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Article

Comparative population genomics reveals genetic basis underlying body size of domestic chickens

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Body size is the most important economic trait for animal production and breeding. Several hundreds of loci have been reported to be associated with growth trait and body weight in chickens. The loci are mapped to large genomic regions due to the low density and limited number of genetic markers in previous studies. Herein, we employed comparative population genomics to identify genetic basis underlying the small body size of Yuanbao chicken (a famous ornamental chicken) based on 89 whole genomes. The most significant signal was mapped to the *BMP10* gene, whose expression was upregulated in the Yuanbao chicken. Overexpression of *BMP10* induced a significant decrease in body length by inhibiting angiogenic vessel development in zebrafish. In addition, three other loci on chromosomes 1, 2, and 24 were also identified to be potentially involved in the development of body size. Our results provide a paradigm shift in identification of novel loci controlling body size variation, availing a fast and efficient strategy. These loci, particularly *BMP10*, add insights into ongoing research of the evolution of body size under artificial selection and have important implications for future chicken breeding.

Keywords: body size, artificial selection, comparative population genomics, domestication

Introduction

Domestic animals are excellent biological models widely used in developmental biology, phenotypic evolution, and medical research studies. They have been developed as different breeds exhibiting remarkable differences in morphology, physiology, behavior, and adaptations (Darwin, 1868; Roots, 2007; Sutter et al., 2007; Menheniott et al., 2013; Gou et al., 2014; Yoon et al., 2014; Wang et al., 2015a, 2016). As an economic character, body size of domestic animals is extremely important for humans and the development of human civilization. An amazing amount of body size variation is seen within domestic animals, which is much higher than that seen in their wild ancestors (Roots, 2007). In addition to breeders, both evolutionary and developmental biologists are interested in discovering and characterizing the mechanisms that underlie the genetic control of variation in body size of domestic animals (Sutter et al., 2007; Makvandi-Nejad et al., 2012; Gou et al., 2014).

Domestic chickens are the most phenotypically variable bird (Darwin, 1868). For instance, bantam and cochin are amazing chicken breeds with adult body sizes at ~0.5 and ~5 kg, respectively, on the two extremes. As the farm animal with the widest distribution globally and raised in the largest number, domestic chickens have also been used in genetic and medical studies (Lawler, 2014). Genetic variants of specific traits, especially for body size, have been characterized, as they have major implications in both research and breeding (Sutter et al., 2007; Makvandi-Nejad et al., 2012; Gou et al., 2014). Several hundreds of quantitative trait loci (QTL) have been mapped and reported to be associated with growth and body weight of chickens (http://www.animalgenome.org/cgi-bin/QTLdb/GG/index).

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Despite these achievements, most of these QTLs are mapped to large genomic regions due to the low resolution of low-density loci and limited number of microsatellite and SNP panel markers. Thus, only a limited number of causative loci have been identified. For example, some genes, including *IGF1*, *TBC1D1*, *FOXO1A*, *KPNA3*, *INTS6*, and *HNF4G*, have been associated with growth and body weight in chickens (Rubin et al., 2010; Gu et al., 2011; Elferink et al., 2012; Xie et al., 2012; Wang et al., 2015b). These studies were mostly based on commercial chickens with very limited variations, and most of the variants controlling body size could have likely been missed. Genome-wide association studies (GWAS) hold a promise for elucidating the quantitative genetic basis of this complex trait (Gu et al., 2011; Elferink et al., 2012; Xie et al., 2012; Wang et al., 2015b), although the difficulty of the methods and the high expense of collecting phenotypic data hamper its wide application. In addition, the great phenotypic diversity among the diverse breeds and their complicated demographic histories (Miao et al., 2013) have also impeded the study for genetic mechanisms underlying the variation of body size in chickens. Fortunately, next-generation genome sequencing data supplemented by comparative population genomics have revolutionized the fields of quantitative genetics and evolution, and thus have proved to be a powerful tool for interpreting the genetic underpinnings of complex traits in domestic animals, e.g. the head crest in the rock pigeon (Shapiro et al., 2013), cold adaptation of high latitude Chinese pigs (Ai et al., 2015), and adaptation to starch-rich foods by dogs (Axelsson et al., 2013).

Yuanbao chicken, a famous Chinese ornamental chicken breed, is known for its miniature body size, with adult male weight ~800 g and adult female ~500 g. It has a long breeding history that can be traced back to the Tang dynasty (Supplementary Figure S1). Both the small body size that makes it easily handled in the palm and the appearance similar to 'Yuanbao', a metallic ingot used in ancient China as money, made Yuanbao chicken be treated as a symbol of wealth at-hand in ancient times (Supplementary Figure S1). To date, Yuanbao chicken is indisputably one of the most esteemed chicken breeds in China. Here, we employed comparative population genomics to study the genetic basis underlying the small body size of Yuanbao chicken. We identified four novel loci that potentially control the variation in body size of domestic chickens.

Results

Analyses of 89 chicken genomes identify >20 million SNPs

In this study, 89 genomes were obtained representing 7 Red Junglefowls, 24 Yuanbao chickens (Supplementary Figure S1), and 58 other domestic chickens, with ~12.2× sequence coverage for each individual (Supplementary Figure S2 and Table S1). Comparisons among the genome sequences identified a total of 21286312 SNPs, with 51.8% of them mapping to intergenic regions, 42.6% to intronic regions, and only a small proportion (1.5%) mapping to exonic regions of the genome (Supplementary Table S2). Functional annotation of the SNPs assigned to protein-coding regions identified 101999 SNPs that produce non-synonymous amino acid substitutions and 226713 SNPs that were synonymous, with 704 genes having SNPs that cause gain or loss of a stop codon (Supplementary Table S3). Further comparisons indicated that 88% and 90% of the SNPs used in the 60 K Illumina BeadChip genotyping array and the 600 K Affymetrix® Axiom® HD genotyping array, respectively, were contained in our new dataset. Our dataset of SNPs is much larger than those available in the chicken SNP database: 14353694 and 8670333 of our SNPs were not reported in BUILD 138 and BUILD 145 of the chicken dbSNP databases (ftp://ftp.ncbi.nih.gov/snp/organisms/chicken_9031), respectively. These novel SNPs potentially supplement the catalog of chicken variants.

Compared with other birds, Yuanbao chicken showed a lower level of nucleotide diversity (mean value: 4.56E-03) (Supplementary Figure S3). A phylogenetic tree of all individuals was constructed using weighted neighbour-joining method (Bruno et al., 2000), which revealed that Yuanbao chicken formed a relatively homogeneous ancestral cluster (Figure 1A). Principle component analysis (PCA) (Figure 1B), admixture (Supplementary Figure S4), and haplotype-based structure analyses (Supplementary Figure S5) indicated that several Yuanbao chickens had mixed ancestry with other chicken breeds.

Comparative population genomics as a strategy to identify loci controlling body size variation in chickens

Comparative analysis of population variants is a powerful tool that has enabled successful investigation into genetic mechanisms underlying complex traits (Axelsson et al., 2013; Kamberov et al., 2013; Shapiro et al., 2013; Ai et al., 2015; Lamichhaney et al., 2015). Since Yuanbao chicken has a remarkably smaller body size compared to the average body size of chickens, comparative genome analysis of Yuanbao and other chickens would be an effective strategy to identify the genetic basis underlying the variation in body size among chickens. Here, we employed F_{ST} and LSBL (Shriver et al., 2004) to evaluate the population differentiation of Yuanbao chicken from other chickens (Figure 1C and D). First, a sliding window analysis was performed, with 50 kb window size and 25 kb step size, identifying 268 and 275 genes from the empirical data with F_{ST} and LSBL, respectively, as candidates based on the outlier approach (99th percentile cutoff). Functional enrichment analysis of these candidate genes did not reveal any pathway specifically associated with the development of body size (Supplementary Tables S4 and S5). By combining the signals of F_{ST} and LSBL, we identified four regions of the genome (chr22:0.25Mb–0.33Mb, chr1:147.55Mb–147.82Mb, chr2:57.05Mb–57.22Mb, and chr24:6.1Mb–6.3Mb) that exhibited extreme population differentiation, likely as the result of artificial selection.

Analysis of chr22:0.25Mb–0.33Mb shows BMP10 potentially controlling the body size of Yuanbao chicken

The genomic region chr22:0.25Mb–0.33Mb stands out as the most extremely candidate selective sweep with the highest level of population differentiation (Figure 1C). There are three genes *GKN1*, *GKN2*, and *BMP10* located in this region (Figure 2A).

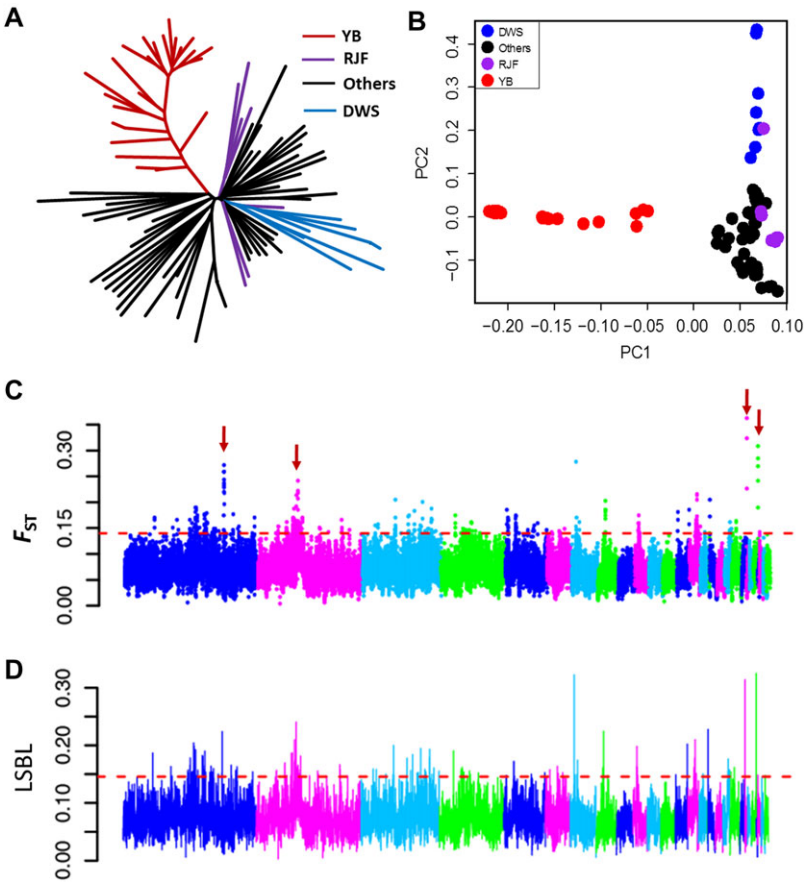

Figure 1 Phylogenetic and positive selection analyses. (**A**) Neighbour-joining tree of 89 chicken genomes. (**B**) PCA. (**C** and **D**) Genomic landscape of population differentiation by F_{ST} (**C**) and LSBL (**D**). Four significant clusters are marked and presented. YB, Yuanbao chicken; RJF, Red Junglefowl; DWS, Daweishan chicken.

GKN1 and *GKN2* are paralogues abundantly and uniquely expressed in the stomach (Menheniott et al., 2013). Both genes have documented functional importance in maintaining integrity and normal function of gastric mucosa, and their anomaly is associated with gastric cancer (Kim et al., 2014; Yoon et al., 2014). In Yuanbao and other chickens, both *GKN1* and *GKN2* exhibited no or extremely low expression levels in the heat, kidney, spleen, muscle, liver, and lung (Supplementary Figure S6).

BMP10, a member of the transforming growth factor β (TGFβ) family, showed consistently higher values in F_{ST} and LSBL analyses and high differences in allele frequencies (Figure 2A–C). A haplotype comparison analysis revealed a consistent differentiation of *BMP10* for Yuanbao chicken from other chickens (Figure 2D). Until now, a role of *BMP10* in body size has not been reported in chickens. In addition, no QTL associated with body size in chickens was mapped to this genomic region.

Further investigation showed that *BMP10* had a high expression in the heart (Supplementary Figure S7), and the expression level was significantly upregulated in Yuanbao chicken compared to other chickens (Figure 3A). Phylogenetic network analysis based on the Sanger resequencing verified data also supported the conclusion that the promoter region of *BMP10* has become highly differentiated in Yuanbao chicken compared to other chickens (Figure 3B). To examine promoter activity, we performed luciferase reporter gene assays with *BMP10* promoter sequence from Yuanbao chicken and the reference sequence. Consistently, we observed an increased reporter activity driven by the *BMP10* promoter of Yuanbao chicken compared to the reference promoter (Figure 3C and D). Next, based on our phylogenetic network, we identified 21 SNPs that were located upstream of *BMP10* and showed high differentiation between Yuanbao and other chickens. These SNPs were further examined to investigate differences in DNA–protein interactions by electrophoretic mobility shift assays (EMSA) with nuclear extracts from chicken hearts. Probes for five of these SNPs showed differences in gel shift between Yuanbao and other chickens (Figure 3E and Supplementary Figure S8). Mutations in Yuanbao chicken at chr22:274606(T→C) and chr22:274758 (C→deletion) led to decrease or loss of interactions between DNA and protein. On the other hand, mutations in Yuanbao chicken at chr22:274670(A→G), chr22:276118(A→G), and chr22:276140 (C→T) increased the DNA–protein interaction. From these observations, we inferred that the differences in protein binding, due to the underlying SNPs, likely contribute to the upregulation of

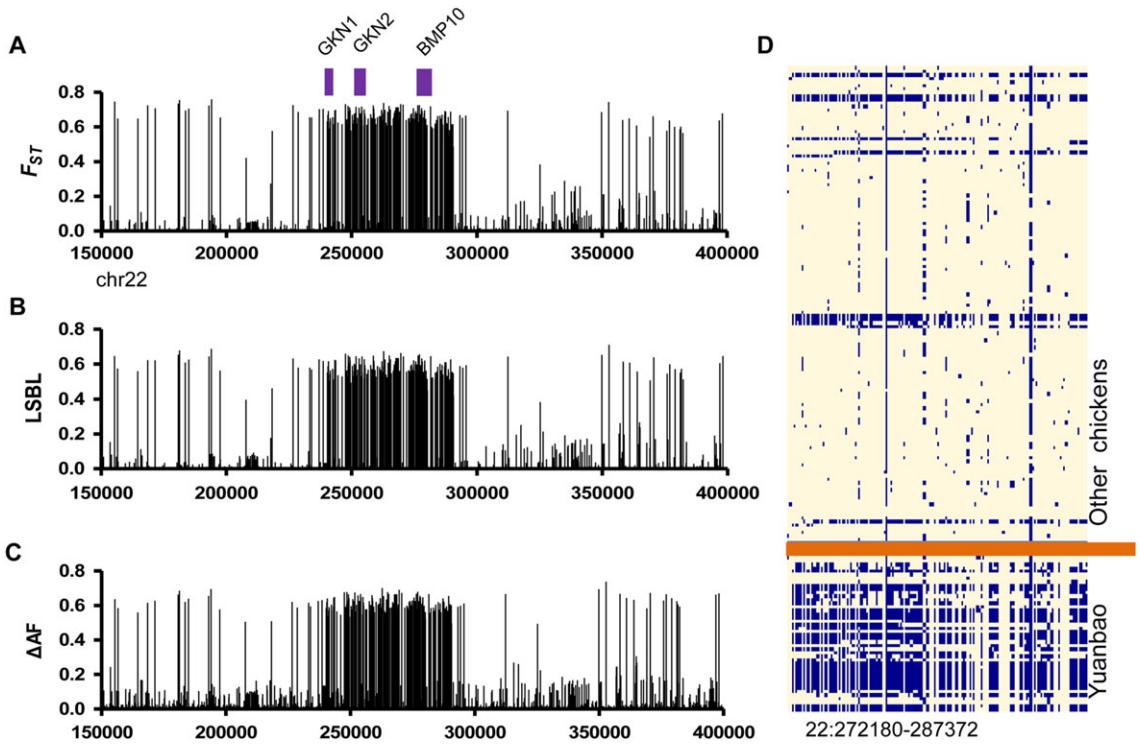

Figure 2 Population differentiation in chr22:0.25 Mb–0.33 Mb between Yuanbao and other chickens. (**A—C**) Landscape of F_{ST} (**A**), LSBL (**B**), and ΔAF (**C**). (**D**) Haplotype comparison between Yuanbao and other chickens. Alternative alleles are labelled in blue.

BMP10 expression in the heart of Yuanbao chicken (Figure 3A). We further genotyped one SNP within this strong linkage region, which showed a significant association with body weight (Figure 3F, P = 3.249E-18). The SNP could explain 22.41% of the overall weight variance in five chicken lines, i.e. Jiningbairi chicken, Luhua chicken, bantam, Yuanbao chicken, and ornamental chicken.

BMP10 overexpression inhibits angiogenesis and induces short body length in zebrafish

Overexpression of *BMP10* induced a decrease in body weight in mice (Chen et al., 2006). To examine whether it is a conserved function of *BMP10* to control body size in vertebrates, we performed an overexpression assay of wild-type *BMP10* in zebrafish. Fertilized one-cell zebrafish embryos from the *fli1a-EGFP* transgenic line were injected with 200 pg wild-type zebrafish *BMP10* mRNA per embryo respectively. Overexpression of *BMP10* in zebrafish resulted in a decreased body length and a curved body axis compared to uninjected control embryos (Figure 4 and Supplementary Figure S9). These results affirm that *BMP10* has an important role in determining body size. Angiogenesis is a normal and vital process in growth and development (Folkman and Shing, 1992). To study the phenotypic consequence of *BMP10* overexpression on angiogenesis in zebrafish, we anesthetized transgenic embryos with 0.016% tricaine methanesulfonate (MS-222) and counted the number of complete intersegmental vessels (ISVs) at 32 h post-fertilization (hpf). Zebrafish with overexpression of *BMP10* showed a larger number of incomplete ISVs and only occasional sprouts of dorsal aorta (DA) compared with the control zebrafish (Figure 5 and Supplementary Figure S10). These results indicate that overexpression of *BMP10* inhibits angiogenic vessel growth in zebrafish, which would inhibit growth and result in a shorter body. All these data suggest that upregulation of *BMP10* likely contributes to a smaller body size of Yuanbao chicken.

Genes at chr1:147.55Mb–147.82Mb, chr2:57.05Mb–57.22Mb, and chr24:6.17Mb–6.25Mb are potentially involved in the development of body size

Chr1:147.55Mb–147.82Mb, harbouring a cluster of selective sweep SNPs, demonstrates significantly higher levels of population differentiation as revealed by F_{ST} (Figure 1C) and LSBL (Figure 1D). No annotated gene is located in this region. However, this region is located within a previously reported QTL associated with 'Body weight' and 'Growth' (Carlborg et al., 2003; Gu et al., 2011). The gene *GPC5,* which plays a role in the control of cell division and growth regulation (Yang et al., 2013), is found adjacent to this mapped location. RNA-seq analysis showed that expression of *GPC5* was downregulated in the heart of Yuanbao chicken (Supplementary Figure S11A, $P < 0.05$).

Similar to that in chr1:147.55Mb–147.82Mb, no protein-coding gene is located in the chr2:57.05Mb–57.22Mb genomic region, although it also displayed strong signals of selection with high levels of population differentiation as revealed by F_{ST} (Figure 1C) and LSBL (Figure 1D). QTL mapping has shown that this region is strongly associated with 'Body weight' and

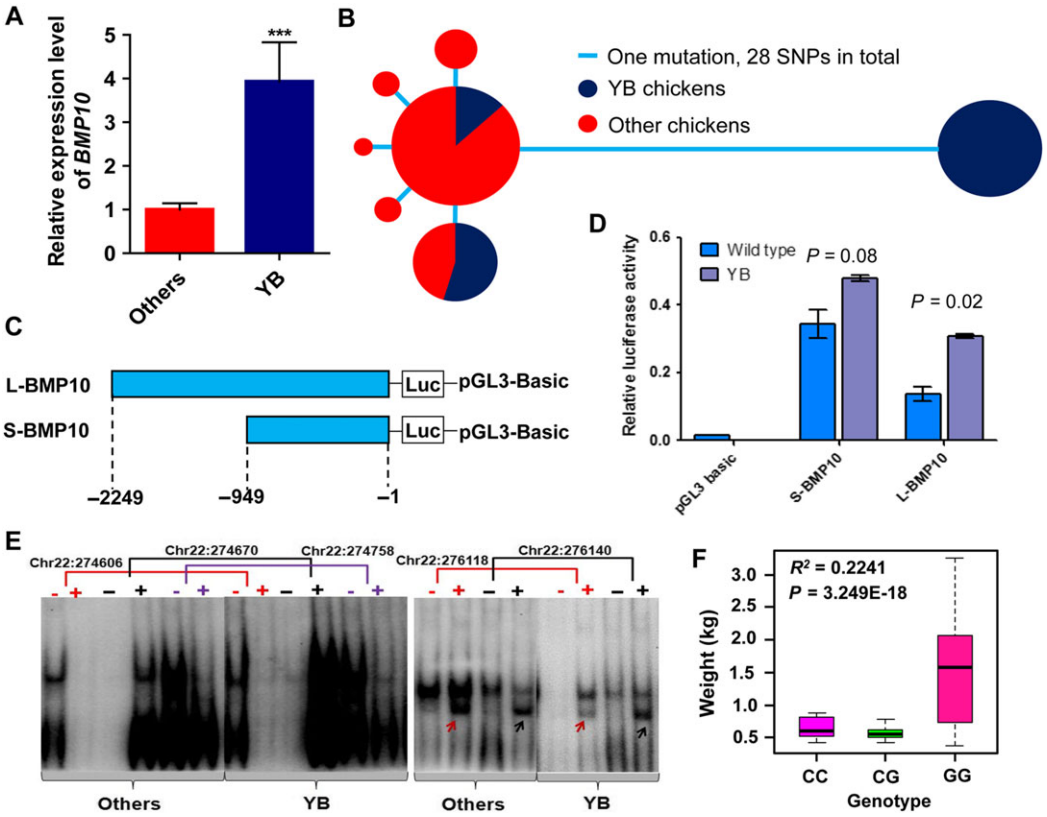

Figure 3 Expression and association analysis of the gene *BMP10*. (**A**) Comparison of the expression of *BMP10* in heart tissue between YB and other chickens. (**B**) Median joining network of the *BMP10* promoter haplotypes. (**C**) Schematic structure of luciferase reporter plasmids with different lengths. (**D**) Comparison of luciferase activity of the promoters between YB and other chickens. (**E**) Five SNPs present differential mobility by EMSA. '+' and '−' refer to probes with the mutated YB allele and reference allele, respectively. (**F**) Association analysis of *BMP10* and weight. G is the reference allele. Statistical significance is measured by Student's *t*-test. Error bar represents SEM. YB, Yuanbao chicken.

'Growth rate' in chickens (Carlborg et al., 2003; Siwek et al., 2004; Tercic et al., 2009). Chr2:57.05Mb–57.22Mb is upstream of the gene spalt-like transcription factor 3 (*SALL3*), a transcription factor that plays a fundamental role in animal development (de Celis and Barrio, 2008). We observed an upregulated expression of *SALL3* in kidneys of Yuanbao chicken compared to village domestic chicken (P = 0.0208) and Red Junglefowl (P = 0.24795) (Supplementary Figure S11B).

The forth mapped region chr24:6.17Mb–6.25Mb contains 13 protein-coding genes (Supplementary Table S6). There is no QTL associated with body weight or growth rate in this region. These 13 genes are involved in various biological processes (Supplementary Table S6). For example, *BCO2* is associated with skin colour in chickens (Eriksson et al., 2008), while *DIXDC1* and *CRYAB* are involved in nervous system (Ousman et al., 2007; Kivimae et al., 2011). *HSPB2*, a heat shock protein (HSPs) gene, is highly expressed in the heart and skeletal muscle, which is essential in maintaining muscle cell integrity in some mouse skeletal muscles. Knockdown of *HSPB2* results in degeneration of skeletal muscle in mice (Brady et al., 2001). In our study, the expression of *HSPB2* was downregulated in the muscle of Yuanbao chicken compared to village domestic chicken (P = 0.0682) and Red Junglefowl (P = 0.06205) (Supplementary Figure S12).

Discussion

Chickens are of integral dietary importance (e.g. egg and meat) in many communities globally. They are also raised for other purposes like biological research and entertainment. For the source of food and economic income, breeders have made great effort to develop chickens with a large body size and rapid growth rate for meat production (Lawler, 2014). Our study reports four loci that potentially control body size, providing important information and candidate genetic markers for chicken breeding. Our study also provides a strategy with comparative population genomics to identify candidate genes/variants accounting for the variation in the body size of chickens. This strategy is much more cost-effective and timesaving than previous methods such as QTL mapping and GWAS analysis.

A great variation in body size has been observed in several domesticated animals, including dogs, pigs, and chickens (Roots, 2007). Body size is not only an important commercial trait for food production, but also a key topic for evolutionary and developmental biology studies (Sutter et al., 2007;

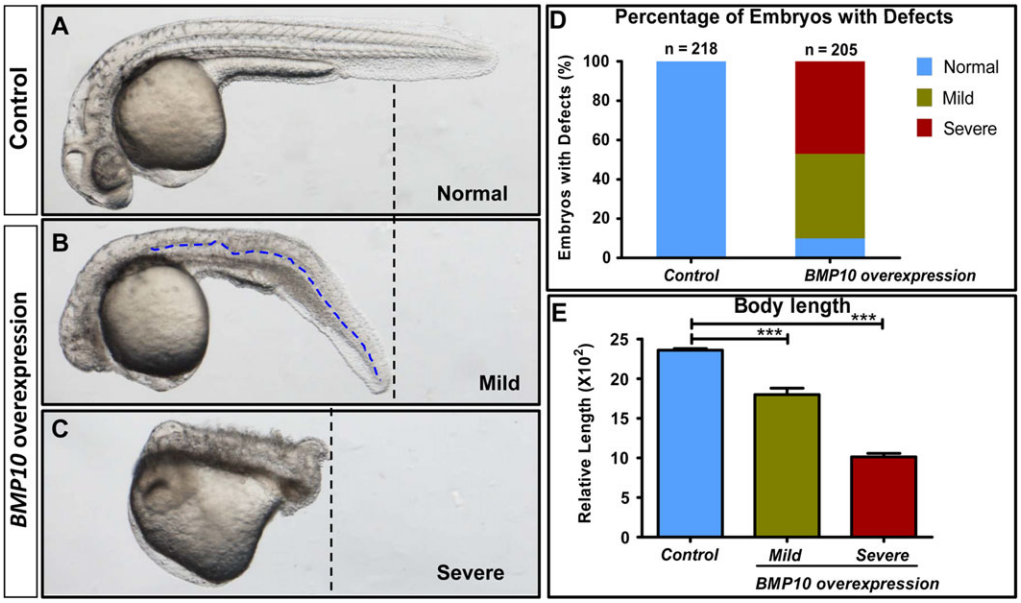

Figure 4 Overexpression of *BMP10* induces developmental defects in Zebrafish. (**A**–**C**) Gross morphology at 32 hpf. Compared with un-injected wild-type control embryos, embryos with wild-type zebrafish *BMP10* overexpression exhibited a decreased body length (indicated by the black dotted line) and a curved body axis (indicated by the blue dotted line). See Supplementary Figure S9 for more samples. (**D** and **E**) Bar graphs show that the *BMP10* overexpression caused higher percentage of embryos with developmental defects and shorter body length.

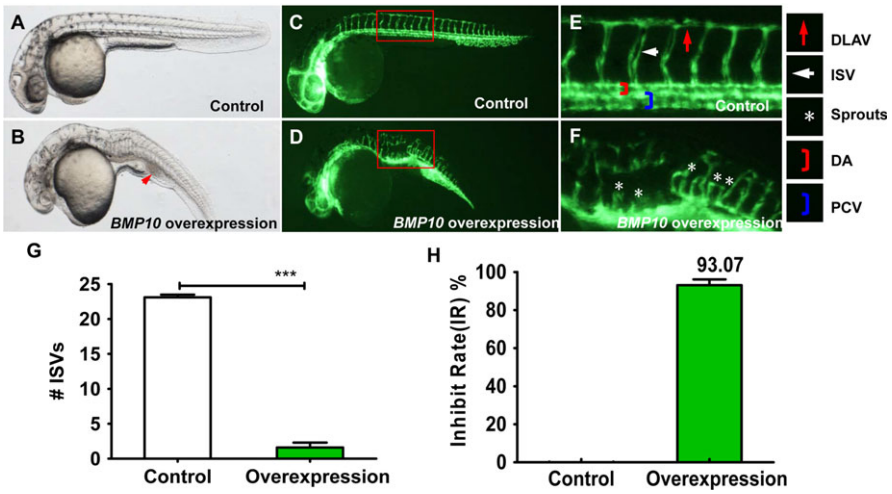

Figure 5 Overexpression of *BMP10* inhibits angiogenesis in Zebrafish. (**A**–**F**) Representative bright field and fluorescent images of zebrafish embryos at 32 hpf. Red arrow indicates haemorrhage in the tail (**B**). (**C**–**H**) Compared to wild-type control, the fish injected with wild-type zebrafish *BMP10* mRNA (200 pg) less incomplete ISVs and occasional sprouts (marked with asterisk) of the dorsal aorta. The regions boxed in red are shown in higher magnification to the right. DLAV, dorsal longitudinal anastomotic vessels; ISV, intersegmental vessels; DA, dorsal aorta; PCV, posterior cardinal vein.

Makvandi-Nejad et al., 2012; Rubin et al., 2012; Rimbault et al., 2013; Qanbari et al., 2014). The investigation in genes/genetic variants controlling variation in body size has attracted attentions from animal breeders, evolutionary and developmental biologists, and even medical scientists (Sutter et al., 2007; Makvandi-Nejad et al., 2012; Gou et al., 2014).

Body size, typical of many complex traits, is commonly believed to be influenced by many genes involved in similar functional pathways (Devlin et al., 2009). For example, in a study of humans, 97 loci associated with body mass index (BMI) were identified, yet these loci only account for ~2.7% of BMI variation (Locke et al., 2015). In stark contrast, very few genes have been reported to be involved in the evolution of body size in domestic animals. For example, derived variants at six genes explain nearly half of the size reduction seen in some dog breeds (Rimbault et al., 2013). In horses, a similar pattern

was reported, where four loci explained 83% of the variation in body size (Makvandi-Nejad et al., 2012). Here, we also found that *BMP10* could explain 22.41% of body size variation in five chicken lines, including Jiningbairi chicken, Luhua chicken, bantam, Yuanbao chicken, and ornamental chicken. These contrasting patterns between humans and domestic animals are likely explained by the differences in natural vs. artificial selection.

In assessing the genetic basis underlying the small body size of Yuanbao chicken, we identified several genomic regions associated with morphogenic genes. A non-genic region was identified on chromosome 1 (chr1:147.55Mb–147.82Mb). This sequence is upstream of the protein-coding gene *GPC5* and showed evidence of positive selection, whose expression was downregulated in the heart of Yuanbao chicken. The human *GPC5* locus has been reported to be associated with height (Lango Allen et al., 2015). A GWAS based on the Illumina 60 K Chicken SNP Beadchip also found evidence for a potential association of *GPC5* with body weight in domestic chickens (Sewalem et al., 2002; Carlborg et al., 2004; Gu et al., 2011). Similarly, no protein-coding gene was found in the selected region of chromosome 2 (chr2:57.05Mb–57.22Mb), which is located upstream of another gene involved in development, *SALL3* (Parrish et al., 2004; Kojima et al., 2013). The expression of *SALL3* was upregulated in kidneys of Yuanbao chicken. Whether *SALL3* has a function in controlling body size is unclear. But SALL3 protein directly binds to DNA methyltransferase 3 alpha (DNMT3A) and reduces DNMT3A-mediated CpG island methylation (Shikauchi et al., 2009). *DNMT3A* is necessary for the control of body weight and energy homoeostasis (Kohno et al., 2014), and is associated with height in humans (Gudbjartsson et al., 2008). In addition, expression of *SALL3* can be induced by *BMP4* (Shikauchi et al., 2009), a bone morphogenetic protein (BMP) that plays an important role in the development of the skeletal system. Mouse mutants with double-null Sall1/Sall3 exhibited malformation in limb morphogenesis (Kawakami et al., 2009). In a selective sweep region in chromosome 24 (chr24:6.17Mb–6.25Mb), there were 13 protein-coding genes with diverse biological functions. *HSPB2*, a gene essential for skeletal muscle development, was downregulated in Yuanbao chicken, likely showing an association with the unique growth properties of Yuanbao chicken. Three protein-coding genes *GKN1*, *GKN2*, and *BMP10* were found in the selected region of chromosome 22 (chr22:0.25Mb–0.33Mb). Both *GKN1* and *GKN2* are highly and uniquely expressed in the stomach, with important roles in maintaining its normal function. *BMP10* is specifically expressed in the heart and plays a crucial role in regulating the development of the heart in mice (Neuhaus et al., 1999). *BMP10*-deficient mice survived through E10.0–E10.5 and then died due to severely impaired cardiac development and function (Chen et al., 2004). Overexpression of *BMP10* in mice leads to myocardial overgrowth and hypertrabeculation in embryos (Pashmforoush et al., 2004). Transgenic mice with postnatal overexpression of *BMP10* in the myocardium display a 50% deduction in the heart size and a reduction in body weight and size at the age of 1 month (Chen et al., 2006). *BMP10* is also reported to have a role in inducing apoptosis, proliferation, and growth of cells (Kawakami et al., 2009; Kim et al., 2014). For example, *BMP10* expression was observed to be decreased or absent in prostate tumours, and forced *BMP10* overexpression decreased *in vitro* growth, cell matrix adhesion, invasion, and migration of prostate cancer cells (Kawakami et al., 2009). Further examination showed that *BMP10* expression was upregulated in the heart of Yuanbao chicken, probably as a result of five potential mutations upstream of *BMP10* that likely increased the promoter activity. Similar to that in mice (Chen et al., 2006), an overexpression of *BMP10* induced a decrease in body length in zebrafish, implying a conserved function of *BMP10* in controlling body size in vertebrates. The four loci identified in our study, with high population differentiation between Yuanbao and other chickens, potentially influence gene expression rather than protein-coding sequence. This potentially supports a model that changes of gene expression contribute significantly to the evolution of body size, a view consistent with the hypothesis that changes in gene expression are particularly important in morphological evolution (Carroll, 2008; Young et al., 2015).

There are still some limitations in our study. First, we supposed that five mutations could have likely changed the promoter activity of *BMP10* leading to higher expression of *BMP10* in Yuanbao chicken. Two mutations chr22:274606(T→C) and chr22:274758(C→deletion) lead to decrease or loss of DNA–protein interactions, while the other three mutations chr22:274670(A→G), chr22:276118(A→G), and chr22:276140 (C→T) increased DNA–protein interactions. Our study could not single out which proteins were involved in these interactions, neither whether all or only some of these sites work together to activate the promoter of *BMP10*. In addition, some miniature domestic chicken breeds have similar body size as Yuanbao chicken, but we only include Yuanbao chicken as a small body-sized chicken line in our study. Furthermore, the chromosomal region containing *BMP10* gene in Daweishan chicken was in the same wild-type state as in Red Junglefowl and other domestic chicken lines. Hence, we cannot definitely conclude that *BMP10* has a common consequence in other domestic chicken lines, especially due to the complex origin and demographic history of domestic chickens (Miao et al., 2013). Broader sampling to include more chicken breeds with small body size and additional work will help address these issues in the future.

Materials and methods

Animal experimental ethics

All animal experimental procedures were performed according to the guidelines approved by the Ethics Committee of Kunming Institute of Zoology.

Sampling and genomic data collection

Up to 42 genomes, including 24 Yuanbao chickens (YB), 8 Daweishan chickens (DWS, a semi-domestic and miniature breed), 1 Red Junglefowl (RJF), 6 Guangzhou local chickens, and

3 Yunnan local chickens were sequenced in this study (Supplementary Table S1). DNA was extracted using the phenol-chloroform extraction method and the quality was measured by electrophoresis and on a NanoDrop spectrophotometer 2000. Only high-quality DNA was used for the construction of genome sequencing libraries according to the Illumina standard genome library preparation pipeline. Sequencing was performed on an Illumina Hiseq 2000 platform with a read length of 101 bp. Genomes for 33 chickens from our previous study (Wang et al., 2015a), 2 chickens from the study by Fan et al. (2013), and 12 chickens from the study by Yi et al. (2014) were integrated into our study (Supplementary Table S1). Overall, 89 genomes for 7 Red Junglefowls and 82 domestic chickens were obtained.

Genomic sequence alignment, SNP calling and annotation

Raw sequence reads were filtered by removing adaptors and low-quality bases using cutadaptor and Btrim software (Kong, 2011). Qualified reads were aligned onto the chicken reference genome (Galgal4) using BWA-MEM with default settings except the '-t 8 -M' options (https://github.com/lh3/bwa). A series of post-processes were then employed to process the alignment BAM format file, including sorting, duplicates marking, local realignment, and base quality recalibration, which were carried out using the SortSam and MarkDuplicates functions in the Picards (picard-tools-1.56, http://picard.sourceforge.net) package, and RealignerTargetCreator, IndelRealigner, and BaseRecalibrator tools in the Genome Analysis Toolkit (GenomeAnalysisTK-2.6-4, GATK) (McKenna et al., 2010). SNPs and indels were called and filtered using UnifiedGenotyper and VariantFiltration command in GATK. Loci with RMS mapping quality <25 and genotype quality <40, for which reads with zero mapping quality constitute >10% of all reads at this site were removed. Loci with >2 alleles and within clusters (>3 SNPs in a 10-bp window) were removed. All SNPs were assigned to specific genomic regions and genes using ANNOVAR based on the ENSEMBL chicken annotations (Wang et al., 2010). Missing SNPs with <10% frequency were imputed, and haplotypes for each chromosome were deduced by BEAGLE (BEAGLE 3.3.2.) (Browning and Browning, 2007).

Population variation and population genetic analyses

Genome-wide genetic diversity (π) was calculated for Yuanbao chicken, Red Junglefowl, Daweishan chicken, and other chicken groups using VCFtools (Danecek et al., 2011) using a 50-kb sliding window with 25-kb stepwise increments. Several methods were applied to infer the population structure of Yuanbao chicken. First, we constructed a neighbour-joining tree using the software PHYLIP (Bruno et al., 2000) based on the pairwise distance matrix derived from the simple matching distance for all SNP sites. The tree was viewed using MEGA5 (Tamura et al., 2011). Second, to minimize the effects of SNPs contributed by regions of extensive strong linkage disequilibrium (LD), we pruned the SNPs according to the observed sample correlation coefficients using PLINK (Purcell et al., 2007) with the parameter '--indep 100 50 0.1', and PCA was performed using GCTA (Yang et al., 2011). Third, admixture analysis was performed to view the population structure by using ADMIXTURE (Alexander et al., 2009) with an ancestor population size ranging from 2 to 5, based on the pruned data. Fourth, we used a haplotype-based approach, ChromoPainter and fineSTRUCTURE, to infer population structure (Lawson et al., 2012).

Genome-wide selective sweep analysis

We employed three tests to investigate the genomic regions harbouring footprints of positive selection in Yuanbao chicken. F_{ST} values for each SNP were estimated between Yuanbao and other chickens as described elsewhere (Akey et al., 2002). LSBL statistics were calculated for each SNP based on the F_{ST} values between the three groups (Shriver et al., 2004). Here we defined Yuanbao chicken as group A, Red Junglefowl and Daweishan chicken as Group B since Daweishan chicken possesses features similar to Red Junglefowl (i.e. appearance, habits, and characters). Other domestic chicken lines were assigned group C. LSBL statistics for each variant was calculated using the formula: $\mathrm{LSBL} = (F_{ST(AB)} + F_{ST(AC)} - F_{ST(BC)})/2$. Sliding window analysis was performed for F_{ST} and LSBL in each 50-kb window with 25-kb stepwise increments. In addition, we computed the absolute allele frequency difference (ΔAF) per SNP between Yuanbao and other chickens to confirm the signal of positive selection (Carneiro et al., 2014). Deduced candidate selective sweeps detected by above methods were annotated using the Variant Effect Predictor available at http://asia.ensembl.org/info/docs/tools/index.html. Functional enrichments of protein-coding genes including Gene Ontology (GO) categories, KEGG pathway, and Human Phenotype Ontologies (HPO) were analyzed using g:Profiler (Reimand et al., 2011).

SNP verification and network construction

Sanger resequencing on an Appled Biosystems ABI 3730XL Genetic Analyzer, was used to verify SNPs in the region upstream and in the first exon of the *BMP10* gene. A total of 54 chickens, including Yuanbao chicken and local indigenous chickens, were used for confirmation. Primers used for amplification and sequencing are listed in Supplementary Table S7. Haplotypes were phased using PHASE program (Stephens and Donnelly, 2003). A median-joining network was constructed using Network (Bandelt et al., 1999).

Genotyping and association analysis

To further define how variation at *BMP10* contributes to the body size, we genotyped one SNP (chr22:276457, G/C) in overall 301 chickens from five chicken lines (Jiningbairi chicken, Luhua chicken, bantam, Yuanbao chicken, and ornamental chicken) with available body weight information using Sanger resequencing method. The proportion of weight variation explained was estimated using PLINK with the linear model (Purcell et al., 2007).

RNA extraction and real-time quantitative PCR assay

Total RNA was isolated from heart, liver, spleen, lung, muscle, kidney, and brain tissues of adult chickens using TRNzol-A+ Reagent

(TIANGEN) and purified using RNeasy Micro Kit (QIAGEN). The concentration and integrity of the RNA was measured using electrophoresis and NanoDrop spectrophotometer 2000. Total RNA (~2 μg) was used to synthesize single-strand cDNA using the PrimeScript RT-PCR Kit in a final volume of 25 μl according to the manufacturer's instructions. Relative mRNA expression levels of *BMP10* in the chicken heart were measured using real-time quantitative PCR (qPCR) with the relative standard curve method and normalization to the housekeeping gene *GAPDH*. Primer pairs used for *BMP10* are listed in Supplementary Table S8. qPCR was performed on the iQ2 system platform (BioRad Laboratories, Hercules) with SYBR® Premix Ex Taq™ II Kit. Student's *t*-test was used to measure the statistical significance.

RNA-seq analysis

For RNA-seq analysis, we included 47 transcriptomes from lung, heart, muscle, spleen, liver, and kidney tissues of Yuanbao chicken, village domestic chicken, and Red Junglefowl, which were generated using Hiseq platform in one of our projects (see Supplementary Table S9). Firstly, poor-quality reads were filtered out using Trimmomatic (Bolger et al., 2014), with parameters set to 'LEADING:5 TRAILING:5 SLIDINGWINDOW:4:10 MINLEN:50'. Secondly, clean reads were aligned onto chicken reference genome (Galgal4) using HISAT2 (Kim et al., 2015) with parameters set to '--sp 1000,1000 --k 20 --no-unal --dta --dta-cufflinks --no-discordant' StringTie (Pertea et al., 2015). Cuffcompare (Trapnell et al., 2012) were then used to assemble new transcripts and compare the assembled transcripts with the annotated reference transcripts to generate a new-merged GTF annotation file. Cuffdiff (Trapnell et al., 2012) was used to measure the significance of the gene expression difference for each tissue between Red Junglefowl and Yuanbao chicken, as well as between village domestic chicken and Yuanbao chicken. *P*-value was calculated based on the Poisson fragment dispersion model (default by cuffdiff program) (Trapnell et al., 2012).

Luciferase reporter analysis

To infer whether the highly differentiated SNPs in the upstream of *BMP10* in Yuanbao chicken increased the *BMP10* promoter activity, we performed a luciferase reporter assay. Two upstream fragments of the *BMP10* gene, a long 2449-bp fragment (L-BMP10, chr22:274094–276542) and a short 949-bp fragment (S-BMP10, chr22:275594–276542), respectively, were generated by PCR and cloned into the pGL3 Basic vector (Promega). *Kpn*I and *Xho*I enzyme sites were used to construct the vectors. Human embryonic kidney 293T cells (HEK 293T) plated in 24 wells were transfected at 60%–70% confluency with the pGL3 reporter plasmids and 80 ng of pRL-TK Renilla luciferase construct in each well using Lipofectamine™ 2000 (Invitrogen). Luciferase activity was measured at 24 h after transfection using the GloMax® 96 Microplate Luminometer (Promega). Ratios of Firefly luminescence/Renilla luminescence were calculated with the Basic vector as the reference. Three technical replicates were performed. Student's *t*-test was used to measure the statistical significance between Yuanbao and other chickens.

Electrophoretic mobility shift assays

A total of 21 SNPs within 2249-bp upstream region from start site of the *BMP10* gene, which were also verified by Sanger sequencing, were selected for a functional assay using EMSA to reveal potential differences in DNA–protein interactions. A total of 20 pairs of 5′Biotin-labelled probes (mutations at chr22:276330 (T→C) and chr22:276331(G→A) shared one probe) were synthesized (Integrated DNA Technologies). Heart tissues from 4 chickens (including one Yuanbao chicken and three Chinese local domestic chickens) were collected after the chickens were sacrificed and stored at −70°C until further analysis. Nuclear extracts were prepared from the heart tissue using NucBuster Protein Extraction Kit (Viagene Biotech). The nuclear extracts (2.1 μg) were added to the binding reaction and then preincubated for 20 min on ice. For the competition reactions, 20 pmol of unlabelled double-strand oligos were added to the reactions. After preincubation, 20 fmol of the biotinylated oligos were added to the reactions and incubated for 20 min at room temperature. DNA–protein complexes were separated by electrophoresis on 6.5% non-denaturing polyacrylamide gel at 120 V for 90 min in 0.5× TBE running buffer. Separated complexes were transferred to binding-membrane (Viagene Biotech) at 390 mA for 40 min in cold 0.5× TBE. DNA–protein complexes were crosslinked using Stratalinker UV Crosslinker, and biotinylated probes were detected using Lighten® HRP-B Substrate Solution A and B (Viagene Biotech).

Zebrafish care and maintenance

Adult zebrafish were maintained at 28.5°C on a 14-h light/10-h dark cycle (Westerfield, 1993). Four to five pairs of zebrafish were set up for natural mating for every cross. On average, 100–200 embryos were generated. Embryos were maintained at 28.5°C in fish water (0.2% instant ocean salt in deionized water). Embryos were washed and staged according to Kimmel et al. (1995). The establishment and characterization of the *fli1a-EGFP* transgenic line has been described elsewhere (Lawson and Weinstein, 2002). The zebrafish facility at the Shanghai Biomodel Organism Science & Technology Development Co., Ltd is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

Zebrafish microinjection

For the overexpression assay, fertilized one-cell embryos were injected with 200 pg wild-type zebrafish *BMP10* mRNA per embryo.

Zebrafish angiogenesis studies

To evaluate blood vessel formation in zebrafish, fertilized one-cell *fli1a-EGFP* transgenic embryos were injected with 200 pg wild-type *BMP10* mRNA per embryo. At 32 hpf, embryos were anesthetized with 0.016% MS-222 (Sigma-Aldrich), and the number of complete ISVs, which connect the DA to the

DLAV, was counted. The anti-angiogenesis effect was determined using the following formula:

$$\%\ \text{inhibition} = \left(1 - \frac{\text{ISV amount of experiment group}}{\text{ISV amount of wild type control}}\right) \times 100 \quad \text{(a)}$$

Image acquisition

Embryos and larvae were examined with a Nikon SMZ 1500 Fluorescence microscope and subsequently photographed with digital cameras. A subset of images were adjusted for levels of brightness, contrast, hue, and saturation with Adobe Photoshop 7.0 software (Adobe) to optimally visualize the expression patterns. Quantitative image analyses were processed using image-based morphometric analysis (NIS-Elements D3.1). Ten animals were quantified for each treatment.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis and graphical representation of the data were performed using GraphPad Prism 5.0 (GraphPad Software). Statistical significance was performed using a Student's *t*-test, ANOVA, or χ^2 test as appropriate. Statistical significance is indicated by *, where $P < 0.05$, and ***, where $P < 0.0001$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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