Investigating the molecular control of deer antler extract on articular cartilage

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Research article

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Abstract

**Background:** Deer antler is considered as a precious traditional Chinese medicinal material, and has been widely used to reinforce kidney's yang, nourish essence and strengthen bone function. The most prominent bioactive components in deer antler are water-soluble proteins that play potential roles in bone formation and repair. The aim of this study was to explore the molecular control and therapeutic targets of deer antler extract (DAE) on articular cartilage.

**Methods:** DAE was prepared as previously described. All rats were randomly divided into Blank group and DAE group (10 rats per group) after 7-day adaptive feeding. The rats in DAE group were orally administrated with DAE at a dose of 0.2 g/kg per day for 3 weeks and the rats in Blank group were fed with drinking water. Total RNA was isolated from the articular cartilage of knee joints. RNA sequencing (RNA-seq) experiment combined with quantitative real-time polymerase chain reaction (qRT-PCR) verification assay were carried out to explore the molecular control and therapeutic targets of DAE on articular cartilage.

**Results:** We demonstrated that DAE significantly increased the expression levels of functional genes involved in cartilage formation, growth and repair, and decreased the expression levels of susceptibility genes involved in the pathophysiology of osteoarthritis.

**Conclusions:** DAE might serve as a candidate supplement for maintaining cartilage homeostasis, and preventing cartilage degeneration and inflammation. These effects were possibly achieved by accelerating the expression of functional genes involved in chondrocyte commitment, survival, proliferation and differentiation, and suppressing the expression of susceptibility genes involved in the pathophysiology of osteoarthritis. Thus, our findings will contribute towards deepening the knowledge about the molecular control and therapeutic targets of DAE on the treatment of cartilage related diseases.

**Background**

Deer antler is considered as a precious traditional Chinese medicinal material, and has been widely used to reinforce kidney's yang, nourish essence and strengthen bone function [1]. The most prominent bioactive components in deer antler are water-soluble proteins that play potential roles in bone formation and repair [2-6]. However, little is known regarding their effects on cartilage development and growth. Deer antler is a fantastic mammalian appendage with characteristics of rapid growth and annually regeneration [7-9]. The longitudinal growth of the antler was considered as a modified endochondral ossification process that is analogous to the long bone growth originated from mesenchymal condensation, chondrocytic differentiation and ossification [10]. During rapid growth stage, deer antler is capable to grow as rapidly as 2 cm per day, which represents the fastest rate of cartilaginous tissue growth in the mammal kingdom [11].
Antler growth is driven by the growth center that is located in the distal tip consisted of mesenchyme and cartilage tissues [12]. According to the application of deer antler in traditional Chinese medicine, the antler is divided into wax slice, powder slice, blood slice and bone slice from distal tip to proximal base, and the wax slice is located in the distal tip of antler (growth center), which has the highest percentage of bioactive components [13, 14]. In our previous studies, we generated the deer antler extract (DAE), and the proportion of protein was 70% according to the protein concentration assay [15].

We also performed a series of analyses to investigate the effects of deer antler extract (DAE) on chondrocyte proliferation, differentiation and apoptosis, and demonstrated that freshly aqueous extracts from sika deer antlers at rapid growth stage could enhance chondrocyte viability and promote chondrocyte proliferation, but inhibit chondrocyte differentiation, maturation and apoptosis. Furthermore, our results suggested that DAE play potential roles in boosting the abilities of chondrocytes against oxidative, inflammatory, and immune stresses [15, 16].

In the present study, we carried out state-of-the-art RNA sequencing (RNA-seq) experiment combined with quantitative real-time polymerase chain reaction (qRT-PCR) verification assay to explore the molecular control and therapeutic targets of DAE on articular cartilage. We demonstrated that DAE significantly increased the expression levels of functional genes involved in cartilage formation, growth and repair, and decreased the expression levels of susceptibility genes involved in the pathophysiology of osteoarthritis. Thus, DAE might serve as a candidate supplement for maintaining cartilage homeostasis, and preventing cartilage degeneration and inflammation. These effects were possibly achieved by accelerating the expression of functional genes involved in chondrocyte commitment, survival, proliferation and differentiation, and suppressing the expression of susceptibility genes involved in the pathophysiology of osteoarthritis.

**Methods**

**DAE preparation**

The DAE used in the following experiments was same as the ones that were prepared as previously described [15]. All experiments were approved by the Institutional Animal Ethics Committee of Changchun University of Chinese Medicine. Briefly, deer antlers in the rapid growth phase were obtained from three 4-year-old adult sika deer. The antlers were chopped into small pieces and thoroughly washed with ice water. The clean antler pieces were completely homogenized with a Tissue Homogenizer (Voshin, China) and centrifuged with an Eppendorf 5804R Refrigerated Centrifuge (Eppendorf, Germany). The supernatant was further clarified by filtering through a Hollow Fiber Membrane Filter Column (GE Healthcare, USA) and lyophilized with a Heto PowerDry LL3000 Freeze Dryer prior to storage at -80 °C (Thermo, USA).

**Experimental animals**
Twenty male Sprague-Dawley (SD) rats (SPF grade, 7 weeks old) were obtained from the Changchun Yisi laboratory animal technology Co, Ltd. (Changchun, China) with production license number SCXK (Ji) 2016-0003. The rats were housed with a constant temperature of 23°C accompanied with a relative humidity of 50% in an air conditioned room, and exposed to a 12/12 h (light/dark) cycle. All animal protocols were approved by the Institutional Animal Care and Use Committee of Changchun University of Chinese Medicine and all experimental procedures were performed in accordance with corresponding standards and guidelines.

**Drug administration and cartilage collection**

All rats were randomly divided into Blank group and DAE group (10 rats per group) after 7-day adaptive feeding. The drug administration was carried out as previously described [17]. The rats in DAE group were orally administrated with DAE at a dose of 0.2 g/kg per day for 3 weeks and the rats in Blank group were fed with drinking water. The administrated dose for DAE in rat experiment was calculated based on the body surface area normalization method [18]. Articular cartilage from each rat was harvested in the early morning after 3 weeks of DAE administration. Briefly, all rats were euthanized with CO$_2$ inhalation and cervical dislocation to assure death. Articular cartilage from either the Blank group or DAE group was carefully removed from the underlying subchondral bone from left knee joint with a scalpel blade accompanied with a stereo microscope (Nikon, Japan) following Katagiri’s methods [19], and stored at -80 °C for RNA extraction.

**RNA isolation and sequencing**

Cartilage from each group was pooled together and pulverised into a powder in liquid nitrogen, respectively. Total RNA was isolated from the cartilage samples with the TRizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The quality of RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Illumina 2 × 150 paired-end mRNA libraries were prepared with the TruSeq Stranded mRNA kit (Illumina, USA) according to the manufacturer’s instructions. Transcriptome sequencing was carried out by RNA-seq method on an Illumina HiSeq 2500 platform (Illumina, USA).

**RNA-seq data analysis**

After RNA-seq, raw reads in FASTQ format were first processed by perl scripts. High-quality clean reads were generated by removing the low-quality reads and adapter sequences. The clean reads from each sample were aligned to the rat (*Rattus norvegicus*) reference genome via HISAT software [20]. Gene expression levels were evaluated by calculating the relative transcript abundance using the FPKM algorithm [21]. Genes with an FPKM smaller than 0.2 were considered not expressed and removed [22]. The transcripts were annotated using the BLAST program against the National Center for Biotechnology Information (NCBI) non-redundant (NR) and Swiss-Prot protein databases [23]. Differentially expressed genes (DEGs) between the Blank group and DAE group were identified using the DEGseq R package [24]. Transcripts with a log$_2$ fold change $\geq$1 or $\leq$-1 and with a $p$ value $\leq$0.001 were defined as DEGs. Gene
ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment was analysed using an R function `phyper`, and the Hypergeometric test and Bonferroni correction were carried out for multiple testing corrections. GO terms or pathways with an adjusted $p$ value (Q value) less than 0.05 were recognized to be significantly enriched [25].

**Verification of RNA-seq data by qRT-PCR**

To verify the RNA-seq data, qRT-PCR was carried out to detect the expression levels of DEGs identified by RNA-seq analysis. Briefly, total RNA was isolated using TRIzol reagent (Invitrogen, USA) following the manufacturer’s instructions. cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad, USA) and amplified using a SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, USA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) under standard amplification conditions. Gene expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method by normalizing to the rat glyceraldehyde 3-phosphate dehydrogenase gene (Gapdh) [26].

**Results**

**Statistic summary of RNA-seq data**

The messenger RNAs (mRNAs) of articular cartilage from rats with or without the treatment of DAE were sequenced using Illumina paired-end sequencing technology. The raw reads were uploaded in the NCBI Sequence Read Archive (SRA) database under BioProject accession number PRJNA642711. As shown in Table 1, 39,698,758 (Blank group) and 40,202,822 (DAE group) clean reads were obtained after trimming away the low-quality and adapter sequences, respectively, and the quality control results showed that the Q30 value was greater than 94%, and the GC content was around 51%. Therefore, the sequencing results were accurate and reliable for further analysis. By performing data mapping and annotation, 37,619,438 (Blank group) and 38,032,508 (DAE group) reads were mapped to the rat (*Rattus norvegicus*) genome, subsequently, 12,821 out of 15,664 (Blank group) and 12,902 out of 15,733 (DAE group) transcripts were obtained by annotating against the non-redundant (NR) NCBI protein database and Swiss-Prot database, respectively.

| Statistics                     | Blank          | DAE            |
|--------------------------------|----------------|----------------|
| Clean reads                    | 39,698,758     | 40,202,822     |
| Q30 percentage                 | 94.78          | 94.86          |
| GC percentage                  | 51.27          | 51.84          |
| Total mapped reads             | 37,619,438     | 38,032,508     |
| Total transcripts              | 15,664         | 15,733         |
| Known transcripts              | 12,821         | 12,902         |
DEGs identification, GO and KEGG enrichment analysis

By comparing the Blank group and DAE group, 308 genes were identified as DEGs under the established criteria \((\log_2 \text{fold change} \geq 1 \text{ or } \leq -1 \text{ and } p \leq 0.001)\), including 208 upregulated genes \((\log_2 \text{fold change} \geq 1 \text{ and } p \leq 0.001)\) and 100 downregulated genes \((\log_2 \text{fold change} \leq -1 \text{ and } p \leq 0.001)\), as shown in Table 2. GO enrichment analyses were carried out to gain insight into the biological functions of DEGs under DAE treatment, as shown in Fig. 1. Under the category of cellular component, the significantly enriched GO terms were mainly classified into extracellular region, extracellular region part, extracellular space, extracellular matrix and proteinaceous extracellular matrix; under the category of molecular function, the significantly enriched GO terms were mainly classified into protein binding, transporter activity, substrate-specific transporter activity, protein heterodimerization activity and tetrapyrole binding; under the category of biological process, the significantly enriched GO terms were mainly classified into single-multicellular organism process, developmental process, single-organism developmental process, anatomical structure development and multicellular organism development. KEGG pathway enrichment analyses were carried out to further explore the possible functional pathways of DEGs under DAE treatment, as shown in Fig. 2, the significant enriched pathways were predominantly mapped to thyroid hormone signaling pathway, protein digestion and absorption, PI3K-AKT signaling pathway, nitrogen metabolism, ECM-receptor interaction and cell adhesion molecules (CAMs).

| Statistics                              | Number |
|----------------------------------------|--------|
| Differentially expressed mRNAs         | 308    |
| Upregulated mRNAs                      | 208    |
| Downregulated mRNAs                    | 100    |

### Table 2

Statistics for the differentially expressed genes (DAE vs. Blank)

DAE significantly increased the expression levels of functional genes involved in cartilage formation, growth and repair

According to the analysis of DEGs, 36 genes involved in cartilage formation, growth and repair were identified, such as hyaluronan and proteoglycan link protein 1 (Hapln1), collagen alpha-1(IX) chain (Col9a1), scrapie-responsive protein 1 (Scrg1), cartilage oligomeric matrix protein (Comp), leukocyte cell-derived chemotaxin 1 (Cnmd), carbonic anhydrase 2 (Ca2), matrilin-3 (Matn3), transferrin receptor protein 1 (Tfrc), vascular endothelial growth factor A (Vegfa) and collagen alpha-1(XXVII) chain (Col27a1), etc., as shown in Table 3.
| Gene name                                           | Expression level (FPKM) | log₂ fold change (DAE/Blank) | p value  |
|----------------------------------------------------|-------------------------|-----------------------------|----------|
| Hyaluronan and proteoglycan link protein 1 (Hapln1) | 301.97                  | 1.58                        | 0        |
| Collagen alpha-1 (IX) chain (Col9a1)               | 142.16                  | 1.87                        | 0        |
| Scrapie-responsive protein 1 (Scrg1)                | 203.52                  | 1.27                        | 1.23E-92 |
| Cartilage oligomeric matrix protein (Comp)         | 230.34                  | 1.09                        | 0        |
| Leukocyte cell-derived chemotaxin 1 (Cnmd)         | 135.34                  | 1.55                        | 1.08E-321|
| Carbonic anhydrase 2 (Ca2)                         | 172.19                  | 1.03                        | 2.61E-159|
| Matrilin-3 (Matn3)                                 | 112.11                  | 1.38                        | 0        |
| Transferrin receptor protein 1 (Tfrc)              | 46.77                   | 1.33                        | 1.65E-274|
| Vascular endothelial growth factor A (Vegfa)       | 51.06                   | 1.07                        | 8.57E-143|
| Collagen alpha-1 (XXVII) chain (Col27a1)           | 41.63                   | 1.12                        | 2.44E-248|
| M-phase inducer phosphatase 2 (Cdc25b)             | 33.93                   | 1.16                        | 1.82E-92 |
| Proliferation marker protein Ki-67 (Mki67)         | 21.76                   | 1.06                        | 6.36E-136|
| Carbonic anhydrase 12 (Ca12)                       | 10.53                   | 1.77                        | 2.03E-95 |
| Radical S-adenosyl methionine domain-containing protein 2 (Rsad2) | 9.91                   | 1.59                        | 1.27E-69 |
| T-cell acute lymphocytic leukemia protein 1 homolog (Tal1) | 10.64                   | 1.14                        | 2.07E-44 |
| Chondroitin sulfate N-acetylgalactosaminyltransferase 1 (Csgalnact1) | 11.15                   | 1.03                        | 3.03E-18 |
| Cartilage matrix protein (Matn1)                   | 4.78                    | 2.18                        | 3.49E-40 |
| Chordin-like protein 1 (Chrdl1)                    | 7.49                    | 1.35                        | 2.56E-41 |
| Thrombospondin-3 (Thbs3)                           | 5.25                    | 1.67                        | 7.21E-37 |
| Complement decay-accelerating factor, GPI-anchored (Cd55) | 7.74                    | 1.04                        | 1.04E-10 |
| Mitotic checkpoint serine/threonine-protein kinase BUB1 (Bub1) | 7.32                    | 1.02                        | 4.70E-23 |
| Gene name                                      | Expression level (FPKM) | log$_2$ fold change (DAE/Blank) | p value     |
|-----------------------------------------------|-------------------------|----------------------------------|-------------|
| G1/S-specific cyclin-E2 (Ccne2)              | 6.42                    | 14.00                            | 1.12        |
| Protein MGARP (Mgarp)                        | 4.50                    | 12.66                            | 1.49        |
| M-phase phosphoprotein 8 (Mphosph8)          | 5.78                    | 12.46                            | 1.11        |
| Noggin (Nog)                                 | 4.80                    | 11.69                            | 1.28        |
| Carbonic anhydrase 9 (Ca9)                   | 5.45                    | 11.63                            | 1.09        |
| ESF1 homolog (Esf1)                          | 5.27                    | 11.61                            | 1.14        |
| Aryl hydrocarbon receptor nuclear translocator-like protein 1 (Arntl) | 3.14                    | 10.98                            | 1.81        |
| H(+)/Cl(-) exchange transporter 3 (Clcn3)    | 4.15                    | 9.18                             | 1.15        |
| G2/M phase-specific E3 ubiquitin-protein ligase (G2e3) | 3.80                    | 7.66                             | 1.01        |
| Dual specificity tyrosine-phosphorylation-regulated kinase 3 (Dyrk3) | 2.45                    | 6.59                             | 1.43        |
| Protein kinase C zeta type (Prkcz)           | 2.98                    | 6.32                             | 1.08        |
| WNT1-inducible-signaling pathway protein 3 (Wisp3) | 2.82                    | 5.89                             | 1.06        |
| Centrosomal protein of 70 kDa (Cep70)        | 2.02                    | 4.72                             | 1.22        |
| Dermatopontin (Dpt)                          | 1.46                    | 3.97                             | 1.44        |
| Murinoglobulin-1 (Mug1)                      | 0.56                    | 1.67                             | 1.58        |

DAE significantly decreased the expression levels of susceptibility genes involved in the pathophysiology of osteoarthritis

According to the analysis of DEGs, 31 genes involved in osteoarthritis susceptibility were identified, such as collagen alpha-1(I) chain (Col1a1), protein S100-A4 (S100a4), fatty acid-binding protein (Fabp4), collagen alpha-1(IV) chain (Col4a1), cyclic AMP-responsive element-binding protein 3-like protein 1 (Creb3l1), C-C motif chemokine 9 (Ccl9), retinoid-binding protein 7 (Rbp7), neurogenic locus notch homolog protein 3 (Notch3), C-X-C motif chemokine 16 (Cxc16) and receptor activity-modifying protein 3 (Ramp3), etc., as shown in Table 4.
Table 4
List of significantly downregulated DEGs involved in osteoarthritis susceptibility

| Gene name                                                                 | Expression level (FPKM) | log\textsubscript{2} fold change (DAE/Blank) | p value     |
|---------------------------------------------------------------------------|-------------------------|---------------------------------------------|-------------|
| Collagen alpha-1(I) chain (Col1a1)                                       | 6244.47                 | -1.19                                       | 0           |
| Protein S100-A4 (S100a4)                                                 | 687.97                  | -1.06                                       | 2.85E-77    |
| Fatty acid-binding protein (Fabp4)                                       | 150.74                  | -1.02                                       | 1.22E-22    |
| Collagen alpha-1(IV) chain (Col4a1)                                      | 60.42                   | -1.22                                       | 1.55E-166   |
| Cyclic AMP-responsive element-binding protein 3-like protein 1 (Creb3l1) | 53.52                   | -1.18                                       | 1.14E-56    |
| C-C motif chemokine 9 (Ccl9)                                             | 34.51                   | -1.60                                       | 2.15E-27    |
| Retinoid-binding protein 7 (Rbp7)                                        | 19.22                   | -1.69                                       | 1.21E-07    |
| Neurogenic locus notch homolog protein 3 (Notch3)                        | 18.11                   | -1.08                                       | 1.69E-47    |
| C-X-C motif chemokine 16 (Cxcl16)                                        | 17.30                   | -1.18                                       | 1.19E-12    |
| Receptor activity-modifying protein 3 (Ramp3)                            | 16.14                   | -1.25                                       | 2.83E-09    |
| Cytochrome P450 1B1 (Cyp1b1)                                             | 15.63                   | -1.19                                       | 1.50E-15    |
| Proprotein convertase subtilisin/kexin type 1 inhibitor (Pcsk1n)         | 15.53                   | -1.19                                       | 1.73E-06    |
| Homeobox protein DLX-3 (Dlx3)                                            | 15.49                   | -1.08                                       | 3.27E-15    |
| Protein naked cuticle homolog 2 (Nkd2)                                   | 14.53                   | -1.14                                       | 1.00E-22    |
| Matrix metalloproteinase-19 (Mmp19)                                      | 12.71                   | -1.07                                       | 2.53E-09    |
| Endosialin (Cd248)                                                       | 11.69                   | -1.21                                       | 2.00E-13    |
| Pentraxin-related protein PTX3 (Ptx3)                                    | 10.83                   | -1.03                                       | 1.16E-07    |
| Fc receptor-like protein 2 (Fcrl2)                                       | 10.76                   | -1.16                                       | 1.76E-10    |
| Serine protease HTR4 (Htra4)                                             | 10.74                   | -1.31                                       | 2.14E-12    |
| Transforming growth factor beta-1-induced transcript 1 protein (Tgfb1i1) | 10.61                   | -1.07                                       | 3.56E-08    |
| Apelin receptor (Aplnr)                                                  | 10.59                   | -1.49                                       | 2.16E-23    |
| Gene name                                 | Expression level (FPKM) | log₂ fold change (DAE/Blank) | p value   |
|------------------------------------------|-------------------------|------------------------------|-----------|
| Tubulin beta-2B chain (Tubb2b)           | 7.26                    | 3.04                         | -1.26     | 1.80E-06  |
| Arginase-1 (Arg1)                        | 5.65                    | 2.37                         | -1.25     | 8.66E-05  |
| Nostrin (Nostrin)                        | 5.43                    | 2.16                         | -1.33     | 1.80E-05  |
| von Willebrand factor (Vwf)              | 3.76                    | 1.63                         | -1.21     | 3.03E-15  |
| Tenascin-X (Tnxb)                        | 2.82                    | 1.27                         | -1.15     | 4.95E-05  |
| Aryl hydrocarbon receptor (Ahr)           | 2.41                    | 1.16                         | -1.05     | 1.62E-05  |
| Interferon-induced protein with tetratricopeptide repeats 1 (Ifit1) | 2.01                    | 0.54                         | -1.90     | 1.02E-04  |
| Interleukin-2 receptor subunit beta (Il2rb) | 1.91                    | 0.59                         | -1.69     | 1.31E-04  |
| Chloride intracellular channel protein 5 (Clic5) | 1.81                    | 0.51                         | -1.83     | 1.62E-09  |
| C-type lectin domain family 9 member A (Clec9a) | 1.47                    | 0.43                         | -1.77     | 9.17E-05  |

**Gene expression levels of DEGs validated by qRT-PCR**

The expression levels of a series of DEGs were validated by qRT-PCR assay, including 6 significantly upregulated genes and 6 significantly downregulated genes. The specific gene primers for qRT-PCR were listed in Table 5. The relative fold change of each gene was normalized to the internal reference gene Gapdh. The expression levels of the selected DEGs validated by qRT-PCR exhibited similar expression patterns as those of the RNA-seq analysis, as shown in Fig. 3.
Table 5  
List of primers used for qRT-PCR validation

| Gene  | Primer       | Sequence                |
|-------|--------------|-------------------------|
| Hapln1| Forward primer | GACAGCTACACTCCGGGATCA  |
|       | Reverse primer | AGCCAAATGCTGTAGGGGTCT  |
| Col9a1| Forward primer | CCAGCACATCAAGCAGGTTT   |
|       | Reverse primer | CCTCCAGGAAGACCAGAAG    |
| Scrg1 | Forward primer | CTTGTACATCCCTCGGCTAA   |
|       | Reverse primer | AACAGGAGAGCGACTTGGAA   |
| Comp  | Forward primer | CAGCTCAAGGCTGTCAAGTC   |
|       | Reverse primer | CTTCCAGCCACATTTCGAG    |
| Cnmd  | Forward primer | TTACCACCAGCAG GAAGGAG  |
|       | Reverse primer | TGGACCGACACCTTTGGTAAT   |
| Ca2   | Forward primer | TCCTTGCTTCCTTCTTCTG    |
|       | Reverse primer | CAGGTCACACATTCACGAG    |
| Col1a1| Forward primer | ACCTCAGGGTATTGCTGGAC   |
|       | Reverse primer | GACCAGGGAACGCTCTCT    |
| S100a4| Forward primer | GCTGCATTCAGAAGCTGAT    |
|       | Reverse primer | CATCATGGGAATGCAGGACA   |
| Fabp4 | Forward primer | ATGTGCAAGATGGGATGGA    |
|       | Reverse primer | GTCAGGCTTTTATGACAG     |
| Col4a1| Forward primer | CCTCCAGGAACGACTCTCC    |
|       | Reverse primer | GCACACCTGCTAATGAAGGG   |
| Creb3l1| Forward primer | CAGCTGCAAGAATCCACAG    |
|       | Reverse primer | CCAGAACAAGACACAAGGCT   |
| Ccl9  | Forward primer | TCGTACATGCGACAGGACA    |
|       | Reverse primer | TGGACCGTGAGGTATAGGA    |

**Discussion**

For many years, RNA-seq has become an essential tool for studying the dynamic and complex characteristics of the transcriptome, especially for the mRNA molecules, which encode proteins following the central dogma of molecular biology [27, 28]. Meanwhile, many scientists in the research field of Chinese medicine have paid more and more attention to explore the molecular control of Chinese herbs and formulations by taking advantage of RNA-seq technology [29, 30]. Our previous studies have shown that DAE play potential roles in regulating chondrocyte proliferation and differentiation [15, 16]. Therefore, in the present study, we further investigated the effects of DAE on articular cartilage using a state-of-the-
art RNA-seq technology accompanied with validation method to obtain the precise molecular mechanism of DAE on cartilage homeostasis.

In total, 308 DEGs were identified, including 208 upregulated genes and 100 downregulated genes by comparing DAE treated group with Blank group (DAE vs. Blank). According to the GO enrichment analysis, the significantly enriched GO terms were predominantly involved in extracellular matrix synthesis, binding activity and developmental process. Those functional gene groups play pivotal roles in regulating cartilage homeostasis [31-33]. Based on the KEGG enrichment analysis, the significant enriched pathways were predominantly involved in thyroid hormone signaling pathway, protein digestion and absorption, PI3K-AKT signaling pathway, nitrogen metabolism, ECM-receptor interaction and cell adhesion molecules (CAMs). Among those enriched signaling pathways, thyroid hormone signaling pathway, PI3K-AKT signaling pathway, ECM-receptor interaction and cell adhesion molecules (CAMs) have been considered to play crucial roles in articular cartilage maintenance and osteoarthritis pathogenesis [34-37]. Thus, these results suggest that DAE play potential role in regulating articular cartilage homeostasis by controlling multiple functional group genes and signaling pathways.

Among the significantly upregulated DEGs under DAE treatment, 36 genes that participate in cartilage formation, growth and repair were identified. For instance, Hapln1, Col9a1, Comp, Cnmd, Matn3, Col27a1, Matn1 and Dpt are extracellular matrix that play key role in regulating chondrocyte metabolism and functions via cell-matrix interaction [38-42]. Scrg1 is a stimulator of chondrogenesis that has a potential role in tissue engineering of articular cartilage [43]. Tfrc, also known as CD71, is a transferrin receptor that is essential for cartilage maturation during embryonic development [44]. Vegfa, a member of the Vegf growth factor family, is a key component to support chondrocyte survival [45]. Ca2, Ca12 and Ca9 are family members of carbonic anhydrases, which are important for cartilage homeostasis. All of them are significantly expressed in the articular chondrocytes, and Ca2 is mainly localized in the proliferating chondrocytes [46]. Rsad2, also known as viperin, is highly expressed in the middle zone of articular cartilage [47]. Tal1 is a basic helix-loop-helix transcription factor in the articular chondrocytes, and serves as a crucial regulator during chondrocyte maturation [48].

Csgalnact1 is a glycosyltransferase that is necessary for the biosynthesis of chondroitin sulfate proteoglycans in cartilage, particularly in the proliferating chondrocytes [49]. Chrdl1, a secreted glycoprotein, is considered to be a juvenile chondrocyte-specific factor that stimulates stem cell growth [50]. Thbs3 is mainly expressed in the proliferating chondrocytes, and inhibits cartilage clarification [51]. Cd55 is a complement decay-accelerating factor in cartilage that play pivotal role in protecting chondrocytes from possible damage [52]. Nog serves as an inhibitor of bone morphogenetic proteins (BMPs), and prevents cartilage degeneration and osteoarthritis development by inhibiting Il1β and Bmp2 expression [53]. Esf1 is an essential nucleolar protein that is required for cartilage formation [54]. Arntl, also known as Bmal1, plays crucial role in controlling cartilage homeostasis through modulating TGF-β signaling [55]. Clcn3 is a highly expressed channel protein in chondrocytes during cartilage development, and plays a key role in cell volume regulation [56]. Wisp3 is a multi-domain protein that maintains cartilage integrity and prevents chondrocyte hypertrophy [57]. Mug1 serves as an inhibitor of proteolytic
enzyme that prevents the degradation of cartilage extracellular matrix [58]. In addition, Cdc25b, Mki67, Bub1, Ccne2, Mphosph8, G2e3, Dyrk3 and Cep70 are considered to be essential genes involved in cell proliferation [59-65]. Thus, these results suggest that DAE might serve as a candidate supplement for maintaining cartilage homeostasis.

Among the significantly downregulated DEGs under DAE treatment, 31 genes involved in osteoarthritis susceptibility were identified. For instance, Col1a1 and Col4a1 are collagen fibers that were associated with the progression of osteoarthritis [66]. S100a4, a member of the S100 protein family, is involved in cartilage degradation of osteoarthritis pathophysiology [67]. Fabp4 is a fatty acid-binding protein that serves as a biomarker for knee osteoarthritis [68]. Creb3l1 is a transcription factor significantly upregulated at the early stage of osteoarthritis [69]. Ccl9, also called macrophage inflammatory protein-1 gamma (MIP-1γ), is a small cytokine belonging to the CC chemokine family that was shown to be highly expressed during osteoarthritis progression [70]. Rbp7, a family member of the cellular retinol-binding proteins, is significantly upregulated in osteoarthritic chondrocytes [71]. Notch3 is a family member of Notch receptors, and genetic deletion of Notch3 or the blockade of Notch3 signaling prevents joint damage and attenuates inflammation of inflammatory arthritis [72].

Cxcl16 (a chemokine ligand), Cd248 (a transmembrane glycoprotein), Pttx3 (an inflammation induced gene), Fcr2l (a subtype of Fc receptor-like molecules), Tgfb1i1 (a transforming growth factor beta 1 induced gene), Nostrin (a nitric oxide synthase traffic inducer), Vwf (an adhesive and multimeric glycoprotein), Tnxb (a member of the tenascin family), Ahr (a ligand-activated transcription factor), Lfif1 (an interferon-induced protein), Il2rb (an interleukin-2 receptor subunit) and Clec9a (a C-type lectin) are involved in the inflammation associated with arthritis pathology [73-84]. Ramp3 is a receptor activity-modifying protein that is highly expressed during joint inflammation [85]. Cyp1b1 (a member of the cytochrome P450 enzyme family), Pcsk1n (an inhibitor of prohormone convertase 1), Dlx3 (a family member of homeobox proteins), Nkd2 (a regulator of inflammatory response), Mmp19 (a subtype of matrix metalloproteinases), Htra4 (a subtype of serine proteases), Apnhr (a G protein-coupled receptor of apelin), Tubb2b (a beta isoform of tubulin), Arg1 (a cytosolic manganese-dependent enzyme) and Clc5 (a chloride intracellular channel protein) are reported to be highly expressed under osteoarthritic condition [86-95]. Thus, these results suggest that DAE might serve as a candidate supplement for preventing cartilage degeneration and inflammation.

In addition to the above findings, we also compared the expression levels of genes that are well known to characterize hyaline cartilage, such as Sox9, Sox5, Sox6, Wwp2, Acan, Col2a1, Col9a1, Col11a1, Hapl, Comp, Matn1, Ptch1, Fgfr3, Runx2 and Runx3. As shown in Table S1, the expression levels of a majority of these genes were slightly upregulated under the DAE treatment. However, the expression level of Sox9 was slightly downregulated, which indicates that DAE might regulate articular chondrocytes through other transcription factors and related signaling pathways.

**Conclusion**
In summary, the present study demonstrated that DAE might serve as a candidate supplement for maintaining cartilage homeostasis, and preventing cartilage degeneration and inflammation. These effects were possibly achieved by accelerating the expression of functional genes involved in cartilage formation, growth and repair, and suppressing the expression of susceptibility genes involved in the pathophysiology of osteoarthritis. Thus, our findings will contribute towards deepening the knowledge about the molecular control and therapeutic targets of DAE on the treatment of cartilage related diseases.

**Abbreviations**

DAE: Deer antler extract; SD: Sprague-Dawley; RNA-seq: RNA sequencing; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: Differentially expressed genes; NCBI: National Center for Biotechnology Information; NR: Non-redundant; SRA: Sequence Read Archive; qRT-PCR: Quantitative real-time PCR; Gapdh: Glyceraldehyde 3-phosphate dehydrogenase; mRNAs: Messenger RNAs.

**Declarations**

**Ethics approval and consent to participate**

All procedures were performed in accordance with the guidelines of the Institutional Animal Ethics Committee of Changchun University of Chinese Medicine (No. ccucm-2017-0015).

**Consent for publication**

*Not applicable.*

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

*The authors declare that they have no competing interests.*

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**Authors’ contributions**
BY, XL and DZ conceived and designed the work; BY and XL helped to coordinate support and funding; BY, ZZ and MZ performed the experiments; BY analysed the data and wrote the original draft; BY, XL and DZ reviewed and revised the manuscript. All authors read and approved the final version of manuscript.

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Figures
Figure 1

Histogram presentation of enriched GO terms in articular cartilage under DAE treatment. The horizontal coordinate represents the number of mapped genes in a category, and the vertical coordinate represents the GO terms significantly enriched (p < 0.05) in the categories of cellular component, molecular function and biological process.
Figure 2

Scatter plot of enriched KEGG pathways in articular cartilage under DAE treatment. The horizontal coordinate represents the rich factor that is calculated as the ratio of the number of DEGs divided by the total gene number in a certain pathway, and the vertical coordinate represents the enriched pathway. The color and size of the dots represent the range of the Q values and the number of DEGs mapped to a certain pathway, respectively.
Figure 3

qRT-PCR validation of RNA-seq data. Data are presented as the mean for technical triplicates in an experiment representative of several independent ones with their corresponding standard deviation. The asterisk *, ** and *** indicate significant differences using Student t-test with p value <0.05, <0.01 and <0.001, respectively. Gene expression levels for individual genes are presented as the ratio of (fold change) the DAE group to the Blank group.

Supplementary Files
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- TableS1.pdf