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Comparative analysis of activin receptor type-IIA gene expression in two distinct chicken lines

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Abstract
Activin Receptor Type-IIA (ACVR2A) is a member of TGF-β super family, which is receptor for MSTN playing inhibitory role in regulating muscular growth in chicken [1]. Expression profile of ACVR2A gene was studied in two contrasting lines of chicken *viz*. control broiler line (CB) having faster growth rate and control layer line (CL) having slower growth. We conducted experiment during post-hatch stage from day 1, day 14, day 28, day 42 and day 56 of age. Expression of ACVR2A gene in pectoral muscle ranged from -0.43 ±0.71 to 1.26±0.71 in CB and 2.13 ±0.71 to 6.29 ±0.71 in CL line. ACVR2A gene expressed differently in both CB broiler and CL.

Keywords: Activin receptor type-IIA, chicken, expression profile

Introduction
The ACVR2A is the heteromeric complexes which transduce signals for MSTN [2, 3]. MSTN first bind with ACVR2A and this MSTN/ACVR2A complex then recruits a type I receptor which in turn phosphorylates the Smad proteins. The activated Smad proteins function as the key intracellular mediators of signaling by entering the nucleus to down regulate MyoD gene expression in muscle cells [4, 5]. The MyoD Plays important role in Myogenesis to regulates the myoblast and satellite cell proliferation and act in a redundant fashion, genetically upstream ofmyogenin and MRF4 to specify myoblasts for terminal differentiation [6]. MSTN/ACVR2A also signals through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K/Akt) pathways to down regulates the myogenesis [7, 8]. MSTN protein involved in inhibiting both satellite cell proliferation and differentiation in chicken and other animals [2, 3].It stops cell cycle of satellite cells by up regulating p21, which inactivates cyclin-dependent kinase activity in the cell. MSTN also regulates satellite cell differentiation by inhibiting the expression of the myogenic growth factor, MyoD, via Smad 3 signaling during embryonic development [4, 5]. In addition, MSTN plays a critical role in regulating muscle homeostasis postnatally by suppressing muscle growth. At the same time, overexpression of MSTN led to the reduction of muscle mass suggesting MSTN to be a negative regulator of skeletal muscle growth. During embryogenesis, MSTN is exclusively expressed in myoblast cells to control the differentiation and proliferation of the myoblast [2], but in adulthood, it is not only restricted to skeletal muscle but also detected in other tissues (e.g. heart, adipose tissue, mammary gland) [2].

Materials and methods
Experimental birds
The study was conducted in two chicken lines namely *viz* control broiler (CB) and control layer (CL) maintained at the ICAR-Directorate of Poultry Research, Hyderabad, India. The CB line was a synthetic colour broiler line that is random bred pedigreed over last 9 generations. The body weight of CB line at 5 weeks of age was 625.5 ± 0.13 g [9]. Control layer was evolved from White Leghorn breed of chicken. The CL line was a random-bred line developed by random mating over 12 generations. The body weight of CL birds at 8 weeks of age was 456 ± 0.16 g [9]. The CB and CL birds were kept in the brooder house till the age of 6 weeks. All the birds were reared on deep litter system in the same shed under intensive management of farming providing same management regimen with ad libitum feeding and watering up to 6 weeks.

Collection of tissue samples

A total of five males and five females each of CB and five males and five females each of CL lines slaughtered to collect pectoral muscles from day 1st, 14th, 28th and 48thof post hatch period. In total, 40 samples were collected for gene expression study. The birds during post-hatch were slaughtered to collect pectoral muscles under aseptic conditions following the approved slaughtered protocol of Ethical Committee of ICAR-Directorate of Poultry Research, Hyderabad. Pectoral muscles were collected in 15 ml DNase, RNase free sterile polypropylene tubes under aseptic conditions using DEPC treated sterile instruments. Samples were immediately chilled on ice to minimize RNA degradation, transferred to lab, and kept at -80°C for RNA extraction and cDNA preparation.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from tissue samples using Trizol (Invitrogen) according to the manufacturer's protocol. The RNA pellet was washed with 1 ml 75% ethanol prepared with DEPC water, then centrifuged at 7,500 rpm for 5 minutes at 4°C. The RNA pellets were dried for five to ten minutes, then re-suspended in DEPC water. The RNA samples were then treated withDNaseI (Fermentas) for removal of possible genomic DNA contamination. cDNA was synthesized using RT enzyme (Thermo scientific, first strand cDNA synthesis Kit, #K1612) in a final volume of 20 µl, having 5X Reaction Buffer, 10 mM of each dNTPs, 20 U of RNase inhibitor (Thermo scientific), Random hexamer primers and 20 U Reverse transcriptase enzyme. Reverse transcription was carried out in thermocycler (Mastercycler, Eppendorf, Germany) following the manufacturer's thermal profiles.

Expression of ACVR2A gene

The qPCR was performed for studying expression of ACVR2A gene along with GAPDH gene as an internal control withthe cDNA using thermal cycler Applied Biosystems® by Life Technologies v2.2.2 machine with SYBR Green (Thermo scientific). For mRNA quantification of ACVR2A and GAPDH genes, two pairs of primers were designed from chicken cDNA sequences of ACVR2A (NCBI Accession No. - NM_205367.1) and GAPDH gene (NCBI Accession No. - NM_204305.1), using DNASTAR software (Lasergene Inc.). GAPDH was used as housekeeping gene for internal control. Primers of ACVR2A gene were ACVR2A5F: GATCCTGGACCACCACCGC and ACVR2A5R: CTGGATAGGGAATATTTTGACTG (Fragment size was 195 bp) while those for GAPDH gene were QGAPDHF: CTGCCGTCCTCTCTGGC and QGAPDHR: GACAGTGCCTTGAAGTGT (Fragment size was 119 bp).

SYBR Green (Thermo scientific, #K0221) was used for preparation of the reaction mixture. All the reactions were run in thermal cycler machine (Applied Biosystems® by Life Technologies v2.2.2) and quantification of data were analyzed by using Step one software, version2.2.1. Each sample was run in duplicate both for target gene as well as internal control. A negative control, containing all the reaction components except the template was also used to check any contamination of foreign DNA in the reaction components.

Analysis of qPCR data

The experiments were repeated twice and statistical analysis was performed by ANOVA using SPSS version 20. Data from representative experiment are presented as Mean ± SE for different samples with differences determined by least significant differences at the 5% level ($p < 0.05$).

Calculation of $2^{-\Delta\Delta Ct}$ was performed as follows:

Arithmetic mean of Ct value (mCt) for each sample was calculated.

The value was normalized by using Ct value of internal control gene.

ΔCt = m Ct (Target gene) – m Ct (Internal control gene)

Calculation of ΔΔCt value:

Relative quantity or fold change in gene expression of target genes at different ages (1stDay, 14thDay, 28thday and 42ndday) with respect to reference genotype was calculated.

ΔΔCt = ΔCt (Expression Target gene at different age) - ΔCt (Expression of target gene at 1th day)

Calculation of Fold change: Fold change = $2^{-\Delta\Delta Ct}$

Results

ACVR2A gene expression was studied on 1st, 14th, 28thand 42ndday post hatch in CB and CL lines. The ΔCt values in CB were -0.429±0.715, -1.08±0.715, 1.064±0.715 and 1.263±0.715 on 1st, 14th, 28thand 48thday, respectively. Corresponding values in CL were 2.132±0.715, 2.24±0.715, 6.293±0.715 and 4.074±0.715 (Fig. 2). The expression of ACVR2A gene was significantly ($p \leq 0.05$) higher at similar age in CL as compared to CB.

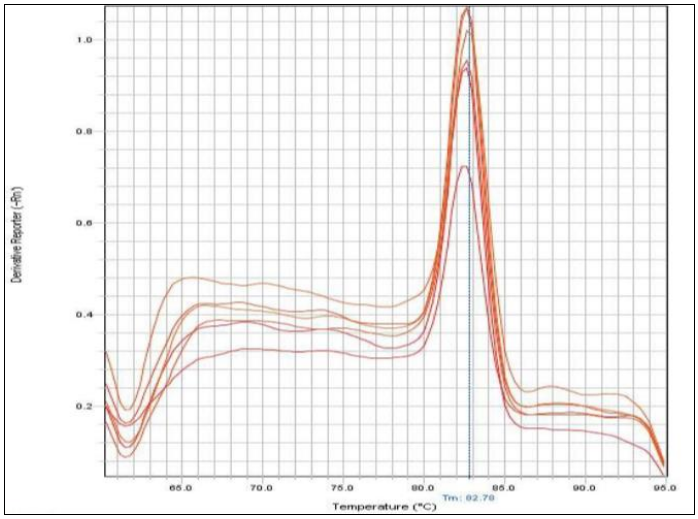

Fig 1: Melting curve analysis

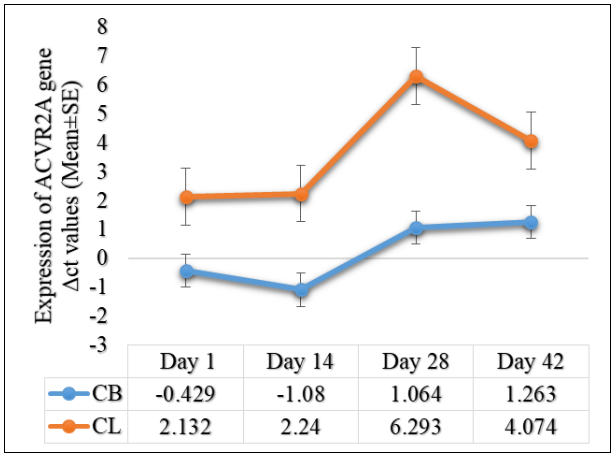

Fig 2: Expression of ACVR2A gene in pectoral muscles of CB and CL lines at different ages

Fold change expression was used to study the up regulation and down regulation of ACVR2A gene in CB and CL during post-hatch period. Post hatch expression of ACVR2A gene at different ages was compared with the expression on 1st day in both the lines. It was found that ACVR2A was up regulated by 8, 2 and 1.48 folds on 14th, 28thand 42ndday in CB. The highest up regulation of ACVR2A was observed on 14th day but level of expression reduced as the day progressed. The ACVR2A gene was up regulated by 6.08 and 1.40 folds on 14th and 42nd days in CL lines. However, it was down regulated by 0.042 folds on 28thday (Fig 3). The highest up regulation of the gene was observed on day 14th in CL too.

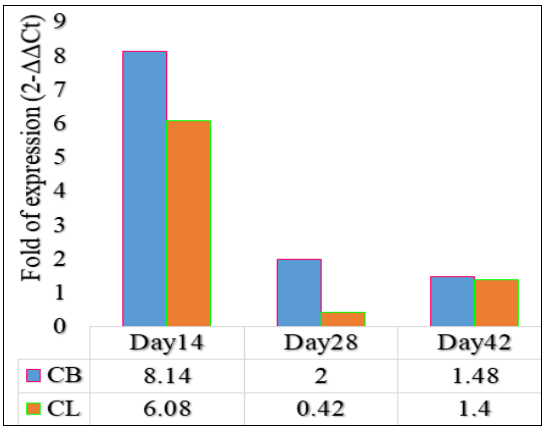

Fig 3: Fold change expression of ACVR2A gene in pectoral muscle of CB and CL lines

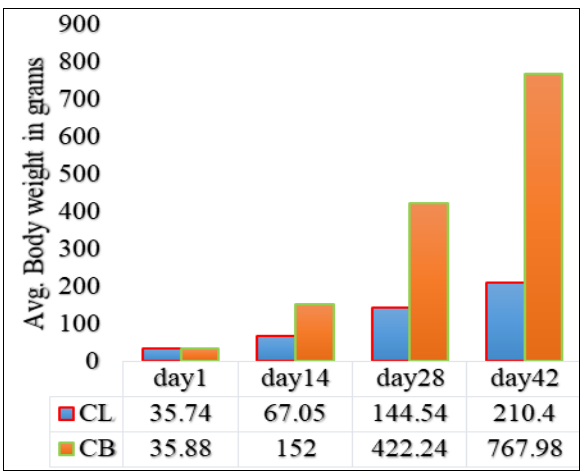

Fig 4: Average body weight of CB and CL lines at different ages

Discussion

We measured body weight of CB and CL during sample collection. The average body weight of chicken 35.88, 152, 422.24 and 767.98g was observed on 1st, 14th, 28thand 42ndday in CB. The average body weight of CL was 35.74, 67.05, 144.54 and 210.4g on 1st, 14th, 28thand 42ndday (Fig 4). We correlate the Weight of bird and Expression of ACVR2A gene in of the same bird. The expression of ACVR2A gene ΔCt values in CB line were -0.429±0.715, -1.08±0.715, 1.064±0.715 and 1.263±0.715 on 1st, 14th, 28thand 48thday, respectively. The expression of ACVR2A gene ΔCt values in CL line were 2.132±0.715, 2.24±0.715, 6.293±0.715 and 4.074±0.715on 1st, 14th, 28thand 48thday, respectively. This study shows the expression of ACVR2A gene is negatively correlated with body weight of both the CB and CL lines. And also that there was lesser expression of ACVR2A gene in CB line compare to CL line. Due to this lessor expression ACVR2A gene which might have helped in more number of muscle fibres formation in CB line resulting into higher body weight with faster growth. At the same time period due to higher expression of ACVR2A gene in CL line resulting into lesser body weight with slower growth.

Conclusion

Finally the results were concluded the expression of ACVR2A gene in Control Broiler and Control Layer lines having significant difference during post hatch juvenile stage. Between lines, the ACVR2A gene was expressed higher in the control broiler line compare to control broiler line were significant level in all age matching days. But within line the expression of ACVR2A gene was not differ during age matching days and the highest expression was noticed during day 14thwhich was gradually decreased day 42ndonwards. Which conforms the second week age of the juvenile stage of chicken life crucial stage for highest expression of ACVR2A. On the muscle cell membrane due to this highest expression of ACVR2A which allows to bind more number of extra cellular proteins of MSTN ligands. More binding of MSTN down regulate the MyoD gene expression in the more number of muscle cells during the second week of age chicks life leads to decreasing the level of proliferation and differentiation of muscle cells results lesser muscle fibre formation with low body weight.

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