Gastrocnemius transcriptome analysis reveals domestication induced gene expression changes between wild and domestic chickens

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ABSTRACT

Artificial selection of chicken for human-preferred traits has manifested great phenotypic differences between wild and domestic chickens. Study on the formation of these phenotypic variations will contribute to comprehensive understanding of the molecular mechanism of animal domestication. We used three kinds of chicken breeds for transcriptome analysis, including the red jungle fowl which was the wild ancestor of chickens, and two other domestic breeds, the chahua chicken and the avian broiler. More than 12,000 genes’ expression levels were compared between different chicken breeds, and hundreds of genes displayed differential expression levels compared with wild chicken. Gene ontology analysis showed that differentially expressed genes in domestic chickens tended to be enriched in extracellular matrix, DNA binding and immune system development, etc. Some genes with important biological functions were differentially expressed in the domestic chickens, including titin, myostatin ubiquitin related genes, and transforming growth factor-beta receptor III, indicating possible selection pressures on these genes.

1. Introduction

Chicken is an important animal that has both economic and cultural significance to humankind. Chicken is widely used for the study of embryology, immunology, development, behavior and reproduction [1]. Domestic chickens are derived from the red jungle fowl, and chickens are thought to have been domesticated about 8000 years ago in South-East Asia [2,3]. Domestication and breeding of chickens have been performed for different purposes, including aggression, appearance, rapid growth and egg production [4]. As many as sixty breeds have resulted from chicken domestication, which can be mainly categorized into four distinct lineages: meat-type, game-type, egg-type and bantam [5]. Animal domestication is accompanied by a range of changes that are both morphological and behavioral, such as faster development, plumage color variety, and reduced fearfulness towards humans [4]. Many genetic or epigenetic alterations have occurred at molecular level, and the changes in gene expression are the direct cause of phenotypic variations and morphological diversity [6–8].

Domestication is an evolutionary process during which animals become accustomed to human environments, with the intention of selection for human-preferred traits [9]. Over-expression of certain genes may cause dramatic alterations in phenotype. Growth hormone (GH) transgenesis in mice increased growth rate up to 2-fold [10]. Evidence suggests that numerous genes show different expression between wild and domestic animals [6,7,11]. Defective control of genomic imprinting by the callipyge (CLPG) gene, resulting in abnormal expression, leads to the formation of callipyge phenotype in sheep [7]. Up-regulation of IGF2 expression in postnatal skeletal and cardiac muscle, but not in prenatal muscle or in liver, directly causes high muscularity, less backfat and a larger heart in certain domestic pig breeds [11,12].

The first livestock species to be completely sequenced, the chicken genome, was released in 2004 [13], which lay the foundation for the comprehensive transcriptome analysis of the chicken. In our study, the red jungle fowl, chahua chicken, and avian broiler were used to analyze genes with altered expression level in the process of domestication. Red jungle fowl is believed to be the wild ancestor of domestic chickens [2]. Chahua chicken is a native breed in southwest China that displays many similar phenotypes and behaviors with the red jungle fowl [14]. Avian broiler is a domestic breed that has been intensively selected for meat production during the last century. Gene expression profiles of the wild and domestic chicken were obtained by RNA-seq, and DEGs among chicken breeds were identified by further data analysis. Numerous DEGs between breeds were identified by pairwise comparison. Among these genes, several involved in muscle growth regulation and lipid metabolism showed differential expression levels between wild and domestic chicken breeds.
indicating possible roles of these genes in the formation of human-preferred traits.

2. Results

2.1. Transcriptome analysis of wild and domestic chickens by RNA-seq

In our study, gastrocnemius of four or five 7-day-old individuals was used for each breed, and gene expression profiling of each sample was obtained by sequencing the cDNA library with Illumina HiSeq™ 2000. A range of 6,955,676 to 7,541,094 of raw reads was generated for each sample. Approximately 60% of the reads were mapped, and about 45% of the reads in each sample were uniquely mapped to the chicken genome (Table S1). The average expression level of each transcript was calculated by RPKM (reads per kb per million) method [15]. In total, 12,204 to 13,355 genes were termed as expressed genes in each sample (Table S2).

To verify the gene expression data generated by RNA-seq, 11 genes were randomly selected for real-time RT PCR validation. Correlation analysis showed that the RNA-seq data was highly consistent with the real-time PCR data (Spearman's rho = 0.84, p < 0.0001, Fig. 1), indicating the accuracy of RNA-seq for measuring gene expression level.

2.2. Differentially expressed genes between wild and domestic breeds

We investigated the influence of genetic selection on gene expression patterns by comparing the gene expression profiles of muscle tissue of different chicken breeds. Genes accorded with the following criteria were termed as DEGs: expression level fold change between two breeds of more than 2, p < 0.05 and FDR < 0.05. In all, we identified 1389 DEGs between RJF and AA (Table S3 and Fig S1), 907 DEGs between RJF and CH (Table S4), and 301 DEGs between CH and AA (Table S5). Compared with RJF, CH and AA are both domestic breeds, although CH is something like the wild one. What attracted our attention most was the genes that showed different expression levels, between RJF and CH, and between RJF and AA. 297 genes were up-regulated in both CH and AA, compared with their wild ancestor (Table S6); in addition, 262 genes were down-regulated in both CH and AA, compared with RJF (Table S7). Genes that up- or down-regulated both in CH and AA were termed as commonly up- or down-regulated genes. Hierarchical clustering was performed for these commonly up- and down-regulated genes (Fig. 2).

2.3. Gene ontology (GO) and pathway analysis of DEGs between chicken breeds

Using the gene ontology annotation and KEGG pathway database, we compared the GO and pathway enrichment of DEGs among the three breeds pairwise. Our results showed that commonly up-regulated genes in domestic chicken breeds tended to be enriched in extracellular matrix, basement membrane and extracellular structure organization, etc. (Table 1). Commonly down-regulated genes were over-represented in DNA binding, hemopoietic or lymphoid organ development and immune system development, etc. (Table 1). The pathway enrichment of commonly up- and down-regulated genes was not as frequent as that of GO enrichment, only phagosome and caffeine metabolism pathways (p < 0.05) were over-represented by these genes.

We also analyzed the GO enrichment of DEGs between other chicken breed comparisons. The DEGs between RJF and CH tend to be enriched in extracellular region part and transcription factor complex, etc. (Table S8). DEGs of AA were enriched in cell surface and immune system development, etc. when compared to RJF, etc. (Table S9). DEGs between AA and CH were overrepresented in the ontology of DNA replication, response to gas transport and DNA metabolic process, etc. (Table S10).

2.4. Biologically important genes differentially expressed in domestic chicken breeds compared with red jungle fowl

Some of the genes that are involved in crucial biological processes, including cellular differentiation, proliferation, skeletal muscle development and fatty acid metabolism, displayed different expression levels in domestic and wild chicken breeds. These were titin, myostatin, insulin-like growth factor-binding protein-5 (IGFBP-5), transforming growth factor-beta receptor III (TGFBR3), follistatin-like 1 (FSTL1), fibronectin type III domain containing 3B (FNDC3B) and fatty acid desaturase 1 (FADS1).

RNA-seq data suggested that titin, myostatin and IGFBP5 were down-regulated in domestic breeds, compared with red jungle fowl (Fig. 3A). We subsequently carried out real-time PCR to validate differential expression of these genes. Although real-time PCR results differed in the extent of expression changes, the general down-regulated trends of these genes were consistent with that of RNA-seq. Myostatin, a gene that is specifically a negative regulator of skeletal muscle growth, was down-regulated 7- and 3-fold in CH and AA, respectively (Fig. 3A). IGFBP5, a key regulator of IGF, was down-regulated 2- and 3-fold in CH and AA (Fig. 3A). Some genes were up-regulated in the domestic breeds, including TGFBR3, FSTL1, FNDC3B and FADS1 (Fig. 3B). Real-time PCR results demonstrated that TGFBR3 was up-regulated 2- and 16-fold in CH and AA, respectively (Fig. 3B). With the function of modulating the action of some growth factors on cell proliferation and differentiation, the expression level of FSTL1 was 1.5- and 27-fold higher in CH and AA, respectively, than in RJF (Fig. 3B).

2.5. Domestication driven differential expression of ubiquitin related genes in domestic breeds

In muscle, the vast majority of intracellular proteins are degraded by the ubiquitin–proteasome system [16]. In this system, proteins are first marked for degradation by chains of the polypeptide cofactor ubiquitin, and then recognized by the 26S proteasome which can degrade ubiquitinated proteins to small peptides [17,18]. In our study we observed a number of ubiquitin-related genes with different expression levels in wild and domestic chicken breeds (Table 2), indicating possible selection pressure on this specific function of genes. There were 14 common genes of CH and AA that were different from RJF. These included E3 ligases, deubiquitinating enzymes, and 26S proteasome subunit (Table 2 and Fig S2). Among these genes, 12 were up-regulated, and 2 de-ubiquitinating enzymes were down-regulated in the domestic breeds.
Fig. 2. Hierarchical clustering of commonly up- and down-regulated genes between wild and domesticated chickens. 297 up-regulated and 262 down-regulated genes in both of CH and AA compared with JRF were used for unsupervised hierarchical clustering. The data was shown in the form of log2AA/RJF or log2CH/RJF.

Fig. 3. Real-time RT PCR validation of biologically important genes that differentially expressed between the wild and domestic chicken breeds. Gastrocnemius from 5 chickens was used in this experiment, and expression levels of genes were normalized to that of GAPDH. Q-PCR results were analyzed with 2−△△Ct method. A: expression of extracellular matrix and extracellular structure organization to that of GAPDH. B: expression of transcription factor complex and negative regulation of cell proliferation to that of GAPDH. Q-PCR results were analyzed with 2−△△Ct method.

### Table 1

<table>
<thead>
<tr>
<th>Gene ontology (GO) term</th>
<th>P-value for enrichment</th>
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<tbody>
<tr>
<td>Up-regulated</td>
<td></td>
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<tr>
<td>Extracellular matrix</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Basement membrane</td>
<td>&lt;0.01</td>
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<tr>
<td>Extracellular structure organization</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Down-regulated</td>
<td></td>
</tr>
<tr>
<td>DNA binding</td>
<td>5.71E−05</td>
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<tr>
<td>Hemopoietic or lymphoid organ development</td>
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<tr>
<td>Immune system development</td>
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<tr>
<td>Regulation of cell proliferation</td>
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<td>Negative regulation of cell proliferation</td>
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<td>Skeletal system development</td>
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3. Discussion

Muscle yield is one of the most economically important phenotypes in animal domestication, and there is little information about the mechanisms underlying the diverse muscle phenotypes in domestic animals. Here we report the use of RNA-seq to decipher the gene profiles of three chicken breeds, including the wild breed and two domestic breeds that have undergone extensive genetic selection. Hundred of genes were identified as DEGs among the breeds, indicating the altered gene expression patterns between wild and domestic chickens, and also between domesticated chickens. AA and RJF had the highest number of DEGs, either up- or down-regulated, while the lowest number of DEGs was detected between the two domestic breeds, AA and CH. The DEGs between wild and domestic chickens enlarge our knowledge of how domestication altered the molecular mechanisms that resulted in the establishment of human-preferred phenotypes.

The formation of a specific phenotype is a result of the interaction of a certain genotype with the environment, and environmental factors exert their influence on phenotype through genetic or epigenetic mechanisms. Both genetic and environmental factors will influence gene expression in the process of animal domestication. Previous studies have revealed the domestication induced selection pressure on genes with certain functions, such as growth regulation, appetite and metabolic regulation, susceptibility to obesity and coat color, reflected on gene expression level, genetic polymorphism, or protein structure [19–22]. Insulin-like growth factor 2 (IGF2) is a well-studied key gene involved in growth regulation and economically important phenotype formation that shows altered expression level in domestic pigs [11,12,23]. Other genes, such as insulin-like growth factor 1 (IGF1), growth hormone receptor (GHR), thyroid hormone receptor (THR), and growth hormone (GH), show elevated expression levels in domesticated strains [24–28]. In our study, we mainly focused on the genes that were concordantly up- or down-regulated, both in CH and AA, compared with their wild counterpart. We found a number of genes, including titin, myostatin, IGFBP5, TGFBR3, follistatin-like 1, FNDCC3B and FADS1. Titin, myostatin and IGFBP5 were down-regulated in domestic chickens (Fig. 2). Titin is the backbone of sarcomeres and is associated with sarcomere extensibility during muscle shortening [29]. As a member of the transforming growth factor-beta (TGF-β) superfamily [6], myostatin is a negative regulator of muscle growth, and loss of its function always results in increased muscle mass in animals [30,31]. IGFBP5 is a critical member of the IGF axis and plays a crucial role in regulation of cell differentiation and apoptosis. It has been shown to either inhibit or stimulate the growth promoting effects of the IGFs [32]. Four key genes are up-regulated in domesticated breeds. These are TGFBR3, follistatin-like 1, FNDCC3B and FADS1. TGF-β/cytokines have been shown to have profound suppressive effects on muscle growth, and TGFBR3 encodes a receptor that may inhibit this pathway [33]. FSTL1 encodes a protein similar to follistatin that may modulate the action of some growth factors on cell differentiation and proliferation [34], FNDCC3B may be a positive regulator of adipogenesis, and FADS1 is a component of a lipid metabolic pathway that catalyzes biosynthesis of highly unsaturated fatty acids [35,36]. It is clear that these genes are all closely related with economically important functions, such as growth regulation and lipid metabolism, indicating possible selection pressures on these genes in domesticated breeds. We speculate that the expression of these genes contributes to the phenotypic diversity of domesticated chicken breeds. Genes under selection are expected to show changes in comparison with unselected genes, and much genetic or epigenetic diversity that is induced by selection pressures may be due to different gene expression.

It is noteworthy that many ubiquitin-related genes showed differential expression levels, compared with their wild counterpart. These genes encoded for E3 ubiquitin ligases, deubiquitinase or the 26S proteasome subunit. Poly-ubiquitination of proteins is an epigenetic modification that functions in gene expression regulation. Fourteen of this kind of genes displayed variable expression between domesticated breeds and their wild ancestor (Table 2); twelve of them were up-regulated and two were down-regulated, but only three genes were down-regulated. The data was shown in the form of log2AA/RJF or log2CH/RJF.
regulated. Our results suggest that parts of the protein degradation systems underwent genetic selection pressures when wild animals adapted to anthropogenic conditions or were selected for human-preferred traits.

Domestication has been widely used to modify phenotypes for human benefit in agricultural animal, and plenty of animal breeds have been formed due to artificial selection. The present study provides significant information about the comprehensive understanding of the mechanisms of animal domestication, and is also useful for the selection of desired phenotypes through traditional breeding or human-intervention approaches such as transgenesis.

4. Materials and methods

4.1. Animals

Four or five 7-day-old female chickens were utilized in this study for each chicken breed (RJF, CH and AA). The chickens were sacrificed according to local standards of animal welfare issues. The study was approved by the animal welfare committee of State Key Laboratory for Agro-biotechnology of China Agricultural University with approval number XK257. Gastrocnemius taken from each animal was frozen in liquid nitrogen and then stored at −80 °C. All the data in this study were generated from Gastrocnemius.

4.2. Total RNA isolation and RNA-seq

A piece of tissue was ground in liquid nitrogen, total RNA was extracted with TRIzol® Reagent (Invitrogen) using the manufacturer’s instructions. At least 4 individuals were used for RNA-seq. mRNA was extracted with TRIzol® Reagent (Invitrogen) using the manufacturer’s instructions. At least 4 individuals were used for RNA-seq. mRNA was enriched by oligo(dT) magnetic beads from total RNA. mRNA was degraded to about 200 bp fragments in buffer. Then the first strand of cDNA was synthesized by using random hexamer-primer, and the second strand was subsequently synthesized based on the first strand. The double stranded cDNA was purified with QiaQuick PCR extraction kit (Qiagen) and washed with elution buffer for end repair and adenine addition. Finally, sequencing adaptors were ligated to the fragments. The required fragments were purified by agarose gel electrophoresis and enriched by PCR amplification. The library products were ready for sequencing analysis via Illumina HiSeq™ 2000.

4.3. Real-time RT PCR assay

First-strand cDNA was reverse transcribed by molony murine leukemia virus reverse transcriptase (Promega) and oligo(dT) primer (Promega) using 1.5 μg of total RNA. The expression of specific genes was quantified by real-time PCR using an ABI 7900HT instrument with the SYBR® Green system (Applied Biosystems). Primers for real-time PCR were designed by Oligo 6.0 software (primer sequence will be available upon request). For RT PCR amplification, cDNA was pre-denatured at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, and 60 °C for 1 min. The expression of the housekeeping gene GAPDH was used as internal control to normalize for input cDNA. The consistency between RNA-seq and real-time RT PCR results was determined by calculating the Spearman’s rank correlation coefficient for the expression of 11 randomly selected genes across 12 samples (4 samples for each breeds, total n = 132).

4.4. GO enrichment analysis

Gene information was downloaded from the public FTP site of Ensembl (ftp://ftp.ensembl.org/pub/) in October, 2008. The information about GO terms was downloaded from the UniProtKB-GOA database. We selected random samples of N different genes at each iteration and computed Fisher’s exact test p-values for over-representation of the selected genes in all GO biological categories. GO terms with p<0.05 were considered as significantly enriched.

Table 2

| Ubiquitin related genes differentially expressed between wild and domestic breeds. |
|---------------------------------|-----------------|-----------------|-----------------|
| Ensembl gene ID | Annotation | Fold change (AA/RJF) | Fold change (CH/RJF) | Possible function (by Genecards) |
| ENSGALT0000033197 | Beta-transducin repeat containing (BTRC) membrane-associated ring finger 6 (MARCH6) cytokine inducible SH2-containing protein (CISH) Valosin containing protein (p97)/p47 complex interacting protein 1 (VCPIP1) Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB) Tripartite motif containing 41 (TRIM41) | 2.053 | 2.08 | Subunit of SCP E3 ubiquitin ligase complex. E3 ubiquitin ligase that promotes ubiquitination of DIO2. Subunit of SCP E3 ubiquitin ligase complex. |
| ENSGALT0000000538 | ENSGALT00000003093 | 2.124 | 2.43 | |
| ENSGALT000000024772 | Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB) | 2.133 | 2.41 | Acts as a deubiquitinating enzyme. |
| ENSGALT00000000084 | Tripartite motif containing 41 (TRIM41) | 2.167 | 2.02 | |
| ENSGALT00000006707 | Proteasome (prosome, macropain) 26S subunit, non-ATPase. 13 (PSMD13) | 2.5 | 2.42 | A regulatory subunit of the 26S proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins. Deubiquitinating enzyme that regulates the degradation of various proteins. E3 ubiquitin ligase that mediates monoubiquitination of histone H2A. Modulates the deubiquitinase activity of UCHL5. |
| ENSGALT0000011032 | Ubiquitin specific peptidase 19 (USP19) | 2.567 | 3.04 | |
| ENSGALT0000037411 | Ubiquitin specific peptidase 2 (USP2) | 2.704 | 2.95 | |
| ENSGALT0000001988 | Ubiquitin specific peptidase 19 (USP19) | 2.786 | 2.48 | |
| ENSGALT0000010909 | Ubiquitin specific peptidase 19 (USP19) | 3 | 2.37 | Component of the E3 ubiquitin ligase DCX DET1-COP1 complex. |
| ENSGALT0000010602 | Ubiquitin associated SH3 domain-containing protein B (UBASH3B) | 3 | 2.59 | Encodes a protein that contains a ubiquitin associated domain at the N-terminus, an SH3 domain, and a C-terminal domain with similarities to the catalytic motif of phosphoglycerate mutase. Deubiquitinating enzyme which may play an important role during spermatogenesis. Specifically deubiquititates histone H2A. |
| ENSGALT0000005338 | Ubiquitin specific peptidase 42 (USP42) | 0.2 | 0.35 | |
| ENSGALT0000025494 | Ubiquitin specific peptidase 16 (USP16) | 0.335 | 0.39 | |
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2012.07.008.

Abbreviations

RJF red jungle fowl
CH Chahua chicken
AA Avian broiler
DEGs differentially expressed genes
RPKM reads per kb per million
GO gene ontology
KEGG Kyoto Encyclopedia of Genes and Genomes
FDR false discovery rate
IGFBP-5 insulin-like growth factor-binding protein-5
TGFBR3 transforming growth factor-beta receptor III
FSTL1 follistatin-like 1
FNDC3B fibronectin type III domain containing 3B
FADS1 fatty acid desaturase 1
RNA-seq RNA-sequencing
IGF insulin-like growth factor

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