) and SB_C (0.003%), respectively.

3.3. Association Analyses of Haplotype Blocks with Reproductive Traits

In this study, the LD between every two SNPs were estimated for gene *IL6R* and *LEPR*. Only one haplotype block was constructed for *LEPR* consisting all screened SNPs, which included 10 haplotypes (Figure 2). As a low LD between SNP g.16210680C/A and g.16177843C/T, two SNPs in *IL6R* were not considered as haplotype blocks.

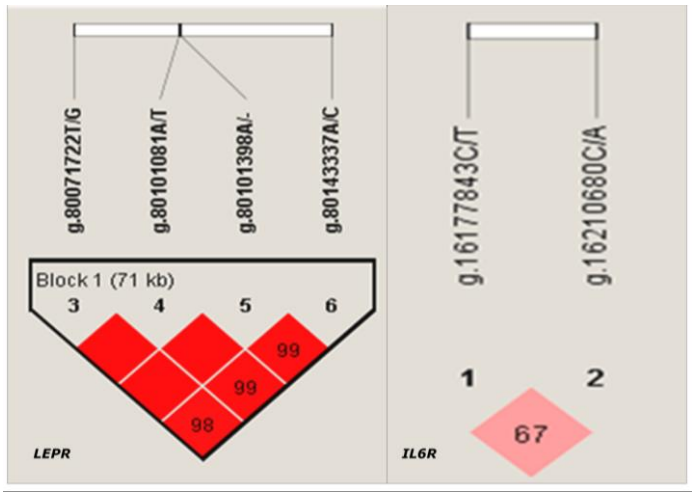

Figure 2. Haplotype blocks constructed based on linkage disequilibrium (LD) for gene *IL6R* and *LEPR* in Chinese Holstein cows.

The significant associations detected between haplotype block and nine reproductive traits are presented in Table 3. Consistent with the results from SNP-based analyses, haplotype block in *LEPR* had significant associations with AFC, ICF, and CE_C. Furthermore, the significant associations were also found with AFS and IFL_C. For traits ICF and CE_C, H1H1 was the most beneficial haplotype.

Table 2. Association of SNPs within gene *IL6R* and *LEPR* with reproductive traits in Holstein cows (least square mean ± standard error).

SNP	Genotype	AFS	AFC	IFL_H	IFL_C	ICF	SB_H	SB_C	CE_H	CE_C
IL6R	GG (155)	−16.718 ± 0.631	−10.085 ± 0.506	5.983 ± 0.499	2.365 ± 0.410	0.544 ± 0.090	0.025 ± 0.003	0.016 ± 0.001	0.009 ± 0.001	0.001 ± 0.000
g.16210680C/A	TG (614)	−17.431 ± 0.317	−9.317 ± 0.254	6.609 ± 0.251	2.585 ± 0.206	0.425 ± 0.045	0.029 ± 0.001	0.015 ± 0.001	0.009 ± 0.001	0.001 ± 0.000
	TT (469)	−17.359 ± 0.363	−9.020 ± 0.291	6.673 ± 0.287	2.475 ± 0.236	0.356 ± 0.052	0.028 ± 0.001	0.014 ± 0.001	0.008 ± 0.001	0.001 ± 0.000
	p value	0.593	0.189	0.466	0.870	0.182	0.450	0.089	0.331	0.162
IL6R	AA (456)	−17.750 ± 0.354	−9.726 ± 0.299	6.464 ± 0.295	2.505 ± 0.237	0.403 ± 0.052	0.027 ± 0.001	0.014 ± 0.001	0.007 ± 0.001 ^{b}	0.001 ± 0.000
g.16177843C/T	AG (684)	−17.392 ± 0.289	−9.129 ± 0.244	6.921 ± 0.241	2.465 ± 0.193	0.355 ± 0.043	0.029 ± 0.001	0.015 ± 0.000	0.009 ± 0.000 ^{a}	0.001 ± 0.000
	GG (260)	−17.199 ± 0.469	−8.731 ± 0.397	6.739 ± 0.391	2.344 ± 0.314	0.469 ± 0.070	0.027 ± 0.002	0.014 ± 0.001	0.009 ± 0.001 ^{ab}	0.000 ± 0.000
	p value	0.598	0.107	0.486	0.917	0.367	0.495	0.690	0.018	0.310
LEPR	GG (440)	−16.878 ± 0.370	−8.753 ± 0.306^{a}	7.114 ± 0.299	2.666 ± 0.243	0.571 ± 0.053^{a}	0.028 ± 0.001	0.015 ± 0.001	0.007 ± 0.001 ^{b}	0.001 ± 0.000 ^{a}
g.80143337A/C	GT (737)	−17.978 ± 0.285	−9.694 ± 0.236^{b}	6.414 ± 0.231	2.605 ± 0.188	0.394 ± 0.041^{b}	0.029 ± 0.001	0.015 ± 0.000	0.009 ± 0.000 ^{a}	0.001 ± 0.000 ^{a}
	TT (291)	−17.232 ± 0.454	−8.942 ± 0.375^{ab}	6.609 ± 0.367	2.260 ± 0.299	0.076 ± 0.065^{c}	0.027 ± 0.002	0.015 ± 0.001	0.010 ± 0.001 ^{a}	0.000 ± 0.000 ^{b}
	p value	0.050	0.033	0.179	0.534	<0.0001	0.794	0.897	0.002	<0.0001
LEPR	119	−16.770 ± 0.707	−8.300 ± 0.590	7.485 ± 0.575	2.950 ± 0.469	0.641 ± 0.103^{a}	0.030 ± 0.003	0.014 ± 0.001	0.007 ± 0.001	0.001 ± 0.001
g.80101398A/-	A- (621)	−17.610 ± 0.308	−9.344 ± 0.257	6.690 ± 0.250	2.701 ± 0.204	0.437 ± 0.045 ^{ab}	0.028 ± 0.001	0.014 ± 0.001	0.008 ± 0.001	0.001 ± 0.000
	AA (723)	−17.570 ± 0.286	−9.305 ± 0.239	6.547 ± 0.232	2.281 ± 0.189	0.294 ± 0.041 ^{b}	0.028 ± 0.001	0.015 ± 0.000	0.009 ± 0.000	0.001 ± 0.000
	p value	0.538	0.250	0.318	0.201	0.002	0.850	0.354	0.388	0.510
LEPR	AA (722)	−17.649 ± 0.286	−9.332 ± 0.239	6.522 ± 0.233	2.277 ± 0.190	0.290 ± 0.042 ^{b}	0.028 ± 0.001	0.015 ± 0.000	0.009 ± 0.000	0.001 ± 0.000
g.80101081A/T	AT (619)	−17.598 ± 0.308	−9.328 ± 0.258	6.723 ± 0.251	2.739 ± 0.205	0.434 ± 0.045 ^{ab}	0.028 ± 0.001	0.014 ± 0.001	0.008 ± 0.001	0.001 ± 0.000
	TT (120)	−16.613 ± 0.703	−8.270 ± 0.588	7.291 ± 0.574	2.854 ± 0.467	0.633 ± 0.103 ^{a}	0.030 ± 0.003	0.015 ± 0.001	0.007 ± 0.001	0.001 ± 0.001
	p value	0.383	0.225	0.445	0.192	0.002	0.741	0.334	0.366	0.470
LEPR	AA (53)	−18.391 ± 1.060	−9.591 ± 0.872	5.843 ± 0.852	2.571 ± 0.702	0.520 ± 0.154 ^{ab}	0.029 ± 0.004	0.017 ± 0.002	0.006 ± 0.002	0.002 ± 0.001
g.80071722T/G	CA (404)	−16.865 ± 0.385	−8.618 ± 0.316	6.833 ± 0.309	2.911 ± 0.254	0.517 ± 0.056 ^{a}	0.028 ± 0.002	0.015 ± 0.001	0.008 ± 0.001	0.001 ± 0.000
	CC (986)	−17.799 ± 0.246	−9.515 ± 0.202	6.641 ± 0.198	2.375 ± 0.163	0.335 ± 0.036 ^{b}	0.028 ± 0.001	0.015 ± 0.000	0.009 ± 0.000	0.001 ± 0.000
	p value	0.090	0.054	0.537	0.207	0.017	0.969	0.448	0.168	0.336

AFS, age at the first calving; AFC, age at the first service; IFL_H, the interval from the first to last insemination in heifers; IFL_C, the interval from the first to last insemination in cows; ICF, the interval from calving to the first insemination; SB_H, stillbirth in heifers; SB_C, stillbirth in cows; CE_H, calving ease in heifers; CE_C, calving ease in cows. In multiple comparisons by employing Bonferroni *t*-test, different genotypes containing same letter superscripts presents no significant difference ($p < 0.05$), and whereas no same letter superscripts mean significant difference ($p > 0.05$).

Table 3. Association of haplotype block within gene *LEPR* with reproductive traits in Holstein cows (least square mean ± standard error).

Haplotype	AFS	AFC	IFL_H	IFL_C	ICF	SB_H	SB_C	CE_H	CE_C
H1H1 (274)	−17.304 ± 0.462	−9.194 ± 0.382	6.493 ± 0.376	2.178 ± 0.308	0.091 ± 0.067 ^{b}	0.027 ± 0.002	0.015 ± 0.001	0.010 ± 0.001	0.000 ± 0.000 ^{b}
H1H2 (379)	−18.234 ± 0.393	−9.720 ± 0.325	6.522 ± 0.320	2.886 ± 0.262	0.396 ± 0.057 ^{a}	0.028 ± 0.002	0.014 ± 0.001	0.009 ± 0.001	0.000 ± 0.000 ^{ab}
H1H3 (215)	−17.282 ± 0.522	−8.786 ± 0.431	6.588 ± 0.425	2.773 ± 0.348	0.449 ± 0.076 ^{a}	0.029 ± 0.002	0.015 ± 0.001	0.008 ± 0.001	0.000 ± 0.000 ^{ab}
H1H4 (108)	−18.748 ± 0.736	−10.791 ± 0.609	6.223 ± 0.600	1.189 ± 0.491	0.270 ± 0.107 ^{ab}	0.029 ± 0.003	0.016 ± 0.001	0.010 ± 0.001	0.002 ± 0.001 ^{a}
H2H2 (115)	−16.655 ± 0.713	−8.191 ± 0.590	7.465 ± 0.581	2.923 ± 0.475	0.652 ± 0.103 ^{a}	0.030 ± 0.003	0.015 ± 0.001	0.007 ± 0.001	0.001 ± 0.001 ^{ab}
H2H3 (144)	−16.422 ± 0.637	−8.239 ± 0.527	7.306 ± 0.519	3.264 ± 0.425	0.578 ± 0.092 ^{a}	0.028 ± 0.003	0.014 ± 0.001	0.007 ± 0.001	0.001 ± 0.000 ^{ab}
H2H4 (62)	−16.877 ± 0.971	−9.634 ± 0.803	7.058 ± 0.791	1.188 ± 0.648	0.474 ± 0.141 ^{ab}	0.027 ± 0.004	0.014 ± 0.002	0.006 ± 0.002	0.002 ± 0.001 ^{ab}
H3H3 (52)	−18.382 ± 1.061	−9.591 ± 0.877	5.872 ± 0.864	2.644 ± 0.707	0.526 ± 0.154 ^{ab}	0.029 ± 0.004	0.017 ± 0.002	0.006 ± 0.002	0.002 ± 0.001 ^{ab}
H3H4 (29)	−17.430 ± 1.420	−9.808 ± 1.175	6.159 ± 1.157	3.277 ± 0.947	0.457 ± 0.206 ^{ab}	0.028 ± 0.006	0.015 ± 0.002	0.008 ± 0.002	0.002 ± 0.001 ^{ab}
H4H4 (9)	−23.454 ± 2.550	−12.237 ± 2.108	9.755 ± 2.077	0.389 ± 1.700	0.568 ± 0.370 ^{ab}	0.013 ± 0.010	0.010 ± 0.004	0.001 ± 0.004	0.001 ± 0.002 ^{ab}
p value	0.044	0.025	0.545	0.012	<0.001	0.942	0.850	0.087	0.001

AFS, age at the first calving; AFC, age at the first service; IFL_H, the interval from the first to last insemination in heifers; IFL_C, the interval from the first to last insemination in cows; ICF, the interval from calving to the first insemination; SB_H, stillbirth in heifers; SB_C, stillbirth in cows; CE_H, calving ease in heifers; CE_C, calving ease in cows. In multiple comparisons by employing Bonferroni *t*-test, different genotypes containing same letter superscripts presents no significant difference ($p < 0.05$), whereas no same letter superscripts mean significant differences ($p > 0.05$).

3.4. Gene Expression Analysis in GCs Isolated from Follicle of Different Developmental Stages

By using RT-qPCR, the relative expression level of gene *IL6R* and *LEPR* was measured in GCs, which were isolated from bovine ovarian follicular presenting different development stages. The relative mRNA expression of *IL6R* showed an increase trend along the development of follicles and reached the highest level in stage 3 (Figure 3A). The significant differences of the relative mRNA expression were observed between stages 1, 2, and 3. In contrast, its relative mRNA expression significantly decreased along the development of follicles for gene *LEPR* (Figure 3B). The significant differences of the relative mRNA expression for *LEPR* were found across different development stages, except for stages 3 and 4.

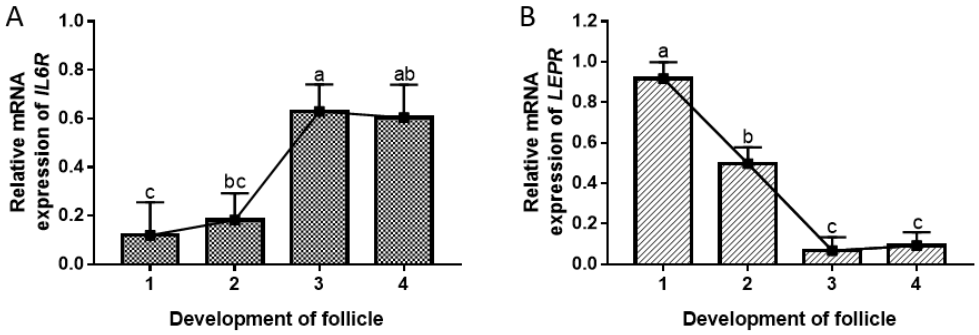

Figure 3. The relative mRNA expression level of the gene *IL6R* (**A**) and *LEPR* (**B**) in isolated granulosa. Based on the diameter (d), follicles were divided into four different development stages, including stage 1 ($d \leq 3$ mm), stage 2 (3 mm $< d \leq 7$ mm), stage 3 (7 mm $< d \leq 10$ mm), and stage 4 ($d > 10$ mm). Different letters are used when the expression levels are significantly different between two stages ($p < 0.05$).

4. Discussion

In this study, we examined two metabolic syndrome pathway genes, *IL6R* and *LEPR*, for their effects on cattle female reproduction by both association analyses at population level and the mRNA expression analyses at the cell level. Among six SNPs of gene *IL6R* and *LEPR* genotyped in resource population, five SNPs were significantly associated with five reproduction traits covering both the ability to recycle after calving (ICF) and the ability to conceive and keep pregnancy when inseminated properly (AFS and AFC), and calving ease (CE_H and CE_C), with the greatest number of significant SNPs in gene *LEPR* for ICF.

In our previous GWAS in Chinese and Nordic Holsteins, the gene *IL6R* was identified as the candidate gene of cattle reproduction [11]. In this study, the SNP g.16177843C/T located in the downstream region of *IL6R* was significantly associated with trait CE_H, which further confirmed our previous findings. In transition dairy cows, gene *IL6R* was differently expressed in subcutaneous adipose tissue at the close-up dry period [37]. Considering the relationship between body condition score (BCS) and incidence of calving ease [38], the *IL6R* may play roles in reproductive traits by regulating the body condition of both cows and calf at calving.

The effects of gene *LEPR* on economically important traits of cattle have been widely investigated, including reproduction [39–44], milk production [39,41–44], BCS [45], somatic cell score [40,46], and energy output and energy storage traits [47] in Holstein, Slovak Spotted, and Pinzgau cows, as well as growth [48] and fat deposition in Chinese indigenous cattle [49]. In this study, we firstly confirmed its genetic associations with reproductive traits in a large population from Chinese Holsteins. Amongst the four SNPs, the SNP g.80143337A/C located in exon 2 of *LEPR* had significant associations with five reproductive traits, including AFS, AFC, ICF, CE_H, and CE_C. The SNP g.80143337A/C is a missense mutation, which can cause changes in protein structure, thereby possibly altering protein function. Previous studies investigated the genetic effect of polymorphisms of *LEPR* gene on AFS in Polish Holstein [46] and AFC in Slovak Spotted and Pinzgau cows [39] and

obtained similar results as our study. In Holstein primiparous cows, *LEPR* was identified to be significantly associated with days open (sum of ICF and IFL), but not for the services per conception [43]. Furthermore, there exists a weak association between the *LEPR* polymorphism and ICF in US Holstein cows ($p = 0.079$) [24]. In the present study, all those four SNPs were significantly associated with ICF, which is similar to the findings of above studies. Although the association between *LEPR* polymorphism and reproductive traits of cattle has been widely reported in various population, less has been conducted in terms of mRNA expression analyses, especially in GC tissue with different development stages.

We observed that the relative expression of *IL6R* in GCs increased with increasing follicle size, which was consistent with the observations from the previous study [50]. Baskind et al. [51] also reported that the expression level of *IL6* gene was high before ovulation. IL6 can inhibit the secretion of progesterone-induced by LH [50] and further inhibits the secretion of estrogen in GCs [52], highlighting the role of *IL6R* in the follicular development process. This trend characteristic of *IL6R* may also be related to follicular atresia at later stages of development [53]. With the increase in follicular size, we observed that the relative expression of *LEPR* in GCs decreased, which is similar to the finding reported by Sarkar et al. (2010) [54]. Additionally, several studies have shown that leptin binds to LEPR to modulate steroid production in GCs in the ovary [14] and improve the developmental competence of oocytes at later stages [55], in which the mechanism of LEPR promoting steroid production remains less studied. Differential expression in varying follicular developmental stages indicates its potential role in follicular development.

5. Conclusions

In this study, we investigated two metabolic-syndrome pathway genes, *IL6R* and *LEPR*, for their effects on reproductive performance in dairy cows. Among the six SNPs we examined, five were significantly associated with at least one reproductive trait, including age at the first service, age at the first calving, the interval from calving to first insemination, and calving ease in heifers and cows. The roles of *IL6R* and *LEPR* in cattle reproduction were further confirmed by observed differences in relative gene expression levels amongst granulosa cells with different developmental stages. Collectively, the functional validation of *IL6R* and *LEPR* performed in this study provided important molecular markers for genetic selection of reproductive traits in high-yielding dairy cattle.

Supplementary Materials: The following supporting information can be downloaded at the figshare repository, accession number https://doi.org/10.6084/m9.figshare.21671198 (accessed on 12 December 2022), Table S1: Primers (38) of the gene *IL6R* and *LEPR* for pooled DNA sequencing; Table S2: Descriptive statistics of EBV for reproductive traits in Chinese Holstein cattle; Table S3: Genetic parameters and variance components for reproductive traits in Chinese Holstein cattle; Table S4: List of primers pairs of employed for RT-qPCR; Table S5: Polymorphic loci information of candidate gene *IL6R* and *LEPR* in Chinese Holstein cattle; Table S6: The percentage of phenotypic variance explained by six SNPs in the Holstein population.

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Institutional Review Board Statement: Ethical review and approval was not required for the animal study because ethical review and approval were waived for this study. Healthy ovaries were collected from the slaughterhouse. The blood samples and frozen semen were collected along with the regular

quarantine inspection of the farms and breeding stations, so no ethical approval was required for this study. Written informed consent was obtained from the owners for the participation of their animals in this study.

Data Availability Statement: The data presented in the study are deposited in the figshare repository, accession number https://doi.org/10.6084/m9.figshare.21971585 (accessed on 28 January2022).

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Conflicts of Interest: Author G.G. is employed by Beijing Sunlon Livestock Development Co., Ltd. and author L.L. is employed by the Beijing Dairy Cattle Center. Both authors are involved in custody and analysis of the data of dairy herd improvement program. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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