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LncRNA-599554 sponges miR-15a-5p to contribute inductive ability of dermal papilla cells through positive regulation of the expression of Wnt3a in cashmere goat

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ABSTRACT

Background: Long non-coding RNAs (lncRNAs), as post-transcriptional regulators, were thought to function in the inductive property of dermal papilla cells (DPCs) in cashmere goat. Previously, lncRNA-599554 was identified in secondary hair follicle (SHF) of cashmere goat, but its functional significance is unknown.
Results: In the present investigation, we verified that lncRNA-599554 had significantly higher expression at the anagen dermal papilla of cashmere goat SHF than that at telogen. Based on overexpression and knockdown techniques, we found that lncRNA-599554 contributes the inductive property of DPCs of cashmere goat, which was assessed by detecting the changes in the expression of several typical indictor genes in DPCs including ET-1, SCF, Versican, ALP, Lef1 and Ptc-1. Based on RNA pull-down assay, we verified that lncRNA-599554 directly interacted with chi-miR-15a-5p. Also, we showed that lncRNA-599554 positively regulated the Wnt3a expression in DPCs but which did not appear to involve its modulating of promoter methylation. Based on the use of Dual-luciferase reporter assays, our data indicated that lncRNA-599554 regulated the Wnt3a expression through chi-miR-15a-5p-mediated post-transcriptional level.
Conclusions: We showed that lncRNA-599554 contributes the inductive property of DPCs in cashmere goat which might be achieved through sponging chi-miR-15b-5p to promote the Wnt3a expression. The results from the present investigation provided a novel insight into the functional mechanism of lncRNA-599554 in the SHF regeneration of cashmere goat along with the formation and growth of cashmere fiber.
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1. Introduction

Cashmere goats are precious livestock resources for the production of natural fiber with high quality. They are prevalent in northern China with more than 10 different breeds, such as Inner Mongolia, Liaoning, Hexi, and Shanbei breeds [1]. Although cashmere goats are multipurpose livestock that provide people cashmere, wool, meat, fur, and other products; cashmere and meat are the main use of cashmere goats [2,3,4]. Like meat, cashmere also has important economic implications for local herdsmen [5]. Cashmere is produced by secondary hair follicle (SHF) of cashmere goat, and its growth is under the control of the photoperiod along with the change of the endocrine system in the body [6,7]. During the production process of cashmere, several important biological events are implicated, such as the regeneration and development of SHF, as well as, the formation and growth of cashmere fiber, where the inductive property of dermal papilla cells (DPCs) was thought to play a significant role [4,8].

In DPCs of human and mice, it was verified that several genes act as important regulators in maintaining the DPC inductive property, including Fgf2, Bmp6, Wnt10b and Wnt5a [9,10,11,12]. It was found that DPCs co-cultured with keratinocytes could promote the inductive

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property of DPC *in vitro* [9]. On the other hand, in mice, it was revealed that many long non-coding RNAs (lncRNAs) were also implicated in the regulation of DPC inductive property, such as lncRNA-RP11-766N7.3, lncRNA-H19, and lncRNA-HOTAIR [13]. In the last few years, interestingly, many lncRNAs have also been identified in skin tissue/SHF of cashmere goat that were thought to play significant roles in the regeneration and development of cashmere goat SHF [14,15,16,17]. Furthermore, the lncRNA-000133 originating from the antisense stand of Son gene of cashmere goat was verified to promote the inductive property of DPCs [4]. Thus, it is apparent that lncRNAs, as a novel regulatory layer, may be implicated in DPC inductive property of cashmere goat.

In our previous study, we identified that a lncRNA transcript (named as lncRNA-599554) has significantly higher expression at anagen SHF of cashmere goat than that of telogen [14]. Considering the fact that DPCs at anagen are in more exuberant status in terms of inductive property than that at telogen [18], we hypothesize that lncRNA-599554 may be implicated in DPC inductive property of cashmere goats. In this investigation, firstly, the expression of lncRNA-599554 was verified in SHF dermal papilla of cashmere goat at two different stages: anagen and telogen, and next, the potential effects of lncRNA-599554 on the DPC inductive property in cashmere goat were investigated through overexpression and siRNA interference techniques. The possible molecular mechanisms of lncRNA-599554 in regulating the DPC inductive property were further explored based on the use of RNA pull-down and Dual-luciferase reporter assays along with methylation analysis. Our results will provide novel evidence for revealing the functional roles and molecular mechanisms of lncRNA-599554 in modulating the DPC inductive property of cashmere goats.

2. Materials and methods

2.1. Sequence, samples, and cell culture

Here, all experimental assays were reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University. The analyzed lncRNA-599554 (465-nt in length) was identified in our previous study that was found to exhibit significantly higher expression at anagen SHF of cashmere goat than that at telogen [14]. The BioEdit program [19] was used for displaying the sequence of lncRNA-599554 whose potential open reading frames (ORFs) were analyzed with the ORF Finder program at NCBI (https://www.ncbi.nlm.nih.gov/orffinder). On lncRNA-599554 sequence, the potential binding sites of different miRNAs were predicted using the miRDB program (http://www.mirdb.org), combining with RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) and miRNA-target programs (http://www.targetscan.org). Using the MEGA program [20], we analyzed the nucleotide composition of lncRNA-599554 along with its frequency distributions of different nucleotide pairs.

Skin tissue were collected from nine adult female individuals in our previous investigation [17]. The procedure is as follows. The skin tissues 1 cm^2 from each goat were first washed and sterilized with 75% alcohol, and then, the samples were cut into 5 mm^2 blocks by ophthalmic scissors and washed using PBS for three times. The sample blocks were treated with 0.25% dispase II at 4°C overnight. Under a stereomicroscope, the SHFs from each sample were isolated *in vitro* by microseparation. After 30 min digestion in DMEM/F12 medium containing 0.2 mg/ml collagenase II (Gibco, Carlsbad, CA, USA) at room temperature, we microdissected the dermal papillae from SHFs with forceps and syringe needles [21]. Subsequently, the dermal papillae from each sample were added to 24-well culture plates, respectively, and cultured in DMEM/F12 (Hyclone, USA) that contained 10% (v/v) fetal bovine serum (FBS) at 37°C with 5% (v/v) CO_2 [22]. Cell migrations from dermal papillae were observed and media were replaced every 2 d.

2.2. Overexpression of lncRNA-599,547 and its siRNA interference

The DPCs of passage 3 were used to perform the overexpression of lncRNA-599554 and its siRNA interference analysis. In DPCs, the overexpression analysis of lncRNA-599554 was performed based on the use of AdEasy Adenoviral vector assay, where recombinant adenovirus was prepared following the method by He et al. [22]. Recombinant adenovirus Ad-lncRNA-599554 and Ad-GFP (negative control) were packaged and amplified in HEK-293A cells. The method: 50% tissue culture infectious dose ($TCID_{50}$) was used to determine the viral titers. DPCs were infected with or without recombinant adenoviruses, and after 24 h the overexpression of lncRNA-599554 in DPCs was verified *via* real-time PCR analysis. The cells with or without recombinant adenoviruses were cultured for 48 h in DMEM/F12 (Hyclone, USA) that contained 10% (*v*/v) fetal bovine serum (FBS) at 37°C with 5% (v/v) CO_2, and then, they were collected respectively for further testing.

Based on the sequence of lncRNA-599554, two specific siRNAs were designed using the online program: DSIR (http://biodev.extra.cea.fr/DSIR/DSIR.html). They were named as Si-lncR1 (5′-GCUUGUCUCUAGAAAUGAAUC-3, 5′-UUCAUUUCUAGAGACAAGCAU-3′), and Si-lncR2 (5′-GGCAUGUUGUGUAUAAUUAGU-3′, 5′-UAAUUAUACACAACAUGCCCA-3′), respectively, and were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The corresponding negative control siRNAs (5'-UUCUCCGAACGUGUCACGUTT-3′, 5′-ACGUGACACGUUCGGAGAATT-3′) were also provided by Invitrogen, and they have no homology with all siRNAs and the known genes. After centrifuging at 12000g/min for 10 min, the siRNAs were dissolved in 1 ml DEPC water. The siRNA interference experiments were performed when the DPCs reached 80% confluence. Using the Lipofectamine® RNAiMAX Reagent (Invitrogen, Shanghai, China), siRNAs were transfected into DPCs for knockdown of lncRNA-599554. The cells were then allowed to grow in the abovementioned conditions of cell culture for 48 h. Subsequently, the cells were harvested for further testing.

2.3. RNA pull-down assay

Here, the RNA pull-down analysis was performed following the assay described in previous publications [23,24]. In brief, the T7-containing primer was used to amply the biotinylated DNA probe that is complementary to lncRNA-599,547. The obtained prober was then cloned into the plasmid vector GV394 (Genechem, Shanghai, China). The resultant plasmid was digested for linearization using restriction enzyme *Xho*I (TaKaRa, Dalian, China). *In vitro,* we reversely transcribed the biotin-labeled RNAs through the use of T7 RNA polymerase (Roche, Indianapolis, IN, USA) and biotin RNA Labeling Mix (Roche, Roche, Indianapolis, IN, USA). The reaction was performed in a 20-μl final volume containing 20 U ribonuclease inhibitor, linearized plasmid DNA 400 ng, 20 U T7 RNA polymerase, and 2.5 mM NTP mixture supplemented with 10% biotin-labeled UTP at 37°C for 1 h. Using the RNase-free DNase I (Invitrogen, Carlsbad, CA, USA), we treated the transcribed products at 37°C for 30 min, followed by a purification process with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). We prepared the cellular extract by cell lysis buffer, followed by an incubation with the RNA probe at 4°C for 1 h. The cell pellets were washed in phosphate buffered saline for five times and were used for RNA isolation. The bound RNAs were further analyzed using real-time PCR assay as described in previous publication [25].

2.4. RNA extraction, primer designing and real-time PCR assay

Using the RNAiso reagent kit (TaKaRa, Dalian, China), we extracted the total RNA from SHF dermal papillae and DPCs following the manufacturer's recommendation. The Premier Primer software (Version 5.0, http://www.premierbiosoft.com) was used for designing the gene-specific primers. After retrieving the mature sequence of miRNAs from miRBase database (http://www.mirbase.org), we

Table 1
Information of the primers used in this study and their annealing temperature for PCR reaction.

Primer name	Reference or accession number in GenBank/miRBase	Primer length (nt)	Sequence (5′–3′)[a]	Amplicon size (bp)	*Ta*[b] (°C)
lncRNA-599554-F	[14]	19	F: TAGAGGCAGGGCAAGAGTG	103	54
lncRNA-599554-R		19	R: CTGGTGGAACAGCAACATC		
REF-UBC-F	[29]	20	F:GCATTGTTGGGTTCCTGTGT	90	52
REF-UBC-R		20	R:TTTGCATTTTGACCTGTGAG		
REF-YWHAZ-F	[29]	22	F:TGTAGGAGCCCGTAGGTCATCT	102	56
REF-YWHAZ-R		25	R:TTCTCTCTGTATTCTCGAGCCATCT		
REF-SDHA-F	[29]	19	F:AGCACTGGAGGAAGCACAC	105	53
REF-SDHA-R		19	R:CACAGTCGGTCTCGTTCAA		
Et-1-F	[4]	21	F:GTTCCCCCAACCATCTTCACT	147	54
Et-1-R		21	R:TCCTTCCTCTCTCTCTCCG		
SCF-F	[4]	21	F:TTGTTGGATAAGCGAGATGGT	197	57
SCF-R		21	R:TGGGTTCTGGGCTCTTGAATG		
Versican-F	[4]	19	F:AGATGGGAAAGGCAGGAGT	113	52
Versican-R		19	R:GGGGACAGTGAGGTGGAAC		
Alp-F	[4]	21	F:CACGCCCTTTGCTTTATCTTG	107	55
Alp-R		21	R:GGTCTGAGCCTCCTCTTTTCC		
Ptc-1-F	[4]	20	F:ACTAAACAGCGTCTGGTGGA	232	55
Ptc-1-R		20	R:CGTTGAGGTAGAAAGGGAAC		
Lef1-F	[4]	21	F:TTCCTTCAACCCCTTCCCCTA	157	58
Lef1-R		21	R:AGCAACGACATTCGCTCTCAT		
Gapdh-F	[4]	21	F:GACACCCACTCCTCCACCTTT	187	56
Gapdh-R		21	R: CTCTTCCTCTTGTGCTCTTGC		
Wnt3a-F	XM_018051579.1 in GenBank	21	F: GAGTTCGGCGGGATGGTGTCT	91	60
Wnt3a-R		21	R: CCTCATTGTTGTGGCGGTTCA		
BSP-F	The present study	21	F: TTTTTTTTAGTGGTGTGTGGG	429	60
BSP-R		21	R: TTACAAAATCCRCAAATATCC		
chi-miR-424-5p	MIMAT0036222 in miRBase	23	F: CGCAGCAGCAATTCATGTTTTGA	Not available	61
chi-miR-497-5p	MIMAT0036259 in miRBase	24	F: CGAGCAGCACACTGTGGTTTGTAC	Not available	60
chi-miR-15a-5p	MIMAT0035990 in miRBase	23	F: TAGCAGCACATAATGGTTTGTGG	Not available.	60

[a] F: forward, R:reverse.
[b] *Ta*: annealing temperature.

designed the miRNA-specific sense primers according to the method recommended by Benes and Castoldi [26]. The universal anti-sense primers were used for the analyzing miRNAs which were obtained from the miRNA detection kit (TaKaRa, Dalian, China). In Table 1, we listed the detailed information of all primers used in this investigation.

For the detecting of lncRNA-599554 and mRNA of indicator genes, we reversely transcribed the first strand cDNA from total RNA using M-MuLV cDNA Synthesis Kit (Sangon, Shanghai, China). For detecting miRNAs, the first strand cDNA was reversely transcribed using One Step PrimeScript miRNA cDNA synthesis kit (TaKaRa, Dalian, China) according the manufacturer's recommendation. On a LightCycler 480 Real Time PCR system (Roche Diagnostics, Mannheim, Germany), real-time PCR reaction was carried out with SYBR Green I assay (TaKaRa, Dalian, China). The following thermal cycling conditions were used in real-time PCR reactions: 95°C for 4 min, followed by 35 cycles of 95°C for 30 s, 52–61°C (Table 1) for 30 s, and 72°C for 30 s. The last cycle was followed by a melting curve analysis ranging from 56 to 95°C with a ramp speed of 0.5°C per 10 s, to verify a single product being amplified for each pair primers. Each sample was conducted in triplicate, and a negative control with cDNA template was incorporated in each measurement.

2.5. Methylation analysis of Wnt3a promoter in DPCs

The sequence of goat Wnt3a gene (under chromosome 7: NC_030814.1) was retrieved from goat genome database (https://www.ncbi.nlm.nih.gov/genome/?term=goat). Subsequently, we performed a search for potential CpG island in the immediate upstream of transcriptional start site (TSS: NC_030814.1:105708614) within range of 1000-nt through the Methyl Primer Express program (Version 1.0, Applied Biosystems, CA, USA). Within the amplification region of 429 nt, the potential binding sites of transcription factors were predicted using online JASPAR procedure (http://jaspar.genereg.net). The JASPAR is an open-access database with its web framework, and it contains a curated, nonredundant set of profiles, which are derived from published and experimentally defined transcription factor binding sites for eukaryotes [27]. The relative score was set to be greater than 93.00%.

The genomic DNA from DPCs was extracted *via* the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China), and the obtained DNA was further treated using MethylCode™ Bisulfite Conversion Kit (Invitrogen, Shanghai, China). Using the Methyl Primer Express program (Version 1.0, Applied Biosystems, CA, USA), a pair of Bisulfite Sequencing PCR (BSP) primers: BSP-F and BSP-R) were designed whose potential amplification region was 429-bp in length targeting part of the CpG island (564-nt) with 32 CpG sites. The BSP reactions were performed using the primer pair BSP-F and BSP-R. After purifying with DNA purification kit (TaKaRa, Dalian, China), the resultant PCR products were ligated to the pMD18-T Vector (TaKaRa, Dalian, China), and propagated into *Escherichia coli* DH5α cells. For each group of DPCs, 10 positive clones were sequenced in both directions with the sequencing results being measured by the QUMA program [28].

2.6. Dual-luciferase reporter assays

In this study, we carried out the dual-luciferase reporter analysis following the method described in previous publication by Yu et al. [24]. In brief, Wnt3a CDS region fragment of goat harboring the putative chi-miR-15a-5p binding sites were inserted into the pGL3 Basic vector (Promega, Madison, WI, USA) that is immediate downstream of coding region of luciferase gene. Based on the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), DPCs were transfected with the goat Wnt3a CDS fragment report vector according to the manufacturer's recommendation. The transfected cells were cultured in the abovementioned conditions of cell culture for 48 h. Subsequently, we consecutively detected the luciferase activity by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Finally, the ratio of Firefly to Renilla luciferase activity was determined to eliminate the potential difference from the transfection efficiencies.

2.7. Statistical analysis

All obtained data was expressed as mean ± SEM. We conducted statistical analysis using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA). For normalizing the gene expression level, the geometric mean of three internal reference genes: UBC, YWHAZ and SDHA were used as recommended in our previous publication [29]. For normalizing the miRNA expression level, the internal reference miRNAs: chi-let-7d-5p, chi-miR-26a-5p, and chi-miR-15a-5p were used based on the recommendation in a previous study [30]. The means between two groups was compared with Student's *t*-test. *P* value less than 0.05 was considered to be statistically significant. All data results were obtained from three replicates with three independent experiments.

3. Results and discussion

3.1. Molecular characterization of lncRNA-599554 in cashmere goat

The cDNA sequence of lncRNA-599554 was provided in Fig. 1a. It is 465-nt long and harbored one potential open reading frame (ORF)

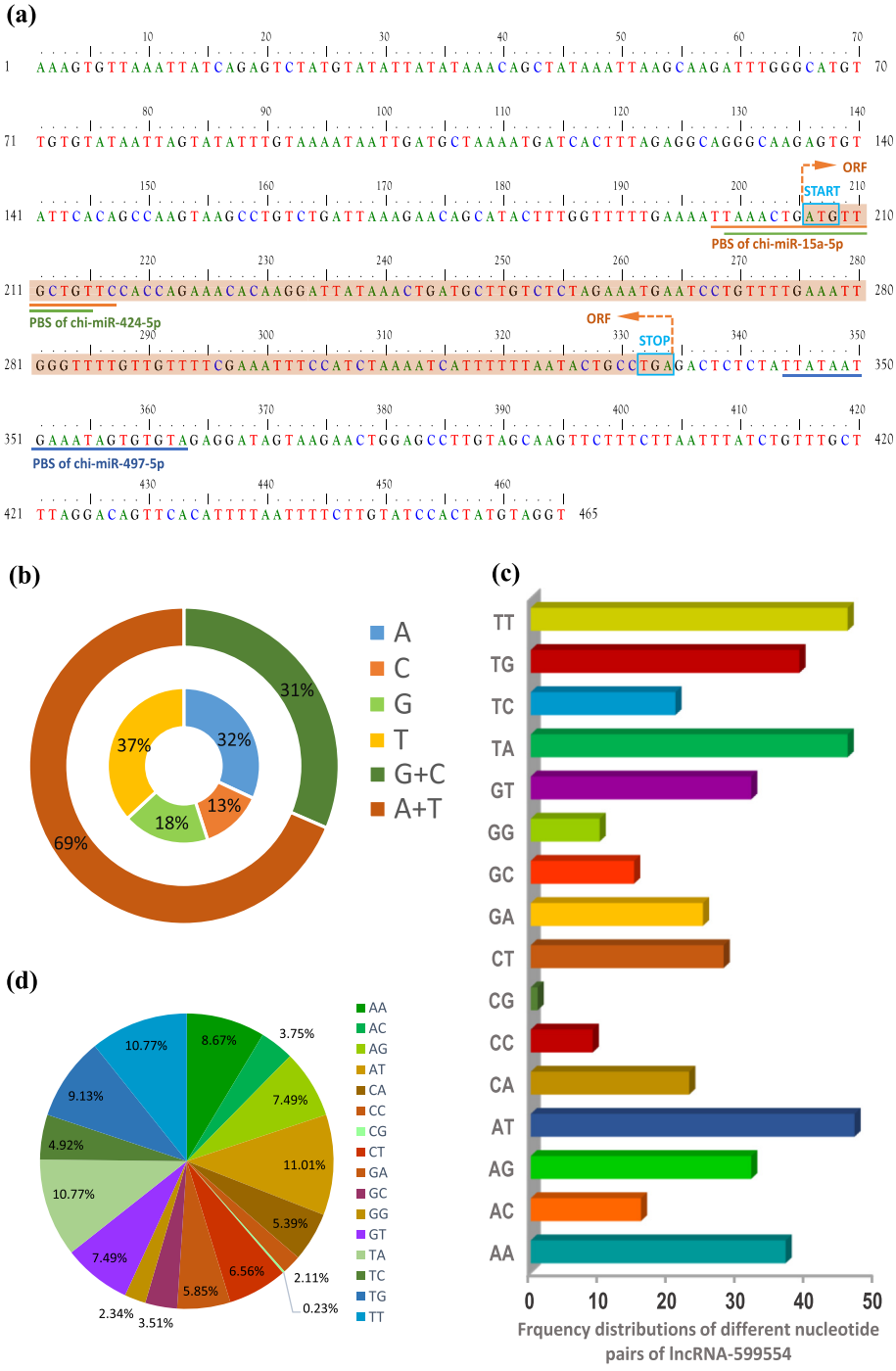

Fig. 1. Molecular characterization analysis of lncRNA-599554 sequence. (a) Display of LncRNA-599,547 cDNA sequence. The ORF Finder at NCBI (https://www.ncbi.nlm.nih.gov) was used for revealing potential ORF that was indicated by shadow regions with yellow colors. The start and stop codons of the ORF were boxed by bright blue color. The region of no significant ORF was indicated by horizontal arrows. The potential binding sites (PBS) of different miRNAs on lncRNA-599554 were indicated by underline sharing the same color with the corresponding miRNA name. (b) Nucleotide composition of lncRNA-599554 with a combinational analysis of guanine (G) + cytosine (C), and adenine (A) + thymidine (T). (c) The distribution of different nucleotide pair of lncRNA-599554 in cashmere goat. (d) The composition percent of different nucleotide pair of lncRNA-599554 in cashmere goat.

which was determined by the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). The revealed ORF is 129-nt long with the start codon ATG and stop codon TGA, which is line with the previous finding that the longest ORF should not be more than 300-nt in a lncRNA molecule [31]. On the other hand, we found that lncRNA-599554 also contains the potential binding sites of three miRNAs: chi-miR-15a-5p, chi-miR-424-5p, and chi-miR-497-5p (Fig. 1a). Through analyzing the nucleotide composition, it is indicated that Adenine (A) and Thymine (T) are the dominant nucleotides in the lncRNA-599,547 of cashmere goat (Fig. 1b), which are consistent with the investigated results from human 6136 lncRNAs [32]. We analyzed the frequency distribution of nucleotide pair of lncRNA-599554 along with the percentage of each pair. As shown in Fig. 1c and d, we found that AT, TA, and TT nucleotide pairs were revealed to be higher in frequency distribution of lncRNA-599554 with a percentage of 11.01%, 10.77%, and 10.77%, respectively. *In vivo*, the functional roles of AT, TA, and TT-rich pair in RNA molecules remains to be further determined; interestingly, several motifs of continuous A or T was observed in lncRNA-599554, such as the continuous A motif at sites 93–96, and the continuous T motif at sites 317-322. It is known that continuous A motif in RNA molecule can be specifically recognized by the PABP protein that is implicated in the stabilization or degradation of RNA molecules [33]. The continuous T motif can be specifically recognized by Sxl protein which is implicated in the alternative splicing event of RNA molecule [34]. Therefore, we speculate that motifs of continuous A or T may mean further biological significances in modulating its target genes that may be implicated in the main physiological process of cashmere goat SHF.

3.2. Expression pattern of lncRNA-599,547 in dermal papilla and its functional roles in regulating the inductive property of DPCs

Two main stages of cashmere goat SHF cycle were investigated to characterize the expression pattern of lncRNA-599554 in dermal papilla including anagen and telogen stages. As observed in Fig. 2a, lncRNA-599554 exhibited significantly higher expression at anagen dermal papilla than in the counterpart of telogen. It is well known that compared with telogen, the dermal papilla of SHF at anagen is highly active, during which the regeneration of SHF along with the formation of new cashmere fiber are thought to deeply rely on the strong inductive property of DPCs [3]. This was also further supported by the significantly higher expression of several indicator genes on inductive property of DPCs at anagen SHF than the counterpart at telogen SHF of cashmere goat including Et-1, Scf, Versican, Alp, Ptc-1 and Lef1 (data not shown). Thus, we speculate that lncRNA-599554 may be implicated in modulating the DPC inductive properties of cashmere goat.

To define this hypothesis, both overexpression and siRNA interference experiments of lncRNA-59554 were carried out in DPCs to determine whether it plays certain roles in regulating the DPC inductive property of cashmere goat. This was evaluated through analyzing the expression changes of the typical indictor genes on DPC

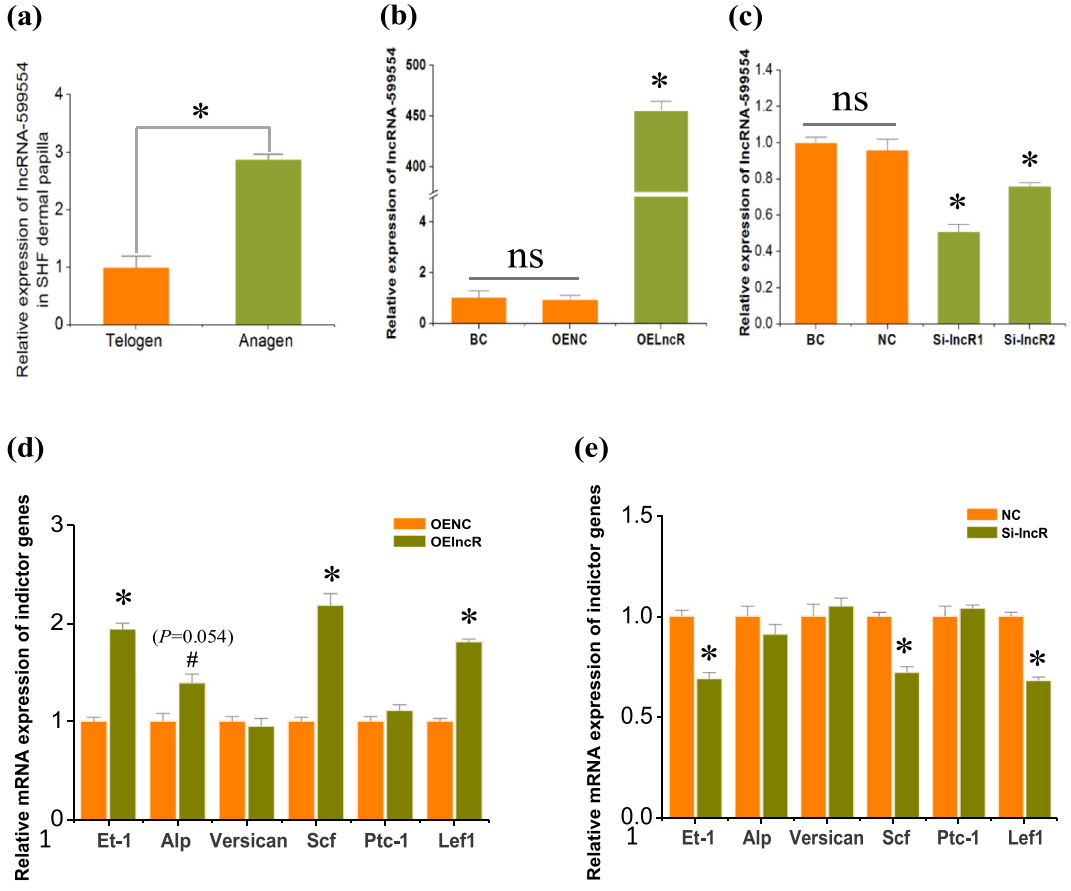

Fig. 2. Expression characterization of lncRNA-599554 in SHF dermal papilla of cashmere goat and its effects on the indictor genes on inductive property of DPCs. (a) Expression detection of lncRNA-599554 in SHF dermal papilla of cashmere goat at telogen and anagen. (b) Efficiency analysis of lncRNA-599554 overexpression in DPCs. (c) Knockdown efficiency analysis of Si-lncR1 and Si-lncR2 to lncRNA-599,547 in DPCs, respectively. (d) Overexpression of lncRNA-599554 led to the significant increase in the expression of indictor genes: Et-1, Alp, Scf and Lef1 in DPCs. (e) Knockdown of lncRNA-599,547 significantly suppressed the expression of indictor genes: Et-1, Scf and Lef1 in DPCs. BC = blank cell group, OENC = overexpression negative control group, OElncR = overexpression lncRNA-599554 groups, NC = negative control group, and Si-lncR = Si-lncR interference group. ns = no significant difference. Error bar represented the standard deviation within the group. The asterisk (*) was used to indicate the significant difference ($P < 0.05$).

inductive property including Et-1, Scf [35], Versican, Alp [9,36,37], Ptc-1 and Lef1 [38]. The overexpression of lncRNA-599554 in DPCs was verified by the quantitative PCR technique. As observed from Fig. 2b, the relative expression of lncRNA-599554 was upregulated approximately 450 times in the overexpression group (OELncR) in comparison to the blank control group (BC) and the negative control group (OENC), whereas no significant difference was recorded in the expression of lncRNA-599554 between the BC group and the OENC group (Fig. 2b). The expression changes of six indictor genes (Et-1, Scf, Versican, Alp, Ptc-1 and Lef1) on DPC inductive property were also analyzed between the OENC group and the OElncR group. Interestingly, we found that the expression of Et-1, Scf and Lef1 was significantly upregulated in the OELncR group compared with the counterpart of OENC group (Fig. 2d). Additionally, the overexpression of lncRNA-599554 also led to an increasing expression of Alp in DPCs compared with the OENC group with a *P*-value of 0.054, although the difference was not significance (Fig. 2d). On the other hand, siRNA interference analysis on lncRNA-599554 was carried out in DPCs of cashmere goat by using two independent siRNAs: Si-lncR1 and Si-lncR2. The Si-lncR1 was revealed to be more efficient (~50.00%) in interference expression on lncRNA-599554 than the counterpart of Si-lncR2 (Fig. 2c). Therefore, the Si-lncR1 was used for the siRNA interference experiments of lncRNA-59,554 in DPCs of cashmere goat. The obtained results are presented in Fig. 2e. As observed from Fig. 2e, the knockdown of lncRNA-599554 led to the significantly decreasing expression of three indictor genes on DPC inductive property, including Et-1, Scf and Lef1.

Previously, based on immunocytochemistry along with *in situ* hybridization techniques, it was demonstrated that the expression level of Et-1 and Scf in DPCs was positively associated with the ability of DPCs to induce hair follicle regeneration [35]. It was thought that the Lef1 might act as a key hair inducing gene in DPCs to promote the regeneration of hair follicle [38]. On the other hand, in several studies, it was revealed that the expression of Alp was implicated in the hair-inducing ability of DPCs, namely, the stronger its expression in DPCs means the better ability of DPCs in inducing hair follicle regeneration [9,36,37]. Therefore, taken with our results from both overexpression and siRNA interference experiments of lncRNA-59,554 in DPCs, it is apparent that lncRNA-599554 may be implicated in positively regulating the inductive property of DPCs in cashmere goat.

3.3. LncRNA-599554 directly interacts with chi-miR-15a-5p and may regulate its expression in DPCs

It is well known that lncRNAs can prevent endogenous miRNAs from binding to their target mRNAs through serving as miRNA "molecular sponge" [39,40]. Through bioinformatics analysis, it was revealed that three miRNAs had potential binding target sites on lncRNA-599554, including chi-miR-15a-5p, chi-miR-424-5p, and chi-miR-497-5p (Fig. 3a). In order to define which miRNAs may directly interact with lncRNA-599554, here, we conducted a biotin-labeled RNA pull-down analysis. As shown in Fig. 3b, in the lncRNA-599554 pulled down pellet, significantly higher enrichment of lncRNA-599554 was verified compared with the negative control prober (Bio-NC-prober) based on the use of real-time PCR technique ($P < 0.05$). Interestingly, we found that the lncRNA-599554 pulled down pellet also contained a significantly higher amount of chi-miR-15a-5p, but not for chi-miR-424-5p and chi-miR-497-5p in comparison to the corresponding Bio-NC-prober which was evaluated by real-time PCR analysis (Fig. 3c). These results suggested that lncRNA-599554 might interact with chi-miR-15b-5p through serving as its "molecular sponge".

In order to determine whether lncRNA-599,547 regulates the expression of chi-miR-15b-5p, overexpression analysis of lncRNA-599554 in DPCs were carried out through AdEasy Adenoviral vector assay. The obtained results are presented in Fig. 3d, from which we noted that the overexpression of lncRNA-599,547 significantly downregulated the expression of chi-miR-15b-5p in DPCs compared with the negative control group ($P < 0.05$). Correspondingly, based on the use of siRNA Si-lncR1, the knockdown of lncRNA-599554 led to a significant increase of chi-miR-15b-5p in expression (Fig. 3e). On the other hand, we found that the overexpression or knockdown of chi-miR-15a-5p did not lead to the significant changes of lncRNA-599554 in DPCs (data not shown). These results indicated that lncRNA-599554 might regulate the expression of chi-miR-15a-5p in DPCs. More recently, in interstitial cells of human primary valve, a similar regulatory pattern was also revealed by Yu et al. [24] where the authors found that the expression of miR-204-5p was negatively regulated by lncRNA-TUG1.

However, several indicated that a single lncRNA might play roles in multiple biological processes in cells, such as cell differentiation, X chromosome inactivation, splicing, and cellular structural maintenance [41,42,43,44]. Moreover, it was thought that lncRNAs could function *via* multiple regulatory mechanisms, such as genomic imprinting [45], gene transcriptional regulation [46], and epigenetic control [47]. In the present work, we found that lncRNA-599554 directly interacts with chi-miR-15a-5p (Fig. 3c), and negatively regulates its expression in DPCs (Fig. 3d and e). However, the other biological significance of lncRNA-599554 should be further investigated in DPCs of cashmere goat, like its potential effects on the DPC proliferation along with differentiation.

3.4. LncRNA-599554 promotes the expression of Wnt3a but not through modulating its promoter methylation level

In previous studies, it was demonstrated that Wnt3a can maintain the inductive properties of DPCs [48]. Moreover, the expression of Wnt3a with a low level by a subset of cells in the enriched DPCs population is sufficient to maintain their inductive activity, which further suggests that Wnt3a plays an essential role in the hair inductive activity of DPCs [49]. These previous findings motivated us to further investigate whether the overexpression or knockdown of lncRNA-599554 can change the expression of Wnt3a in DPCs. The obtained results are presented in Fig. 4. As observed from Fig. 4a, the overexpression of lncRNA-599554 in DPCs led to the significant increase of Wnt3a in expression. Conversely, the knockdown of lncRNA-599554 with Si-lncR1 in DPCs led to a significant decrease of Wnt3a in expression (Fig. 4b). Thus, it appears to become apparent that lncRNA-599554 was positively implicated in the expression of Wnt3a in DPCs through certain mechanisms.

It is well established that DNA methylation within the promoter region of many genes acts as essential regulatory elements in modulating their transcriptional expression but no alteration in nucleotide sequence [50]. This promotes us to ask whether lncRNA-599554 modifies the methylation level of Wnt3a gene promoter region, thereby to change the expression level of Wnt3a in DPCs. In order to define this hypothesis, we further investigated the potential effects of lncRNA-599554 on the methylation modification of Wnt3a promoter in the DPCs treated with overexpression or siRNA interference assay of lncRNA-599554. As observed from Fig. 4c, a CpG island of 564-nt in length exists in the upstream of transcription starting site of goat Wnt3a gene. Using the primer pair: BSP-F and BSP-R, we amplified a fragment of 429-bp in size that harbors 32 CpG sites (Fig. 4c) and spanned potential binding sites of multiple transcriptional factors such as ZEB1, TCF3/4, YY1, TFAP2A and TCG3 (Fig. 4d).

In Fig. 4e and f, we provided the methylation detecting results on Wnt3a promoter in DPCs treated with lncRNA-599554 overexpression or siRNA interference assay. As shown in Fig. 4e, the ratios of methylated CpG sites of Wnt3a prompter is 17.19% and 18.44% in the DPCs of negative control and overexpression groups, respectively. Unexpectedly, highly similar results were observed within the analyzed promoter region of Wnt3a in the DPCs treated with lncRNA-599,547 siRNA interference assay where the ratios of methylated CpG sites is 16.56% and 17.50% in siRNA interference and negative groups,

(a)

lncRNA /miRNA	Structure	Position	Site type	ΔG (Kcal/mol)
lncRNA-599554	5' U G U C 3' UAAACU AUG UGCUGUU GUUUGG UAC ACGACGA	198-217	7 mer	-20.00
chi-miR-15a-5p	3' GGU UAA U 5'			
lncRNA /miRNA	Structure	Position	Site type	ΔG (Kcal/mol)
lncRNA-599554	5' U U 3' AAAC UGA UGUUGCUG UUUG ACU ACGACGAC	199-215	7 mer	-19.80
chi-miR-424-5p	3' AGU U UA 5'			
lncRNA /miRNA	Structure	Position	Site type	ΔG (Kcal/mol)
lncRNA-599554	5' U UGAA A 3' UAUAA AUAGUGUGU AUGUU UGUCACACG	344-363	4 mer	-19.30
chi-miR-497-5p	3' UGG ACGA 5'			

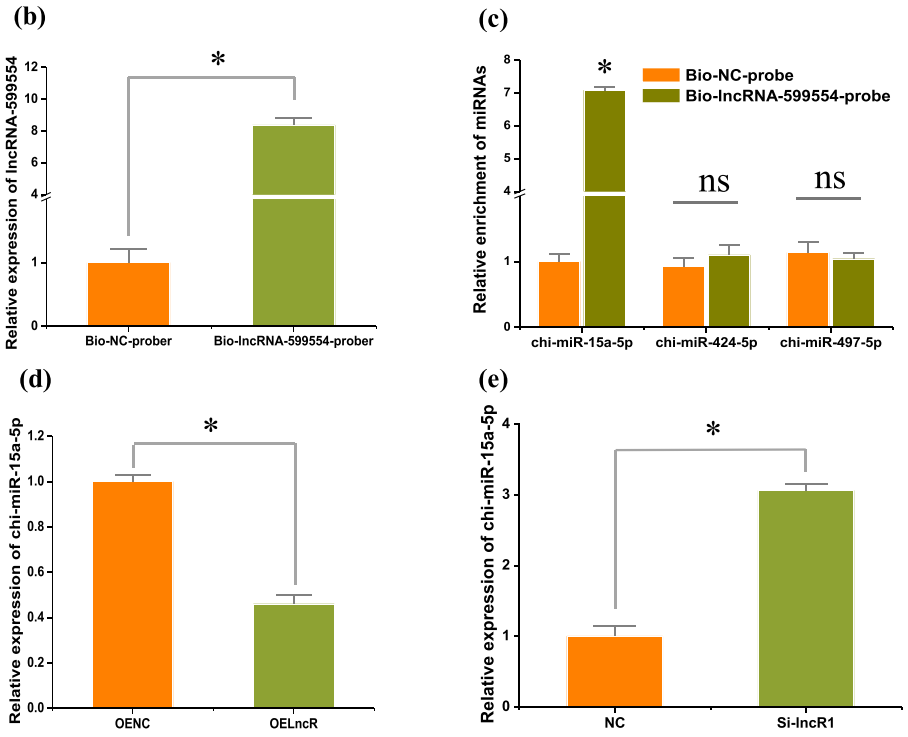

Fig. 3. LncRNA-599554 directly interacts with chi-miR-15a-5p, and modifies its expression in DPCs. (a) The analysis of potential binding sites of chi-miR-15a-5p, chi-miR-424-5p, and chi-miR-497-5p on lncRNA-599554 sequence. (b) Analyzing results of lncRNA-599554 in the same sample pulled down by Bio-lncRNA-599554 probe and Bio-NC-probe (c) Analyzing results of chi-miR-15a-5p, chi-miR-424-5p, and chi-miR-497-5p in the sample pulled down by the Bio-lncRNA-599554 probe and Bio-NC-probe, respectively. (d) Analyzing results of chi-miR-15a-5p in DPCs treated with lncRNA-599554 overexpression assay. (e) Analyzing results of chi-miR-15a-5p in DPCs treated with Si-LnR1 assay. Bio-NC-probe = negative control probe group, Bio-lncRNA-599554 probe = biotinylated lncRNA-599554 probe group, OENC = negative control group, OELncR = overexpression lncRNA-599554 groups, NC = negative control group, and Si-lncR1 = knockdown group of lncRNA-599554. Error bar represented the standard deviation within the group. The asterisk (*) was used to indicate significant difference ($P < 0.05$).

respectively (Fig. 4f). Thus, it appears to become apparent that lncRNA599554 positively regulates the expression of Wnt3a in DPCs which is not through modifying the methylation degree of its promoter region. Namely, the methylation modulation of Wnt3a promoter is not involved in the observed regulation of lncRNA-599554 on Wnt3a expression in DPCs of cashmere goat. However, a functional mechanism has been identified that lncRNA can regulate its target gene expression which can be achieved *via* modulating the promoter methylation degree of its target gene. For example, in the Wnt signaling pathway, it was demonstrated that lncRNA-HOTAIR, as an essential regulator of hair inductive property of DPCs, can lead to the decrease of Wif-1 in expression through modulating its promoter methylation degree [13,51,52]. Thus, it should be pointed out that herein our experiments were performed in the DPCs *in vitro*, and further investigation would be needed *in vivo* for defining whether the methylation modulation of Wnt3a promoter might be implicated in the positive regulation of lncRNA-599554 on its transcriptional expression in DPCs.

3.5. LncRNA-599554 positively regulates the transcriptional expression of Wnt3a via chi-miR-15a-5p

Studies indicate that lncRNAs may sponge miRNAs from their nature target mRNAs which leads to a decrease of active miRNAs, thereby de-inhibits their target mRNAs [53,54,55]. This draws us to ask whether the observed positively regulation of lncRNA-599554 on Wnt3a expression may be achieved through miRNA-mediated pathway. In this study, we have demonstrated that lncRNA-599554 directly interacted with chi-miR-15a-5p (Fig. 3c). Based on bioinformatics analysis, a screening was performed for potential binding sites of chi-miR-15a-5p in Wnt3a mRNA sequence. A potential binding site was

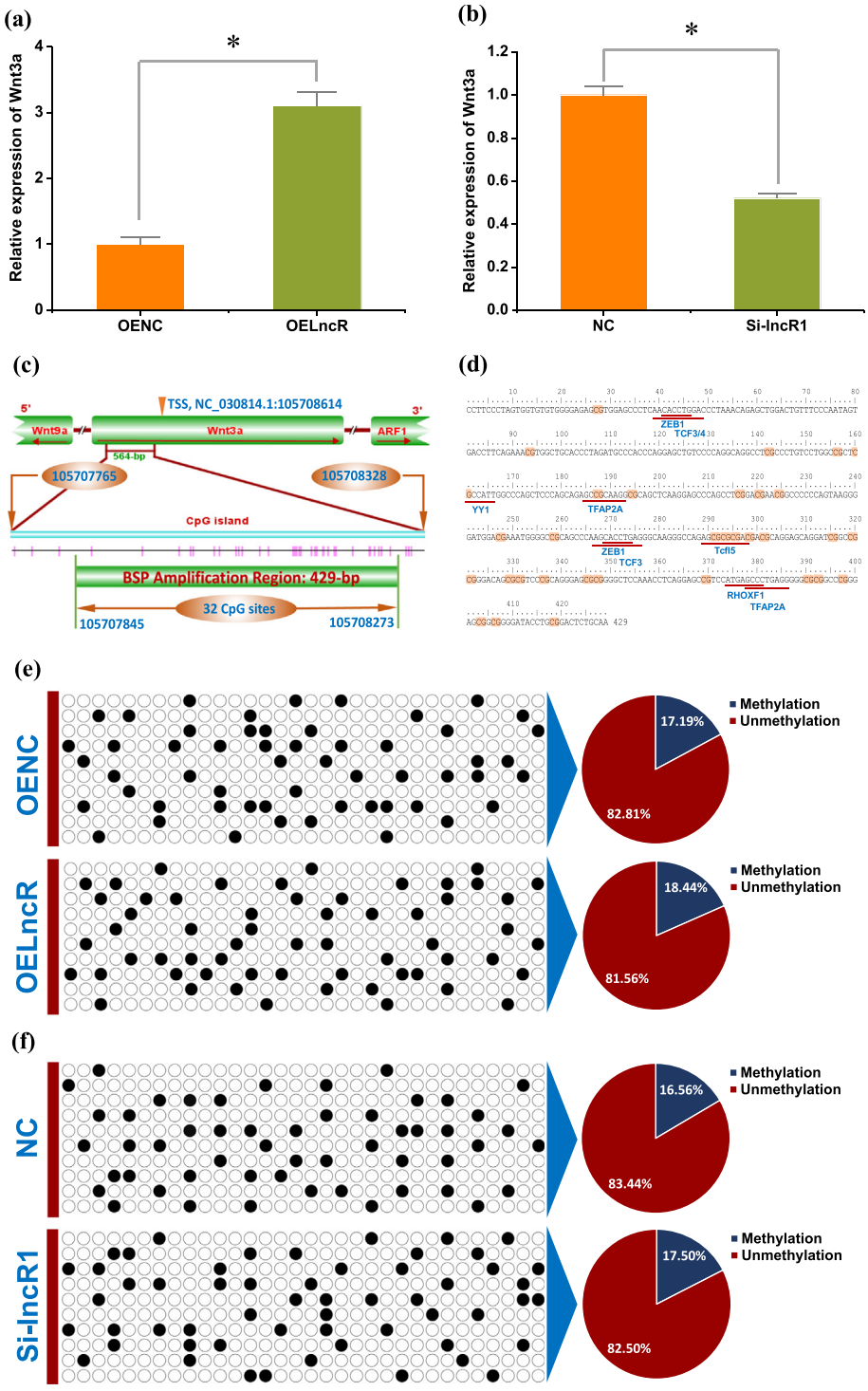

Fig. 4. Effects of LncRNA-599554 on Wnt3a expression and its promoter methylation in DPCs. (a) Analyzing results of Wnt3a mRNA in DPCs treated with lncRNA-599554 overexpression assay. (b) Analyzing results of Wnt3a mRNA by in DPCs treated with Si-lnR1 assay. Error bar represented the standard deviation within the group. The asterisk (*) was used to indicate the significant difference ($P < 0.05$). (c) A graph of CpG islands in promoter region of Wnt3a gene. The corresponding nucleotide positions within Wnt3a gene are determined by the NC_030814.1 sequence on chromosome 7 of goat genome at NCBI databank (https://www.ncbi.nlm.nih.gov/genome/?term=goat).The CpG sites in analyzed region were indicated with pink short vertical lines. (d) Transcription factors having potential binding sites in the BSP amplification region of Wnt3a promoter of cashmere goat. The CpG sites were highlighted by yellow shadow, and the potential binding sites of transcription factors were underlined with corresponding name of the transcription factor. (e)The methylation analysis of Wnt3a promoter in DPCs treated with overexpression of lncRNA-599554 assay. (f) The methylation analysis of Wnt3a promoter in DPCs treated with Si-lnR1 assay. A total of 32 CpG sites were analyzed within the amplification region of 429-bp with ten clones being sequenced for each group. For each clone, the analyzing results were presented in a line. The methylated CpG sites were indicated with filled black circles, whereas, the unmethylated CpG sites was indicated with unfilled white circles. Pie charts were applied to indicate the methylation percentage of analyzed region of Wnt3a promoter for different DPC groups. The site-specific methylation degree was displayed with the QUMA program (Kumaki et al., 2008). OENC = negative control group, OELncR = overexpression lncRNA-599554 groups, NC = negative control group, and Si-lncR1 = knockdown group of lncRNA-599554.

revealed within the Wnt3a mRNA CDS that is rather close to its 3'-UTR with a 6 mer binding type in seed region of chi-miR-15a-5p (Fig. 5a).

Therefore, the Dual-luciferase Reporter Assays were used to verify the predicted binding of chi-15a-5p on lncRNA-599554 sequence where the reporter vector of goat Wnt3a mRNA CDS fragment was used that contained the predicted binding site for chi-miR-15b-5p. We presented the obtained results in Fig. 5b and c. Compared with the negative control, the overexpression of lncRNA-599554 led to a significant increase in the relative Wnt3a CDS luciferase activity in DPCs (Fig. 5b). In contrast, a significant decrease in luciferase activity was recorded in the DPCs treated with Si-lncR1 knockdown assay of lncRNA-599554 in comparison to the negative control DPCs (Fig. 5c). Thus, we confirm that the Wnt3a expression is regulated by lncRNA-599554 through the chi-miR-15a-5p mediated pathway. Taken together with the above results from this study, it can be drawn that lncRNA-599554 sequesters chi-miR-15b-5p to upregulate the Wnt3a expression, hereby, further contribute the inductive property of DPCs. In a previous investigation, several lncRNAs are also implicated that they contribute the inductive property of mouse DPCs, such as H19, HOTAIR and RP11-766 N7.3 [13]. In cashmere goat, we showed that lncRNA-000133 could contribute the inductive property of DPCs [4]. Thus, it can be suggested that a larger range of lncRNA molecules might be implicated in the inductive property of DPCs of cashmere goat.

On the other hand, based on the interactional mechanism of lncRNA-miRNA-mRNA, it was widely accepted that a single lncRNA might modulate several mRNA targets, and conversely, a single mRNA targets might be cooperatively modulated by multiple different lncRNAs [56,57,58]. Here, we showed that Wnt3a expression was positively modulated by lncRNA-599554 through the chi-15a-5p mediated pathway in DPCs of cashmere goat. However, it should be further determined that whether lncRNA-599,547 could modulate other key regulators implicated in the inductive property of DPCs in cashmere goats, or the expression of Wnt3a might be regulated by other lncRNAs identified in SHF of cashmere goat.

In conclusion, we verified that lncRNA-599554 had significantly higher expression at anagen dermal papilla than the counterpart at telogen of cashmere goat. Further, we showed that lncRNA-599554 could contribute the inductive property of DPCs which might be achieved through sponging chi-miR-15a-5p to upregulate the expression of Wnt3a in DPCs. Our results provided novel evidence for revealing the functional mechanism of lncRNA-599554 in the SHF regeneration and the formation and growth of cashmere fiber in cashmere goat.

Ethical approval

In this study, the entire experimental protocols were approved by Ethics Committee of Shenyang Agricultural University (Shenyang, China) with the approval number: No. 201606005.

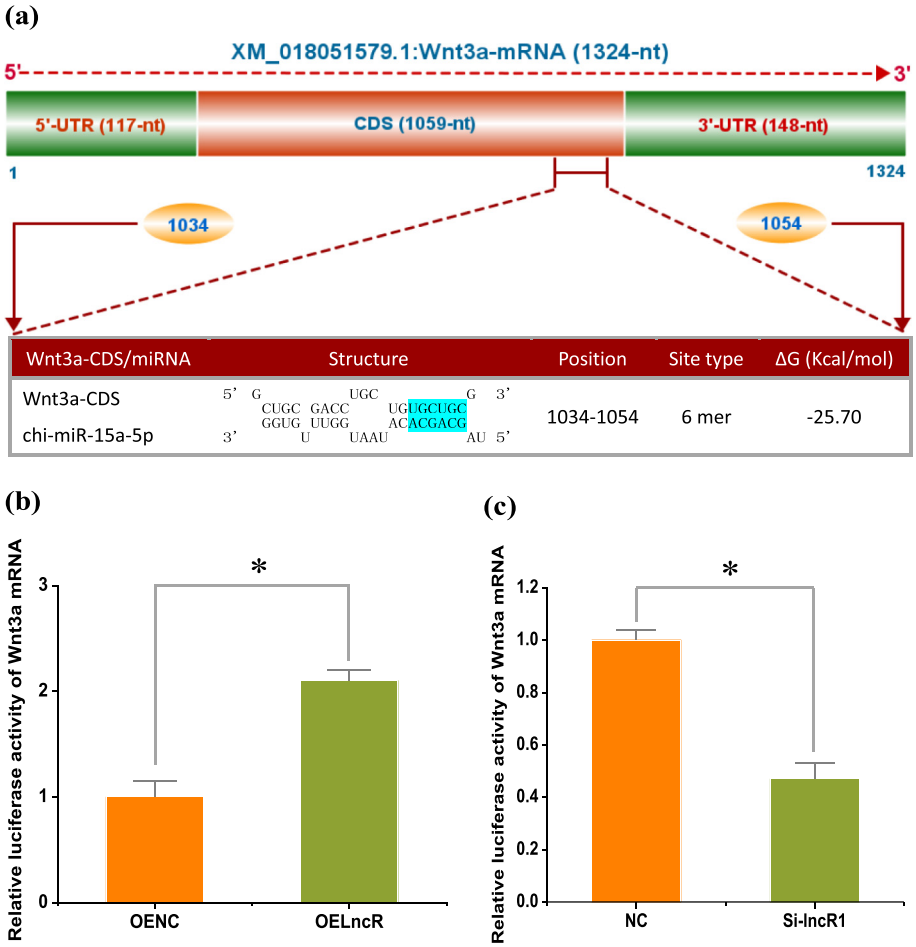

Fig. 5. The effect of chi-miR-15a-5p on Wnt3a expression which was analyzed using Dual-luciferase Reporter Assays. (a) A structural graph of goat Wnt3a mRNA and the analysis of potential binding sites of chi-miR-15a-5p on its mRNA sequence where the nucleotide positions are determined based on the goat Wnt3a mRNA sequence under accession number: XM_018051579.1 at GenBank (https://www.ncbi.nlm.nih.gov). (b) Relative luciferase activities of Wnt3a mRNA reporter in DPCs transfected with OENC or OElncR. (c) Relative luciferase activities of Wnt3a mRNA reporter in DPCs transfected with NC or Si-lncR1. OENC = negative control group, OELncR = overexpression lncRNA-599554 groups, NC = negative control group, and Si-lncR1 = knockdown group of lncRNA-599554. The asterisk (*) was used to indicate significant difference ($P < 0.05$).

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Conflict of interest

The authors declare that there are no conflicts of interest.

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