Relationships of a Transforming Growth Factor-β2 Single Nucleotide Polymorphism and Messenger Ribonucleic Acid Abundance with Bone and Production Traits in Chickens

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ABSTRACT Osteoporosis is a serious problem for the laying hen industry with economic, production, and welfare consequences. Transforming growth factor-β2 (TGFβ2) has been implicated as an important factor in coupling bone resorption and formation in bone remodeling. The current study was designed to determine if TGFβ2 was associated with variation in bone mineralization in chickens, using 2 complementary experimental approaches. First, an intronic single nucleotide polymorphism (SNP) present in TGFβ2 was investigated in an F_2 population to determine its association with bone, growth, and egg traits of importance to the layer and broiler industries. The TGFβ2 SNP was significantly associated ($P < 0.05$) with bone mineral density and content. However, these associations became nonsignificant when BW was included as a covariate in analyses. The TGFβ2 SNP was also significantly associated ($P < 0.05$) with BW from 1 to 6 wk of age and egg production from 46 to 55 wk of age. To further explore the relationship between TGFβ2 and bone strength, bone marrow TGFβ2 mRNA abundance was compared between broiler and layer chickens at 15, 35, and 60 wk of age. Bone and egg traits were measured along with mRNA abundance at each age and found to differ significantly between lines. The TGFβ2 mRNA abundance was approximately 4-fold greater in broiler compared with layer hens at 15 wk of age but was similar between lines at later ages. Thus, even though the TGFβ2 SNP will likely not be an effective marker for improving bone strength independently of changes in BW, further research is warranted to investigate the relationship of TGFβ2 mRNA abundance to bone strength in laying hens.

Key words: chicken, gene expression, osteoporosis, single nucleotide polymorphism, transforming growth factor-β2

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INTRODUCTION

Osteoporosis is an important issue in the laying hen industry with significant economic and welfare consequences. The hallmarks of osteoporotic bones, including increased porosity, fragility, and fracture susceptibility, are caused by a decrease in structural bone content. The presumed pain experienced by laying hens due to bone fracture represents the main welfare issue for osteoporosis (Whitehead and Fleming, 2000). The economic losses associated with osteoporosis are related to decreased production (Cransberg et al., 2001) and loss of market opportunity for spent hens due to bone fragmentation during processing (Gregory and Wilkins, 1989).

Although nutrition, exercise, and handling are all important factors in bone health, improvements in these management considerations alone will not solve the osteoporosis problem in modern flocks (Rennie et al., 1997; Rath et al., 2000; Whitehead and Fleming, 2000; Webster, 2004; Whitehead, 2004). Selection in the layer industry for traits of economic importance, such as feed efficiency and productivity, has revealed an unfavorable correlated response in bone integrity and has played an important role in the establishment of osteoporosis as a problem in layers. Bishop et al. (2000) used selection in layers to demonstrate that bone traits are moderately to highly heritable and respond quickly to selection. Therefore, selection is a tool that could be used to affect long-term improvement in bone integrity.

A challenge in selecting birds for improved bone strength is identifying the superior animals for breeding purposes. One option is to use retrospective selection based on phenotypic measurements from end-of-lay hens (Bishop et al., 2000), but performing matings ahead of selection generates excess animals. A second option is to perform in vivo measurements of bone density, such as dual energy X-ray absorptiometry, in potential breeders. However, this option is poorly suited for large-scale breeding programs, because it is time-consuming and expensive (Hester et al., 2004). An alternative option is to utilize genetic markers to facilitate the identification

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of animals for breeding purposes in a MAS program. However, a limited number of candidate genes and QTL that influence bone integrity traits in chickens have been identified to date (Li et al., 2003; Schreiweis et al., 2005a; Zhou et al., 2005).

One candidate gene that has been associated with bone strength in chickens is transforming growth factor-β2 (**TGFβ2**). Transforming growth factor-β2 is an important cytokine in bone remodeling and has been implicated as a coupling factor between osteoclastic bone resorption and osteoblastic bone formation. In addition to a candidate gene analysis reported by Li et al. (2003), a genome-wide QTL scan conducted on the F_2 population used in the current study reported a suggestive QTL on chromosome 3 for 35-wk tibial bone mineral density (**BMD**) and tibia bone breaking force (Schreiweis et al., 2005a). The TGFβ2 gene is located near the peak of this QTL.

The first objective of the present study was to determine the effect of a single nucleotide polymorphism (**SNP**) in the TGFβ2 gene on bone trait phenotypes in an F_2 population of chickens generated from a broiler × layer cross. A second objective of the current study was to investigate the mRNA abundance of TGFβ2 in the bone marrow of layer and broiler hens that differ for traits related to bone mineralization and strength.

MATERIALS AND METHODS

Investigation of a TGFβ2 SNP

The TGFβ2 SNP published by Li et al. (2003) was evaluated but was not informative in the current resource population (unpublished data). Therefore, a novel SNP was identified and utilized. The TGFβ2 forward (5′-CTCGCTCTTTATGCTGGTGGTCC-3′) and reverse (5′-CCTCTGTGATGGAGCCATTCATGTA-3′) primers were designed from Ensembl (www.ensembl.org/Gallus_gallus/) sequence data for the TGFβ2 transcript ENSGALT00000015664 located on chromosome 3. Genomic DNA was amplified from layer and broiler grandparents of the resource population by PCR in 10-μL reactions including 25 to 50 ng of DNA, 0.25 μ*M* of each primer, 2.5× Eppendorf MasterMix (Eppendorf AG, Hamburg, Germany), and 1.25 m*M* Mg^{2+} (Eppendorf AG). Samples were heated to 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min and then 72°C for 10 min. One SNP was identified in the TGFβ2 gene after comparing the layer and broiler sequences. This polymorphism was genotyped, along with other SNP previously described (Bennett et al., 2006), using the ABI Prism SNaPshot Multiplex kit (Applied Biosystems, Foster City, CA). Individual gene fragments were amplified from genomic DNA in 10-μL PCR reactions as previously described. The suggested protocols of the manufacturer were followed to design a SNP genotyping primer (5′-GCTGGTGGTCCACTTTACTGCAAATCTG-CTAAATATTAACTTG-3′), pool and purify PCR products, and process SNaPshot reactions using a 3100 Genetic Analyzer (Applied Biosystems). Genotypes were identified using GeneScan 3.0 software (Applied Biosystems).

An F_2 resource population developed for a QTL scan, as described by Schreiweis et al. (2005b, 2006), was used to investigate the relationship between the TGFβ2 SNP and phenotypic traits of interest. Briefly, the 513 animal F_2 population resulted from the cross of 2 founder lines represented by 16 pedigree line Hy-Line White Leghorn layer hens and 5 commercial line Cobb-Cobb broiler roosters. Fifty-five traits associated with bone strength, growth, egg production, and egg quality were measured in the F_2 resource population (see Schreiweis et al., 2005a and 2006, for a complete description of traits). Association of the TGFβ2 SNP with all traits was evaluated by ANOVA using a model including hatch (1 to 7 hatches), genotype, and their interaction as fixed effects and F_1 family as a random effect. Results were evaluated with and without the inclusion of BW at the time of bone mineral content (**BMC**) and BMD measurements as a covariate. Significant associations were defined by $P < 0.05$.

Investigation of Bone Marrow TGFβ2 mRNA

Phenotypic data were collected for production and bone traits on 27 female pedigree line Hy-Line White Leghorn layers and 30 female commercial line Cobb-Cobb broilers. The broilers and layers used in this objective represented the same lines as the founders of the F_2 population, except the lines had continued to undergo industry selection between these separate experiments. Birds were obtained on day of hatch and fed a starter diet to 6 wk of age, a grower diet from 6 to 8 wk, a developer diet from 8 to 15 wk, a prelay diet from 15 to 18 wk, and a breeder diet from 18 wk until termination of the experiment. Diet compositions have been reported previously (Schreiweis et al., 2005b). Layers were provided feed and water ad libitum; however, daily feed restriction beginning at 6 wk of age, based on average BW collected at monthly intervals, was necessary to prevent obesity in the broilers. All animal management procedures were approved by the Purdue University Animal Care and Use Committee.

Ten layers and 10 broilers were randomly chosen at each of 3 ages (15, 35, and 60 wk; only 7 layers were collected at 60 wk) and euthanized with CO_2 gas. The BMD and BMC measurements of the left humerus and tibia (including fibula) were obtained from densitometric scans using dual energy X-ray absorptiometry (476D014, Norland Medical Systems, Fort Atkinson, WI) on the day before euthanasia for birds sampled at 15 wk of age (Schreiweis et al., 2003, 2004, 2005b). The larger body size of birds at 35 and 60 wk of age required these measurements to be taken after euthanasia on the severed left wing and leg, with all soft tissues intact. Individual BW were recorded when BMD and BMC measurements were taken.

Additional phenotypes measured in all hens included bone breaking force, daily egg production, and egg com-

ponent measurements. The left tibia was collected following euthanasia, cleaned of all soft tissues, wrapped in 0.85% saline-soaked gauze, and frozen in a –20°C freezer until bone breaking measurements were taken as described by Schreiweis et al. (2003). Egg measurements were taken from eggs laid 2 wk before euthanasia as previously described by Schreiweis et al. (2006).

Following euthanasia, the right tibia was excised and cracked with a hammer. Bone marrow was scraped from the entire length of the bone and immediately frozen in liquid N. Total RNA was extracted from entire bone marrow samples with Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the recommended protocol of the manufacturer. Contaminating DNA was removed by digestion with DNase (DNA-FREE, Ambion, Austin, TX). The RNA quantity was determined by spectrophotometry (NanoDrop Technologies, Wilmington, DE), and quality was evaluated by gel electrophoresis. One microgram of total RNA was reverse-transcribed to cDNA using the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA). The abundance of TGFβ2 mRNA transcripts was measured by quantitative real-time PCR (**QPCR**) using the absolute quantitation method to estimate the number of transcripts in the starting reaction, based on a standard curve. All QPCR assays were carried out in the BioRad iCycler (BioRad Laboratories) in a 25-μL reaction volume with the iQ SYBR Green 2× supermix (BioRad Laboratories), 5 μ*M* forward and reverse primers, and 2 μL of cDNA template. The TGFβ2 forward (5′-TGCTAATGTTGTTACCCTCC-3′) and reverse (5′-ATAA AGTGGACGCAGGCAGC-3′) primers were designed to span the intron between exons 5 and 6. Duplicate reactions were carried out for each experimental sample. Each PCR run also included duplicate reactions for standards representing log dilutions (10^8 to 10^2) of the TGFβ2 PCR product. These standards were generated from purified plasmid DNA containing the TGFβ2 PCR product as an insert. The known log of the starting copy number (**LSCN**) of TGFβ2 transcripts in control samples was regressed on cycle threshold to establish a standard curve for predicting LSCN for each experimental sample. Resulting data are presented as the LSCN of TGFβ2 transcripts.

Phenotypic and expression data were analyzed by ANOVA using the mixed model procedure of the SAS Institute (2003), with genotype (layer or broiler) as a fixed effect. The BW at time of measurement was considered as a covariate for BMD and BMC traits. Quantitative PCR expression data also included animal within genotype as a random effect. Significance was defined as $P < 0.05$.

RESULTS

Investigation of a TGFβ2 SNP

The TGFβ2 SNP is a T to C polymorphism in the intron between exons 3 and 4 (base 71 of GenBank accession number X59081.1). Allelic frequencies of the T allele were 0.00, 0.84, and 0.43 in the broiler founder line, layer founder line, and F_2 population, respectively. Significant associations were found between TGFβ2 and BMD and BMC of the tibia at 35 and 55 wk ($P < 0.05$; Table 1) when no BW covariate was included in the analysis. However, when BW was included in the model as a covariate, associations with BMD and BMC became nonsignificant. Significant associations were found between the TGFβ2 SNP and all BW growth traits from 1 to 6 wk ($P < 0.04$). Finally, the effect of genotype on egg production from 46 to 55 wk of age was also significant ($P = 0.03$). In general, the CC genotype had higher BMD, BMC, and BW but lower egg production from 46 to 55 wk of age, consistent with the high frequency of the C allele from the broiler grandparent line.

Investigation of Bone Marrow TGFβ2 mRNA

Phenotypic data collected on broiler and layer lines showed significantly ($P < 0.0001$) higher BW, BMD, BMC, and bone breaking force in broilers across all ages. Eggs produced by broilers had significantly higher yolk weights. Broilers, however, came in to lay later and had lower rates of egg production compared with layers, as assessed by the total number of eggs laid by 35 and 60 wk of age, divided by the number of days since the date of first egg (Table 2). The QPCR analysis showed a significant difference in TGFβ2 ($P = 0.0002$) mRNA abundance at 15 wk of age, with a 4-fold increase in bone marrow TGFβ2 mRNA in broilers relative to layers (Figure 1). At this age, all birds were in a prelay stage. Although broilers had higher TGFβ2 mRNA abundance at 15 wk of age, no difference was found between the lines at 35 and 60 wk of age.

DISCUSSION

Previously, Li et al. (2003) investigated associations between a TGFβ2 SNP and bone characteristics in a resource population generated from a broiler × Leghorn cross. The first objective of the current study was to replicate this work in an independent resource population that was managed under different conditions. Because broad regions of linkage disequilibrium are maintained in F_2 populations such as those used by Li et al. (2003) and the current study, the SNP evaluated should be interpreted as being in linkage with, not causal for, the underlying QTL. Li et al. (2003) studied BMD and BMC in the tibia of male birds managed as broilers in floor pens at 8 wk of age. In contrast, the current study assessed these traits in female birds raised in wire cages at 35 and 55 wk of age. Despite these differences, both studies report a similar association in that individuals homozygous for the broiler allele had significantly higher BMD and BMC when data were not corrected for BW. It should be recognized that many traits (55) were evaluated in the present study and up to 3 significant associations would be expected by chance alone. However, the fact that associations between TGFβ2 genotypes and bone traits were similar across 2

Table 1. Significant statistical associations between transforming growth factor-β2 single nucleotide polymorphism and bone, egg production, and BW traits in an F_2 resource population

Trait	Age of measurement (wk)	*P*-value[2]	Genotype[1]		
			TT	TC	CC
Tibial mineral density (g/cm^2)	35	0.006 (0.05)	0.238 ± 0.005	0.240 ± 0.004	0.252 ± 0.004
Tibial mineral content (g)	35	0.02 (0.16)	4.09 ± 0.11	4.17 ± 0.08	4.39 ± 0.09
Tibial mineral density (g/cm^2)	55	0.005 (0.08)	0.316 ± 0.009	0.311 ± 0.006	0.334 ± 0.007
Tibial mineral content (g)	55	0.02 (0.42)	5.7 ± 0.2	5.7 ± 0.1	6.1 ± 0.1
Egg production (no. of eggs)	46 to 55	0.03	42 ± 2	39 ± 1	37 ± 2
BW (g)	1	0.04	86.7 ± 1.2	88.6 ± 0.8	90.1 ± 1.0
BW (g)	2	0.02	179 ± 4	188 ± 3	190 ± 3
BW (g)	3	0.02	319 ± 6	331 ± 4	339 ± 5
BW (g)	4	0.01	504 ± 9	523 ± 7	537 ± 8
BW (g)	5	0.02	734 ± 12	752 ± 9	770 ± 10
BW (g)	6	0.01	937 ± 16	972 ± 11	990 ± 12

[1]Values represent the least square means ± the SE.

[2]The *P*-values in parentheses for tibial mineral density and content represent the significance level observed when BW was included in the model as a covariate.

independent populations lends credibility to this relationship.

The importance of BW on the BMD phenotype must be carefully considered, because BW is an important factor that influences BMD of load-bearing bones such as the tibia. In both the experiment reported by Li et al. (2003) and the current study, the association between the TGFβ2 genotype and BMC and BMD became nonsignificant after accounting for variation in BW. Additionally, the TGFβ2 genotype was significantly associated with BW from 1 to 6 wk of age in the current population. Thus, it is difficult to dissect the complex relationships among BW, BMD, and the TGFβ2 genotype. Therefore, a second objective of the present study was to further explore the relationship between TGFβ2 and bone strength by determining whether bone marrow TGFβ2 mRNA abundance differed between layer and broiler hens.

The TGFβ family contains 3 isoforms, including TGFβ2, that elicit a variety of effects on cellular proliferation and differentiation. Although TGFβ has been shown to facilitate differentiation of monocytes into bone-resorbing osteoclasts (Lovibond et al., 2003), it also plays an important role in osteoblast-mediated suppression of osteoclast formation. Bone serves as an important reservoir for latent TGFβ, which can be released and activated upon osteoclastic bone resorption (Oreffo et al., 1989; Oursler, 1994). When this occurs, TGFβ stimulates increased production of osteoprotegrin (Takai et al., 1998) and decreased production of receptor activator for nuclear factor κ B ligand (Quinn et al., 2001) by osteoblasts, leading to a reduced rate of osteoclast differentiation. In the present study, greater TGFβ2 mRNA abundance was observed in broiler compared with layer bone marrow at 15 wk of age. Thus, it is hypothesized that broilers produce and deposit more TGFβ2 in the bone matrix before sexual maturity compared with layers. This increased reservoir of TGFβ2 may then aid in the prevention of excessive bone resorption to support eggshell production once sexual maturity is reached, thereby contributing to stronger bones in broiler compared with layer hens.

Table 2. Means of bone and egg traits of broilers and layers at 15, 35, and 60 wk of age

Trait[1]	Broiler line			Layer line		
	15 wk	35 wk	60 wk	15 wk	35 wk	60 wk
BW, g	2,251***	3,771***	4,123***	992	1,524	1,435
Bone breaking force, kg	24.4***	42.0***	44.3***	14.1	16.1	12.1
BMD humerus, g/cm^2	0.290***	0.255***	0.256***	0.122	0.178	0.159
BMC humerus, g	4.88***	3.35***	2.86***	0.97	1.80	1.24
BMD tibia, g/cm^2	0.196*	0.280***	0.333***	0.131	0.170	0.162
BMC tibia, g	4.23*	6.17***	6.82***	1.56	2.45	2.02
Age at first egg,[2] d	—	182*	186*	—	165	173
Eggs per day in lay, %	—	79*	65*	—	90	80
Egg weight, g	—	62.12	70.20	—	61.65	67.70
Yolk weight, g	—	17.24*	21.64***	—	15.07	17.32
Albumen weight, g	—	39.26	42.47	—	40.92	44.58
Shell weight, g	—	5.39	6.04	—	5.63	5.80
Shell thickness, mm	—	0.34	0.35	—	0.35	0.34
Shell, %	—	8.70	8.63	—	9.15	8.57

[1]BMD = bone mineral density; BMC = bone mineral content.

[2]Data for different birds within each line were used to calculate age at first egg at 35 and 60 wk, resulting in some nonsignificant within-line variability between ages for this trait.

*$P < 0.05$; ***$P < 0.001$.

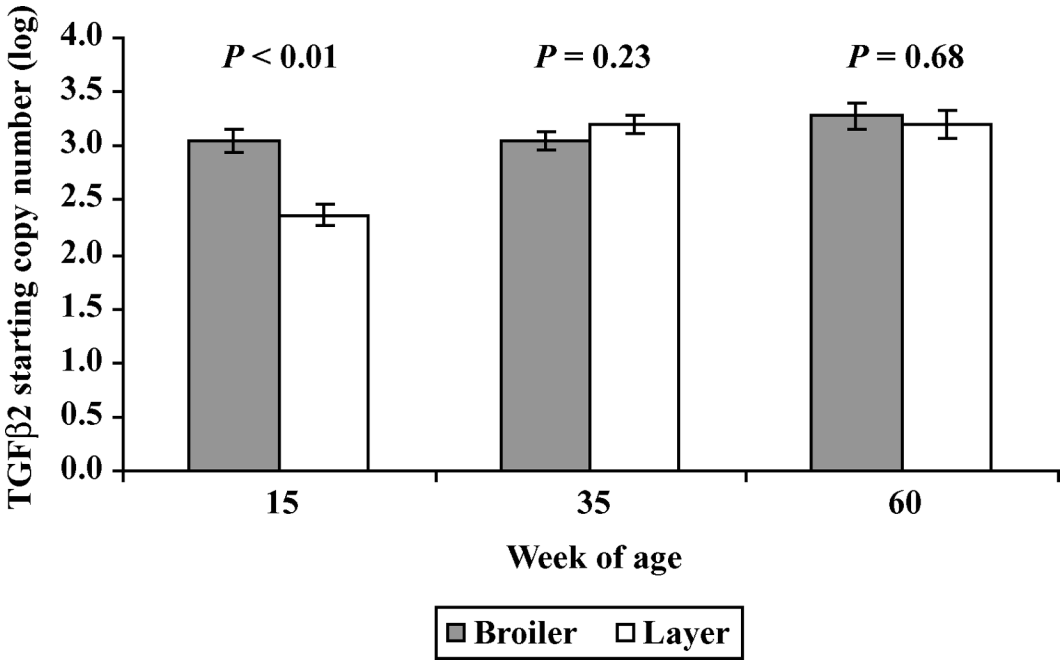

Figure 1. Transforming growth factor-β2 (TGFβ2) mRNA transcript abundance in bone marrow at 15, 35, and 60 wk of age in broiler and layer lines of chickens. The *P*-values given above columns represent the level of significance between the 2 lines at each age.

It is particularly interesting that the difference in TGFβ2 mRNA abundance was observed at 15 wk but not at later ages. Body weight is an unlikely explanation for the difference in TGFβ2 mRNA, because broilers were significantly heavier than layers across all ages. Egg production also does not account for the difference in TGFβ2 mRNA, because birds were not sexually mature at the time the difference was observed. One potential explanation for the age-specific difference in TGFβ2 mRNA is that other cytokines and hormones influencing the regulation of bone metabolism become relatively more important after sexual maturity. For example, TGFβ2 may be most important as a prelay cytokine in chickens to establish the foundation of structural integrity of bone but becomes less influential after other factors, such as estrogen, begin to regulate Ca demand for eggshell production. Finally, as with all investigations of mRNA abundance, it should be recognized that TGFβ2 mRNA abundance may not be a reliable indicator of active TGFβ2 protein. How the abundance of TGFβ2 mRNA in avian bone marrow samples relates to the levels of latent TGFβ2 in bone matrix or the levels of available active protein is currently unknown.

In summary, TGFβ2 has been implicated as an important functional and positional candidate gene for bone integrity traits. Results from the current study confirm that a TGFβ2 SNP association with BMD and BMC is largely due to confounding effects of BW but describe important differences in TGFβ2 mRNA abundance between lines of chickens that differ significantly for bone phenotypes. Thus, even though the TGFβ2 SNP will likely not be an effective marker for improving bone strength independently of changes in BW, further research is warranted to investigate the relationship of TGFβ2 mRNA abundance to bone strength in laying hens.

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