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Novel circRNAs from cashmere goats: Discovery, integrated regulatory network, and their putative roles in the regeneration and growth of secondary hair follicles

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Abstract: Circular RNAs (circRNAs) were identified as noncoding RNAs with covalently closed structures differing from other types of RNA molecules. It was thought that circRNAs were implicated in the development of secondary hair follicles (SHFs) of cashmere goats. In this investigation, a total of 21 novel circRNAs were identified from the skin tissue of cashmere goats. Of them, nine circRNAs were found to be significantly higher in expression at anagen skin tissue than those at telogen of cashmere goats. Based on bioinformatics analysis, a complicated regulatory relationship was revealed among the nine upregulated circRNAs at anagen with related signalling pathways, such as Wnt signalling pathway, TGF-beta signalling pathway, mTOR signalling pathway, MAPK signalling pathway, and axon guidance. Further, the expression pattern analysis of the nine upregulated circRNAs at anagen along with their host genes in SHFs suggested their potential functional roles in the regeneration and growth of anagen SHFs of cashmere goats. Our results provided novel significant information for elucidating the molecular regulatory mechanisms understanding the regeneration and growth of SHFs in cashmere goats, which will be essential for artificially regulating the growth of cashmere to increase cashmere yield.

Keywords: circular RNA; ceRNA; miRNA; skin tissue; signalling pathway

Cashmere is a kind of natural protein fibre from secondary hair follicles (SHFs) of cashmere goats. Cashmere fibres are characterized by smoothness, softness, and lightness with the good warmth retention. As high-end textile materials, its products have always been very popular among consumers. As well known, the growth of SHF cashmere fibres is periodically controlled by the SHF activity being a cyclic biological process with three main stages: anagen, catagen, and telogen. Among them, the anagen, as a highly active stage of SHFs, is typically characterized by a considerable change of endoge-

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nous regulatory factors related with morphogenesis and growth of cashmere fibres in cashmere goats (Geng et al. 2013).

Over the past few decades, endogenous regulatory factors in the growth of cashmere fibre in goats were extendedly investigated at multiple levels including functional genes, microRNAs (miRNAs), and long non-coding RNAs (lncRNAs) (Jiao et al. 2019). Also, it was thought that several signalling pathways were implicated in the SHF regeneration and cashmere fibre growth of cashmere goats, such as Wnt/β-catenin, BMP, Notch, MAPK, TGF-β and Hedgehog (Zhang et al. 2020). Besides, it was reported that another kind of non-coding RNAs, named as circular RNAs (circRNAs), was isolated from SHFs of cashmere goats with differential expression between anagen and telogen stages (Yin et al. 2019). Moreover, it was demonstrated that circRNA-1926 promotes the differentiation of goat SHF stem cells into hair follicle lineage by miR-148a/b-3p/CDK19 axis (Yin et al. 2020). This finding suggested that circRNAs, as a novel kind of regulatory factors, might be essentially involved in the SHF development and cashmere fibre growth of cashmere goats.

CircRNAs, at the outset, were identified as non-coding RNAs (ncRNAs) with covalently closed structures differing from other types of RNA molecules. Increasing evidences have shown that circRNAs are endogenous, abundant, conserved, and stable with the tissue-type specific features in mammalian cells (Memczak et al. 2013). It was thought that circRNAs could function at multiple levels of gene expression during biological processes, such as transcription, epigenetic modification, and RNA splicing. Also, accumulating evidences have shown that many circRNAs have one or more open reading frames (ORFs) that can be translated into functional peptides with biological activity (Yang et al. 2017). To date, however, we still have a poor understanding of the expression profiles and functional regulatory characteristics of circRNAs in SHFs of cashmere goats. The aim of this study is to identify and characterize novel circRNAs from the skin tissue of cashmere goats, and reveal their regulatory network and the expression pattern in SHFs of cashmere goat during SHF cycles including anagen, catagen, and telogen. Our results will provide novel and essential information for revealing the regulatory features and biological significance of the circRNAs in the regeneration and growth of SHFs in cashmere goats.

MATERIAL AND METHODS

Sources of putative circRNA sequences and total RNA samples

In this study, the experiment protocol was reviewed and approved by the Animal Experimental Committee of Shenyang Agricultural University (Shenyang, Liaoning, China) with an ethical code of 201606005. All experiments were carried out according to the approved protocol guidelines. Here, 21 analyzed sequences of putative circRNAs were from full-length transcriptome sequencing data on skin tissue at anagen of cashmere goat, which was described well in a previous investigation (Yin et al. 2019). In Table S1 in electronic supplementary material (for the supplementary material see the electronic version), we provided the approved names of the host genes of the 21 analyzed putative circRNAs. For preliminarily characterizing the expression of the 21 putative circRNAs in the skin tissue of cashmere goat at SHF anagen and telogen, the total RNA was used which was extracted from the skin tissue of cashmere goat as described in a previous investigation (Bai et al. 2016a). In order to further characterize the expression pattern of the nine anagen upregulated circRNAs along with their corresponding host genes in SHFs of cashmere goats, three stages of SHF cycles were analyzed including anagen, catagen, and telogen. We used the total RNA extracted from SHFs of cashmere goat in our another study (Zhu et al. 2018).

Sequence analysis of putative circRNA and integrated regulatory network with the signalling pathway enrichment

The encoding potential of the 21 putative circRNAs was analyzed using the Coding Potential Assessment Tool (CPAT, http://lilab.research.bcm.edu/cpat/index.php). The CPAT program, an alignment-free method, was recognized as more accurate and sensitive than the alignment-based method (Yin et al. 2019). In CPAT analysis, a logistic regression model is used to differentiate coding and non-coding RNA sequences via pure sequence-based linguistic features considering the Fickett TestCode statistic, hexamer usage bias, and ORF size along with its coverage. We set the cutoff score as 0.1

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for a more reliable analysis of coding probability. To obtain the genomic location information of the analyzed circRNAs, we mapped them into the reference genome of goat (assembly ARS1 with NCBI annotation release: 102, https://www.ncbi.nlm.nih.gov/genome/?term=goat).

The regulatory network of each putative circRNA was generated based on their interacting mechanism of competitive endogenous RNAs (ceRNAs). In the ceRNAs analysis, we predicted the potential target miRNAs of each putative circRNA through the use of an online program miRDB (http://www.mirdb.org). The miRDB was developed for the specific prediction of miRNA targets within noncoding RNA (ncRNA) or mRNA sequences. The miRDB predicts all target miRNAs within the given RNA sequence (ncRNA or mRNA) via analyzing thousands of interactions between miRNAs and ncRNAs or mRNAs molecules resulting from sequencing data using a high-throughput technique. Furthermore, the miRDB performs the prediction of target miRNAs through a machine learning approach, where common characterizations related with both the binding of miRNA and the downregulation of target have been recognized as prediction basis. Also, the miRDB was used to further predict the target genes of the resultant miRNAs. Finally, the Cytoscape program v2.8 (https://cytoscape.org/) was used to generate and visualize the ceRNA regulatory network of the analyzed circRNAs.

The CluePedia built-in plugin of Cytoscape (http://www.ici.upmc.fr/cluepedia/) was used to perform the pathway enrichment on potential regulatory genes of putative circRNAs through ceRNA model. According to the claims from the developers, the CluePedia can calculate linear and nonlinear statistical dependencies from experimental data, and reveal novel markers potentially related with the pathways. Therefore, the CluePedia can connect genes and integrate them into a network which may reveal novel potential relationships among the analyzed genes and pathways.

Reverse transcription-quantitative real-time PCR (RT-qPCR)

The reverse transcriptions were performed on total RNA using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's recommendation.

In the qPCR reactions a total of 33 pairs of primers were used. To analyze the expression of putative circRNAs, 21 pairs of divergent primers were designed through the use of the CircPrimer procedure (http://www.bioinf.com.cn). To detect the expression of the host genes of the analyzed circRNAs, nine pairs of convergent primers were designed through the use of the Primer Premier v5.0 procedure (http://www.premierbiosoft.com). Besides, to normalize the expression of the analyzed circRNAs along with their corresponding host genes, the UBB, YWHAZ, and SDHA genes were used as combined internal references, which was recommended in a previous investigation (Bai et al. 2014). Thus, the corresponding primers of the UBB, YWHAZ, and SDHA genes were directly cited from the publication (Bai et al. 2014). The three reference genes are from sheep, but the nucleotide sequences of the primers are identical with those of goat. Here, all the primers were commercially synthesized by Sangon Biotechnology Co., Ltd. (Sangon, Shanghai, China), and corresponding sequences for the primers are listed in Table 1.

The qPCR reactions were performed in a LightCycler 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany) to detect the expression of the putative circRNA along with their corresponding host genes in the skin tissue and SHFs of cashmere goats. In a 20 µl final volume, qPCR reactions were conducted with SYBR® Green assay containing 10 µl PCR Supermix, 0.8 µl each primer (10 mM), 2.0 µl first-strand cDNA, and 6.4 µl PCR-grade ddH_2O. We set the thermal cycling reactions as initial single cycle (95 °C for 4 min), followed by 40 cycles: 95 °C for 30 s, 52–57 °C (Table 1) for 30 s, and 72 °C for 30 seconds. A melting curve analysis was set for all reactions with 56 °C to 95 °C at a ramp speed of 0.5 °C per 10 s to verify only a single amplified product for each pair of primers. All reactions were performed with three replicates. Finally, we calculated the relative expression levels of each analyzed circRNA or gene by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Statistical analysis

All measured data was provided as mean ± standard deviation. Student's *t*-test was used to compare the differences between two groups, or a one-way analysis of variance (ANOVA) along with the Bonferroni test was used to compare the dif-

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Table 1. Detail of the primers used in this study and their real-time PCR reaction conditions

Gene	Primer type/reference in GenBank	Sequence (5'–3')	Amplicon size (bp)	*Ta* (°C)
circRNA-BOC	divergent/this study	F: CTGAATGGGAGGGAGCTGAA, R: TCCAAAGCCAACGTTCCAAG	235	56
circRNA-PARL	divergent/this study	F: ACTGTTGGTCTTCATGGGGT, R: GATCATTGTCCGCTGCAGAG	224	56
circRNA-TIAM1	divergent/this study	F: CAGGCGGTTGATGACATTGT, R: GGGTCAGAGGGCTTCATCTC	218	56
circRNA-IFFO2 (L)	divergent/this study	F: GGAGAGGCTAAGCTGACACA, R: GTCGGCTCCATGAACATCAC	218	55
circRNA-IFFO2 (S)	divergent/this study	F: GGAGCCTCGGAGTGAATGAA, R: GTCGGCTCCATGAACATCAC	236	57
circRNA-ZBTB40	divergent/this study	F: TGCACTCTTGATTACCCAGGA, R: CTCCATCGTGTCAGCTCTGT	151	56
circRNA-KMT2C	divergent/this study	F:CCATGACATGCTGCACAGTT, R: TGTCACACACCAGGCAATTG	226	56
circRNA-LOC102174373	divergent/this study	F: TGCCATCTCCTTCCTGTCAG, R: AAAATGCGTGATGGGAGCTC	243	55
circRNA-PLIN2	divergent/this study	F: AAGTGTCTGATGGCCTCCTC, R: TTCTCTTCCACTCCAGCCAG	203	57
circRNA-TULP4	divergent/this study	F: GGGACAGAAACACTCCACAG, R: CACACTCTTCAAACGGCACA	151	55
circRNA-TENT2	divergent/this study	F: TGTGAAAGCTGAACTGTGCA, R: GAGAAATCACACTGCCTGAGG	198	54
circRNA-CAMSAP1	divergent/this study	F: CTTTGAGGATGAGGCTGCAC, R: CGAGGATGCCATGGTCTTCT	162	56
circRNA-ODF2	divergent/this study	F: AGCTGTGTAGGGAAGTGACC, R: TCTGCGTGAGACTCGTTACT	211	56
circRNA-THSD1	divergent/this study	F: TAGTCACAGAGCACCACCAG, R: ACTTTGGTGTTTCAAGCCGA	208	55
circRNA-VWA8	divergent/this study	F: ATCCTGACAAGCAACCCTCA, R: TTTGCTTGTTGTCCTTGGGG	152	54
circRNA-ZC3H13	divergent/this study	F: GGATGGGCAGAAGTAGAGCA, R: GGCGAGGAGAAGGAGAATGT	151	54
circRNA-TENT4A	divergent/this study	F: TCATGGCAGCTTCTTCAGGA, R: GGACTCTTCAAAGCCGCAAT	250	55
circRNA-SNRK	divergent/this study	F: AGGGAAGAAGCTCACCACAA, R: ACTCCAAGACATACCCTGCA	164	54
circRNA-TXNRD3	divergent/this study	F: GGATCCCGCGTGTTTAAGTT, R: ACTTCTTCGTGCAGGAGTCA	245	54
circRNA-TNFRSF21	divergent/this study	F: TGGTGATAGTGGTGTGCAGT, R: GGTCGACGTGGTGGTATTTG	234	55
circRNA-ERCC6	divergent/this study	F: CAGAGACGCCAAGTTTGAGG, R: TGTACACCGTCACCTGCTTT	194	55
BOC	convergent/XM_018066620.1	F: GGCGGAGAATGAAGTTGGGA, R: ACTGGAGGTGTGACGGTGGC	114	57
TIAM1	convergent/XM_018048380.1	F: ACTGCCATCCACTCTGCTT, R: TCGCCCATTTTCTTCATCT	137	53
PLIN2	convergent/NM_001285596.1	F: TTACCGCAGAACATTCAAGA, R: GGATAAAAGGGACCTACCAG	218	52

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Table 1 to be continued

Gene	Primer type/reference in GenBank	Sequence (5'–3')	Amplicon size (bp)	*Ta* (°C)
TENT2	convergent/XM_013966728.2	F: CTCTGTCCGATTGTTGTTTACC, R: CAGTTTCTTTTTTGTTCCCCTT	230	52
CAMSAP1	convergent/ XM_018056072.1	F: CTACTACCCCGATACCGAG, R: GGGATGAGGCTGAACTGCT	123	53
VWA8	convergent/ XM_018056795.1	F: TAAAGGAAGGTGGTTTGAGAAG, R: CAGAGTGGAGGGAAAAGATAAG	234	53
TENT4A	convergent/XM_018065610.1	F: CAGTTTGTCTGCGGTTCGTGAT, R: CCGTGCTTCTTTAGTGGAGGGT	166	57
TXNRD3	convergent/ XM_018067233.1	F: AGCACCACAAAGTCAAGGCA, R: CAGTAGGGCAGCGAGAACAG	160	54
ERCC6	convergent/XM_018042328.1	F: CGTCCGTGTTTGTGTTTCTT, R:ATCTTTTCCTCAATGGTGCC	202	55
*REF-UBB**	convergent/NM_001009202.1	F: GCATTGTTGGGTTCCTGTGT, R: TTTGCATTTTGACCTGTGAG	90	52
*REF-YWHAZ**	convergent/AY970970	F: TGTAGGAGCCCGTAGGTCATCT, R: TTCTCTCTGTATTCTCGAGCCATCT	102	56
*REF-SDHA**	convergent/DQ386895	F: AGCACTGGAGGAAGCACAC, R: CACAGTCGGTCTCGTTCAA	105	53

F = forward; R = reverse; *Ta* = annealing temperature

*The primers were cited from our previous study (Bai et al. 2014)

ferences between multiple groups. When a *P*-value was less than 0.05, the difference was considered to be statistically significant. All statistical analyses in this investigation were carried out using the SPSS v17.0 program (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Discovery and characterization of novel circRNAs in skin tissue of cashmere goat

In this study, a total of 21 novel circRNAs were analyzed that were from full-length transcriptome sequencing data on skin tissue at anagen of cashmere goats, which was described well in a previous publication (Yin et al. 2019). The genomic information on the 21 analyzed circRNAs was defined through mapping them into the goat reference genome (assembly ARS1 with NCBI annotation release: 102, https://www.ncbi.nlm.nih.gov/genome/?term=goat). In Table 2, we presented the relevant mapping information on the 21 analyzed circRNAs in goat genome. As shown in Table 2, several putative circRNAs are mapped onto the same chromosome, such as circRNA-BOC, circRNA-TIAM1, and circRNA-PARL on chromosome 1, as well as circRNA-VWA8, circRNA-ZC3H13, and circRNA-THSD1 on chromosome 12, but they do not have any shared overlap region, which can be determined by the matching position information on the corresponding chromosomes. Although the meaning of multiple circRNAs localized on the same chromosome still needs to be further elucidated, we speculate that these circRNAs may have similar or related functions in the SHF physiological process of cashmere goat. On the other hand, we also noted that the circRNA-IFFO2 (S) and circRNA-IFFO2 (L) were transcribed from the same host gene *IFFO2*, moreover, there was an overlap region being shared between them which can be defined through the mapped locations on their corresponding regions on *IFFO2* gene. The remaining 19 analyzed circRNAs were found to result from differential host genes in goats (Table 2).

Bioinformatically, we evaluated the protein-encoding potential of 21 circRNAs based on the use of CPAT program. The resultant data revealed that all the analyzed circRNAs exhibited a coding probability score being greater than the set cut-off value of 0.1 (Figure 1A). These results provide a novel insight into the protein-coding probability of the analyzed circRNAs in the skin tissue of cashmere goats. Therefore, we strongly suggested that the potential active peptides encoded by these

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Table 2. Genomic information on 21 novel circRNAs from the skin tissue of cashmere goats

circRNA name	Length of spliced sequence (nt)	No. of chromosome	Sequence ID in goat genome	Location on chromosome	Host gene
circRNA-BOC	1122	chromosome 1	NC_030808.1	57744493 to 57745614	*BOC*
circRNA-PARL	3533	chromosome 1	NC_030808.1	82964031 to 82967563	*PARL*
circRNA-TIAM1	2050	chromosome 1	NC_030808.1	2843808 to 2845857	*TIAM1*
circRNA-IFFO2(L)	4115	chromosome 2	NC_030809.1	2308670 to 2312784	*IFFO2*
circRNA-IFFO2(S)	2773	chromosome 2	NC_030809.1	2308670 to 2311442	*IFFO2*
circRNA-ZBTB40	1344	chromosome 2	NC_030809.1	5631520 to 5632863	*ZBTB40*
circRNA-KMT2C	4920	chromosome 4	NC_030811.1	5177551 to 5182470	*KMT2C*
circRNA-LOC102174373	1223	chromosome 6	NC_030813.1	10852125 to 10853347	*LOC102174373*
circRNA-PLIN2	1674	chromosome 8	NC_030815.1	25000463 to 25002136	*PLIN2*
circRNA-TULP4	2204	chromosome 9	NC_030816.1	82114863 to 82117066	*TULP4*
circRNA-TENT2	3982	chromosome 10	NC_030817.1	90657472 to 90661453	*TENT2*
circRNA-CAMSAP1	1161	chromosome 11	NC_030818.1	102883878 to 102885038	*CAMSAP1*
circRNA-ODF2	1395	chromosome 11	NC_030818.1	98569888 to 98571282	*ODF2*
circRNA-THSD1	4545	chromosome 12	NC_030819.1	65012157 to 65016701	*THSD1*
circRNA-VWA8	1356	chromosome 12	NC_030819.1	74848510 to 74849865	*VWA8*
circRNA-ZC3H13	3305	chromosome 12	NC_030819.1	70487015 to 70490319	*ZC3H13*
circRNA-TENT4A	4106	chromosome 20	NC_030827.1	66422778 to 66426883	*TENT4A*
circRNA-SNRK	4298	chromosome 22	NC_030829.1	15088417 to 15092714	*SNRK*
circRNA-TXNRD3	3533	chromosome 22	NC_030829.1	60040814 to 60044346	*TXNRD3*
circRNA-TNFRSF21	2669	chromosome 23	NC_030830.1	28430416 to 28433084	*TNFRSF21*
circRNA-ERCC6	1381	chromosome 28	NC_030835.1	2123183 to 2124563	*ERCC6*

The genomic detail of each circRNA was determined by mapping its linear sequence into the reference genome of goat (assembly ARS1 with NCBI annotation release: 102, https://www.ncbi.nlm.nih.gov/genome/?term=goat)

circRNAs should be further investigated in the skin tissue of cashmere goats through an appropriate assay which might mean essential significance to the development and physiology of SHFs in cashmere goats.

It is widely accepted that the RT-qPCR is an appropriate technique in detecting the expression of various types of RNA molecules. Here, through the use of RT-qPCR, we detected the relative expression of 21 putative circRNAs in the skin tissue of cashmere goat at both anagen and telogen of the SHF cycle (expressions in all three phases, anagen catagen and telogen, were studied in nine upregulated circRNAs; see below). As a result, we found that the 21 analyzed circRNAs were transcribed in the skin tissue of cashmere goats at detected anagen and telogen stages. There is no significant difference in the expression level of circRNA-LOC102174373, circRNA-ZBTB40, circRNA-ODF2 and circRNA-TULP4 between anagen and telogen (Figure 1B). The remaining 17 analyzed circRNAs show significant differences in the expression level between anagen and telogen (Figure 1B). Among them, interestingly, the expression of nine circRNAs was significantly upregulated at anagen compared with those of telogen, including circRNA-BOC, circRNA-CAMSAP1, circRNA-VWA8, circRNA-ERCC6, circRNA-PLIN2, circRNA-TIAM1, circRNA-TXNRD3, circRNA-TENT2, and circRNA-TENT4A (Figure1B).

As well known, the anagen of the SHF cycle in cashmere goats is a key dynamic period for the formation and growth of cashmere fibres, within which many cashmere-related molecules are specifically expressed in SHFs, such as various keratins and components of the inner root sheath (Yin et al. 2019). Thus, we speculate that the nine circRNAs having higher expression at anagen of the SHF cycle may be essentially involved in the formation of cashmere fibre along with its growth. This draws us to conduct further research on the nine upregulated circRNAs at anagen including

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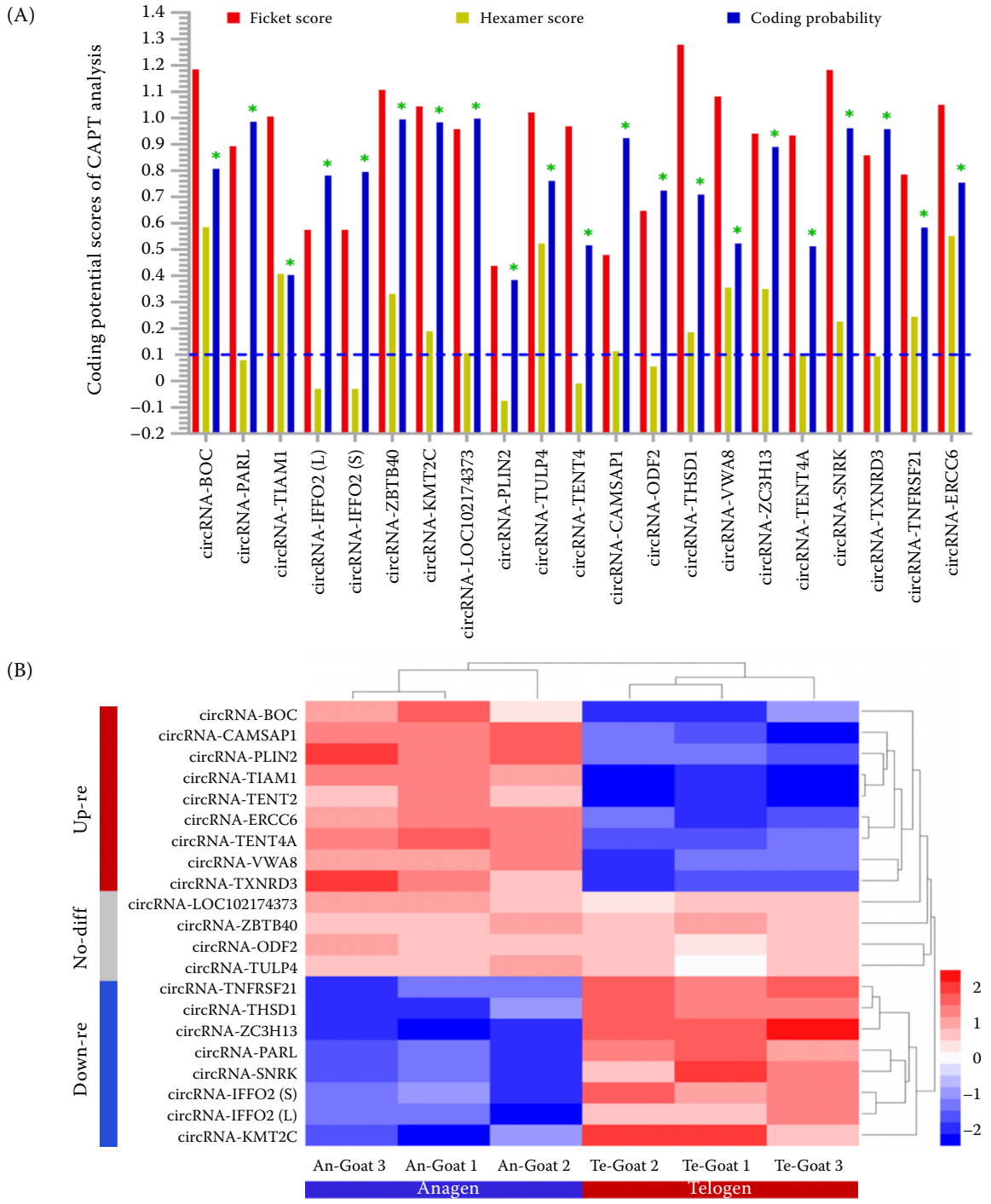

Figure 1. Identification of 21 novel circRNAs from cashmere goats and their expression features in skin tissue at both anagen and telogen stages

(A) Coding potential analysis of 21 circRNAs by CPAT program with the cutoff value being set as 0.1, where the asterisk (*) represents the coding probability score of each analyzed circRNA being more than the cutoff value of 0.1. (B) Clustering expression heat map of 21 circRNAs in the skin tissue of cashmere goats between anagen and telogen stages which was created using the RT-qPCR data obtained in this investigation

Down-re = represents the expression mean of circRNAs being significantly downregulated at the anagen skin tissue of cashmere goats (compared with telogen, $P < 0.05$); No-diff = no significant difference in the expression mean of circRNAs in the skin tissue of cashmere goats between anagen and telogen stages; Up-re = expression mean of the circRNAs being significantly upregulated at the anagen skin tissue of cashmere goats (compared with telogen, $P < 0.05$)

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potential regulatory network, involved signalling pathways, and their expression characterizations in SHFs of cashmere goats during different stages of the SHF cycle.

Potential regulatory network of upregulated circRNAs at anagen skin tissue of cashmere goats

As known, circRNAs can function as a "molecular sponge" of miRNAs, and finally increasing the translated protein level of their target mRNAs, which can be achieved through a regulatory pathway: circRNA-miRNA-mRNA (Yin et al. 2020). In order to understand the regulatory characterizations of the nine upregulated circRNAs at anagen, a ceRNA network was generated for each analyzed circRNA via the use of bioinformatics tools. As shown in Figure 2, each analyzed circRNA has multiple regulatory pathways with its target miRNAs along with their regulatory mRNAs. As an example, the circRNA-TENT2 may bind with six miRNAs, namely, miR-190a-3p, miR-5011-5p, miR-664a-3p, miR-888-3p, miR-361-5p, and miR-374a-3p, thereby further individually or cooperatively regulating the expression of multiple target genes such as *ADCYAP1*, *VEGFA*, *PTP4A2* and *KLF3* (Figure 2D).

In order to more comprehensively understand the integrated regulatory relationships among the analyzed circRNAs, miRNAs and target mRNAs, we incorporated nine fractional networks (Figures 2A–2I) into an integrated network (Figure 3) where the circRNAs, miRNAs, and target mRNAs are represented by red rhombuses, yellow "V" shapes, and green circles, respectively. As shown in Figure 3, a richer and more complicated regulatory relationship was revealed among the nine circRNAs and implicated miRNAs along with their target genes. As an example, *MINDY2* may be regulated coordinately by miR-29a-5p and miR-670-3p, which may further be subjected to the regulation by both circRNA-TIAM1 and circRNA-ERCC6 (Figure 3). Interestingly, several miRNAs potentially interacting with the single or multiple analyzed circRNAs were found to have significantly lower expression at anagen hair follicle than those at telogen, such as miR-24-3p ($P < 0.05$) (Bai et al. 2016b), miR-7 ($P \leq 0.000\ 01$), miR-29a ($P \leq 0.000\ 01$), miR-374a-3p ($P = 0.013\ 43$) (Yuan et al. 2013). Thus, it can be suggested that these miRNAs might be required for the optimal expression of related genes at anagen SHFs of cashmere goats.

On the other hand, within this incorporated network, we also noted that many genes were verified to play important functional roles in the morphogenesis and development of hair follicle. The knockdown of *IGF1R* led to an earlier enter into anagen and a delay in the anagen/catagen switch, along with the inhibited expression of *BMP4* in mouse hair follicles (Castela et al. 2017). This suggests that *IGF1R* is heavily implicated in the hair cycle. Also, the *KLF3* is involved in the effects of miR-21 on hair follicle development in sheep (Zhai et al. 2019). In addition, the *ZNF* family is also implicated in the SHF physiology of cashmere goats (Su et al. 2018). Here, several members of *ZNF* family were contained in this incorporated ceRNA network, such as *ZNF189*, *ZNF773*, and *ZNF37A* (Figure 4). Thus, it can be implied that the circRNAs in this incorporated network might play essential functional roles in regulating the expression of their corresponding target genes in cashmere goat SHFs via the ceRNA regulatory pathways.

Pathway enrichment analysis on the regulatory target genes of upregulated circRNAs at anagen skin tissue of cashmere goats

As well known, a biological process in cells is often regulated integrally by multiple pathways and related regulatory genes. To understand the functional roles and regulatory characterizations of the nine up-regulated circRNAs at anagen, a pathway enrichment analysis was conducted on their ceRNA regulatory genes via the use of CluePedia built-in plugin of Cytoscape program. As shown in Figure 4, the regulatory genes of the upregulated circRNAs at anagen were significantly enriched into multiple different pathways, such as Wnt signalling pathway, TGF-beta signalling pathway, mTOR signalling pathway, MAPK signalling pathway, axon guidance, insulin secretion, GnRH secretion, and signalling pathway regulating the pluripotency of stem cells (Figure 4). Of them, several pathways were verified to have functional roles in hair follicle physiology. As an example, it has been demonstrated that the Wnt signalling pathway plays important roles in the formation of new hair fibre, and the inhibiting of Wnt sig-

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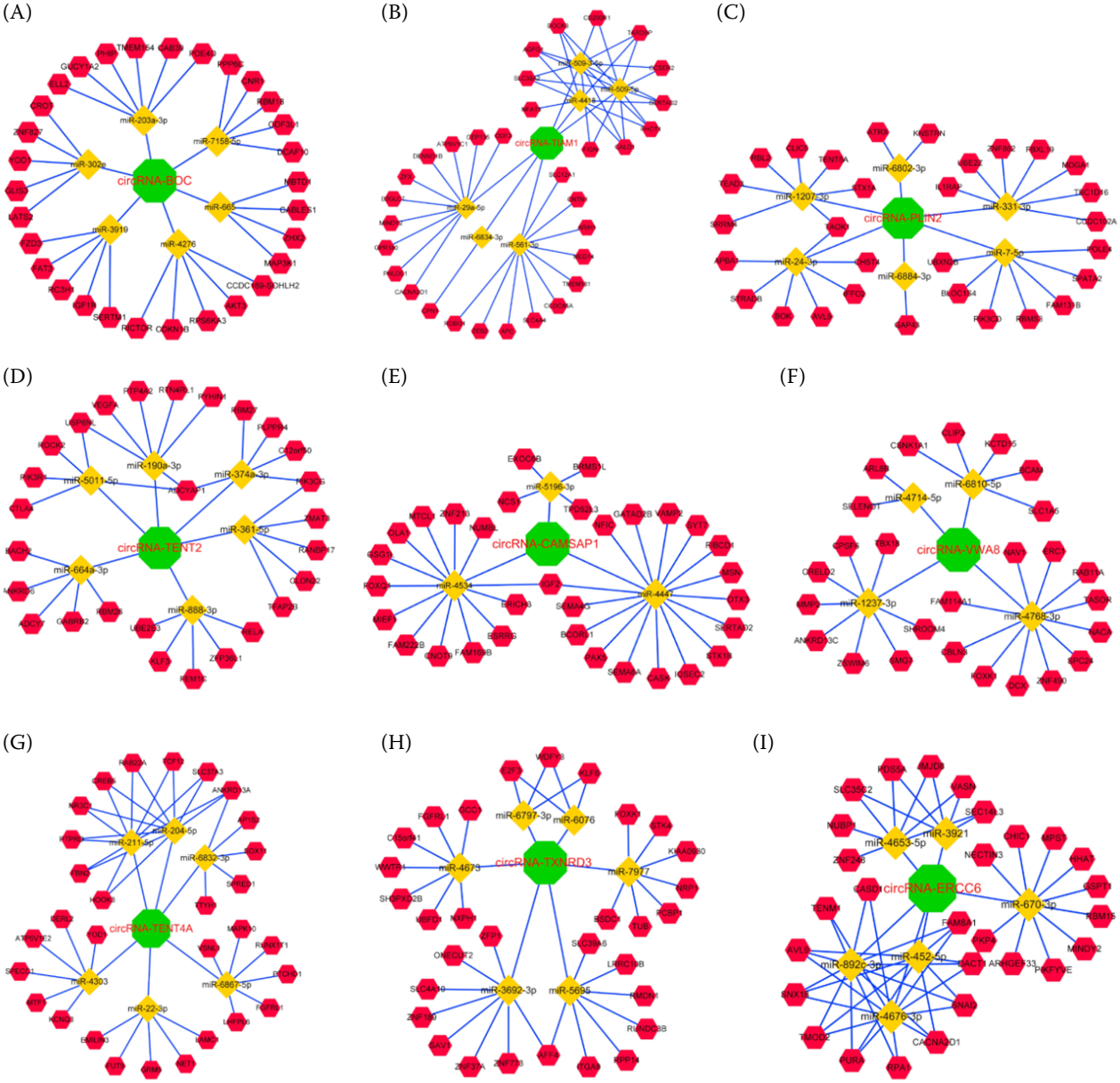

Figure 2. The ceRNA regulatory network of each upregulated circRNA in the anagen skin tissue of cashmere goats (compared with telogen)

(A) circRNA-BOC; (B) circRNA-TIAM1; (C) circRNA-PLIN2; (D) circRNA-TENT2; (E) circRNA-CAMSAP1; (F) circRNA-VWA8; (G) circRNA-TENT4A; (H) circRNA-TXNRD3; (I) circRNA-ERCC6

Green octagons = circRNAs; red hexagons = target genes; yellow rhombi = miRNAs

nalling pathway can lead to a failure in maintaining hair follicle structures (Park et al. 2012). Also, it was reported that paracrine TGF-beta signalling counterbalanced BMP-mediated repression in the activation of hair follicle stem cells (Oshimori and Fuchs 2012). Similarly, the mTOR signalling promoted the activation of hair follicle stem cells via counterbalancing BMP-mediated repression during hair regeneration (Deng et al. 2015). The MAPK signalling pathway was implicated in hair growth with enhancing expression of related growth factors (Kim et al. 2020). The axon guidance signalling is required for hair follicle formation through driving located cellular rearrangements (Sennett et al. 2015). To this end, we suggest that the enriched genes in Figure 4 may be implicated in the SHF development and cashmere fibre growth of cashmere goats in which their functional roles may be finally

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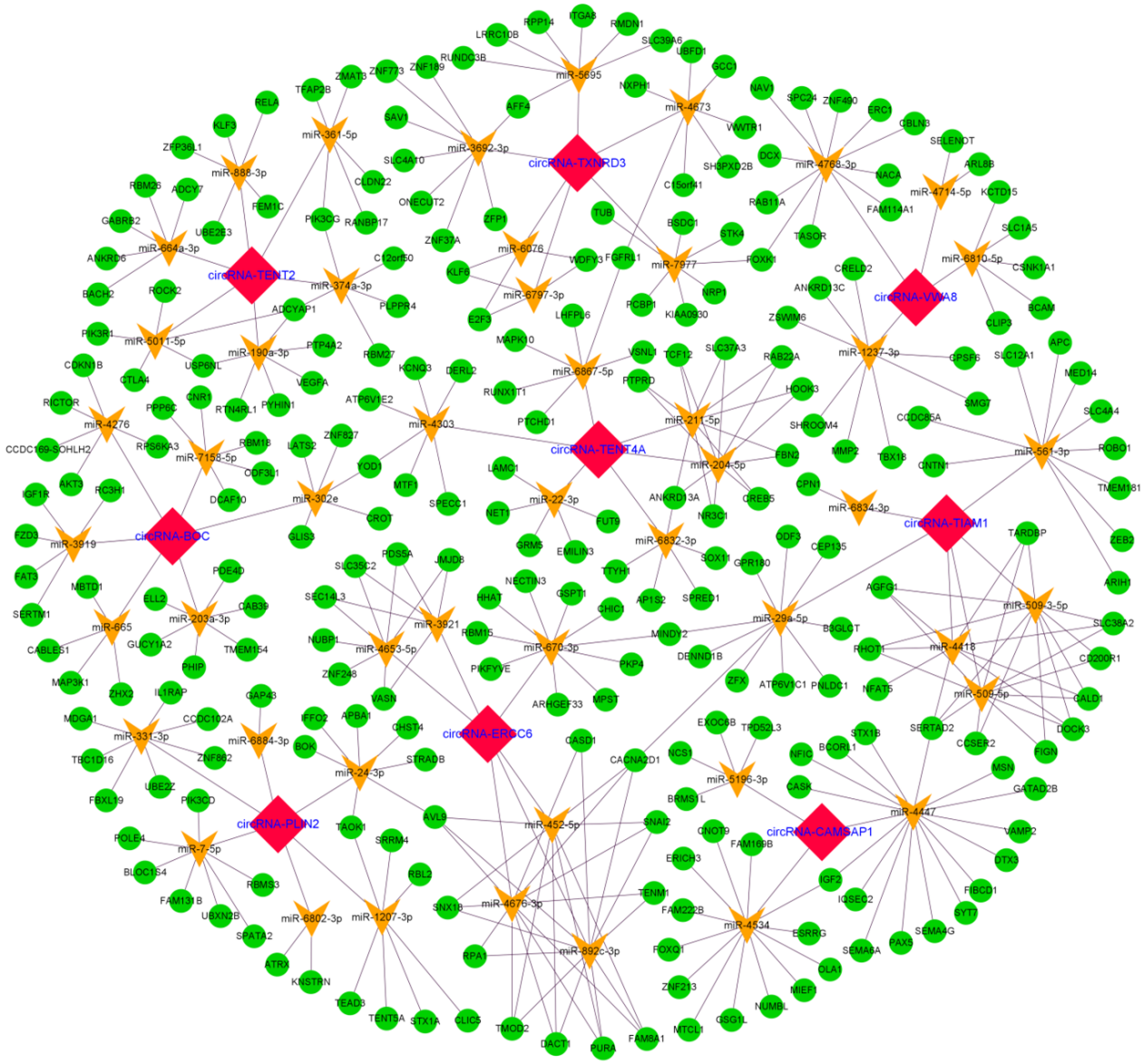

Figure 3. Integrated regulatory network of nine upregulated circRNAs in the anagen skin tissue of cashmere goats (compared with telogen)

Green circles = target genes; red rhombi = circRNAs; yellow V-shapes = miRNAs

This integrated network was generated and visualized by the Cytoscape program v2.8 (https://cytoscape.org/)

subjected to the regulation of the corresponding circRNAs in cashmere goats.

Expression characterizations of upregulated circRNAs at anagen along with their host genes in SHFs of cashmere goats during hair follicle cycle

In order to further understand the functional significance and regulatory characterizations of the nine upregulated circRNAs at anagen, we investigated the expression characterizations of the nine circRNAs along with their host genes in SHFs of cashmere goats at three main stages of the SHF cycle: anagen, catagen and telogen. As shown in Figure 5, the circRNA-BOC, circRNA-CAMSAP1, circRNA-VWA8, and circRNA-ERCC6 exhibited a highly similar expression pattern to their respective host gene in SHFs during the investigated stages (Figures 5A, 5E, 5F and 5I). These observations are consistent with the previous reports that the expression abundances of many circRNAs were revealed to be positively related with

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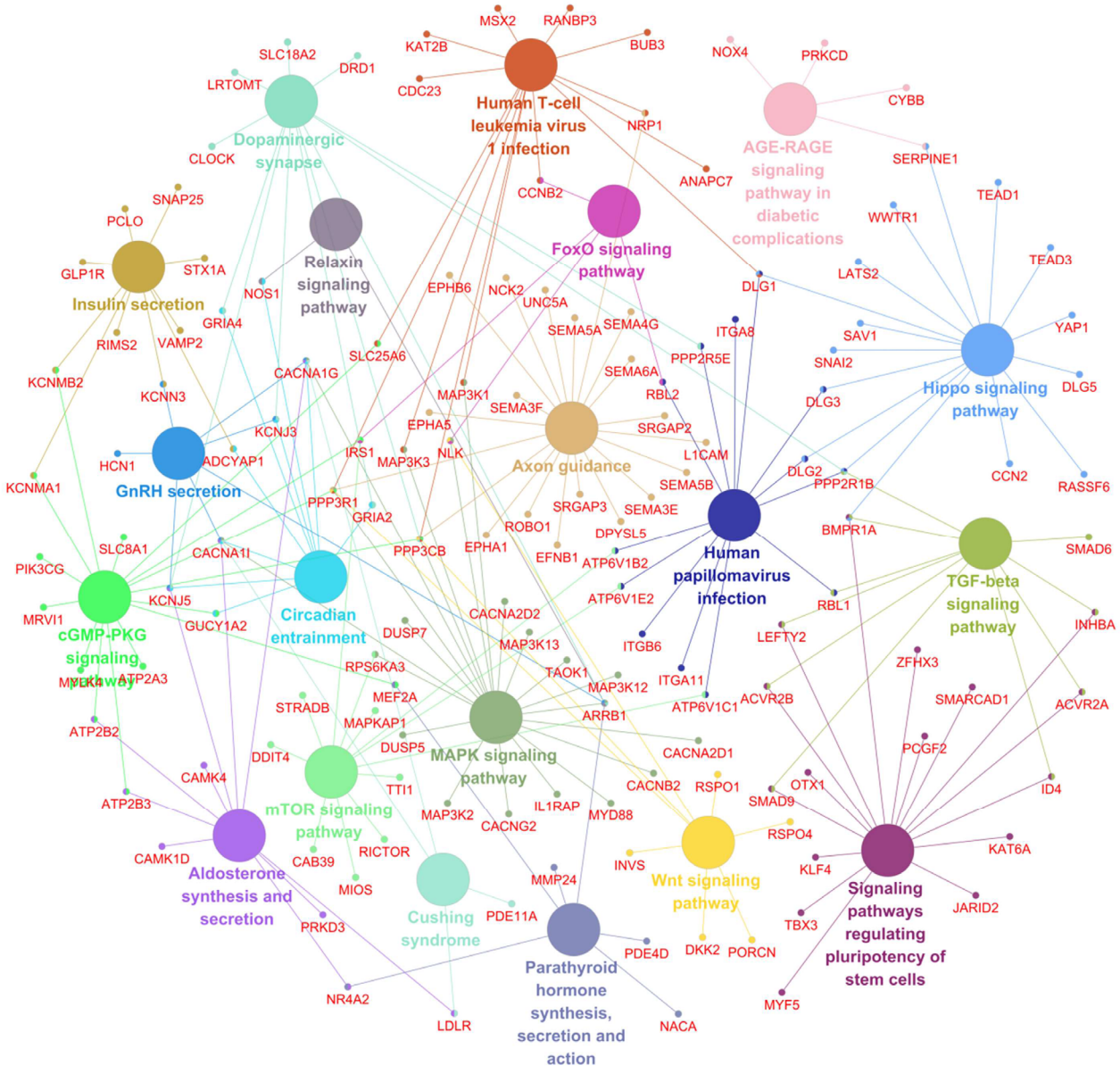

Figure 4. Signalling pathway enrichment for the ceRNA regulatory genes of nine upregulated circRNAs in the anagen skin tissue of cashmere goats (compared with telogen)

The enrichment analysis was carried out by the CluePedia built-in plugin within Cytoscape program v2.8 (http://www.ici.upmc.fr/cluepedia/). The enriched genes along with their corresponding pathways were simultaneously visualized in the generated network where pathway terms/nodes and their related gene nodes/edges share the same colour

their mRNA abundances from the same host gene (Rybak-Wolf et al. 2015). However, we also found that the expression pattern of five circRNAs deviated from those of their respective host gene during the analyzed stages including circRNA-TIAM1, circRNA-PLIN2, circRNA-TENT2, circRNA-TENT4A, and circRNA-TXNRD3 (Figures 5B, 5C, 5D, 5G and 5H). These results further supported the finding that some circRNAs even go against the mRNA counterparts of their host gene with dynamic changes (Yin et al. 2019). On the other hand, our data indicated that the nine analyzed circRNAs exhibited a significantly higher expression at anagen SHFs than those at telogen (Figure 5), which was highly similar to those of the skin tissue of cashmere goats (Figure 1B). Furthermore, we found that the respective host gene of the nine analyzed circRNAs was also the case in the expression trend between anagen and telogen SHFs of cashmere goats (Figure 5).

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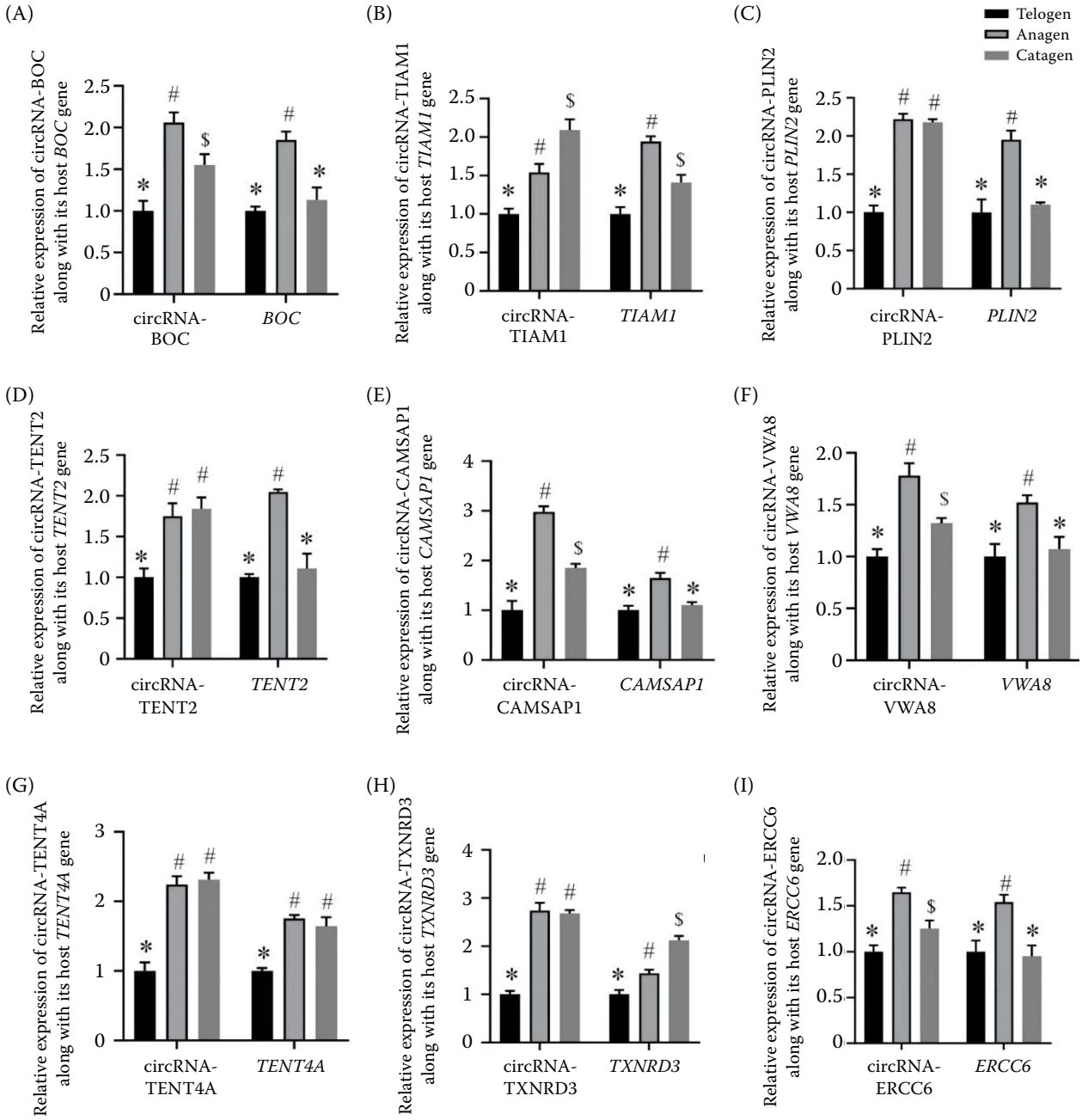

Figure 5. Expression features of nine upregulated circRNAs at anagen (compared with telogen) along with their corresponding host genes in SHFs of cashmere goats during different stages of the SHF cycle: anagen, catagen, and telogen
Error bar indicates standard deviation within the group. Different symbols among telogen, anagen, and catagen stand for significant difference ($P < 0.05$)

The *TIAM1* (the host gene of circRNA-TIAM1), a guanine nucleotide exchange factor for Rac, is implicated in regulating local Rac-PAK signalling in hair cells, thereby maintaining centrosome anchoring, the normal V-shape of hair bundles, the pericentriolar matrix and microtubule organization (Sipe et al. 2013). The *ERCC6* (the host gene of circRNA-ERCC6) is a member of the nucleotide excision repair (NER) family. Although its functional role in hair follicle physiology needs to be further investigated, several members of NER family were revealed to be expressed in both the hair bulb and the upper hair sheaths (containing the bulge region) including *ERCC1*, *ERCC2*, *ERCC3*, *ERCC4*, *ERCC5*, *XPA*, *NTPBP*, *HCNP*, *DDB2* and *POLH* (Yu et al. 2012). This implies that the significantly higher

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expression of *ERCC6* at anagen SHFs of cashmere goat might mean further essential significance. Thus, we speculate that these two circRNAs (circRNA-ERCC6 and circRNA-TIAM1) may be essentially involved in the SHFs development and cashmere growth of cashmere goats via establishing balanced regulatory pairs with their respective host gene (*TIAM1* and *ERCC6*).

It is well known that the anagen is a highly dynamic stage of SHFs in cashmere goats, accompanied by an extensive proliferation of hair follicle cells and growth of SHFs and cashmere fibres (Geng et al. 2013). During this active stage, several signalling pathways are implicated to complete the reconstruction of SHFs along with the formation and growth of cashmere fibres, including Wnt/β-catenin, Notch, JAK-STAT, IGF, NF-κB, SHH, and BMP (Suen et al. 2020). In the present study, we showed that *BOC*, *PLIN2*, *TENT2*, *CAMSAP1*, and *VWA8* exhibited significantly higher expression in anagen SHFs than those in telogen SHFs (Figures 5A, 5C, 5D, 5E and 5F). Among them, although the specific functional roles of VWA8 and TENT2 are unknown in hair follicle physiology, it has been demonstrated that BOC can form SHH receptor complexes with PTCH1, and is required for SHH-mediated cell proliferation, whereas PTCH1 plays a role in the differentiation of hair follicle lineage (Villani et al. 2010). CircRNA-CAMSAP1 acted as the sponge of miR-328-5p and upregulated *E2F1* expression to promote cell proliferation (Zhou et al. 2020), whereas E2F1 was also identified as a key transcript factor to regulate the proliferation of dermal papilla cells (Shen et al. 2017). Also, it was shown that the knockdown of *PLIN2* suppressed the activation of Wnt coreceptor pathways implying that *PLIN2* mediates the Wnt signalling pathway (Liu et al. 2015). Interestingly, the Wnt signalling pathway plays important roles in promoting hair follicle development and the differentiation of bulge stem cells into hair follicle lineage with hair formation (Lowry et al. 2005). Thus, taken together with our results, it can be suggested that *BOC*, *CAMSAP1*, and *PLIN2* along with the respective circRNA (circRNA-BOC, circRNA-CAMSAP1, and circRNA-PLIN2) might form a new regulatory layer in the proliferation of hair follicle cells and the growth of anagen SHFs of cashmere goats.

In addition, we found that circRNA-TIAM1 exhibited significantly higher expression at catagen SHFs of cashmere goats than those of both anagen and telogen (Figure 5B), and the *TXNRD3* was also the case in SHFs of cashmere goats (Figure 5H). While, compared with telogen, the circRNA-TENT4A and its host gene *TENT4A* were continuously upregulated from anagen to catagen (Figure 5G). As well known, in fact, catagen is a short transitional period of the cashmere fibre growth cycle (between anagen and telogen) that lasts approximately 30 days in cashmere goats, during which the lower portion of SHFs shrinks dramatically and cashmere fibre growth ends (Yin et al. 2019). Thus, we speculate that the TXNRD3, circRNA-TIAM1, and circRNA-TENT4A along with its host gene *TENT4A* might play significant roles in regulating the transition of SHFs from catagen to telogen in cashmere goats.

CONCLUSION

A total of 21 novel circRNAs were identified from skin tissues of cashmere goats. Of them, nine circRNAs exhibited significantly higher expression at anagen than those at telogen in skin tissues of cashmere goats. The expression pattern of the nine circRNAs along with their host genes in SHFs suggests their potential functional roles in the regeneration and growth of SHFs in cashmere goats.

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Conflict of interest

The authors declare no conflict of interest.

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