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RESEARCH ARTICLE

Comparative Analysis of the Heart Tissue Transcriptomes Between Low-altitude Reared and High-altitude Reared Bar-headed Geese *(Anser indicus)*

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Abstract

The bar-headed geese *(Anser indicus)* are renowned for high-altitude migratory flights and they must fly over the Qinghai-Tibetan Plateau for their annual migration. Through comparing the high-altitude bar-headed geese with the other closely related low-altitude species, many efforts have been made to reveal the unique adaptations at physiological, biochemical, and behavioral levels that help bar-headed geese living and flying in high-altitude conditions. Nonetheless, little is known about the transcriptome level changes of the bar-headed geese adaptation to low-altitude environment. To explore the variations of gene expression that were induced by low-altitude environment in the bar-headed geese, we conducted the first comparative transcriptomic analysis of heart tissues between bar-headed geese reared in high-altitude regions (~3000 m), and the bar-headed geese reared at the low-altitude regions (~30 m) for nearly three years. A total of 76 differentially expressed genes (DEGs) were detected in the low-altitude bar-headed geese compared with the high-altitude bar-headed geese. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that these DEGs were mainly involved in the focal adhesion, extracellular matrix (ECM) - receptor interaction, the mammalian target of rapamycin (mTOR) signaling pathway, wingless-type (Wnt) signaling pathway, and glycosaminoglycan degradation etc. The results will be useful for understanding the divergent adaptation of the bar-headed geese to different altitude environment, and the transcriptome data provides a valuable resource for future functional studies.

Keywords: *Anser indicus, Transcriptome, Adaptation, Altitude, Heart*

Düşük ve Yüksek İrtifalı Alanlarda Yetiştirilen Çubuk Başlı Kazlarda *(Anser indicus)* Kalp Dokusu Transkriptomlarının Karşılaştırmalı Analizi

Öz

Çubuk başlı kazlar *(Anser indicus)*, yüksek irtifada göçmen uçuşlarıyla ünlüdür ve yıllık göçleri için Tibet Platosu üzerinden uçmaları gerekir. Yüksek irtifaya adapte çubuk başlı kazları, diğer yakından ilişkili alçak irtifalı türlerle karşılaştırarak, bu kazların yüksek irtifa koşullarında yaşamasına ve uçmasına yardımcı olan fizyolojik, biyokimyasal ve davranışsal seviyelerde benzersiz adaptasyonlarını ortaya çıkaracak birçok çalışma yapılmıştır. Bununla birlikte, çubuk başlı kazların alçak irtifalı ortamlara adaptasyonlarının transkriptom seviyesi değişiklikleri ile ilgili çok az şey bilinmektedir. Çubuk başlı kazlarda düşük irtifa ortamı tarafından indüklenen gen ekspresyon varyasyonlarını araştırmak için, yüksek irtifa bölgelerinde (~3000 m) yetiştirilen çubuk başlı kazlar arasındaki kalp dokularının ilk karşılaştırmalı transkriptomik analizini gerçekleştirdik ve bu kazlar yaklaşık üç yıl boyunca alçak irtifalı bölgelerde (~30 m) yetiştirildi. Yüksek irtifalı çubuk başlı kazlarla karşılaştırıldığında, alçak irtifalı çubuk başlı kazlarda farklı eksprese edilmiş (DEG) toplam 76 gen tespit edildi. Gen ontolojisi (GO) ve Kyoto Genler ve Genom Ansiklopedisi (KEGG) analizi, bu DEG'lerin temel olarak fokal adezyon, ekstrasellüler matris (ECM) - reseptör etkileşimi, rapamisin protein kompleksinin memeli hedefi (mTOR) sinyal yolu, wingless-tip (Wnt) sinyal yolu ve glikozaminoglikan degredasyonu vb. ile ilişkili oduğunu göstermiştir. Çalışma sonuçları, çubuk başlı kazların farklı irtifa ortamlarına farklı adaptasyonlarını anlamak için faydalı olacaktır ve elde edilen transkriptom verileri gelecekteki fonksiyonel çalışmalar için değerli bir kaynak imkanı sunacaktır.

Anahtar sözcükler: *Anser indicus, Transkriptom, Adaptasyon, İrtifa, Kalp*

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Introduction

The bar-headed goose *(Anser indicus)* is endemic to Asia, breeding in the high-altitude plateau wetlands of central Asia [1-4], wintering mainly in the south-central Tibet (approximately 56.88% of the world populations) and India (around 27.84% of the world populations) [1,4]. This species is famous for extremely high-altitude flying over the Himalayan Mountains twice a year on the Central Asian flyway between the wintering areas in India subcontinent and the summering grounds on the Qinghai-Tibetan Plateau [5]. The bar-headed geese were tracked flying as high as 7.290 meters up [6], and mountaineers even reported seeing them fly over the summit of Mount Everest (8.850 meters above sea level) [7]. At these heights, the partial pressure of oxygen is one-third that of sea level. Incredibly, bar-headed geese are able to maintain the high metabolic and oxygen consumption rates necessary for flapping flight under severe hypoxia conditions [8]. Not surprisingly, there has been increasing interest among researchers in understanding the physiological, molecular, and behavioral adaptations of bar-headed geese that allow for the exceptional high-altitude flight performances [9,10].

Through comparing the high-altitude bar-headed geese with the other closely related low-altitude species, a growing body of literature has identified several unique adaptations that help bar-headed geese flying in low oxygen conditions. For example, physiological adaptations have evolved at every step in the oxygen transport cascade of bar-headed geese to help them accomplish the high-altitude success. Such adaptations include an effective breathing pattern [11], larger lungs [12], an enhanced hypoxic ventilatory response [13], hemoglobin (Hb) with a higher oxygen affinity [14], an increase in flight and cardiac muscle capillarity [15], and subcellular redistribution of the mitochondrial reducing oxygen diffusion distances [15]. The increased affinity between Hb and O_2 plays a critical role in adaptation of bar-headed geese to hypoxia through enhancing pulmonary O_2 loading. A few studies have found that in the bar-headed goose the major isoform of Hb has significantly higher O_2-affinity than that of the closely related low-altitude species due to the single and large-effect substitutions in the hemoglobin gene [16,17]. In addition, a roller coaster strategy was reported to take by bar-headed geese, rising and falling with the relief of the terrain, to minimize energy expenditure during their trans-Himalayan flights [18]. Overall, these studies have greatly improved our understanding of the adaptive mechanism of bar-headed goose for high-altitude conditions.

Recent advances in genomics have opened a huge opportunity to study the genetic basis of high-altitude adaptation characteristics [19,20]. In our previous study, we reported the first *de novo* whole genome sequencing, assembly, gene prediction and annotation of the bar-headed goose [21]. Transcriptome is a useful tool to analyze changes in gene expression, and has been widely used to explore high-altitude adaptations in a group of birds [22]. Therefore, in this study, we aimed to compare the transcriptome profiles of heart tissues between bar-headed geese reared in low- and high-altitude environment. Unlike previous studies that compared high-altitude bar-headed geese with the other lower-altitude birds, our current study focused on comparison between the low-altitude reared bar-headed geese, where these geese were transported to and there is no distribution of wild bar-headed geese, with the high-altitude reared bar-headed geese. The major advantage of this experimental setup is that the genetic backgrounds of the subjects being compared are the same. The results will enrich the transcriptomic resources of bar-headed geese and laid a foundation for the functional study of genes related to low altitude adaptation in this species.

Material and Methods

Ethics Statement

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006-398). The research protocol was reviewed and approved by the Ethical Committee of Qinghai University.

Animal Sample Collection

Eggs of wild bar-headed geese were collected and hatched at the Fei Yan specialized breeding and rearing farming cooperative (elevation 3.000 meter) in Huangzhong District, Xi'ning City, Qinghai Province, China. After hatch, three individuals were reared at this cooperative for three years until the experiment began and labeled as high-altitude group (HA group, n=3) *(Fig. 1)*. Another three individuals were transported to and reared at the He Ming breeding Co., LTD. (elevation 30 meter) in Hengshui City, Hebei Province, China. The latter three individuals were also reared for three years until the experiment began and labeled as low-altitude group (LA group, n=3) *(Fig. 1)*.

These bar-headed geese lived freely in both wild and artificially reared environments. These birds were healthy during experimental period. The heart tissues were sampled rapidly from each carcass, and immediately frozen in the liquid nitrogen. All heart samples were stored at -80°C until used.

RNA Extraction and Transcriptome Sequencing

For each sample, total RNA was isolated using Trizol reagent (Invitrogen, California, USA) following the manufacturer's instructions. Residual genomic DNA was digested by RNase-free DNase (Qiagen, Germany). The RNA concentration and overall quality were assessed using a Qubit 2.0 Fluorometer (Invitrogen, California, USA), and an Agilent 2100

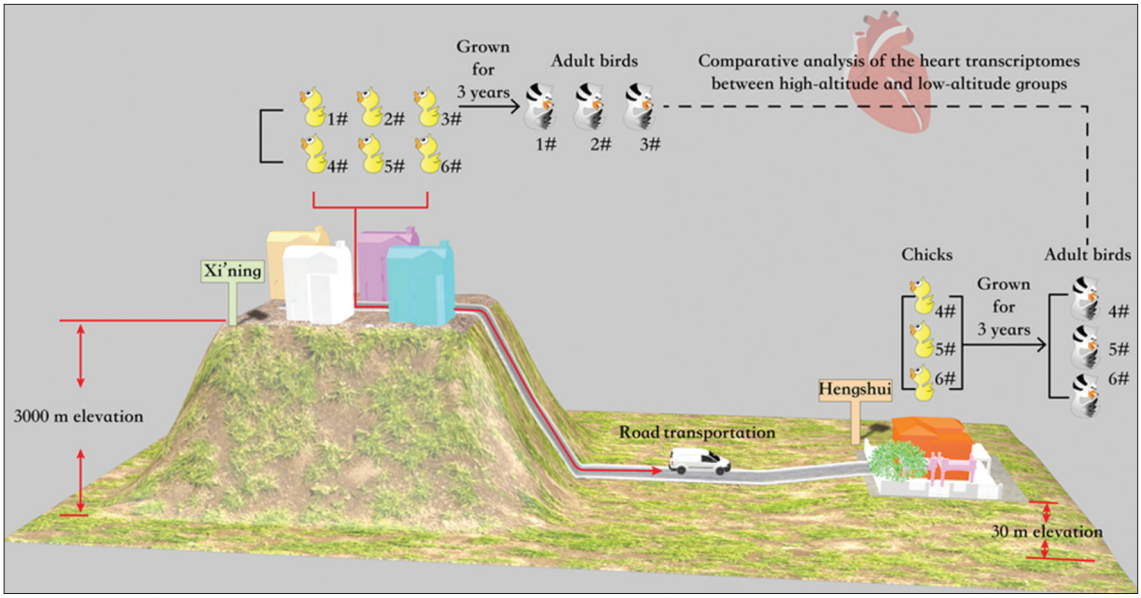

Fig 1. The schematic diagram of our experiment

bioanalyser (Agilent Technologies, USA), respectively. The RNA integrity number threshold was set at 7.0 for the construction of library. mRNA was further purified using poly-T oligo-attached magnetic beads. Finally, six libraries with 350 bp insert sizes were constructed using the NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). The constructed libraries were then sequenced by the Illumina NovaSeq 6000 platform at Novogene Bioinformatics Technology Co. Ltd (Beijing, China). The whole process followed a standardized procedure and was monitored by Novogene's Quality Control System.

Data Accessibility

The raw sequencing data has been deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) with the bioproject number PRJNA612653.

Bioinformatics Analyses

- Quality Control

To produce the high-quality clean reads, raw reads were assessed for quality using FastQC v.0.11.9 *(https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)* and filtered for low-quality reads according to the following rules: 1) removing the reads with adapter contamination; 2) removing the reads with unknown nucleobases; 3) removing the low-quality reads (reads with a Q_{PHRED} <= 20 base number accounting for more than 50% of the total read length). At the same time, Q20, Q30 and GC content of the clean reads were counted. The clean reads with high quality were used for further analysis.

- Genome Mapping

The bar-headed goose reference genome (DDBJ/ENA/GenBank under the accession VDDG01000000) [21] was used as the reference genome for our transcriptome sequencing data. Genome mapping was conducted using HISAT2 v2.0.5 software [23].

- Quantification of Gene Expression Level

FeatureCounts v1.5.0-p3 [24] was used to calculate the number of mapped reads to each gene, which were then normalized for the gene length and library size. FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) of each gene was counted based on the length of the gene and reads count mapped to this gene.

- Differential Expression Analysis

Genes with very low expression levels were filtered out of the dataset. Then, differentially expressed genes were analyzed using DESeq2 R package (1.16.1) [25] between HA and LA groups. The DEGs were identified using the thresholds of |log2 fold change| ≥1.0 and Padj <0.05. Clustering analysis of the identified DEGs was implemented by the heatmaps in R software.

- GO Function and KEGG Pathway Enrichment Analysis

GO functional enrichment and KEGG pathway analysis of DEGs was conducted by the clusterProfiler R package [26], in which gene length bias was corrected. GO terms or KEGG pathways with P<0.05 were significantly enriched.

- Real-time Quantitative PCR (RT-qPCR) Verification

To verify the repeatability of DEGs derived from transcriptome data, 16 DEGs were selected randomly for RT-qPCR validation. The primer sequences were shown in *Table 1*. RNAiso Pure RNA Isolation Kit (TaKaRa, Dalian, China) was used to extract RNA. A PrimeScript RT reagent

Table 1. *Primers used in this study for real-time quantitative PCR (RT-qPCR)*

Gene	Regulation	Primer Sequences (5'-3')	Amplicon Size (bp)
DUSP1	Up	Forward: CGATGGAGGAAGGGTGTTTG	152
		Reverse: TGAAGTTTGGGGAGATGATGC	
NR4A1	Up	Forward: CCCTCCAAGCCCAAGCA	238
		Reverse: CGGCGAAGCCCTGAATC	
FOS	Up	Forward: TTCTATGCGTCGGACTGGG	113
		Reverse: AAGGTGGAGGTGTAGGTGCTG	
JUN	Up	Forward: GGAAAAGGAAGTTGGAAAGGAT	155
		Reverse: CTGGCACCCGCTGTTGA	
HSP70	Up	Forward: AGCGTAACACCACCATTCCC	94
		Reverse: TCACCCTCATACACCTGGACC	
FABP	Up	Forward: CCAAGCCCAATGTAACTATCAGC	237
		Reverse: CGACCAGGTTCCCATCCAC	
APOA1	Up	Forward: GACCCTCGCCCTGCTCTT	209
		Reverse: GCGTGTCCAGGTTGTCGG	
SCD	Up	Forward: TCAACCCACGGGAGAACC	213
		Reverse: TCCGCATTTTCCGAGCC	
ACACB	Down	Forward: TGAGGTGGGGATGGTTGC	96
		Reverse: TTGTGCGTGATGTCGTTGC	
TNC	Down	Forward: CCAAGGGGCACCAAACAA	103
		Reverse: CGGAAGCCGTCTGGAGTAGC	
COL4A1	Down	Forward: ACAAGGCAATGAAAGAGCACA	150
		Reverse: GGCGTTGACAGCCAGTAGG	
MYLK	Down	Forward: CGCCTACCAGCCCGATAA	177
		Reverse: CAGGGTCAGGATAGCCTTCAA	
FLNC	Down	Forward: CATCAAGAACGACAACGACACC	108
		Reverse: AACGGGCTGCTGGGGAT	
GK	Down	Forward: ATGTAACCAATGCCAGTAGAACG	185
		Reverse: GTCACCCAAGCACCCAGAA	
GPCPD1	Down	Forward: TGGATGCTGGGTGGCTGAC	193
		Reverse: TTCTGAGGAACTGTAGGGGATGT	
SLC9A1	Down	Forward: GCAGTCGCCAGAGTCCGT	110
		Reverse: GCTCCTTCACCCGCATCA	
TUBULIN	-	Forward: ATCAGGTGGTCCCCAAGAGC	143
		Reverse: GGTGATGAGATGGCGGAGG	

DUSP1: *Dual Specificity Phosphatase 1;* ***NR4A1:*** *Nuclear Receptor Subfamily 4 Group A Member 1;* ***FOS:*** *Fos Proto-Oncogene, AP-1 Transcription Factor Subunit;* ***JUN:*** *Jun Proto-Oncogene, AP-1 Transcription Factor Subunit;* ***HSP70:*** *Heat Shock Protein 70;* ***FABP:*** *Fatty Acid Binding Protein;* ***APOA1:*** *Apolipoprotein A1;* ***SCD:*** *Stearoyl-CoA Desaturase;* ***ACACB:*** *Acetyl-CoA Carboxylase Beta;* ***TNC:*** *Tenascin C;* ***COL4A1:*** *Collagen Type IV Alpha 1 Chain;* ***MYLK:*** *Myosin Light Chain Kinase;* ***FLNC:*** *Filamin C;* ***GK:*** *Glycerol Kinase;* ***GPCPD1:*** *Glycerophosphocholine Phosphodiesterase 1;* ***SLC9A1:*** *Solute Carrier Family 9 Member A1*

kit (TaKaRa, Dalian, China) was used to synthesize the single stranded cDNA. qPCR was conducted on Gene9600 RT-PCR detection system (Hangzhou Bioer Technology Co. Ltd., Hangzhou, China) and TB Green Fast qPCR Mix (TaKaRa, Dalian, China). The 6 samples were run in triplicate for the 16 genes. *TUBULIN* was used as an internal control in this study. The method of $2^{-\Delta\Delta CT}$ [27] was used to count the relative expression of the 16 genes.

RESULTS

Overview of Transcriptome Sequencing Data

Six sequencing libraries from the two distinct groups (high- and low-altitude) were constructed and sequenced using the Illumina HiSeq platform. The overall sequencing results were shown in *Table 2*. A total of 391,970,972 raw

Table 2. *Summary of read statistics from the transcriptome sequencing*

Samples	Raw Reads	Clean Reads	Clean Bases (Gb)	Error Rate (%)	Q20 (%)	Q30 (%)	GC (%)
HA_h1	59,914,010	58,248,934	8.74	0.03	96.34	90.93	50.84
HA_h2	64,648,766	62,867,252	9.43	0.03	96.62	91.39	50.56
HA_h3	67,105,524	65,327,764	9.80	0.03	96.05	90.13	50.42
LA_h1	68,834,484	67,275,492	10.09	0.03	96.76	91.65	50.20
LA_h2	66,169,684	64,443,694	9.67	0.03	96.29	90.65	50.56
LA_h3	65,298,504	63,595,928	9.54	0.03	96.49	91.15	50.46

Table 3. *Reads coverage mapping to the reference genome of bar-headed goose*

Samples	Total Reads	Total Map	Unique Map	Multiple Map	Exon	Intron	Intergenic
HA_h1	58,248,934	36,857,118(63.28%)	36,275,462(62.28%)	581,656(1.0%)	3,699,777,153(67.2575%)	571,953,754(10.3974%)	1,229,188,619(22.3451%)
HA_h2	62,867,252	40,369,734(64.21%)	39,737,652(63.21%)	632,082(1.01%)	4,014,112,346(66.5661%)	614,605,303(10.192%)	1,401,548,436(23.2419%)
HA_h3	65,327,764	40,977,873(62.73%)	40,401,956(61.85%)	575,917(0.88%)	4,066,706,910(66.433%)	654,328,150(10.689%)	1,400,487,751(22.8781%)
LA_h1	67,275,492	41,840,766(62.19%)	41,197,140(61.24%)	643,626(0.96%)	4,245,606,572(67.9315%)	648,989,314(10.3841%)	1,355,233,098(21.6843%)
LA_h2	64,443,694	40,178,319(62.35%)	39,525,864(61.33%)	652,455(1.01%)	4,097,059,115(68.2738%)	607,198,221(10.1184%)	1,296,665,508(21.6078%)
LA_h3	63,595,928	39,515,638(62.14%)	38,940,133(61.23%)	575,505(0.9%)	3,805,155,967(64.4771%)	696,974,108(11.81%)	1,399,431,085(23.7129%)

reads were generated and were deposited in the NCBI SRA database (accession number SRP252835). All error rates were less than 0.04%, and the quality of the base values were Q20 ≥ 96% and Q30 ≥ 90% in the six samples, suggesting that the transcriptome data were technically qualified. After filtering the adapter sequences, the ambiguous N nucleotides, and the low-quality reads, we got a total of 381,759,064 clean reads. These clean reads were then used for subsequent analysis. The percentage of clean reads among raw reads ranged from 97.22% to 97.74% in each sample. The guanine + cytosine (GC) values ranged from 50.20% to 50.84%.

The mapping results showed that more than 62% of the clean reads (total mapped reads) matched to the bar-headed goose reference genome *(Table 3)*. Among the mapped reads, more than 61% of the clean reads were matched to a unique genomic location, and less than 1% of the clean reads were matched to multiple genomic locations *(Table 3)*. For the high-altitude group and low-altitude group, 66.75% and 66.89% were mapping to the exon region, 10.43% and 10.77% belonged to the intron region, and 22.82% and 22.34% were in the intergenic sequence respectively *(Table 3)*. These results suggested that the transcriptome data were good enough for further analyses.

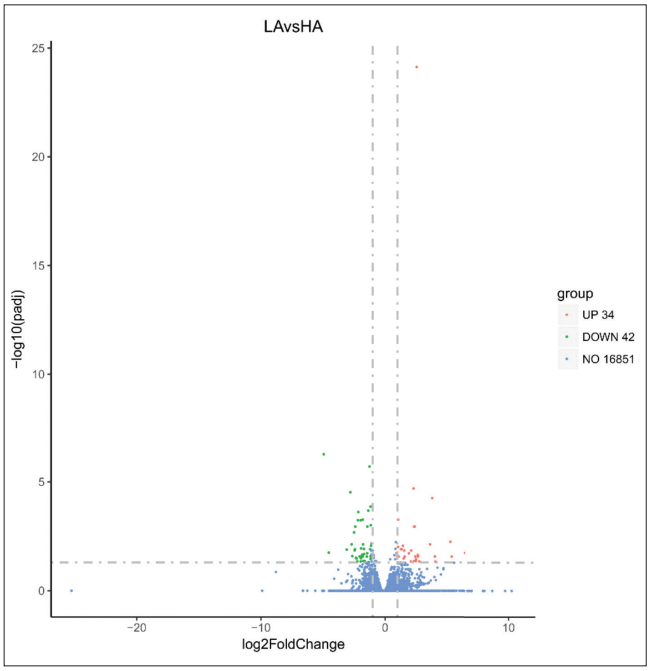

Fig 2. Differentially expressed genes (DEGs) in low-altitude hearts (LA_h) versus high-altitude hearts (HA_h). Red indicates up regulated genes, green indicates down regulated genes, and blue indicates genes with unchanged expression

DEGs Analysis and RT-qPCR Validation

DEGs between the low- and high-altitude bar-headed geese hearts were analyzed by the DESeq2 package, with the criteria of |log2 fold change| ≥ 1.0 and Padj < 0.05. A total of 76 DEGs were identified, of which 34 showed up-regulation, and 42 showed down-regulation in the low-altitude group compared with the high-altitude group *(Fig. 2)*.

A heat map of the DEGs between the low- and high-altitude groups was shown with hierarchical clustering of the samples and genes in *Fig. 3*. Hierarchical clustering result clearly separated the high- and low-altitude groups, while samples within each group showed strong correlations *(Fig. 4)*.

In order to verify the reliability of the candidate DEGs, we selected and quantified the expression levels for 16 DEGs (8 up-regulated DEGs and 8 down-regulated DEGs)

by using the RT-qPCR technique *(Fig. 5)*. We successfully confirmed the differential expression for all the selected DEGs *(Fig. 5)*. The results revealed the tendency of gene expression was in line with the transcriptome results, suggesting the transcriptome sequencing results were reliable for following functional analysis.

Functional Analysis for DEGs

To further explore the possible biological functions and metabolic pathways of the identified DEGs, GO and KEGG enrichment analyses were performed.

The DEGs in the comparison group (LA_h vs. HA_h) were first annotated by the GO function database. The DEGs were significantly enriched in 1 terms of BP (biological process), 1 terms of CC (cellular component), and 11 terms of MF (molecular function) *(Fig. 6)*. Under the molecular function category, the genes mainly enriched in the functions involved in "extracellular matrix structural constituent", "GTPase activity", "hydrolase activity", and "nucleotide/ nucleoside binding" *(Fig. 6)*.

We then performed a KEGG analysis of the DEGs. In our study, 76 DEGs were mapped to 32 KEGG pathways. A total

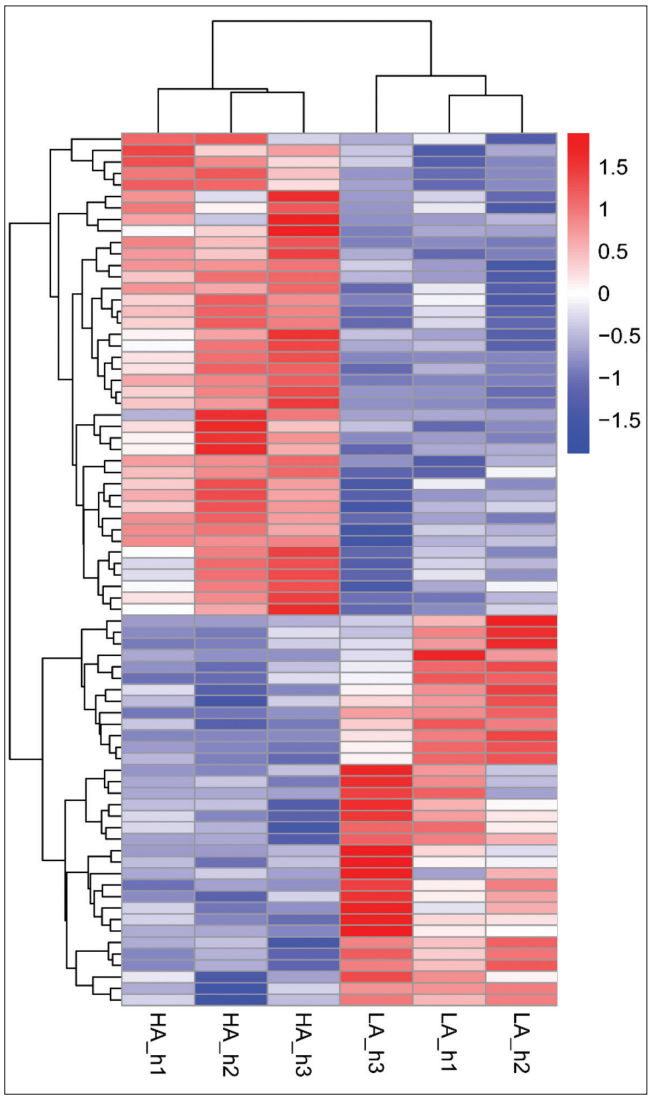

Fig 3. Heat map of the 76 differentially expressed genes (DEGs) in the two groups. Colors represent the normalized gene expression values of DEGs

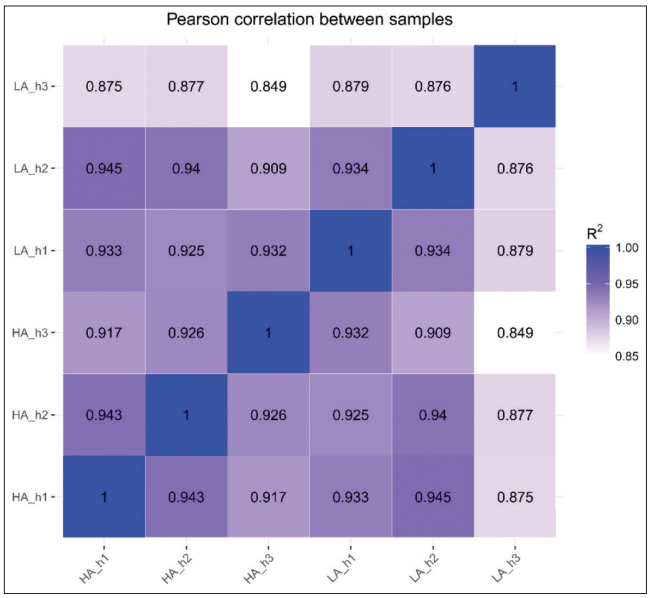

Fig 4. Hierarchical clustering (Pearson correlation) of transcriptome sequencing results of low-altitude hearts (LA_h, n=3) and high-altitude hearts (HA_h, n=3)

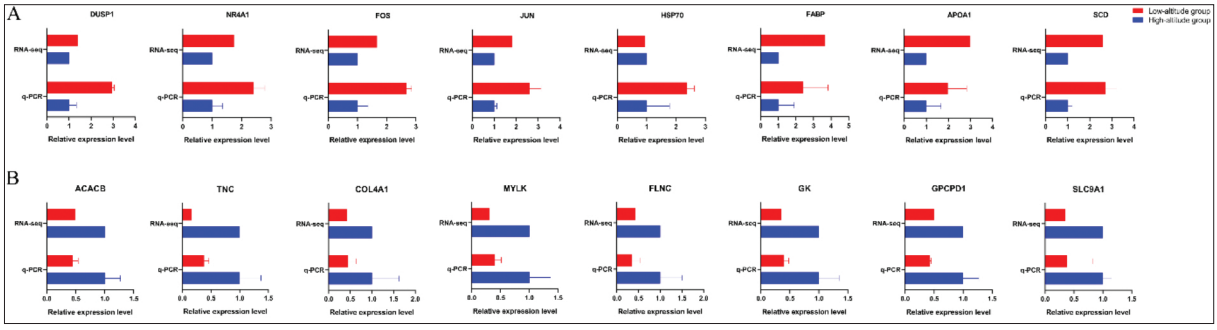

Fig 5. Validation of RNA-seq results by Real-time quantitative PCR (RT-qPCR). **(A)** validation of up-regulated differentially expressed genes (DEGs) by RT-qPCR, **(B)** validation of down-regulated differentially expressed genes (DEGs) by RT-qPCR. The RT-qPCR results are the means ± standard deviations (± SDs) of three replicates

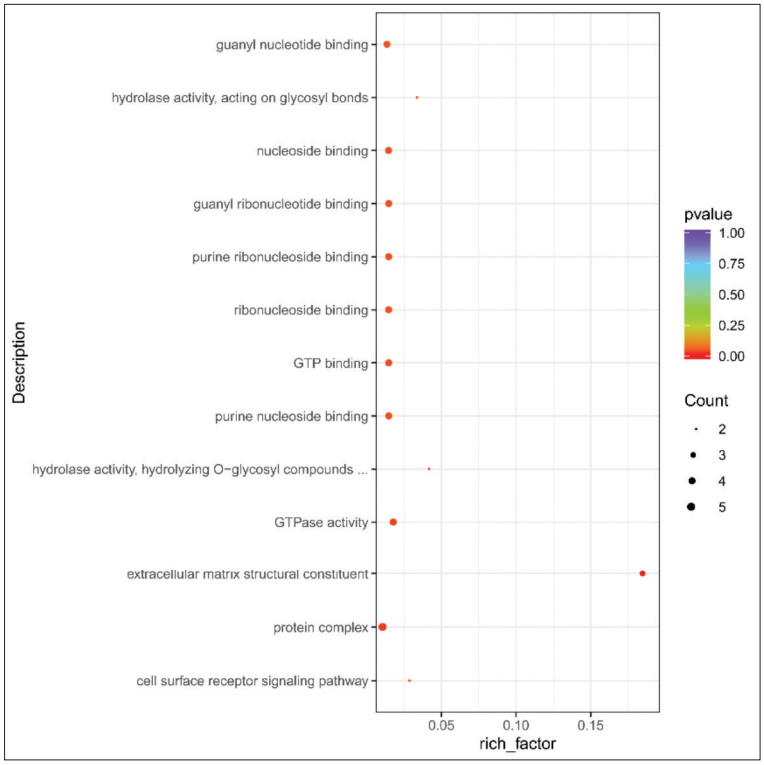

Fig 6. Gene Ontology enrichment scatter plot for differentially expressed genes between low-altitude hearts (LA_h) and high-altitude hearts (HA_h)

Table 4. *Differentially expressed genes for the six significant KEGG signal pathways*

KEGG Signal Pathway	Up_regulated Genes	Down_regulated Genes
ECM-receptor interaction	-	TNC, COL4A1, COL4A2, THBS1, COL4A5
Focal adhesion	-	TNC, COL4A1, MYLK, COL4A2, THBS1, COL4A5
AGE-RAGE signaling pathway in diabetic complications	EGR1	COL4A1, COL4A2, COL4A5
Glycosaminoglycan degradation	-	HYAL2
mTOR signaling pathway	FZD8, FZD2	ULK2, ATP6V1C2
Wnt signaling pathway	FZD8, FZD2, AXIN2	-

of 6 statistically significant pathways were identified when comparing LA_h group with HA_h group, including three in environmental information processing, one in cellular processes, one in human diseases, and one in metabolism categories, respectively *(Table 4)*. Within the upregulated DEGs, the most enriched pathway term was "Wnt signaling pathway" *(Table 4)*. For the downregulated DEGs, the dominant pathways were as follows: "focal adhesion", "ECM - receptor interaction","Advanced glycation end-product (AGE) - role of receptor for advanced glycation end-product (RAGE) signaling pathway in diabetic complications", and "glycosaminoglycan degradation" *(Table 4)*.

Discussion

The bar-headed goose provides an extraordinary opportunity to investigate the mechanism of high-altitude adaptation for their migratory flight at extreme altitude, and attracted many experts from various fields [5,9,10,12]. However, the adaptive mechanism of bar-headed geese migrating to low altitude environment has not been reported. We newly sequenced a bar-headed goose genome to provide a useful resource for detection of genomic adaptive changes at the DNA sequence level associated with this species [21]. Transcriptome intermediate between DNA sequences and physiological traits can extend the genomic information by concentrate on gene expression and molecular pathways involved in different altitude adaptation. Here, for the first time, we performed transcriptome analysis in bar-headed geese reared in both high-altitude and low-altitude environment for one hypoxia-sensitive tissue, heart. In this study, bar-headed geese were transported to low-altitude areas for artificial rearing, and then comparative transcriptome analysis was conducted with high-altitude bar-headed geese. This kind of comparison, rather than comparing high-altitude bar-headed geese with the relative geese species living at low-altitude, will better explore the adaptability of bar-headed geese to low-altitude environment.

In this study, each sample obtained more than 8.74 GB of high-quality clean sequencing data, and the base quality

value (error rate, Q20, and Q30) indicating good quality and high reliability. Eight up-regulated DEGs and 8 down-regulated DEGs were chosen for RT-qPCR test to confirm the expression levels calculated by FPKM values. The similar changing trends were found between RT-qPCR validation and transcriptome sequencing. Overall, these results indicate that the results of our transcriptome data are reliable.

In this work, GO annotation results showed that most DEGs were found to be related to cell surface receptor signaling pathway, extracellular matrix structural constituent, GTPase and hydrolase activity, and nucleotide / nucleoside binding. This suggests that when bar-headed geese migrate from high altitude to low altitude, the interactions between membrane receptors and extracellular ligands of the heart were changed. The results were similar to those of Qi et al. in the study of yak heart transcriptome [28]. The effects of these changes on the health status of low-altitude bar-headed geese need to be studied and determined with the help of more morphological, physiological, and pathological data in the future. The annotation analysis of metabolic pathways of DEGs is helpful for further interpretation of gene function. KEGG database is the main public database on metabolic pathways, and six KEGG pathways were significantly enriched in this study. "Wnt signaling pathway" *(FZD8, FZD2, AXIN2)* was the only upregulated KEGG pathways in the hearts of low-altitude bar-headed geese. A large body of evidence shows that the Wnt signaling pathway plays an important role in various stages of heart development [29]. While this pathway is quiescent in adult hearts, and activation occurs upon pathological stress [30]. In the future, it is necessary to further study the correlation between the activation of this signaling pathway and heart disease of bar-headed geese reared at low altitude. "focal adhesion" and "ECM-receptor interaction" were found to be downregulated in the hearts of low-altitude bar-headed geese. Collagens that encode cell-ECM (including downregulated *COL4A1*, *COL4A2*, and *COL4A5)* are the major structural components of the basement membrane and are important during cardiac morphogenesis by promoting cardiomyocyte proliferation [31]. We also identified three DEGs in focal adhesion pathways (including *TNC*, *MYLK*, and *THBS1)* with significantly downregulated in the hearts of low-altitude bar-headed geese. Those genes usually provide a force-transmitting physical link between the EMC and cytoskeleton, and are helpful for the species to adapt to high altitude [28]. But we hypothesized that the expression of these genes was suppressed in low-altitude environment where oxygen was abundant.

Glycosaminoglycans are a class of anionic linear periodic polysaccharides, which play a key role in tissue homeostasis not only by providing mechanical load resistance but also signaling mediators that play an important physiological function in cell proliferation, differentiation, metastasis, and recognition [32]. The glycosaminoglycan degradation pathway in the hearts of low-altitude bar-headed geese is involved in the regulation of the structure and function of glycosaminoglycans in the hearts. The down-regulated expression of *HYAL2* in this pathway was detected by transcriptome data. Hyaluronidase is an endogenous glycosidase that can degrade glycosidic bonds in hyaluronic acid polymers (one form of glycosaminoglycans). We hypothesized that high-altitude bar-headed geese need hyaluronidase and other hyaluronidase to maintain the permeability of extracellular matrix of heart, so as to facilitate oxygen exchange and transportation in anoxic environment. However, when high-altitude bar-head geese migrate to low-altitude environment for growth, oxygen supply is sufficient, and hyaluronic acid in the extracellular matrix of heart does not need to be greatly degraded, so the glycosaminoglycan degradation pathway is inhibited. All these differentially expressed signal pathways and metabolic pathways in the hearts of bar-head geese intercrossed with each other, and thus forming a huge and complex network. The specific regulatory mechanism and biological effects of these differentially expressed signaling pathways remain to be further studied. Our study has a limitation that also suggests direction for further research. We did not do biochemical, physiological, and histological tests, and how these observed differently expressed genes and pathways may translate into functional and phenotypic changes remains unclear. However, the transcriptome data obtained in this study provides us with a useful candidate pathway and gene resource for future functional studies of altitude adaptation.

In conclusion, this study lays the first foundations for comparative analysis of transcriptional and signaling pathway changes in bar-headed geese heart tissue under low-altitude environment. A total of 76 DEGs were identified, of which 34 showed up-regulation and 42 showed down-regulation in the low-altitude bar-headed geese compared with the high-altitude bar-headed geese. Nine KEGG pathways and 13 GO terms were significantly enriched in low-altitude bar-headed geese. Further studies are required to confirm the functions of these different pathways and genes reported here between low-altitude bar-headed geese and high-altitude bar-headed geese, and the extent to which these differences may contribute to the low-altitude adaptation.

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Declaration of Conflicting Interests

The authors declare that they have no conflicts of interest.

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Author Contributions

WW conceived and designed the study. WW, and KS critically revised the manuscript. YL, FW, XLG, and LLZ performed the experiments, analyzed the data. All authors read and approved the final manuscript.

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