Journal of Animal Research: v.8 n.4, p. 549-554. August 2018

DOI: 10.30954/2277-940X.08.2018.1

Relative Expression Profile of AA Genotype of BMP4 Gene in Broiler and Layer Chicken

D. Divya, M. Gnana Prakash[1], R.N. Chatterjee[2], V. Ravinder Reddy[1], Y. Narasimha Reddy[1] and T.K. Bhattacharya[2]*

[1]Depratment of Animal Genetics and Breeding, C.V.Sc., PVNR TVU, Hyderabad, INDIA
[2]ICAR-Directorate of Poultry Research, Hyderabad, INDIA

**Corresponding author: TK Bhattacharya; Email: bhattacharyatk@gmail.com*

Received: 22 Feb., 2018 **Revised:** 15 June, 2018 **Accepted:** 19 July, 2018

ABSTRACT

Bone morphogenetic protein 4 (BMP4) gene is primarily involved in regulation of bone development and is considered as positive regulator for osteogenesis. The objectives of the study were to explore the polymorphism of BMP4 gene and to determine the expression profile of the predominant genotype observed in broiler (81%) and layer (95%). PCR-SSCP revealed differential pattern with AA genotype being most frequent over AB and AC genotypes. The fast-growing birds displayed higher magnitudes of growth traits over their counter layers. The mRNA expression level was relatively higher in broiler at day 42 and in layer on day 1 and lower on day 28 of both the lines. Significant difference ($p \leq 0.05$) in gene expression was observed during different stages of growth in layer line only. It can be concluded that the BMP4 gene was polymorphic and the mRNA expression varied distinctly over different developmental stages of juvenile period in broiler and layer chicken.

Keywords*:* Bone morphogenetic protein4, polymorphism, expression profile.

Bone morphogenetic protein 4 (BMP), a member of transforming growth factor-β superfamily found predominantly in mesenchymal cells, soft bone cells, periosteum, bone marrow cavity and the muscle cells in the nearby regions of bone fracture site (Nakase *et al*., 1994). BMP4 are secretory, hydrophobic and acidic glycoprotein with mature polypeptide encompassing 114 amino acids and signal peptide region (1st 19 amino acids) at N-terminal site with 4 possible glycosylation sites (144th, 208th, 347th and 362nd). They form a close cluster with BMP2, a positive regulator for osteogenesis. They are one of the key players in osteogenesis where they act as stimulating factor for soft bone ossification (Yaoita *et al*., 2000) at early stages and inhibits osteoclastogenesis process. Various studies using gene knock-in-knock-out technology disclosed the functional diversity of BMP4 gene i.e. in the processes of craniofacial morphogenesis, limb morphogenesis, chondrogenesis and osteogenesis. Their variable effect irrespective of tissue and stage of development was well documented in chicken (Miyazaki *et al*., 2003; Onagbesan *et al*., 2003; Shimizu *et al*., 2004; Weber *et al*., 2008 and Perry *et al*., 2009). However, a detail comprehension in the expression at various stages of development and the associated-based study of expression is lagging in chicken. Thus, an attempt was made to explore the polymorphism of BMP4 gene and to study the expression pattern of most predominant genotype at different stages of growth period in broiler and layer chickens.

MATERIALS AND METHODS

Experimental birds

The birds of control broiler and layer chicken population maintained at ICAR-Directorate of Poultry Research, Hyderabad was used for the study where all managemental and biosecurity measures with respect to watering, feeding, health, vaccination *etc.* were taken besides summer management.

Sample collection

Bone marrow tissues of 40 birds (in total) of broiler and layer lines of 1, 14, 28 and 42 days of age (i.e. 10 birds/ day/line) was collected after the approval of IAEC, DPR, Hyderabad in 1.5 ml sterile polypropylene tube (formerly treated with DEPC) and preserved until processing at -80ºC for the purpose of gene expression study. Congruently, blood from broiler (286) and layer (270) birds was collected in to an anticoagulant added sterile polypropylene tube (2.7% EDTA i.e. 60-70 µl/ 1ml of blood) and stored at -20ºC till succeeding for DNA isolation.

Genomic DNA and RNA isolation process

Phenol-chloroform method (Sambrook and Russell, 2001) was used to extract genomic DNA from blood samples of each bird under study. The purity was checked in terms of quality and quantity and the ones with good DNA were subjected to further procedures. Trizol (Qiagen, Invitrogen) method of RNA isolation was done from the bird's bone marrow tissue and stored at -80°C.

cDNA synthesis

Isolated RNA samples of each bird of different age group of both the lines were converted in to cDNA by using a high capacity cDNA Reverse transcription kit (ABI, USA). The conversion mix prepared as per the manufacturer's protocol, blended with RNA sample (10 µl) and placed in thermocycler (Mastercycler, Eppendorf) for reverse transcription @ 25ºC/10 min (denaturation), 37ºC/120 min (annealing), 85ºC/5 min (extension) and held at 4ºC. The resulted cDNA's were kept at -20ºC till use for expression studies.

Polymerase chain reaction of partial promoter region of BMP4 gene

A 536 bp 5' upstream region (partial promoter) of BMP4 gene was amplified by using genomic DNA as a template. The master mix constituting 10X dream taq buffer (1 µl), 2.5 mM dTNPs mix (0.5 µl), 1 µl gene specific forward and reverse primers (20ng/µl), 0.2 µl taq DNA polymerase (5U/µl) and genomic DNA (1 µl) was prepared and runned [@ 96˚C (10 min), {95˚C (45 sec), 58˚C (30 sec), 72˚C (30 sec)} for 35 cycles, 72˚C (5 min), 15˚C hold and stored at 4˚C] in standard PCR (Mastercycler, Eppendorf) with primers forward (BMP4PF: CCTGAGGAGTGCAGGTG) and reverse (BMP4PR: TCTCCAGGCGCAGTGC) designed by using DNAstar software (Lasergene Inc., USA) and produced by IDT primers, Biosquarebio. The resulted PCR products (5 µl) and DNA molecular marker (1 Kb) were separately mixed with 6X gel loading dye (1 µl) and were loaded in 1% w/v agarose gel submarined in horizontal electrophoresis. The whole apparatus was electrophoresed @ 100 V (40 min), viewed under UV transilluminator, compared with standard DNA molecular marker (1 Kb) and documented.

Single stranded conformation polymorphism (SSCP) and Statistical analysis

The populations of broiler and layer birds of different age groups were screened for the presence of polymorphism by using single stranded conformation polymorphism (SSCP). Native PAGE was prepared with the ingredients such as 49:1 Acrylamide:Bisacrylamide (9.6 ml), 1X TBE buffer (28.3 ml), Glycerol (1.5 ml), 10% APS (100 µl) and TEMED (75 µl). Individual samples in formamide dye were denatured by means of heating and were not allowed for renaturation by means of immediate snap cooling. The chilled products were then electrophoresed @ 195 V (8 hrs) in vertical gel electrophoresis, silver stained and observed for differential pattern. The samples that showed variation in expression pattern was sequenced by using automated dye-terminator cycler in ABI PRIZM 377 DNA sequencer (Perkin-Elmer), ScieGenom lab. The association study of genotype with expression was performed using Univariate General Linear Model of SPSS 16.0 with the model: $Y_{ijk} = \mu + G_i + A_j + S_k + e_{ijk}$ where, Y_{ijk} = dependent variable; μ = overall population mean; G_i = fixed effect of genotype; A_j = fixed effect of age; S_k = is the fixed effect of sex and e_{ijk} = random error with NID (0, σ^2). Significant differences were calculated between least-squares means of genotype by Duncan's method (5% LS).

BMP4 gene expression profiling

The gene expression levels were quantified using real time PCR (Applied Biosystems® Step One RealTime PCR, Life Technologies v2.2.2 machine) where template (cDNA) and SYBR® Green JumpStart™ TaqReadyMix™ (Thermo Scientific, #K0221) as a dye. The respected forward and

reverse primers used are CCTGGTAACCGAATGCTGAT and GCTCTGCGACTTTCTTCCT for BMP4 (106 bp) and CTGCCGTCCTCTCTGGC and GACAG TGCCCTTGAAGTGT for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 119 bp) where the latter pair was used as a normalizer for the present expression study. The conditions used for amplification of both the genes was 95˚C/10 min, 35 cycles of denaturation at 94˚C/30 sec, 58˚C/30 sec and 72˚C/30 sec and finally 72˚C/10 min. Thermal cycling condition involved in real time PCR machine is (a) Stage 1: taq polymerase (95°C /10 min); (b) Stage 2: denaturation (94°C/15 sec), annealing (58°C/1 min) and extension (72°C/15 sec) for 40 cycles and (c) Stage 3: dissociation curve analysis (95°C/15 sec, 60°C/1 min and 95°C /15 sec) with the reaction containing 2x SYBR Green mix (5 µl), forward and reverse primer (1 µl; 20 ng), nuclease free water (2 µl) and template cDNA (1 µl).

So obtained Ct (comparative threshold) values of BMP4 (target) and reference (GAPDH) genes were quantified using the formula:

$$\Delta Ct = \text{maen Ct of target gene} - \text{mean Ct value of endogenous control}$$

$$\Delta\Delta Ct = \Delta Ct \left(\begin{matrix}\text{Expression of target}\\ \text{gene at different ages}\end{matrix}\right) - \Delta Ct \left(\begin{matrix}\text{Expression of target}\\ \text{gene at day 1}\end{matrix}\right)$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

Statistical analysis

The expression data was analyzed with ANOVA following univariate GLM with Duncan's as post-hoc test (between different age groups) using SPSS 16.0 version. The data were being presented in mean ± SE with LSD at 5% level of significance ($p<0.05$). The statistical model for the present study was:

$$Y_{ijk} = \mu + G_i + A_j + S_k + e_{ijk}$$

Where, Y_{ijk} is expression of each individual at each age group in the population, µ is the overall mean, G_i is the fixed effect of genotype, A_j is the fixed effect of age, S_k is the fixed effect of sex and e_{ijk} is the random error distributed normally with mean 0 and common variance σ^2 (NID- 0, σ^2).

RESULTS AND DISCUSSION

A 536 bp 5' upstream region (partial promoter) of BMP4 gene was amplified using gene specific primers and was subjected to SSCP to determine polymorphism in the fragment (Fig. 1).

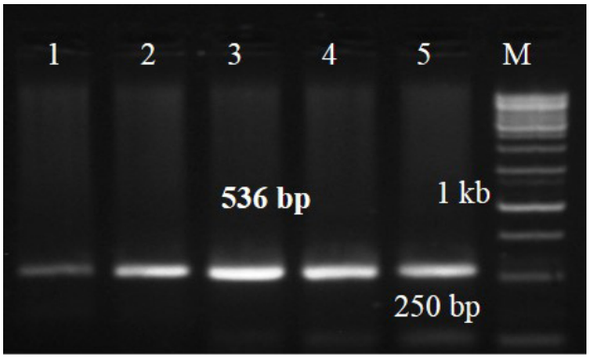

Fig. 1: AGE showing PCR amplified product from genomic DNA of BMP4 gene promoter

The study revealed the presence of polymorphism in the fragments of both broiler and layer chicken lines (Fig. 2) with AA genotype being predominant one having frequency of >80% over other two genotypes (AB and AC with combined frequency of <13%).

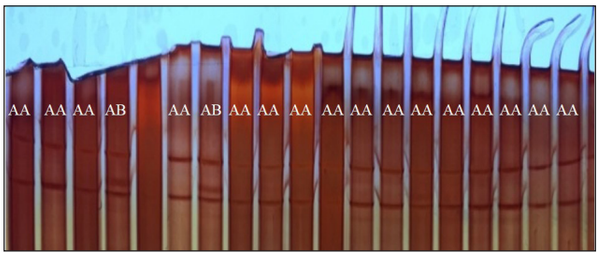

Fig. 2: PAGE displaying SSCP patterns of BMP4 gene promoter in broiler and layer

The mean weights of growth and conformation traits were tabulated in table 1 where the broiler birds recorded significantly higher ($p\leq0.05$) magnitude of body weights and conformation traits such as shank length and breast angle at 6 weeks of age. The occurrence of polymorphisms in the promoter region may influence the binding of

Table 1: Mean (±SE) values of growth and conformation traits

Lines	Body weights at different ages (g)				Weights at D42 age (g)			Conformation traits	
	D1	D14	D28	D42	Breast	Leg	Wing	BA (º)	SL (mm)
Broiler	39.08 ± 0.4[a]	169.49 ± 6.5[a]	475.00 ± 17.6[a]	950.70 ± 27.4[a]	125.65 ± 4.3[a]	222.01 ± 5.4[a]	83.84 ± 4.1[a]	70.71 ± 3.2[a]	70.07 ± 2.1[a]
Layer	34.05 ± 0.72[b]	67.88 ± 3.8[b]	115.12 ± 8.4[b]	192.11 ± 11.8[b]	21.97 ± 2.68[b]	36.21 ± 3.1[b]	14.97 ± 1.02[b]	56.83 ± 1.9[b]	30.64± 3.9[b]

Means bearing different superscripts in the same column are significantly different (P≤0.05)

D1=Day1; D14=Day14; D28=Day28; D42=Day42; BA=Breast angle; SL=Shank length.

transcription factors with promoters and ultimately interfere the gene expression, which may thereby impact the bone development and skeletal integrity in chicken. The bone marrow tissues of the predominant AA genotype at respective age groups (day 1, 14, 28 and 42) were subjected to expression profiling. In the present study, the amplified products of BMP4 and GAPDH genes through real time PCR was run in 0.8% agarose gel and the respective bands have been detected in Fig. 3.

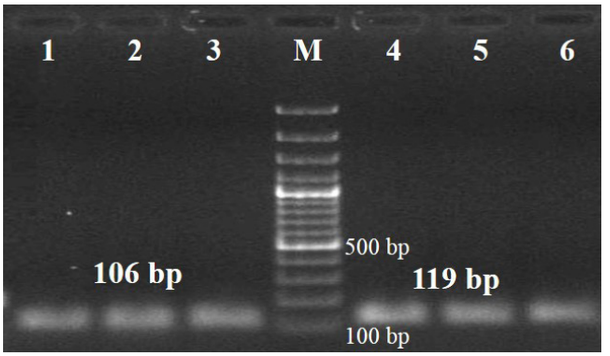

Fig. 3: AGE showing PCR amplified product from cDNA of BMP4 and GAPDH genes

[Lane 1-3 → 106 bp (BMP4); 4-6 → 119 bp (GAPDH); M: 100 bp ladder]

The mean Cq values were recorded separately and the relative standard curve was made by taking cDNA on X-axis (independent variable; in log) and quantification cycle on Y-axis (dependent variable) where a coefficient of determination (R^2) of 0.9583 and 0.9316 was observed in respective BMP4 and GAPDH genes. Correspondingly, the relative expression of BMP4 gene was analyzed up to day 42 in broiler and layer chicken lines wherein the 40-ΔCt value directly corresponds to the gene expression i.e. the higher the 40-ΔCt value, the higher will be the expression of gene and *vice-versa*.

A non-significant effect of sex was observed in both the lines on gene expression. The highest and lowest BMP4 gene expression was found with the magnitude of 42.29 ± 0.71 on day 42 (D42) and 39.03 ± 0.70 on day 28 (D28) in AA genotype of broiler and 39.84 ± 0.4 on D42 and 38.01 ± 0.5 on D28 in AA genotype of layer lines, respectively (Table 2).

Table 2: 40-ΔCt values of BMP4 gene in broiler and layer birds

Lines	D1	D14	D28	D42
Broiler	40.69 ± 0.70[abx]	39.50 ± 1.7[bx]	39.03 ± 0.70[bx]	42.29 ± 0.71[ax]
Layer	39.96 ± 0.40[bx]	39.56 ± 3.8[bx]	38.01 ± 0.50[bx]	39.84 ± 0.4[ay]

[a,b]Means bearing different superscripts in the same row are significantly different (P≤0.05)

[x,y]Means bearing different superscripts in the same column are significantly different (P≤0.05)

D1=Day1; D14=Day14; D28=Day28; D42=Day42; BA=Breast angle; SL=Shank length.

In other words, the broiler population exerted a slightly higher mRNA expression than their counter parts implicating the role of BMP4 expression in regulating higher growth in the broiler line. The significant differences (p≤0.05) in gene expression were observed during different ages of growth (D1, D14, D28 and D42) in the layer line only. Zhen *et al*. (2006) revealed the significant effect of genotypes on gene expression for Hsp70 gene in chicken. However, the expression trend was found to be decreased in both the fast (broiler) and slow growing

(layer) lines from D1 to D28 with further increase after D28. However, the fold change was calculated where the expression values in each line at different stages were compared by taking the expression value on D1 as a calibrator/standard. A down regulation by 0.44 and 0.32 on D14 and D28 and upregulation by 3.03 folds on D42 in broiler and down regulation by 0.75, 0.26 and 0.92 folds in layers on D14, D28 and D42 of age was observed, respectively as compared to expression on D1. The varied expression at different ages was reported by other workers in other animal species (Kingsley, 1994; Winnier *et al*., 1995; Chang *et al*., 2002; Bandyopadhyay *et al*., 2006; Shu *et al*., 2011). Tissues where BMP4 is expressed were osteoblastic cell, hippocampus, brain, astrocyte, oligodendrocyte and ependymal cells of spinal cord in rat (Harris *et al*., 1994; Fan *et al*., 2003; Mikawa *et al*., 2006; Miyagi *et al*., 2012), branchial arches, retinal pigment epithelium and embryo in chick (Francis-West *et al*., 1994; Zhang *et al*., 2013; Judge *et al*., 2016), alveolar bone, hair follicle and digit in mouse (Zhang *et al*., 2002; Han *et al*., 2003; Ou *et al*., 2015), embryo (MartÍnez-Barberá *et al*., 1997) in zebra fish and bone marrow tissue in chicken. Their over expression displayed ectopic bone formation (Alden *et al*., 1999; Jane *et al*., 2002; Kubota *et al*., 2002) and under expression caused osteogenic impairment characterized by spontaneous bone fractures. In this study, we observed differential growth performance of broiler and layer birds, which could be attributed to the breed differences and effect of genotypes of candidate genes related to controlling growth in chicken. The expression of BMP4 gene in AA genotyped birds of broiler was found to be relatively higher than that of layer birds indicating its role in regulating growth and skeletal conformation traits.

CONCLUSION

The polymorphism was observed in 5'upstream region of BMP4 gene in chicken in which the predominant AA genotype showed differential expression between breeds and among different age groups with mRNA expression being higher at D42 in broiler and at D1 in layer line.

ACKNOWLEDGMENTS

The work has been done under National Fellow project, Directorate of Poultry Research (DPR), Hyderabad. The authors thank the Indian Council of Agricultural Research (ICAR) for their support and the Director, ICAR-DPR for providing the necessary facilities during the course of experiment.

REFERENCES

Alden, T.D., Pittman, D.D., Hankins, G.R., Beres, E.J., Engh, J.A., Das, S., Hudson, S.B., Kerns, K.M., Kallmes, D.F. and Helm, G.A. 1999. In vivo endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. *Hum. Gene Ther.,* **10**: 2245–2253.

Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B.D., Rosen, V. and Tabin, C.J. 2006. Genetic analysis of the roles of BMP2, BMP4 and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.,* **2**(12): 2116–2130.

Chang, H., Brown, C.W. and Matzuk, M.M. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr. Rev.,* **23**: 787–823.

Fan, X., Xu, H., Cai, W., Yang, Z. and Zhang, J. 2003. Spatial and temporal patterns of expression of Noggin and BMP4 in embryonic and postnatal rat hippocampus. *Dev. Brain Res.,* **146**: 51–58.

Francis-West, P.H., Tatla, T. and Brickell, P.M. 1994. Expression patterns of the bone morphogenetic protein genes bmp-4 and bmp-2 in the developing chick face suggest a role in outgrowth of the primordia. *Dev. Dyn.,* **201**: 168–178.

Han, M., Yang, X., Farrington, J.E. and Muneoka, K. 2003. Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development,* **130**: 5123–5132.

Harris, S.E., Sabatini, M., Harris, M.A., Feng, J.Q., Wozney, J. and Mundy, G.R. 1994. Expression of bone morphogenetic protein messenger RNA in prolonged cultures of fetal rat calvarial cells. *J. Bone Miner. Res.,* **9**: 389–394.

Jane Jr J, A., Dunford, B.A., Kron, A., Pittman, D.D., Sasaki, T., Li, J.Z., Li, H., Alden, T.D., Dayoub, H., Hankins, G.R., Kallmes, D.F. and Helm, G.A. 2002. Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer. *Mol. Ther.,* **6**: 464–470.

Judge, L., Giles, S. and Brennan. 2016. Exploring the effects of ethanol exposure on the expression of bone morphogenetic protein 4 using a chick embryo model of fetal alcohol syndrome. *Reprod. toxicol.,* **64**: 29–49.

Kingsley, D.M. 1994. What do BMPs do in mammals? Clues from the mouse short-ear mutation. *Trends Genet.,* **10**(1): 16–21.

Kubota, K., Iseki, S., Kuroda, S., Oida, S., Iimura, T., Duarte, W.R., Ohya, K., Ishikawa, I. and Kasugai, S. 2002. Synergistic effect of fibroblast growth factor-4 in ectopic bone formation induced by bone morphogenetic protein-2. *Bone,* **31**: 465–471.

MartÍnez-Barberá, J.P., Toresson, H., Rocha, S.D. and Krauss, S. 1997. Cloning and expression of three members of the Zebrafish BMP family: BMP2A, BMP2B and BMP4. *Gene,* **198**: 53–59.

Mikawa, S., Wang, C. and Sato, K. 2006. Bone morphogenetic protein-4 expression in the adult rat brain. *J. Comp. Neurol.,* **499**: 613–625.

Miyagi, M., Mikawa, S., Sato, T., Hasegawa, T., Kobayashi, S., Matsuyama, Y. and Sato, K. 2012. BMP2, BMP4, NOGGIN, BMPRIA, BMPRIB and BMPRII are differentially expressed in the adult rat spinal cord. *Neuroscience,* **203**: 12–26.

Miyazaki, Y., Oshima, K., Fogo, A. and Ichikawa, I. 2003. Evidence that bone morphogenetic protein 4 has multiple biological functions during kidney and urinary tract development. *Kidney Int.,* **63**: 835–844.

Nakase, T., Nomura, S., Yoshikawa, H., Hashimoto, J., Hirota, S., Kitamura, Y., Oikawa, S., Ono, K. and Takaoka, K. 1994. Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. *J. Bone Miner. Res.,* **9**: 651–659.

Onagbesan, O.M., Briggeman, V., Van As, P., Tona, K., Williams, J. and Decuypere, E. 2003. BMPs and BMPRs in chicken ovary and effects of BMP-4 and -7 on granulose cell proliferation and progesterone production in vitro. *Am. J. Physiol. Endocrinol. Metab.,* **285**: 973–983.

Ou, M., Zhao, Y., Zhang, F. and Huang, X. 2015. Bmp2 and Bmp4 accelerate alveolar bone development. *Connect. Tissue Res.,* **56** (3): 204–211.

Perry, J.M., Harandi. O.F., Porauette, P., Hedge, S., Kannan, A.K. and Paulson, R.F. 2009. Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood,* **113**: 911–918.

Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor laboratory press, Cold Spring Harbor, New York.

Shi, Y. and Massagué, J. 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. *Cell,* **113**(6): 685–700.

Shimizu, T., Yokoo, M., Miyake, Y., Sasada, H. and Sato, E. 2004. Differential expression of bone morphogenetic protein 4-6 (BMP-4, -5, and -6) and growth differentiation factor-9 (GDF-9) during ovarian development in neonatal pigs. *Domest. Anim. Endocrinol.,* **27**: 397–405.

Weber, S., Taylor, J.C., Winyard, P., Baker, K.F., Sullivan-Brown, J., Schild, R., Knuppel, T., Zurowska, A.M., Caldas-Alfaonso, A., Litwin, M., Emre, S., Ghiggeri, G.M., Bakkaloglu, A., Mehls, O., Antignac, C., Network, E., Schaefer, F. and Burdine, R.D. 2008. SIX2 and BMP4 mutations associate with anomalous kidney development. *J. Am. Soc. Nephrol.,* **19**: 891–903.

Winnier, G., Blessing, M., Labosky, P.A. and Hogan, B.L. 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.,* **9** (17): 2105–2116.

Yaoita, H., Orimo, H., Shirai, Y. and Shimada, T. 2000. Expression of bone morphogenetic proteins and rat distal-less homolog genes following rat femoral fracture. *J. Bone Miner. Metab.,* **18**(2): 63–70.

Zhang, J., Tan, X., Contag, C.H., Lu, Y., Guo, D., Harris, S.E. and Feng, J.Q. 2002. Dissection of promoter control modules that direct Bmp4 expression in the epithelium-derived components of hair follicles. *Biochem. Biophys. Res. Commun.,* **293**(5): 1412–1419.

Zhang, Y., Liu, Y., Ho, C. and Wildsoet, C.F. 2013. Effects of imposed defocus of opposite sign on temporal gene expression patterns of BMP4 and BMP7 in chick RPE. *Exp. Eye Res.,* **109**: 98–106.

Zhen, F.S., Du, H.L., Xu, H.P., Luo, Q.B. and Zhang, X.Q. 2006. Tissue and allelic-specific expression of hsp70 gene in chickens: basal and heat-stress-induced mRNA level quantified with real-time reverse transcriptase polymerase chain reaction. *Br. Poult. Sci.,* **47**(4): 449–455.