

Article

DDIT3 Governs Milk Production Traits by Targeting IL-6 to Induce Apoptosis in Dairy Cattle

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Abstract: The mechanisms of modulating milk production traits remain largely unknown. Based on our previous RNA-seq, *DDIT3* was presumed as a novel, promising candidate gene for regulating milk protein and fat traits in dairy cattle. To further detect the genetic effect of *DDIT3* and its potential molecular mechanisms in regulating milk production traits in dairy cattle, here, we performed a genotype-phenotype association study. Two SNPs, g.-1194 C>T and g.-128 C>T, were significantly associated with MY ($p = 0.0063$), FY ($p = 0.0001$) and PY ($p = 0.0216$), respectively. A luciferase assay demonstrated that the allele T of g.-128 C>T increased the promoter activity by binding the HSF2, while allele C did not. To further reveal the molecular regulatory mechanisms, the *DDIT3*-knockdown MAC-T cells were established. It was observed that *DDIT3* silencing could induce apoptosis and increase the number of PI-positive cells. Meanwhile, *DDIT3* silencing led to increased expression of inflammatory markers, such as *IL-6*, *IL6R*, *IL1B*, *IL7R*, *IL1RL2*, *IL1A*, *STAT1-5*, *MYC*, *IGFBP4*, and *IGFBP5*, and especially for *IL-6* ($\log_2FC = 4.22$; $p = 3.49 \times 10^{-112}$). Additionally, compared with the control group, increased lipid accumulation was found in the *DDIT3*-knockdown MAC-T cells. Thus, our results proved that lower expression of *DDIT3* could result in increased lipid accumulation and apoptosis via up-regulating the expression of *IL-6*. These findings provided clues about the regulatory mechanisms of milk production traits in dairy cattle.

Keywords: *DDIT3*; genetic effect; milk production traits; apoptosis; dairy cattle



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1. Introduction

The milk production traits of dairy cattle, including milk yield (MY), fat yield (FY), protein yield (PY), fat percentage (FP), and protein percentage (PP), are the most important economical traits, which are influenced by many genes [1]. Therefore, the most profitable breeding goal for milk-producing traits remains improvement [2]. As is known, RNA sequencing (RNA-seq) has enabled gene discovery and expedited the genetic improvement of dairy cattle [3,4]. Our previous RNA-seq study identified 31 differentially expressed genes associated with milk production traits in the mammary glands of lactating Holstein cows [5]. Of these, DNA damage-inducible transcript 3 (*DDIT3*) was significantly down-regulated ($p = 4.01 \times 10^{-5}$) in the Holstein cows [5]. Moreover, it was found that *DDIT3* located only 0.46 Mb, from the SNP (ARS-BFGL-NGS-14781), which was significantly associated with fat percentage [6]. In addition, *DDIT3* was also adjacent to a previously reported QTL for PP and FP [7]. These findings indicated that the *DDIT3* gene may be a promising key gene for milk production traits in dairy cattle.

DDIT3, known as the CAAT/enhancer binding protein homologous transcription factor (*CHOP*) or growth retardation and DNA damage-inducible gene 153 (*GADD153*),

is involved in various biological processes, including differentiation, proliferation, expression, and energy metabolism [8]. As a transcription factor, *DDIT3* has specifically been shown to regulate DNA damage and ER stress-induced apoptosis by regulating the transcription of antiapoptotic and proapoptotic genes [9,10]. Recent publications have indicated that an overexpression of *DDIT3* promotes apoptosis in several cell lines [11–13]. Overexpression of *DDIT3* in human cells led to cell death and growth arrest at the G1/S phase in murine 3T3 cells [14]. *DDIT3*^{-/-} mouse experiments revealed that *DDIT3*-mediated apoptosis contributes to the pathogenesis of ER stress-related diseases in murine 3T3 cells [10]. Current evidence suggests that *DDIT3* is an important mediator of cellular functions. However, the effects of *DDIT3* on cellular functions have not been tested and clarified in dairy cattle.

To explore the potential causal genetic variants associated with milk production traits, we detected the genetic effects with an independent dairy cattle population. We also generated *DDIT3* knock-out bovine MAC-T cells using RNA interference (RNAi) and applied RNA sequencing technology to investigate the biological function of *DDIT3*. Subsequently, the differentiation model was established to understand the molecular mechanisms of *DDIT3*. Our results may lead to a better understanding of the biochemical mechanisms by which *DDIT3* regulates milk production ability and can be used via marker-assisted breeding.

2. Materials and Methods

2.1. Ethics Statement

The experimental programs and protocols were approved by the Institutional Animal Care and Use Committee of Hefei University of Technology (approval number HFUT20200518005).

2.2. Animals and Phenotypic Data

A daughter design was used in this study. A total of 717 Chinese Holstein cows from 12 corresponding sires were collected to construct the study population. The dairy cattle were from 16 dairy farms in Sanyuanlvhe Dairy Farming Center (Beijing, China). The estimated breeding values (EBVs) for milk yield (MY), protein yield (PY), fat yield (FY), protein percentage (PP), and fat percentage (FP) were predicted by the Dairy Data Center of the Dairy Association of China.

2.3. DNA Extraction and SNP Identification

Blood samples were collected from the dairy cows and stored at -20°C . Genomic DNA was extracted using the Tiangen DP (318) Blood DNA Kit (Beijing, China). A DNA pool was constructed with equal DNA concentration for each. The results of the isolated DNA were detected by a NanoDrop™ ND-2000c Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Based on the genomic sequence of the bovine *DDIT3* gene (ENSBTAT0000044712.2), 8 pairs of primers were designed by Primer Premier 5.0 to amplify all four exons and 1500 bp of 5' flanking sequences (Table S1).

2.4. Genotyping and Haplotype Analysis

The SNPs of all individuals were genotyped using the SEQUENOM MassArray MALDI-TOF mass spectrometer (Sequenom, San Diego, CA, USA). To further explore the LD extent, haplotypes of the *DDIT3* gene were inferred using the software BEAGLE 3.2 program, and, where necessary, sporadic missing genotype data were also imputed. The measure of pairwise LD for all three SNPs was performed using the software Haploview 4.2 (Broad Institute of MIT and Harvard, Cambridge, MA, USA). Accordingly, the LD block was generated using the linkage disequilibrium coefficient D' . Haplotypes within these blocks were applied to test their associations with the milk production traits.

2.5. Association Analyses

The pedigrees of the dairy cows detected in the present study were traced back for three generations to create a genetic matrix. Based on the estimated matrices, associations

between genotypes and/or haplotype combinations and the five milk production traits were evaluated by the mixed model in SAS 9.1.2 (University of Notre Dame, Notre Dame, IN, USA). A linear mixed regression model was shown as follows:

$$y = 1\mu + bx + Za + e$$

where y is the EBVs value of the five milk production traits, and μ is the overall mean for the values. B is the regression coefficient, x is a design vector of the SNP genotype or haplotype combination, a is the vector of residual polygenetic effects, Z is a diagonal incidence (0 or 1) matrix that links each animal additive genetic value to its “phenotype”, and e is the vector of residual errors.

The additive (a), dominance (d), and allele substitution (α) effects were further evaluated as follows: $a = (AA - BB)/2$, $d = AB - (AA + BB)/2$, and $\alpha = a + d(q - p)$, where AB represents heterozygous genotype, AA and BB indicate the two homozygous genotypes, and p and q are the A and B frequencies, respectively (Falconer and Mackay, 1996). For both analyses above, the Bonferroni method was used according to the number of SNP or haplotype blocks tested. A p value $< 0.05/N$ was considered significant; here, N is the SNP loci numbers or haplotype blocks tested in the analyses.

2.6. Construction of Recombinant Plasmid and Luciferase Assay

To directly detect the allele-specific effects of the SNP g -128 C>T on the promoter activity, three luciferase fragments, which correspond to the *DDIT3* promoter (−281 to +52, +1 represents transcription start site) were synthesized. Two restriction enzyme recognition sites, KpnI and BglIII, were used at the 5′ and 3′ termini, respectively. The fragments were then cloned into the Luciferase Assay Vector pGL4.14 (Promega, Madison, WI, USA).

Then, these recombinant plasmids were co-transfected to HEK-293 cells. Transfection reactions were performed using 490 ng of plasmid DNAs and 10 ng of pRL-TK as an internal control, containing Lipofectamine 2000 (Invitrogen, CA, USA), with pGL4.14 vector as a negative control. After 48 h transfection, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega).

2.7. siRNA Design, Synthesis, and Assessment

Three siRNAs′ sequences targeting *DDIT3* mRNA and a non-specific siRNA were designed and synthesized by GenePharma (GenePharma, Suzhou, China) (Table 1). MAC-T bovine mammary epithelial cells were cultured in DMEM/F12 basal medium (Gibco Life Technologies, Grand Island, NY, USA) supplied with 10% FBS (Gibco) and maintained with 5% CO₂ at 37 °C. When the cells were 60–80% confluent, 100 pmol of siRNA was transfected into cells in one well using 5 μL of Lipofectamine™ 2000 (Invitrogen), and the cells were then incubated (37 °C, 5% CO₂) for 48 h. Next, the total RNA of the MAC-T cells was extracted with a TRIzol reagent (Invitrogen), and a qRT-PCR analysis was explored to detect the *DDIT3* expression in the cells transfected by siRNAs. Each of the siRNAs was detected in triplicate.

Table 1. Associations of the two SNPs in the *DDIT3* gene with the five traits (LSM ± SE).

SNPs	Genotype (No. Individuals)	MY	FY	FP	PY	PP
g-1194 C>T	CC(313)	426.07 ± 63.03 ^a	9.29 ± 2.81 ^A	−0.04 ± 0.029	11.34 ± 1.97 ^a	−0.014 ± 0.009
	TC(321)	302.21 ± 62.33 ^b	2.90 ± 2.78 ^B	−0.06 ± 0.029	9.16 ± 1.95 ^{ab}	0.001 ± 0.009
	TT(83)	232.53 ± 82.51 ^b	−0.96 ± 3.05 ^B	−0.07 ± 0.036	6.20 ± 2.45 ^b	−0.004 ± 0.012
	<i>p</i> value	0.0063 ^{**}	0.0001 ^{**}	0.5409	0.0216 [*]	0.1536
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	<i>p</i> value	0.0063 ^{**}	0.0001 ^{**}	0.5409	0.0216 [*]	0.1536

^{A, B} means $p < 0.01$; ^{a, b} means $p < 0.05$. * p indicates the significant association after Bonferroni correction for multiple testing at the significance level of 0.05. ** p indicates the extremely significant association after Bonferroni correction for multiple testing at the significance level of 0.01.

2.8. Construction of shRNA Vectors and Transfection in MAC-T cells

Based on the qRT-PCR results, the siRNA *DDIT3-287* was found to reduce the *DDIT3* expression by at least 60%. Hence, the DNA sense and antisense sequences for this shRNA were synthesized, and restriction sites were used for connecting synthetic DNA fragments into the LV3 shRNA lentiviral expression vectors (GenePharma)—namely LV3-287. With the appropriate lentiviral expression vectors, the HEK-293T cells (GenePharma) were transiently transfected, then following packaging vectors pGag/Pol, pRev, and pVSV-G to obtain lentiviral particles.

The MAC-T cells were propagated and incubated in DMEM/F12, with 10% FBS (Gibco), and 1×Penicillin-*Streptomycin* (Gibco) at 37 °C and 5% CO₂. One day before infection, the cells were seeded in T25 culture flasks and infected when reaching approximately 80% confluence. The 500 µL mixed solution was as follows: 50 µL of lentiviral particles (1 × 10⁹ TU/ml) carrying shRNA for *DDIT3-287*, 25 µg of Polybrene (Sigma), and 450 µL of Opti-MEM I Reduced-Serum Medium (Gibco). After one day, the cells were cultured in fresh growth media without lentiviral particles for another two days.

2.9. RNA Sequencing

Three parallel samples (cells from 3 culture flasks) of the experimental group (*DDIT3-287* shRNA) and control group (NC shRNA) were prepared, respectively. The integrity of the obtained RNA was confirmed using Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). A total of 5 µg of RNA with RIN value ≥ 7.0 was used to standardize between the RNA samples. Using the Kit NEBNext[®] Ultra™ RNA Library Prep Kit (NEB), a sequencing library was prepared. Then, clustering of the index-coded samples was carried out by the TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, these paired-end reads were generated, and RNA-seq libraries were sequenced using an Illumina HiSeq 2000 platform. Meanwhile, FASTQ sequence files were generated by CASAVA ver.1.8.2 (Illumina), accompanied by the removal of failed reads.

2.10. Differential Expression Analysis and Pathway Enrichment Analysis

The mapping of reads to the reference genome (*Bos Taurus* ARS-UCD1.2) was performed, and a differential expression analysis of the data was then performed using the package Bowtie v0.12.8 and paired-end and TopHat v2.0.0. To make estimates and determine differentially expressed genes, DESeq2 relies on the negative binomial distribution by using a generalized linear regression model. The Log₂ fold-change (L2FC) values, *p*-values, and *q*-values (*p*-adjusted) of the differentially expressed genes were obtained in the output data. A *q*-value of 0.05 was considered significant.

Gene Ontology (GO) enrichment was analyzed by the Goseq R package. The enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were evaluated by KOBAS software. The GO term and KEGG pathway analysis results with corrected *p* values < 0.05 were considered to be significantly enriched.

2.11. Validation of RNA-Seq Results by qPCR

Eight DEGs were randomly selected to validate the RNA sequencing data (Table S1 shows the primers that were used in sequencing). A qRT-PCR was carried out using a SYBR Green I Master Kit using a LightCycler 96 real-time PCR system (Roche Applied Science, Penzberg, Germany), and the program was 95 °C for 5 min, 40 cycles of 95 °C for 12 s, 58 °C for 12 s, 72 °C for 12 s, and 72 °C for 6 min. The relative expression level was normalized to the *GAPDH* and *ACTB* by the 2^{-ΔΔCt} method.

2.12. Oil Red O Staining and TG Content Measurement

After washing with PBS, the cells in the chamber slides were then fixed with 4% paraformaldehyde for 30 min, incubated for 5 min with Oil Red O at room temperature, and then visualized using ECLIPSE Ti-S light microscopy (Nikon Corporation, Tokyo,

Japan). Additionally, the TG content was determined by a triglyceride detection kit (Sangon Biotech Co., Ltd., Shanghai, China).

2.13. Calcein-AM/Propidium Iodide (PI) Staining

After different stimulation, the MAC-T cells were then collected by trypsinization, centrifuged at $1500\times g$ for 2 min at room temperature to collect the cell pellet, and washed once with $1\times$ Assay Buffer. Then, the cells were mixed with $1\times$ assay buffer and stained with the mixture by $2\ \mu\text{M}$ of calcein-AM and $4.5\ \mu\text{M}$ of PI per well for 15 min at room temperature. The images of the cells were collected immediately and analyzed using a Nikon eclipse Ti-S fluorescence microscope.

3. Results

3.1. SNP Identification and Its Associations with the Five Milk Traits

Through resequencing of the whole coding and 5' regulatory regions, two SNPs were discovered. These two variants, g.-1194 C>T and g.-128 C>T, were in the 5' regulatory regions (Figure 1a,b). Chi-squared testing showed that the SNPs of the DDIT3 gene were in Hardy–Weinberg equilibrium ($p > 0.05$). The allele frequencies and locations of the SNPs are shown in Table S2.

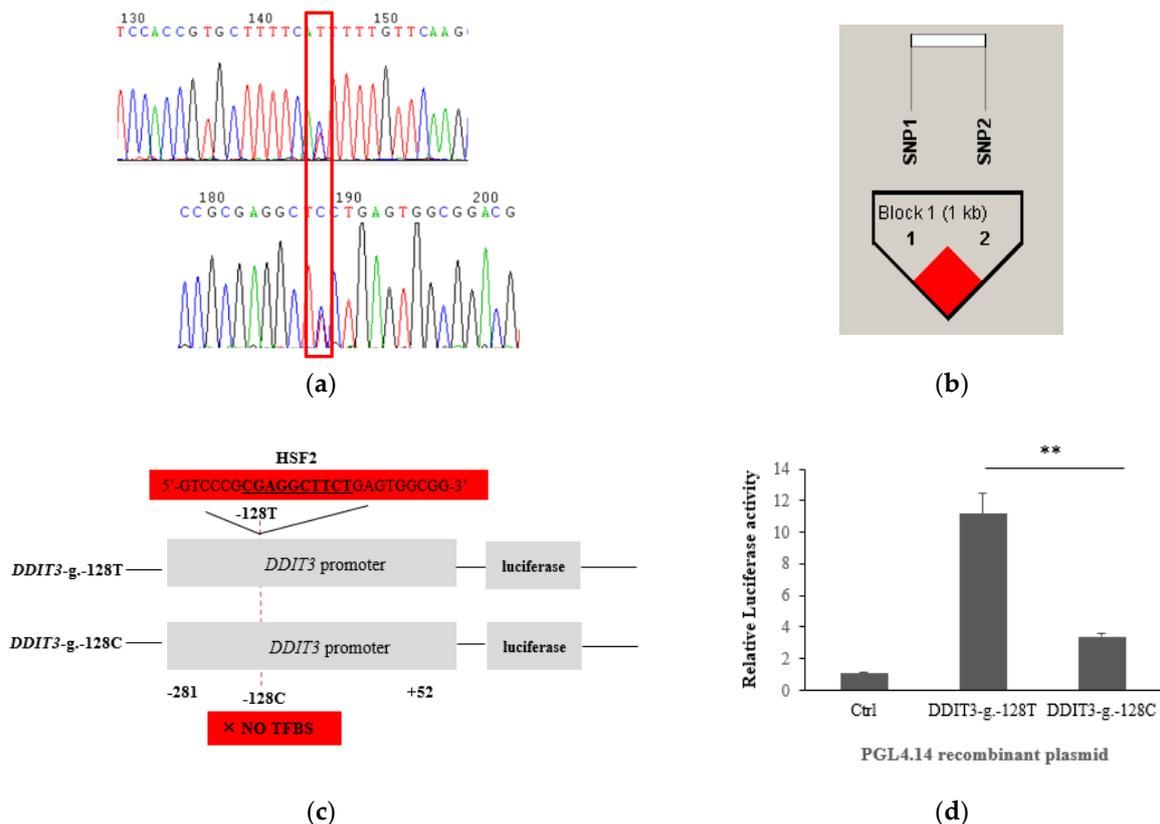


Figure 1. The variants of DDIT3 and luciferase activity analysis in HEK293 cells. Note: (a) The variants g.-1194 C>T and g.-128 C>T in the 5' regulatory regions. (b) The haplotype block and LD pattern formed by g.-1194 C>T and g.-128 C>T in the DDIT3; the darker shading indicates higher LD. The length of the block is provided in kilobases (kb), and pairwise linkage disequilibrium (D') is given for each SNP combination. (c) TFBS modified from TFSEARCH software output; underlined nucleotides denote the TFBS sequences. (d) Luciferase activity analysis; the value was shown as the mean \pm SEM. p values are from a t -test (two-tailed). ** $p < 0.01$.

The results of association between the EBVs of the five milk production traits and the two identified SNPs are shown in Table 1. After applying the Bonferroni correction for multiple t-testing, both the SNPs, g.-1194 C>T and g.-128 C>T, were significantly associated

with MY ($p = 0.0063$), FY ($p = 0.0001$) and PY ($p = 0.0216$), and these two SNPs showed the same results. Correspondingly, significant dominant effects, additive effects, and allele substitution effects were observed (Table S3). On the other hand, one haplotype block from the HAPLOVIEW program, composed of g.-1194 C>T and g.-128 C>T, was observed in Figure 1a.

3.2. Prediction of TFBSs and Its Effect on Transcriptional Activity

The online software TFSEARCH was conducted to predict the putative differential TFBSs of the two regulatory SNPs, g.-1194 C>T and g.-128 C>T. It was shown that the allele of T in g.-128 C>T generated a putative TFBS for the transcription factors HSF2 (Heat shock factor protein 2), while that was abolished upon its substitution by the C allele (Figure 1c). However, there were no significant differences between the allele of T or C for the SNP g.-1194 C>T. Meanwhile, as shown in Figure 1d, the allele of T showed 68% higher luciferase activity compared with the allele of C ($p < 0.01$). Therefore, g.-128 C>T mutation can be considered as a functional SNP for gene expression via the alteration of transcription factor binding sites.

3.3. Interference Efficiency of Designed siRNAs in Bovine MAC-T Cells

A total of three siRNAs were designed against *DDIT3* mRNA (Table 2; Figure S1). With the qRT-PCR assay, only siRNA D287 was found to decrease the mRNA expression of the *DDIT3* gene in the MAC-T cells compared with the controls ($p < 0.05$), while D82 and D394 siRNAs did not have an obvious effect ($p > 0.05$) (Figure 2A). Of them, D287 was chosen for further RNAi experiments, which reduced the *DDIT3* expression by about 50%. The shRNA lentiviral vector for D287, LV3-287, was constructed and transfected into the MAC-T cells. Green fluorescence signals of GFP were seen after 72 h of culturing by transient transfection of shRNA virus particles. As shown in Figure 2B, the surviving cells simultaneously expressed GFP and shRNA. Correspondingly, the mRNA levels of the *DDIT3* gene in the knockdown lines were found to decrease by about 50% compared with the NC group using the qRT-PCR ($p < 0.01$).

Table 2. Sequences and positions of 3 siRNAs against *DDIT3* gene.

siRNA Name	Sense (5'-3')	Target Sites in <i>DDIT3</i>	Target Sites in <i>DDIT3</i> Gene
D394	GCTGGCTGAAGAGAATGAACG	394–415 bp	Exon 4
D287	GACCAAGGAAGAACCAGAAAA	287–308 bp	Exon 4
D80	GTGCTGTCCTCAGATGAAAAT	80–101 bp	Exon 3
NC	GATCCGTTCTCCGAACGTGTCACGT		

A

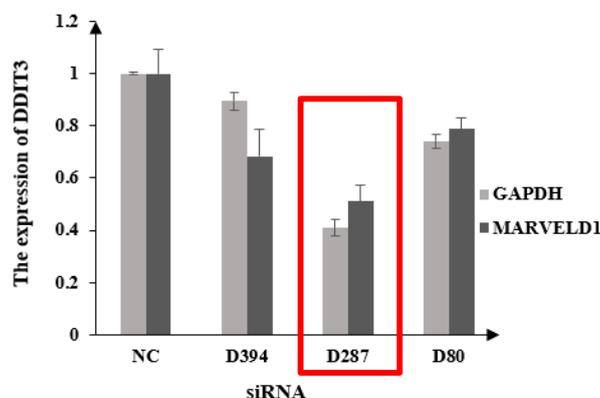
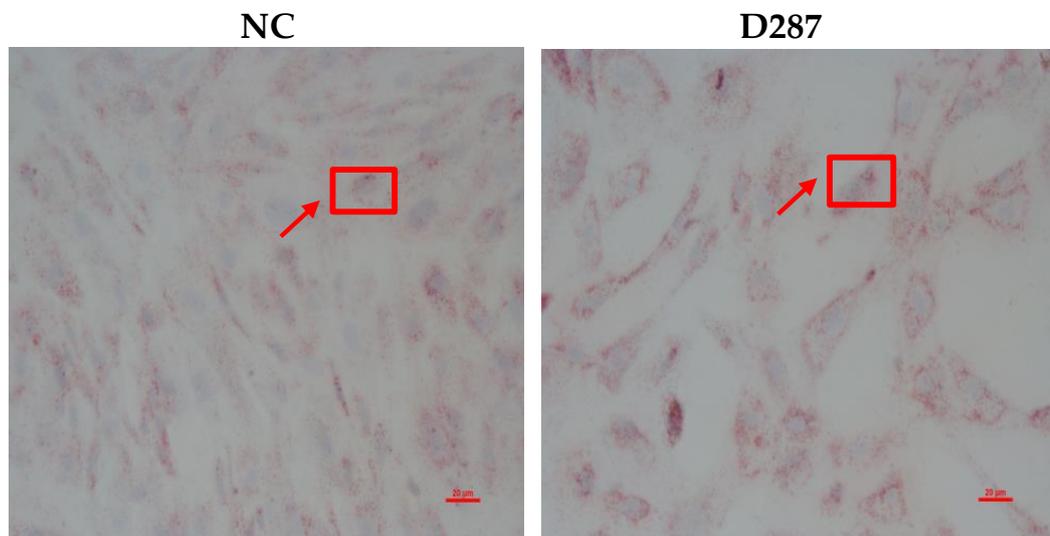


Figure 2. Cont.

B



C

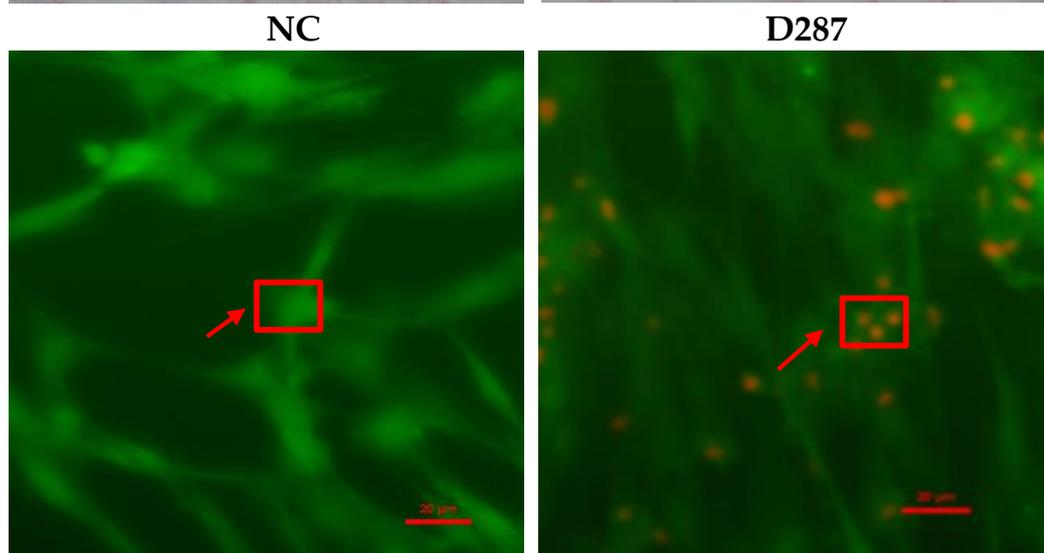


Figure 2. shRNA interference of *DDIT3* and its effect on MAC-T cells. **Note:** (A) The shRNA interference effects of LV3-DDIT3-D287 in MAC-T cells. (B) Lipid accumulation of NC and LV3-DDIT3-D287 groups in MAC-T cells by Oil Red O staining; NC-versus Ctrl group and D287-LV3-DDIT3-D287 group. (C) Calcein-AM/PI staining assays.

3.4. DEGs and Functional Annotation

In total, 3838 differentially expressed genes were identified (Table S4). Of these, 1940 genes were down-regulated and 1898 were genes up-regulated. The volcano plot of the DEGs is displayed in Figure S2. Moreover, the top 10 genes associated with the regulation of fat metabolism are shown in Table 3. Of them, *IL6* was the most significantly up-regulated gene (Log_2 fold change = 4.22). The 3838 common DEGs were then processed by GO and KEGG enrichment analysis. We found that the expression pattern significantly changed the genes involved in cellular senescence, cell cycle and death, DNA replication and lysosome, etc. (Table S5).

Table 3. Top 10 differentially expressed genes between knockdown and control groups.

Symbol	Gene Name	Regulation	Log ₂ Fold Change	padj	Gene Function
<i>IL6</i>	interleukin 6	up	4.22	3.49×10^{-112}	plays important roles in fatty acid biosynthesis
<i>CXCL8</i>	C-X-C motif chemokine ligand 8	up	3.01	2.04×10^{-90}	catalyzes the first step in the hydrolysis of triglycerides
<i>GRO1</i>	chemokine (C-X-C motif) ligand 1	up	2.68	1.21×10^{-87}	involved in the biosynthesis of unsaturated fatty acids
<i>TNFAIP3</i>	TNF alpha-induced protein 3	up	2.08	6.41×10^{-80}	linoleic acid metabolism, n-3, and n-6 fatty acid metabolism
<i>EGR1</i>	early growth response 1	up	3.03	7.80×10^{-78}	involved in sterol biosynthesis
<i>PHLDA1</i>	pleckstrin homology-like domain family A1	up	1.72	7.19×10^{-53}	involved in adipocyte differentiation and glucose homeostasis
<i>SERPINE1</i>	serpin family E member 1	up	1.48	7.74×10^{-49}	plays a key role in lipid biosynthesis and fatty acid degradation
<i>CYR61</i>	cysteine rich angiogenic inducer 61	up	1.66	8.90×10^{-49}	catalyzes the carboxylation of acetyl-CoA to malonyl-CoA
<i>CCL20</i>	C-C motif chemokine ligand 20	up	4.27	2.00×10^{-46}	involved in lipid synthesis and energy generation
<i>IFI27</i>	putative ISG12(a) protein	down	-3.01	1.06×10^{-46}	plays critical roles in lipid metabolism

Meanwhile, these results indicated that not only the regulation of autophagy, cellular senescence, and cell cycle, but also those involved in lipid metabolic signaling pathways could be regulated by *DDIT3*, for their role in cell growth, proliferation, and differentiation.

3.5. *DDIT3* Knocking down Caused Lipid Accumulation and Apoptosis in Bovine MAC-T Cells

The differentiation of MAC-T cells was detected using Oil Red O staining and triglycerides content assay. It showed that the number and size of the lipid droplets in the *DDIT3* knock-down group were significantly increased compared with the NC group (Figure 2B). Furthermore, the concentration of TG was significantly lower in the *DDIT3* knock-down group than that of the control group. We then determined the role of *DDIT3* in cell viability and apoptosis. Calcein-AM/propidium iodide (PI) staining showed that the transfection of *DDIT3* siRNA could induce apoptosis and increase the number of PI-positive cells (Figure 2C). *DDIT3* silencing by D287 led to increased expression of inflammatory markers.

3.6. The Expression Alteration of the Key Genes

Here, we first detected the expression of these genes (*IL-6*, *IL6R*, *IL1B*, *IL7R*, *IL1RL2*, *IL1A*, *STAT1-5*, *MYC*, *IGFBP4*, and *IGFBP5*), which were acquired by our RNA-seq and pathway analysis. Compared with the control group, the mRNA expression levels of these genes in the *DDIT3* knock-down group altered significantly. As shown in Figure 3A,C,D, *DDIT3* knocking down can markedly increase the mRNA expressions of *IL-6*, *IL6R*, *IL1B*, *IL7R*, *MYC*, and *IGFBP4* ($p < 0.01$), especially for *IL-6*, which was known as pro-inflammatory cytokine. Moreover, the expression levels of *STAT1-5*, *IL1RL2*, *IL1A*, and *IGFBP5* mRNA were lower in the *DDIT3* knock-down group than those in the control group ($p < 0.01$) (Figure 3A,B,E). All the expressions of these detected genes were consistent with our RNA-seq data. Altogether, these results indicated that these key genes may play a critical role in regulating milk composition traits, which were induced by knocking down *DDIT3*. Then, based on the mRNA expression, we proposed that *DDIT3* might modulate the lipid metabolism and apoptosis in dairy cattle through regulating the expression of *IL6*. Thus, these results demonstrated that *DDIT3* knocking down caused lipid accumulation and apoptosis in bovine MAC-T cells.

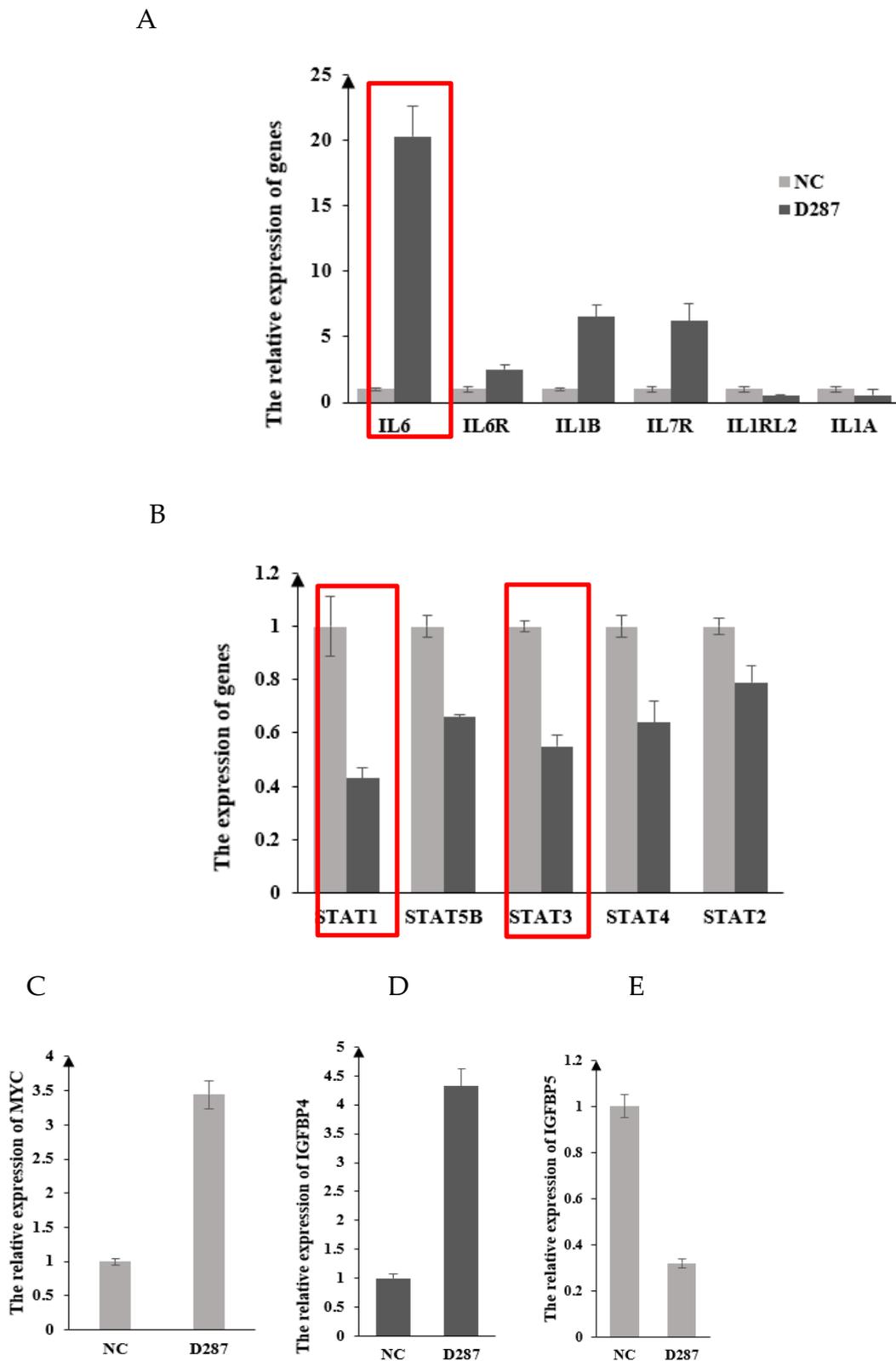


Figure 3. Effects of *DDIT3* knocking down on the mRNA expression of genes regulating apoptosis in MAC-T cells. **Note:** (A). The mRNA expression of genes regulating inflammatory response. (B). The expression of STAT1-5. (C–E). The mRNA expression of MYC, IGFBP4, and IGFBP5, respectively.

4. Discussion

In the present study, we confirmed that the *DDIT3* gene was significantly associated with five milk production traits. *DDIT3* was initially identified in our previous RNA-seq, where it was found to be significantly differentially expressed in the high and low PP and FP of lactating Holstein cows [5]. Genetic variations in key promoter regions may cause significant potential phenotype diversity [15]. In the present study, we found that the SNPs g.-1194 C>T and g.-128 C>T, which are located in the promoter region, were significantly associated with MY, FY, and PY. Cows with the TT genotype had lower MY, FY, and PY than those with the TT genotype. In the association analyses, the EBVs of daughters were used as phenotypic observations. By using phenotypic values for association analyses, Li et al. (2019) found that, in the first lactation, the SNP g.56284880C>T (which was g.-128 C>T in our study) was significantly associated with milk MY, FY, and PY; likewise, in the second lactation, it was significantly associated with milk MY and PY. These results were consistent with our current study. Hence, the findings for the association analyses based on the two different variables (phenotypic values and EBVs) basically overlapped. Thus, *DDIT3* was considered to be a major gene influencing milk production traits.

Meanwhile, the promoter activity analysis clearly demonstrated that the allele T of g.-128 C>T of the *DDIT3* gene was influenced by the increased promoter activity by binding the HSF2 factor, while allele C did not, indicating that the HSF2 (heat shock factor) transcription factor may up-regulate the expression of *DDIT3*. HSF2, as a transcription factor, participated in the expression of heat shock genes by interacting directly with HSF1 or HSF4 [16–18]. Additionally, HSF2 plays important roles in regulating the HSF1-mediated stress response [19]. Numerous studies have shown that the SNPs in the promoter region are associated with the changes of gene expression by altering putative transcription factor-binding sites [20]. Considering the significant association effects of g.-128 C>T on the milk fat and protein production traits, it is possible that this SNP regulates the *DDIT3* expression by changing the binding status of the transcription factor, HSF2, to affect the formation of milk and protein traits. Additionally, there was no TFBS for the loci g.-1194 C>T; the significant association of g.-1194 C>T with MY, FY, or PY may be due to the very strong LD between g.-1194 C>T and g.-128 C>T ($D' = 1.00$). Therefore, the locus g.-128 C>T is directly responsible for the *DDIT3* expression. Based on these findings, HSF2 can be used for marker-assisted selection to modulate the expression of the *DDIT3* gene in dairy cattle to improve milk production.

It has been widely reported that *DDIT3* affects ER stress and induces cell apoptosis or autophagy [21–23], but its relationship with milk production traits remains unclear in dairy cattle. In the present study, for the loci g.-128 C>T, cows with the TT genotype had lower MY, FY, and PY than those with the TT genotype. Therefore, *DDIT3* negatively regulated milk production traits, which were all consistent with our previous studies [5,24]. Furthermore, the concentration of TG in the *DDIT3* knock-down group showed significantly higher content than that of the NC group. In addition, *DDIT3*-knockdown induced an increased number of PI-positive cells and could induce apoptosis by calcein-AM/ PI staining in the MAC-T cell line. This is the first report on *DDIT3* inhibiting lipid accumulation and inducing apoptosis in the bovine MAC-T cell line.

To further exploit the effects of *DDIT3* on the apoptosis of the bovine MAC-T cell line, we knocked down *DDIT3* in bovine MAC-T cells and found that interleukin-6 (IL6) was regulated by *DDIT3*. It has been reported that IL6 regulates inflammatory responses, can be produced for a short period of time after the body is stimulated by inflammation and independently of stimulation by tumor necrosis factor-alpha ($\text{TNF}\alpha$) and accompanied by the increase in other inflammatory substances [25,26]. In addition, IL6 mediates cellular hypertrophy through the activation of the JAK/STAT pathway in different types of cells [27,28]. In our present study, *DDIT3* knockdown promoted the expression of IL6, and induced apoptosis of the bovine MAC-T cell line, indicating that the regulatory effects of *DDIT3* on the vitality of MAC-T cells is mediated by IL6. As is known, the JAK/STAT pathway is stimulated in the early stage of diabetic nephropathy [26,29,30]. Our present

results only showed that IL6 could further stimulate STAT3-related pathways, resulting in an aggravating effect on inflammatory responses, whereas JAK2 was not induced significantly. The different results probably stemmed from the differences between the in vivo and in vitro studies, as well as the heterogeneity of the cells used in the study. Collectively, these findings and our present results suggest that the *DDIT3*/IL6/STAT3 signaling pathway may be involved in affecting milk production traits. Thus, further studies of animal models with IL6/STAT3 double deficiency will be required to provide additional evidence for this possibility.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agriculture13010117/s1>, Table S1. Primers used for pooled DNA sequencing for the *DDIT3* gene; Table S2. Genotypic and allelic frequencies of the two SNPs in the *DDIT3* gene; Table S3. Genetic effects of g.-1194 C>T and g.-128 C>T on milk production traits in Chinese Holsteins; Table S4. DEGs within two different comparison groups; Table S5. Significantly enriched KEGG terms of DEGs; Figure S1. Sequencing of recombinant plasmid; Figure S2. DEGs among the *DDIT3*-KDvsNC groups. Note: FC presented in this figure is fold change.

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