miR-2478 inhibits TGFβ1 expression by targeting the transcriptional activation region downstream of the TGFβ1 promoter in dairy goats

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In a previous study, miR-2478 was demonstrated to be up-regulated in dairy goat mammary glands during peak lactation compared with the dry period. However, the detailed mechanisms by which miR-2478 regulates physiological lactation and mammary gland development in dairy goats remain unclear. In this study, we used bioinformatics analysis and homologous cloning to predict the target genes of miR-2478 and selected INSR, FBXO11, TGFβ1 and ING4 as candidate target genes of miR-2478. Subsequently, by targeting the 5′UTR of the TGFβ1 gene, we verified that miR-2478 significantly inhibited TGFβ1 transcription and the Pearson’s correlation coefficient between miR-2478 expression and TGFβ1 expression was −0.98. Furthermore, we identified the potential promoter and transcription factor binding regions of TGFβ1 and analyzed the potential mechanisms of interaction between miR-2478 and TGFβ1. Dual-luciferase reporter assays revealed that two regions, spanning from −904 to −690 bp and from −79 to +197 bp, were transcription factor binding regions of TGFβ1. Interestingly, the miR-2478 binding sequence was determined to span from +123 to +142 bp in the TGFβ1 gene promoter. Thus, our results have demonstrated that miR-2478 binds to the core region of the TGFβ1 promoter and that it affects goat mammary gland development by inhibiting TGFβ1 transcription.

microRNAs (miRNAs) are 18–25-nucleotide-long, endogenous, single-stranded, non-coding RNA molecules that base-pair with target mRNAs in order to post-transcriptionally modulate gene expression through actions such as, translational repression or mRNA destabilization1,2. miRNAs are important mediators of numerous biological processes, including cell proliferation and apoptosis3, hormone secretion4, and tumor formation5. A number of studies investigating miRNAs in farm animals have shown that several miRNAs play important roles in muscle development6, fat deposition7, oocyte maturation8 and early embryonic development9. However, functional studies of miRNAs in mammary tissues have only recently emerged. Indeed, in 2004, Liu et al.10 used microarrays to identify 23 novel, specific miRNAs in human mammary tissues, highlighting the need for studies of the mechanisms by which miRNAs regulate mammary gland development and lactation. Subsequently, numerous studies on the regulation of mammary gland development by miRNAs emerged, particularly studies focusing on the use of miRNAs as molecular markers of breast carcinogenesis. However, few studies have examined the normal regulatory functions of miRNAs in mammary glands, particularly in those of farm animals. In particular, human miR-125b has been reported to be expressed at high levels during all stages of mammary gland development but to be expressed at a low level in breast carcinoma tissue, suggesting that suppression of this miRNA negatively affects the differentiation of mammary gland epithelial cells11. Moreover, mouse miR-126–3p inhibits the proliferation of mammary gland epithelial cells and the expression of casein by binding to a target site in the 3′ untranslated region (UTR) of progesterone receptor (PGR)12. Further, in one study of ruminants, 59 miRNAs were isolated from bovine adipose and mammary gland tissues, among which miR-21, miR-23a, miR-24 and miR-143 were highly expressed in bovine mammary gland tissues7, and suppression of endogenous

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miR-24 expression reduced the rate of high-temperature-induced apoptosis of bovine mammary gland epithelial cell. In addition, some researchers have explored the molecular mechanisms of miRNAs in the regulation of lactation physiology and mammary gland development in the dairy goat. In a previous study, we conducted comparative transcriptome profiling of miRNAs in dairy goat mammary gland tissues during both the dry period and peak lactation and identified 8 miRNAs, including miR-2478 (fold-change >1 and P-value < 0.01), that were up-regulated in the mammary gland during peak lactation compared with the dry period. However, the roles and detailed mechanisms of miR-2478 in regulating physiological lactation and mammary gland development in dairy goats remain unclear.

In the present study, we predicted and screened four genes (insulin receptor, INSR; F-box protein 11, FBXO11; transforming growth factor beta 1, TGFβ1; and inhibitor of growth family member 4, ING4) as candidate targets of dairy goat miR-2478. Subsequently, we verified TGFβ1 as a target gene of miR-2478 and inferred that this miRNA might participate in the negative regulation of dairy goat mammary gland development by targeting the 5’UTR of the TGFβ1 gene. In addition, we identified the potential promoter and transcription factor binding regions of TGFβ1 and examined the potential mechanisms of the miR-2478/TGFβ1 interaction. The present study provides evidence that miR-2478 binds to the region of transcriptional activity downstream of the TGFβ1 promoter and that it might synergistically regulate the inhibition of TGFβ1 via the transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBPJ) to affect goat mammary gland development.

Materials and Methods

Protocol approval. The experiments and animal care were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, 2004) and were approved by the Institutional Animal Care and Use Committee of the Institute of Animal Science and Technology, Northwest A&F University, China. All of the experiments and methods were carried out in “accordance” with the approved guidelines. The goats were allowed access to feed and water ad libitum under normal conditions, and all efforts were made to minimize their suffering.

Identification of goat miR-2478 target genes. miRNA target prediction. miRNA target genes were predicted using TargetScan (http://www.targetscan.org)15. The parameters were set according to the methods of Allen et al.16 and Schwab et al.17. The following criteria were applied for target gene prediction: (1) no more than four mismatches between the miRNA and target; (2) no more than two adjacent mismatches in the miRNA/target duplex; (3) no more than 2.5 mismatches at positions 1–12 of the miRNA/target duplex (5’ of the miRNA); (4) no mismatches at positions 10–11 of the miRNA/target duplex; (5) no adjacent mismatches at positions 2–12 of the miRNA/target duplex; and (6) minimum free energy of the miRNA/target duplex of more than 75% of that of the miRNA bound to its perfect complement. No data regarding mRNA:miRNA interactions in goats or sheep are available in the current version of TargetScan software. Therefore, the potential target genes of miR-2478 were predicted by referring to bovine mRNA:miRNA interactions and were screened according to the sequence characteristics of miR-2478 and the associated Gene Ontology (GO) terms18.

Cloning and verification of the corresponding target gene sequences. Seven primers for target genes were designed based on the sequences of bovine steroid receptor RNA activator 1 (SRA1, GenBank: AC_000164.1), FBXO11 (GenBank: AC_000168.1), nuclear receptor co-repressor 1 (NCO1, GenBank: AC_000162.1), TGFβ1 (GenBank: AC_000176.1), ING4 (GenBank: AC_000162.1), inhibitor of growth family member 2 (ING2, GenBank: AC_000184.1) and INSR (GenBank: AC_000164.1). The primers, annealing temperatures, and fragment sizes are provided in Table S1. The corresponding target sequences were amplified from caprine genomic DNA. PCR amplification was performed in a 25 μL reaction mixture with the following cycling parameters: 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 40 s, and a final extension step at 72 °C for 10 min. The amplification products were sub-cloned into a pMD18-T vector (Takara, Dalian, China), and then sequencing was performed. The caprine sequence identities of the miR-2478 binding sites were analyzed using BioXM 2.6 software (Nanjing Agricultural University, Nanjing, China).

Plasmids. The potential target genes were further screened based on the results of sequence identity analysis of the miR-2478 binding sites. To generate wild-type and mutant vectors containing the miR-2478 binding sites of the candidate target genes (FBXO11, TGFβ1, INSR and ING4), primers were designed based on the results of candidate target gene sequences in goat (Table 1). miR-2478 binding site mutants were generated via mutation of the target sequences of the miR-2478 seed region in the candidate target genes by overlap PCR. The PCR products for the wild-type and mutant target genes were cloned into multiple cloning regions of the vector psiCHECK-2 (Promega, Madison, WI, USA) using the NotI and Xhol restriction sites. The constructed vectors were sequenced to verify the base sequences of the miR-2478 binding and mutant sites.

HEK293T cell culture and co-transfection. HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin. The cells were then seeded into 24-well plates. Next, a miR-2478 mimic (2.0 μM) exhibiting miR-2478 activity was used to examine the effects of miRNA over-expression. The mimic was co-transfected into HEK293T cells with wild-type plasmids (psiCHECK-2-INSR, psiCHECK-2-FBXO11, psiCHECK-2-TGFβ1 and psiCHECK-2-ING4) or mutant plasmids (psiCHECK-2-INSR-mut, psiCHECK-2-FBXO11-mut, psiCHECK-2-TGFβ1-mut and psiCHECK-2-ING4-mut) (0.25 ng) according to the manufacturer’s instructions. An empty psiCHECK-2 plasmid was used
as a negative control. Each sample was transfected in triplicate. At 48 h after transfection, cells were collected for use in subsequent dual-luciferase reporter assays.

**Dual-luciferase reporter assay.** Cells were lysed in passive lysis buffer (Promega, USA), and the lysates were assayed for reporter gene activity using a dual-luciferase assay system (Promega, USA) according to the manufacturer’s instructions. Luciferase signal was detected with a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA). Renilla luciferase signal was normalized to firefly luciferase signal, and normalized Renilla luciferase activity was compared between samples using the independent sample t-test.

**Transfection with miR-2478 mimic or inhibitor and quantitative PCR (qPCR).** Dairy goat primary mammary gland epithelial cells (provided by Professor Jun Luo) were cultured in DMEM/F-12 medium supplemented with 10% FBS, 5 μg/mL bovine insulin, 10 ng/mL epidermal growth factor, 100 U/mL penicillin/streptomycin and 5 μg/mL hydrocortisone. The cells were grown at 37 °C and 5% CO2 and were transfected with 25 nM of a miR-2478 mimic or inhibitor or a negative control (NC) sequence (Qiagen, Hilden, Germany) using HiPerFect HTS Reagent according to the manufacturer’s instructions. Sterile ultra-pure water was employed as a negative control. Each sample was transfected in triplicate. Cells were directly collected after 24, 48 and 72 h, and total RNA was isolated from the cells using RNAiso Plus reagent (TaKaRa, Dalian, China). The RNA samples were subsequently reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China), according to the manufacturer’s instructions. Real-time PCR was performed using a standard SYBR Green PCR Kit (Takara, Dalian, China) and a BioRad CFX96 Real-Time PCR Detection System. Caprine miR-2478 expression patterns were verified via stem-loop RT-PCR according to Li *et al.*14. The miR-2478 mimic sequences were 5′-GUAUCCCACUUGUAGCAGCUGAUC-3′ (sense) and 5′-UGGGUUGUGCUACUGUGUGAACUU-3′ (antisense), and the miR-2478 inhibitor sequence was 5′-UGGGUUGUCAGAAGUGGAUAUC-3′. In addition, the relative TGFβ1 levels were determined using the following primers: caprine GAPDH: F: 5′-GCAAGTTCCACGGCACAG-3′ and R: 5′-GGTTCAGCAGCGCAAGAATG-3′; and caprine TGFβ1: F: 5′-GTGCTAATGGTGGAATACGG-3′ and R: 5′-CGCCAGGAATTGTTGCTATA-3′. 18S-snRNA and GAPDH were used as endogenous controls. Relative gene expression levels were determined using the 2−ΔΔCt method.

**Analysis of the mechanism of TGFβ1 regulation by miR-2478.** Cloning of the caprine TGFβ1 gene and construction of 5′UTR sequence deletions. In a previous experiment, we showed that miR-2478 targeted

| Gene    | Primers (5′−3′)                                      | Sizes (bp) |
|---------|-----------------------------------------------------|------------|
| INS7    | F1: cccCTCGAGGCTTATGCGGAAATCAACTC                  | 301        |
|         | R1: attGCGGCCGCAAAATACCACACCC                       |            |
| FBXO11  | F1: cccCTCGAGGAGCAAGATACAAAGGAAGAAGG               | 491        |
|         | R1: attGCGGCCGAAATACCCACACCC                       |            |
| TGFβ1-mut| F3: cccCTCGAGGCTTATGCGGAAATCAACTC                  | 343        |
|         | R3: attGCGGCCGCAAAATACCACACCC                       |            |
| ING4-mut| F4: cccCTCGAGGCTTATGCGGAAATCAACTC                  | 289        |
|         | R4: attGCGGCCGCAAAATACCACACCC                       |            |

Table 1. Primers used to construct wild-type and mutant vectors containing the miR-2478 binding sites in candidate target genes. Note: The attached nucleotides are indicated in lowercase, and the restriction sites for NotI and XhoI are underlined.
the 5′UTR of TGFβ1. Therefore, to explore the mechanisms underlying the miR-2478/TGFβ1 interaction, we cloned and sequenced fragments containing the entire coding region and partial intron of the caprine TGFβ1 gene, as well as parts of the 5′ and 3′ flanking sequences. In addition, the promoter sequence of TGFβ1 and the regulatory effects of miR-2478 binding to the 5′UTR were determined. Based on the bovine TGFβ1 sequence (GenBank: NC_007316.5), seven pairs of primers for amplifying different fragments of the caprine TGFβ1 gene were designed. The primers, annealing temperatures, and fragment sizes are shown in Table S1.

To verify the basic core promoter of the caprine TGFβ1 gene, specific primers were designed based on the acquired caprine TGFβ1 sequence to construct pGL3 vectors harboring deletions of varying lengths in the 5′UTR (Table S2). The PCR products were cloned into multiple cloning sites of a pGL3-Basic luciferase vector (Promega, WI, USA) using the KpnI and HindIII restriction sites. Then, the constructed vectors were sequenced to verify the deleted sequences.

Cell culturing and transfection. The culture conditions for HEK293T cells were the same as described above. Prior to transfection, pGL3-Basic recombinant plasmids (500 ng) carrying sequences with deletions of different lengths were diluted with serum-free DMEM without antibiotics and mixed with pRL-CMV endogenous control plasmids (10 ng). Subsequently, the plasmid mixtures were transfected into HEK293T cells using Lipofectamine 2000.

Six groups were included in the experiment: a negative control group (pGL3-Basic and pRL-CMV), treatment group 1 (pGL3-TGFβ1 A and pRL-CMV), treatment group 2 (pGL3-TGFβ1 B and pRL-CMV), treatment group 3 (pGL3-TGFβ1 C and pRL-CMV), treatment group 4 (pGL3-TGFβ1 D and pRL-CMV) and treatment group 5 (pGL3-TGFβ1 E and pRL-CMV). Each sample was transfected in triplicate. At 48 h after transfection, cells were collected for use in subsequent dual-luciferase reporter assays.

Bioinformatics analysis of the 5′UTR of TGFβ1. Potential cis elements in the TGFβ1 5′UTR were predicted using the following four online software platforms for the prediction of transcription factors and promoters: PROSCAN (http://www-bimas.cit.nih.gov/molbio/proscan/), Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter), TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html) and MatInspector (http://www.genomatix.de/matinspector.html).

Statistical analysis. Statistical analyses of normalized Renilla luciferase activity and the relative expression levels of miR-2478 and TGFβ1 were performed using SPSS version 20.0. The correlation between miR-2478 expression and TGFβ1 mRNA expression was assessed using Pearson's correlation analysis. In addition, the sample data were compared using the independent sample t-test and one-way ANOVA, followed by Dunnett's test, in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The data are expressed as the mean ± SE.

Results
Prediction of miR-2478 target genes and experimental verification of miR-2478 binding sites in these genes in goat. To further elucidate the role of miR-2478 in regulating mammary gland development in dairy goats, we used TargetScan15 (http://www.targetscan.org/) to predict the target genes regulated by miR-2478. Based on their high scores, sequence characteristics and GO terms, we selected seven potential targets: SRA1, FBXO11, NCOA1, TGFβ1, ING2, ING4 and INSR. TGFβ1 is associated with the GO terms "transforming growth factor beta receptor signaling pathway" and "secretion by cell," and ING2 is associated with the terms "transforming growth factor beta receptor signaling pathway," "regulation of biosynthetic process" and "biological regulation." Notably, the TGFβ3 receptor signaling pathway plays important roles in mammary gland development, differentiation and degeneration. In addition, NCOA1 is associated with the GO terms "lipid metabolic process," "lipid transport," "lipid localization" and "fatty acid transport," which play important roles during lactation.

As the prediction of target genes was based on bovine genomic sequences, we suspected that the corresponding sequences in the caprine genome might exhibit some differences. Thus, the corresponding sequences of the miR-2478 binding sites in the potential target genes were amplified from caprine genomic DNA and sequenced by homologous cloning. Nucleotide sequence alignment analysis indicated base mismatches in the miR-2478 binding sites in goat SRA1, NCOA1 and ING2 compared with the corresponding sequences in the bovine genes, while the miR-2478 binding sites in the other four potential target genes were the same in goat and cattle (Fig. 1). Therefore, the remaining four genes (INSR, FBXO11, TGFβ1 and ING4) were screened as candidate targets of dairy goat miR-2478. The miR-2478 binding sites of three of the four candidate targets were located in the 5′UTR, whereas that of TGFβ1 was located in the 5′UTR.

miR-2478 binds to the 5′UTR of the TGFβ1 gene. A dual-luciferase reporter system was used to identify the target genes of miR-2478. The luciferase activity of HEK293T cells co-transfected with the miR-2478 mimic and psiCHECK-2-TGFβ1 5′UTR vector was significantly decreased compared with that of cells transfected only with the negative control psiCHECK-2-TGFβ1 5′UTR vector (P < 0.01), while luciferase activity was recovered in cells transfected with the psiCHECK-2-TGFβ1-mut vector (P > 0.01) (Fig. 2A and B), indicating that miR-2478 directly targets TGFβ1 by binding to its 5′UTR. INSR, FBXO11 and ING4 were not found to be positive target genes, as no significant differences in luciferase activity were observed between cells co-transfected with the mimic and psiCHECK-2-INSR, psiCHECK-2-FBXO11 or psiCHECK-2-ING4 and negative control cells (P > 0.05) (Fig. 3). A previous study has reported that miR-2478 expression is up-regulated in dairy goat mammary glands during peak lactation, whereas TGFβ1 gene expression is down-regulated44. These results demonstrate that miR-2478 binds to the 5′UTR of the TGFβ1 gene (from +123 bp to +142 bp) (Fig. 2A).
miR-2478 significantly inhibits TGFβ1 transcription. The dual-luciferase reporter assay confirmed the interaction between miR-2478 and the 5′UTR of the TGFβ1 gene. Thus, we inferred that miR-2478 might affect the transcription of TGFβ1. To verify this hypothesis, we examined changes in TGFβ1 mRNA expression in dairy goat primary mammary gland epithelial cells after transfection of a miR-2478 mimic or inhibitor for 24, 48 or 72 h. qPCR assays showed that the expression of mature miR-2478 was significantly higher at 24 and 72 h after transfection of the miR-2478 mimic than that in control cells (P < 0.01), indicating that the miR-2478 mimic was successfully transfected into the mammary gland epithelial cells (Fig. 2C). In addition, the expression of endogenous mature miR-2478 was significantly lower at 24, 48 and 72 h after transfection of the miR-2478 inhibitor than that in the control (P < 0.01 or P < 0.05), indicating that the miR-2478 inhibitor suppressed the expression of endogenous miR-2478 in mammary epithelial cells (Fig. 2C). qPCR after 48 h of over-expression of miR-2478 in dairy goat mammary gland epithelial cells revealed that the TGFβ1 mRNA level was significantly down-regulated compared with that in the control cells (P < 0.01) (Fig. 2D). In addition, the level of TGFβ1 mRNA was significantly up-regulated at 48 and 72 h after transfection of the miR-2478 inhibitor (P < 0.01) (Fig. 2D). These findings demonstrate that miR-2478 significantly inhibits TGFβ1 transcription by binding to the 5′UTR of the TGFβ1 gene (from +123 bp to +142 bp).

Expression analysis and investigation of the conservation of the TGFβ1 gene in goat. In a previous study, we investigated the expression patterns of miR-2478 in the skeletal muscle (Mu), heart (He), liver (Li), kidney (Ki), spleen (Sp), lung (Lu), inner fat (Fa), ovary (Ov) and mammary gland at four developmental stages (at 30 days after lambing (DAL), 75 DAL, 200 DAL, and 320 DAL)44. We also performed profiling analysis of miR-2478 targeting of the TGFβ1 gene in these tissues. TGFβ1 gene expression was higher at 30 and 75 DAL (peak lactation) than at 320 DAL (dry period) (Fig. 4), revealing that this gene is related to mammary gland development. Furthermore, the correlation between miR-2478 expression and TGFβ1 mRNA expression was assessed by Pearson's correlation analysis using SPSS yielding a correlation coefficient of −0.98 (P = 0.017). These findings demonstrate that the expression levels of miR-2478 and TGFβ1 mRNA in the goat mammary gland are inversely correlated during lactation, implying that miR-2478 may be a major regulator of TGFβ1 mRNA expression during lactation.

The caprine TGFβ1 gene (length of 1971 bp) was cloned and sequenced, with the length of 1971 bp. Alignment of the caprine TGFβ1 cDNA with the corresponding coding sequences (CDSs) from other mammalian species showed similarities of 98.04% with sheep, 98.30% with cattle, 89.86% with humans and 83.38% with mice. At the protein level, the caprine amino acid sequence shared 99.12, 99.12, 97.83 and 95.37% identity with sheep, cattle, humans and mice, respectively, as shown in Fig. 5. These results indicate that the TGFβ1 gene is highly conserved, implying that the function of this gene is consistent among mammals.
The mechanism of miR-2478 in inhibiting TGF\(\beta\)1 transcription. While the above results demonstrated that miR-2478 binds to the 5′UTR of TGF\(\beta\)1 to exert a regulatory function, the mechanisms underlying the miR-2478/TGF\(\beta\)1 5′UTR interaction remained unclear. Thus, to examine this interaction, potential promoters and transcription factor binding regions in the 5′UTR of TGF\(\beta\)1 were assessed. Various deletion vectors for the goat TGF\(\beta\)1 5′UTR were constructed and designated pGL3-TGF\(\beta\)1 A, pGL3-TGF\(\beta\)1B, pGL3-TGF\(\beta\)1 C, pGL3-TGF\(\beta\)1D and pGL3-TGF\(\beta\)1E. The luciferase activities of HEK293T cells co-transfected with the pGL3-TGF\(\beta\)1 A/B/C/D/E recombinant vectors or the endogenous control vector pRL-CMV were significantly higher than that of cells transfected with the negative control vector (pGL3-Basic and pRL-CMV; \(P < 0.05\) or \(P < 0.01\)), indicating that the corresponding sequences all initiated reporter gene transcription, but to different extents (Fig. 6). Among the five recombinant vectors, pGL3-TGF\(\beta\)1B resulted in the highest luciferase activity (8.32 ± 0.71), and pGL3-TGF\(\beta\)1D resulted in the lowest activity (2.57 ± 0.07). Considering the luciferase activity of pGL3-TGF\(\beta\)1 A as a reference (100%), the relative luciferase activities of the other four recombinant vectors (pGL3-TGF\(\beta\)1B-E) were 109.04%, 91.87%, 33.68% and 56.09%, respectively (Table 2). Statistical analysis showed that the transcriptional activity of pGL3-TGF\(\beta\)1D was significantly lower than the activities of pGL3-TGF\(\beta\)1A/B/C (\(P < 0.01\)) and pGL3-TGF\(\beta\)1E (\(P < 0.05\)). In addition, the transcriptional activity of pGL3-TGF\(\beta\)1E was significantly lower than that of pGL3-TGF\(\beta\)1A/B/C (\(P < 0.05\)) but was significantly higher than that of pGL3-TGF\(\beta\)1D (\(P < 0.05\)). No significant differences in transcriptional activity were observed among pGL3-TGF\(\beta\)1A/B/C. The results of dual-luciferase reporter assays revealed that two regions spanning from −904 to −690 bp and from −79 to +197 bp were transcription factor binding regions in the TGF\(\beta\)1 5′UTR. The above-mentioned results demonstrated that miR-2478 bound to the region from +123 to +142 bp in the TGF\(\beta\)1 5′UTR (GGTTTTTTCCTCGGGTACG) (Fig. 2A). It is interesting that this miR-2478 binding region (+123 to +142 bp) is located in the core region of the TGF\(\beta\)1 gene promoter (from −79 to +197 bp) (Fig. 7). Taken together, our results demonstrate that miR-2478 binds to the core region of the TGF\(\beta\)1 promoter and affects goat mammary gland development by inhibiting TGF\(\beta\)1 transcription.

Transcription factor prediction for the TGF\(\beta\)1 5′UTR and regulatory analysis of miR-2478. The TGF\(\beta\)1 promoter was predicted using three online software platforms: PROSCAN, Promoter 2.0 and MatInspector (Table S3). The overlapping predictions of PROSCAN and MatInspector showed a region of transcriptional activity downstream of the TGF\(\beta\)1 transcription start site, and those of PROSCAN and Promoter 2.0 showed a region of transcriptional activity approximately −1000 bp upstream of the transcription start site. These
two predictions are approximately consistent with the previous experimental identification of transcription factor binding regions in the TGFβ1 5′UTR (two regions from −904 to −690 bp and from −79 to +197 bp). Next, we predicted the transcription factor binding sites in the two regions of the TGFβ1 5′UTR using TFSEARCH. A total of 21 binding sites were predicted, including 8 Sp1 sites, 3 GCF sites, 2 T-Ag sites, 1 PEBP2 site, 1 ETF site and 1 AP-2 site, spanning from −731 to −981 bp. The region from +29 to +279 bp was predicted to contain 9 Sp1 sites, 2 AP-2 sites, 1 GCF site, 1 NF-κB site and 1 T-Ag site. The prediction results indicated that these two regions are enriched in transcription factor binding sites. Moreover, a cis-acting element of the transcription factor RBPJ was identified in the core region from +135 to +138 bp (TGGG), while the binding seed region of miR-2478 was detected at +134 to +142 bp (GTGGGATAC) (Fig. 7). Previous studies have shown that RBPJ is a critical transcription factor in mammals. Thus, these results indicate that the binding of miR-2478 to TGFβ1 (seed sequence: GTGGGATAC) may prevent the transcription factor RBPJ from binding to the promoter (cis-acting element: TGGG), thereby inhibiting TGFβ1 transcription.

Discussion
miRNAs have been identified in mice, humans, bovines and goats and in mammalian mammary glands at various developmental stages, suggesting that multiple miRNAs play important roles in the differential expression of genes during mammary development and lactation. miR-155 affects cell proliferation and apoptosis by targeting RhoA, and this process is mediated by TGFβ signaling pathways. Jie has confirmed that the targeting of 3′ UTR sequences in GHR by miR-139 in bovine mammary gland epithelial cells might play a role in the activity of ductal epithelial cells during lactation. However, the function and potential mechanism of miR-2478 in regulating mammary gland development in dairy goats have not previously been explored.

In the present study, we predicted the target genes of goat miR-2478 using the TargetScan program. Previous predictions of miRNA target genes have been focused on the 3′UTR. However, studies have recently shown that
in addition to traditional 3′UTR targeting, a number of 5′UTR and amino acid CDS sites are also targeted in mammals. Although high percentages of reads mapping to CDS sites (>25%) and 5′UTRs (>1%) have been reported, most reads match to the 3′UTR, confirming that miRNAs preferentially bind to targets in the 3′UTR but that 5′UTR/CDS-mediated interactions are also significant. Therefore, we considered the three potential miRNA target regions in the prediction of goat miR-2478 targets. We preliminarily selected seven potential targets and ultimately screened four of these genes (INSR, FBXO11, TGFβ1, and ING4), which negatively regulate mammary gland development and physiological lactation, as candidate targets of dairy goat miR-2478 by PCR verification using goat genomic DNA and by sequence identity analysis of miR-2478 binding sites in goat and bovine (Fig. 1).

miR-2478 binding sites were identified in the 5′UTR of TGFβ1, while in the other three target genes, traditional

Figure 5. Comparison of sequence homology among goat, sheep, cattle, human, and mouse TGFβ1 amino acid sequences. The amino acid sequences were derived from the following reference sequences: NP_001009400.1 (sheep), NP_001159540.1 (cattle), NP_000651.3 (human), and NP_035707.1 (mouse).

Figure 6. Luciferase activity assays for a series of truncated sequences of the TGFβ1 5′UTR in HEK293T cells. pGL3-TGFβ1A/B/C/D/E or a pGL3-Basic vector was co-transfected with a pRL-CMV vector into HEK293T cells. Then, the cells were lysed, and luciferase assays were performed after 48 h. The pGL3-Basic vector was used as a negative control. The data are presented as the mean ± SE (n = 3). (*P < 0.05; **P < 0.01).
sites in the 3′UTR were detected. These results demonstrate that TGFβ1 is a target gene of miR-2478 (Fig. 2A and B). Subsequently, we found that over-expression of miR-2478 in dairy goat mammary gland epithelial cells resulted in significant down-regulation of the TGFβ1 mRNA level, while inhibition of endogenous miR-2478 expression resulted in significant up-regulation of TGFβ1 mRNA (Fig. 2C and D), demonstrating that miR-2478 significantly inhibited TGFβ1 transcription by binding to its 5′UTR. The potential regulation of mRNAs by miRNAs through binding sites in their 5′UTRs was demonstrated early on25. Many validated examples of naturally occurring 5′UTR miRNA targets exist in the literature to date21–28. Likewise, the present study has provided additional data in support of 5′UTR targeting by a miRNA, miR-2478, which resulted in a reduction in TGFβ1 mRNA abundance.

We also investigated the conservation of the TGFβ1 gene in mammals, including goat, sheep, cattle, humans and mice (Fig. 5). These results showed that the TGFβ1 gene is highly conserved, implying that the function of this gene is consistent, among mammals. TGFβ1 plays important roles in mammary gland development, differentiation and degeneration29. In microfabricated mammary gland tissue, branching has been shown to be inhibited at sites with high concentrations of TGFβ130. WNT5A acts downstream of TGFβ1 in vivo and is required for TGFβ3-mediated inhibition of mammary branching morphogenesis31–33. Increasing evidence indicates that TGFβ1 functions as a paracrine or autocrine epithelial cell inhibitory factor and as an apoptosis-inducing factor in mammary glands. In mice, TGFβ1 is a potent inhibitor of cell proliferation, and it has been shown to suppress alveolar development and lactogenesis34,35. Over-expression of TGFβ1 has been reported to lead to inhibition of mammary gland development in vivo36. In addition, TGFβ1 has been demonstrated to be an antiproliferative and apoptotic factor in dairy cattle mammary epithelial cells37. Thus, TGFβ1 overexpression may decrease the autophagic apoptosis of mammary epithelial cells in cattle. TGFβ1 belongs to a group of intramammary autocrine/paracrine inhibitors of bovine mammary epithelial cell growth and inducers of apoptosis38. Moreover, TGFβ3 induces apoptosis of bovine mammary epithelial cells through the ALK-5-Smad2/3 pathway and promotes bovine mammary fibroblast proliferation through the ERK1/2 pathway to maintain normal development and lactation in the mammary glands39,40. Thus, TGFβ1 may act as a growth suppressor, exerting negative effects on mammary epithelial cell growth and also suppressing milk secretion41.

In addition, Wareski et al.42 investigated the expression of apoptosis-related proteins in the mammary glands of dairy goats during the lactation cycle (peak lactation, late lactation and drying off) and observed that the expression of the examined proteins was increased during late lactation and the dry period, suggesting that these proteins are involved in the induction, regulation and execution of programmed cell death during mammary gland involution. Based on these data, we inferred that miR-2478 might play an important role in mammary gland development in dairy goats by targeting the 5′UTR of the TGFβ1 gene.

To further explore how miR-2478 regulates the transcription of TGFβ1 by binding to its 5′UTR, we identified the potential core promoter and transcription factor binding regions of TGFβ1 and analyzed the potential mechanisms of interaction between miR-2478 and TGFβ1. The experimental results indicated that two sequences spanning from −904 to −690 bp and from −79 to +197 bp might act as transcription factor binding regions in TGFβ1 (Fig. 6), and this was also confirmed through promoter/transcription factor prediction for this gene (Table S3). It is interesting that the binding region of miR-2478 (+123 to +142 bp) is located in the core region of the TGFβ1 gene promoter (−79 to +197 bp). In addition, we observed that the miR-2478 binding sequence was

| Group       | Relative luciferase activity | Normalized to pGL3-TGFβ1 A |
|-------------|------------------------------|---------------------------|
| pGL3-TGFβ1A | 7.63 ± 0.41*                 | 100%                      |
| pGL3-TGFβ1B | 8.32 ± 0.73*                 | 109.04%                   |
| pGL3-TGFβ1C | 7.01 ± 0.51*                 | 91.87%                    |
| pGL3-TGFβ1D | 2.57 ± 0.39*                 | 33.68%                    |
| pGL3-TGFβ1E | 4.28 ± 0.12*                 | 56.09%                    |
| pGL3-Basic  | 0.23 ± 0.16                  | 3.01%                     |

Table 2. Relative luciferase activity assays for TGFβ1 5′UTR sequences in HEK293T cells. Note: compared with pGL3-Basic, a: P < 0.01, b: P < 0.05; compared with pGL3-TGFβ1 A, c: P < 0.01, d: P < 0.05.
located in the region of transcriptional activity downstream of the TGFβ1 promoter. Moreover, our predictions showed that the seed sequence of miR-2478 that binds to TGFβ1 (TGGG) is a cis-acting element of the inhibitory transcription factor RBPJ. Thus, we speculate that miR-2478 inhibits TGFβ1 expression at the transcriptional level by preventing the transcription factor RBPJ from binding to the promoter.

Accumulating evidence indicates that some miRNAs bind to the promoter regions of target genes to regulate their mRNA levels. We and others have demonstrated that miRNAs complementary to gene promoters are potent silencers of target gene expression in mammalian cells at the transcriptional level. The miRNA-promoter interaction resource is a public database containing over 15 million predicted miRNA target sites located within human promoter sequences. For example, the scanning of miRNA target sequences in transcription start sites using bioinformatic techniques has revealed that miR-320 acts at the POLR3D promoter and that it plays a cis-regulatory role in the silencing of gene expression at the transcriptional level. In addition, Place has provided evidence of a promoter-targeting miRNA by demonstrating that the transfection of cells with miR-373 induces the expression of CDH1 and CSDC2, which possess complementary promoter sequences. Sequence-specific recognition of PGR gene promoters by miR-423 may occur simultaneously with binding by protein transcription factors. Moreover, miR-130a functions as a suppressor that modulates promoter activity even in the presence of other direct or indirect transcriptional modulators. These observations suggest that miRNA-promoter interactions may be a natural and general mechanism for regulating gene transcription. In our study, miR-2478 bound to the promoter region of its target gene, TGFβ1, and regulated its expression at the transcriptional level. Moreover, a cis-acting element of the transcription factor RBPJ was predicted to be located in the TGFβ1 promoter region.

Knowledge of the mechanisms underlying the regulatory effects of miRNAs on target genes is increasing. Transcription factors and miRNAs have been frequently detected in numerous transcriptional regulatory network motifs. As cancer research expands, transcription factors, miRNAs and their equivalent target genes are emerging in an increasing number of studies. Thus, we infer that the transcription factor RBPJ, miR-2478 and their shared target gene TGFβ1 may interact to achieve transcriptional regulation. However, this hypothesis requires further verification.

In conclusion, we have revealed that miR-2478 significantly inhibits TGFβ1 transcription by targeting the 5′UTR of the TGFβ1 gene. Furthermore, we have identified the potential promoter and transcription factor binding regions of TGFβ1 and have analyzed the potential interaction mechanisms of miR-2478/TGFβ1. Previous studies have indicated that the transcription factor binding regions of TGFβ1 include two regions spanning from −904 to −690 and from −79 bp to +197 bp. In addition, based on the prediction that the seed sequence of miR-2478 that binds to TGFβ1 (TGGG) is a cis-acting element of the inhibitory transcription factor RBPJ, we suggest that the binding of miR-2478 to TGFβ1 (seed sequence: GTGGGATAC) may prevent the transcription factor RBPJ from binding to the promoter (cis-acting element: TGGG) and thereby inhibiting TGFβ1 transcription.

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Author Contributions

Z.L. designed the study and wrote the main manuscript text; R.H. prepared the figures and table; J.W., W.G., J.S. and Y.H. performed the experiments; and X.L. and H.C. analyzed the data. All authors revised the article and approved the final version for publication.

Additional Information

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The original PDF version of this Article contained an error in the order of corresponding authors. This has now been corrected in the PDF version of this Article; the HTML version was correct from the time of publication.

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