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High-Throughput Sequencing Reveals miRNAs Affecting Follicle Development in Chicken

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Abstract: As the derivative of chicken skin, hair follicle is capable of self-renew. Its proliferation and differentiation result in hair formation. MicroRNAs (miRNAs) can effectively regulate gene expression at the post-transcriptional level and play a critical role in tissue growth, development. In this study, we used next generation sequencing technology sequenced miRNAs of the hair follicle derived from the 13 day-old chicken (*Gallus gallus*) embryos in which from Kirin chicken and Huaixiang chicken that feathers having morphogenesis with significantly different curling. A population of conserved miRNAs was identified. These conserved miRNAs were derived from 638 homologous hairpin precursors across 5 animal species. We identified a total of 645 miRNAs in the chicken embryos. Among them, 11 differentially expressed miRNAs were identified (>±2 Fold, p value <0.05) by comparing Kirin chicken and Huaixiang chicken. Several gene ontology (GO) biology processes and the WNT, BMP and TGF-β signaling pathways were found to be differentially expressed miRNAs as part of hair follicle development process. The miR-1623 has an effect on WNT4 and involved in hair follicle cell development. This study has identified miRNAs that associated with the chick embryonic hair follicle development and identified some target miRNAs for further research into their role played in feather growth.

Keywords: Chicken Embryo, Follicle, Mirnas

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that suppress gene expression post-transcriptionally, and function important roles in diverse biological processes [1]. Hundreds of miRNA genes have been found in diverse animals, and many of these are phylogenetically conserved [2]. In addition to endogenous presence in cells, miRNAs can also be actively released into extracellular fluids through exosomes or microvesicles [3, 4]. Consequently, miRNA research has become a hot spot in the field of biological for explaining molecular formation mechanisms [5] and important traits of animals [6-9]. The skin plays an important protection role in animal existence and it evolves with the animal bifurcation.

The feather is one of the most complex integumentary appendages due to the extensive diversity in shape, size, arrangement and pigmentation, and is therefore an excellent model for evolutionary and developmental biology as variations can occur at each step of development and differentiation [10-13]. The Kirin chicken can adapt to high-temperature environment because of unique frizzled feather branching structure characteristic, rachis stout and outwardly curved, barbs short sparse, feather hook can't connect with the back edge of the adjacent twig lead to pinna can not closed. Recent advances that frizzled feather is caused by *KRT75* mutation reside in autosomal, belong to incomplete dominant inheritance [14]. Feathers develop from the hair follicle, therefore the hair follicles numbers, diameter with feathers growth have a direct relationship [15, 16]. And miRNAs connected with hair follicles developmental processes [5, 17] and regulate hair follicle development and hair growth [18, 19]

In this study, we investigated the expression profile of miRNAs in the follicles of 13-day chicken embryos from the Kirin chicken (KRC) and Huaixiang chicken (HXC). The results demonstrate that chicken embryonic follicle contains large amounts of miRNAs.

2. Materials and Methods

2.1. Ethics Statement

All chicken embryos experiments were approved and reviewed by the local ethical committee and the procedures in this study followed the guidelines of the Guangdong Ocean University Animal Care and Use Committee. To minimize the suffering of animals, sodium pentobarbital anesthesia was used before the collection of chicken skin hair follicles samples.

2.2. Collection of Chicken Embryonic Follicle Samples

Fertile eggs were collected from 45-wk-old KRC and HXC. Fertile eggs were incubated at 37.8°C with 65 to 75% humidity and intermittent rotations, which to provided 2-3cm skin tissue were obtained from the back of the body of 13-day chicken embryos, separately. Samples of hair follicle stored at −80°C until used.

2.3. Small RNA Library Preparation and Sequencing

Collected feather follicle from six chicken embryos at the age of 13-day were used to construct two small RNA libraries in this study. These samples included three KRC ones and three HXC, respectively.

Total RNA was extracted from the follicle using TruSeq Small RNA Sample Pre Kits (Illumine, San Diego, USA) according to the manufacturer's instructions. Total RNA quality was checked with a Bioanalyzer 2100 (Agilent Technologies, USA). The RIN was > 8.0 and A260/A280 was > 2.1 for all samples. The equal concentration total RNAs of six samples were constructed small RNA libraries by TruSeq Small RNA Sample prep Kit of illumina. The overall flow of the sequencing procedure is as follows: small RNAs ranging from 18 to 35nt in length was purified from 15% polyacrylamide gels, then ligated to 5' and 3' adapters. Reverse transcription was performed, and followed by PCR amplification. The purified PCR products (~140bp) were used directly for cluster generation and sequencing analysis using the Illumina's 2000 Sequencer according to the manufacturer's instructions (Personalbio, ShangHai, China).

2.4. Sequence Data Analysis

Sequence data analysis was done using AGGT101-miR tool. After deleting poor quality reads, adaptor pollution reads and reads less than 18nt, the clean reads were obtained.

The clean reads of small RNAs were aligned to the reference chicken (G. gallus) genome to identify known miRNAs. The sequences that matched perfectly to known miRNAs (miRBase V21.0) were determined as conserved miRNAs. Other small RNAs (rRNA, tRNA, snRNA and snoRNA) were annotated by blasting against the Rfram, Repbase and ncRNA databases.

The unannotated small RNA sequences were aligned to the reference chicken (G. gallus) genome to find potential precursor sequences for novel miRNAs. Novel miRNAs were predicted by RNA-fold tools following the criteria: (1) number of nucleotides in one bulge in stem (< = 12); (2) number of base pairs in the stem region of the predicted hairpin (> = 16); (3) cutoff of free energy (kcal/mol< = -15); (4) length of hairpin (up and down stems + terminal loop> = 50); (5) length of hairpin loop (< = 20); (6) number of nucleotides in one bulge in mature region (< = 8); (7) number of biased errors in one bulge in mature region (< = 4); (8) number of biased bulges in mature region (< = 2); (9) number of errors in mature region (< = 7); (10) number of base pairs in the mature region of the predicted hairpin (> = 12); (11)percent of mature in stem (> = 80). Furthermore, the raw reads > = 10 at least.

To identify differentially expressed miRNAs, the number of conserved miRNAs was normalized to the total number of reads in each sample that matched the chicken (*Gallus Gallus*) genome. P-values for differentially expressed miRNAs (KRC/HXC) were calculated by Fisher's exact-test and Chi square (2×2) test.

2.5. Mirna Target Prediction and Functional Analysis

Target genes of differentially expressed miRNAs were predicted by Target Scan and miRanada. To acquire higher prediction accuracy, only common target genes were considered. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were retrieved using DAVID (http://david.abcc.ncifcrf.gov/).

2.6. Quantitative RT-PCR

Total RNAs of sampled follicle were reverse-transcribed by PrimeScript® RT reagent Kit (TAKARA, DRR037A). The primers (Table 1) were designed by Primer 5.0 (ABI). 5ul RT reaction system included: denatured RNA and RT primer (2 uM) 3.0ul, 5×PrimeScript®Buffer 1.0ul, RNase Free dH_2O 0.6ul, PrimeScript® RT Enzyme Mix I 0.4ul. The RT reactions were performed as follows: 42°C for 15 minutes, 85°C for 5 seconds and hold at 4°C. 20ul real-time PCR reaction system included: 2×SYBR Green Mix with ROX 10.0ul, ddH_2O 8.2ul, Primer mix (10 uM) 0.8ul, cDNA 1ul. The PCR reactions were performed as follows: 50°C for 2 minutes, 95°C for 2 minutes, then 40 cycles with 94°C for 15 seconds and 60°C for 30 seconds.

Table 1. *The list of RT-qPCR primer.*

Primer	sequences (5'to3')	bp
miR-1623	ACCGCAGGCACAGACAGGCAGT	22
miR-U6	TGCTTTGGCAGCACATATACCAA	23
Reverse Primer	GATCGCCCTTCTACGTCGTAT	21
WNT4-F	TCTACGCCATCTCTTCAGCA	20
WNT4-R	AGGCAATGTTATCGGAGCAG	20
β-actin-F	TGCCAGGGTACATTGTGGTA	20
β-actin-R	TGCGTGACATCAAGGAGAAG	20

All experiments were performed on ABI 7900 HT sequence detection system. Each reaction was carried out with 3 replicates. snRNA U6 was used as the control for RT-qPCR. The relative expression level of each miRNA to U6 snRNA was normalized as ΔCp = Cq miRNA - Cq U6RNA [20]. Comparison of relative expression level in different stages

was determined using the $2^{-\Delta\Delta Cp}$ method [21]. Statistical significance analysis of the expression change was performed by one-way ANOVA in SPSS 20.0.

3. Results

3.1. Small RNA Library Construction and Sequencing

To investigate the miRNA expression profile in chicken follicle, High-throughput HiSeq 2000 sequencing yielded 9,714,611 (HXC) and 10,875,477 (KRC) raw reads on average for each group small RNA libraries. After filtered low quality sequences, 9,134,499 (HXC) and 7,919,788 (KRC) clean reads for each group were obtained respectively (Table 2). The histograms of the reads length distribution showed majority were 20nt ~ 24nt (Figure 1). Of these, 35,705 (HXC) and 52,201 (KRC) unique small RNAs were identified.

Table 2. *The hair follicles small RNA sequencing reads of Kirin chicken and Huaixiang chicken.*

Sample	HXC1	HXC2	HXC3	KRC1	KRC2	KRC3
raw reads	9,873,177	9,556,045	11,410,76	11,278,066	11,337,531	10,010,835
clean reads	9,318,257	8,950,741	10,596,63	8,282,543	8,141,768	7,335,054
15-30nt reads	8,881,208	7,869,359	9,238,098	7,733,489	7,519,340	6,329,447
unique reads	36,938	31,801	38,376	56,408	53,568	46,628
miRNA	540	525	572	524	543	520

HXC: wild feather of Huaixiang chickens; KRC: frizzle feather of Kirin chickens.

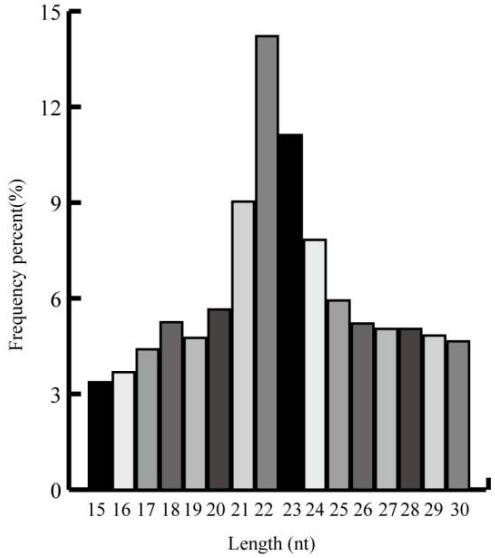

Figure 1. *The histograms of the RNA-seq reads length distribution from 15nt to 30nt.*

3.2. Identification of Conserved Mirnas

To identify conserved miRNAs in chicken follicle, the small RNAs were aligned to current miRBase (Release V21.0). Sequences with perfect matching to known chicken (*Gallus Gallus*) miRNAs were considered as conserved miRNAs. In total, 645 conserved sequences were annotated as chicken miRNAs. To obtain higher reliable results, only the miRNAs with raw reads >10 at least were considered. Then 290 were sorted as common miRNAs, with only one KRC-specific miRNA (gga-miR-1682). All 291 conserved miRNAs detected by sequencing were listed in Table 3.

Table 3 *The conserved miRNAs expressed in the chicken follicle*

miRNA_name	miRNA_seq
gga-miR-6586-5p	TGCTGCCAGATAGAAGTTCACCT
gga-miR-1467-5p	TCTCAGCTACATCGGTGTAAATC
gga-miR-101-1-5p	CGGTTATCATGGTACCGGTGCTGT
gga-miR-1329-3p	CCTCGTAGCTTGATCACGATAT
Continuous the above Table 3	
gga-miR-3607-5p	TACATATGATGAGCTTTGCAGT
gga-miR-6544-5p	TTCAGAAAAGGATATGAATTGT
gga-miR-6555-5p	GATCTGCAGAGCCCACAACTAG
gga-miR-6694-3p	TTAAGAGTAGGGATTCTGTTCC
gga-miR-1800	ACTACGTGATGCGATCTGATG
gga-miR-1737	CAGCACTGCTGCGCTCGGTG
gga-miR-1559-3p	AGTTACATGTATGCATCGAGCA
gga-miR-429-5p	GTCTTACCAGGCAAAGTTAGA
gga-miR-6550-3p	GCTCCACCCTCGGCTGCTTTGA
gga-miR-19b-5p	AGTTTTGCAGGTTTGCATCCCAGC
gga-miR-6548-5p	AACAACAGCTGCGTGCCATGCC
gga-miR-29a-3p	TAGCACCATTTGAAATCGGTTA
gga-miR-6590-3p	TTACTTCTGTTCCTGATCATCA
gga-miR-23b-5p	TGGGTTCCTGGCATGATGATTT
gga-miR-2128	CAGTGACGTCTCTTCCCCGCAGT
gga-miR-6575-5p	TTGTCAGCTTGGGGAAGCTCTT
gga-miR-1727	AAGCTGCTCTAATGAACTGAAG
gga-miR-1591-5p	TGATTCATTGCCTGGCTCTGCA
gga-miR-128-2-5p	GGGGGCCGTTACACTGTAAGAGA
gga-miR-1781-3p	TTTAAATCATGCAGCTGTTGA
gga-miR-6548-3p	CAGAGGTGCCCCGCTGTTGCTGT
gga-miR-6516-3p	TGGTCATGTATGATACTGCACA
gga-miR-1626-3p	TCTGGAAGTTGCCCTGGACGTGT
gga-miR-30b-3p	CTGGGGGGTGGATGTTTACTTC
gga-miR-1666	TAACGCCACGGGGCTGAGGCTG
gga-miR-1644	TCTGTTGTGCAGGGCTGTGCTCT
gga-miR-135a-3-3p	ATGTAGGGCGAAAAGCCATGGG
gga-miR-7460-3p	CCTGACTGAGCTCTGCTTTCTC
gga-miR-1698	CGAGGCTGCGGCAATCCCTGCGC
gga-miR-6582-3p	CACTCTGGGTGTTTCTCTTGCAG
gga-miR-1808	TGTTGGGAATGAATACATATTGT
gga-miR-6566-5p	TGAGGCCCGATGTGTCATTCCTGGA
gga-miR-6565-3p	TCTGTGCTTGTGTACTCATAGT
gga-miR-33-3p	CAATGTTCCTGCAGTGCAGTA
gga-miR-6631-5p	GAAGAGAATGCTGTGGTTCTGC
gga-miR-1649-5p	TCCTGCAGAAGGTGCGGCTGTGT
gga-miR-6648-3p	TCCGGCATTCTGAACGCTCCT
gga-miR-16-2-3p	CCCAATATTATTGTGCTGCTT
gga-miR-6669-3p	TGCAGCCTGGCCGTATCTCAGT
gga-miR-1684a-3p	AAGTATGAGGAAATGGAGCTCT
gga-miR-3532-3p	TTGGAGGCTGCAGTGTCATGGT
gga-miR-1651-3p	TTGCTTTTGTTGGCCTCTGCTGT
gga-miR-1805-3p	TGTATTGGAACACTACAGCTCC
gga-miR-1707	TTTGAGCGGGATCTGTTATCGTG
gga-miR-1769-3p	AGTGTGAAATCTGCCTGAAAGT

Continuous the above Table 3	
gga-miR-1558	CTGCTGTGATGGGAGCTCTGAGCAG
gga-miR-365-1-5p	AGGGACTTTTGGGGGCAGATGTG
gga-miR-133b	TTTGGTCCCCTTCAACCAGCTA
gga-miR-1574-3p	ACAGGAGGATGTCAGGAAGCTTC
gga-miR-1623	ACCGCAGGCACAGACAGGCAGT
gga-miR-1663-3p	TGGCATCCAGAACAGCGGTAC
gga-miR-460b-5p	TCCTCATTGTACATGCTGTGTG
gga-miR-153-3p	TTGCATAGTCACAAAAGTGATCGT
gga-miR-1716	AGCGGGGCGGCTGTGAGCTGAGCT
gga-miR-6549-5p	AGCCTTCTGTTGTGCATCTGAGA
gga-miR-551-3p	GCGACCCATACTTGGTTTCAGT
gga-miR-138-5p	AGCTGGTGTTGTGAATCAGGC
gga-miR-6710-3p	AAACTGTTCTCTTCCATCTAGT
gga-miR-1458	TTCCTGTGATGCTCATGAGA
gga-miR-1729-3p	CTACTCGGTGAGTAAGGATAGC
gga-miR-1663-5p	TACCGCTGTCCTGGGTGCCTGG
gga-miR-34c-5p	AGGCAGTGTAGTTAGCTGATTGT
gga-miR-6543-5p	GCTGTTACCTGAGAGAAGACGCTG
gga-let-7g-3p	CTGTACAGGCCACTGCCTTGC
gga-miR-3594-3p	TCTGCATCGCTGGGCTGTGTCCT
gga-miR-135a-2-3p	TGTAGGGATGGAAGCCATGA
gga-miR-9-3p	TAAAGCTAGAGAACCGAATGTA
gga-miR-196-1-3p	ACAAGAACATCAAACTACCTGA
gga-miR-6670-5p	TGGAATGATGATCTATATTCTGAGT
gga-miR-138-1-3p	GCTACTTCACAACACCAGGGT
gga-miR-130c-5p	CCTTTTTATGTTGTACTACTAG
gga-miR-1747-5p	TGCACTTGAATGGAGTTCTGGGT
gga-miR-1563	GCACATGATGAGGAAGCACTGAAACTGAC
gga-miR-6660-3p	CTGACATGGCTCTGCTCCGCAGT
gga-miR-1569	TGTTTGGGACGTTGCTCTGCAG
gga-miR-21-3p	AACAACAGTCGGTAGGCTGTCT
gga-miR-6615-3p	TGGCACTGATGTGTTCTCCACA
gga-miR-1703-5p	AGAGGCTGTAGGTCCCGTGCTTT
gga-miR-137-3p	TTATTGCTGGAGAATACGCGTAG
gga-miR-1635	TGCCCAGGCTGTGCTGTGCTCTGG
gga-miR-460a-3p	CACAGCGCATACAATGTGGATT
gga-miR-181b-2-3p	CTCACTGATCAATGAATGCAAA
gga-miR-1724	TGCTGAGCGTTGGCTGCGCTGC
gga-miR-15c-3p	CAGACCATTCTGGGCTGCCTCA
gga-miR-1662	TTGACATCATCATACTTGGGAT
gga-miR-6604-5p	TGGCACGGTGCTAGGGATTTCTGT
gga-miR-1731-5p	ACTTGACTGCAGGCACTGCTGCT
gga-miR-146b-3p	TGCCCTATGGATTCAGTTCTGC
gga-miR-1451-3p	CGTAACTCGCTGCTGTGAGAGGT
gga-miR-6543-3p	CCTCCTTTCAGGTCACTGTAGG
gga-miR-133a-5p	AGCTGGTAAAATGGAACCAAAT
gga-miR-138-2-3p	GCTATTTCACTACACCAGGGT
gga-miR-1551-5p	CTAGCAGCAAAAAGAACTTCAGA
gga-miR-490-3p	CAACCTGGAGGACTCCATGCTG
gga-miR-489-3p	TGACATCATATGTACGGCTGCT
gga-let-7f-3p	CTATACAATCTATTGCCTTCCT
gga-miR-3538	GTTCGGTGATGAAACCATGGAATA
gga-miR-147	GTGTGCGGAAATGCTTCTGCTA
gga-miR-194	TGTAACAGCAACTCCATGTGGAC
gga-miR-24-5p	GTGCCTACTGAGCTGATATCAGT
gga-let-7j-3p	CTATACAGTCTATTGCCTTCCT
gga-miR-6557-3p	CGCGCCGATTGTCTCCTCCGGGCA
gga-miR-16-1-3p	CCAGTATTAACTGTGCTGCTGAA
gga-let-7c-3p	CTGTACAACCTTCTAGCTTTCC
gga-miR-3529	AGGCAGACTGTGACTTGTTGT
gga-miR-99a-3p	CAAGCTCGCTTCTATGGGTCTGT
gga-miR-15b-3p	CGAATCATTATTTGCTGCTTTA
gga-miR-3607-3p	ATACTGTAAACGCTTTCTGATG
gga-miR-1712-3p	TTCAGTTATCAGTGGAGTTTGG
gga-miR-199b	CAGTAGTCTGCACATT
gga-let-7a-2-3p	CTGTACAACCTCCTAGCTTTCC
gga-miR-6615-5p	TTGGGGACACCATCAGAACAGCCACA

Continuous the above Table 3	
gga-miR-92-5p	AGGTTGGGATCAGTTGCAATGCT
gga-miR-22-5p	AGTTCTTCAGTGGCAAGCTTT
gga-miR-301b-3p	CAGTGCAATAGTATTGTCAAAGCATT
gga-miR-6599-3p	TGACGGATCCTGGCTCCCTCCG
gga-miR-33-5p	GTGCATTGTAGTTGCATTGC
gga-miR-193a-3p	AACTGGCCTACAAAGTCCCAGT
gga-miR-1786	ATTCTTTTCTGCTGTGTTACT
gga-miR-20b-3p	ACTGTAATGTGGGCACTTAC
gga-miR-142-5p	CATAAAGTAGAAAGCACTACT
gga-miR-3523	CCGCGCAGTGCCTCGTCCTCGA
gga-miR-3525	CAGCCATTCTGCGATTCTGTGA
gga-miR-1744-3p	ACTTCAACAGGAGCAAGACTGA
gga-miR-181b-1-3p	CTCACTGAACAATGAATGCAA
gga-miR-19a-5p	๛TAGTTTTGCATAGTTGCACT
gga-miR-1684b-3p	AAGTATGAGGAAATGGAGATCT
gga-miR-2188-3p	GATATATGTGGTCGGACCTAT
gga-miR-142-3p	TGTAGTGTTTCCTACTTTATGG
gga-miR-1625-5p	TGGACCAGGGCTCTTCCTGCTGGCT
gga-miR-1560-3p	GCATCTCTGGACGCGCTCGTTC
gga-miR-1674	GGGCTATGATGCTGGATTTTCTGAGCA
gga-miR-1677-5p	TCCTGCACCGCTGAAGTCAAT
gga-miR-17-3p	CTGCAGTGAAGGCACTTGTAGCT
gga-miR-130c-3p	CAGTGCAATGTTAAAAGGGCATT
gga-miR-26a-3p	CCTATTCTTGGTTACTTGCACT
gga-miR-32-3p	CAATTTAGTGTGTGCGATACT
gga-miR-1664-3p	TTCTGTGACCTCATTTACCTCC
gga-miR-18a-3p	ACTGCCCTAAGTGCTCCTTCTGG
gga-miR-15a	TAGCAGCACATAATGGTTTGTG
gga-miR-190a-5p	TGATATGTTTGATATATTAGGTTG
gga-miR-2954	CATCCCCATTCCACTCCTAGCAGTT
gga-miR-128-1-5p	CGGGGCCGTAACACTGTCTGAGA
gga-miR-146a-3p	ACCCATGGGGCTCAGTTCTTCA
gga-miR-133c-3p	TTTGGTCCCCTTCAACCAGCTG
gga-miR-383-5p	AGATCAGAAGGTGATTGTGGCT
gga-miR-130a-5p	GCCCTTTTTCTGTTGTACTACT
gga-miR-1779	AGACGTGGACTGGAACACCTGAG
gga-miR-130a-3p	CAGTGCAATATTAAAAGGGCA
gga-miR-15b-5p	TAGCAGCACATCATGGTTTGCA
gga-miR-27b-5p	AGAGCTTAGCTGATTGGTGAACA
gga-miR-375	TTTGTTCGTTCGGCTCGCGTTA
gga-miR-1416-5p	TCCTTAACTCATGCCGCTGTG
gga-let-7d	AGAGGTAGTGGGTTGCATAGTT
gga-miR-301b-5p	GCTCTGACTTTATTGCACTACT
gga-miR-10b-3p	ACAGATTCGATTCTAGGGGAAT
gga-miR-301a-3p	CAGTGCAATAATATTGTCAAAGCATT
gga-miR-6542-3p	ACGGGACAGTGCTGAAGACT
gga-miR-184-3p	TGGACGGAGAACTGATAAGGGT
gga-miR-2131-5p	ATGCAGAAGTGCACGGAAACAGCT
gga-miR-1306-5p	CCACCTCCCCTGCAAACGTCCA
gga-miR-18b-3p	TACTGCCCTAAATGCTCCTTCT
gga-miR-1456-5p	AAAGGACGGAGGCGGCCCGCGC
gga-miR-135a-5p	TATGGCTTTTTATTCCTATGTG
gga-miR-187-3p	TCGTGTCTTGTGTTGCAGCCAGT
gga-miR-18b-5p	TAAGGTGCATCTAGTGCAGT
gga-miR-34a-5p	TGGCAGTGTCTTAGCTGGTTGT
gga-miR-365-2-5p	AGGGACTTTCAGGGGCAGCTGTG
gga-miR-200b-5p	CATCTTACTGGGCAGCATTGGA
gga-miR-219b	CACAAGAATTGCGTTTGGACAAT
gga-miR-193b-3p	AACTGGCCCACAAAGTCCCGCT
gga-miR-1306-3p	TGGACGTTGGCTCTGGTGGTGA
gga-miR-2131-3p	CTGTTACTGTTCTTCTGATGG
gga-miR-458a-3p	ATAGCTCTTTGAATGGTACTGC
gga-miR-122-5p	TGGAGTGTGACAATGGTGTTTG
gga-miR-223	TGTCAGTTTGTCAAATACCCCA
gga-miR-144-5p	GGATATCATCATATACTGTAAGT
gga-miR-1451-5p	TCGCACAGGAGCAAGTTACCGC
gga-miR-301a-5p	GCTCTGACAATGTTGCACTACT

Continuous the above Table 3

gga-miR-215-5p	ATGACCTATGAATTGACAGACT
gga-miR-133a-3p	TTTGGTCCCCTTCAACCAGCTGT
gga-miR-1329-5p	TACAGTGATCACGTTACGATGGAT
gga-miR-30c-2-3p	CTGGGAGAAGGCTGTTTACTCT
gga-miR-148a-5p	AAAGTTCTGTGACACTCAGACT
gga-miR-1711	GGTGCAGTGCTGCATCTCTGG
gga-miR-183	TATGGCACTGGTAGAATTCACT
gga-miR-1729-5p	ATCCCTTACTCACATGAGTAGT
gga-let-7k-3p	CTATACAATCTACTGTCTTTCT
gga-miR-32-5p	TATTGCACATTACTAAGTTGCA
gga-miR-206	TGGAATGTAAGGAAGTGTGTGG
gga-miR-10a-3p	CAAATTCGTATCTAGGGGAAT
gga-miR-1b-3p	TGGAATGTTAAGAAGTATGTAT
gga-miR-30a-3p	CTTTCAGTCGGATGTTTGCAGC
gga-miR-499-5p	TTAAGACTTGTAGTGATGTTTA
gga-miR-221-5p	ACCTGGCATACAATGTAGATTTCTGT
gga-miR-455-3p	GCAGTCCATGGGCATATACACC
gga-miR-19a-3p	TGTGCAAATCTATGCAAAACTGT
gga-miR-193a-5p	TGGGTCTTTGCGGGCGAGATGA
gga-miR-1552-3p	CTAGCTGCTCTGCACTGACTGT
gga-miR-6606-5p	GAGGAGCGGGAGGAGCGGGA
gga-miR-181a-3p	ACCATCGACCGTTGATTGTACC
gga-miR-15c-5p	TAGCAGCACATCATGGTTTGTA
gga-miR-30c-1-3p	TGGGAGAGGATTGTTTACGCCT
gga-miR-18a-5p	TAAGGTGCATCTAGTGCAGATAG
gga-miR-31-5p	AGGCAAGATGTTGGCATAGCTG
gga-miR-1552-5p	TTAGTGCGCGGTAAGCTAGGGTG
gga-miR-146c-3p	GTCCATGGTATTCAGTTCTCTA
gga-miR-146b-5p	TGAGAACTGAATTCCATAGGCGTT
gga-miR-30b-5p	TGTAAACATCCTACACTCAGCT
gga-miR-9-5p	TCTTTGGTTATCTAGCTGTATGA
gga-miR-1559-5p	TTCGATGCTTGTATGCTACTCC
gga-miR-107-3p	AGCAGCATTGTACAGGGCTATCA
gga-miR-455-5p	TGTGCCCTTGGACTACATCGTG
gga-miR-30e-3p	CTTTCAGTCGGATGTTTACAGC
gga-miR-140-5p	CAGTGGTTTTACCCTATGGTAG
gga-miR-126-5p	CATTATTACTTTTGGTACGCG
gga-miR-2188-5p	AAGGTCCAACCTCACATGTCCT
gga-miR-106-3p	ACTGCAGTATAAGCACTTCTGGC
gga-miR-130b-5p	CCTCTTTCCCTGTTGCACTACT
gga-miR-144-3p	CTACAGTATAGATGATGTACTCT
gga-miR-200a-5p	CATCTTACTAGACAGTGCTGGA
gga-let-7b	TGAGGTAGTAGGTTGTGTGGTT
gga-miR-125b-3p	ACAAGTCAGGCTCTTGGGACT
gga-miR-1677-3p	TTGACTTCAGTAGGAGCAGGATT
gga-miR-365-3p	TAATGCCCCTAAAAATCCTTAT
gga-miR-146a-5p	TGAGAACTGAATTCCATGGGTTG
gga-miR-19b-3p	TGTGCAAATCCATGCAAAACTG
gga-miR-16-5p	TAGCAGCACGTAAATATTGGTG
gga-miR-211	TTCCCTTTGTCATCCTATGCCT
gga-miR-460a-5p	CCTGCATTGTACACACTGTGTG
gga-miR-17-5p	CAAAGTGCTTACAGTGCAGGTAG
gga-miR-196-5p	TAGGTAGTTTCATGTTGTTGGG
gga-miR-22-3p	AAGCTGCCAGTTGAAGAACTGT
gga-miR-221-3p	AGCTACATTGTCTGCTGGGTTTC
gga-miR-222a	AGCTACATCTGGCTACTGGGTCTCT
gga-miR-7	TGGAAGACTAGTGATTTTGTTGTT
gga-miR-1a-3p	TGGAATGTAAAGAAGTATGTAT
gga-miR-214	ACAGCAGGCACAGACAGGCAGT
gga-miR-454-3p	TAGTGCAATATTGCTTATAGGGTCT
gga-miR-106-5p	AAAAGTGCTTACAGTGCAGGTAG
gga-miR-140-3p	ACCACAGGGTAGAACCACGGAC
gga-miR-429-3p	TAATACTGTCTGGTAATGCCGT
gga-miR-181b-5p	AACATTCATTGCTGTCGGTGGGT
gga-let-7c-5p	TGAGGTAGTAGGTTGTATGGTT
gga-miR-205b	CCCTTCATTCCACCGGAATCTG
gga-let-7k-5p	TGAGGTAGTAGATTGAATAGTT

Continuous the above Table 3

gga-miR-23b-3p	ATCACATTGCCAGGGATTTCCA
gga-miR-20b-5p	CAAAGTGCTCATAGTGCAGGTAG
gga-miR-7b	TGGAAGACTAGTGATTTTTGTTGTT
gga-miR-181a-5p	AACATTCAACGCTGTCGGTGAGT
gga-miR-451	AAACCGTTACCATTACTGAGTTT
gga-miR-30c-5p	TGTAAACATCCTACACTCTCAGCT
gga-miR-218-5p	TTGTGCTTGATCTAACCATGT
gga-miR-24-3p	TGGCTCAGTTCAGCAGGAACAG
gga-miR-20a-5p	TAAAGTGCTTATAGTGCAGGTAG
gga-miR-30e-5p	TGTAAACATCCTTGACTGGAAGCT
gga-miR-125b-5p	TCCCTGAGACCCTAACTTGTGA
gga-miR-130b-3p	CAGTGCAATAATGAAAGGGCGT
gga-miR-30a-5p	TGTAAACATCCTCGACTGGAAGCT
gga-miR-456-3p	CAGGCTGGTTAGATGGTTGTCT
gga-miR-103-3p	AGCAGCATTGTACAGGGCTATGA
gga-miR-128-3p	TCACAGTGAACCGGTCTCTTT
gga-miR-101-3p	GTACAGTACTGTGATAACTGAA
gga-miR-16c-5p	TAGCAGCACGTAAATACTGGAG
gga-miR-27b-3p	TTCACAGTGGCTAAGTTCTGC
gga-let-7j-5p	TGAGGTAGTAGGTTGTATAGTT
gga-let-7g-5p	TGAGGTAGTAGTTTGTACAGTT
gga-miR-126-3p	TCGTACCGTGAGTAATAATGCG
gga-miR-30d	TGTAAACATCCCCGACTGGAAGCT
gga-miR-200a-3p	TAACACTGTCTGGTAACGATGTT
gga-miR-200b-3p	TAATACTGCCTGGTAATGATGAT
gga-let-7i	TGAGGTAGTAGTTTGTGCTGTT
gga-miR-199-5p	CCCAGTGTTCAGACTACCTGTTC
gga-let-7f-5p	TGAGGTAGTAGATTGTATAGTT
gga-miR-92-3p	TATTGCACTTGTCCCGGCCTGT
gga-miR-26a-5p	TTCAAGTAATCCAGGATAGGCT
gga-miR-21-5p	TAGCTTATCAGACTGATGTTGAC
gga-miR-199-3p	ACAGTAGTCTGCACATTGGTT
gga-miR-10b-5p	TACCCTGTAGAACCGAATTTGT
gga-miR-99a-5p	AACCCGTAGATCCGATCTTGT
gga-miR-146c-5p	TGAGAACTGAATTCCATGGACTG
gga-miR-100-5p	AACCCGTAGATCCGAACTTGTG
gga-miR-10a-5p	TACCCTGTAGATCCGAATTTGT
gga-miR-205a	TCCTTCATTCCACCGGAGTCTG
gga-miR-148a-3p	TCAGTGCACTACAGAACTTTGT
gga-miR-203a	GTGAAATGTTTAGGACCACTTG
gga-miR-1682*	TGGGTCAGATGGAGCTGAGGGT

* represent the KRC-specific miRNA.

3.3. Differential Expression Profiles of Conserved Mirnas Between HXC and KRC

To compare the differential expression of miRNAs in the follicle of HXC versus KRC chickens, the numbers of miRNAs in each group samples were normalized to the total number of reads. The expression of one KRC-specific miRNAs was not significant in KRC chickens compared to HXC counterparts. So only the differentially expressed miRNAs in follicle were showed in table 4. In total, 11 miRNAs were considered to be differentially expressed ($P< 0.05$), with 5 up-regulated and 6 down-regulated. Nine miRNAs had more than two fold expression changes ($|\log2(\text{fold-change})| >= 1.0$) from KRC to HXC (Table 4). We also confirmed the expression patterns by RT-qPCR which of four differentially expressed miRNAs in follicle (Figure 2), the results of which correlated to the RPKM values estimated by RNA sequencing ($r = 0.81$).

Table 4. *The differentially expressed miRNAs between HXC and KRC.*

miR_name	miR_seq	Fold Change (KRC/HXC)	log2(Fold Change)	P-value
gga-miR-1623	ACCGCAGGCACAGACAGGCAGT	9.09	3.18	0.00000
gga-miR-6544-3p	AGTTGTATTTCTTTTCTGACAG	3.30	1.72	0.00871
gga-miR-1458	TTCCTGTGATGCTCATGAGA	3.04	1.60	0.00281
gga-miR-1559-3p	AGTTACATGTATGCATCGAGCA	2.81	1.49	0.00536
gga-miR-199b	CAGTAGTCTGCACATT	2.34	1.23	0.00628
gga-miR-144-5p	GGATATCATCATATACTGTAAGT	0.59	-0.77	0.04962
gga-miR-6599-3p	TGACGGATCCTGGCTCCCTCCG	0.51	-0.98	0.03224
gga-miR-1731-5p	ACTTGACTGCAGGCACTGCTGCT	0.50	-1.01	0.03513
gga-miR-184-3p	TGGACGGAGAACTGATAAGGGT	0.36	-1.46	0.03880
gga-miR-1798-5p	AACGTGACACTTTAGAAAACCT	0.26	-1.95	0.03156
gga-miR-1798-3p	TTTTCAGAAGTGTAGCGTTTA	0.12	-3.11	0.00003

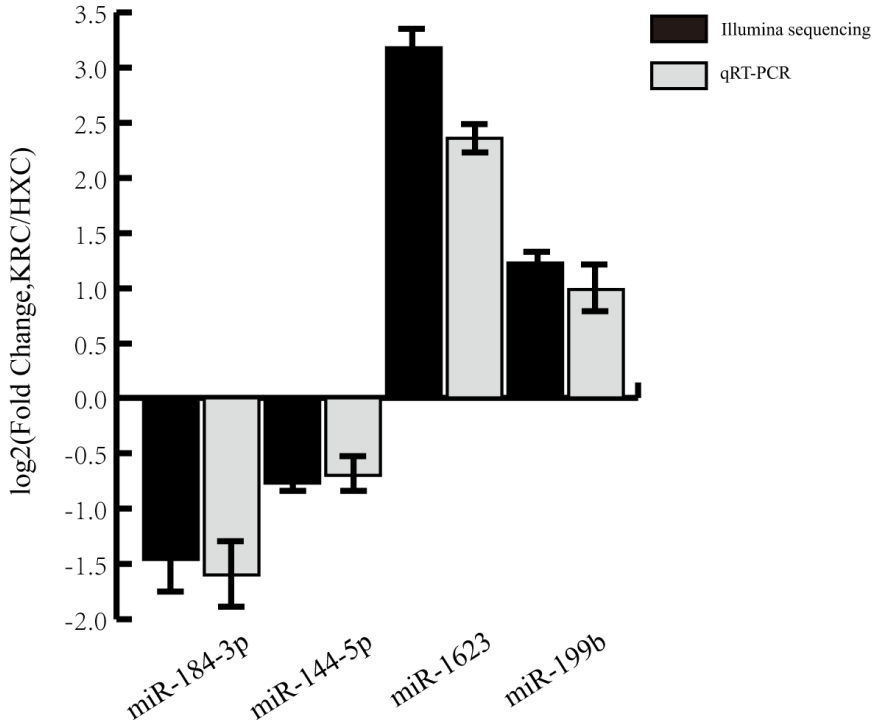

Figure 2. *The expression patterns by RT-qPCR compared with RNA-seq of differentially expressed miRNAs.*

3.4. Target Prediction and Functional Analysis of Differential Expression Mirnas

To further explore the roles of differentially expressed miRNAs, putative target genes of the most differentially expressed 9 miRNAs (|Log2 (fold-change)|> = 1.0) were predicted by integrating TargetScan and miRanda. In total, 8250 common target genes were found (data is not shown) which include *FZD4*, *WNT4, BMP* and *EGF* of which related hair follicle development (Table 5).

Table 5. *The differentially expressed miRNAs target genes annotation.*

Gene Name	Ensembl Gene ID	Involved KEGG pathways	Associated miRNAs
FZD4	ENSGALG00000017242	Wnt signaling pathway	gga-miR-1623, gga-miR-184-3p, gga-miR-1458
WNT4	ENSGALG00000004790	mTOR signaling pathway	gga-miR-1623
EGF	ENSGALG00000012155	MAPK signaling pathway	gga-miR-184-3p, gga-miR-199b
BMP2/4	ENSGALG00000012429	TGF-beta signaling pathway	miR-184-3p, miR-1559-3p

GO annotation showed the putative target genes were significantly enriched (counts > 30, $P< 0.05$) in biological processes (BP) (Table 6), Cellular component (CC) (Table 7) and Molecular function (MF) (Table 8). The KEGG analysis (Table 9) suggested that Focal adhesion, Phosphatidylinositol signaling system, ECM-receptor interaction, Inositol phosphate metabolism, Oocyte meiosis and Ubiquitin mediated proteolysis were the most enriched

pathways (counts > 50, $P < 0.01$).

Table 6. *GO analysis of the putative target genes in biological processes.*

GO term in biological processes	gene count	P-value
cell migration	78	0.00003
protein autophosphorylation	76	0.00006
signal transduction	166	0.00006
intracellular protein transport	106	0.00013
peptidyl-tyrosine phosphorylation	33	0.00017
peptidyl-serine phosphorylation	70	0.00028
activation of GTPase activity	41	0.00035
cell-matrix adhesion	36	0.00066
positive regulation of protein kinase B signaling	37	0.00300
positive regulation of JNK cascade	30	0.00450
positive regulation of I-kappaB kinase/NF-kappaB signaling	68	0.00460
cell adhesion	97	0.00500
axon guidance	54	0.00740
extracellular matrix organization	49	0.00760
cilium assembly	62	0.00980
positive regulation of NF-kappaB transcription factor activity	43	0.00990
single organismal cell-cell adhesion	30	0.01000
positive regulation of canonical Wnt signaling pathway	35	0.01100
positive regulation of transcription from RNA polymerase II promoter	273	0.01200
positive regulation of protein binding	32	0.01200
positive regulation of cell migration	74	0.01300
transmembrane receptor protein tyrosine kinase signaling pathway	46	0.01500
intracellular signal transduction	148	0.01600
positive regulation of peptidyl-serine phosphorylation	36	0.01600
integrin-mediated signaling pathway	36	0.01600
positive regulation of proteasomal ubiquitin-dependent protein catabolic process	33	0.01900
xenophagy	40	0.02000
positive regulation of phosphatidylinositol 3-kinase signaling	35	0.02100
positive regulation of neuron projection development	30	0.02100
post-embryonic development	37	0.02300
positive regulation of transcription, DNA-templated	134	0.02600
innate immune response	75	0.03200
protein ubiquitination involved in ubiquitin-dependent protein catabolic process	68	0.03600
protein polyubiquitination	44	0.03700
cellular response to lipopolysaccharide	30	0.03900
positive regulation of cell proliferation	110	0.04200
regulation of Rho protein signal transduction	34	0.04500
endocytosis	45	0.04700
protein phosphorylation	60	0.04700
negative regulation of transcription, DNA-templated	111	0.04700

Table 7. *GO analysis of the putative target genes in Cellular component.*

GO term in Cellular component	gene count	P-value
nucleoplasm	685	0.00000
cytoplasm	1337	0.00000
centrosome	183	0.00000
plasma membrane	726	0.00001
PML body	47	0.00002
cytosol	494	0.00013
early endosome	82	0.00048
proteinaceous extracellular matrix	100	0.00052
extracellular exosome	959	0.00054
perinuclear region of cytoplasm	175	0.00069
cell junction	100	0.00082
cell-cell junction	63	0.00150
spindle pole	34	0.00210
centriole	42	0.00210
receptor complex	59	0.00230
cell surface	169	0.00240
extrinsic component of membrane	40	0.00380
trans-Golgi network	66	0.00620
basement membrane	35	0.00750
postsynaptic membrane	54	0.00120
ciliary basal body	38	0.01200
Golgi apparatus	237	0.01400
endomembrane system	44	0.01500
cytoplasmic vesicle	51	0.02300
apical plasma membrane	76	0.03100
membrane	417	0.03600
recycling endosome	40	0.03700
lamellipodium	60	0.04300
blood microparticle	35	0.04300

Table 8. *GO analysis of the putative target genes in Molecular function.*

GO term in Molecular function	gene count	P-value
ATP binding	679	0.00000
metal ion binding	416	0.00000
zinc ion binding	471	0.00002
phosphatidylinositol binding	50	0.00031
protein serine/threonine kinase activity	122	0.00044
ubiquitin-protein transferase activity	90	0.00065
protein kinase activity	67	0.00069
non-membrane spanning protein tyrosine kinase activity	30	0.00250
GTPase activator activity	100	0.00280
transcription regulatory region DNA binding	57	0.00460
receptor activity	47	0.00580
ligase activity	39	0.00760
thiol-dependent ubiquitin-specific protease activity	39	0.00760
ATPase activity	62	0.01000
receptor signaling protein serine/threonine kinase activity	35	0.01200
extracellular matrix structural constituent	30	0.01300
receptor binding	37	0.02200
Rab GTPase binding	30	0.02400
chromatin binding	145	0.02700
microtubule motor activity	31	0.03300
signal transducer activity	63	0.03900

Table 9. *KEGG analysis of the putative target genes.*

KEGG Term	Count	P-value
Focal adhesion	137	0.00001
Phosphatidylinositol signaling system	73	0.00003
ECM-receptor interaction	59	0.00015
Inositol phosphate metabolism	55	0.00026
Oocyte meiosis	67	0.00110
Ubiquitin mediated proteolysis	87	0.00690
Cell cycle	77	0.01700
Endocytosis	153	0.03700
p53 signaling pathway	44	0.04100

4. Discussion

In this study, we detected 11 differential expressed miRNAs that were enriched in the KRC and HXC libraries and obtained several predicted target genes that may play different roles in

hair follicles formation and development. We also identified several pathways associated with hair follicle cell development, including Focal adhesion, Phosphatidylinositol signaling system, ECM-receptor interaction.

Our analysis of the most abundant mature miRNAs with raw reads>=10 in the KRCs and HXCs identified 290 miRNAs that were common in the two groups. Our analysis of the differential expressed miRNAs revealed 11 miRNAs (table 2) in two groups. These differential expressed miRNAs including miR-1623, miR-184-3p, miR-199b. The miR-184 inhibition argonaute 2 protein expression [22] and the growth of hair follicles [23]. And miRNA-199b has an important role in skin and hair follicle development [24, 25]. The miR-1623 target genes that played important roles in Wnt/β-catenin pathway [19]. Through qRT-PCR, we confirmed miR-1623 target gene the *WNT4* in chicken and *WNT4* showed the higher expression in HXCs (Figure 3). Thus, the miR-1623 has an effect on *WNT4* and involved in hair follicle cell development. We also identified several target genes, such as those encoding *FZD4* and *EGF*, which are related to hair follicle development in chicken. These miRNAs target genes involved several signaling pathways, including WNT, TGF-β, EGF, FGF, BMP, Hox signaling pathway [26-30]. These signaling pathways regulated and transformed hair follicle development in different stages. The WNT and BMP signaling pathways related to differentiation of keratin cell and regulation of the hair shaft formation [31]. And the TGF-β pathway control growth of hair follicles [27].

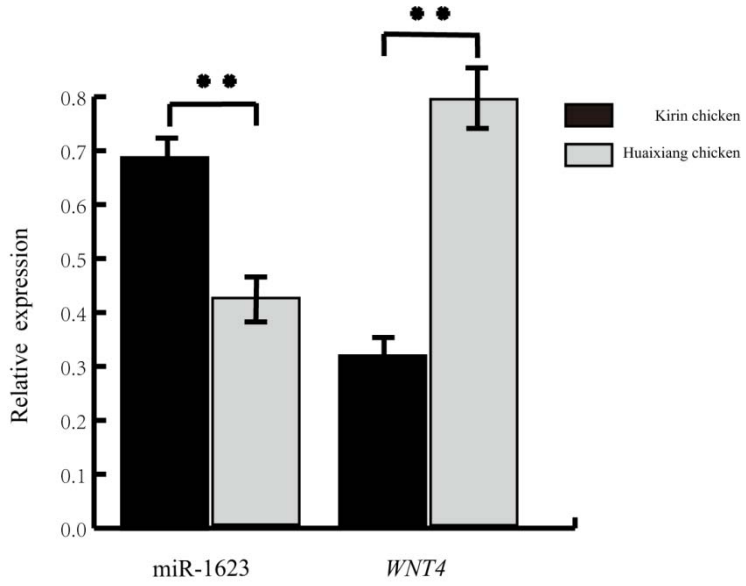

Figure 3. *The miR-1623 target gene expression.*

Many complex factors support hair follicle cell development. To further investigate the functions of miRNAs and their target genes more experiments need to be performed and miRNA knockdown to identify target genes expression levels.

5. Conclusion

In conclusion, we identified several miRNAs such as miR-1623, miR-184-3p, miR-199b, and their target genes *WNT4*, *FZD4* and *EGF* which may be involved in hair follicle development in chicken. These data provide a strong foundation for the study of hair follicle development in chicken at the molecular levels.

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