

# Osteogenesis in Chicken (*Gallus gallus domesticus*) and Expression of VEGF in this Process between 5<sup>o</sup> to 19<sup>o</sup> Days of Incubation

Paula Fratini<sup>1\*</sup>, Dayane Alcântara<sup>1</sup>, Marcio Nogueira Rodrigues<sup>1</sup>, Fernanda Menezes de Oliveira e Silva<sup>1</sup>, André Luis Rezende Francioli<sup>1</sup>, Erika Toledo da Fonseca<sup>1</sup>, Valdir Pavanelo Junior<sup>1</sup>, Carlos Alberto Palmeira Sarmento<sup>1</sup>, Antonio José Piantino Ferreira<sup>2</sup> and Maria Angelica Migliano<sup>1</sup>

<sup>1</sup>Departamento de Cirurgia, Setor de Anatomia, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Brazil

<sup>2</sup>Departamento de Patologia, Setor de Ornitopatologia, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Brazil

## Abstract

Poultry production is of great economic importance nowadays and it is important to constantly improve the production quality. In this context, the bird growth and adequate bone development are necessary for successful production.

In this paper we evaluate the expression of VEGF and its importance in the osteogenesis process in embryonic and fetal tissues of *Gallus gallus domesticus* at different gestational ages.

We observed that the Vascular Endothelial Growth Factor (VEGF) is essential in the formation of cartilaginous tissue and bone in the embryo and fetus of *Gallus gallus domesticus*.

## Introduction

The economic importance of poultry and eggs production in the world is unquestionable. The development of genetic lineages with rapid growth and high egg production, associated with technological development in nutrition, management and sanity, led poultry production to industrial levels [1].

To better assess the current strategies of the poultry industry, it is necessary to expand the knowledge about its behavior and well-being, regardless of culture systems. Thus it is possible to intervene appropriately, proposing new production systems capable of providing adequate facilities and management. With the increase of genetic improvement researches, fast-growing strains have been obtained, with earlier and greater muscular development. However, bone tissue development does not have accompanied these physiological processes, thereby increasing the incidence of problems in the hind limbs and bone weakness [2].

The bone tissue is the second to have its development prioritized by the body, behind only the nervous system and followed by the muscle or fat tissues. It has several important functions such as body support, locomotion, protection of internal organs and metabolic reserves (lipids and minerals), also working as a hematopoietic organ [3,4].

According to [5], fundamental bone components are osteoblasts, osteocytes and osteoclasts. The bone tissue formation can occur through two distinct processes: endochondral ossification (cartilage growth) or intramembranous ossification (membrane growth) [6,7]. When chondrocytes, involved in the endochondral process, are hypertrophic, they produce angiogenic factors (VEGF) which induce the formation of blood vessels from the perichondrium. With the emergence of these blood vessels chondrogenic cells become osteogenic, originating osteoblasts. Morphologically, the bone has two forms, cortical or compact (for mechanical protection) and cancellous (with metabolic function) [6].

During bone growth, development and remodeling, angiogenesis and osteogenesis are closely associated processes. Proliferation and differentiation of osteoblast cells lineage occur under the influence of a number of hormones, transcription and growth factors [8]. The limited capacity of bone to heal and regenerate, particularly in the craniofacial

region, represents a serious clinical problem. Several issues need to be thoroughly addressed in order to establish therapeutic approaches resulting in effective and extensive reconstruction of bone. A key step is the identification of the molecules that play major roles in survival and differentiation of skeletogenesis precursors in order to appropriately modulate their expression or mimic their function in a clinical context. Growth factors and extracellular matrix components are of particular interest since they can be more easily manipulated than intracellular proteins. Among animal models used to address some of these issues, the chicken presents several advantages, being easily accessible hence amenable to embryonic manipulation and having cellular origins of the craniofacial skeleton thoroughly mapped [9].

The problems related to poor bone formation became worrisome for poultry, mainly due to the significant rate of rejection of carcasses poorly developed at slaughterhouses. Therefore, this study aimed to evaluate the expression of VEGF and its importance in the formation and osteogenic differentiation in embryonic and fetal tissues of *Gallus gallus domesticus* during the most critical phases of development and cell differentiation, thereby obtaining data for a better understanding of their development that can be used to improve poultry production techniques.

## Materials and Methods

30 fertilized eggs from domestic chickens, between 5 and 19 days of incubation, were used. The eggs were packed and stored at 26 ± 0.5°C and 45 ± 2% relative humidity.

\*Corresponding author: Paula Fratini, Departamento de Cirurgia, Setor de Anatomia, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Brazil, Email: [fratini@usp.br](mailto:fratini@usp.br)

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Every 24 hours, two eggs from each age period were collected, identified, evaluated in an egg scope for embryos identification and weighed. Each egg was opened carefully by its apical region (air chamber) using dissection tweezers. Then, the yolk sac was excised and the embryos were removed, identified, weighed, measured and fixed in 4% paraformaldehyde.

For macroscopic analysis, a stereo microscope magnifier (Zeiss® model Stemi DV4) was used. Biometrics was performed with a stainless steel caliper, determining their diameter, measurements of lower and upper beak and third finger, as described by [10]. All results description followed as described by [11,12]. For analysis by light microscopy, embryos fixed in paraformaldehyde 4% were washed in Phosphate-Buffered Saline (PBS) and dehydrated in a series of increasing ethanol concentrations (from 70 to 100%), diaphanized in xylene and included in paraffin (Histosec). Sections 5 µm thick were obtained in an automatic microtome (Leica RM2165) and stained with Hematoxylin-eosin. The morphological characteristics found were photo documented under a light microscope (Nikon Eclipse E-800).

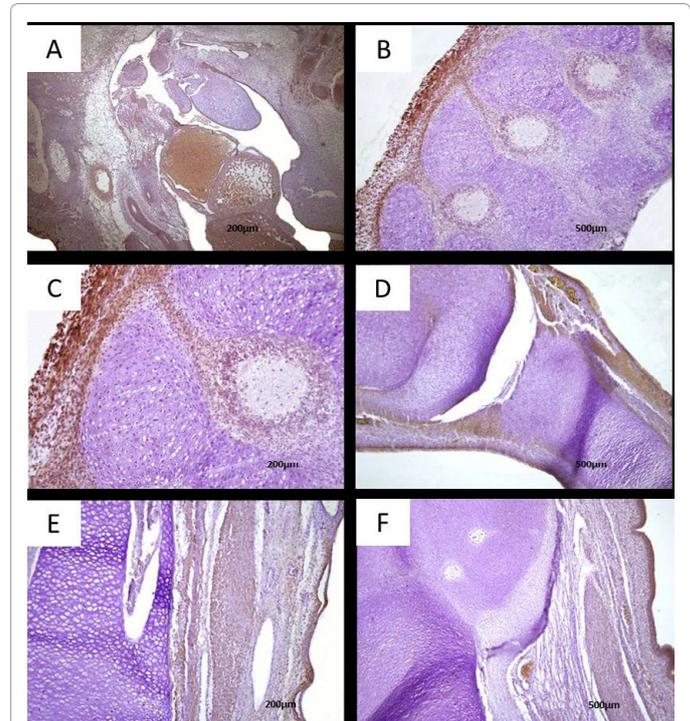
In VEGF analysis, sections were deparaffinized and rehydrated to perform immunohistochemical analysis. Antigen retrieval, as well as dilution and clones, for the monoclonal antibodies CD34 (Dako, Carpinteria, USA) and VEGF-A (Dako, Carpinteria, USA) to block endogenous peroxidase, was performed using 3% hydrogen peroxide for 15 minutes, in darkroom. The blockage of tissue proteins was performed using a specific solution at room temperature for 10 minutes (TM Protein Blocking Solution, Dako, Carpinteria, USA).

## Results

From the mesoderm located along the embryo back and notochord and neural tube sides, an epimer or the dorsal mesoderm was observed, being divided into blocks called somites, after 5 days of incubation. At 7 days there was a cross segmentation of the epimer into somites. At this stage they were visualized as paired structures consisting of a mesenchymal tissue mass lined by a columnar epithelium. At 9 days sclerotome cells begin to condense and differentiate into cartilage. Vertebral ossification begins at 11 days with the merger of primordial cartilage forming the vertebral bodies. The beginnings of the ribs, which are also derived from sclerotomes and somites, were initially observed as a segmented condensation at day 9. At day 11, at which vertebrae ossification begins, the ribs were visualized separate from the vertebrae in the same age.

VEGF acts during the osteogenesis process in the somites differentiation by mediating the angiogenesis and endochondral ossification processes. At 5 days, VEGF positive expression was not observed and low expression was noted until day 8. At day 9 and 10 of incubation, a higher intensity of VEGF expression was observed accompanied by the differentiation process of somites into cartilage (Figure 1A and 1B). This expression was clearly demonstrated in cells that surrounded the area where the somite was developing in the cervical region (Figures 1C and 1D). At 14 days, when there is cartilage differentiation into osteoblasts and subsequently osteocytes, an intense VEGF expression was observed (Figure 1E and 1F).

In fetuses of 14 days of incubation, an advanced endochondral ossification process was checked, in which a membrane of pre-existing cartilage tissue origin the mold of the future bone tissue, via a primary ossification center (Figures 2A and 2B) Structurally, three distinct regions were observed during bone formation in the fetal skull region, at 14 days of incubation: cartilage tissue, proliferation and primary



**Figure 1:** Development of somites in *Gallus gallus domesticus* embryos and fetuses, 5-14 days of incubation. Immunolocalization of VEGF in embryonic and fetal tissues. In A: 5 days embryo, 4x. B: 9 days embryo, 10x, with somite formation. In D: hindlimb of a 10 days embryo, 10x. In E: 14 days embryo, forelimb (thigh area), 10x.

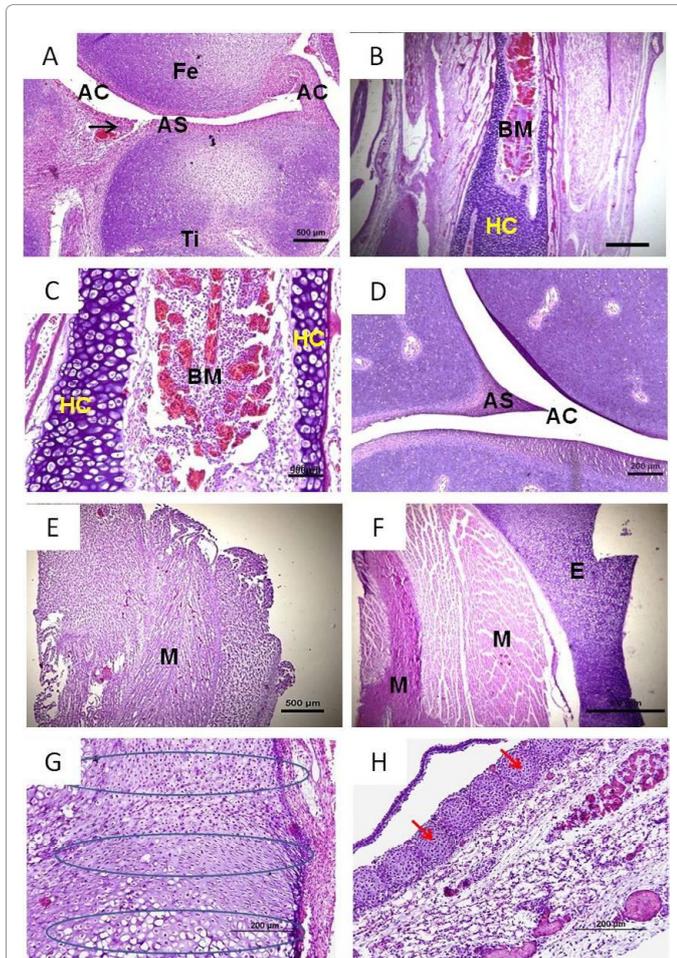
ossification zone (Figure 2C). They also observed the formation of secondary ossification centers, which are similar to the primary, but the growth occurs radially (Figure 2C), rather than longitudinally.

It was also noted that, at this age, skullcap consists of a thin layer of epithelial tissue that formed skin layers (dermis and epidermis), followed by skull bones in formation and underlying connective tissue which carries blood vessels (Figure 2D).

Limb buds were observed after 7 days and consisted of an aggregate of mesenchymal cells embedded in an extracellular matrix composed of a collagen fibers' net. As the bud elongates, a differentiation into cartilage begins. At 9 days there was a initial differentiation of future pelvic limbs and wings. After 12 days of incubation it was possible to observe the beginning of the endochondral ossification process. The cartilaginous mold previously existing in this phase begins to be replaced by bone tissue.

Histologically, mature chondrocytes have a cytoplasm strongly stained at this developmental stage and blood vessels were observed in the tissue adjacent to the bone and cartilaginous tissues in formation (Figure 2). In a medial view of the stifle joint from 14 days embryos, femur and tibia were identified, as well as regions of the articular surface, joint cavity and capsule (Figure 2). At that stage the histological composition of the bone marrow in long bones of the pelvic limb was observed (Figure 2B and 2C).

The development of the forelimb and wings in *Gallus gallus* was studied focusing on the development and growth of their articular and bone components at different ages. Under light microscopy, phalanges region in 9 days embryos showed cartilage components with late-stage



**Figure 2:** Development of bone and cartilaginous structures of the pelvic limb in *Gallus gallus* fetuses at 12 days of incubation and the development of bone and cartilaginous structures of the cranial and cervical region in *Gallus gallus* fetuses at 14 days of incubation. Bone and articular development. A. Medial view of the stifle joint of 12 days embryos: Femur (FE), Tibia (TI), Articular Surface (AS), Articular Cavity (AC), Articular Capsule (AC). B and C. Long bone (femur): Bone Marrow (BN). D. Articular Surface (AS), Articular Cavity (AC). Note in A, B and C the presence of ossification zone still with a cartilage mold, shown by stars. Observe the skull region of an embryo with 14 days showing the process of endochondral ossification. 20x. G: Photomicrograph of the cervical region of a 14 days embryo, showing the secondary ossification. H: Note that the skull at this stage generally consists of a thin layer of epithelial tissue, corresponding to the skin layers, followed by forming skull and underlying connective tissue, where it is observed a large number of blood vessels.

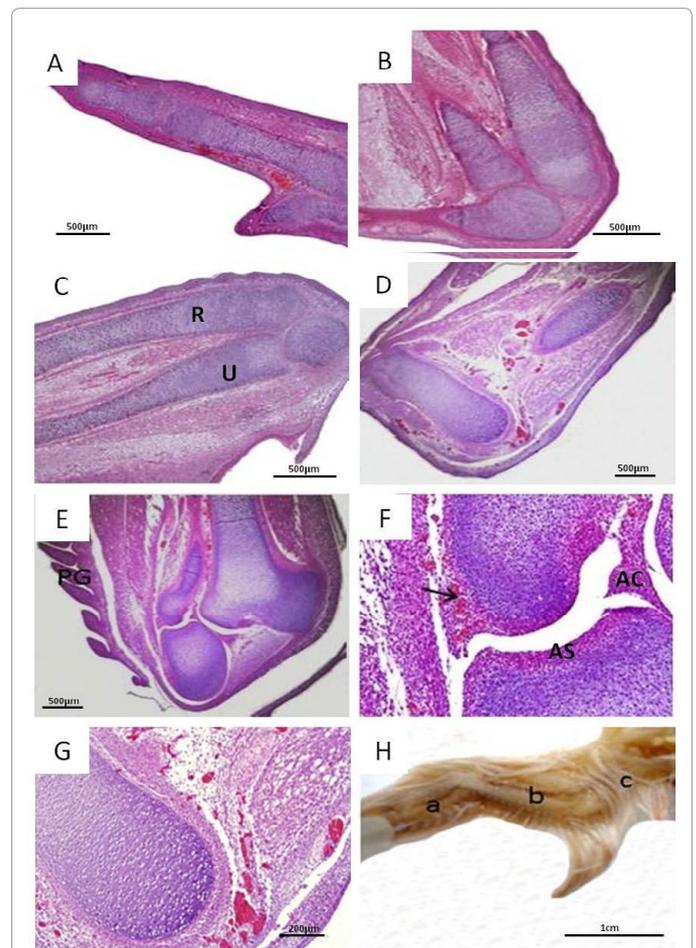
development with cells already differentiating into bone tissue. Blood vessels were observed in the region of the connective tissue underlying the cartilage and bone, promoting their irrigation. It was also noted an intense amount of skeletal muscle fibers surrounding the components of the appendicular skeleton (Figures 3A and 3B).

At day 10, molds of ulna and radius could be easily identified. It was noted the fast cartilaginous tissue replacement by bone tissue and the intense local vascularization. In the bone ends it was observed the formation of structures which will give rise to synovial joints (Figures 3C-3F). Overlying layers of skin in fetuses of 12 days of incubation, the formation and development of corneal attachments (feathers) were also observed by light microscopy (Figure 3D). At this stage, there is a more advanced degree of ossification of the forelimb bone components. After 19 days of development, forelimb was fully formed, and it is possible to

identify key bone and articular areas of the forelimb, and the feathers are already formed (Figure 3D).

## Discussion

The skeleton is formed by mesenchymal cells, derived from cranial neural crest, somites and lateral plate mesoderm. In 5 days of incubation was observed the transversal segmentation of the epimer in the somites and around day 9 occurs the condensation and differentiation of the sclerotome cells in cartilage according to [13,14] these cells condense to form the future skeletal elements. [15] cited that the osteogenic condensations are formed first in the caudal region at around 9 days and later in the more rostral regions similar to observed in our study. Secondary ossification centers of ossification appear within the cartilaginous epiphysis by a mechanism that is similar to the formation of the primary center [16] At 11 days the primordial cartilage fusion could be seen by us forming the vertebral bodies. [15] described that the first site of mineralization is seen around day 9 in the articular, angular and supra-angular bones in the caudal region of the



**Figure 3:** Development of cartilage and bone structures of the forelimb in *Gallus gallus* embryos and fetuses, between 9 and 19 days of incubation. A and B. Histological section of phalanges (Ph) in embryos with 9 days. C. Radio (r) and ulna (U) at day 9. D-F: Forelimb at 10 days. In D, the articulation humerus, ulna and radius. Notice in E the presence of feathers germ (FG). In F: note the intense blood flow (arrow) in the region of the humerus articular surface (AS). AC: Articular Capsule. G: wing development in embryos with 12 days. H: Medial view of the wing joint of 19 days embryos: a. phalanges, b. carpus. c. radius and ulna.

developing bone mandible. The caudal bones enlarge and fuse followed by mineralization of the bones in more rostral regions that occurs at around stage day 11 in the chicken like observed in our study.

The craniofacial skeleton in the first branchial arch and other regions of the developing head develops largely from neural crest cells originating from the dorsal neural tube; some cranial bones are derived from the cephalic mesoderm [9,17]. In Chicken the initial craniofacial skeleton development was observed in 12 days of incubation. The ribs and vertebrae form from the sclerotomal part of the somites, and the appendicular skeleton is derived from the lateral mesoderm. Although the embryologic origin of various skeletal elements is different, the precursor cells will eventually differentiate into three specific cell types that are the same in every skeletal element. The chondrocyte in cartilage is of mesodermal origin [18].

Endochondral ossification is regulated by a complex relationship of local growth factors and systemic subordinate to the endocrine system of the animal. Among these factors is the Vascular Endothelial Growth Factor (VEGF) produced by hypertrophic chondrocytes, which is blamed for vascular invasion of the growth plate [19]. Matrix-mediated tissue interactions have been implicated in the skeletogenesis differentiation of crest cells, but little is known of the role that growth factors might play in this process [20]. Found that neural crest responsiveness to FGF in avians exists before migration and that it is required for neural crest cell survival.

At 5 days we didn't note VEGF positive expression in our analysis. This result can be explained by the fact that the cells still are immature in this age supported by [21] when cited that the immature and proliferating chondrocytes secrete and express angiogenic inhibitors. At 9 and 10 high VEGF expression were observed, however in this age the initial process condensation and differentiation of the sclerotome cells in cartilage will be occur [19,21]. Report that chondrocytes in the cartilage template and later in the growth plate first proliferate and then differentiate into mature hypertrophic cells are the first place that could be observe the initial of the VEGF expression. The VEGF expression in these ages represent an important phase of osteogenic development in chicken, once that the vessel invasion has a key role in endochondral bone formation and VEGF plays an important role in this phenomenon. VEGF may mediate interactions between endothelial cells and bone cells, endothelial cells, chondrocytes [22], and osteoblasts [23] secrete endogenous VEGF that may stimulate chondrocytes [22], osteoblasts [24] and osteoclasts [25]. In endochondral ossification, an avascular cartilage model is first formed.

The VEGF expression, in our study, has been observed in the margin of the somites where the chondrocytes were developing it. These region express high levels of VEGF according to Gerber et al. (1999). VEGF expression in the hypertrophic cartilage causes the upregulation of VEGFR1 and VEGFR2 expression in the perichondral endothelium [26] and there upon induces vessels to invade the hypertrophic cartilage from the perichondrium [27]. Zelzer and Olsen [14] have speculated that high levels of VEGF near hypertrophic chondrocytes maintain endothelial cells in a sprouting mode. This has been explained by the fact, that sprouting growth plate capillaries contain no basement membranes or pericytes but, in the region where bone matrix is being synthesized, endothelial cells are surrounded by BMs and pericytes.

We can noted that the chondrocyte hypertrophy cause an increase in cell size, cessation of proliferation, and synthesis of a new repertoire of differentiation-specific gene products [28-31]. The example that we can

cite is the matrix protein collagen X, which represents the predominant biosynthetic product of hypertrophic cartilage [32,33].

In summary, changes in the expression of VEGF in different ages in chicken can shift the balance between proliferation and skeletogenesis differentiation. This indicates that a fine tuning of VEGF signalling, and possibly of other growth factors implicated in skeletal morphogenesis, is required for the progression of skeletogenesis differentiation. The VEGF can be a key role for determining the skeletogenesis fate of neural crest cells and in modulating the skeletogenesis differentiation. Finally, it clearly indicates that induction of bone regeneration either using growth factors, or cell therapy, or a combination of the two, is most likely to succeed if such mechanisms are thoroughly understood and strategies to reproduce them devised when the animals are affected by bone diseases or defects during development.

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