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# *RUNX2* Plays an Essential Role in the Manifestation of Femoral Head Necrosis in Broilers

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**ABSTRACT:** Economic losses due to the increase of leg disorders in broilers have become one of the major concerns from the poultry industry. Despite the efforts to select animals against skeletal abnormalities, insufficient progress was obtained. The genetic pathways and genes involved in most of the bone pathologies, including femoral head necrosis, remains unclear. In this study, bone samples were collected from broilers with femoral head necrosis (FHN) and with normal femurs. The qPCR reactions for the *TNFRSF11B*, *RUNX2*, *CALB1* and *SMAD1* genes were submitted to relative quantification analysis. Only *RUNX2* was differentially expressed, being down regulated ($p<0.05$) in the affected group, with its expression reduced four times when compared to the non-affected group. This result may indicate that the repression of *RUNX2* gene product could contribute to an increased incidence of femoral head necrosis in chickens.

**Keywords:** Leg disorders; gene expression; qPCR

## Introduction

In the last decades, the poultry production system has focused in the intense selection for heavier and faster growing broilers (Havenstein et al. (2003)). However, at the same time, the incidence of bone problems have increased significantly, causing a negative impact on welfare, feed efficiency, growth performance and other traits. These problems led to annually economic losses over $100 million (Cook (2000)), being considered one of the main concerns to the poultry industry (Oviedo-Rondón (2007)). Despite the efforts to select animals against skeletal abnormalities, insufficient progress has been made. Besides that, the genetic pathways involved in most of the bone pathologies remain unclear.

One of the major leg disorders is the femoral head necrosis (FHN). This disorder is associated with poor bone mineralization and can affect broilers in the growing phase. The main clinical sign is the difficulty to stand, and during necropsy, the proximal end of the femurs are not fully calcified (Cook (2000)). Many factors, including nutrition, are involved with this disorder. Heritability estimates are shown to be moderate ($h^2=0.4$), indicating an important role of genetic factors (Bishop et al. (2000)) in the occurrence of leg problems. Functional candidate genes are known to be involved with the formation, development and maintenance of the bone tissue. Some of these genes have been described in the chicken genome, but most of functional pathways are unknown, since the studies are available only in humans and rodents. To obtain insights on the genetics involved in the incidence of the FHN, the aim of this study was to evaluate the expression profile of bone-specific candidate genes in commercial broilers with healthy and injured bones.

## Material and Methods

**Animals.** A total of 20 commercial male broilers (Cobb-500) with 45 days of age were used in this study. Body conditional score was evaluated in all the animals prior the necropsy. Femurs were collected and classified according to the presence or absence of necrosis of femoral head. Femoral samples were collected from 10 healthy animals (control group) and from 10 animals with first stage of femur necrosis (case group). After collection, samples were stored in liquid nitrogen and then transferred to -80ºC freezer until the RNA extraction.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from 100mg from femoral head using Trizol Reagent® (Life Technologies), following the manufacturer's instructions. Total RNA was quantified in Nanodrop spectrophotometer and integrity was confirmed in 1.5% agarose gel. The cDNA was synthetized from 3 ug of total RNA using SuperScript III First-Strand Synthesis SuperMix ® (Life Technologies) kit.

**Gene expression analysis.** The candidate genes analyzed in this study were: *TNFRSF11B* (osteoprotegerin), *RUNX2* (Runt-related transcription factor 2), *CALB1* (Calbindin 1, 28kDa) and *SMAD1* (SMAD family member 1). The *HBMS* was used as housekeeping gene. Primers to amplify the gene fragments were designed in exon-exon junctions using *Primer-Blast* software (Table 1). The qPCR analysis was performed in the ABI Prism 7500 Sequence Detection Systems (Applied Biosystems) with the SYBR Green fluorescent dye.

**Table 1. Primers used in the qPCR analysis**

| Gene | Primer | Accession |
|---|---|---|
| *TNFRSF11B* | F ACGCTTGTGCTCTTGGACAT<br>R:CAGCGTAGTACTGGTCTGGG | NM_001033641.1 |
| *RUNX2* | F: GATTACAGACCCCAGGCAGG<br>R: TGGCTCAAGTAGGACGGGTA | NM_204128.1 |
| *CALB1* | F: GGCAGGCTTGGACTTAACAC<br>R: GCTGCTGGCACCTAAAGAAC | NM_205513.1 |

| | | |
|---|---|---|
| *SMAD1* | F: GTTTTGTAAAGGGTTGGGGAGC<br>R: AATGCAGGAGCTTGGGACCTTA | NM_0012<br>01455.1 |
| *HBMS* | F: ACTAGTTCACTTCGGCGAGC<br>R: CTCAGGAGCTGACCTATGCG | XM_4178<br>46.3 |

Real Time PCR Ct (threshold cycle) values were obtained from each sample in duplicate. Means of Ct were collected and the REST (*Relative Expression Software Tool*®) software was used to carry out the statistical analysis (Pfaffl et al. (2002)).

## Results and Discussion

Out of the four evaluated genes involved in bone metabolism: *TNFRSF11B*, *RUNX2*, *CALB1* and *SMAD1,* only *RUNX2* was differentially expressed between the case and control groups. This gene was four times down regulated ($p<0.05$) in the affected group when compared to the non-affected group (Figure 1).

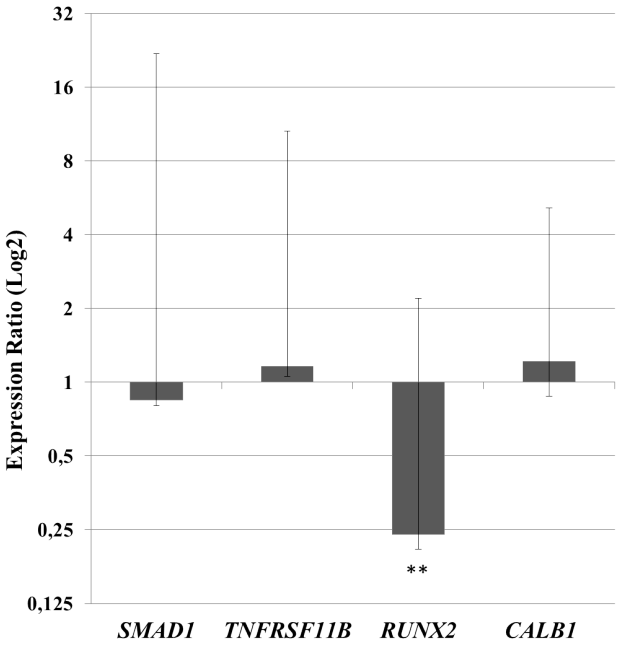


**Figure 1. Ratio of bone related gene expression in femur between affected and control group, normalized by the housekeeping gene *HBMS*. $^{**}p<0.05$.**

The FHN could be caused by two main factors: presence of bacteria as *E. coli* and *S. aureus*, known as bacterial chondronecrosis (BCO), and Avascular Necrosis of Femoral Head (AFHN), induced by the suppression of blood vessels during the ossification (McNamee and Smyth (2000)). In humans, AFHN is associated with a reduction of progenitor cells in the proximal femur. In adults, red bone marrow persists in the proximal femur shafts, while hematopoietic marrow is absent in the femoral head (Sen (2009)). *RUNX2* belongs to the *Runt transcription factor family* and its expression is fundamental to osteoblast differentiation (Provot (2005)). It has been reported that *RUNX2* is expressed in the fetal growth plate inducing cell proliferation. This gene stimulates bone morphogenic proteins (BMPs) via SMAD signaling (Lee et al. (2012)). Furthermore, *RUNX2* stimulates the vascular endothelial growth factor (VEGF) gene, which is responsible to increase blood vessels invasion in the growth plate and cartilage. Therefore, *VEGF* is a strong candidate to avoid AFHN.

In chickens, information on *RUNX2* function in bone metabolism is still scarce. Our findings suggest that the down regulation of *RUNX2* expression could promote the necrosis of femoral head in commercial broilers. This can possibly be explained by a reduced expression of *VEGF*, causing lack of blood vessels invasion. Similar results were demonstrated by Kido et al. (2014), which observed tibial bone problems in mice when *RUNX2* was down regulated. Moreover, it has been shown that the expression of *RUNX2* was involved with bone matrix protein-related genes and mineralization in immature mesenchymal cells (Fugita et al. (2004)). Hence, low levels of *RUNX2*, as found in this study, might reduce bone mineralization, increasing the skeletal problems incidence in broilers.

## Conclusion

The *RUNX2* was four-fold less expressed in affected broilers, than in healthy chickens. These results indicate a possible involvement of this gene with the incidence of femoral head necrosis in poultry production.

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