Comprehensive Analysis of Coding and Non-coding RNA Transcriptomes Related to Hypoxic Adaptation in Tibetan Chickens

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Research

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Abstract

Background: Tibetan chickens, a unique native breed in the Qinghai-Tibet Plateau of China, have a suite of adaptive features to tolerate the high-altitude hypoxic environment. Increasing evidence suggests that long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) have roles in the hypoxic adaptation of high-altitude animals, although their exact involvement remains unclear.

Results: This study aimed to elucidate the global landscape of mRNAs, lncRNAs, and miRNAs using transcriptome sequencing in order to construct a regulatory network of competing endogenous RNAs (ceRNAs) and thus provide insights into the hypoxic adaptation of Tibetan chicken embryos. In total, 354 differentially expressed genes (DEGs), 389 differentially expressed lncRNAs (DELs), and 73 differentially expressed miRNAs (DEMs) were identified between Tibetan (TC) and Chahua chickens (CH). Functional analysis revealed that several important DEMs and their target DELs and DEGs are involved in angiogenesis (including blood vessel development and blood circulation) and energy metabolism (including glucose, carbohydrate, and lipid metabolism). The ceRNA network was then constructed with the predicted DEGs-DEMs-DELs interactions, which further revealed the regulatory roles of these differentially expressed RNAs during hypoxic adaptation of Tibetan chickens.

Conclusions: These transcriptomic data revealed several key candidate ceRNAs that may play high-priority roles in the hypoxic adaptation of Tibetan chickens by regulating angiogenesis and energy metabolism. These results provide insight into the molecular mechanisms of hypoxic adaptation regulatory networks from the perspective of coding and non-coding RNAs.

Background

MicroRNAs (miRNAs) are small non-coding RNA molecules that inhibit gene expression by binding to specific mRNAs. lncRNAs are another type of non-coding RNA that are approximately 200 nucleotides in length and regulate gene expression through a number of mechanisms. Both of these non-coding RNAs play different roles in various aspects of cellular function, including the regulation of hypoxia-related genes. Through their activity on target transcripts, some critical miRNAs regulate angiogenesis, the cell cycle, as well as lactate transport under hypoxic conditions [1–3]. HIF-1 and VEGF are regulated by miR-17-92, miR-20b, and miR-199a, which play roles in tumor development, cancer cell proliferation, and cardiac myocytes [4–6]. Various studies have shown that lncRNAs can directly bind to HIF-1 and activate its expression, in turn modulating hypoxic responses as done through suppression of cancer cell adaptation to hypoxia, which provides important insights into how tumor cells sense and adapt to hypoxic stress [7–9]. Although there have been many studies on genes involved in high-altitude adaptation in humans and animals, the regulatory mechanism of non-coding RNAs involved in hypoxic adaptation remains largely unknown.

Tibetan chickens, which have lived in Tibetan highlands and have undergone long-term natural selection, exhibit stable genetic adaptation to high-altitude environments characterized by high hatchability under hypoxic incubation when compared to lowland chickens [10, 11]. This indicates that Tibetan chickens have special physiological and genetic mechanisms to adapt to extreme high-elevation environments ensuring their normal hatching, reproduction, and growth in plateau environments [12–14]. Previous studies using genomic, transcriptomic, and proteomic profiles have identified several functional genes related to hypoxic adaptation in Tibetan chickens [15–17]. However, the mechanisms involved in the non-coding RNA regulation of hypoxic adaptation are unclear.

In this study, RNA sequencing (RNA-seq) was performed to explore the regulation of ncRNAs and candidate genes responsible for hypoxic adaptation traits in Tibetan chickens via comparison to Chahua chickens using embryos. These findings will help us further understand the molecular mechanism of adaptability to hypoxic conditions in Tibetan chickens from an ncRNA perspective.

Methods

Sample collection and preparation

The experiments and animal care protocol were approved by the animal welfare committee of the State Key Laboratory for Agro-Biotechnology of the China Agricultural University (approval number, XK257), and all methods were performed in accordance with the relevant guidelines and regulations.

We collected eggs from Tibetan chickens (TCs) and Chahua chickens (CHs) from the Experimental Station for Poultry Genetic Resources and Breeding, China Agricultural University (CAU). The eggs were incubated in a hypoxic incubator ($13\% \pm 0.2\% \text{ O}_2$) and a normoxic incubator ($21\% \pm 0.2\% \text{ O}_2$), and the temperature and humidity of the incubators were 37.8°C and 60%, respectively. Eighteen chorioallantoic membrane (CAM) samples from Tibetan ($n = 9$) and Chahua chickens ($n = 9$) under hypoxic conditions were collected from female embryos at day 11 of incubation, immediately frozen in liquid nitrogen, and stored at -80°C for sequencing.

RNA extraction, library construction, and sequencing

Total RNA from eighteen CAM tissues of day 11 embryos was extracted using an RNA pure Tissue Kit (Tiangen Biochemical Technology Beijing Co., Ltd) according to the manufacturer's protocol. Six RNA-seq libraries (TC1, TC2, TC3, CH1, CH2, and CH3) were constructed, and each sample was mixed from three individuals of the same breed.

Library preparation and sequencing of mRNA and lncRNA

The Epicentre Ribo-Zero™ kit was used to remove sample rRNA, and fragmentation buffer was added to randomly interrupt rRNA-depleted RNA. Using rRNA-depleted RNA as a template, the first cDNA strand was synthesized with random hexamers. Thereafter, buffer, dATP, dUTP, dCTP, dGTP, RNase H, and DNA polymerase I were added to synthesize the second cDNA strand. AMPure XP beads were used to purify cDNA. The double-stranded cDNA was repaired at the ends, adding poly-A and connecting the sequencing adapters. AMPure XP beads were then used for fragment size selection. The U chain was degraded and the cDNA library was obtained by PCR enrichment. The library was sequenced on an Illumina HiSeq platform and 150 paired-end reads were generated.

Library preparation and sequencing for sRNA

After the sample was qualified, a linker was added to the RNA, which was reverse transcribed to synthesize cDNA, followed by PCR amplification. Gel separation technology was employed to screen the target fragment, and the fragment recovered by gel cutting was the small RNA library. The library was sequenced on an Illumina HiSeq platform, and 50 single-end reads were generated.

mRNA and lncRNA data analysis

Quality control was performed using in-house Perl scripts. Raw reads were filtered by removing reads containing adapters, poly-N, and low-quality reads, in order to obtain clean reads. The Q20, Q30, and GC content of the clean data were calculated. Clean reads were aligned to the chicken reference genome Gallus-gallus-6.0 (GRCg6a-96.Gallus-gallus.GRCg6a-96.genome.fa) using the HISAT2 software [18]. LncRNA prediction was performed through basic screening and coding potential screening. The basic screening included: (1) Selecting the class-code of transcripts as "i", "x", "u", "o", "e" [19]; (2) Selecting transcripts with length ≥ 200 bp and exon number ≥ 2 [20]; (3) Selecting transcripts with FPKM ≥ 0.1 [20]; and using four tools to further screen the selected candidate lncRNA in order to predict its coding potential: CPC2 (Coding Potential Calculator) [21], CNCI (Coding-Non-Coding Index) [22], CPAT (Coding Potential Assessment Tool) [23], pfam protein domain analysis [24]. Based on the mode of action of lncRNAs and their target genes, two methods are used for predicting the target genes of lncRNAs: (1) lncRNA regulates the expression of its neighboring genes, which is mainly predicted based on the position between lncRNA and the gene. The neighboring genes within 100 kb of the lncRNA are target genes; (2) To predict the trans-target genes of lncRNA by analyzing the correlation between lncRNA and mRNA expression among samples. The mapped reads of each sample were assembled, and the gene and lncRNA expression levels were quantified based on fragments per kilobase of transcript per million fragments mapped (FPKM) using the StringTie software [25]. The DESeq2 software package was used to detect differentially expressed lncRNAs (DELs) and differentially expressed genes (DEGs) with criteria of $\log_2$ |fold change| > 1 and $P < 0.05$ [26].

miRNA data analysis

The raw data were subjected to quality control in order to obtain clean reads as described hereafter. Sequences with a low quality value were removed from each sample. Reads with an unknown base N (N is an unrecognizable base) content greater than or equal to 10% were removed. Reads without a 3' linker sequence were removed. The 3' linker sequences were cut off. Sequences shorter than 18 or longer than 30 nucleotides were removed. Clean reads were aligned with the Silva, GtRNAdb, Rfam, and Repbase databases to filter ncRNA, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and repeating sequences, in order to obtain unannotated reads containing miRNA. Unannotated reads were mapped to the reference genome (Gallus-gallus.GRCg6a-96) using Bowtie software [27]. Known miRNAs were identified by mapping the matched mature miRNAs in miRBase (v22) to the reference genome. For unannotated reads that matched the reference genome sequences miRDeep2 was used to predict novel miRNAs [28]. The expression of miRNA in each sample was quantified and normalized by the TPM algorithm ($\text{TPM} = \text{Readcount} \times 1,000,000 / \text{Mapped Reads}$) [29]. Target genes were predicted by miRanda and TargetScan based on miRNA and gene sequence information [30, 31]. The DESeq2 software package was used to detect differentially expressed miRNAs (DEMs) with the criteria of $\log_2$ |Fold change| > 1 and $P < 0.05$ [26].

Functional annotation analysis of differentially expressed RNA

Gene Ontology (GO) enrichment analysis of the DEGs was performed using the Goseq R packages based on the Wallenius' non-central hypergeometric distribution. The KEGG is a database resource for understanding high-level functions and utilities of a biological system, such as the cell, organism, and ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (<http://www.genome.jp/kegg/>). We used the KOBAS software to test the statistical significance of DEG enrichment in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Construction of the ceRNA Network

Competitive endogenous RNAs (ceRNAs) are novel transcriptional regulators that can modulate each other's expression through competitive binding. For example, miRNA can silence gene expression by binding to target mRNA, while lncRNAs competitively bind miRNA and affect its function, in turn downregulating gene expression [32]. Based on the regulatory pairs of differentially expressed miRNA-mRNA and miRNA-lncRNA, ceRNA networks (lncRNA-miRNA-mRNA) of the interaction between lncRNA and mRNA through shared miRNAs were predicted, and the following criteria had to be met for ceRNAs: (1) the same number of miRNAs among ceRNAs should be greater than 5; (2) $P < 0.01$, and $FDR < 0.01$ [33].

Verification of DELs, DEGs, and DEMs

To confirm the differentially expressed RNAs identified through RNA-seq, qRT-PCR was carried out for six DELs, six DEMs, and seven DEGs. We used the PrimeScript RT reagent kit with gDNA (Tiangen Biotech Co. Ltd., Beijing, China) to convert total RNA to cDNA, with random hexamers for mRNA, lncRNAs, and mRNAs, and stem-loop RT primers for miRNAs. The qPCR was performed using the SYBR Green PCR kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for lncRNAs, chicken 5S for miRNAs, and HPRT (hypoxanthine guanine phosphoribosyl transferase) for mRNAs were used as internal controls. All primers used in RT-qPCR are shown in Additional file1: Table S1. BioRad CFX96 (Bio-Rad, CA, USA) was used to perform RT-qPCR with SYBR Green PCR Master Mix (Tiangen Biotech Co. Ltd.). Each qPCR experiment was performed in triplicate, relative RNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, and ANOVA testing via SPSS 25.0 was applied. The results were expressed as mean $\pm$ SE, and $P \leq 0.05$ was considered statistically significant.

Results

Overview of RNA transcriptomic profiles of chicken CAM tissues

After low-quality reads were removed, 110.3–114.1 M paired-end reads (150 bp in length) for the mRNAs and lncRNAs, and 23.8–29.2 M single-end reads (50 bp in length) for the miRNAs, were obtained from the CAM of Tibetan (TC) and Chahua chicken (CH) samples (Additional file 2: Table S2). The majority of the lncRNAs (60.7%) were intergenic long-stranded non-coding RNA (lincRNA), followed by intronic-lncRNA (17.7%), antisense-lncRNA (12.4%), and 9.2% sense-lncRNA (Fig. 1a). The length of mRNAs ranged from 51 to 30,0721 bp, lncRNAs ranged from 202 to 223,626 bp, with the majority of mRNAs and lncRNAs being 201–400 bp and > 3000 bp long, respectively (Additional file 3: Fig. S1). In total, 20,912 expressed genes, 15,523 lncRNAs (3,194 known), and 1,439 miRNAs were identified in the CAM tissues of TC and CH. The 1,439 miRNAs included 471 known and 968 novel miRNAs, and most of the miRNA reads were between 20 and 24 nt in length (Fig. 1b, Additional file 4: Table S3). The values of FPKM of mRNA/lncRNA and TPM of miRNA distribution were similar among the six samples (Fig. 1c, d, e).

Differential expression analysis of RNAs between TC and CH

We found 389 DELs, 73 DEMs, and 354 DEGs between the TC and CH groups, among which 194 DELs, 46 DEMs, and 180 DEGs were upregulated, whereas 195 DELs, 27 DEMs, and 174 DEGs were downregulated in the Tibetan chickens compared to Chahua chickens (Fig. 2, Additional file 5: Table S4). The DELs, DEMs, and DEGs were classified into two categories for the six samples in which the expressed quantity of the DELs, DEMs, and DEGs had good repeatability within groups respectively (Additional file 3: Fig. S2).

Functional analysis of differentially expressed non-coding RNAs (DELs and DEMs)

For 389 DELs, we predicted 722 cis- and trans-target genes that were mainly involved in the GO terms of angiogenesis, blood vessel development, regulation of vasoconstriction, regulation of blood pressure, response to oxygen levels, ATP metabolic process, fatty acid metabolic process, glucuronate metabolic process, enzyme binding, and ATP binding (Fig. 3a, Additional file 6: Table S5a). The KEGG analysis revealed involvement of target genes in carbon metabolism, fatty acid metabolism, vascular smooth muscle contraction, Wnt, mTOR, MAPK, VEGF, and calcium signaling pathways (Fig. 3b, Additional file 6: Table S5b).

A total of 1,766 target genes were predicted for 73 DEMs. The target genes were mainly enriched in the GO terms of vasculogenesis, positive regulation of fatty acid oxidation, glycogen metabolic process, angiogenesis, vasculature development, response to hypoxia, oxygen metabolic process, vasodilation, ATP binding, and enzyme binding (Fig. 3c, Additional file 7: Table S6a). Notably, the enriched KEGG pathways were cysteine and methionine metabolism, amino sugar and nucleotide sugar metabolism, apoptosis, vascular smooth muscle contraction, fatty acid metabolism, carbon metabolism, vitamin B6 metabolism, MAPK, Notch, VEGF, mTOR, and calcium signaling pathways (Fig. 3d, Additional file 7: Table S6b).

Comparative parsing of lncRNAs and mRNAs and functional analysis of DEGs

To compare the exon number, open reading frame (ORF), and expression levels of lncRNA and mRNA, we need to consider the differences in structure and sequence. The number of exons corresponding to lncRNAs was mainly less than 8, whereas the number of exons of mRNA was relatively large, ranging from 1 to > 30 (Additional file 3: Fig. S3a, b). The majority lncRNA ORFs were mainly between 50 and 250 nt long, while the length of the ORFs of mRNAs was mainly between 100 and 1,000 nt (Additional file 3: Fig. S3c, d). Expressed mRNAs and lncRNAs were located on all chromosomes of the chicken genome (Fig. 4a). The average and maximum values of mRNA expression were higher compared to those of lncRNA expression (Fig. 4b).

The 354 DEGs were mainly enriched in GO terms of defense response, homeostatic process, regulation of lipid metabolic process, glucose homeostasis, blood microparticle, oxygen transport, blood vessel endothelial cell migration, blood circulation, and respiratory system development. Enriched KEGG pathways included fatty acid metabolism, vascular smooth muscle contraction, calcium, as well as PPAR and MAPK signaling pathways (Fig. 4c, Additional file 8: Table S7). Twelve DEGs (ACTC1, KCNMB4, NCS1, NGFR, ADAM8, CASQ2, IRF4, PTPRZ1, CALML3, ERBB4, ARR3, and NTSR1) were mainly associated with angiogenesis, blood circulation, hematopoiesis, as well as calcium and MAPK signaling pathways. Fifteen DEGs (SSTR5, NR1H4, HTR2C, APOA1, KCNB1, P3H2, CHST8, LYZ, HAO2, ACER1, ACSBG1, ELOVL2, ELOVL3, GBE, and NOX3) were related to glucose and carbohydrate metabolism, fatty acid metabolism, and lactate oxidation (Table 1).

Table 1

Potential key differentially expressed mRNAs (DEGs), their targeted differentially expressed miRNAs (DEMs), differentially expressed lncRNAs (DELs), and their functions related to hypoxic adaption in the Tibetan chicken.

	Gene	Log2FC (TC/CH)	P value	Target miRNA	Target lncRNA	Functional description
Angiogenesis	ACTC1	-3.4644	0.0122	gga-miR-6606-5p	MSTRG.19949.13, MSTRG.128588.3, MSTRG.73129.28, MSTRG.32082.2	Blood circulation
	KCNMB4	2.3558	0.0001	gga-miR-6606-5p	MSTRG.29252.7	Vascular smooth muscle contraction
	NCS1	-1.0050	0.0197	gga-miR-6606-5p, novel_miR_815	MSTRG.33345.5, MSTRG.140060.1, MSTRG.18943.13	Calcium ion transport
	NGFR	1.5519	0.0036	novel_miR_676	MSTRG.118362.24, MSTRG.12318.1, MSTRG.25780.4, MSTRG.128839.3, MSTRG.115756.46, MSTRG.41646.22, MSTRG.80622.27, MSTRG.44286.2, MSTRG.41646.23	Blood vessel morphogenesis, blood vessel development, vasculature development, angiogenesis
	ADAM8	-1.3727	0.0470			Response to hypoxia, blood vessel development, vasculature development, angiogenesis
	CASQ2	-1.0736	0.0315	novel_miR_589, novel_miR_676	MSTRG.73129.28, MSTRG.18943.3	Blood circulation
	IRF4	-1.8880	0.0201			Hemopoiesis
	PTPRZ1	1.3653	0.0000			Hemopoiesis, hematopoietic progenitor cell differentiation
	CALML3	-1.1958	0.0015			Vascular smooth muscle contraction
	ERBB4	-1.2949	0.0102	novel_miR_676, novel_miR_587	MSTRG.147643.141, MSTRG.128534.3, MSTRG.44329.1, MSTRG.29590.15, MSTRG.79818.8, MSTRG.135465.5, MSTRG.30620.3	Calcium and MAPK signaling pathways
	ARR3	1.0513	0.0013			MAPK signaling pathway

	Gene	Log2FC (TC/CH)	P value	Target miRNA	Target lncRNA	Functional description
	NTSR1	-1.5401	0.0140	novel_miR_669, novel_miR_676, novel_miR_567	MSTRG.63187.27, MSTRG.71117.21, MSTRG.133793.2, MSTRG.29590.15, MSTRG.52146.15, MSTRG.72741.32, MSTRG.148631.1	Regulation of respiratory gaseous exchange, Calcium signaling pathway
Energy metabolism	SSTR5	-3.8881	0.0054	novel_miR_819	MSTRG.25881.3	Glucose homeostasis
	NR1H4	-1.1224	0.0179	novel_miR_676	MSTRG.99917.37, MSTRG.64717.15, MSTRG.99917.39, MSTRG.99917.35	Glucose homeostasis, response to oxygen- containing compound
	HTR2C	-1.3476	0.0000			Regulation of lipid metabolic process
	APOA1	1.1034	0.0068			Regulation of lipid metabolic process
	KCNB1	-1.1800	0.0000			Glucose homeostasis, response to oxygen- containing compound
	P3H2	-1.0044	0.0135			Carbohydrate binding
	CHST8	-1.9553	0.0013	novel_miR_693, novel_miR_775		Carbohydrate metabolic process
	LYZ	-1.0365	0.0050			Carbohydrate metabolic process
	HAO2	-1.5483	0.0085	novel_miR_669		Carbon metabolism, lactate oxidation
	ACER1	1.5111	0.0032			Regulation of lipid metabolic process
	ACSBG1	1.1154	0.0000			Fatty acid metabolism
	ELOVL2	-5.1386	0.0480			Fatty acid metabolism

Gene	Log2FC (TC/CH)	P value	Target miRNA	Target lncRNA	Functional description
ELOVL3	2.0872	0.0146	novel_miR_867		Fatty acid metabolism
GBE	-1.0082	0.0072	novel_miR_85	MSTRG.128588.4	Oxygen transport, oxygen binding, reactive oxygen species metabolic process
NOX3	3.0828	0.0000			Reactive oxygen species metabolic process

Construction of the ceRNA network

We constructed a ceRNA network that yielded 529 pairs of candidate ceRNAs (lncRNA-miRNA-mRNA) from 162 DELs and 108 DEGs through 25 miRNA target-mediated relationships (Additional file 3: Fig. S4). Considering that the network contains enormous information, and each relationship cannot be displayed in the figure, we constructed a mini-ceRNA network of important RNAs. We identified 10 known mRNAs that were related to hypoxic adaptation and involved in angiogenesis and blood circulation (NGFR, ACTC1, CASQ2, ERBB4, KCNMB4, NCS1, and NTSR1) (Fig. 5a), as well as energy metabolism (SSTR5, NR1H4, and GBE) (Fig. 5b). A total of 39 lncRNA-miRNA-mRNA interactions were identified in the mini-ceRNA network constructed with these 10 DEGs and their targeted 37 DELs and 9 DEMs (Table 1, Additional file 9: Table S8).

Validation of DELs, DEMs, and DEGs

Six DELs (MSTRG.118362.24, MSTRG.25780.4, MSTRG.19949.13, MSTRG.29590.15, MSTRG.32082.2 and MSTRG.73129.28), six DEMs (gga-miR-726-3p, gga-miR-6608-3p, gga-miR-6606-5p, novel-miR-676, gga-miR-460b-3p, and gga-miR-7442-5p), and seven DEGs (ARR3, NGFR, NTSR1, ADAM8, ACTC1, CASQ2, and CALML3) were measured using quantitative real-time PCR (qRT-PCR) to validate expression differences identified through RNA-seq. The results indicated that the expression levels of all RNAs were significantly different between TC and CH ($P < 0.05$), and fold changes in expression followed the same trend in qRT-PCR and RNA-seq (Fig. 6).

Discussion

Through evolution, Tibetan chickens have developed genetic adaptability to survive in low-oxygen environments. Previous reports have shown that Tibetan chickens exhibit unique phenotypic and physiological characteristics, including enhanced blood oxygen binding capacity and increased blood circulation [11, 16]. Because it was first reported a few years ago, the ceRNA hypothesis has been widely accepted, leading to an improved understanding of human disease, as well as important advances in animal research [32, 34]. The recently discovered ncRNAs represent a new type of master regulator that affects gene expression and modulates a variety of cellular processes. Based on these theories, we constructed a ceRNA regulatory network in order to clarify the molecular mechanism of Tibetan chicken embryo CAM adaptation to hypoxic environments.

The CAM, an important respiratory and circulatory organ for chicken embryo development, contains a large number of blood vessels, and hypoxia can induce increased blood vessel density [17, 35]. From the constructed ceRNA network, we identified several miRNAs, including down-regulated novel-miR-589, novel-miR-815, novel-miR-85, novel-miR-669, as well as up-regulated miR-6606-5p, novel-miR-676, novel-miR-589, novel-miR-815, novel-miR-85, and novel-miR-567. Among these, miR-6606-5p was significantly up-regulated in the Tibetan chicken, and its target differentially expressed mRNAs (ACTC1, KCNMB4, and NCS1) were shown to be involved in vascular development under hypoxic conditions. ACTC1 was enriched in the GO term of blood circulation, while KCNMB4 has been shown to regulate blood pressure and was enriched in the GO terms of regulation of vasoconstriction, ion transport, and vascular smooth muscle contraction pathway [36]. Previous studies also found that NCS1 regulates calcium ion transport and that Ca^{2+} is necessary for the activity of HIF-1, which is a major transcriptional regulator of cells and development in response to hypoxia [37, 38]. It is worth mentioning that with the exception of miR-6606-5p, other differentially expressed miRNAs, including miRNA-155, miRNA-302a, miR-302b-3p, miR-302b-5p, miR-302c-3p, miR-460b-3p, and miR-460b-5p, also play important roles in angiogenesis under hypoxia. The miRNA-155 promotes HIF-1 α activity during prolonged hypoxia and participates in the PI3K/AKT pathway [39, 40]. Previous studies have shown that the miR-302 family (miR-302a/b/c/d) suppresses the proliferation, migration, and angiogenesis of vascular endothelial cells by targeting VEGFA [41–43]. The miR-460b-3p and miR-460b-5p were identified in animal models of hypoxic pulmonary hypertension and were involved in the regulation of HIF-1 α [44, 45]. Moreover, other differentially expressed mRNAs (NGFR, ADAM8, CASQ2, IRF4, PTPRZ1, CALML3, ERBB4, ARR3, and NTSR1) were enriched in blood vessel development, angiogenesis, blood circulation, hematopoiesis, response to hypoxia, oxygen transport, vascular smooth muscle contraction, calcium and MAPK signaling pathways. For example, NGFR is the receptor of NGF, which can induce chick CAM neovascularization [46]. Studies have shown that ADAM8 is significantly induced by hypoxia and plays a role in the proliferation and migration of endothelial cells during angiogenesis [47, 48]. Therefore, we speculate that the enhanced tolerance of Tibetan chicken embryo CAM under hypoxic conditions is ascribed to miRNA-mediated modulation of the related target mRNAs, further regulating HIF, which enables it to maintain hypoxic adaptation via the promotion of angiogenesis and blood circulation.

Glucose uptake and carbohydrate metabolism are the basis for the maintenance of normal physiological functions in humans and animals. Under hypoxic conditions, oxygen and carbon dioxide metabolism mainly depend on mitochondrial respiration and make use of adenosine triphosphate (ATP). At present, a number of studies have shown the key regulatory role of energy metabolism, including glucose, carbohydrate, and lipid metabolic processes, among others, during hypoxic adaptation [49–52]. In the current research, from the constructed ceRNA network, we identified seven differentially expressed miRNAs (novel-miR-819, novel-miR-676, novel-miR-85, novel-miR-693, novel-miR-775, novel-miR-669, and novel-miR-867) and sixteen differentially expressed mRNAs related to energy metabolism including glycosylation, glucose metabolism process, carbohydrate metabolism process, fatty acid metabolism, and ATP binding. For example, SSTR5 and NR1H4 are essential for glucose homeostasis and play a pivotal role in glucose metabolism in animals [53, 54]. Cortisol regulates the metabolism of mouse adipose cells through the serotonin receptor gene HTR2C, and genetic variation of the APOA1 gene is linked to lipid metabolism and cardiovascular disease risk [55, 56]. It should be considered that more differentially expressed mRNAs (KCNB1, P3H2, CHST8, LYZ, HAO2, ACER1, ACSBG1, ELOVL2, ELOVL3, GBE, and NOX3) were targeted by key miRNAs, and several studies have shown that these mRNAs are also involved in energy metabolism. Therefore, we speculate that Tibetan chickens have enhanced energy metabolism due to the function of these RNAs, allowing for adaptation to hypoxic conditions.

Accumulating evidence indicates that lncRNAs play the role of ceRNAs (or miRNA sponges) in a variety of biological processes, including high-altitude adaptation. Such as LINC-PINT and LINC00599 polymorphisms are associated with high-altitude pulmonary edema in Chinese populations [57]. Another study reported the expression profiles of lncRNAs in mice with high-altitude hypoxia-induced brain injury and provided new insights into the molecular mechanism of its treatment [58]. In addition, a previous study reported the expression profiles of lncRNAs responsible for fatness and fatty acid composition traits in Tibetan pigs [59]. In the current work, we found that the aforementioned differentially expressed mRNAs involved in angiogenesis and energy metabolism were targeted by 37 differentially expressed lncRNAs in the ceRNA network, suggesting that these lncRNAs may also function as miRNA sponges and may play a role in the hypoxic adaptation of Tibetan chicken embryos with regard to angiogenesis and energy metabolism.

Based on the ceRNA theory and the ceRNA network constructed in this study, we proposed the mode of action of differentially expressed lncRNAs, miRNAs, and mRNAs during hypoxic adaptation of Tibetan chicken embryos (Fig. 7). Under hypoxic conditions, miRNAs act as key regulators to modulate the up-regulation or down-regulation of important differentially expressed mRNAs. As a consequence, the angiogenesis/blood circulation of chorioallantoic capillaries and energy metabolism, such as glucose/carbohydrate metabolism, were stimulated, leading to the enhanced hypoxia adaptability of Tibetan chicken embryos. In this process, some lncRNAs act as ceRNAs to competitively bind the MRE of miRNAs, which may indirectly affect the expression of mRNA.

Conclusions

In conclusion, 389 DELs, 73 DEMs, and 354 DEGs were identified between Tibetan and Chahua chickens, the transcriptomic data revealed several key candidate ceRNAs that may play high-priority roles in the hypoxic adaptation of Tibetan chickens by regulating angiogenesis and energy metabolism. These results provide insight into the molecular mechanisms of hypoxic adaptation regulatory networks from the perspective of coding and non-coding RNAs.

Abbreviations

lncRNAs: long non-coding RNA; miRNA: microRNA; TC: Tibetan chicken; CH: Chahua chicken; CAM: Chorioallantoic membrane; DEG: Differentially expressed gene; DEL: Differentially expressed lncRNA; DEM: Differentially expressed miRNA; ceRNA: Competing endogenous RNA; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; ORF: Open reading frame

Declarations

Ethics approval and consent to participate

All animal work was conducted following the guidelines for the care and use of experimental animals. The experiments and animal care protocol were approved by the animal welfare committee of the State Key Laboratory for Agro-Biotechnology of the China Agricultural University (approval number, XK257).

Consent for publication

Not applicable.

Availability of data and materials

All RNA-seq data were deposited in the Gene Expression Omnibus under accession number GSE160324. Other supporting materials are included as additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.Z., and H.Z. designed the research; Y.Z., W.S., and B.Z. collected samples, extracted RNAs and performed qPCR verification experiments; Y.Z. performed most of the bioinformatics studies; Y.Z., H.Z., and W.K. wrote and revised the manuscript; All authors reviewed the manuscript.

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Figures

Figure 1

Comparison of the features of RNAs in the CAM of chicken embryos. (a) Statistics of long non-coding RNA, including intergenic non-coding RNAs (lincRNAs), anti-sense lncRNAs, intronic lncRNAs, and sense lncRNAs; (b) Length distribution of 1439 miRNAs; (c) Distributions of mRNA expression values (FPKM) of six samples; (d) Distributions of lncRNA expression values (FPKM) of six samples; (e) Distributions of miRNA expression values (TPM) of six samples. TC, Tibetan chicken; CH, Chahua chicken.

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Figure 2

Identification of DELs, DEMs, and DEGs between TC and CH. Number of differentially expressed lncRNAs, miRNAs, and genes (a); Volcano plot displaying DELs (b), DEMs (c), and DEGs (d) between TC and CH. Upregulated and downregulated genes are shown in red and blue, respectively. Black dots represent genes with similar expression levels.

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Figure 3

Functional analysis of DELs and DEMs. GO enrichment analysis of DELs (a) and DEMs (c) between TC and CH. KEGG enrichment analysis scatter plot of DELs (b) and DEMs (d) between TC and CH; the rich factor is the ratio of DEL/DEM numbers annotated in the pathway term to the total gene numbers annotated in this pathway term. Smaller P-values indicate higher significance. TC, Tibetan chicken; CH, Chahua chicken.

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Figure 4

Comparative parsing of lncRNAs and mRNAs and functional analysis of DEGs. (a) Distribution of the expression of lncRNAs and mRNAs on the different chromosomes; (b) A comparison of expression between lncRNAs and mRNAs. The box chart statistics from top to bottom are the maximum value, upper quartile, middle value, lower quartile, and the minimum value; (c) GO and KEGG enrichment analysis of DEGs between TC and CH. TC, Tibetan chicken; CH, Chahua chicken.

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(a)

Figure 5

Mini-ceRNA network constructed with 10 DEGs and their 37 target DELs and 9 target DEMs, involved in angiogenesis (a) and energy metabolism (b). Color represents the up-regulated (red color) and down-regulated (blue color) levels.

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The expression of 6 DELs (a), 6 DEMs (c) and 7 DEGs (e) validated by qRT-PCR in chicken embryo chorioallantoic membrane (CAM) tissue. Note: The vertical axis represents the expression value of RNA in chicken embryo CAM and the horizontal axis represents names of RNAs. Error bars represent SE of expression. * on the bars indicate significant differences ($P < 0.05$) and ** indicate extremely significant differences ($P < 0.01$) between TC and CH breeds. TC = Tibetan chicken ($n = 8$). CH = Chahua chicken ($n = 8$). The fold changes of the 6 DELs (b), 6 DEMs (d) and 7 DEGs (f) showed that RT-qPCR results were consistent with the RNA-seq data.

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Figure 7

The proposed model of hypoxic adaptation in Tibetan chicken embryos. Under hypoxic conditions, miRNAs act as key regulators that directly target important DEGs. In this process, some lncRNAs can act as ceRNAs to competitively bind the MRE of miRNAs, indirectly affecting the expression of mRNAs.

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