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Investigating Molecular Mechanisms behind
Bacterial Chondronecrosis with Osteomyelitis (BCO)
Pathogenesis in Modern Broilers

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

Alison Ramser
The Pennsylvania State University
Bachelor of Science in Animal Science, 2019

May 2022
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

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Abstract

Bacterial chondronecrosis with osteomyelitis (BCO), a leading cause of lameness in broiler chickens, is characterized by infection, inflammation, and bone attrition. There are currently no effective treatments and positive diagnosis is only possible through necropsy evaluations. Lameness is also a rising animal welfare and economic concern, making prevention and detection of BCO all the more critical. These challenges are exacerbated by a lack of mechanistic understanding of BCO's etiology. The question I asked during my dissertation was how bacteria induce bone attrition in BCO pathology. My research has shown that mitochondrial dysfunction is characteristic of BCO conditions along with autophagy machinery dysregulation. This autophagy dysregulation is also seen to a result of *in vitro* infection with known BCO-isolates and affecting bone cell viability. The local bone and systemic blood profile of cytokines, chemokines, inflammasomes, and relevant FGFs were also evaluated. This revealed a unique signature of BCO detectable within circulation and in local bone. Additionally, this signature was made up of factors which negatively affect bone cell viability. It was also shown that primary avian chondrocytes exhibiting optimal phenotypes could be successfully isolated form chicks. These primary cells could provide an improved, highly relevant model for *in vitro* analysis of avian bone diseases and infections. Finally, the potential roles of two factors regulating energy and lipid metabolism were preliminarily explored as a future target for BCO research. These findings provide novel insight into mechanisms of etiology and means of non-invasive detection while also improving upon current methods of avian growth-plate research.

Acknowledgements

I would like to acknowledge Dr. Dridi for his advice, teaching, and demand for my best. As well as Dr. Elizabeth Greene for her expertise, skills, and support. I would like to thank all the many researchers and peers I have had that inspired, helped, and educated me and all my committee members for their mentorship and advocacy.

I am grateful for my husband for being a home to me through anything and to my stepson for learning with me through new challenges.

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Chapter 2 – Bacterial chondronecrosis with osteomyelitis (BCO) in modern broilers: impacts, mechanisms, and perspectives (published – *CAB Reviews.* 15;2020.)

Chapter 3 – Evidence of Mitochondrial Dysfunction in Bacterial Chondronecrosis with Osteomyelitis–Affected Broilers (published – Frontiers in Veterinary Science. 8:640901;2021.)

Chapter 4 – Autophagy machinery and bone homeostasis: friend or foe? (Accepted – CAB Reviews)

Chapter 5 – AUTOPHAGY & BROILER LAMENESS: Role of Autophagy Machinery Dysregulation in Bacterial Chondronecrosis with Osteomyelitis (BCO) (Under Review – Poultry Science)

Chapter 6 – Cyto(Chemo)kines: Roles in Skeletal Physiology, Bacterial Pathology, and When those Roles Collide (Accepted – CAB Reviews)

Chapter 7 – Local and Systemic Cytokine, Chemokine, and FGF Profile in Bacterial Chondronecrosis with Osteomyelitis (BCO)-affected Broilers (Published – Cells. 10(11),3174; 2021.)

Chapter 8 – Primary Growth Plate Chondrocyte Isolation, Culture, and Characterization from the Modern Broiler (In Submission – Journal of Animal Science)

Chapter 10 – Hormonal regulation of visfatin gene in avian Leghorn male hepatoma (LMH) cells (Published – Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology. 240:110592; 2020.)

Chapter 11 – Regulation of avian uncoupling protein (av-UCP) expression by cytokines and hormonal signals in quail myoblast cells (Published – Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology. 248:110747; 2020.)

Chapter 1 – Introduction

The modern broiler is a prodigy of genetic investigation, a beneficiary of nutritional and husbandry innovations, and a cornucopia of scientific intrigue. Never has a bird grown as fast and produced as much yield while improving its feed efficiency and resisting disease, infection, and disorders. There is much to celebrate about the efficient animal that globally provides one of the most popular animal protein options. However, the broiler is not finished with its work. While much has been achieved, more needs to be attained. As global population rises and more individuals crave affordable quality animal protein grown with less resources and better conditions, the broiler will need help [1, 2].

One challenge is the maintenance of the broiler's welfare and bodily integrity so that it can continue to perform at its peak. Lameness and skeletal disorders have become an increasingly concerning obstacle to broiler welfare and performance. Specifically, bacterial chondronecrosis with osteomyelitis (BCO), also known as femur head necrosis (FHN) and tibial head necrosis (THN), is a leading cause of lameness with multiple factors at play. BCO primarily affects the proximal growth plates of the long bones, areas within the skeletal system that undergo additional stress and dynamic remodeling during fast growth [3, 4]. These areas are also highly vascularized leading to potential exposure to opportunistic bacteria within the blood stream to weakened or damaged sites within the growth plate [5]. Once infection occurs, inflammatory immune responses and necrosis sets in leading to bone attrition and often lameness [6, 7]. While lameness is a major animal welfare and performance concern as lame birds feel more pain and access less feed and water, the true danger behind BCO is its subclinical persistence [5, 8, 9]. To date, BCO is only diagnosable through necropsy and inspection of the proximal femur and tibial heads [5]. Without lameness, BCO birds may go undetected in breeding programs and

production. Therefore, defining the molecular mechanisms involved in this disorder is critical to improving prevention, detection, and potentially treatment of BCO.

Researchers have successfully demonstrated spontaneous induction of BCO in broilers reared on wire flooring [10, 11]. This model has become the foundation for studying BCO. It works on the principal of increased mechanical stress in addition to a more stressful environment conditions that are optimal for decreased bone integrity and increased bacterial load. Indeed, there is a fairly agreed upon mechanism in which BCO occurs within the long bones, however what is yet to be understood is how bacterial infection creates the symptoms and bone condition seen and what the systemic immune and metabolic factors are that contribute to or are the result of BCO. Recently, it was shown that DICER1 dysregulation coupled with double stranded ribonucleic acid (dsRNA) accumulation led to NLRP3 inflammasome activation in BCO bone and human bone affected by osteomyelitis [12]. This is the first research to implicate a cellular process in the pathogenicity of BCO and set the stage for further in-depth molecular analysis to reveal pathways and processes involved in BCO. In continuum to the above-mentioned study, I demonstrated the presence of mitochondrial dysfunction in BCO (Chapter 3) and outlined its involvement in bacterial infection and cell survival (Chapter 2). Additionally, I evaluated the role of autophagy machinery manipulation in BCO etiology (Chapter 5). It was demonstrated that this dysregulation seen in tissue was also seen under challenge with known BCO-isolates. This dysregulation was sufficient to affect bone cell viability and therefore could be contributing to bone attrition and lack of healing. The systemic effects of BCO were investigated by defining the cytokine, chemokine, inflammasome, and FGF profiles in bone and blood of BCO-affected birds compared to their healthy counterparts (Chapter 7). BCO was shown to have a unique signature of cytokines, including TNFα, and chemokines, such as IL-8, contributing to the systemic

inflammatory state. This signature could be used as a non-invasive detection and could also contribute to inflammatory bone loss through affecting bone cell viability.

One of the limiting factors in the study of BCO is the lack of an avian bone cell line for *in vitro* analysis. To address this limitation, I successfully isolated, cultured, and characterized primary growth plate chondrocytes from chicks (Chapter 8). These cells showed optimal phenotype for mimicking avian growth-plate tissue by day 3 in culture and proven secretion of collagen type II by day 7. Finally, the roles of two regulators of lipid metabolism and energy homeostasis was preliminarily investigated. First, the regulation of visfatin were evaluated within hepatic cells in order to better understand its role in avian systems (Chapter 10). Avian-uncoupling protein (av-UCP) and its regulation by growth factors, hormones, and pro-inflammatory markers, was evaluated in avian muscle cells (Chapter 11). These factors could be essential to understanding the systemic effect and causes of BCO and have shown to be regulated through several means in their major cell types.

Taken as a whole, these novel findings demonstrate complex molecular networks involved in cell stress response, cell survival, bone homeostasis, and systemic inflammation to be implicated in BCO etiology. These pathways and their outcomes create the unique systemic effects seen in BCO and provide a basis for non-invasive markers, potential targets for treatment or selection, and a foundation for future research to better understand BCO in the broiler.

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Chapter 2 –

Title: Bacterial chondronecrosis with osteomyelitis (BCO) in modern broilers: impacts, mechanisms, and perspectives

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Abstract: Bacterial chondronecrosis with osteomyelitis (BCO) is one of the leading causes of lameness and welfare concerns in the modern broiler. The avian bone physiology and remodeling process is complex and dependent on numerous bone and vascular cell types and pathways working harmoniously. The rapid growth rate of the leg bones in the modern broiler coupled with high body weight gain and high metabolic demands have predisposed the broiler to damage within the bone, opportunistic bacterial infection, and subsequent BCO. The maturation and ossification of chondrocytes at the growth plate epiphyseal cartilage junction is critical for leg soundness and defense against mechanical stressors and is ongoing throughout a broiler's life span. The mitochondria play a critical role in stem cell maturation and differentiation in bone and their dysfunction has been implicated in numerous diseases, including type 2 diabetic osteomyelitis and osteoporosis. An understanding of the dynamics between bone physiology and bacterial infection along with mitochondrial dynamics and function in disease states could give more insights into the etiology of BCO and subsequent development of effective strategies to preventing/treating BCO pathology. This review will address the impacts of BCO as well as the physiology involved while highlighting the need for mechanistic understanding of BCO at the

cellular level which could address the potential role of mitochondrial dysfunction and BCO pathogenicity.

Keywords: broilers, lameness, osteomyelitis, osteochondrosis, mitochondria, mitochondrial dysfunction, avian bone physiology

Review Methodology: Data for this review were compiled using databases such as NCBI PubMed, University of Arkansas Library Quick Search, run by WorldCat, Science Direct, and JSTOR. Search terms used included "bacterial chondronecrosis with osteomyelitis (BCO)", "broilers", "femur head necrosis", "femur head separation", "lameness in broilers", "avian bone physiology", "avian anatomy", "avian bone remodeling", "endocrinology of bone remodeling", "causative agents of BCO", "*S. aureus* infection", "mitochondrial dysfunction", "mitochondrial function", "mitochondrial dysfunction and bone", "mitochondrial dysfunction and disease", and "mitochondria and bacterial infections". Manual of Ornithology, by Noble Proctor and Patrick Lynch, was sourced from the author's personal library.

Introduction: In order to address lameness in modern broilers, a basic understanding of bone physiology is necessary. Although genetics has successfully selected for a faster growing bird, selection for leg health traits such as gait scores is novel, and the avian bone consists of numerous cell types and structures that genetic selection has yet to explore. In the case of BCO, there are numerous hypotheses surrounding the involvement of the skeletal system and the ultimate bacterial infection of the femur head. This review will explore not only the impact of BCO on the poultry industry in terms of production losses due to lameness, but also the increasingly more pressing loss in animal welfare. The physiology behind BCO will be reviewed before exploring the causative agents and current theories on BCO pathogenicity. In this review, we will summarize recent evidence supporting the pivotal role of mitochondria in bone disease

and highlighting the need of further in depth mechanistic studies delineating mitochondria dysfunction and BCO development, which might allow the discovery of new metabolic and signaling pathways as effective preventive/therapeutic targets.

Impacts of BCO on the poultry industry

The modern broiler is the most efficient meat bird in history in terms of feed conversion and muscle development. This has been achieved through not only improved husbandry and nutrition, but also intense genetic selection for performance traits. The success of the broiler in performance is marred by numerous health and welfare disorders. Recent studies put the prevalence of birds with severe gait impairment anywhere from 5.5 to 48.8%. BCO is the most common cause of severe leg disorders in broilers and a 2017 study found 28% of necropsied birds from 20 commercial farms exhibited BCO lesions [1, 2]. Although lameness has shown to be associated with BCO, it has also shown to not be a sufficient indicator of BCO as numerous studies show incidence of BCO in sound/normal gait, unchallenged birds [3–6]. The greatest threat of BCO is the subclinical birds. Currently, there is no method for diagnosing BCO in live birds. Therefore, BCO has persisted in breeding programs and a portion of BCO affected birds go undetected in commercial flocks. BCO has also become a serious welfare concern as it is considered painful for the bird even when subclinical but especially when lameness is exhibited [7, 8]. This concern is not only for the betterment of the birds, but also the success of producers as lameness has increasingly become a measurement of welfare in audits of poultry producers [9]. A better understanding of the mechanism behind BCO could provide targets for treatment and prevention to improve overall production and welfare of the broiler.

Overview of avian physiology involved in BCO

Bone structure/anatomy

Avian bone is a dynamic and complex tissue essential for not only overall growth of the bird, but also for immune health, reproductive effectiveness, and proficient movement. The avian leg consists of four bones, not including the metatarsals of the foot, and demonstrates avian skeletal evolution. In birds, the tibia and tarsals have fused forming a tibiotarsus joined at the distal end of the femur along with the fibula forming the knee joint. This fusion is also seen below the ankle or "heel" joint where the tarsometatarsus is the result of fusion between tarsals and metatarsals [10]. This fusion of the lower bones within the leg is what makes birds digitigrade, meaning they walk on their toes. Fusion of bones is seen throughout the avian skeletal system and aids in shock absorption from flight takeoff, landing, and running in the legs [10, 11]. The proximal end of the femur joins the pelvic girdle at the acetabulum in an area known as the trochanter of the femur. The distal end joins the patella and cnemial crest of the tibiotarsus (hereafter referred to as the tibia) to form the knee joint [12].

The structure of the avian bone starts with an outer shell of cortical bone which surrounds the marrow space and trabecular or cancellous bone. The trabecular bones have higher turnover rates due to the increased surface area of their lattice-like organization [13]. Bone can either be lamellar or woven, with lamellar being a combination of corticoid and cancellous bones. As a mature form of bone, lamellar bone is deposited more slowly than woven bones, which is deposited during healing processes and intramembranous ossification [14–16]. A single leg bone has several regions starting with the largest section, the diaphysis which spans the center of the bone and multiple bone sections. Within the diaphysis region, there is a medullary cavity which develops from trabecular bone thinning and resorbing [3, 4]. Surrounding the medullary cavity is

endosteal bone leading into the metaphysis region above the diaphysis shaft of the bone toward the proximal and distal ends. Here, the resorptive zone is where the trabecular bone is resorbed and is surrounded by periosteal bone. The metaphysis region continues through a bone cell calcifying zone until the growth plate or physis, where elongation of the bone occurs due to cell division and maturation. Within the growth plate, the hypertrophic zone is followed by a prehypertrophic zone, a proliferating zone and ends at a resting zone up against the epiphyseal region consisting of a hyaline zone and articular cartilage cap [3, 4].

These zones and regions are often delineated by the vascular structures within them (Figure 1). Unlike mammals, avian growth plates are highly vascularized and are primarily supplied by the ischiadic artery and partly by the external iliac artery [10, 22]. Blood enters the bone in two regions, the epiphysis and the diaphysis. In the epiphysis, the epiphyseal vascular supply travels through epiphyseal canals through the hyaline zone or junctional canals through the hyaline zone and down toward the growth plate. The terminal branches of this vasculature form epiphyseal vascular capillary complexes if they terminate within the hyaline zone or penetrating vascular capillary plexus if they are penetrating epiphyseal vessels traveling through junctional canals ending at the growth plate. The penetrating vascular capillary plexus brings blood to the resting, proliferating, and prehypertrophic zone within the growth plate and serve as direct physiological links between the ever-shifting growth plate and the harder articular cartilage cap. In the diaphysis region, the nutrient artery enters at the nutrient foramen and splits into ascending and descending branches that continue to divide inside the diaphysis forming metaphyseal vessels that penetrate the metaphysis (Figure 1). These vessels terminate as metaphyseal vascular capillary plexus and bring blood to the calcifying zone. In addition to the vasculature terminating in specific regions, there is some evidence that occasional or even putative transphyseal vessels

penetrate through both the hyaline cartilage and the entire growth plate although it also resembles scar tissue left by retreating penetrating epiphyseal vessels. These conflicting observations warrant further investigation into communication between arterioles and veins within the physeal-metaphyseal interface [17–20].

Cell types and functions

The regions of the bone also correlate with differing cell types and stages of cellular maturation. The major cell types that make up the mineralized connective tissue that is bone are osteoblasts, lining cells, osteoclasts, and osteocytes [23–25].

On the surface of the bone, cuboidal cells, mature osteoblasts, derive from mesenchymal stem cells of the bone marrow in which the commitment toward osteoprogenitor cell lineage requires specific gene expression and synchronized synthesis of specific proteins [24, 25]. Osteoblasts are primarily found in the areas of active bone formation as their primary function is to synthesize components of the extracellular matrix in bone. To accomplish this, a complex arrangement of osteoblasts in contact via adherens and gap junctions connect to microfilaments and enzymes involved in intracellular secondary messenger systems [24]. Along with numerous secretory vesicles, osteoblasts contain abundant rough endoplasmic reticulum and Golgi apparatus for the purposes of protein synthesis, modification, and transportation during bone matrix formation [25].

Osteoclasts differ from osteoblasts in several ways. Foremost, osteoblasts' main function is of bone resorption for the purpose of bone remodeling in times of growth or mechanical stressors [24]. Additionally, osteoclasts are terminally differentiated multinucleated cells with origins from mononuclear cells in the hematopoietic stem cell lineage [25]. The commitment of

hematopoietic stem cells is influenced by multiple factors, some of which are secreted by osteoblast and osteoprogenitor mesenchymal cells. This relationship to osteoblasts, as well as others, constitutes the balance of bone resorption and formation needed for effective remodeling of the skeletal structure due to growth, metabolic needs, or in response to mechanical stressors. Osteoclasts are also highly secretory cells which form either denovo multinucleated osteoclasts through fusing together or a precursor pool by remaining mononuclear cells for future recruitment. During bone remodeling, osteoclasts polarize and have four distinct membrane domains, two of which are in contact with the bone matrix, the sealing zone, and ruffled border. These domains only form when osteoclasts are in direct contact with extracellular mineralized matrix and result in depressions known as Howship's lacunae due to resorption [24, 25].

When neither bone resorption nor formation is occurring, flat, elongated cells called lining cells cover the surface of bone and are considered post proliferative osteoblasts. The lining cells act as a barrier preventing osteoclasts from contacting bone matrix and beginning resorption. Within the bone, osteocytes make up most of the cells found in bone and are defined by their location in the bone and morphology. Their dendritic morphology is elongated in cortical bone and rounded in trabecular bone. Osteocytes are "entrapped osteoblasts" incorporated in the matrix. These cells make up the majority of the diaphysis region in the bone and contribute to the remodeling of bone within the shaft.

The metaphysis and growth plate regions contain cellular structure and cell types responsible for longitudinal growth. Longitudinal growth is accomplished through endochondral ossification which involves the replacement of the cartilage within the growth plate with bone tissue. Chondrocyte cells are the most prominent cell within cartilage and secrete most of the components in the cartilage extracellular matrix. Cartilage extracellular matrix consists primarily

of aggregates of aggrecan and glycosaminoglycan hyaluronan all packed among collagen type II fibrils [26, 27]. These components contribute greatly to growth through not only mechanical stability, but also the ability of the growth plate to withstand compression [28]. The zones within the growth plate and metaphysis region correspond to the stages of chondrocyte hypertrophy, cell growth, and proliferation, cellular division. Immature chondrocytes make up the resting zone of the growth plate directly beneath the epiphyseal region. Below the resting zone is the proliferating zone were post-hypertrophic chondrocytes proliferate before entering the pre-hypertrophic layer. After prehypertrophic, enlarged chondrocytes, hypertrophic chondrocytes make up the hypertrophic zone which is nonvascular. The calcifying zone consists of chondrocytes organized in columns in numerous cellular states. Within the calcifying zone, chondrocytes become degenerative before undergoing apoptosis and subsequent calcification. Also within the calcifying zone, metaphyseal vascular capillary plexus and metaphyseal vessels run along either side of the chondrocyte columns. Once in the hypertrophic zone, extracellular matrix separates the chondrocytes before they undergo ossification. Chondrocytes can also be found in small amounts the hyaline cartilage of the epiphyseal region which is primarily comprised of dense cartilage extracellular matrix [29]. The function for the articular cartilage, found at the most proximal or distal end of the long bone, is to provide lubrication and decreased friction for articulation of the joint and aid in transmission of weight [30].

Bone remodeling

The fast growth and abundant meat yield seen in the modern broiler may be the focus of performance, but it is the rapid remodeling of skeletal structures that most contributes to growth in any organism. In birds, bone remodeling is best known to occur in the medullary cavity to provide calcium needed for eggshell formation [31]. However, in the modern broiler, selective

breeding has resulted in an accelerated early skeletal development along with rapid muscle growth and weight gain. As early as 1950, researchers established the relationship between weight gain and skeletal development with over 98% of the variability in femur length attributable to weight [32]. In 1997, it was determined that as weight increases, bone remodeling escalates leading to thicker bone [33]. In particular, the modern broiler's leg bones have a distinctly rapid rate of elongation via growth plates and expansion via remodeling of the cortical bone. The long bones in the modern broiler increase four-fold in length and three- to five-fold in mid shaft diameter within 6 weeks of age [34].

In addition to the rapid growth, longitudinal bone growth continues in broiler breeders until 25 weeks of age and broilers reach market weight within 5–8 weeks [16]. This indicates that broilers do not reach a stage of homeostasis and remodeling but instead undergo development of bone through endochondral ossification while simultaneously reaching peak muscular growth within their short lifespan. Bone elongation and endochondral ossification occur due to chondrocyte proliferation and hypertrophy along with extracellular matrix secretion [28]. Proliferating chondrocytes are responsible for new growth and secrete large aggregating sulfated proteoglycans, including aggrecan, and become embedded in fibers consisting of primarily type II collagen [35–37]. These components, along with many other, including growth factors, cytokines, and prostaglandins, make up the extra cellular matrix which encases proliferating chondrocytes. As they mature, the secretions and morphologies of the chondrocytes change and become hypertrophic chondrocytes, enlarging and eventually undergoing terminal differentiation [38, 39]. Secretions of collagen type X, in addition to type II, along with fibronectin, annexin, and small molecular proteoglycan components such as decorin and byglycan are produced by chondrocytes within the hypertrophic zone [40–42]. There is also a high level of alkaline

phosphatase activity within this zone that is associated with mineralization. Mineralization occurs in the epiphyseal plates and is regulated spatially and temporally [35]. Aging chondrocytes that have used up all remaining glycogen begin to concentrate calcium via their mitochondria. The mitochondria simultaneously shift to anaerobic metabolism along with changes in oxygen tension and glycolytic enzymes [43]. Matrix vesicles containing alkaline phosphatase, phosphatidyl serine, a complex of calcium and phosphorus, and annexins II, V, and VI are shed by the hypertrophic chondrocytes [44–46]. The complex acts as the nucleation site for calcification, while annexins are collagen binding, acting as anchors to the extra cellular matrix and mediating calcium influx into the vesicles [45, 47]. The inclusion of zinc and copper into the matrix vesicles is thought to aid in maintaining an undefined shape of the mineral until hydroxyapatite crystallization is initiated [45, 48]. The initiation occurs within the vesicles and ultimately ruptures the vesicle to begin growth along the collagen type I fibers that were previously synthesized by the hypertrophic chondrocytes [39]. Studies have shown that some chondrocytes secrete angiogenic factors like vascular endothelial growth factor and stimulate invasion of the vascular system into the cartilage matrix [49, 50]. The vasculature that invades the cartilage simultaneously brings osteoblasts to initiate bone formation through binding to the mineralized cartilage matrix and secretion of components of osteoid which provides the crosslinking for hydroxyapatite crystals to grow along [25, 51]. As the osteoid is laid and bone forms, vessels which previously supplied areas of new growth must retreat timely to allow the transition of tissue within the bone and maintain blood supply to the new regions of growth. The retreat of epiphyseal vessels from the growth plate during growth results in eosinophilic streaks which were once thought to be means of communication between epiphyseal and metaphyseal vessels across the growth plate [20].

Physiologically, bone remodeling is regulated and influenced by several growth and metabolic pathways related to nutritional state, weight, and age. Growth hormones are some of the key factors influencing proliferation of chondrocyte cells in the proliferation zone of the growth plate. Thyroid hormones regulate fibroblast growth factor receptor signaling during chondrogenesis [52]. Endochondral ossification is regulated by several transcription factors including runt-related transcription factor 2 (RUNX2) and Osterix (SP7) [53]. RUNX2 has a connection with chordate, channel-forming protein known as Pannexin 3 (PANX3) in osteoblasts and chondrocytes. Pannexins are homologous to invertebrate gap junction proteins known as Innexins [54–58]. PANX3 has been shown to form hemichannels that may be required for the differentiation of hypertrophic chondrocytes to osteoblasts in late stage bone growth [59]. Fibroblast growth factor receptors (Cek1, Cek2, Cek3, and FREK) are present in proliferating chondrocytes and known to be key regulators of endochondral bone growth. Fibroblast growth factor ligands (FGF-2 and FGF-4) are present in the epiphyseal growth plate and have been shown to stimulate chondrocyte proliferation [60]. Nutritionally, polyunsaturated fatty acids of the n-3 series (n-3 PUFAs) have shown beneficial effects on bone formation such as stimulation of osteoblast function in cell-culture studies and fish oil is often fed to improve bone growth and as a preventative to certain leg disorders. Calcium, phosphorus, and vitamin D are also fed to broilers to improve and aid in bone formation since both minerals are needed in abundance and vitamin D3 acts on both osteoblasts and chondrocytes and stimulates production of osteoclasts all for bone remodeling [61, 62].

Current theories on the pathogenesis of BCO and modeling BCO

Tissue damage and bacteria infection

The BCO pathogenicity has been extensively studied, starting in the 1930s with hypotheses surrounding routes of infection by bacteria [63]. The causative route of infection that leads to the bacterial colonization of BCO has been the focal point for many studies and been hypothesized to be through wounds and abrasions on the feet and skin, insect bites, nasal cavity adhesion of particular *Staphylococcus* strains, latent staphylococci being triggered by stressful rearing conditions, the blood, and mechanical damage or a disease state that favors bacterial colonization [63–71]. The use of "floor eggs" has also been implicated in the development of BCO through exposure to feces in the litter resulting in increased bacterial infection of staphylococcal strains compared to chicks hatched from eggs in the nest boxes. This coincided with increased incidence of BCO in floor-egg-hatched birds [72].

In an attempt to develop an experimental model of BCO, these potential routes of infection were further investigated and utilized. In the case of respiratory transmission, aerosol inoculation with causative bacteria was investigated as a model for BCO. Aerosol inoculation has been both successful and unsuccessful at inducing osteomyelitis, but has proven to be dependent on other factors such as simultaneous viral infection and virulence factors of bacterial strains used [68, 70]. However, a successful model using aerosol found that slow-growing genotypes were unaffected by aerosol inoculation [72].

In 1973, it was shown that intravenous injections of *Staphylococcus aureus* resulted in osteomyelitis lesions matching the spontaneous disease in turkeys [73]. In the continued search for an effective experimental model, this method was used in broilers, first as a model of

osteomyelitis in infants, and later for BCO [74–76]. The success of this model indicates that BCO is a result of bacterial infection via the growing ends of metaphyseal blood vessels within the growth plate in the hypertrophic region responsible for longitudinal growth of the bone. The attenuated endothelium of the capillary tips of these blood vessels are known to be fenestrated and often discontinuous and believed to provide the contact point between bacteria in the blood stream and the cartilage matrix [20, 77]. Spread of bacterial infection has been hypothesized to be due to transphyseal blood vessels which connect the metaphyseal and physeal vessels to the epiphyseal vessels within growing broilers [75].

An immunosuppression and stress-mediated model for inducing BCO has also been investigated and shown successful. Immunosuppression has been implicated in commercial outbreaks of BCO [68, 70, 72, 78–82]. In 2012, Wideman and Pevzner successfully induced spontaneous BCO through dexamethasone injections, a synthetic glucocorticoid, and a model already proven to induce turkey osteomyelitis complex. Also, in 2012, Wideman developed a wire flooring model for spontaneously inducing BCO in boilers without the use of bacterial or glucocorticoid injection. This model works under the hypothesis that fundamental weakness at the cartilagegrowth plate junction, coupled with mechanical stressors, in the form of unstable footing on wire floors, leads to chondrotic cleft formation, or “wound sites” [3, 4]. These wound sites expose the cartilage matrix to opportunistic, collagen binding bacteria and often intersect blood vessels within the growth plate leading to exposure to circulating bacteria and areas of decreased blood flow [3, 4]. This model also is believed to spontaneously induce immunosuppression through stimulating chronic stress by depriving birds’ access to floor liter [4, 83]. The ability to spontaneously induce BCO without introducing a specific bacterium gives the wire-flooring model better mimics commercial environments as numerous opportunistic bacteria have been

identified in BCO affected tissue, which will be discussed in more detail in the proceeding section. The efficacy of this model is further supported by physiological studies of broiler bone remodeling and growth rates.

In the modern broilers, bone remodeling, as previously described, occurs at a rapid rate. Applegate and Lilburn demonstrated how the length of the femur and tibia in the modern broilers increases approximately four-fold along with the diameter of the mid-shaft increasing anywhere from three- to five-fold within the first 6 weeks post-hatch [34]. Broiler's rapid growth has been extensively documented. Along with the proclivity of broilers to develop lameness when compared to egg laying breeds [21, 84–89]. A recent RNASeq study sampled 42-day-old male broilers with separation of the articular cartilage cap from the growth plate and "initial," non-necrotic BCO lesions in both tibias as an affected group and attached cartilage cap and absence of lesions as unaffected. The results showed osteochondral genes downregulated in BCO affected tibia compared to unaffected bone. One-twenty nine downregulated genes were investigated and found to be associated with nine biological processes involving skeletal system, connective tissue and cartilage development along with organ and skeletal system morphogenesis [90]. Similar findings were found in a RNASeq study of the femur head, specifically femur head separation (FHS). With similar sampling parameters, 160 downregulated and 154 upregulated genes were found in FHS affected birds and associated with biological processes such as cell adhesion, extracellular matrix, bone development, blood circulation, lipid metabolism, cell death, and immune response [91]. These findings further support the hypothesis that a structural weakness at the growth plate epiphyseal cartilage junction leads to a predisposition to wound sites and bacterial infection seen in BCO. This would suggest that bacterial infection is secondary and corresponds with a late, necrotic stage of BCO.

Current identified causative agents and potential underlying mechanisms in BCO

The bacterial component of BCO has been associated with development of necrotic bone and subsequent lameness in the modern broilers. The most commonly found bacterium associated with BCO is *Staphylococcus aureus*. However, numerous opportunistic bacteria have been identified including *Staphylococcus hyicus*, *Staphylococcus xylosus*, *Staphylococcus simulans*, *Staphylococcus agnetis*, *Mycobacterium avium*, *Salmonella spp.*, *E. coli*, and *Enterococcus* [82, 92–94]. These bacteria are often in mixed cultures and form complex microbial communities in bone, including bone that appears to be normal [4, 95]. Studies of these communities have found that microbial communities differ from the tibia to the femur as well as among severity of lesions. In the presence of BCO lesions, the microbial community is comprised of primarily *Staphylococcus*, *Enterobacter*, and *Serrotia* indicating that BCO is associated with decreased microbial diversity in the bone [4, 95].

The genus *Staphylococcus* is a Gram-positive, halotolerant bacteria that are prevalent on skin and mucosal surfaces [96]. *Staphylococcus aureus* is the most pathogenic species within the genus and is the only species that produces a protein called coagulases which affect fibrinogen [96]. *S. aureus* has been well studied in the case of osteomyelitis and has been found to be one of the most prevalent in avian BCO. Another member for the *Staphylococcus* genus that has been associated with BCO is *Staphylococcus agnetis*. The *S. agnetis* 908 strain was successfully isolated from BCO affected birds, and its genome has been investigated for virulence factors affecting its pathogenicity [97, 98]. *S. agnetis* 908 has also been used in *in vitro* models for BCO using bone cells [99]. *Staphylococcus* species are known to incite bone destruction, soft tissue inflammation in the surrounding area, and spread throughout the bloodstream. *S. aureus* invades and persists within osteoblasts causing physiological changes that promote bone resorption. Bone

resorption is promoted through increased expression of the osteoclast-activating molecule receptor activator of NF-$_K$B ligand (RANK-L) and pro-inflammatory cytokines. In a second mode of favoring bone resorption, *S. aureus* within osteoblasts results in cell death. The specific mechanism for cell death is not fully understood; however, microcomputed tomography of BCO affected femurs showed that the bacterial regulatory locus of *S. aureus* Sae was critical to pathogenesis. Sae-regulated factors not only favor bacterial survival within the bone, but also promote pathological bone remodeling [100]. Osteoblast cell death and bone destruction are triggered by aureolysin, which is a Sae-regulated secreted protease that self-activates when secreted and initiates the proteolytic activation cascade [100, 101]. *S. aureus* also produces toxins such as poreforming toxins (PFTs), α-toxins, and leukotoxins designed to avoid elimination by host defense using different mechanisms [102,103]. Leukotoxins act in both a receptormediated and receptor-independent manner to target white blood cell lysis [102]. PFTs not only disrupt membrane barriers of eukaryotic cells to cause host cell lysis, but also exert subtle changes to cell activity and physiology through toxin receptors [103].

The role of the bacterial communities in the pathogenicity of BCO in the avian species is unclear. The presence of communities in healthy bone as well as the spontaneous development of BCO on wire flooring, as well as RNASeq showing differentially expressed genes involved in cartilaginous and skeletal development, point to a need for bone abnormalities to be present for the bacterial effects to take hold. Regardless of the specific role bacteria play on BCO of the modern broiler, opportunistic bacteria overwhelm the mechanically and/or metabolically stressed femoral head of the growth plate leading to decreased bacterial diversity and severe necrosis of the bone.

Mitochondrial dysfunction and disease

The mitochondria are organelles found in almost every eukaryotic cell and are responsible for not only energy production, but also a cell death regulator [104], with some of the key pathways illustrated in Figure 2. Functioning mitochondria can change their shape, position, and number within the cell in order to facilitate numerous cellular processes including calcium signaling, cellular proliferation and differentiation, thermogenesis, and cell death [105, 106]. This is done through three major facets of mitochondria: mitochondrial biogenesis, function, and dynamics (fission or fusion) [107]. These three facets work harmoniously in healthy cells and their dysregulation has been implicated in numerous diseases and disorders and termed mitochondrial dysfunction.

Mitochondrial dysfunction can be defined as alterations in the function of the electron transport chain, the maintenance of the electrical and chemical transmembrane potential or a decrease in the transport of metabolites into the mitochondria [108]. This dysfunction leads to reduced oxidative phosphorylation efficiency and subsequently reduced adenosine-5′-triphosphate (ATP) (Figure 2). Mitochondrial efficiency and function are dependent on a balance of mitophagy and biogenesis [107, 109]. Biogenesis and other mitochondrial processes are controlled and conducted by proteins encoded by both nuclear and mitochondrial genomes [109]. Proteomic and genomic analysis has shown that the mitochondria are comprised of over 1,000 proteins with the number varying between species [110, 111]. Mitochondrial biogenesis not only results in variations in mitochondrial number but also size and mass [109]. The transcription cascade of mitochondrial biogenesis is regulated by numerous transcription factors including PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), nuclear respiratory factors (NRF1 and NRF2), and mitochondrial transcription factor A (Tfam). Biogenesis is stimulated through

PGC-1α through cold exposure and exercise and its dysregulation has been shown in disease states [112–114].

Mitochondria are dynamic organelles that divide and fuse to meet the metabolic and stress needs of the cell. Mitochondrial fusing (fusion) and division (fission) counteract aging through completion of gene products after fusion of impaired mitochondria and acts as organelle quality control by dividing and removing damaged mitochondria and is also a step in apoptosis [115, 116]. Fusion of mitochondria mitigates stress through mixing damaged components as a form of complementation and fission enables removal of damaged mitochondria as well as apoptosis at high levels of cellular stress [116]. Fission is mediated by cytosolic dynamin family member (Drp1/ Dnm1) which is recruited by MFF1 (mitochondrial fission factor) and then fission is supported by mitochondrial fission process 1 (MTFP1), a protein found of the inner membrane [117–119]. Mitochondrial fission regulator 1 protein (MTFR1) not only regulates fission, but also been shown to regulate antioxidant activity in vivo [120,121].

Mitochondrial function involves the efficiency of oxidative phosphorylation, through maintenance of mitochondrial membrane potential and ATP-producing machinery, and the absence of mitochondrial DNA (mtDNA) damage. Dysfunction of oxidative phosphorylation leads to oxidative stress as reactive oxidative species accumulates which can lead to mtDNA damage and dysfunction, as well as apoptosis [122, 123]. Decreased production of ATP through loss of mitochondrial basic functions leads to activation of AMP-activated protein kinase (AMPK) signaling which stimulates biogenesis through PGC-1α [124]. All mitochondrial processes work cooperatively to meat metabolic demands and adapt or respond to environmental stressors and without this cooperation and function, disease states can be reached.

Mitochondrial dysfunction has been found to underly more than 50 inborn errors of metabolism [125, 126]. In the broiler, mitochondrial dysfunction has been implicated in pulmonary hypertension syndrome (ascites), heat stress effects, high dietary copper effects, and ammonia inhalation damage to the bursa of fabricius [127–132]. Growing evidence of its implications in the etiologies of several diverse diseases, including type 2 diabetes, neurodegenerative diseases such as Alzheimer's, and cancer, point to the farreaching effects of the mitochondria on cellular processes in all tissues [133–135]. Type 2 diabetes is one of the most common conditions associated with osteomyelitis and often occurs in the foot of type 2 diabetes patients [136, 137]. It has also been shown that chronic osteomyelitis increases the incidence of type 2 diabetes and an estimated 60% of diabetic foot ulcers are infected and more than two thirds of diabetic patients with foot ulcers have lower limb amputations due to infection [138]. Mitochondria are key players in bone homeostasis through their involvement in regulation of stem cell differentiation and maturation which occurs rapidly and in multiple cell types of growing bone. As previously described, the maturation and ossification of chondrocytes is key to proper cartilage and bone formation and mitochondrial damage has been linked to osteoporosis [36, 139]. The mechanism behind potential role mitochondrial dysfunction plays in bone related diseases has not been fully investigated. In BCO, a study has shown ROS accumulation coinciding with inflammasome activation in BCO affected tissue and *in vitro* model [92]. As ROS is a key indicator of mitochondrial dysfunction and modern broiler bones demand mitochondrial function during rapid growth, further investigation into this disease and mitochondrial function is warranted. Mitochondrial dysfunction has become a key player in the etiology of numerous metabolic and physiological diseases with far-reaching implications and dynamic mechanisms.

Conclusions and perspectives

The modern broiler's high performance of growth and muscle development has pushed the bounds of physiology to a point that is vulnerable to environmental stressors and disease. A fundamental weakness in growth plate and cartilage junctions in fast-growing bone predisposes broilers to circulating opportunistic bacteria and subsequent necrosis in what is described as BCO. The mechanism of BCO is not yet fully understood although implications of osteochondrotic genes in the pathogenicity of BCO coupled with mitochondrial involvement in stem cell maturation warrants investigation into the potential role mitochondria play in BCO in the modern broiler.

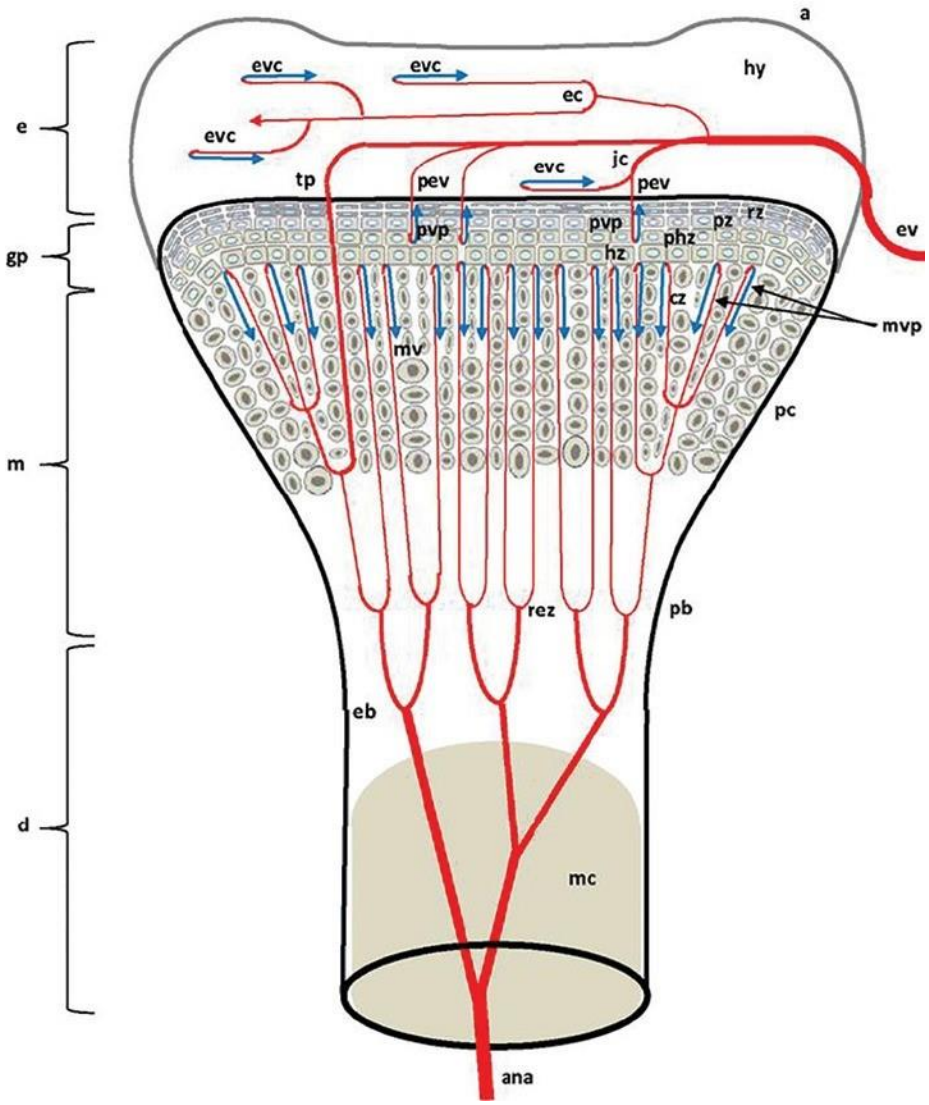

Figure 1. Proximal head of growing bone. Epiphyseal vascular supply (ev); epiphyseal vascular canals (ec); hyaline zone (hy); epiphysis (e); junctional canals (jc); growth plate (gp) or physis; epiphyseal vascular capillary complexes (evc); penetrating epiphyseal vessels (pev) that terminate as a penetrating vascular capillary plexus (pvp) supplying the resting zone (rz), proliferating zone (pz), and prehypertrophic zone (phz); nutrient artery (ana) forms metaphyseal vessels (mv) within the metaphysis (m) which then terminate as the metaphyseal vascular capillary plexus (mvp) supplying the calcifying zone (cz); the diaphysis (d); hypertrophic zone (hz); transphyseal vessels (tp). This figure was first published in Wideman RF, Prisby RD "Bone circulatory disturbances in the development of spontaneous bacterial chondronecrosis with osteomyelitis: a translational model for the pathogenesis of femoral head necrosis" in Frontiers Endocrinology, 2012 and was made through references of original sources [17–21] (Wise and Jennings, 1973; Howlett, 1979, 1980; Duff, 1984; Thorp, 1986; Ali, 1992; Farquharson and Jefferies, 2000).

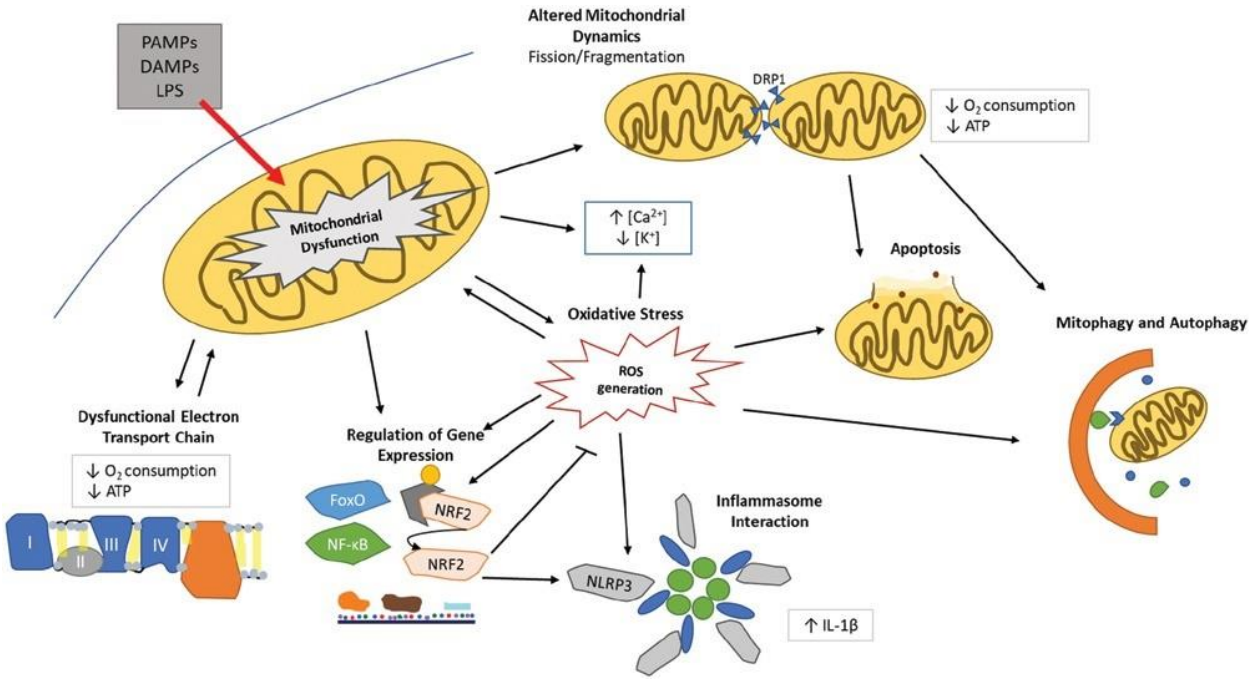

Figure 2. Mitochondrial dysfunction and its affects. Mitochondrial dysfunction in relation to environmental and foreign stimuli resulting in numerous effects such as gene regulation, ATP production, and cell survival. DAMP, damage-associated molecular partners; DRP1, dynamin-related protein 1; Foxo, forkhead box O; NLRP3, NLR family pyrin domain containing 3; ROS, reactive oxygen species

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Chapter 3 –

Title: Evidence of Mitochondrial Dysfunction in bacterial chondronecrosis with osteomyelitis (BCO)-affected broilers

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Abstract: A leading cause of lameness in modern broilers is bacterial chondronecrosis with osteomyelitis (BCO). While it is known that the components of BCO are bacterial infection, necrosis, and inflammation, the mechanism behind BCO etiology is not yet fully understood. In numerous species, including chicken, mitochondrial dysfunction has been shown to have a role in the pathogenicity of numerous diseases. The mitochondria is a known target for intracellular bacterial infections, similar to that of common causative agents in BCO, as well as a known regulator of cellular metabolism, stress response, and certain types of cell death. This study aimed to determine the expression profile of genes involved in mitochondrial biogenesis, dynamics and function. RNA was isolated form the tibias from BCO-affected and healthy broilers and used to measure target gene expression via real-time qPCR. Mitochondrial biogenesis factors PGC-1α and PGC-1β were both significantly upregulated in BCO along with mitochondrial fission factors OMA1, MTFR1, MTFP1, and MFF1 as well as cellular respiration related genes FOXO3 and FOXO4 and av-UCP. Conversely, genes involved in mitochondrial function, ANT, COXIV and COX5A showed decreased mRNA expression in BCO affected tibia. This study is the

first to provide evidence of potential mitochondrial dysfunction in BCO bone and warrants further mechanistic investigation into how this dysfunction contributes to BCO etiology.

Key words: mitochondrial dysfunction, lameness, broilers, chondronecrosis, osteomyelitis, BCO

Introduction

Bacterial chondronecrosis with osteomyelitis (BCO) is a prominent cause of lameness in the poultry industry which predominantly affects fast growing, large broilers. Lameness in modern broilers poses both an animal welfare and a production concern as it has been shown that higher incidence of lameness is associated with suboptimal environments leading to other associated economic and health concerns including condemnations postmortem and footpad dermatitis [1]. In the case of BCO related lameness, points of high mechanical stress within the avian skeletal system include the proximal femur head and are the most common areas for BCO to occur [2,3]. Histological changes in the proximal head of femurs affected by BCO show evidence of wound sites within the epiphyseal region of the growth plate with clear chondronecrosis. These sites often transect vasculature within the highly vascularized avian growth plate leading to decreased blood flow to surrounding areas and an entry point for any circulating, collagen-binding bacteria [2-4]. Numerous opportunistic bacterial species have been identified as causative agents in BCO, the most common being *Staphylococcus aureus* [3,4]. It is unclear if wound sights develop before bacterial infection occurs, but once infected, chondronecrosis is observed at varying degrees. A study of the microbiome within chicken growth plates showed a decrease in the diversity of bacterial communities in BCO affected femurs compared to normal with *Staphylococcus* species predominating, indicating a shift in the make-up of the microbiome with tissue damage and inflammation [5]. Research into mitigating BCO in large broilers has primarily centered around probiotic and feed supplement treatments during production. While

some of these supplements show to be promising, their proposed mechanisms target the bacterial load or entry by either improving gut integrity or improving the overall immune system response without fully investing the etiology of bacterial lead necrosis at the site of infection [6,7]. While further investigation into management and nutritional preventions are valuable, an understanding of the molecular mechanisms involved in BCO has yet to be elucidated and would provide greater insight into treatment and prevention from both a management and potentially genetic standpoint. Although the exact pathology of BCO is debated, the three major components of BCO are clear, bacterial infection coinciding with attrition and inflammation of the bone.

The mitochondria play a pivotal role in energy homeostasis, bone growth and remodeling, and host-cell response to bacterial infection [8]. Mitochondrial dysfunction can be a disruption in mitochondrial biogenesis, ATP production/bioenergetics, mitochondrial fission and fusion pathways, or mitochondrial driven cellular responses to stimuli such as environmental stress, cellular damage, bacterial infection, and inflammation [8]. In the case of intracellular bacterial infection, like that of *S. aureus*, the mitochondria is not only a bacterial target, but also a mode of host-cell defense through accumulation of reactive oxidative species (ROS) [8,9]. Mitochondrial dysfunction has been reported in neurological degenerative diseases such as Alzheimer's and Parkinson's disease as well as metabolic diseases such as type 2 diabetes and associated osteomyelitis [8, 11-13]. For these reasons, we sought to investigate the state of mitochondria in BCO-affected bone of modern broilers. To that end, we assessed the expression of key regulatory genes for mitochondrial biogenesis, dynamics, and function. Our results show, for the first time, evidence of mitochondrial dysfunction in the form of increased mitochondrial biogenesis coactivators and mtDNA, as well as increased expression of known fission regulators and imbalance of gene expression for genes involved in cellular respiration and antioxidant response.

Materials and Methods

Collection of *BCO and Normal Bone Samples*.

All animal experiments were approved by the University of Arkansas (Fayetteville, AR) Animal Care and Use Committee (protocol number 15043) and were in accordance with recommendations in NIH's Guide for the Care and Use of Laboratory Animals. The BCO model and healthy counterparts were conducted as previously described [3,4]. Cobb 500 males were the same animals used previously [14]. Briefly, animals had ad libitum access to fresh water and feed (3.9 Mcal metabolizable energy $kg^{-1}$and 180 g crude protein kg^{-1}) while the experiment took place in the Poultry Environmental Research Laboratory at the University of Arkansas Poultry Research Farm. Ambient temperature was lowered gradually from 32˚C to 25˚C by 21 days of age. A light cycle of 23 hours light/1 hour dark was maintained along with an approximately 20% relative humidity until 56 days of age. At the end of the 56 days, animals were weighed, humanely euthanized, and immediately necropsied to determine presence of subclinical lesions in the proximal heads of both the femora and tibiae. Bone was selected macroscopically based on previously reported scale (3,4). Normal bone was considered free of any necrosis or lesion and BCO-affected bone consisted of bone which had either tibial head necrosis (THN) or severe tibial head necrosis (THNs). Proximal portions of bone, primarily consisting of the growth plate, from both affected and unaffected animals were snap frozen in liquid nitrogen and stored at -80˚C for later analysis.

Real-Time Quantitative PCR

From the normal and BCO-affected bone samples (N=6), total RNA was isolated in accordance with the protocol of Carter et al [15]. Cellular RNA was isolated using Trizol reagent (Life

Technologies, Carlsbad, CA), based on manufacturer's instructions. RNA concentrations were determined using Synergy HT multimode microplate reader and total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithsburg, MD). Amplification was achieved using Power SYBRGreen Master Mix (Life Technologies, Carlsbad, CA) and real-time quantitative PCR (7500 Real Time System; Applied Biosystems, Foster City, CA). The sequences for oligonucleotide primers for *r18s, av-UCP, ANT, NRF1, Ski, FOXO1, PPARα, PPARγ, PGC-1α, PGC-1β, COXIV, COX5a, D-loop (mtDNA), SSBP1, TFAM, MFN1, MFN2, DNM1, OPA1, OMA1, MTFR1, MTFP1,* and *MFF1* were previously published (16). Novel primers used were *FOXO3* (forward, 5'-GCTCCCGGACAAATTCGA-3'; and reverse, 5'-TCGCCAAAATCGGTGACAA-3'), *FOXO4* (forward, 5'-CTGGGATACCGGGTCTTGAG-3', and reverse, 5'-GGCTATCTGTCGATTTGAGTAATGAA-3'), *Keap1* (forward, 5'-CGCCATCTGTTACAACC -3'; and reverse, 5'- GCGTAGATCCCGTCGAT -3').

Real-time quantitative PCR cycling conditions were 50˚C for 2 minutes, 95˚C for 10 minutes and 40 cycles of a two-step amplification (95˚C for 15 seconds followed by 58˚C for 1 minute). The dissociation protocol from the sequence detection system was used for melting curve analysis to exclude potential contamination of non-specific PCR products. Negative controls that were used as templates contained no reverse transcription products. Relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method and healthy bone tissue or untreated cells were used as calibrators [16].

Statistical Analysis

Data were analyzed by Student t-test using GraphPad version 7.03 (GraphPad Software, Inc., LaJolla, CA). Results are expressed as means ±SEM, with P-value < 0.05 set as statistically significant.

Results

Mitochondrial biogenesis-associated genes PGC-1α and PGC-1β were both significantly upregulated in BCO affected tissue as well as mitochondrial displacement loop (D-loop) ($P < 0.05$, Figure 1 a, c, d). Mitochondrial transcription factor A (TFAM) and Ski followed a similar trend although not significantly upregulated (Figure 1e, b). Single-stranded DNA-binding protein 1 (SSBP1) mRNA expression was not significantly different between healthy control and BCO-affected group (Figure 1f). Mitochondrial dynamics indicated several differentially expressed genes including fusion related gene optic atrophy protein 1 (OPA1) expression being significantly down regulated (Figure 2a). Mitofusin 2 (MFN2), another fusion regulatory gene, was significantly upregulated, but MFN1 remained unchanged ($P < 0.05$, Figure 2b, c). The fission related genes, including overlapping with the M-AAA protease 1 homolog (OMA1), mitochondrial fission factor (MFF1) and mitochondrial fission regulator 1 (MTFR1), were not significantly upregulated in BCO-affected tissue (Figure 2d, g, h). Although mitochondrial fission process 1 (MTFP1) expression was decreased, it was also not statistically significant (Figure 2e). Dynamin 1 (DNM1) expression was not affected (Figure 2f).

Gene expression was measured for regulators of mitochondrial function in ATP synthesis and overall respiratory activity (Figure 3). The gene for adenine nucleotide translocator (ANT) as well as cytochrome *c*-oxidase IV and 5A (COXIV, COX5A) were both significantly downregulated in BCO-affected tibia which coincided with a significant upregulation of avian uncoupling protein (av-UCP) ($P <0.05$, Figure 3a, b, c, d). Although Kelch-like ECH-associated protein 1 (Keap1) and Forkhead box O3 and 4 transcription factors (FOXO3 and FOXO4) were not significantly upregulated in the case of BCO, they followed a similar positive trend (Figure 3 e, h, i). Peroxisome proliferator activated receptors α and γ (PPARα and PPARγ) as well as

nuclear factor erythroid 2-related factor 1 (NRF1) were nonsignificant, but upward trending (Figure 3 f, j, k).

Discussion

The role of mitochondria, its biogenesis, dynamics, and function, in disease states has become increasingly more poignant in the research community. In human disease, mitochondrial dysfunction, in the form of mtDNA mutations or disruption in overall mitochondrial biology, has been implicated in diabetes, Parkinson's disease, Alzheimer's disease, dementia, coronary artery disease, chronic fatigue syndrome, and ataxia [8-13,18]. In broiler (meat-type) chickens, research has shown evidence of mitochondrial dysfunction related to susceptibility to ascites and to be influenced by orexin in broiler muscle [19,20]. Mitochondria are also direct targets for some bacterial infections including *Staphylococcus aureus*, one of the most common causative agents in BCO [9, 21]. Mitochondrial dysfunction is believed to be associated with so many disease states due to its relation to apoptotic and inflammatory pathways [10]. This study found evidence of mitochondrial dysfunction in the form of biogenesis regulation, fission or fractionation, and potential disruption of oxidative pathways.

Transcriptional control of mitochondrial biogenesis is heavily regulated via PGC-1α and PGC-1β [22]. These coactivators both target transcription factors as well as act as targets for gene expression in mitochondrial biogenesis pathways [23]. Extracellular signals such as metabolic changes and cell growth are known to modulate their expression, and upregulation of PGC-1α has been shown to coincide with increased mitochondrial biogenesis and respiration in inflammatory states [24,25]. In BCO-affected tissue, both PGC-1α and PGC-1β were significantly upregulated along with a nonsignificant but increased trend of TFAM. This increased expression suggests potential inflammatory or metabolic stressors influencing

increased mitochondrial biogenesis to offset deleterious effects of these states. D-Loop, a measurement of mtDNA, was also significantly upregulated, indicating an increase in mitochondrial DNA in BCO tissue [26]. Although the state and quality of the mtDNA is not known, increased expression of key co-activators, along with increased mtDNA, could point to increased mitochondrial biogenesis in the presence of BCO. Since BCO is a known bacterial infection, its inflammatory response could be the cause in increased need for mitochondria as ROS accumulation and metabolic shifts occur. SSBP1 has been shown to regulate mitochondrial mass in cancer states and to be involved in DNA damage response pathways [27]. SSBP1 expression was not significantly altered in BCO compared to healthy tibia. SSBP1 being not affected decreases the possibility that DNA damage repair is needed in BCO or that mitochondrial production is needed not just an increased mass of mitochondria.

Mitochondria are dynamic organelles with their shape and size shifting in response to numerous stimuli to maintain homeostasis or alter cellular energy states [28]. Mitochondrial fusion is a state in which separate mitochondrial organelles fuse into a larger network and is often seen during stress conditions as it has the capacity to partially mix damaged mitochondria for complementation [28,29]. OPA1 is a key component of mitochondrial fusion found in the inner mitochondrial membrane and its knock down has resulted in blocked mitochondrial fusion and cellular defects including decreased cellular respiration and heterogeneity of mitochondrial membrane [29]. In an ascites susceptible selected line of broilers, OPA1 expression was shown to decrease under ascites conditions in the lungs, thigh, and breast compared to an ascites resistant selected line (19). In BCO, significant decrease in OPA1 mRNA expression was observed although MFN2 was upregulated. Mitofusins (MFN1 and MFN2) are found on the outer mitochondrial membrane and also are necessary for mitochondrial fusion. However,

without OPA1, the inner membrane is unable to fuse and the membrane potential can be implicated [29]. This demonstrates that there exists a potential pathway of mitochondrial dysfunction via interruption of inner membrane fusion through OPA1 dysregulation in BCO. This dysregulation could be due to a shift in cell death pathways in which fracturing of the mitochondria is a key component, or it could be the result of mitophagy as dysfunctional mitochondria are processed by the cells via fractionation [30]. The decrease in OPA1 expression was coupled with a trending increase expression of OMA1, a regulator of mitochondrial fission via "cutting" OPA1 at specific sites to render it inactive [31,32]. Mitochondrial fission enables the removal of damaged mitochondria through fragmentation when complementation via fusion is no longer possible, but also facilitates apoptosis in response to high levels of cellular stress [32]. Although none were statistically significant, all fission gene expression followed a positive trend and analysis of protein expression could further elucidate the potential role of these genes. This increased expression of fission related genes further supports a cellular shift towards fission of mitochondria rather than fusion and suggests a potential state of mitochondrial turn-over in response to the high cellular stress during BCO. MTFR1 overexpression has been shown to promote mitochondrial fission [33]. MTFP1 overexpression has been linked to increased ROS production and it has been shown to aid in mitochondrial recruitment of DNM1 along with MFF1 [34-37]. These results show a decreased expression of a significant inner membrane fission factor OPA1 and point to potential fission/fragmentation of mitochondrial networks in BCO. Further research is needed to understand the potential impacts of this fission favored state in relation to BCO pathogenicity and whether it is a response to bacterial infection or a result of cellular removal and repair of necrotic tissue [37].

The main function of the mitochondria is to produce ATP for the cell. This is done through oxidative phosphorylation via the electron transport chain. Mitochondrial membrane potential as well as function cytochrome-c complexes are necessary for ATP production [38-41]. Key proteins in this process, including ANT, COXIV, and COX5A, were significantly down regulated in BCO affected tissue. Conversely, av-UCP, an uncoupler of ATP production via movement of hydrogen ions across the mitochondrial inner membrane without ATP production, was significantly upregulated in BCO [42,43]. Av-UCP has been shown to be upregulated by pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) via the transcription factors PGC-1α and PPARs [42]. This imbalance of gene expression when compared to unaffected tissue points to potential dysfunction in ATP production in BCO via uncoupling of ATP production a potential loss of respiratory machinery and transporters such as COXIV and ANT. Beyond the machinery directly needed for ATP production, increased FOXO3 expression, although not significant, indicates possible reduction of respiratory activity through its repression of nuclear encoding mitochondrial genes [44]. FOXO4 is a known activator of antioxidant genes, suggesting potential increased ROS as cellular respiration is impaired [45]. PPARα and PPARγ were slightly upregulated, however not significant. PPARs are involved in mitochondrial function and have been implicated in diabetes-related mitochondrial dysfunction [46,47]. Overall, mitochondrial ATP production could be affected in BCO via increased av-UCP as an inflammatory response and decreased expression of mitochondrial transport proteins. This decreased in genes needed for proper mitochondrial ATP production could also be the result of mitochondrial turnover, as indicative of the fission pathways being activated in BCO. Whether a cause for or result of mitochondrial turnover, gene expression suggests dysregulation of ATP production pathways in BCO affected tissue.

Taken together, these results are the first to implicate potential mitochondrial dysfunction with BCO in modern broilers. Based on the expression of key mitochondrial related genes in BCO affected bone, upregulation of mitochondrial biogenesis, a known stress response, as well as mitochondrial fission pathways indicate latent mitochondrial turn over or fractionation coupled with disruption of ATP production machinery and transporters. More research is needed in order to elucidate the relationship between mRNA and protein expression in BCO as well as potential varying gene expressions in the severity of lesions or progression of BCO. In the future, a cell model of BCO would need to be developed in order to investigate and demonstrate the effects of differentially expressed genes on the pathogenicity of BCO including mechanisms of cell death and the extent of bacterial involvement.

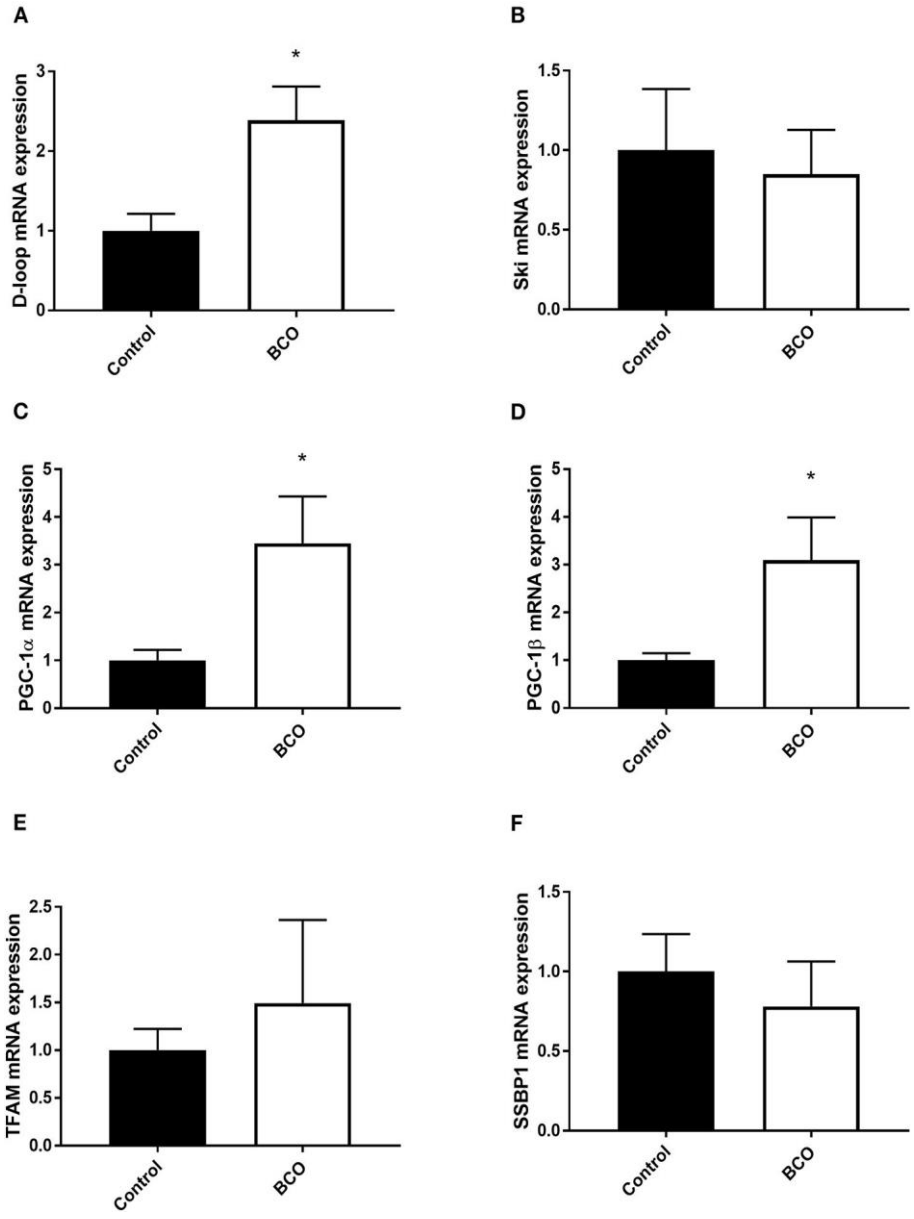

Figure 1. Mitochondrial Biogenesis
RT-qPCR results for a measurement of mitochondrial DNA, D-loop (a) as well as mitochondrial biogenesis regulators Ski (b), PGC-1α (c), PGC-1β (d), TFAM (e), and SSBP1 (f). Student t-test was conducted with significance set at p-value < 0.05. * indicates significant differences between Control and BCO groups.

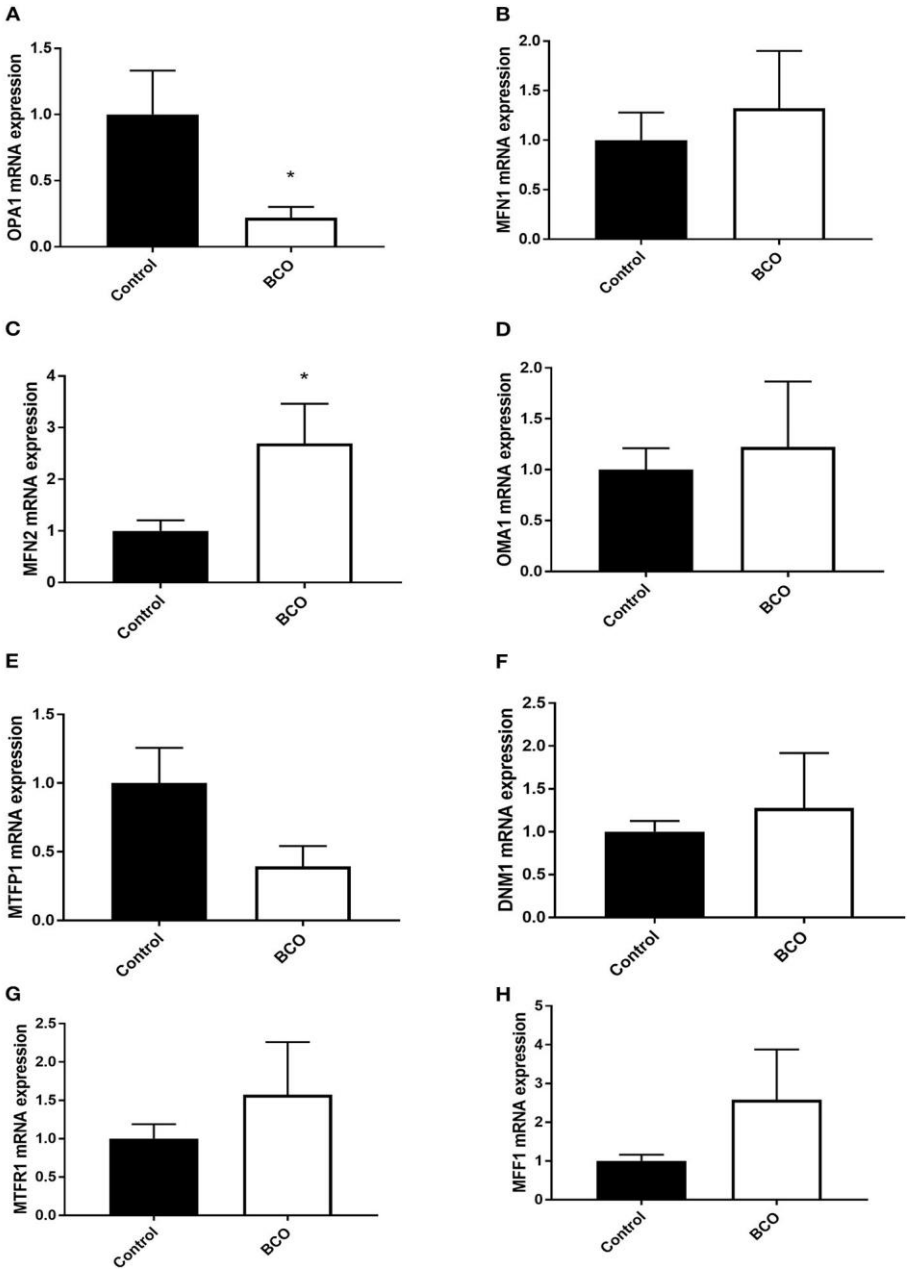

Figure 2. Mitochondrial Dynamics

Gene expression for genes regulating mitochondrial fusion, OPA1 (a), MFN1 (b), MFN2 (c), and fission, OMA1 (d), MTFP1 (e), DNM1 (f), MTFR1 (g), MFF1 (h). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Control and BCO expression.

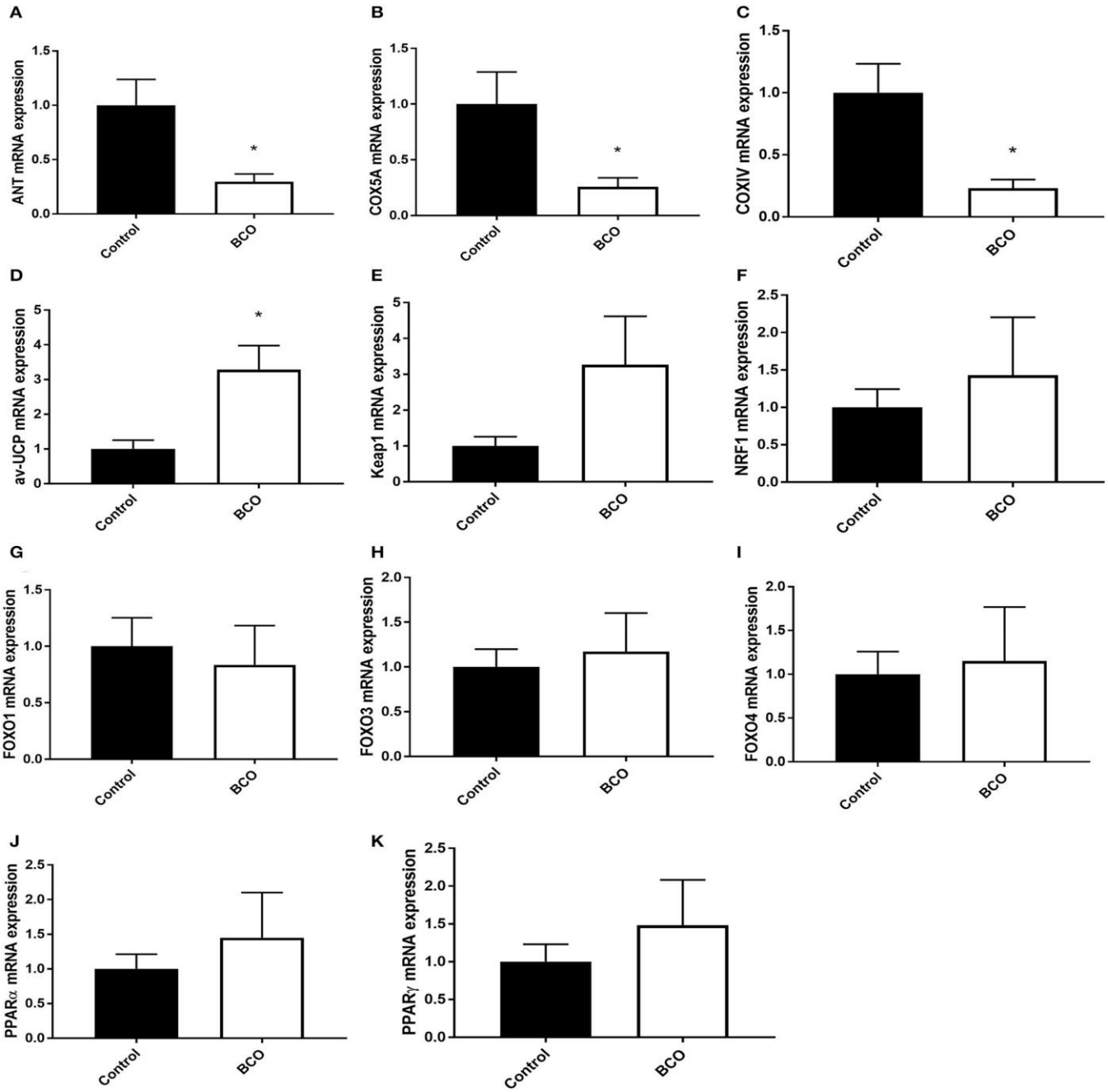

Figure 3. Mitochondrial Function

Gene expression for genes regulating mitochondrial function, ANT(a), COX5A (b), COXIV (c), av-UCP (d), Keap1 (e), NRF1 (f), FOXO1 (g), FOXO3 (h), FOXO4 (i), PPARα (j), and PPARγ (k). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Control and BCO.

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Chapter 4 –

Title: Autophagy machinery and bone homeostasis: friend or foe?

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Key Words: autophagy, macroautophagy, bacterial challenge, skeletal system, bone, osteomyelitis

Methodology: Data for this review was compiled using databases such as NCBI PubMed, University of Arkansas Library Quick Search, run by WorldCat, Science Direct, and JSTOR. Search terms used included “autophagy”, “autophagy and bone”, “autophagy stages”, “bacterial infection and autophagy”, “skeletal diseases and autophagy”, “cell survival and autophagy”, “macroautophagy”, “autophagy manipulation”, autophagy dysregulation”, “staphylococcus infection and autophagy” and “skeletal growth and autophagy”.

Abstract: Since its discovery, autophagy has proven to be influential in numerous physiological processes and states, including growth, stress, disease, and infection, for various cell types. After decades of research, novel roles for autophagy are still being found as the intricacies of the autophagy pathway and the balance between degradation and survival are explored. For the skeletal system, autophagy promotes bone growth through facilitating cell differentiation and survival within the ossifying bone. Under certain bacterial challenges, cells rely on autophagy for targeting and degrading pathogens and their secretions. Yet, cells can fall prey to bacterial means

of autophagy manipulation or hijacking, resulting in persistent infection and potential cell death. In this review, the molecular mechanisms of autophagy will be explained along with autophagy's role in the skeletal system. Furthermore, insights into the role of autophagy in skeletal diseases and bacterial infections will be explored as well as considerations for future areas of research where these physiological states overlap.

Introduction

The maintenance of body tissue and organs involves the function and collaboration of several different cell types along with appropriate response to environmental stimuli such as stress, disease, and infection. Autophagy is a means of cellular recycling that has a diverse and broad range of purposes. In bone tissue, autophagy facilitates cell differentiation in order to maintain the ratios of cell types needed for proper bone growth, healing, and turn-over. Under normal conditions, it used for the regular maintenance of needed protein turn-over and degradation. In disease states, or under bacterial challenge, the role of autophagy becomes more complex. If balanced properly, autophagy promotes cell survival and aids in immune response. However, if dysregulated, autophagy can be a means of bacterial proliferation and cell death stimulation. It is for these reasons that investigation into the role of autophagy in bacterial infections of the bone is worth pursuing.

Overview of Autophagy

Autophagy is a mechanism for the maintenance of cellular homeostasis whose name is derived from the Greek words "auto", meaning self, and "phagy", meaning eating. Through its involvement in both cell survival and death pathways, autophagy is a key component of a cell's reaction to different external and internal environmental stimuli. It was first discovered in 1955

when biologist Christian de Duve recognized organelles containing hydrolytic enzymes and dubbed them lysosomes [1 – 2]. Simultaneously, Novikoff and colleagues were capturing electron microscopy images of dense intracellular bodies containing components of the cytoplasm [2]. Both researchers had found evidence of a portion of the process now called autophagy and their findings catalyzed decades of research undertaken to understand and investigate autophagy. It is now understood that autophagy is a degradation process for cellular components, organelles, and various molecules. Its function is that of maintaining homeostasis via endogenous and exogenous waste disposal or the breakdown of entire cellular organelles/components if damaged or faulty [3].

The autophagy pathway is activated via several stimuli, including, but not limited to, amino acid starvation, endoplasmic reticulum and heat stress, changes in hormone levels, and infectious pathogens [4 – 10]. These stimuli involve a variety of autophagy modulators to achieve activation and therefore result in differing cellular consequences due to the numerous and diverse signaling pathways involved. For example, in chicken, autophagy is shown to be partially regulated by leptin signaling in a tissue specific manner [11]. A disruption in the autophagy mechanism or its modulators has been shown to have pathological consequences which will be discussed in more detail in this review [3]. A synopsis of the different types of autophagy can be seen in Figure 1 and will be elaborated on in the proceeding section.

Types of Autophagy

Autophagy can be further defined by the cellular components it targets, or the mechanism by which it performs membrane development and lysosomal degradation. This characterization results in the many types of autophagy such as macroautophagy, microautophagy, and chaperone mediated autophagy.

Macroautophagy

If large cellular contents and cytoplasm, such as aggregated proteins, damaged organelles, and intracellular pathogens, need to be degraded, macroautophagy is the major pathway involved [12]. There are four stages to macroautophagy, initiation, nucleation, elongation, and fusion (Figure 1a) [12]. During the initiation stage of macroautophagy (Figure 1a), a double-membraned, crescent-shape phagophore forms, isolating the components [13]. Nucleation allows the sequestering of proteins needed for phagophore growth and maturation during elongation. Fusion between the lysosome, which contains lysosomal enzymes capable of degradation, and the autophagosome is the final stage in macroautophagy [13]. This process has been described as either non-selective or cargo specific depending on the components, receptors, and stimuli involved [14].

A type of macroautophagy that is specific to the organelle it degrades is mitophagy. In this process, the mitochondria is the specific target via specific outer mitochondrial membrane proteins [14]. Mitophagy occurs primarily when there is a decline in the demand for metabolic capacity or when the mitochondria is damaged [15].

This review will primarily focus on macroautophagy (called autophagy from now on) its stages, key proteins and regulators, and its role in physiology and disease.

Microautophagy and Chaperone Mediated Autophagy

Lysosomal fusion with a pre-formed and matured autophagosome is not the only means by which cellular components can be degraded. Chaperone-Mediated Autophagy (CMA) is a selective form of autophagy involving only proteins with a C-terminal pentapeptide KFERQ motif. The HSP70 cochaperone identifies these proteins in the cytoplasm and delivers them to

the lysosome (Figure 1b). There, a receptor allows for translocation of the protein into the lysosomal lumen for degradation [16]. In microautophagy lysosomes directly engulf and digest cellular components that have formed multi-vesicular bodies (Figure 1c). Microautophagy interacts with both autophagy and endocytic machinery as well as relies on interactions between endosomal sorting complexes and heat-shock protein 70 (Hsp70) [14, 17].

Autophagy Machinery and Stages

The autophagy pathway is complex and detailed, involving several different protein complexes and reactions. The pathway is best explained in four stages, initiation, nucleation, elongation, and fusion.

Initiation

The primary proteins involved in initiation of autophagy compose the unc51-like autophagy activating kinase 1 (ULK1) complex and the class III phosphatidylinositol 3-kinase (PI3K) complex I as seen in Figure 2 [17]. ULK1 is the most important isoform in the family of ULK kinases in autophagy [18 – 21]. Assembly of the ULK1 complex then leads to phosphorylation of autophagy and Beclin regulator 1 (AMBRA1) and activation of the PI3K complex [18, 21].

The ULK1 complex is composed of ULK1 and noncatalytic subunits FAK family-interacting protein of 200 kDa (FIP200), autophagy-related protein 13 (Atg13), and autophagy related protein 101 (Atg101) [18]. The complex must be activated in three ways, the protein kinase activity must be actuated, the kinase must then be recruited to the phagophore assembly site (PAS), and the noncatalytic scaffolding structures must be functioning [21]. Through autophosphorylation of the protein kinase's activation loop at Thr180 of ULK1 the first step in complex activation is achieved. Autophosphorylation is induced by autophagy inducing

conditions such as starvation, as well as co-assembly with the other subunits which increases the concentration of ULK1 promoting autophosphorylation [21].

The mechanism by which starvation triggers activation of the ULK1 complex has been extensively researched yet is still debated. What is understood is that mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of metabolism and is known to phosphorylate Atg13 under normal conditions [22]. Phosphorylation of Atg13 inhibits its assembly into the ULK1 complex. It is through this pathway that when mTORC1 is inactivated during amino acid depletion and stress, autophagy is then triggered [7, 22].

Another regulatory mechanism that mediates autophagy via the ULK1 complex is the Adenosine monophosphate (AMP)–activated protein kinase (AMPK) pathway [19]. Direct phosphorylation of ULK1 by AMPK at multiple sites leads to its activation and subsequent upregulation of autophagy [18].

Targeted autophagy involves numerous receptors for the recognition and selection of large protein aggregates for autophagosome degradation. Among these receptors, sequestosome-1 (SQSTM1/p62) is one of the most important as a regulator of autophagy through its action as a reporter of and receptor for autophagy activity [23]. SQSTM1 functions through its C-terminal ubiquitin-binding domain interacting with ubiquitin non-covalently in order to recognize ubiquitinylated proteins. It also possesses a short LC3-interacting region (LIR) sequence which interacts with LC3 as a connection to the autophagy machinery and pathway [23]. When autophagy is inhibited, SQSTM1 will accumulate due to lack of degradation of its targets. Therefore, SQSTM1 is used as a marker for autophagy flux or activity and can be used as an indicator of a disruption in normal autophagy pathways [24].

Nucleation

The process of sequestering small group molecules to the phagophore assembly site is nucleation. A key protein involved is Atg9 which cycles from peripheral sites to the PAS and has been speculated to bring lipids to the growing membrane [25 – 26]. Atg9 recruitment requires a functioning Phosphoinositide 3-kinase (PI3K) complex I [25, 27]. Through their recruitment, subsequent Atg proteins can be recruited to the PAS such as Atg8-PE conjugate and the Atg5-12/16L complex which will be discussed in more detail in the next section [21].

Elongation

Two ubiquitin-like conjugation systems are required for elongation of the phagophore, microtubule-associated protein 1 light chain 3 (LC3) and the Atg5-12/16L1 complex (Figure 2) [28 – 30].

First, LC3 is cleaved by the cysteine specific protease Atg4 which exposes the C-terminal cysteine. Atg7 and Atg3 then conjugate LC3 to the membrane lipid phosphatidylethanolamine (PE) resulting in the transition from the cytosolic LC3 type I (LC3-I) to the membrane bound LC3 type II (LC3-II) as seen in Figure 2 [31]. The ubiquitin-like protein Atg12 covalently conjugates to Atg5 in the second ubiquitin like system. This conjugation requires the activity of Atg7 and Atg10. Once conjugated, noncovalent association with Atg16L1 creates the Atg5-12/16lI complex which forms the scaffold of the maturing phagophore [32]. This complex also acts as an E3-like protein during LC3 lipidation by aiding in recruitment and activation of the Atg7/Atg3 proteins [28, 30]. The Atg5-12/16L1 complex dissociates from the phagophore once the autophagosome membrane fully forms [21].

Fusion

Once the autophagosome has fully enclosed, fusion with a lysosome is possible. This fusion results in an autolysosome that is fully matured. The fusion machinery consists of the Rab-SNARE (Soluble NSF Attachment Proteins Receptor) system including Ras-related protein Rab-7a (Rab7), Vesicle-associated membrane protein 3 (Vamp3), Vacuolar morphogenesis protein 7 (Vam7), and Vesicle transport through interaction with t-SNAREs homolog 1 **(**Vti1) [33 – 34]. Rab7 is known as a key regulator for autophagosome-lysosome fusion and is essential for the progression of autophagy as well as being highly conserved in eukaryotic cells. This is achieved through the regulation of bidirectional transport of autophagosomes along microtubules via the interaction of Rab7 and specific effectors [35 – 37]. In addition to regulating fusion, the subsequent maturation of the autolysosome is also partly regulated by Rab7 function under nutrient rich conditions [37]. The majority of SNARE proteins are tail-anchored transmembrane proteins allowing vesicular traffic-mediated connection to the source membrane. Fusion is also regulated via Class C vacuolar protein sorting (Vps) / Homotypic fusion and protein sorting (HOPS) proteins which contain a tethering protein complex [38 – 39]. The HOPS complex binds to UV radiation resistance associated (UVRAG) which is, in turn, bound to beclin-1 and studies have shown Beclin may be involved as a facilitator for fusion [21, 40].

Lysosomal proteins are also regulators of fusion, such as lysosome-associated membrane glycoprotein 2 (LAMP2). LAMP2 is a key transmembrane protein in chaperone mediated autophagy by binding with heat shock protein complex but is also required for lysosomal fusion in macroautophagy [41]. Additionally, LAMP2 acts as a receptor for DNA and RNA degradation via autophagy [42]. Successful fusion is also dependent on the pH of the compartments within the cell and regulated via vacuolar-type ATPase (V-ATPase) enzymes [43, 44].

Roles of Autophagy

Since it's discovery, autophagy has been implicated in numerous cellular and phenotypic states. The role autophagy plays in these states depends on numerous factors and stimuli, both internal and external. The outcome for both the cell or larger organism is also dependent on autophagy's role and its ability or inability to carry out that specific role.

Autophagy and the Skeletal System

The development and maintenance of the skeletal system relies on the function and cooperation of multiple cell types within the bone as well as various stimuli such as growth activation, mechanical load, and nutritional state. These cell types include the bone-forming osteoblasts, bone-resorbing osteoclasts, and growth plate chondrocytes. Autophagy has been implicated as necessary for bone maintenance, homeostasis, and growth as both a cell survival and intracellular degradation mechanism [45 – 47]. An overview of the roles autophagy plays in bone can be seen in Figure 3 and will be discussed further in the following sections.

Autophagy has been shown to be key in chondrocyte survival and maturation. When key autophagy genes Atg5 or Atg7 are knocked out in mice, enhanced caspase-dependent cell death is seen in isolated chondrocytes as well as reduced growth capacity [48]. This suggests that autophagy is a key regulator for chondrocyte survival during endochondral ossification within the growth plate. Similar studies have found that fibroblast growth factor FGF18 through FGFR4 are mediators of chondrocyte autophagy and subsequent function [49]. It is through chondrocyte differentiation and maturation that longitudinal bone growth is achieved. Therefore, their survival is key to bone development and growth.

Another bone cell type affected by autophagy is the osteoclast. Pro-inflammatory cytokine, interleukin 17A, has been shown to regulate osteoclast differentiation and increase bone resorption via autophagy activation of bone marrow macrophages [50]. The interplay between signaling molecules and autophagy activation in the differentiation of bone cell types leads to alterations in the ratio of bone forming to bone-resorbing cells and therefore can have pathological consequences.

Under normal conditions, increased rates of autophagy have been observed in differentiating osteoblasts and suppression of autophagy has been shown to inhibit terminal differentiation [50 – 52]. Autophagy also pays a role in osteoblast mineralization by autophagic vacuoles trafficking and secreting apatite crystals in bone [51].

The involvement of autophagy in bone development and homeostasis is not surprising given autophagy's recycling capabilities and bone's growth and turn-over. Therefore, a disruption in the balance of autophagy in bone leads to changes in cell differentiation and ultimately, the structure and function of bone. Autophagy is also implicated in the aging process, further demonstrating its significance in maintaining proper cell and tissue function [53 – 54]. Age-related decline in autophagy has been seen in various tissues, with suppression of autophagy in osteocytes mimicking skeletal aging [55].

Autophagy and Disease

Skeletal Diseases/Disorders

Autophagy has been implicated in numerous diseases including neurodegenerative diseases, such as Alzheimer's disease, gastro-intestinal diseases such as Crohn's Disease, and skeletal diseases [56]. In humans, avascular necrosis of the femur head (ANFH) is a result of insufficient blood

supply and characterized by attrition and reduced bone repair in the early phases, followed by necrosis. Increased tumor necrosis factor -α (TNF-α) and autophagy markers in ANFH affected bone as well as increased autophagy and apoptosis in osteoblast cells via TNF-α manipulation indicate that autophagy also plays a role in osteoblast survival [57].

Glucocorticoids are often used to treat inflammatory and autoimmune diseases; however, this can lead to glucocorticoid induced osteoporosis (GIO) via autophagy dysregulation [58 – 59]. In the case of osteoporosis, correlations, both positive and negative, between several autophagy-regulatory genes and bone mineral density were determined in a genome-wide association study [60]. Other bone related diseases in which autophagy dysregulation has been implicated include Paget disease of the bone, osteopetrosis, osteogenesis imperfects, and osteomyelitis. In the case of osteomyelitis, the causative bacterial agent has been shown to manipulate the autophagy pathway leading to persistence within cells and cell death [61]. This pathology could be a contributing factor to the bone attrition which accompanies necrosis and bacterial colonization in osteomyelitis.

Bacterial Infection

Autophagy intervention as Cell Survival Mechanism

Autophagy not only degrades pathogens during bacterial infection, but also acts to activate the immune response. It is in this way that autophagy both directly and indirectly enhances cell survival by combating infection (Figure 3). When autophagy is employed to degrade intracellular pathogens such as *Salmonella enterica, Serovar typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* it is called xenophagy [62]. Cells unable to exhibit xenophagy through membrane-associated, cellular and cytoplasmic modulators are more susceptible to infection [61,

63 – 65]. For example, B-cell lymphoma-extra-large protein (Bcl-xL)-knockout Hela cells are more susceptible to *Streptococcus pyogenes* infection [66]. Additionally, internalized bacterial peptidoglycans are detected by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) which both interact with Atg16L1 and activate an inflammatory response. This type of intracellular bacterial sensing aids in recruitment of autophagy machinery necessary for nucleation and elongation around invading bacteria [67 – 69].

The autophagic degradation of pathogens is heavily relied upon in macrophages as they function in the immune response. Cytoplasmic bacteria such as *Listeria monocytogenes* have been shown to be targeted by autophagy and immunolabeled with antibodies to markers for the rough endoplasmic reticulum in macrophage [70]. This method of removal from the cytoplasm and following deliverance to the endocytic pathway reveals host mechanisms that utilize the autophagy pathway as a means of cell survival via defense against pathogens. Another example of autophagy targeting a cytosolic bacterium is in the case of *Salmonella Typhimurium*. In both *Caenorhabditis elegans* and *Dictyostelium discoideum* damaged *Salmonella*-containing vacuoles are detected and targeted by LC3 and Atg proteins for the purpose of autophagosome formation and lysosomal degradation [71]. While the exact mechanism by which *S. Typhimurium* has yet to be fully elucidated, it is clear that autophagy targeting restricts its growth and protects the cytosol from bacteria colonization [61, 72].

Similarly, *Mycobacterium tuberculosis* is also targeted by autophagy but has also been shown to block phagosome maturation and replicate within it. A study of *M. tuberculosis* infection found that approximately 30% of the phagocytized mycobacteria were selectively labeled with LC3 and Atg12 within 4 hours of infection [73]. It is often the case that particular bacterial secretions are necessary for further targeting via autophagy and in the case of *M. tuberculosis* and *S.*

typhimurium, damage to the vacuoles containing them via toxins also aids in autophagy targeting [74 – 76].

Staphylococcus aureus is a Gram-positive bacterium which leads to numerous infections including sepsis, pneumonia, and osteomyelitis which is found in both humans and other species such as poultry [77 – 78]. Autophagy has been implicated as a means of cell survival through enhancing the cell's tolerance to the bacterial pore forming toxin, α-toxin [79]. This protection is dependent upon the presence and function of major autophagy proteins including LC3 and Atg16L. This has been shown via transgenic KO mice for both LC3 and Atg16L being susceptible to severe *S. aureus* infection of the lungs and blood to the point of lethal [79]. The recycling and degradative property of autophagy which makes it so useful in bone homeostasis also aids in pathogen and pathogen secretion defense. A disruption in this defense system would not only result in decreased cell survival, but also benefit the invading bacteria.

Bacterial hijacking of Autophagy

Although autophagy is an effective mean of degrading intracellular bacterial pathogens, many bacteria have evolved mechanisms and weapons to hijack many biological processes, including the autophagy machinery (Figure 3) [80 – 81]. Multiple mechanisms exist that either enhance or prevent phagosome formation, subvert autophagy machinery, or prevent autophagosome and lysosomal fusion. For example, the causative agent of anaplasmosis, *Anaplasma phagocytophilum,* can enhance autophagosome formation and consequently procure nutrients from within the autophagosome [82 – 83]. *Shigella flexneri*, another bacterial pathogen, has outer membrane protein IcsA autotransporter (IscA) which promotes actin-based motility and can bind to Atg5 making it a target for autophagy degradation. However, the bacterial

protein IscB competitively binds to IscA in place of Atg5, masking the bacteria form the autophagy pathway [84].

While some bacteria evade autophagy through inhibiting its initiation, others are able to utilize the autolysosome for replication before inhibiting processes further down the autophagy pathway. An example of such a bacterium is *S. aureus.* In fact, it has been shown that *S. aureus* will replicate within the LC3+ labeled double-membrane for three to twelve hours before escaping into the cytosol to continue cell death and infection [85 – 87]. *S. aureus* avoids autophagic degradation via inhibition of lysosomal fusion [88]. This was determined by the accumulation of autophagosomes coupled with changes in autophagy gene and protein expression over time of infection in bovine epithelial cells. Both the ratio of LC3 type I:II and the accumulation of p62 were increased, indicating a block of autophagy flux [88]. If autophagy flux is impaired, it is an indicator of disruption of proper fusion and lysosomal degradation, which would allow the bacteria to replicate within the double membrane, causing increased severity of infection [87 – 89]. This type of strategical misuse of autophagy by *S. aureus* calls into question its use of such mechanisms in other cell and tissue types. Further research needs to be done to determine the potential role of autophagy in other diseases caused by *S. aureus*, such as osteomyelitis especially given bone's dependency on autophagy for homeostasis.

Conclusions and Perspectives

Autophagy has far reaching and diverse effects on development, maintenance, and survival at cellular levels. In bone, it is responsible for homeostasis through facilitating cell differentiation into the necessary bone-forming and bone-degrading cell types, as well as providing means of cell survival for chondrocytes in conditions found during longitudinal bone growth. Dysregulation of the autophagy pathway is associated with decreased bone growth and several

skeletal diseases involving bone attrition. In bacterial infections, autophagy is pivotal for survival and also the target of bacterial manipulation for the purpose of infection. One of the most common agents of bone infection such as osteomyelitis, *S. aureus* is known to manipulate autophagy to persist within the cell, leading to intense infection. As of now, the connection between the delicate balance of functioning autophagy in bone and its manipulation under bacterial infection has yet to be investigated. However, given the scope and significance of autophagy's role in bone maintenance and bacterial infection, future studies are warranted.

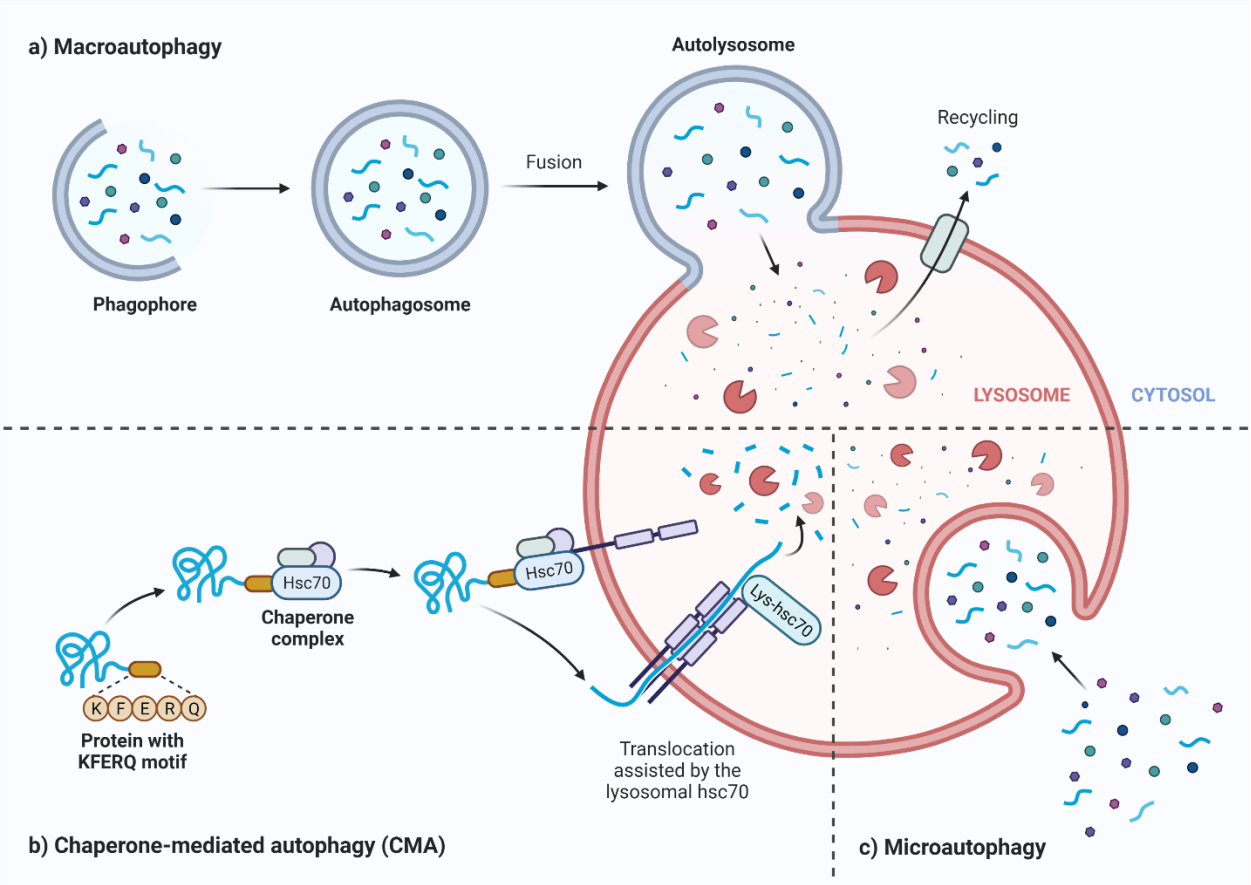

Figure 1: The types of autophagy: a) Macroautophagy characterized by phagophore and autophagosome formation and the focus of this review; b) Chaperone-mediated autophagy involving chaperone complex recognition of proteins for future lysosomal degradation; c) Microautophagy, which involves direct lysosomal engulfment and degradation.

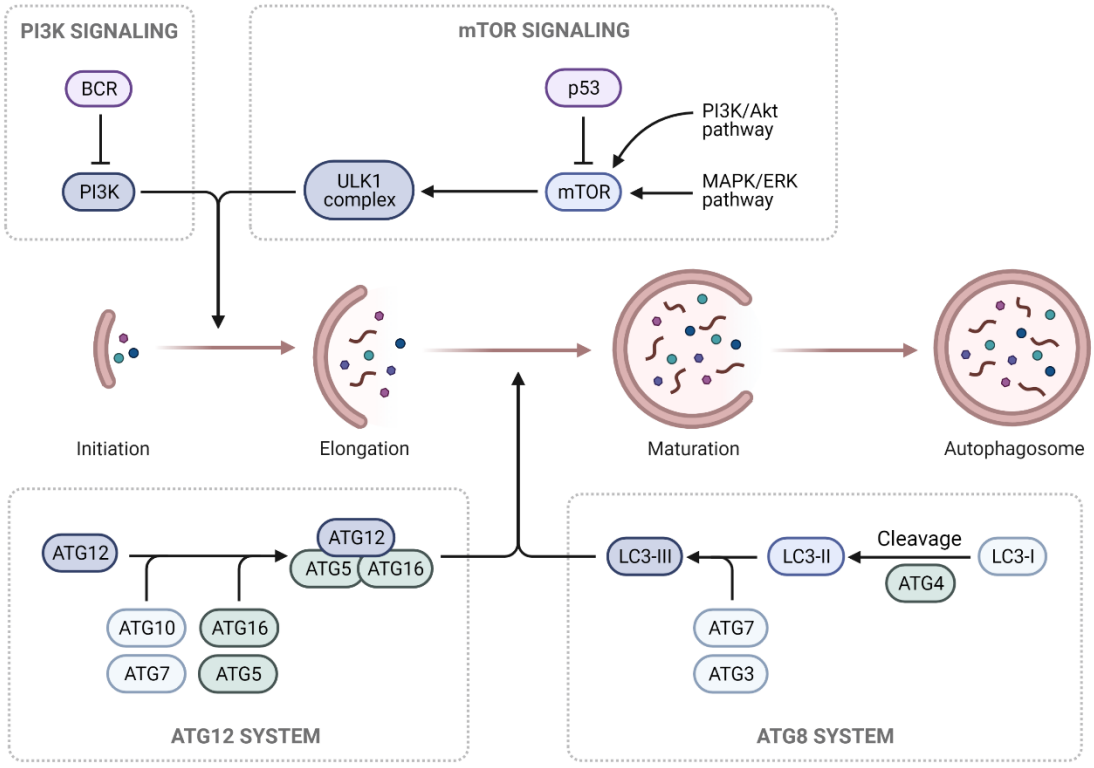

Figure 2: The stages of autophagy and key molecular regulators involved. Initiation is primarily regulated by two complexes: ULK1 and PI3K. Elongation is regulated by the cleavage and alteration of LC3 and the formation of the Atg5-12/16lI complex.

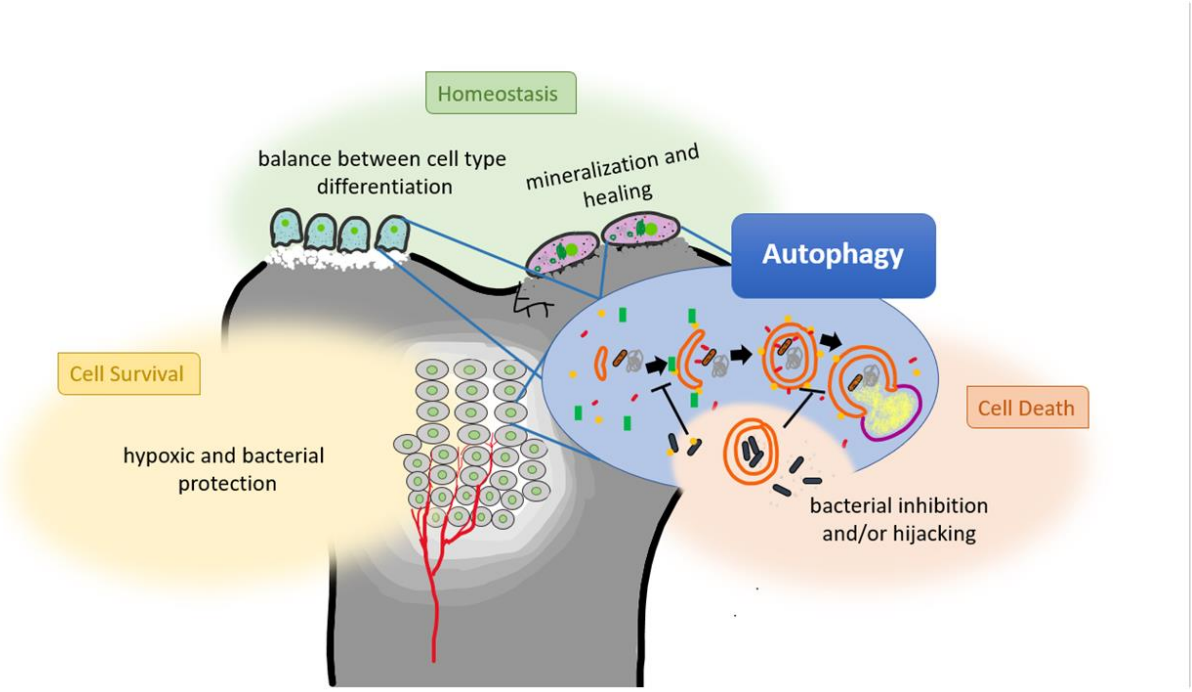

Figure 3: Autophagy machinery and its current/prospective roles in bone: Homeostasis characterized by the influence on osteoclast and osteoblast cell differentiation as well as facilitating osteoblast mineralization during potential disease states and under normal conditions. Cell survival for osteoblast in hypoxic conditions during endochondral ossification and bacterial challenge. Cell death via bacterial inhibition or hijacking and subsequent infection of the cell.

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Chapter 5 –

Title: AUTOPHAGY & BROILER LAMENESS: Role of Autophagy Machinery Dysregulation in Bacterial Chondronecrosis with Osteomyelitis (BCO)

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Abstract: Autophagy is a cell survival and homeostasis mechanism involving lysosomal degradation of cellular components and foreign bodies. It plays a role in bone homeostasis, skeletal diseases, and bacterial infections as both a cell-survival or cell-death pathway. This study sought to determine if autophagy played a role in bacterial chondronecrosis with osteomyelitis (BCO). BCO is a prominent cause of lameness in modern broilers and results from bacterial infection of mechanically stressed leg bone growth plates. The protein and gene expression of key autophagy machinery was analyzed in both normal and BCO-affected broilers using real-time qPCR and immunoblot, respectively. Gene expression showed a significant downregulation of key target signatures involved in every stage of autophagy in BCO-affected bone, such as ATG13, SQSTM1 (p62), ATG9B, ATG16L, ATG12, LC3C, and RAB7A. Additionally, protein expression for LC3 was also significantly lower in BCO. An *in vitro* study using hFOB cells challenged with BCO isolate, *Staphylococcus agnetis* 908, showed a similar

dysregulation of autophagy machinery along with a significant decrease in cell viability. When autophagy was inhibited via 3-MA or CQ, comparable decreases in cell viability were seen along with dysregulation of autophagy machinery. Together, these results are the first to implicate autophagy machinery dysregulation in the pathology of BCO.

Key words: autophagy, broiler, bacterial chondronecrosis with osteomyelitis, lameness**,** osteoblasts, bone

Introduction

Autophagy is a key component of cellular homeostatic, survival, defense, and death. It is a process by which cells are able to degrade cellular debris, damaged organelles, or foreign bodies, via membrane isolation and lysosomal fusion and degradation [1, 2]. There are four stages in autophagy, initiation, nucleation, elongation, and fusion [3]. Initiation involves the activation and trigger of autophagy machinery. Nucleation is characterized by the sequestering of key proteins and complexes to the site of autophagosome formation. Elongation occurs as the autophagosome expands and eventually closes. Fusion refers to the fusion of the mature autophagosome with a lysosome to undergo lysosomal degradation of its contents [4, 5]. Autophagy aids in cellular survival through regular maintenance as well as aid in cellular processes and functions. In bone, functioning autophagy machinery aid in bone growth and homeostasis via promotion of chondrocyte survival in hypoxic environments, regulation of osteoblast and osteoclast differentiation, and contribution to osteoblast mineralization [6-9]. Under bacterial challenge, autophagy's effectiveness has shown to increase cell survival and decrease bacterial loads [10-12]. While its ineffectiveness or inhibition has shown to have damaging effects on cellular viability and function. For example, some bacteria, such as Staphylococcus aureus, have been shown to manipulate autophagy via inhibition of fusion in order to persist within the cell under

membrane protection while replicating and ultimately lysing within the cell and causing cell death [13-15]. It is the dynamic nature of autophagy's involvement in physiological states that has led it to be increasingly investigated in different diseases and disorders. In the case of BCO, the involvement of bacterial infection and bone inflammation as well as cellular necrosis make autophagy a pathway worth investigating given its significance to both the function of bone and response to bacteria.

BCO, also known as femur head necrosis (FHN), is a common cause of lameness in the modern broiler affecting fast-growing, higher yielding birds more frequently [16]. It is characterized by bacterial infection in primarily the proximal head of rapidly-growing leg bones of a broiler, resulting in chondronecrosis and osteomyelitis [17]. It is theorized that collagen-binding bacteria, entering the blood stream via the respiratory or gastrointestinal tract, are able to come into contact with preexisting wound sites within the mechanically-stressed, highly-vascularized growth plates and colonize [18, 19]. These wound sites also often transect blood supply leading to hypoxic conditions. As infection persists, inflammation and necrosis lead to bone attrition and associated lameness can become detectable, although subclinical BCO can also occur [17, 20]. Understanding and combating BCO, and associated lameness, has become a high priority for both improving both animal welfare and production.

Although it is better understood how bacteria infect the bone and what the end-point symptoms of BCO are, what is still yet to be fully elucidated is the mechanism by which bacteria cause BCO. It has been shown the mitochondrial dysfunction exists in BCO affected tissue, but molecular pathways linking bacterial effects and what is seen in BCO tissue have yet to be discovered. To that end, this study sought to elucidate the potential involvement of autophagy in BCO.

Materials and Methods

Collection of BCO and Normal Bone Samples.

All animal experiments were approved by the University of Arkansas (Fayetteville, AR) Animal Care and Use Committee (protocol number 15043) and were in accordance with recommendations in NIH's Guide for the Care and Use of Laboratory Animals. The BCO model and healthy counterparts were conducted as previously described [16, 17]. Briefly, animals were placed on either litter or wire-flooring and had ad libitum access to fresh water and feed (3.9 Mcal metabolizable energy $kg^{-1}$and 180 g crude protein kg^{-1}) while the experiment took place at the University of Arkansas Poultry Research Farm. Ambient temperature was lowered gradually from 32˚C to 25˚C by 21 days of age. A light cycle of 23 hours light/1 hour dark was maintained along with an approximately 20% relative humidity until 56 days of age. At the end of the 56 days, animals were weighed, humanely euthanized, and immediately necropsied to determine presence of subclinical lesions in the proximal heads of both the femora and tibiae. Bone was selected macroscopically based on previously reported scale [16, 17]. Normal bone was taken from animals raised on litter only and when considered free of any necrosis or lesion and BCO-affected bone was from birds raised on wire- flooring and exhibiting lameness, and consisted of bone with FHN. Proximal portions of bone, primarily consisting of the growth plate, from both affected and unaffected animals were snap frozen in liquid nitrogen and stored at -80˚C for later analysis.

Cell Culture

Human fetal osteoblast (hFOB) 1.19 cells (CRL-11372; ATCC, Manassas, VA) were cultured in a 1:1 mixture of Ham's F12 medium/Dulbecco's modified Eagle's medium, 10% fetal bovine

serum, and 0.3 mg/mL G418. Cells were grown at 34ºC in a humidified atmosphere of 95% air and 5% CO_2.

Bacterial Challenge

Staphylococcus agnetis strain 908 (a generous gift from Dr. Douglas Roads) was isolated from the affected bone of BCO model, and was grown overnight in Luria broth at 37˚C [18]. To determine cell density, cultures were diluted using phosphate-buffered saline before absorbance at 650 nm was measured. Bacteria at a multiplicity of 50:1 were added to cell cultures in antibiotic-free media and left to attach for 1 hour. Cells were washed three times with phosphate-buffer saline and complete media was added. Cells were maintained for an additional 24 hours and then processed for protein expression (N=3) [21].

Autophagy Inhibitor Treatments

hFOB cells were treated with either 3-Methyladenine at a concentration of 5mM (Sigma Aldrich, St. Louis, MO), Chloroquine at a dose of 10 µM (InvivoGen, San Diego, CA), or vehicle control for 24 hours before being lysed (N=3). Pilot studies were used to determine effective inhibitor dose.

Cell Viability

Cell viability was performed as previously described [22]. Briefly, hFOB cells were seeded at 1×10^4 cells per well of a 96-well plate before being transfected as described above. CellTiter 96 AQueous One Solution CellProliferation Assay (Promega, Madison, WI) was used, according to manufacturer's recommendations, and results were obtained using a Synergy HT multimode microplate reader (BioTek, Winooski, VT). All sample readings were background corrected, and results were reported relative to control.

RNA isolation, reverse transcription, and real-time quantitative PCR

From the normal and BCO-affected bone samples (N=6), total RNA was isolated in accordance with the protocol of [23]. Cellular RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA), based on manufacturer's instructions. RNA concentrations were determined using Synergy HT multimode microplate reader and total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithsburg, MD). Amplification was achieved using Power SYBRGreen Master Mix (Life Technologies, Carlsbad, CA) and real-time quantitative PCR (7500 Real Time System; Applied Biosystems, Foster City, CA). The sequences for oligonucleotide primers for *r18s, Beclin1, ATG7, ATG3, LC3A, LC3B,* and *LC3C* were previously published [24, 25]. As well as *ATG13, ATG9A, ATG14, UVRAG ATG16L, ATG12, ATG4A, ATG4b,* and *ATG10* [26]. Additional primers used were *ATG2B* (forward, 5'-CCGTTCTCGGAGTCCATCA -3'; and reverse, 5'- GAGCCGGTGCCCTGGTA -3'), *ATG9B* (forward, 5'- TCACCCCTGAAGATGGAGAGA -3'; and reverse, 5'-TTTCCAGCATTGGCTCAATC -3'), *RAB7A* (forward, 5'- GTGCCAAGGAGGCCATTAAC -3'; and reverse, 5'- AAGTGCATTTCGTGCAATCG -3'), *SQSTM1 (p62)* (forward, 5'-TTACGTGCAGGACGGAGTTTT -3'; and reverse, 5'- CACGCCTGCACTCCTTTTTC -3'), and *LAMP2* (forward, 5'- TCAATAGCTGAAGAATGCTTTGCT -3'; and reverse, 5'-TGCCAACTGCGACTGGAATA -3').

Real-time quantitative PCR cycling conditions were 50˚C for 2 minutes, 95˚C for 10 minutes and 40 cycles of a two-step amplification (95˚C for 15 seconds followed by 58˚C for 1 minute). The dissociation protocol from the sequence detection system was used for melting curve analysis to exclude potential contamination of non-specific PCR products. Negative controls that were used as templates contained no reverse transcription products. Relative expression of target genes was

determined using the $2^{-\Delta\Delta CT}$ method and healthy bone tissue or untreated cells were used as calibrators [27].

Western Blot Analysis

Bone samples and cell lysate were homogenized in lysis buffer (10 mmol/L Tris base, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.1% Triton X-100; 0.5% Nonidet P-40; and protease and phosphatase inhibitors) and stainless-steel beads, using the Bullet Blender Storm (NextAdvance, Averill Park, NY). Cells were homogenized in lysis buffer after being washed with phosphate-buffered saline. Total protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA), and ran in 4%-12% gradient Bis-Tris gels (Life Technologies) and then transferred to polyvinylidene difluoride membranes. Once transferred, membranes were blocked using a Tris-buffered saline (TBS) with 5% nonfat milk and Tween 20 at room temperature for 1 hour. The membranes were washed with TBS and Tween 20 and then incubated with primary antibodies at a dilution of either 1:500 or 1:1000 overnight at 4˚C. Primary antibodies used were rabbit anti-Beclin1 (Cell Signaling Technology, Danvers, MA), rabbit anti-ATG7 (Cell Signaling Technology, Danvers, MA), rabbit anti-ATG16L (Aviva Systems Biology, San Diego, CA), rabbit anti-ATG12 (Cell Signaling Technology, Danvers, MA), rabbit anti-ATG3 (Thermo Fisher Scientific, Waltham, MA), rabbit anti-ATG5 (Cell Signaling Technology, Danvers, MA), rabbit anti-LC3 (Cell Signaling Technology, Danvers, MA), and rabbit anti-RAB7 (Santa Cruz Biotechnology, Dallas, TX). After another wash, secondary antibodies diluted to 1:5000 were added to 5% nonfat milk in TBS and Tween 20 and incubated with the membranes at room temperature for 1 hour. The protein signals were visualized using chemiluminescence (ECL Plus; GE Healthcare, Pittsburg, PA) and images were captured using the FluorCHem M MultiFluor System (ProteinSimple, San Jose, CA). Prestained

molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (Bio-Rad, Hercules, CA). Protein loading was assessed by immunoblotting using rabbit antieglyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Dallas, TX). Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993–2011; ProteinSimple).

Statistical Analysis

Data were analyzed by Student t-test or One-way ANOVA, as appropriate, using GraphPad version 7.03 (GraphPad Software, Inc., LaJolla, CA). Results are expressed as means ±SEM, with *P*-value < 0.05 set as statistically significant.

Results

Key Autophagy Machinery Expression was Downregulated in BCO affected Tissue

Key autophagy machinery genes, and corresponding protein, involved in initiation were measured in normal and BCO affected tissue. Both protein and gene expression were analyzed in normal and BCO affected femurs for Coiled-Coil Myosin-Like BCL2-Interacting Protein (Beclin1) with no significant difference seen (Figure 1 a, b, c). Autophagy-related protein 13 (ATG13) and Sequestosome I (*SQSTM1*), also known as Autophagy Receptor P62 (*p62*), mRNA expressions were significantly downregulated in BCO affected tissue (Figure 1 d, e) ($P < 0.05$).

In investigating the nucleation stage of autophagy, Autophagy-related protein 5 (ATG5) protein expression was measured with no significant difference seen when comparing BCO affected and normal bone (Figure 2 a, b). However, the mRNA expression of ATG14 and ATG9B were significantly downregulated in BCO, when compared to normal bone (Figure 2 c, e) ($P < 0.05$).

Autophagy-related protein 9A mRNA expression showed no significant differences between normal and BCO affected bone (Figure 2 d).

The elongation stage of autophagy saw several significant differences in expression of autophagy molecular signatures. While protein expression of ATG7, ATG16L, ATG12, and ATG3 were not significantly different between BCO and normal bone, LC3 protein expression was significantly lower in BCO-affected bone (Figure 3a-f) ($P < 0.05$). Gene expression of *ATG16L*, *ATG12*, *LC3C*, *ATG2B*, *ATG4B*, and *ATG10* were also significantly downregulated in BCO-affected bone compared to normal (Figure 3h, i, m, n, p, q) ($P < 0.05$). *ATG7*, *ATG3*, *LC3A*, *LC3B*, and *ATG4A* showed no significant difference between BCO and normal tissue (Figure 3g, j, k, l, o).

Autophagy machinery involved in the fusion stage showed decreased mRNA abundances of member RAS Oncogene Family (*RAB7A*) in BCO-affected bone (Figure 4c) ($P < 0.05$). While protein expression of RAB7 as well as mRNA expression of Lysosomal Associated Membrane Protein 2 (*LAMP2*) and UV Radiation Resistance-Associated Gene (*UVRAG*) were not significantly different between normal and BCO (Figure 4a, b, d, e).

S. agnetis 908 Challenge Decreased Viability and Key Autophagy Machinery Expression in hFOB cells

Exposure to *S. agnetis* 908 resulted in significant decreased cell viability in hFOB cells (Figure 5 a) ($P < 0.05$). Protein expression of ATG7, ATG16L, Beclin1, ATG12 and ATG3 were significantly lower in bacterially challenged cells compared to control (Figure 5b-e, g, h) ($P < 0.05$). The ratio of LC3 type II to LC3 type I was significantly increased in cells under bacterial challenge (Figure 5b, j) ($P < 0.05$). ATG5 and RAB7 protein expression were unaffected by exposure to *S. agnetis* 908 (Figure 5 b, f, i).

Inhibition of Autophagy in hFOB Decreased Cell Viability and Autophagy Machinery Expression

Treatment of hFOB cells with either 3-methyladenine (3-MA) or Chloroquine (CQ) resulted in significantly decreased cell viability in hFOB cells (Figure 6 a) ($P < 0.05$). Protein expression of ATG7 and Beclin1 was significantly decreased in cells exposed to both inhibitors compared to control (Figure 6 b - d) ($P < 0.05$). ATG12 and ATG3 protein levels were lower in cells treated with CQ compared to control, but not 3-MA (Figure 6 b, e, f) ($P < 0.05$). The ratio of LC3 type II to LC3 type I was significantly increased in both 3-MA and CQ treated hFOB cells, with CQ treatment resulting in the highest ratio (Figure 6 b, g) ($P < 0.05$).

Discussion

In the pursuit of identifying potential mechanisms responsible for the bacterial infection, necrosis, and inflammation of the bone in BCO, the autophagy pathway was investigated. Autophagy has been increasingly implicated in several skeletal disorders such as avascular necrosis of the femur head, glucocorticoid induced osteoporosis and osteomyelitis [28-32]. In osteomyelitis, autophagy has been shown to be manipulated by causative agents allowing the persistence within the cell and subsequent cell death [33, 34]. Its dual role in both cellular defense and cell death under bacterial challenge as well as its susceptibility to bacterial manipulation, warrant in depth analysis into autophagy in BCO.

The initiation stage of autophagy has been shown to be both upregulated and inhibited under bacterial challenge, depending on the bacterium involved [14, 35]. A key component of autophagy initiation is formation of the ULK1 complex, which requires the involvement of ATG1 proteins such as ATG13 [36-38]. BCO affected femurs were shown to have significantly

decreased mRNA expression of *ATG13*. Phosphorylation of ATG13 by mechanistic target of rapamycin complex 1 (mTORC1) occurs under normal conditions, inhibiting autophagy [39]. It is through the inactivation of mTORC1 that stimuli, such as amino acid depletion and stress, activate the autophagy pathway within a cell [40]. Another key component of autophagy initiation is the function of SQSTM1, a reporter of and receptor for autophagy activity. *SQSTM1* mRNA expression as also significantly decreased in BCO femurs. An accumulation of SQSTM1 protein has been used as an indicator of inhibited autophagy [41, 42]. While the mRNA expression does not tell us the state or quantity of protein expression in BCO tissue, it does indicate potential overall decreased SQSTM1 availability. The downregulation of *ATG13* and *SQSTM1* in BCO bone could be indicative of a dysregulation of autophagy initiation under BCO conditions.

Two key machinery involved in autophagy nucleation were significantly downregulated in BCO affected femurs, *ATG14* and *ATG9B.* ATG9 is believed to be involved in bringing lipids to the newly forming membrane in the phagophore assembly site (PAS) [43-45]. ATG9 is critical to survival of mice in the early neonatal starvation period in which functioning autophagy is essential [46]. In the case of bacterial infection, ATG9 has been shown to be essential in the formation of the double membrane around *Salmonella* [47]. ATG14 is a subunit of the Phosphoinositide 3-kinase (PI3K) complex I which aids in directing the complex to the PAS where the complex plays a major role in membrane elongation of the forming autophagosome [48]. Autophagy studies involving yeast and mammals has shown that overexpression of ATG14 increases autophagic activity [49, 50]. Additionally, deletion of ATG14 has been shown to affect the localization of other key autophagy machinery such as AG8 and the ATG5-ATG12/ATG16L complex and, therefore, may indirectly regulate autophagosome size [51-53]. Both ATG9 and

ATG14 dysregulation in BCO bone suggest that the nucleation stage of autophagy may be affected under BCO conditions.

Elongation involves the expansion and closure of the phagophore to form an autophagosome. This process begins with the cleavage of LC3 by ATG4. LC3 is then conjugated from LC3 type I to membrane-bound LC3 type II by ATG7 and ATG3 [54]. The ATG5-12/ATG16L complex comes into play again during elongation by recruiting ATG7 and ATG3 and forming the scaffold by which the maturing phagophore elongates and it requires the activity of ATG7 and ATG10 [55]. In BCO affected tissue, there was significant decreased gene expression of *ATG12*, *ATG16L*, *LC3C*, and *ATG4B*. In the case of LC3, both LC3 protein and *LC3C* gene expression were significantly lower in BCO bone. Additionally, ATG2B and ATG10 were also significantly decreased. ATG2 ATG2 acts as a tether between the pre-autophagosome membranes and the endoplasmic reticulum during elongation to aid in expansion and closure [56, 57]. It has been shown that depletion of ATG2A and ATG2B leads to an accumulation of open and immature phagophore structures in mammalian cells [58, 59]. The formation of the ATG5-ATG12 conjugation of the ATG5-ATG12/ATG16L complex is dependent on initial formation of an ATG12-ATG10 thioester intermediate [60]. Indeed, mutations in the ATG10 gene have shown to lead to dysfunction of the ATG5-ATG12 conjugation and lack of autophagic bodies [61]. Decreased protein and gene expression of key elongation factors could contribute to BCO pathogenicity through dysregulation of autophagy machinery necessary for cellular homeostasis and defense.

The fusion of the fully formed autophagosome with the lysosome and subsequent maturation of the autolysosome involves the Rab-SNARE (Soluble NSF Attachment Proteins Receptor) system including Ras-related protein Rab-7a (RAB7A) [4, 62, 63]. RAB7A is responsible for the

transport of autophagosomes along microtubules for proceeding fusion via interaction with specific effectors [4, 64]. In BCO affected bone, RAB7A mRNA expression was significantly downregulated indicating potential dysregulation of this key fusion machinery.

Taken as a whole, the *in vivo* results of this study suggest dysregulation of key machinery in every stage of autophagy in BCO-affected tissue. In order to determine if this dysregulation was a direct result of the bacterial component of BCO, an *in vitro* experiment was conducted with hFOB cells and known BCO isolate, *S. agnetis* 908. Not only did challenged cells show significant decreased viability, but also similar autophagy machinery dysregulation resulting from bacterial challenge. Key initiation protein Beclin1, as well as elongation machinery, ATG7, ATG16L, ATG12, and ATG3, were significantly decreased in *S. agnetis*-infected hFOB cells. Most notably, the ratio of LC3 type I to LC3 type II significantly increased under bacterial challenge. LC3 has been used as an indicator of autophagy function with the accumulation of LC3 type II in relation to LC3 type I occurring due to lack of LC3 type II lysosomal degradation when autophagy is dysregulated [65, 66]. These results could be indicative of a potential decrease in autophagic activity in bacterially challenged cells. In bone tissue, autophagy is essential in osteoblast differentiation with suppression of autophagy also playing a role in decreased cellular function via reduced autophagic vacuoles containing apatite crystals inhibiting mineralization [6]. These results present the ability of a known BCO isolate to not only impact cell viability, but also affect the autophagy machinery within cells in a manner which could inhibit autophagy.

To elucidate whether inhibition of autophagy could be the mechanism by which the bacterial exposure decreased cellular viability, two different autophagy inhibitors were used, 3-MA and CQ. 3-MA inhibits class II PI3Ks thereby inhibiting autophagy, primarily in the nucleation stage

[67]. While CQ inhibits autophagy via inhibition of lysosomal function and thereby acting on the fusion stage [68, 69]. Both treatments resulted in significant decreased cell viability and increased ratio of LC3 type II to type I. Additionally, decreased expression of ATG7 and Beclin1 was also seen in cells treated with 3-MA and cells treated with CQ. ATG12 and ATG3 protein was significantly lower in CQ treated cells compared to control. These results suggest that inhibition of autophagy, in either earlier or later stages, results in decreased cell viability comparable to that seen when cells are challenged with a known BCO isolate. Autophagy machinery were also significantly affected via these inhibitors comparable to bacterially challenged cells and BCO tissue.

Taken together, the results from this study are the first to implicate autophagy machinery dysregulation in the pathogenicity of BCO in modern broilers. While more research into the exact mechanism and machinery involved in BCO is needed, clearly, autophagy machinery dysregulation is present in BCO and could be caused by bacterial influence. This dysregulation has the capacity to reduce cell viability and potentially contribute to the etiology and symptoms of BCO. These findings give a new perspective into potential targets for treatment and prevention of BCO via genetics, probiotics, or pharmacological means.

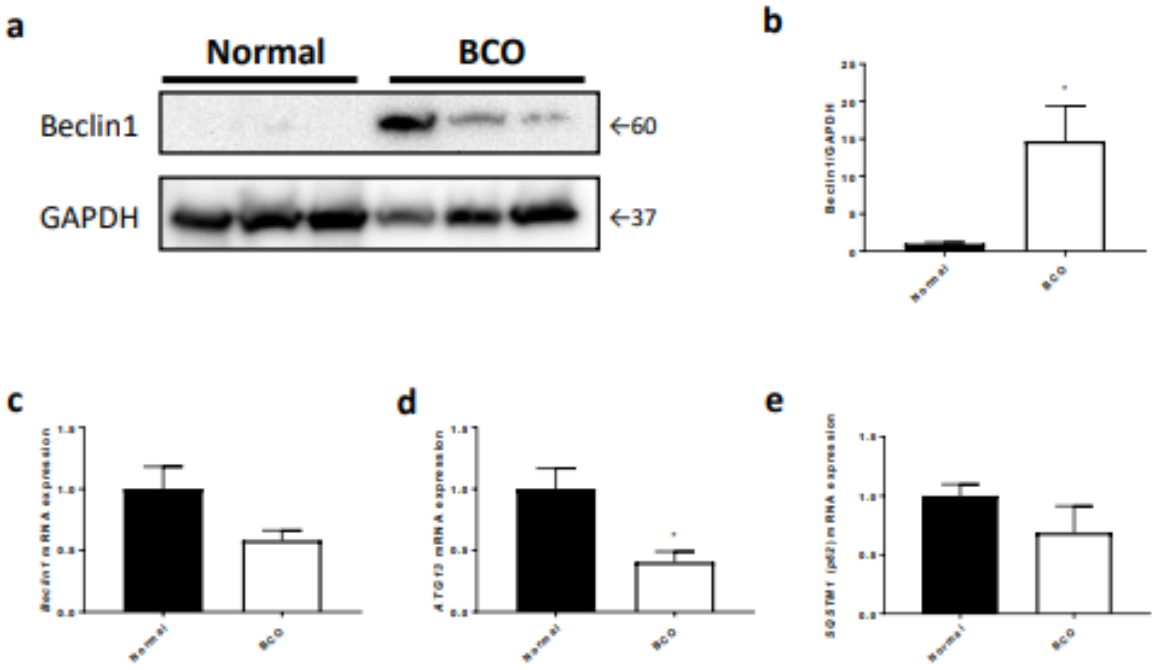

Figure 1. Autophagy Initiation Machinery in Normal and BCO affected Bone
Protein expression for initiation machinery Beclin1 (a) with corresponding statistical analysis (b). Gene expression for genes regulating autophagy initiation, Beclin1 (c), *ATG13* (d), and *SQSTM1* (*p62*) (e). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

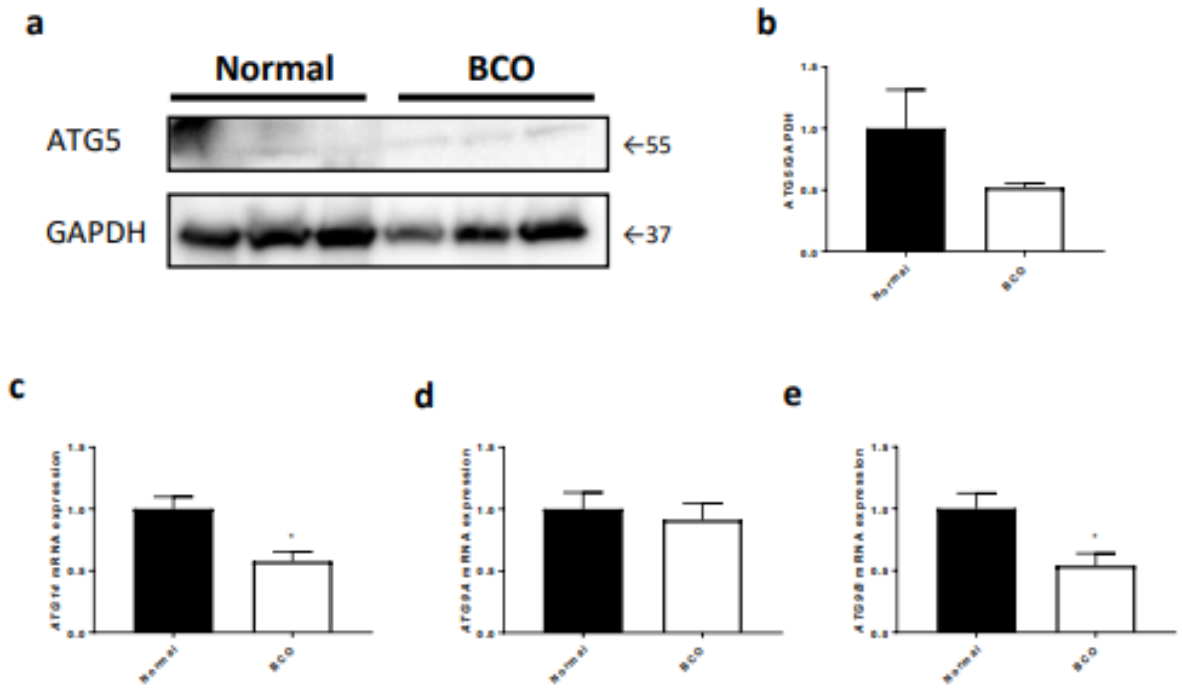

Figure 2. Autophagy Nucleation Machinery in Normal and BCO affected Bone
Protein expression for ATG5 (a) and statistical analysis of the Western blot results (b). Gene expression for key nucleation machinery, *ATG14* (c), *ATG9A* (d), and *ATG9B* (e). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

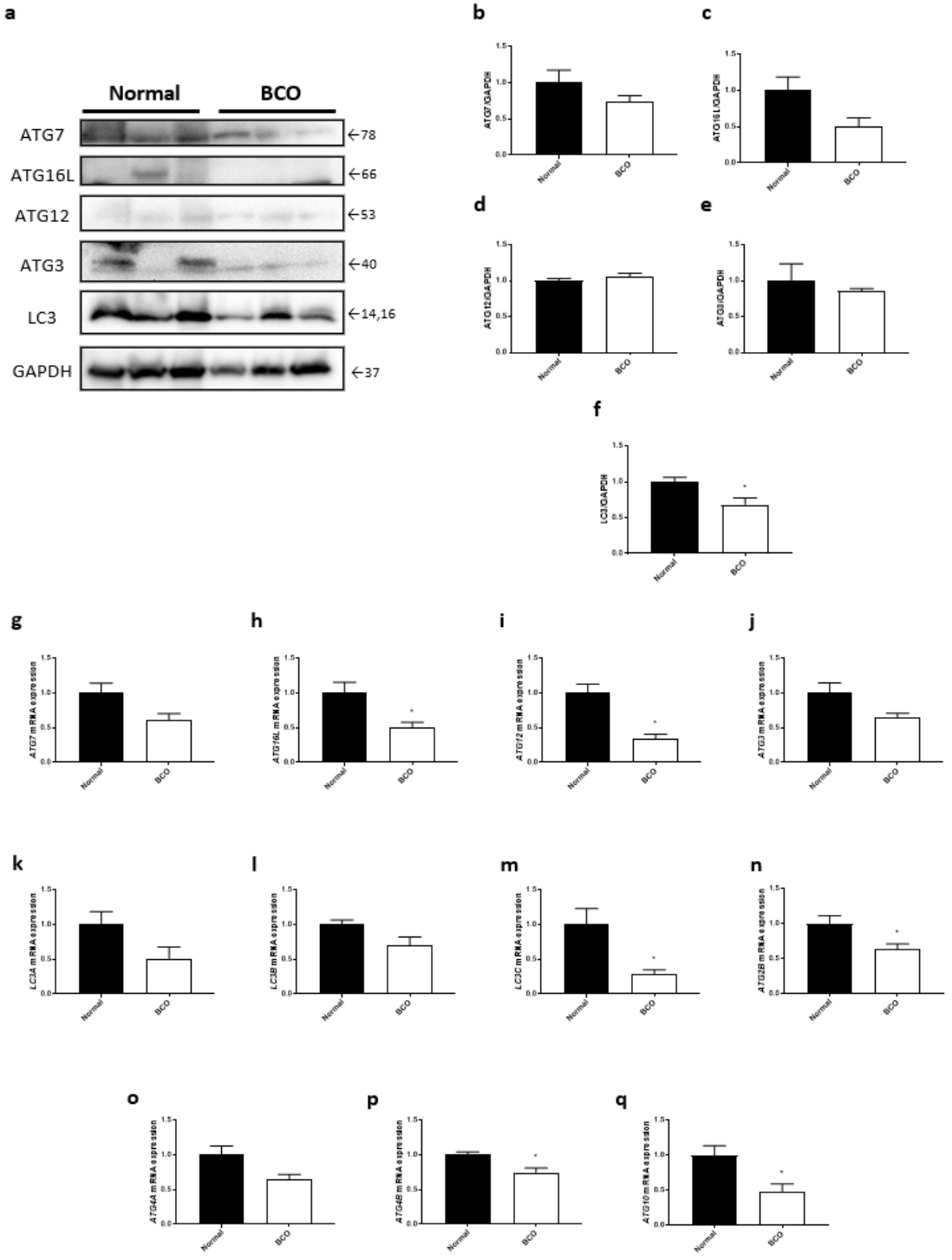

Figure 3. Autophagy Elongation Machinery in Normal and BCO affected Bone
Protein expression for ATG7, ATG16L, ATG12, ATG3, and LC3 (a) and statistical analysis of the results as compared to GAPDH (b, c, d, e, f). Gene expression for key elongation machinery, *ATG7* (g), *ATG16L* (h), *ATG12* (i), *ATG3* (j), *LC3A* (k), *LC3B* (l), *LC3C* (m), *ATG2B* (n), *ATG4A* (o), *ATG4B* (p), and *ATG10* (q). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

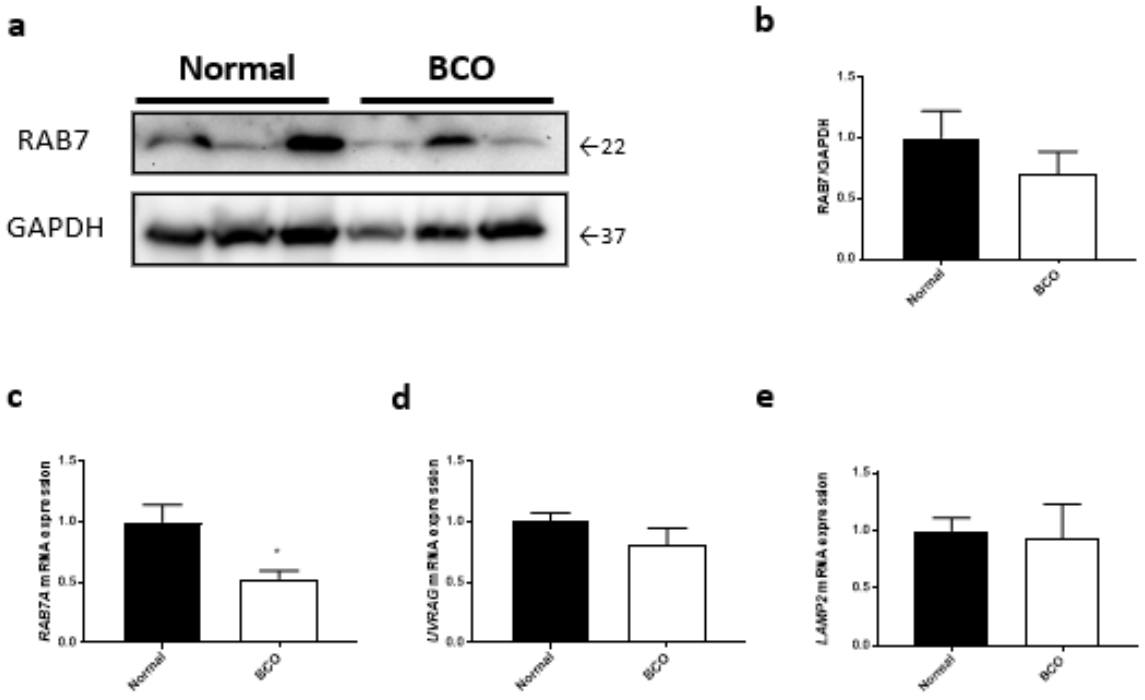

Figure 4. Autophagy Fusion Machinery in Normal and BCO affected Bone
Protein expression for RAB7 (a) and corresponding statistical analysis (b) as compared to GAPDH. Gene expression for *RAB7A* (c), *UVRAG* (d), and *LAMP2* (e), key autophagy fusion machinery. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

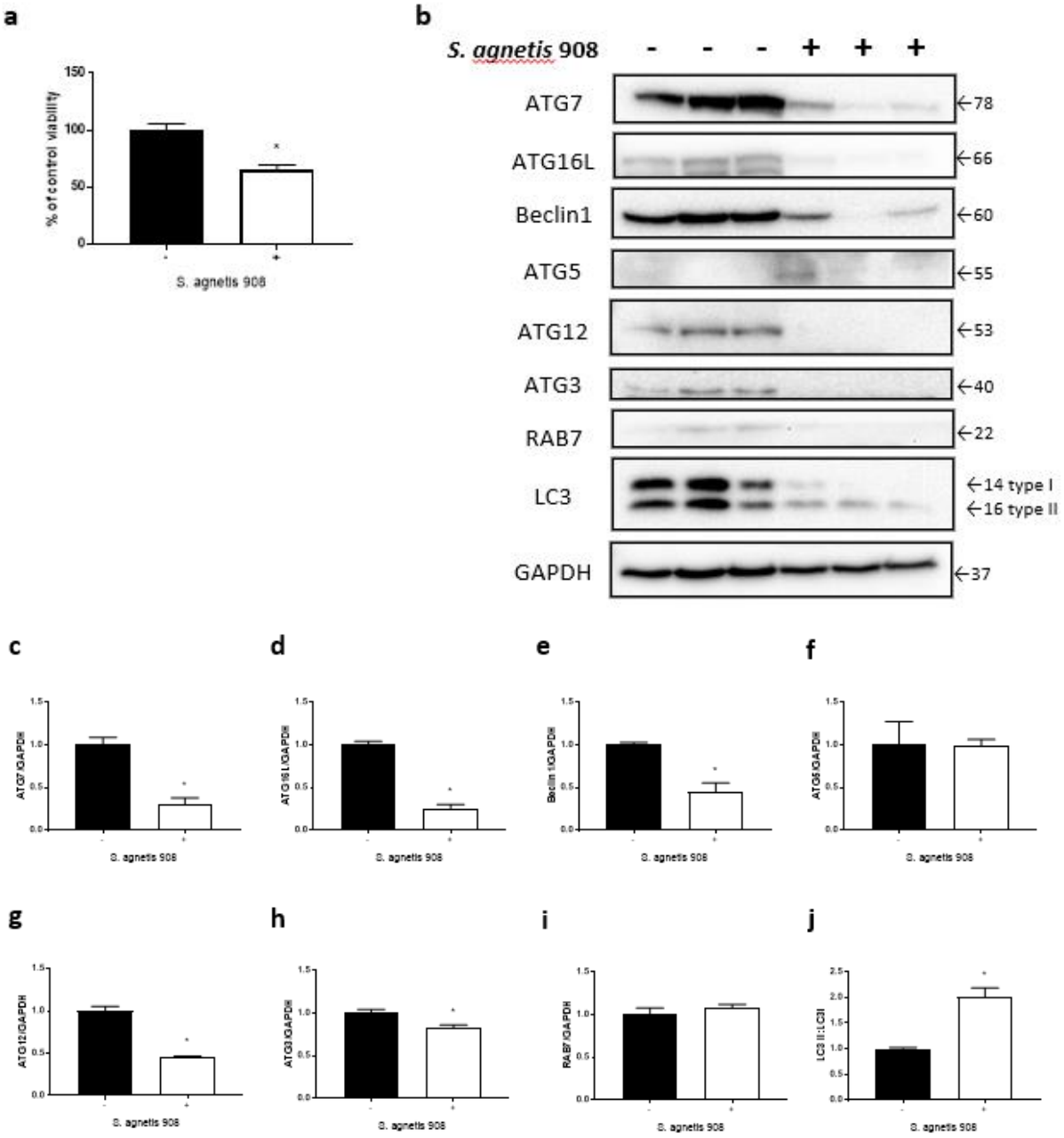

Figure 5. Effect of *S. agnetis* 908 Challenge on Cell Viability and Autophagy Machinery in hFOB cells

MTT assay results from hFOB cells treated with S. agnetis 908 at a MOI of 50:1 or a vehicle control for 24 hours (a). Western blot results (b) and statistical analysis for ATG7 (c), ATG16L (d), Beclin1 (e), ATG5 (f), ATG12 (g), ATG3 (h), RAB7 (i), and LC3 type II: type I (j) as compared to GAPDH. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between control and infected cells.

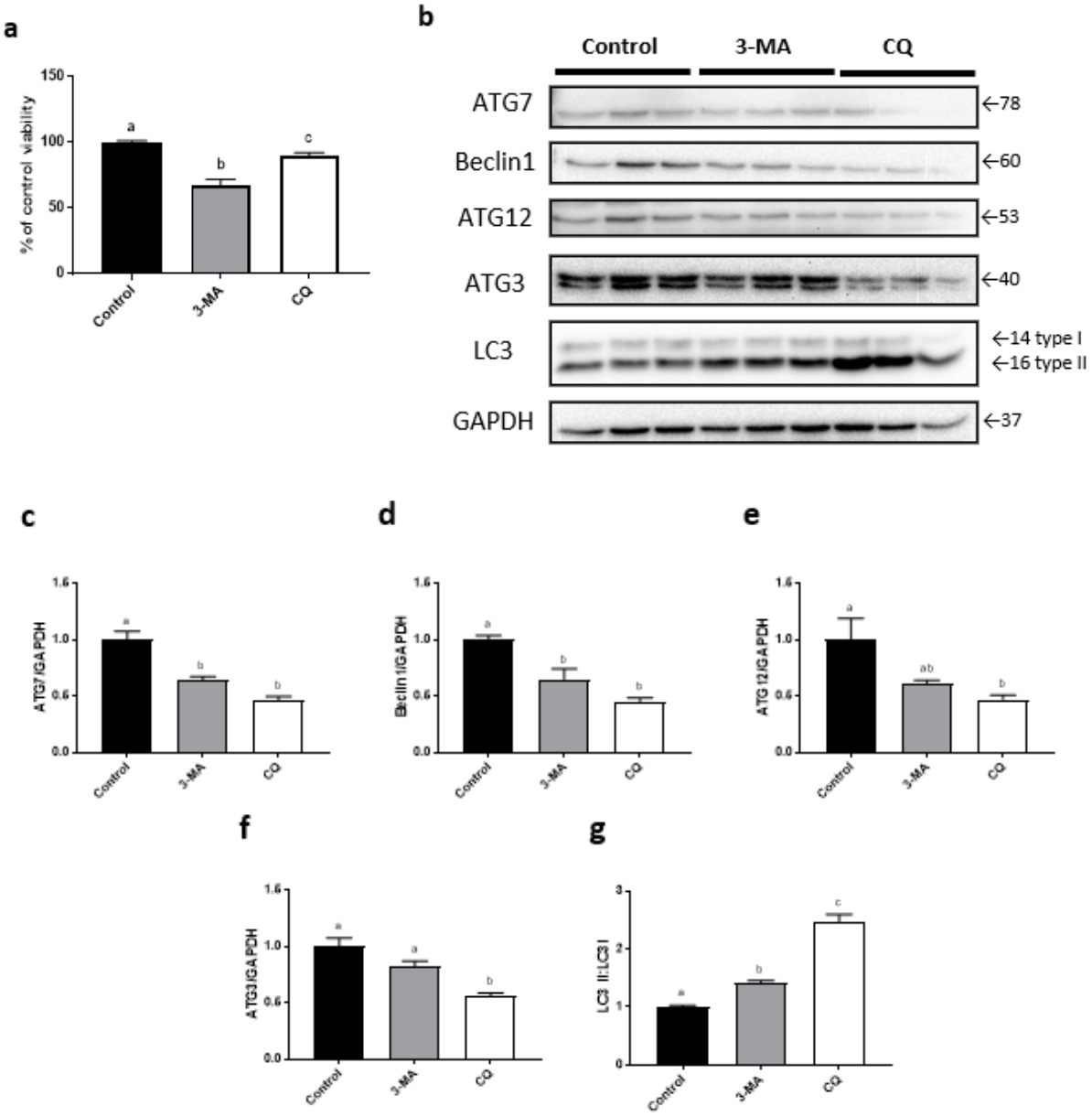

Figure 6. Effect of Autophagy Inhibition via 3-MA and CQ on Cell Viability and Autophagy Machinery in hFOB cells

MTT assay for cell viability presented as percent of control viability under treatment of 3-MA (5 mM), CQ (10 µM), or a vehicle control on hFOB cells for 24 hours (a). Western blot results for autophagy machinery proteins (b) and statistical analysis for ATG7 (c), Beclin1 (d), ATG12 (e), ATG3 (f), and LC3 type II: type I (g) as compared to GAPDH. Significance was determined using One-way ANOVA with p-value < 0.05. Different letters indicate significant differences between treatments.

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Chapter 6 –

Title: Cyto(Chemo)kines: Roles in Skeletal Physiology, Bacterial Pathology, and When those Roles Collide

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Key words: cytokine, chemokine, bone, bacteria, osteomyelitis, immunology

Methodology: Data for this review were compiled using databases such as NCBI PubMed, University of Arkansas Library Quick Search, run by WorldCat, Science Direct, and JSTOR. Search terms used included “cytokine”, “cytokines and bone”, “chemokines”, “bacterial infection and cytokines/chemokines”, “skeletal diseases and cytokines”, “immune system and cytokines”, “skeletal growth and cytokines/chemokines”, “bone and cytokines/chemokines”, “role of cytokines/chemokines in bone infection”, “bone physiology and cytokines/chemokines” and “cytokines/chemokines in circulation”.

Abstract: Molecular biology has revealed the complex interplay and overlap of molecules across biological systems such as the immune system and its relation to and influence on the skeletal system. Cytokines and chemokines are signaling molecules responsible for immune cell function as well as chemotaxis under inflammatory conditions. It has become clear that these molecules have influence on processes beyond inflammation, including cellular differentiation and

maturation, growth, and homeostasis, particularly in bone. In this review, key molecular families of cytokines and chemokines will be described as well as their function in healthy, growing or remodeling bone. Additionally, the current understandings of cyto(chemo)kines' involvement in inflammation and bacterial infections of bone will be expounded and provide a basis for considerations of future research.

Introduction

The skeletal system is home to the generation of immune cells as well as the site of intense growth and constant remodeling. The complexity of bone contributes to its susceptibility to disease and infection. Notoriously difficult infections to treat, osteomyelitis and periodontitis, present a unique challenge to researchers to understand the molecular mechanisms behind the symptoms of necrosis, bone loss, and inflammation to improve treatments and aid in diagnosis. Key modulators of inflammation as well as influential players in bone growth and remodeling, cytokines and chemokines represent a link between pathology and physiology within an organism. The expression pattern of cyto(chemo)kine dictates the immune response, anti- or pro-inflammatory, as well as the cell types involved under that condition. This requires cyto(chemo)kines to have systemic influence within a biological system and a unique profile within circulation of an inflammatory or infectious state. In the skeletal system, these cyto(chemo)kine profile has far reaching implications on cell differentiation, bone growth, and the delicate balance of bone resorption and formation in addition to regulating the bacterially-induced inflammatory response. Therefore, further research into the role of cyto(chemo)kines in both local and systemic immunoregulation and the etiology of bone infection is warranted and could provide novel treatments and means of diagnosis in the fields of human and veterinary medicine.

Overview of Cytokines and Chemokines

Cytokines

Inflammation is an immune process modulated via molecules such as cytokines. These modulators act in a complex network of signaling pathways and interactions resulting in a spectrum of physiological responses and states. The responses differ depending on the cytokine involved, the nature of the immune response, and the cell or tissue type within an organism. Primarily, cytokine driven inflammation involves heating and swelling as well as redness and subsequent pain or loss of function at the tissue level [1,2]. At the cellular level, inflammation involves the recruitment, activation, and modulation of the major immune system cells such as macrophage, neutrophils, monocytes, T-cells and B-cells. Cytokines play a role in the diverse cellular signaling, modulation, and downstream effects seen in inflammatory states. In addition to inflammation, cytokines are involved in cellular processes such as growth and survival [3,4]. They exist within certain organs, such as liver and bone, at low concentrations and can act in polarizing ways with both pro- and anti-inflammatory functions in addition to their influence over homeostatic processes. Cytokines can be grouped by their prospective molecular family, their target cell/function, and whether they act in primarily anti- or pro-inflammatory pathways. In this review, cytokines will be grouped and discussed first by their molecular family and then further defined by their mode of action and effects.

Types of Cytokines and their functions

There are many different cytokines, but two major types are interleukins and tumor necrosis factors. Interleukins can be both pro- and anti-inflammatory and are expressed by numerous cell types such as leukocytes and macrophages [5,6].

A pro-inflammatory interleukin cytokine is interleukin-1β (IL-1β). It is produced primarily in response to microbial molecules via pattern recognition receptors [1]. Once synthesized, IL-1β must then be cleaved to be activated and secreted. Once secreted, IL-1β binds to interleukin-1 receptor 1 (IL-1R1) found on the surface of target cells. This forms a subsequent signaling complex which then interacts with adaptor molecules leading to the activation of mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor kappa B (NF-κB) [7]. This activation leads to further production of pro-inflammatory cytokines and other downstream pro-inflammatory effects. Other pro-inflammatory interleukins include those in the same family as IL-1β, such as IL-18, as well as IL-6 and IL-17 [8,9]. IL-6 can have both pro- and anti-inflammatory effects and is produced by numerous cell types including endothelial cells, fibroblasts, and smooth muscle cells, as well as immune cells such as mononuclear phagocytes and both T- and B-cells. Its production can be induced by other cytokines such as TNFs and IL-1s, but also bacterial stimuli such as lipopolysaccharides (LPS) [9,10]. IL-6 has many roles in host immune response, most notably, it facilitates the final differentiation of B-cells to produce antibodies and stimulates production of acute phase proteins (APPs) in hepatocytes and other cell types [11]. IL-6 functions through a cell-surface receptor complex (IL-6R) with both a ligand binding glycoprotein and a signal transducing component, similarly to IL-1β [12]. IL-6R exists in both a soluble and membrane bound form. The soluble form creates a dimer with IL-6 that travels throughout the body while the membrane bound form executes normal physiological functions in certain cell types mediated via IL-6 [9]. The membrane bound signaling is considered classical signaling pathway which primarily occurs in leukocytes and liver cells and is anti-inflammatory in nature. In contrast, the signaling pathway induced by soluble receptor binding can occur in any cell expressing the soluble receptor within the cytoplasm and elicits

pro-inflammatory responses [11]. IL-6 has been shown to be detectable in circulation under experimental conditions such as induced endotoxinaemia as well as in septic conditions at high concentrations during initial phase. In the case of septic patients, the level of circulating IL-6 has been shown to correlate with severity of disease [13,14]. The dueling roles of IL-6, anti- and pro-inflammatory as well as physiological and pathological, regarding the skeletal system and skeletal diseases is covered in more detail in following sections.

While it is clear cytokines' roles may include both pro- and anti-inflammatory regulation, some cytokines tend to perform anti-inflammatory functions more regularly. Anti-inflammatory interleukins, such as IL-10 and IL-12, act to decrease pro-inflammatory cytokine production and change certain cellular activation or expression [15,16]. For example, IL-10 acts to shift the cytokine expression of T-cells to the T helper 2 (Th2) cytokine profile rather than T helper 1 (Th1) profile as well as decrease macrophage production of pro-inflammatory cytokines. The paradigm of Th2 and Th1 cytokine profiles and their effect on inflammatory and disease states has been extensively reviewed previously [17-20]. IL-10 is produced by numerous cell types in both the adaptive and innate immune system. The receptor for IL-10 is IL-10RA and it signals via the Janus tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs) similarly to other cytokines [21]. Ligand binding allows for recruitment of STATs which form homo- and hetero-dimers capable of nuclear translocation and subsequent transcription of target genes [22-24]. Within the IL-10 family, there are numerous cytokines and a diverse array of receptors. It is the unique expression patterns of cytokine-receptor combinations that result in diverse physiological and pathological responses [15]. So is the case with IL-12, where within the IL-12 family, distinct ligand-receptor combinations result in a range of responses including both pro-inflammatory and immunosuppressive effects depending

on pathological or physiological conditions. The IL-12 cytokine and IL-12R combination functions similarly to IL-10, using JAK and STAT signaling transduction pathways, but primarily acting on natural killer (NK) and T cells [25]. In the IL-12/IL12R signaling pathway, STAT4 and its transcription targets lead to the potentiation of Th1 differentiation. Notably, this signaling pathway has also bene shown to provide negative feedback queues which eventually reduce IL-12 signaling [16,23]. Interleukins are cytokines with diverse and sometimes combating roles which act via a network of receptors and signaling molecules to have both physiological and pathological effects.

Another major group of cytokines are tumor necrosis factors (TNFs). The TNF superfamily consists of transmembrane proteins including receptors and ligands with numerous and diverse functions. Identified in the 1970s, tumor necrosis factor α (TNFα) is a prototypic member of the TNF super family and an endotoxin-induced, monocyte-derived serum factor responsible for necrosis of certain tumors. Since its discovery, TNFα has proven to be an essential component of the innate immune system via its potent pro-inflammatory regulation. Its many roles include inducing cytokine production, stimulating growth, and activating adhesion molecules [26-28]. TNFα is first synthesized as a transmembrane precursor transported by the rough endoplasmic reticulum and Golgi complex to the cell surface for secretion. The soluble form of TNFα results from cleavage by the metalloprotease TNFα converting enzyme (TACE) with the trimers acting as the potent ligand for TNF receptors on target cells[27]. Further cleavage leads to translocation to the nucleus where pro-inflammatory cytokine signaling is induced. The more ubiquitously expressed TNF receptor 1 (TNFR1) is the primary mediator for TNFα pro-inflammatory effects. In addition to immunoregulatory effects, TNFα has functions related to lipid metabolism, insulin

resistance, skeletal growth, and coagulation and has been implicated in numerous disease states [28].

Chemokines

A major tenant of the immune system is its ability to recruit necessary cells to sights of infection or injury and those cells' ability to interact [29]. Chemokines are molecules with the specific function of directing of the circulation of lymphoid cells and recruiting them under necessary conditions. Like cytokines, they function both physiologically, also known as homeostatic chemokines, and pathologically, or inflammatory chemokines. Additionally, the distinction between these two functional groups is not concrete and overlaps in chemokine function exists. A common way of grouping chemokines is using either CC, CXC, XC, or CX3C to indicate the spacing of the first two cysteines on the amino terminals of the proteins [7]. Chemokine ligands are denoted as one of these four groups followed by "L" whereas receptors are denoted as "R". This review will focus primarily on CCL and CXCL chemokines.

Homeostatic CC chemokines include CCL20, CCL17, CCL19, and CCL21 [30]. CC chemokine ligand 20 (CCL20) binds to receptor CCR6 and was one of the first chemokines to be discovered via bioinformatics-based DNA database searching and sequencing techniques [31]. It was given three names from three different research groups. The first name was based on its expression in the liver leading to the name liver and activation-regulated chemokine (LARC) [32]. The second group cloned the cDNA from an activated human monocyte cDNA library and designated it CC chemokine macrophage inflammatory protein-3α (MIP-3α) [33]. Now recognized as CCL20, the protein structure is similar to other chemokines with three-stranded antiparallel β-sheet and a COOH- terminal α-helix [34]. There is also a loop region believed to be involved in initial binding of CLL20 with its receptor [33]. CCL20 has been shown to have effects on T- and B-

lymphocytes and dendritic cells. For dendritic cells, it is clear that CCL20 and tis receptor CCR6 play a role in recruitment of immature dendritic cells and precursors to sites of infection or injury [35]. It has been shown that LPS as well as some viruses and cytokines, such as TNFα and IL-4, are able to induce CCL20 expression in several cell types. It has also been shown that CCL20 has constitutive expression in several tissues which contributes to its more homeostatic function [7]. However, CCL20 follows the same overlapping pattern seen in many chemokines in its involvement in both homeostatic and pathological processes. In particular, pathologies involving endothelial surfaces have demonstrated CCL20 involvement including atopic dermatitis, inflammatory bowel disease, and rheumatoid arthritis [36-38].

Inflammatory CC chemokines include CCL4 and CCL5. CCL4 has a very similar structure to CCL20 with triple-stranded antiparallel β-sheets and the CCL4 dimer is cylindrical in general and elongated. It was first found in the medium of LPS-activated macrophages and has been shown to induce calcium mobilization in several cell types including NK cells, monocytes, leukocytes, and vascular smooth muscle cells [39,40]. CCR5 is the most widely known receptor for CCL4 on the cell surface and is a member of the G-protein-coupled receptor family. CCL4 expression has been associated with type 2 diabetes mellitus and CCR5 expression is associated with inflammatory states and infections in addition to diabetes [29].

CXCL chemokines that are primarily homeostatic include CXCL13 and CXCL14. CXCL14 has been coined the “Swiss army knife” of chemokines due to its role in homeostatic migration of dendritic and NK cells as well as its proven antimicrobial activity against certain bacterial pathogens [41]. For example, decreased constitutive CCL14 expression has been observed in cases of atopic dermatitis lesions [42]. Its expression has also been seen in circulating blood cells as well as skin and saliva barriers which all point to its involvement in innate, and potentially

adaptive, immune response including chemotaxis in addition to its angiogenic and homeostatic functions.

A major inflammatory chemokine is CXCL8, known as α chemokine or IL-8, with the orthologs in avian species being CXCLi1 and CXCLi2 [4,43]. Originally discovered due to its role in chemoattraction of neutrophils, IL-8 also acts on migration and activation of monocytes, lymphocytes, and other cell types at sites of infection and is considered a key inflammatory mediator [44]. Beyond inflammation, IL-8 has been shown to be an angiogenic factor on human microvascular endothelial cells [45]. IL-8 binds to two receptors, CXCR1 and CXCR2 resulting in a diverse array of intracellular signaling cascades inducing chemotaxis and degranulation. The chemotactic signaling induced by IL-8 is primarily through binding with CXCR1. These signaling cascades involve activation of G-proteins which ultimately stimulate enzymes that activate MAPK signaling. MAPK signaling can induce cell survival, proliferation, and inflammation via modifying a cell's gene expression. Some target genes include adhesion molecules such as integrin for chemotaxis as well as production of 3,4,5-inositol triphosphate (IP3) stimulating the release of intracellular calcium stores and affecting degranulation [46,47]. Chemokines act as chemotaxis modulators affecting the make-up of the cellular community involved in inflammation and the function of those cell types. They have diverse effects ranging from homeostasis to inflammation and adaptive immune responses.

Cyto(Chemo)kines Roles in Physiological and Pathological Responses

Due to their diverse roles and scope of influence, cytokines and chemokines have been implicated in both injury and infection as well as normal growth and homeostasis within an organ system, such as the skeletal system[48-50]. Cytokines and chemokines alike have shown significant involvement in the immune response to bacterial pathogens.

Cyto(Chemo)kine Signaling in Skeletal Growth/Remodeling

Skeletal growth is a complex process involving primarily endochondral ossification, a process in which cartilage is an intermediate to bone formation [51]. This process begins in the center of the bone and is followed by growth plate endochondral ossification from either end. The primary cells involved in this process are chondrocytes which undergo differentiation, hypertrophy, and maturation/mineralization, followed by invasion of bone forming osteoblasts and bone absorbing osteoclasts [52]. Vascularization is needed to simultaneously retreat and remodel within the areas of bone growth for the process to continue. Even after skeletal growth is complete, bone remodeling occurs throughout life and relies on a complex network of cell signaling, differentiation, proliferation, and function [53].

The connection between bone and the immune system starts at the very beginning of cellular stages. The progenitors of osteoblasts and osteoclasts are located in the bone marrow where they exist in direct contact with progenitors of immune cells. Osteoclasts themselves are a highly specialized immune cell as they derive form hematopoietic progenitor cells [54,55]. Additionally, immune cells are known producers of key osteogenic and osteoclastogenic factors and several inflammatory disorders have shown to have significant effects on bone mass [56,57]. Indeed, several cytokines have shown to promote osteoclastogenesis including TNFα, IL-1, IL-17, and IL-6 [48,56]. A major player in bone growth and remodeling is the transmembrane protein receptor activator of nuclear factor κB (RANK) or RANK ligand (RANKL). RANKL is key to osteoclast differentiation and therefore bone resorption [58]. IL-17 is known to indirectly promote osteoclastogenesis via induction of RANKL production in certain immune cells and osteoblast lineage cells [3,8]. Influencing RANKL within bone acts as a means of shifting the

balance of bone resorption and bone mineralization and can have effects on bone mass and growth.

Chemokines have also been implicated in physiological conditions of bone remodeling as well as pathological conditions. Both CCL20 and CCR6 deficient mice, showed decreased trabecular bone mass that was attributed to decreased bone formation due to lower numbers of osteoblasts [59]. The pathological effects of CCL20 in bone will be discussed in a subsequent section.

Another chemokine that has shown to have potential effects on bone remodeling and growth is IL-8. *In vitro* studies have shown that both osteoclasts and osteoblasts express IL-8 under treatment with inflammatory mediators, including IL-1β and TNFα [60,61]. IL-8 has also been shown to influence the differentiation of circulating monocytes as conditioned media from IL-8 stimulated primary human osteoblasts lead to osteoclast formation of peripheral blood monocytes [62]. These findings indicate that IL-8 can affect bone resorption via several means. Notably, it has also been shown that breast cancer patients with bone metastases had elevated levels of plasma IL-8 which correlated with increased bone resorption [63].

Overall, cytokines and chemokines have an integrated relationship with bone during growth, remodeling, and homeostasis. This relationship becomes even more complex when the cytokines and chemokines become involved in an immune response to infection or disease.

Cyto(Chemo)kine and Inflammation of the Skeletal System

Cytokines and chemokines participate in numerous cellular processes and affect an array of cell types which overlap both physiology and pathology. In regard to the skeletal system, this complex network of cell signaling, and chemotaxis becomes central to inflammatory response and disease symptoms.

Inflammatory states, especially when chronic, coincide with decreased bone mass as well as potentially decreased longitudinal bone growth [57]. The mechanism by which this association occurs is activated and regulated by the cytokine and chemokine during inflammation. Transgenic mice, overexpressing TNFα and IL-6, experience growth retardation resulting from decreased insulin-like growth factor 1 (IGF1) and IGF1-binding protein (IGFBP3) which are integral to longitudinal bone growth at the growth plate [64]. It has been shown that cytokines can have a systemic effect on skeletal growth via suppression of IGF1 [65,66]. Conversely, TNFα and IL-1β act to suppress growth by decreasing chondrocyte proliferation and hypertrophy while increasing apoptosis within the growth plate [65]. A common disease involving inflammation of the growth plate is rheumatoid arthritis (RA). In RA, bone is rapidly degraded leading to bone erosions due to inflammation [37]. As the main drivers of inflammation, including mediators and chemotaxis of immune cells, cytokines have been implicated in RA pathogenesis [67,68]. The mechanism by which cytokine induce bone degradation is via their influence on differentiation and activity of osteoclasts as previously described. The balance of bone-forming to bone-resorbing cell types is off-balance under inflammatory conditions leading to increased bone resorption and overall bone mass loss. IL-17 has also been implicated in RA inducing bone loss by similar means as other cytokines. Notably, inhibition of IL-17 in animal models of inflammatory arthritis showed reduced bone loss potentially based on upregulation of regulatory cytokines including IL-4 and IL12 [69]. While pro-inflammatory molecules clearly possess primarily osteoclastogenic functions, especially in inflammatory diseases of the bone, anti-inflammatory cytokines such as IL-10 and IL-4 are negative regulators of osteoclast formation. These cytokines are involved in the Th2 differentiation of the T-cell lineage and show to have regulatory effects on the skeletal system in opposition to their pro-inflammatory

counterparts [70,71]. Under both cancer-induced bone metastasis as well as inflammatory RA, the chemokine CCL20 was shown to be correlated to bone loss as well as metastasis and patient survival. Increased CCL20 expression was found in subchondral bone of RA patients while in a mouse model for breast cancer bone metastasis, treatment with anti-CCL20 inhibited metastasis and osteolysis [72,73]. In skeletal inflammatory diseases, cytokines and chemokines act by influencing the ratio of osteoblast to osteoclast and thereby affecting bone mass and homeostasis. Another cell type affected by inflammatory state of the bone is the chondrocyte. Chondrocytes are the main cell type of the growth plate and responsible for endochondral ossification in longitudinal bone growth [74]. Osteoarthritis is an inflammatory degenerative disease affecting the articular cartilage and the chondrocytes therein. It is characterized by decreased extracellular matrix formation by chondrocytes as well as increased matrix metalloproteinases and aggrecanases [75,76]. Both symptoms have been associated with the expression of pro-inflammatory cytokines such as TNFα and IL-1β. It has been shown that metalloproteinases and aggrecanases resulting from these cytokines inhibits the expression of cartilage-specific markers, type II collagen and aggrecan [77]. This shift away from the anabolic processes within the chondrocytes necessary for endochondral ossification contributes to osteoarthritic cartilage degradation and shows how inflammatory states have the potential to produce detrimental cytokine profiles within bone.

The cell types involved in bone growth and remodeling are all influenced by cyto(chemo)kines under inflammatory states. Consequently, a common symptom of inflammation is bone loss and degeneration due to lack of bone formation and dysfunctional cellular processes.

Cyto(Chemo)kines and Bacterial Infection in Bone

In the event of severe bacterial, and even viral, infections, there is a robust production of cytokines and resultant immunopathology that has been called a “cytokine storm” [78,79]. It has been shown that morbidity and mortality in infectious states can be linked to the cytokine and chemokine levels that make up the “storm” [80]. The diverse array of cytokines and chemokines produced as well as their numerous receptor binding and signaling effects results in complex immune and inflammatory responses under bacterial challenge or infection.

In bone, bacterial infections often involve bacteria with receptors for collagen or bone sialoprotein, such as *Staphylococcus aureus* [81,82]. The means by which bacteria cause pathological bone loss are still being investigated. However, it is clear that the bone cell type affected as well as the bacteria involved play a role. Cytokines have been implicated as potential means of bacteria to induce cellular pathways that promote bone resorption rather than formation [83,84]. As previously described, certain cytokines influence stages of bone cell differentiation and maturation. These cytokines are also induced under bacterial infection, resulting in similar pathways of osteoclastogenic and osteolytic influence. Additionally, surface proteins of *S. aureus* have been shown to induce bone loss via potent osteolytic effects which are inhibited by IL-1 receptor or TNF antibodies [85]. Similar trends have been seen under LPS stimulation in bone as well [85]. This indicates bacterial influence on bone resorption via modulation of cytokines and their pathways leading to increased osteoclast activity and decreased bone formation. Bone cells such as osteoblasts have been shown to produce cytokines, such as IL-6, under bacterial infection such as osteomyelitis conditions, potentially exacerbating the effects of bacterial infection, inflammation, and bone loss [86,87]. Research using other bacterial species responsible for bone infections, such as *Escherichia coli* and *Porphyromonas gingivalis*, have shown similar effects of

LPS and surface proteins inducing shifts in bone-resorbing factors and osteolytic factors including IL-1, IL-6, nitric oxide, and granulocyte-macrophage colony stimulating factors [88-95]. In implant-associated osteomyelitis, the gene expression of chemokines CCL3 and CXCL2 were increased in osteolytic sites. It was also shown that osteoblasts produced both CCL3 and CXCL2 under bacterial challenge and CCL3 induced monocyte differentiation to osteoclasts [96].

In addition to stimulating bone resorption and osteolytic factors, endotoxin-LPS has also shown to inhibit bone collagen and non-collagenous protein synthesis [97,98]. Additionally, surface-associated proteins from causative agents of periodontitis inhibited bone matrix synthesis [99]. For example, *Bordetella bronchiseptica*, a bacterium responsible for oral bone lesions exhibiting impaired osteoblastic function, produces a dermonecrotic toxin which lead to decreased capacity of murine osteoblasts to produce both alkaline phosphatase and collagen *in vitro* [100-102]. Notably, exposure to dermonecrotic toxin also resulted in multinucleated osteoblasts indicating this toxin's potential ability to inhibit normal cellular division [103]. The mechanism by which bone matrix synthesis is inhibited, and its relation to potential cell cycle inhibition, is still being investigated. Studies have shown a range of bacterial proteins interfere with cellular functions such as protein synthesis, maturation, differentiation, and potentially cellular division. These effects seem to be carried out in-part by the modulation and activation of the bacterial-induced, cytokine "storm".

The effect of the bacterial infection in bone is seen beyond the tissue level as well, with evidence of shifts in circulating cytokine profiles in osteomyelitis. Indeed, tuberculous osteomyelitis is associated with elevated circulating IL-6 concentrations [104]. Other studies have found a distinction between early and late-stage chronic osteomyelitis, with initial increases of IL-4 and

TNFα in peripheral mononuclear cells followed by increased IL-10, IL-2, and IL-6 [105]. A similar study involving LPS stimulation of whole blood from patients with chronic osteomyelitis as well as evaluation of infected bone found increased expression of TNF, IL-6, and IL-8 locally in the bone with no significant difference in blood after stimulation [106]. Cytokines play an integral role in bacterial infections in bone, their influence on a systemic level and their detection continue to be investigated.

The targeting of cytokines and chemokines in bacterial infections has gone beyond human medicine to reach animal agriculture as well. Bacterial chondronecrosis with osteomyelitis (BCO), also called femur head necrosis (FHN), poses a significant threat to the poultry industry as a leading cause of lameness and production loss in modern broilers [107,108]. Recent studies analyzing the transcriptome of the proximal femur head in broilers with femur head separation, a perceived indicator early-stage BCO, revealed genes involved in the immune system, including cytokine response to be differentially expressed [109]. Notably, some cytokines were also differentially expressed including CCL19 and CCL21 along with their receptor CCR7. These cytokines have also been implicated in rheumatoid arthritis pathogenesis via upregulation of osteoclast activity as previously discussed [110]. In an experimental model of FHN using methylprednisolone intramuscular injections, broilers with FH had increased serum levels of catabolic cytokines such as IL-1β, which was concluded to contribute to a disruption of extracellular matrix homeostasis. This disruption could lead to increased susceptibility to BCO/FHN via a shift to resorption over bone formation [111]. Analysis of BCO affected and healthy bone revealed the involvement of dsRNA accumulation and NLRP3 inflammasome activation in the presence of increased IL-1β expression in BCO affected tissue. Of interest, this inflammasome activation was shown to be induced by IL-1β in an *in vitro* model [112].More

research is needed in order to fully elucidate the role cytokines play in BCO and whether their presence could be used for treatment as well as diagnosis which could improve prevention, detection, and treatment in modern broilers.

Conclusions and Perspectives

Cytokines and chemokines are biological bridges between physiology and pathology, especially within the skeletal system. The unique and diverse ligand-receptor combinations among cytokines and chemokines present a myriad of signaling pathways and functions at the local tissue and systemic circulation level. Continued research into the overlap of immune response and homeostasis in which cytokine sand chemokines are involved could shed light on previously unknown links and mechanisms in bone inflammatory diseases and bacterial infections. These potential links could provide a foundation for improved diagnosis and treatment of bone related diseases in both human medicine and animal agriculture.

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Chapter 7 –

Title: Local and Systemic Cytokine, Chemokine, and FGF Profile in Bacterial Chondronecrosis with Osteomyelitis (BCO)-affected Broilers

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Abstract: Complex disease states, like bacterial chondronecrosis with osteomyelitis (BCO), not only result in physiological symptoms, such as lameness, but also a complex systemic reaction involving immune and growth factor responses. For the modern broiler (meat-type) chickens, BCO is an animal welfare, production, and economic concern involving bacterial infection, inflammation, and bone attrition with a poorly defined etiology. It is, therefore, critical to define the key inflammatory and bone-related factors involved in BCO. In this study, the local bone and systemic blood profile of inflammatory modulators, cytokines, and chemokines, was elucidated along with inflammasome and key FGF genes. BCO-affected bone showed increased expression of cytokines IL-1β, while BCO-affected blood expressed upregulated TNFα and IL-12. Chemokine profile revealed increased IL-8 expression in both BCO-affected bone and blood in

addition to inflammasome NLRC5 being upregulated in circulation. The key FGF receptor, FGFR1, was significantly downregulated in BCO-affected bone. Exposure of two different bone cell types, hFOB and chicken primary chondrocytes, to plasma from BCO-affected birds, as well as recombinant TNFα, resulted in significantly decreased cell viability. These results demonstrate an expression of pro-inflammatory and bone-resorptive factors and their potential contribution to BCO etiology through their impact on bone cell viability. This unique profile could be used for improved, non-invasive detection of BCO and provides potential targets for treatments.

Keywords: Cytokines; FGF; BCO; FHN; lameness; broilers

Introduction

Modern broiler chickens are characterized by fast growth and high meat yield resulting in a highly efficient means of producing animal protein. This achievement in performance has been met with physiological obstacles such as lameness which leads to animal welfare concerns and production loss [1]. A common cause of lameness is bacterial chondronecrosis with osteomyelitis (BCO), also known as femur head necrosis (FHN). BCO involves bacterial infection, bone attrition, and lack of healing in the proximal head of the femur and tibia [2]. These points are known to be key mechanical stress points within the skeletal system resulting in increased susceptibility to bacterial infection [3]. The highly vascularized avian growth plate coupled with occurrence of mechanically induced “wound sites” within the highly dynamic network of cells of the growth plate creates the perfect storm for BCO [3]. The necrosis and attrition of bone with simultaneous bacterial infection leads to rapid deterioration of animal welfare which is exacerbated by the inability to detect BCO without necropsy, especially when subclinical. While it is clear that BCO-affected broilers are under multiple stressors, both

immune and physiological, it has yet to be investigated whether BCO has systemic effects on inflammatory pathways which may contribute to the etiology seen locally which could also be used as a means of detection via a circulating biomarker.

The key characteristics of BCO, infection, inflammation, and lack of healing or regeneration, provide a roadmap of potential pathways involved. As mediators of the inflammatory response under both stress and bacterial infections, cytokines and chemokines have been implicated in numerous skeletal diseases including rheumatoid arthritis, osteoarthritis, periodontitis, and osteomyelitis [4-9]. Their involvement in inflammasome activation has also been well documented and links their roles to cell survival and homeostasis [10]. Additionally, these molecules have key physiological functions in bone development and homeostasis [11,12]. Fibroblast growth factors regulate healing and development as well as immune cell states and function in both bone and on a systemic level [13,14]. Specifically, FGF23 is a key factor in the Bone-Kidney-Cardiac-immune axis as well as macrophage transition under inflammatory states [15]. FGF pathways have also been implicated in healing and bone regeneration which is a major tenant lacking in BCO-affected bone [13]. Therefore, this study was undertaken to investigate whether systemic effects of BCO exist as well as profile the local and systemic expression of cyto(chemo)kines and key FGFs and evaluate their potential role in BCO etiology.

Materials and Methods

Bone and blood sample collection

All animal experiments were approved by the University of Arkansas (Fayetteville, AR) Animal Care and Use Committee (protocol number 15043) and were in accordance with recommendations in *NIH's Guide for the Care and Use of Laboratory Animals*. The BCO model

and healthy counterparts were conducted as previously described [16,17]. Briefly, animals were placed on either litter or wire-flooring and had ad libitum access to fresh water and feed for 56 days. The wire-flooring environment consisted of a raised floor made of wire covered panels to create unsteady footing. This design has been shown to induce spontaneous BCO in broilers in addition to increasing the occurrence of lameness [16]. Ambient temperature and lighting conditions followed industry standards, as previously described [10]. At the end of the 56 days, animals were assessed for presence or absence of lameness. First, blood was drawn from the wing vein of lame birds that were reared on wire-flooring and non-lame birds reared on litter. Next, birds were humanely euthanized and immediately necropsied to determine presence of subclinical lesions in the proximal heads of both the femora and tibiae. Bone was selected macroscopically based on a previously reported scale [16]. Normal blood and bone came from non-lame birds, reared on litter that did not have BCO lesions. BCO-affected blood and bone were from lame birds reared on wire-flooring with severe BCO lesions in both legs. Proximal portions of bone, primarily consisting of the growth plate, were snap frozen in liquid nitrogen and stored at -80˚C for later analysis. Blood was either stored in heparin treated tubes for cell treatment or Trizol reagent (Life Technologies, Carlsbad, CA) for RNA isolation.

Cell Culture and Treatment

Human fetal osteoblast (hFOB) 1.19 cells (CRL-11372; ATCC, Manassas, VA) were cultured in a 1:1 mixture of Ham's F12 medium/Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 0.3 mg/mL G418. Cells were grown at 34ºC in a humidified atmosphere of 95% air and 5% CO_2. Chicken primary chondrocytes were grown in a media of DMEM with 4.5g glucose/L, glutamine, HEPES, and sodium pyruvate supplemented with 10 ng/mL ascorbic acid, 10% FBS, and 1% penicillin/streptomycin. Primary chondrocyte cells were

grown at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. At 80% confluence, cells were synchronized using serum-free media overnight before being treated with human recombinant IL-1β (100 ng/mL) (PreproTech, East Windsor, NJ), IL-8 (100 ng/mL) (R&D Systems, Minneapolis, MN), TNFα (10ng/mL) (PreproTech, East Windsor, NJ), or dimethyl sulfoxide (DMSO) as control for 24 hours (n=3/group). Following treatment, cells were processed for viability as described below. Additionally, hFOB cells were washed three times with phosphate-buffer saline (PBS) and treated with media containing either 5% plasma from healthy or BCO affected birds or PBS (n=3/group). Cells were maintained for an additional 4 hours before being processed for cell viability.

Cell Viability

Cell viability for hFOB cells was performed as previously described [18]. Briefly, cells were seeded at 1×10^4 cells per well of a 96-well plate before being treated as described above. CellTiter 96 AQueous One Solution CellProliferation Assay (Promega, Madison, WI) was used, according to manufacturer's recommendations, and results were obtained using a Synergy HT multimode microplate reader (BioTek, Winooski, VT). All sample readings were background corrected, and results were reported relative to control.

Cell viability for primary chondrocyte cells was conducted using Trypan Blue live-dead stain (0.4%) (Life Technologies, Carlsbad, CA) and quantified using Countess II FL (Life Technologies, Carlsbad, CA).

RNA isolation, reverse transcription, and real-time quantitative PCR

From the normal and BCO-affected bone and blood samples (n =4/group), total RNA was isolated in accordance with the protocol of previous work [19]. Total RNA was isolated using

Trizol reagent (Life Technologies, Carlsbad, CA), based on manufacturer's instructions. RNA concentrations were determined using Synergy HT multimode microplate reader and total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithsburg, MD). Amplification was achieved using Power SYBRGreen Master Mix (Life Technologies, Carlsbad, CA) and real-time quantitative PCR (7500 Real Time System; Applied Biosystems, Foster City, CA). The sequences for oligonucleotide primers for *r18s, TNFα, IL-1β, IL-18, IL-3, IL-4, IL-10, IL-6, CRP, CCLL-4, CXCL-14, CCL-4, CCL-20, NLRP3, NLRC5, NLRX1,* and *NLRC3* were previously published [10,20,21]. Additional primers used were *IL-17* (forward, 5'-CCTTGCTCCTGCTTGCTTTC -3'; and reverse, 5'- AACCAATCGCTCCCCATTTT -3'), *IL-12B* (forward 5' – TGCCCAGTGCCAGAAGGA - 3'; and reverse, 5' – TCAGTCGGCTGGTGCTCTT - 3'), and chemokines *IL-8L1* (forward 5' - CAGAACCAAACCCAGGTGACA - 3'; and reverse, 5' - ACAGCCTTGCCCATCATCTT - 3'), *IL-8L2* (forward 5' - TCCTGGTTTCAGCTGCTCTGT - 3'; and reverse, 5' - CGCAGCTCATTCCCCATCT - 3'), *CCL-5* (forward 5' - TTTCTACACCAGCAGCAAATGC - 3'; and reverse, 5' - GCCCCTTCCTGGTGATGAA - 3'). Also measured were *FGF23* (forward 5' - CTGCTTGTGCTCTGTATCCTGAA - 3'; and reverse, 5' - CAGCAGCGGAGAGGAGTTG - 3'), *FGFR1* (forward 5' - GCCCCGGAGGCTCTGT - 3'; and reverse, 5' - CCGAAGGACCAAACATCACTCT - 3'), and *Klotho* (forward 5' - TGGCGATGTCCCGGTTTAT - 3'; and reverse, 5' - ATATACTCTGAGCTTATCGTGCACCAT - 3').

Real-time quantitative PCR cycling conditions were 50˚C for 2 minutes, 95˚C for 10 minutes and 40 cycles of a two-step amplification (95˚C for 15 seconds followed by 58˚C for 1 minute). The dissociation protocol from the sequence detection system was used for melting curve analysis to

exclude potential contamination of non-specific PCR products. Negative controls that were used as templates contained no reverse transcription products. Relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method and healthy bone tissue or untreated cells were used as calibrators [22].

Statistical Analysis

Data were analyzed by Student t-test or One-way ANOVA, as appropriate, using GraphPad version 7.03 (GraphPad Software, Inc., LaJolla, CA). Results are expressed as means ±SEM, with *P*-value < 0.05 set as statistically significant.

Results

Plasma from BCO-affected broilers significantly decreased hFOB cell viability

hFOB cells were exposed to fresh plasma from bird both lame and affected by BCO as well as plasma from non-lame and apparently normal bone for 4 hours. Cell viability was significantly decreased in cells exposed to plasma from BCO affected birds when compared to normal plasma (Figure 1) ($P < 0.05$).

Unique expression profile of inflammatory and bone-related cytokines and chemokines in the bone and blood of BCO-affected broilers

The local cytokine profile was determined through mRNA expression in normal and BCO-affected bone. Only one significant difference was seen between BCO and normal bone with IL-1β being significantly upregulated in BCO by approximately 4 times the fold change (Figure 2a) ($P < 0.01$). The systemic cytokine profile was determined by determining gene expression of cytokines within the whole blood and showed significant upregulation of TNFα and IL-12B

(Figure 2b) ($P < 0.05$). In both bone and blood, expression of *IL-4*, *IL-18*, *IL-6*, *IL-10*, and *IL-17* were not significantly affected (Figure 2 a, b). In blood, *IL-3* was also measured but its expression remained similar between normal and BCO (Figure 2b).

The chemokine profile was also evaluated in bone and blood of BCO-affected and normal birds. In bone, *IL-8L2* was significantly upregulated in BCO compared to bone without lesions (Figure 3a) ($P < 0.05$). While expression of *IL-8L1* was not significantly different in bone, *IL-8L1* was significantly upregulated in the blood of BCO-affected birds compared to normal group (Figure 3b) ($P < 0.05$). Additionally, the chemokine *CCL-20* was significantly upregulated in blood, but not locally in the bone. *CCL-4*, *CCLL-4*, *CXCL-14*, *CCL-5*, and *CRP* expression was not significantly different in bone nor blood (Figure 3 a, b) ($P > 0.05$).

Circulating levels of inflammasome NLRC5 were significantly increased in BCO

While there were no significant differences in inflammasome expression in bone, blood from BCO-affected birds had significantly higher NLRC5 mRNA expression compared to normal birds (Figure 4 a, b) ($P > 0.05$; $P < 0.05$).

Key receptor in the FGF23 pathway was significantly down regulated in BCO-affected bone

In the local bone, FGFR1 expression was significantly decreased in BCO-affected tissue (Figure 5a) ($P< 0.05$). No significant differences were seen in blood between the two groups (Figure 5b).

Recombinant IL-1β and TNFα proteins significantly decreased cell viability in both human and chicken bone cells

Treatment with human recombinant IL-1β or TNFα significantly reduced cell viability in hFOB cells by approximately 18% ($P = 0.0036$ for IL-1β and $P = 0.0249$ for TNFα, Figure 6). Similar results were seen in chicken primary chondrocyte cells (data not shown).

Discussion

Modern medical research has revealed intense networks of molecular signaling and action under disease and infectious states with far-reaching, systemic impacts. In BCO, the suspected delivery of bacteria to the bone via the high vascularization in the growth plate coupled with the evidence of chronic inflammation and lack of healing could drive systemic effects within the broiler detectable within circulation. If true, this could act as a non-invasive biomarker for BCO. Thus, the potential systemic effects in BCO were first investigated via exposure of hFOB cells to plasma from normal and BCO-affected birds. The resulting decreased cell viability points to potential circulating factors which affect bone cellular function and viability.

Under an inflammatory and infectious state, such as BCO, cyto(chemo)kines play an integral role via their both local and systemic action and stimuli [23]. Cytokines specifically are cell signaling molecules which drive inflammation via their effects on immune cell activation and modulation as well as downstream effects within a multitude of cell types [24]. In BCO-affected bone, there was clear increase in interleukin-1β (IL-1β) mRNA expression while other cytokines measured were unaffected. This corroborates previous work done in BCO which showed that increased IL-1β expression was coupled with dysregulation of Dicer 1 Ribonuclease III and Leucine-rich containing protein 3 (NLRP3) inflammasome activation [10]. IL-1β is a well-studied pro-inflammatory cytokine with roles in not only inflammasome activation and apoptotic pathways [24,25]. Additionally, both IL-1β and TNFα have been shown to decrease chondrocyte proliferation and hypertrophy, necessary steps in endochondral ossification and proper growth

plate homeostasis [26]. In osteoarthritis, IL-1β expression was shown to increase in conjunction with decreased extracellular matrix formation by chondrocytes contributing to osteoarthritic cartilage degradation [27]. Serum levels of IL-1β were increased in broilers with femur head necrosis in an experimental model involving methylprednisolone intramuscular injections which also coincided with disruption of extracellular matrix homeostasis [28]. These results support the finding of increased expression of IL-1β in BCO-affected bone in this study. In BCO, IL-1β could be acting not only as a pro-inflammatory influencer, but as a contributor to decreased cartilage stability and maturation increasing susceptibility to mechanical stress and bacterial infection.

Cytokines upregulated in circulation included tumor necrosis factor α (TNFα) and IL-12B. Like IL-1β, TNFα is a well-studied, potent pro-inflammatory cytokine of the TNF super family. It acts via induction of cytokine production, stimulation of growth and activation of adhesion molecules [29-31]. Additionally, TNFα has roles in metabolism such as insulin resistance and lipid metabolism as well as skeletal growth [30]. Within bone, TNFα has been shown to induce osteoclastogenesis thereby impacting the ratio of bone resorbing to bone mineralizing cells. Indeed, overexpression of TNFα in mice resulted in growth retardation resulting from decreased insulin-like growth factor 1 (IGF1) and IGF1-binding protein (IGFBP3) [32]. This coupled with its effects on chondrocytes previously described could have effects on bone healing and growth plate integrity under BCO conditions. It is well documented that bone healing is driven through a shift from bone-resorbing pathways and cell types, such as osteoclasts, to bone-mineralizing, such as osteoblasts. Interestingly, surface proteins of *Staphylococcus aureus,* one of the most common causative agents in BCO, have been shown to induce bone loss via potent osteolytic effects. These effects were inhibited by IL-1 receptors and TNFα antibodies [33]. Both IL-1β and

TNFα act via catabolic mechanisms within bone and pro-inflammatory mechanisms systemically which could be contributing to the lack of healing seen in BCO. Other studies investigating these cytokines in osteomyelitis found increased TNFα in early-stage of chronic osteomyelitis rather than late stage [34]. Another study involving stimulating blood from patients with osteomyelitis with lipid polysaccharides (LPS) did not affect the expression of TNFα or other cytokines in the blood [35].

While the cytokines implicate thus far have been pro-inflammatory, IL-12B is a primarily anti-inflammatory cytokine [36]. Anti-inflammatory cytokines act by decreasing pro-inflammatory cytokine production and changing immune cell activation or expression, such as the T helper 1 and T helper 2 profiles [37-41]. However, in the case of IL-12B, the combination of cytokine ligand and receptor, as well as the physiological conditions, determines whether its function is anti- or pro-inflammatory [36,38]. In the case of BCO, further investigation into cytokine receptor expression as well as protein expression is necessary to fully elucidate the role of IL-12B in circulation.

Another key molecule in inflammatory states that have been implicated in disease states is chemokines. Chemokines act by directing the circulation of lymphoid cells and recruiting them under conditions such as injury and infection [42,43]. Nomenclature for chemokines involves denoting the spacing of the first two cysteines on the amino acid terminals of the proteins with either CC, CXC, XC, or CX3C followed by “L” to indicate it is the ligand [24]. CCL-20 was significantly upregulated in the blood of BCO-affected broilers and it has been shown to be induced by TNFα in several cell types [24]. It is primarily a homeostatic chemokine with constitutive expression in several tissues. However, it has been shown that CCL-20 plays a role in recruitment of immature dendritic cells and precursors to sites of infection and injury and that

CCL-20 is involved in pathologies of endothelial surfaces, such as inflammatory bowel disease and rheumatoid arthritis [44-46]. In rheumatoid arthritis, CCL-20 expression correlated to bone loss and patient survival [47]. Its presence in circulation of BCO-affected broilers could be due to the simultaneous increased expression of TNFα leading to downstream effects in both immune cell recruitment and bone loss.

In both bone and blood of BCO-affected birds, IL-8 was increased. Granted IL-8L2 was increased in bone and IL-8L1 was increased in blood, the two avian orthologs have shown to have similar functions and comparable homology to human CXCL8 [48,49]. IL-8 acts primarily in activation and migration of monocytes, lymphocytes, and other immune cells to sites of infection and injury [50]. It is considered a key inflammatory mediator and shown to have angiogenic effects [50,51]. In bone, IL-8 expression by osteoblast and osteoclasts has been shown to be induced by cytokines IL-1β and TNFα, both shown here to be upregulated in BCO [52,53]. IL-8 has also been shown to trigger circulating monocytes to undergo osteoclast formation [54]. Notably, breast cancer patients with bone metastases were shown to have elevated levels of plasma IL-8 which also correlated with increased bone resorption [55]. The results of this study corroborate with these studies and suggest further evidence of an imbalance in bone resorption to bone-forming pathways in BCO.

Given the clear upregulation of certain cyto(chemo)kines both in local bone and systemic blood circulation of BCO-affected broilers, the inflammasome profile was also evaluated. NLRP3 has already been shown to be increased in BCO bone [10]. Results showed that NLRC5 was also significantly increased in the blood of BCO affected broilers. NLRC5 has been shown to have potential influences on the nuclear factor kappa B (NF-κB) pathway as well as NLRP3 inflammasome activation by working in the complex forming step [56]. Inflammasome

complexes are a part of the innate immune system initiating inflammation. Their activation leads to the activation of caspase-1 which is responsible for the cleavage and subsequent activation of pro-inflammatory cytokines such as IL-1β [57]. Its expression has been seen in the primary cells of myeloid and lymphoid origin as well as bone marrow and B cells and to be upregulated in chicken macrophages under inflammatory states [58,59]. Interestingly, NLRC5 is seen to play an active role in the first stage of inflammation and a diminished role with the process of inflammation [60]. The upregulation of blood NLRC5 expression in BCO-affected birds could indicate its involvement in induction of the other pro-inflammatory cytokines upregulated and as an indicator that BCO represents a persistent inflammatory state.

In addition to inflammasome complexes, cyto(chemo)kines have influences on and are affected by FGFs [61]. FGFs have far reaching effects on both immune response and healing or homeostasis of bone [13,62]. Indeed, the role of FGF signaling in skeletal formation has been demonstrated via gain-of-function mutations in the FGF receptor (FGFR) genes [63]. Expressions of key FGFs involved in bone processes as well as healing and immune response were evaluated. Notably, FGFR1, which has a diverse and expansive repertoire of ligands and effects in both bone and immune responses, was significantly down regulated in BCO-affected bone. FGFR1 is the receptor for FGF23 which is primarily produced by the bone and involved in immune and homeostasis functions [14]. FGFR1 knock-out mice showed no difference to control in bone mineral density or soft X-ray imaging of the femur, however, the lack of FGFR1 in the bone coincided with decreased serum phosphate, serum FGF23, and expression of FGF23 within the bone in addition to decreased body weight and a shorter lifespan [15]. Extracellular phosphate is involved in the induction of processes necessary within hypertrophic chondrocytes for normal endochondral bone development as well as the development of vascular diseases

[64,65]. Therefore, lack of FGFR1 could be contributing to a loss of bone integrity by disrupting hypertrophic chondrocyte function. Further research into the protein expression and modification state of FGFR within BCO-affected bone is needed to fully understand what its down regulation could mean for BCO etiology. However, this si the first the FGF pathway has been implicate din BCO in modern broilers and warrants further investigation.

Given the clear profile of pro-inflammatory cytokines and chemokines in BCO, recombinant proteins were used to determine if these could be the factors within BCO plasma responsible for the decreased cell viability in hFOB cells. To that end, both hFOB cells and chicken primary chondrocyte cells were challenged with IL-1β, IL-8, and TNFα. Of the three treatments, TNFα significantly reduced cell viability in both cell types. Decreased viable osteoblast-type cells due to the circulating levels of TNFα could be contributing to the etiology of BCO by shifting the ratio of bone-resorbing and bone-forming cells. Additionally, decreased viability of chondrocytes within the growth plate could contribute to its susceptibility to infection and mechanical stress. However, it is unclear if the TNFα expression is the result of or a contributor to the bacterial infection and bone loss. An essential piece missing in the understanding of BCO is its progression at both a cellular and systemic level and warrants further research. Identifying the culpable circulating cell types is also warranted for future research as it could elucidate the means in which BCO exerts systemic effects.

Conclusions

In this study, plasma from BCO-affected broilers had significant effects on the viability of hFOB cells demonstrating circulating factors which influence bone cell function and survival. This is the first study profiling cyto(chemo)kines and key FGFs in BCO bone and blood and demonstrating that elevated TNFα expression in the blood of BCO-affected birds could be the

causative agent in bone cell viability. The unique circulating profile of BCO-affected birds provides a potential means of non-invasive detection and potential targets for treatment. Additionally, this is the first study to investigate FGFs in relation to BCO and showed decreased FGFR1 in the BCO-affected bone. Taken together, these results indicate increased expression of pro-inflammatory and bone-resorptive factors which could be contributing to bone loss and necrosis seen in BCO. Further research is needed to elucidate the direct mechanisms behind these factors and their effects.

Acknowledgments: We thank Dr. Adnan Alrubaye (University of Arkansas) for allowing access for sample acquisition.

Author Contributions: Conceptualization, S.D.; Data curation, A.R., E.G., R.W. and S.D.; Formal analysis, A.R.; Methodology, A.R., E.G., R.W. and S.D.; Supervision, S.D.; Writing-original draft, A.R.; Writing-review and editing, S.D. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: Authors declare no conflict of interest.

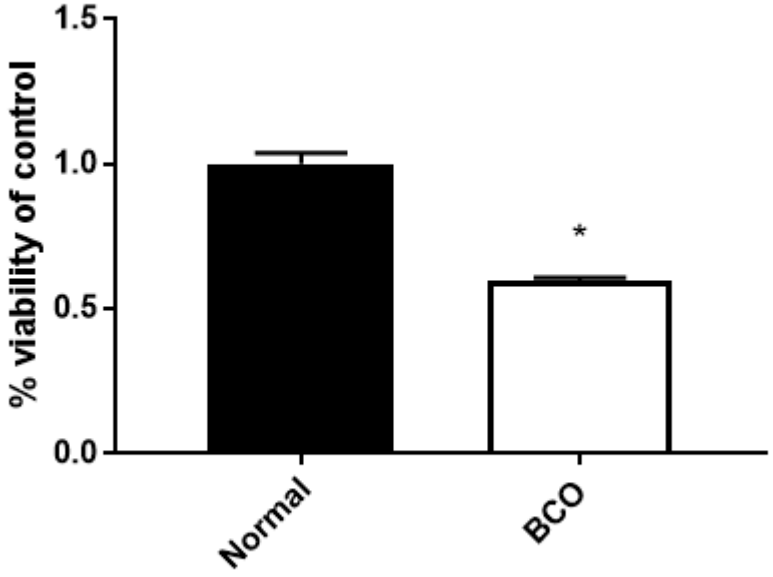

Figure 1. Effect of Plasma from Normal and BCO-affected Broilers on hFOB Cell Viability MTT assay results from hFOB cells treated with media containing 5% plasma form wither normal (non-lame and no lesions of the bone) or BCO-affected (lame with lesions in both legs) broilers for 4 hours. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between groups.

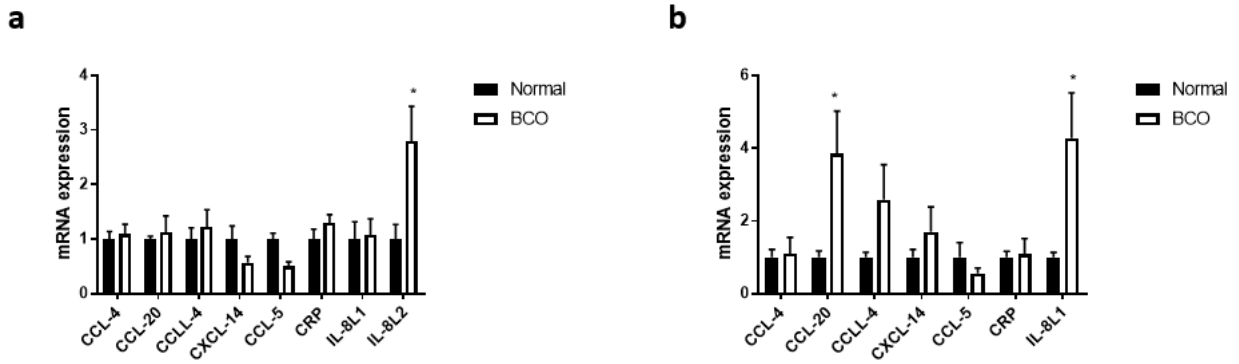

Figure 2. Local and Systemic Cytokine Profile in Normal and BCO-affected broilers
Gene expression for *TNFα, IL-4*, *IL-18*, *IL-1β, IL-6*, *IL-10*, *IL-12B*, *IL-17*, and *IL-3* in bone (a) and blood (b) of normal and BCO-affected broiler chickens. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

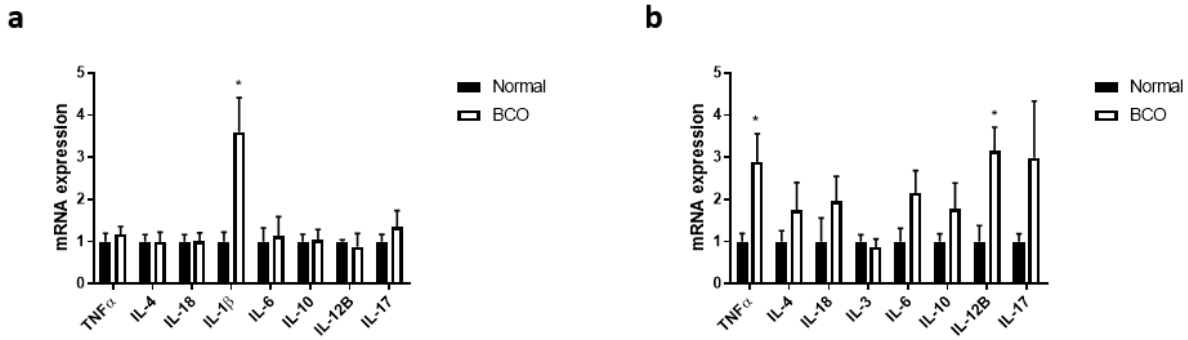

Figure 3. Local and Systemic Chemokine Profile in Normal and BCO-affected broilers
Gene expression for *CCL-4*, *CCL-20*, *CCLL-4*, *CXCL-14*, *CCL-5*, *CRP*, and *IL-8L1/IL-8L2* in bone (a) and blood (b) of normal and BCO-affected broiler chickens. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

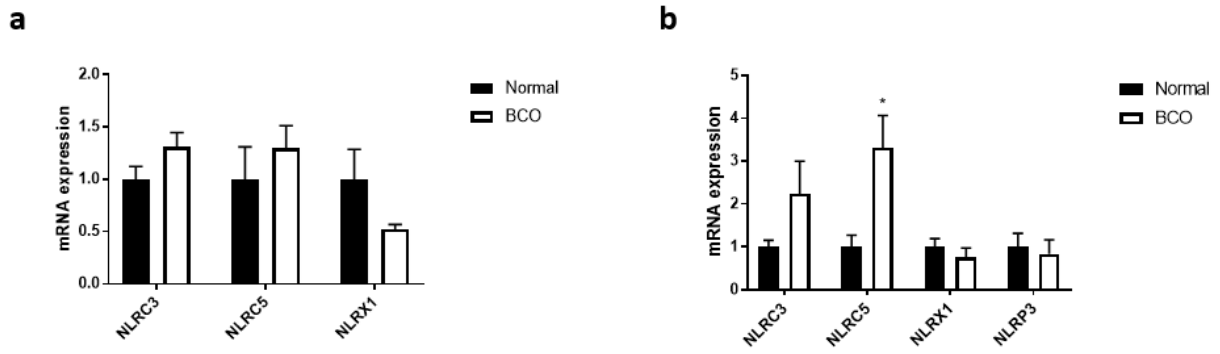

Figure 4. Local and Systemic Inflammasome Profile in Normal and BCO-affected broilers
Gene expression for *NLRC3, NLRC5,* and *NLRX1* in bone (a) and blood (b) of normal and BCO-affected broiler chickens. As well as expression of *NLRP3* in blood (b). Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

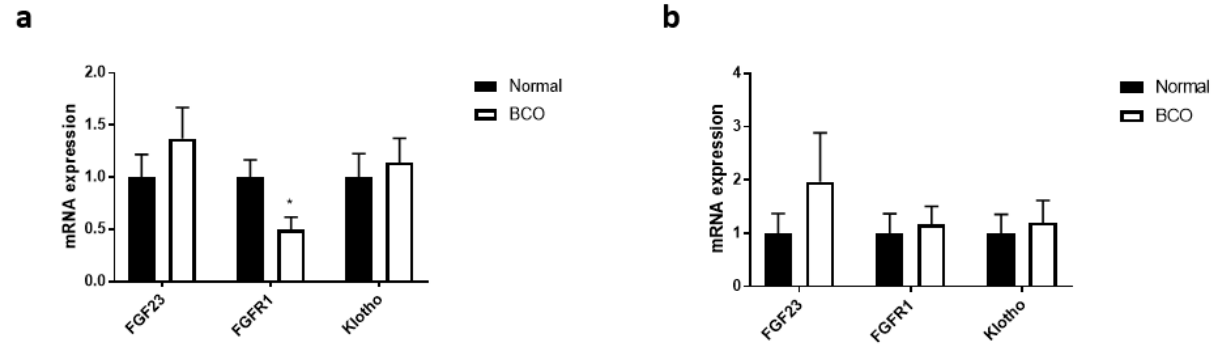

Figure 5. Local and Systemic Expression of key FGFs in Normal and BCO-affected broilers
Gene expression for *FGF23, FGFR1,* and *Klotho* in bone (a) and blood (b) of normal and BCO-affected broiler chickens. Significance was determined using a student t-test with p-value < 0.05. * indicates significant difference between Normal and BCO.

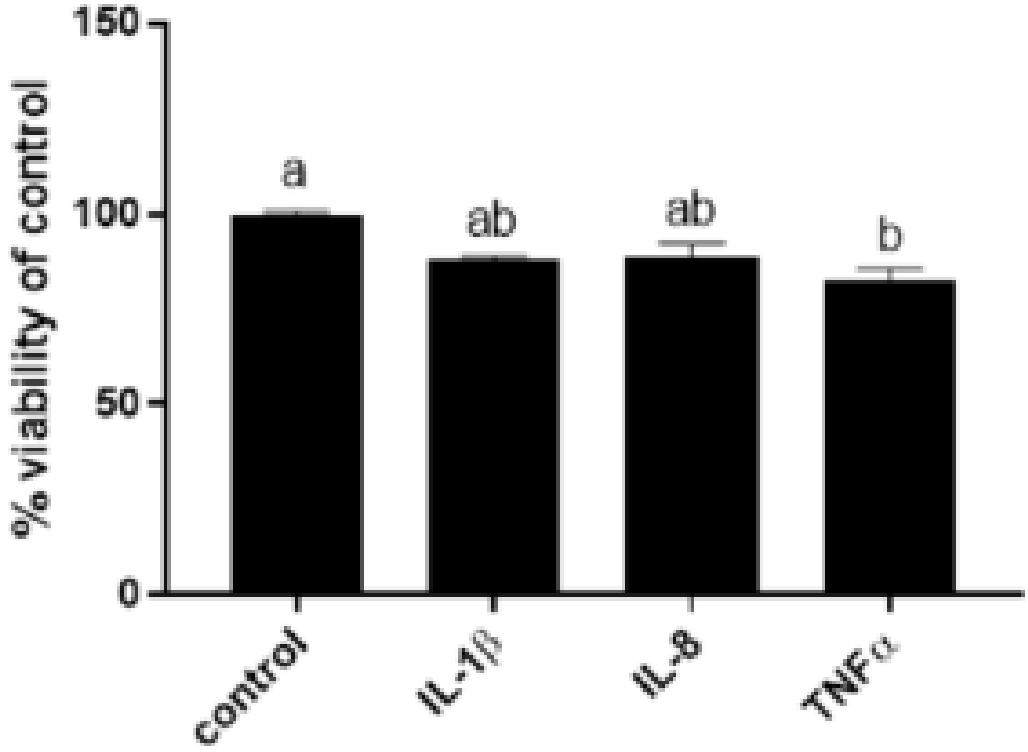

Figure 6. Effect of Human Recombinant IL-1β, IL-8, and TNFα on hFOB Cell Viability
MTT assay results from hFOB cells treated with human recombinant IL-1β (100 ng/mL), IL-8 (100 ng/mL), and TNFα (10 ng/mL) for 24 hours. Significance was determined using a student t-test with *p*-value < 0.05. * indicates significant difference between treated groups and the control.

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Chapter 8 –

Title: Primary Growth Plate Chondrocyte Isolation, Culture, and Characterization from the Modern Broiler

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Abstract: Lameness is a leading cause of animal welfare and production concerns for the poultry industry as fast-growing, high-yielding broilers seem to be more susceptible to bone disease and infections. A major limitation for functional studies of these disorders is the lack of a chicken immortalized chondrocyte cell. Primary chondrocyte isolation is a valid and complex method for establishing a reliable *in vitro* model for diseases. In this study, isolation and high-density culturing of primary chondrocytes from 10-day old chicks was followed by confirmation of cell type, identification of optimal phenotypic expression, and evaluation of cells functionality. By evaluating mRNA abundances as well as protein expression and secretion of collagen type I

(COLI), collagen type II (COLII), SRY-Box Transcription Factor 9 (Sox9), aggrecan (ACAN), and collagen type 10 A1 (COLXA1) on day 3 (d3), d7, d11, d14, d18, and d21 in culture it was shown that avian growth plate chondrocytes under these conditions exhibit optimal phenotypes from d3 to d7. This is evident by a shift from COLII dominant expression in early-culture to COLI dominant expression by late-culture in conjunction with a loss of other chondrocyte markers such as Sox9, ACAN, and COLXA1. Additionally, morphological changes seen through live cell imaging coincide with the shift of phenotype in mid- to late-culture periods indicating a dedifferentiated phenotype. The functionality of the cultured cells was confirmed using Brefeldin-A treatment which significantly reduced secretion of COLII by d7 chondrocytes. These results provide a foundation for future research utilizing avian primary chondrocytes with optimal phenotypes for disease modeling or passaging.

Key Words: broiler, primary chondrocyte, growth plate, chicken, characterize

Introduction

The growth plate is home to intense molecular remodeling from embryonic to early development and beyond. Longitudinal bone growth is the direct result of endochondral ossification done by chondrocyte cells within the growth plate. The junction where the dynamic growth plate meets the harder articular cartilage cap is often the site of infection, disease, and stressors [2, 3]. In addition to mechanical stress from weight bearing and movement, these locations in the skeletal system are under immense metabolic and conformational stress as cells progress through stages needed for bone growth to occur [4]. In the modern chicken, the growth plate is home to the leading causes of lameness, bacterial chondronecrosis with osteomyelitis (BCO) and tibial dyschondroplasia (TD) [5-7]. These disorders are the result of weak points within the growth plate being either exploited by opportunistic bacterium or crippled by hypoxia [8, 9]. Either way,

detection of these two disorders is only possible through necropsy. Additionally, it has been shown that their presence does not always coincide with visible lameness [10]. Regardless of the clinical status, leg disorders and lameness in general, are a major animal welfare and production concern to the poultry industry [11, 12]. Thus far, studies have shown molecular pathways such as dsRNA accumulation and mitochondrial dysfunction to be implicated in BCO etiology through investigation of affected bone tissue and *in vitro* analysis using a human osteoblast cell line [13, 14]. However, a major limitation in the study of this disease is the lack of a chicken specific and growth-plate relevant *in vitro* model. To that end, this study was undertaken to isolate, culture, and characterize primary growth plate chondrocytes from modern broiler chicks.

In order to evaluate the success and effectiveness of primary cell cultures, it is important to understand the in vivo environment and cell stages. There are several key stages to the chondrocyte cell cycle, starting with condensation of the mesenchymal stem cells into chondroprogenitor cells [15]. Condensation is regulated by transcription factors such as Sox9 and results in increased COLII expression[16]. Following condensation, chondroprogenitors go through proliferation and differentiation into chondrocytes exhibiting COLII and ACAN expression and secretion [17]. The final stage chondrocytes undergo is hypertrophy where COL10A1 is a specific marker [18]. Expression of COLI is seen before condensation and in dedifferentiated chondrocytes, therefore, COLI is commonly used as a negative marker for chondrocytes [19]. These markers were evaluated at different time-points during primary chondrocyte cell culture in order to characterize the cells and determine the moment of optimal phenotype for utilizing primary chondrocytes as *in vitro* models for diseases.

Materials and Methods

Cell Isolation and Culture

Primary chondrocytes were isolated from the proximal tibia heads of 10-day-old broiler chicks. The method of isolation was modified from previous work [20]. All animal experiments were approved by the University of Arkansas (Fayetteville, AR) Animal Care and Use Committee (protocol number 21050) and were in accordance with recommendations in NIH's Guide for the Care and Use of Laboratory Animals. Chicks were reared in standard conditions, 32 ºC ambient temperature and 23 hours light/1-hour dark cycles, for ten days with ad libitum access to food and fresh water. Leg quarters were aseptically separated from culled chicks before careful isolation of the proximal tibia head from skin and surrounding muscle. A diagonal cut was made into the tibia head and the articular cartilage cap was removed, exposing the growth plate. A 5 mm curette was used to shave the top layers of the growth plate and shavings were placed in serum-free media, DMEM containing 4.5g glucose/L, glutamine, 25 mM HEPES, 1 mM sodium pyruvate, and 10 ng/mL added ascorbic acid. This process was repeated for all chicks. The shavings were then transferred to digestion media composing of all aforementioned media with additional 10% FBS, 1mg/ml hyaluronidase (Sigma Aldrich, St. Louis, MO), and 10mg collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ) before being incubated overnight at 37˚C. Cells were then seperated from tissue debris using ficoll gradient of Histopaq-1077 density gradient medium and centrifugation separation before being plated at a density of 2 x 10^5 cells/cm^2 in complete media. Complete media contained all components previously mentioned, except for the hyaluronidase and collagenase. Media was changed every 48 hours and the cells were maintained in 37˚C and 5% CO_2.

Live Cell Imaging

Cells were imaged at day 3 (d3), d7, d11, d14, d18, and d21 in culture using the Cytation 3 Cel Imaging Multi-Mode Reader (BioTek, Winooski, VT) at a magnification of 20x.

RNA isolation, reverse transcription, and real-time quantitative PCR

Total RNA was isolated from primary chondrocytes on each time point (n=3/group) in accordance with the protocol of previous work [22]. Cellular RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA), based on manufacturer's instructions. RNA concentrations were determined using Synergy HT multimode microplate reader and total RNA was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithsburg, MD). Amplification was achieved using Power SYBRGreen Master Mix (Life Technologies, Carlsbad, CA) and real-time quantitative PCR (7500 Real Time System; Applied Biosystems, Foster City, CA). The sequences for oligonucleotide primers for *r18s* was previously published [14]. Additional primers used were *COLIA1* (forward, 5'- GTCGCCATCCAACTGACCTT -3'; and reverse, 5'- TGCAGTGGTAGGTGACGTTCTG -3'), *COLIA2* (forward 5' - GCAACACAAGGAGTCTGCATGT - 3'; and reverse, 5' - AAATCCGCGTATCCACAAAGC - 3'), *COLII* (forward 5' - ACCTGCCGCGACATCAA - 3'; and reverse, 5' - GTCAATCCAGTAATCTCCGCTCTT - 3'), *ACAN* (forward 5' - CAGAGCCGTGGAGAATGATTTC - 3'; and reverse, 5' - GGCCGCCAGTTCTCAAATT - 3'), *Sox9* (forward 5' - CCCCTGTGCCGCTTTCT - 3'; and reverse, 5' - GTCCTGTTCTTCTGTCATTTTCATGA - 3'), and *COL10A1* (forward 5' - CAGCTGCCAAATTCAGAATCC - 3'; and reverse, 5' - GGAAACCTGAGAAAGAAGAATGAACA - 3').

Real-time quantitative PCR cycling conditions were 50˚C for 2 minutes, 95˚C for 10 minutes and 40 cycles of a two-step amplification (95˚C for 15 seconds followed by 58˚C for 1 minute). The dissociation protocol from the sequence detection system was used for melting curve analysis to exclude potential contamination of non-specific PCR products. Negative controls that were used as templates contained no reverse transcription products. Relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method and healthy bone tissue or untreated cells were used as calibrators [23].

Protein isolation and Western Blot visualization and analysis

Media and cell lysate from each time point (d3, d7, d11, d14, d18, d21) were homogenized in lysis buffer (10 mmol/L Tris base, pH 7.4; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA; 0.1% Triton X-100; 0.5% Nonidet P-40; and protease and phosphatase inhibitors) and sonicated. Total protein concentrations were determined using a Bradford assay kit (Bio-Rad, Hercules, CA), ran in 4%-12% gradient Bis-Tris gels (Life Technologies, Carlsbad, CA) and then transferred to polyvinylidene difluoride membranes. Once transferred, membranes were blocked using a Tris-buffered saline (TBS) with 5% nonfat milk and Tween 20 at room temperature for 1 hour. Membranes were then washed with TBS and Tween 20 before being incubated with primary antibodies at a dilution of either 1:500 or 1:1000 overnight at 4˚C. Primary antibodies used were rabbit anti- COLI (Origene, Rockville, MD), mouse anti-COLII (Novis Biologicals, Littleton, CO), rabbit anti-ACAN (ABClonal, Woburn, MA), rabbit anti-Sox9 (ABClonal, Woburn, MA), and rabbit anti-COL10A1 (ABClonal, Woburn, MA). After another wash, secondary antibodies diluted to 1:5000 were added to 5% nonfat milk in TBS and Tween 20 and incubated with the membranes at room temperature for 1 hour. The protein signals were visualized using chemiluminescence (ECL Plus; GE Healthcare, Pittsburg, PA) and images

were captured using the FluorChem M MultiFluor System (ProteinSimple, San Jose, CA). Prestained molecular weight marker (Precision Plus Protein Dual Color) was used as a standard (Bio-Rad, Hercules, CA). Protein loading was assessed by immunoblotting using rabbit antieglyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Dallas, TX) or Ponceau S Stain (G-Biosciences, St. Louis, MO). Image acquisition and analysis were performed by AlphaView software (version 3.4.0, 1993–2011; ProteinSimple, San Jose, CA).

Immunofluorescence

Primary chondrocytes were plated on gelatin coated Nunc Lab-Tek II chamber slides (Thermo Fisher Scientific, Waltham, MA) until predetermined time-points in culture. Immunofluorescence staining was performed as previously described [24]. Briefly, cells were fixed using paraformaldehyde for 10 minutes at room temperature before being permeabilized with Triton-X 100. The cells were then blocked with serum-free protein block (Dako, Carpinteria, CA) for 1 hour at room temperature followed by blocking with primary antibodies at a dilution of 1:200 in antibody diluent (Dako) overnight at 4ºC. the signal was visualized using either DyLight 488- or 594-conjugated secondary antibodies (Thermo Fisher Scientific). Vectashield with DAPI (Vector Laboratories, Burlingame, CA) was used as mounting media before slides were cover slipped. Images were obtained using Zeiss Imager M2 and AxioVision software version LE2019 (Carl Zeiss Microscopy, Oberkochen, Germany).

Brefeldin-A treatment

Primary chondrocyte cells were maintained until d7 in culture. Complete media was replaced with media either treated with 1 µg/mL of brefeldin-A (BFA, Sigma Aldrich, St. Louis, MO) or dimethyl sulfoxide (DMSO) as vehicle control (n=3/group). Cells were maintained for another

24 hours at 37˚C and 5% CO_2 before both media and cell lysate were collected for protein isolation and analysis.

Statistical Analysis

Data were analyzed by Student *t*-test or One-way ANOVA, as appropriate, using GraphPad version 7.03 (GraphPad Software, Inc., LaJolla, CA). Results are expressed as means ±SEM, with *P*-value < 0.05 set as statistically significant.

RESULTS

Cell Morphology during Culture Period

Cytation 3 images, taken at 20x magnification, show cell size and shape change throughout the culture (Figure 1). Most notably, the change from smaller rounded cells to more geometric in shape, creating a "cobble-stone" appearance visible starting at d7 until d21. Aggregates of cell scan also be seen more in mid-culture (d11 and d14) as compared to late-culture conditions (d18 and d21) (Figure 1).

Expression profile of chondrocyte phenotype-associated markers

COLIA1 and *COL1A2*, which are markers for both condensation and dedifferentiation of chondrocytes, significantly increased during the culture period with and lowest expression on d3 and the highest expression at d18 ($P < 0.05$) (Figure 2 a, b). *Sox9*, a marker for the condensation stage of chondrocyte cell cycle, was significantly upregulated in the early period of isolation and significantly decreased from d3 to d14, d18, and d21 ($P < 0.05$) (Figure 2 c). *COLII*, a chondrocyte proliferation and dedifferentiation marker, showed the highest mRNA expression in the beginning of the culture period (d3, d7, and d11) and significant decreases in late-culture

(d21) ($P < 0.05$, Figure 2 d). Similarly, *ACAN* showed highest expression at d3 and d7 in culture and significantly dropped at d11 ($P < 0.05$, Figure 2 e). *COLX*, a marker of hypertrophic chondrocytes, significantly decreased from d3 to d7 ($P < 0.05$) and then significantly decreased again at d11 ($P < 0.05$) and remained low throughout the duration of the culture period (Figure 2 f).

At the protein levels, immunoblot blot analysis of the cell lysate showed significantly higher expression of COLI on d18 compared to d3, d7, and d11 ($P < 0.05$) (Figure 3a, b). Expression of Sox9 was also significantly higher on d3 compared to d14, d18, and d21 ($P < 0.05$) (Figure 3a, b). In media, COLII was significantly higher on d7 compared to d3, d14, d18, and d21 ($P < 0.05$) (Figure 3a, c). ACAN was significantly higher on d3 in media of cultured cells compared to all other time points ($P < 0.01$) (Figure3 a, c).

In line with the immunoblot data, immunofluorescence staining in chondrocyte monolayer culture detected spot-like shapes of COL I and COL II on d7 (arrowheads, Figure 3d) and network-like shapes on d18 (arrows, Figure 3d).

Next, chondrocytes were treated with BFA and showed significantly reduced secretion of COLII into the media, as evident by western blot analysis of the cell lysate and media from control and treated cells ($P < 0.05$) (Figure 4a, b).

DISCUSSION

Inflammatory bone diseases, especially those affecting the growth plates, are notoriously difficult to detect and treat due to the extensive layers of skin, muscle, and ligaments surrounding the general region of infection [25, 26]. In modern broilers, lameness is a serious and growing threat to both production and animal welfare. Common causes of lameness include BCO and TD,

which both primarily affect the proximal growth plates of the long bones [27, 28]. As no current immortalized chicken bone cell line exists, this study sought to successfully isolate, culture, and characterize primary growth plate chondrocytes from modern broilers for their potential future use as *in vitro* models of bone infection and disease.

Primary chondrocytes were seeded in accordance with high density plating methods for retaining chondrocyte phenotypic expression [29]. This is believed to be done through the dense layers of chondrocyte cells mimicking a 3D culture environment which has been proven to aid in retention of proper chondrocyte phenotypes as opposed to monolayer culture techniques [29]. Under this environment, the cells presented a commonly seen morphological change starting around d7 and becoming more prominent by d11 and onward. The change in cell size and shape has been shown to coincide with shifts in expression of chondrocyte markers and ECM secretions [30, 31].

Our results show that avian growth plate chondrocytes in high density culture exhibit shape changes accompanied by an early-culture peak in *COLII* mRNA expression and protein secretion followed by a decline in COLII and increase in COLI production into late-culture when the shape change is most prominent. This switch to COLI production is often seen in *in vitro* cultures as a monolayer environment results in dedifferentiation of chondrocytes. Indeed, live cell imaging shows decreased layering of cells by d18 and d21 indicating a loss of high-density, 3D-mimicking culture which is more conducive for retention of chondrocyte phenotype. This pattern of loss of COLII production is seen in other monolayer primary chondrocyte cultures. Human articular chondrocytes seeded at a density of 2.5×10^4 cells/cm^2 in monolayer showed decreased COLII and Sox9 mRNA expression after passaging [32]. Additionally, the morphological changes observed in this study corroborate previous work. Embryonic chick

chondrocytes seeded at 2.5 x 10^5 cells/cm^2 in comparable media showed attachment by d3 and a "polygonal" shape by d9 of culture, similar to the "cobblestone" shape observed in this study [33].

Within early-culture, Sox9 protein expression was highest compared to mid- and late-culture periods, which corroborated with mRNA expression as well. Sox9 is a key transcription factor in chondrogenesis, driving mesenchymal condensation into chondroprogenitors, and therefore was expectedly undetectable in media from cultured cells [34]. Specifically, Sox9 has been shown to regulate COLII, COL9A1, COL11A1, and ACAN gene expression in murine chondrocytes [16, 35]. Chondroprogenitor cells have been shown to express Sox9 and drive COLII production in mice [36]. The early expression of Sox9 protein within the cells indicates the potential presence of chondroprogenitor cells and precedes increased expression of COLII in the cultured chondrocytes and media by d7. Murine growth plate chondrocytes showed high levels of Sox9, ACAN, COLII mRNA expression and significantly higher expression of COLXA1 on d3 in culture when compared to costal chondrocytes [31]. COLXA1 mRNA expression as also significantly higher on d3 compared to all later culture time points. CCOLXA1 is a common product of hypertrophic chondrocytes and its presence in the earliest time point could be the result of sampling. The scraping of the growth plate results in sections deep enough to include layers of condensing, proliferating, and hypertrophic chondrocytes within the digestion and culture. The inclusion of these different staged chondrocytes into the culture could be the causative factor in *COLXA1* mRNA expression in early-culture. Furthermore, ACAN protein secretion was also highest on d3 which indicates the presence of proliferating chondrocytes within culture. This further supports the idea that multiple different cell stages are represented in the first few days of culture. However, the addition of ascorbic acid in the media is noteworthy as ascorbic acid has been shown to induce chondrocyte proliferation in culture [37, 38]. Its

inclusion is due to its ability to aid in chondrocyte phenotype retention and ECM secretion while decreasing oxidative stress [39, 40].

Once an established phenotype was determined, functional analysis of the cells was undertaken using BFA. BFA acts to inhibit protein secretion at a pre-Golgi apparatus stage through blocking guanine nucleotide exchange [41]. Chondrocytes at d7 in culture treated with BFA showed significantly decreased secretion of COLII compared to untreated controls. This indicates the active secretion of COLII by the cultured cells and further defines their functionality and phenotypic relevance for *in vitro* modeling of growth plate diseases and infections.

Taken together, these findings suggest that chondrocyte markers are at their most optimal between d3 and d7 in culture and that the shape change observed starting on d7 and fully present on d11 is likely due to dedifferentiation of chondrocytes. This is supported by a clear shift in collagen type secretion, from COLII dominant to COLI, in addition to loss of chondrocyte specific ECM markers such as ACAN and COLXA1 (Figure 5). Further research is needed into whether this phenotype is maintainable through culture or if these cells could act as an *in vitro* model for already established molecular pathways involved in bone diseases and infections.

Acknowledgements

We thank Dr. Sara Orlowski at the University of Arkansas for providing chicks for this study.

We are additionally grateful to Dr. Fiona Goggin at the University of Arkansas for allowing access to the Cytation 3 Cell Imaging Multi-Mode Reader.

Author Contributions

Conceptualization, S.D.; Data curation, A.R.; Formal analysis, A.R.; Methodology, A.R., E.G., N.R., and S.D.; Supervision, S.D.; Writing-original draft, A.R.; Writing-review and editing, S.D. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

Authors declare no conflict of interest.

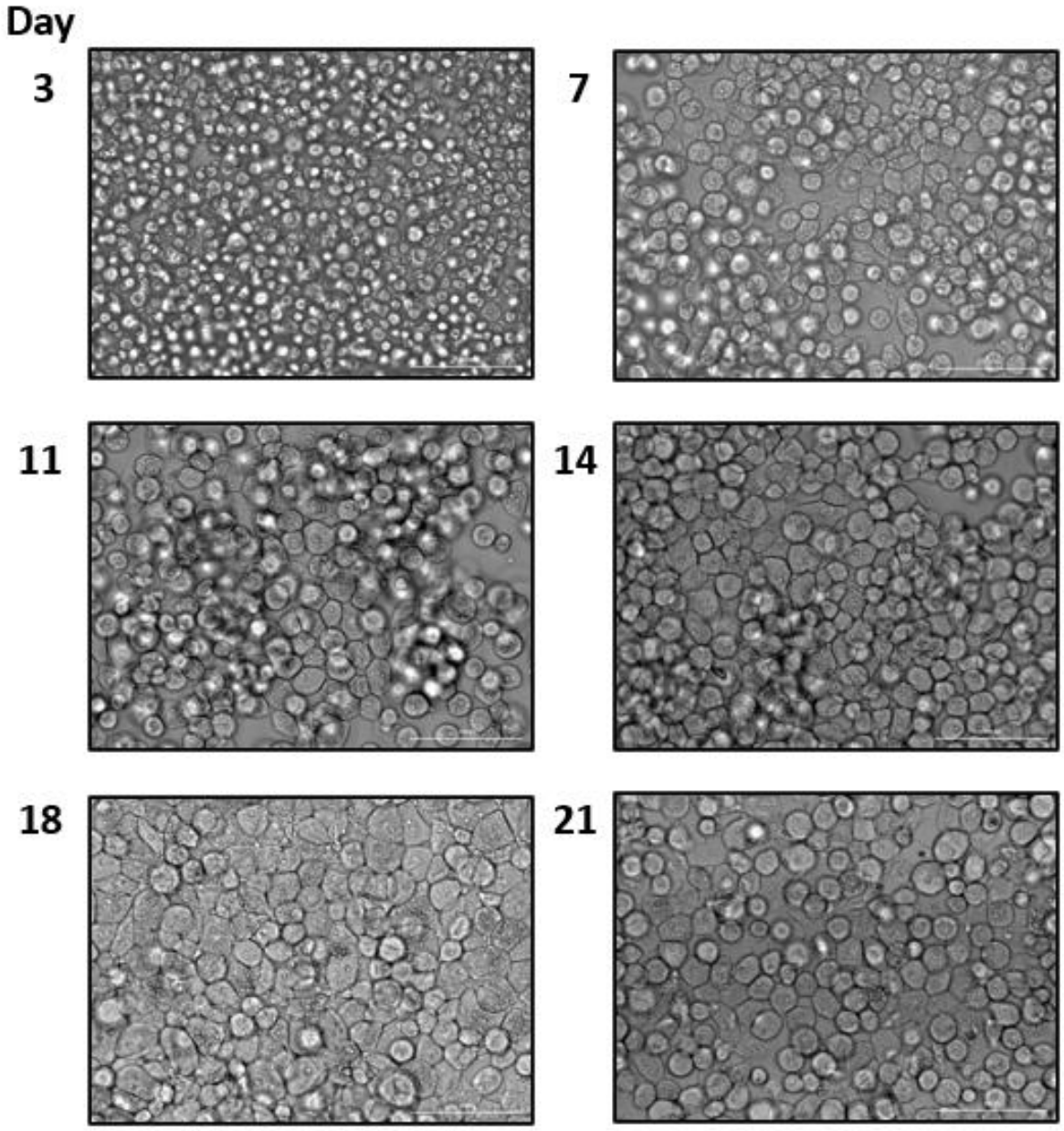

Figure 1. Live-cell imaging of chondrocytes in culture
Live cells seeded at a density of 2 x 10^5 cells/cm^2 were imaged using Cytation 3 Cell Imaging Multi-Mode Reader at 6 different time points, d3, d7, d11, d14, d18, and d21 in culture at the same magnification (20x). The same wells were imaged at each time point.

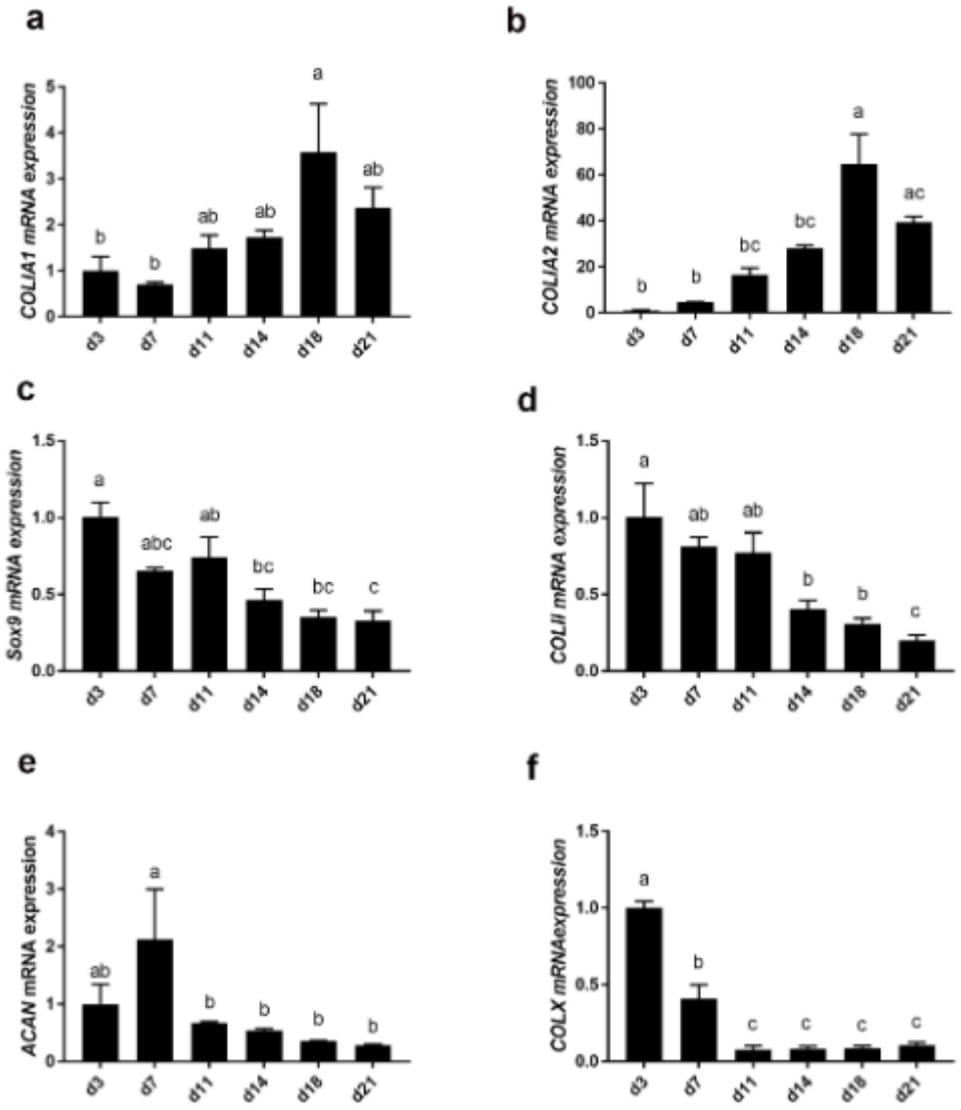

Figure 2. mRNA expression of chondrocyte markers within cultured primary cells throughout the culture period

Gene expression for *COLIA1* (a), *COLIA2* (b), *Sox9* (c), *COLII* (d), *ACAN* (e), and *COLX* (f) in chondrocyte cells at d3, d7, d11, d14, d18, and d21 in culture. Significance was determined using a one-way ANOVA with p-value < 0.05. Lowercase letters indicate significant difference between time points. ACAN, aggrecan; COL, collagen; Sox, SRY-Box.

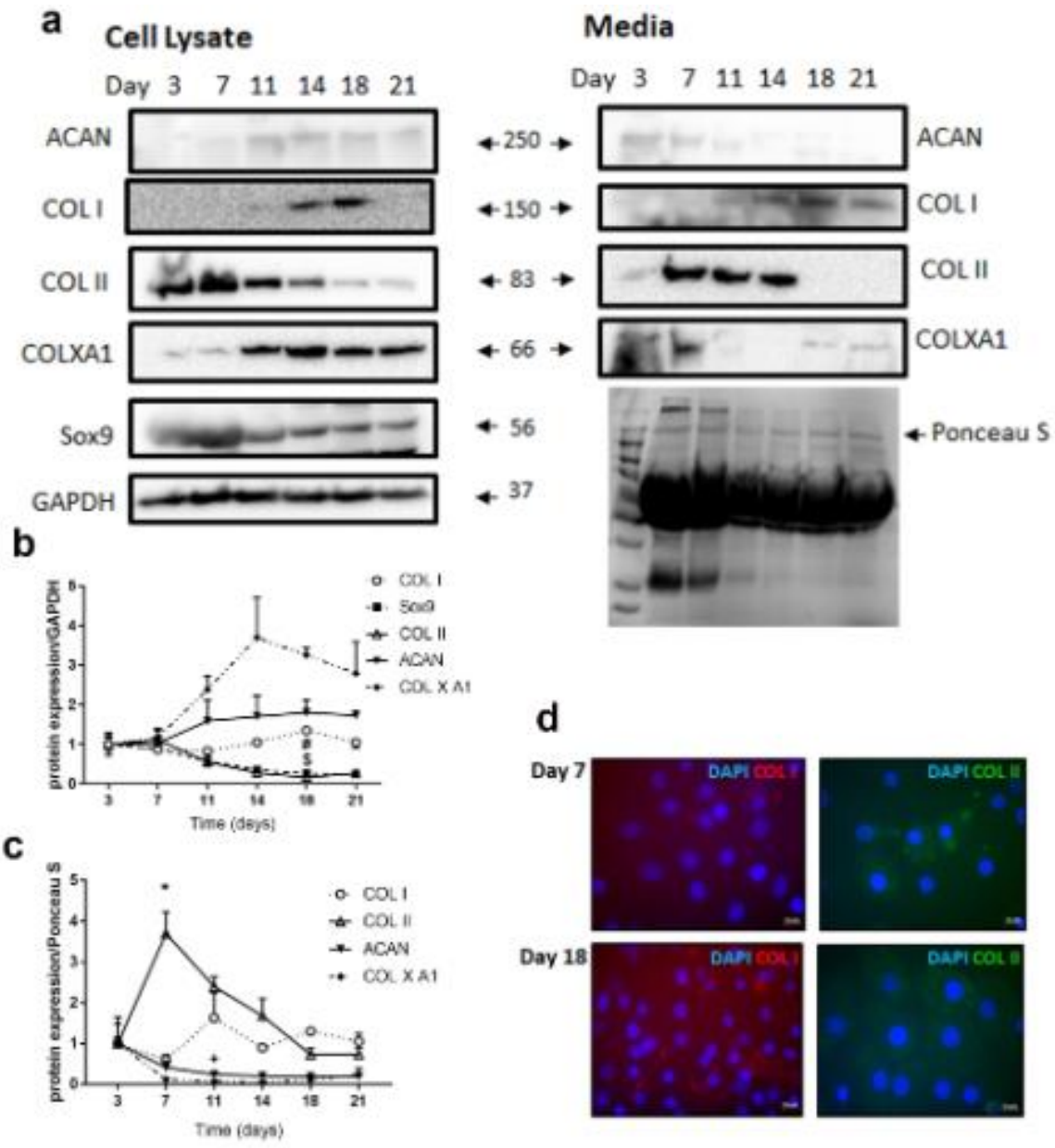

Figure 3. Evaluation of protein production and secretion by primary chondrocytes in culture

Protein expression for COLI, COLII, COLXA1, ACAN, Sox9, and GAPDH in cell lysate and media in addition to Ponceau S stain of the media blot and subsequent statistical analysis in culture (a, b, c). Significance was determined using a one-way ANOVA with p-value < 0.05. (b) $ indicates significant difference between Sox9 expression on d14, d18, and d21 compared to d3. # indicates significant difference between COLI expression on d18 compared to d3, d7, and d11. (c) + indicates significant difference between ACAN secretion on d11, d14, d18, and d21 compared to d3. * indicates significant difference in COLII secretion on d7 compared to d3, d14, d18, and d21. (d) Immunofluorescence staining of COLI (594-conjugated) and COLII (488-conjugated), along with DAPI nuclear dye, in chondrocytes d7 and d18 in culture. ACAN, aggrecan; COL, collagen; Sox, SRY-Box.

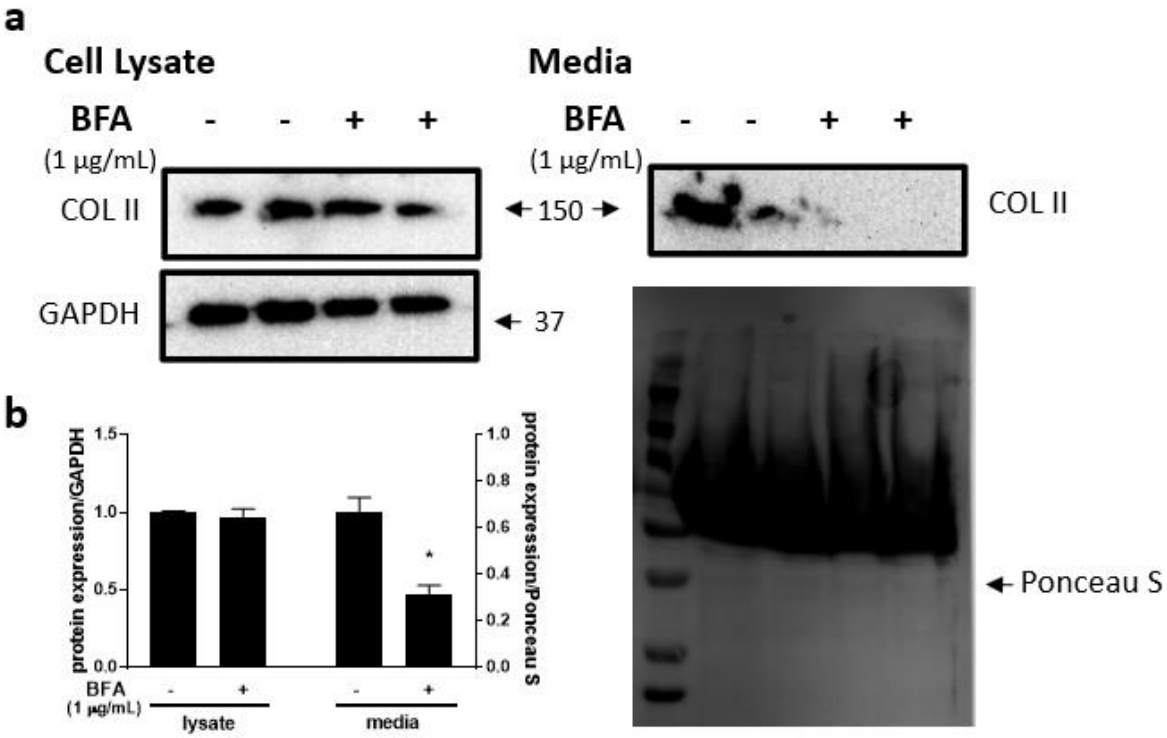

Figure 4. Effect of BFA treatment on d7 chondrocyte COLII secretion
Protein expression of cells and their media secretions treated with either BFA (1 µg/mL) or vehicle control at d7 in culture (a). Significance was determined using a student *t*-test with *p*-value < 0.05 (b). * indicates significant difference between control and treated cells.

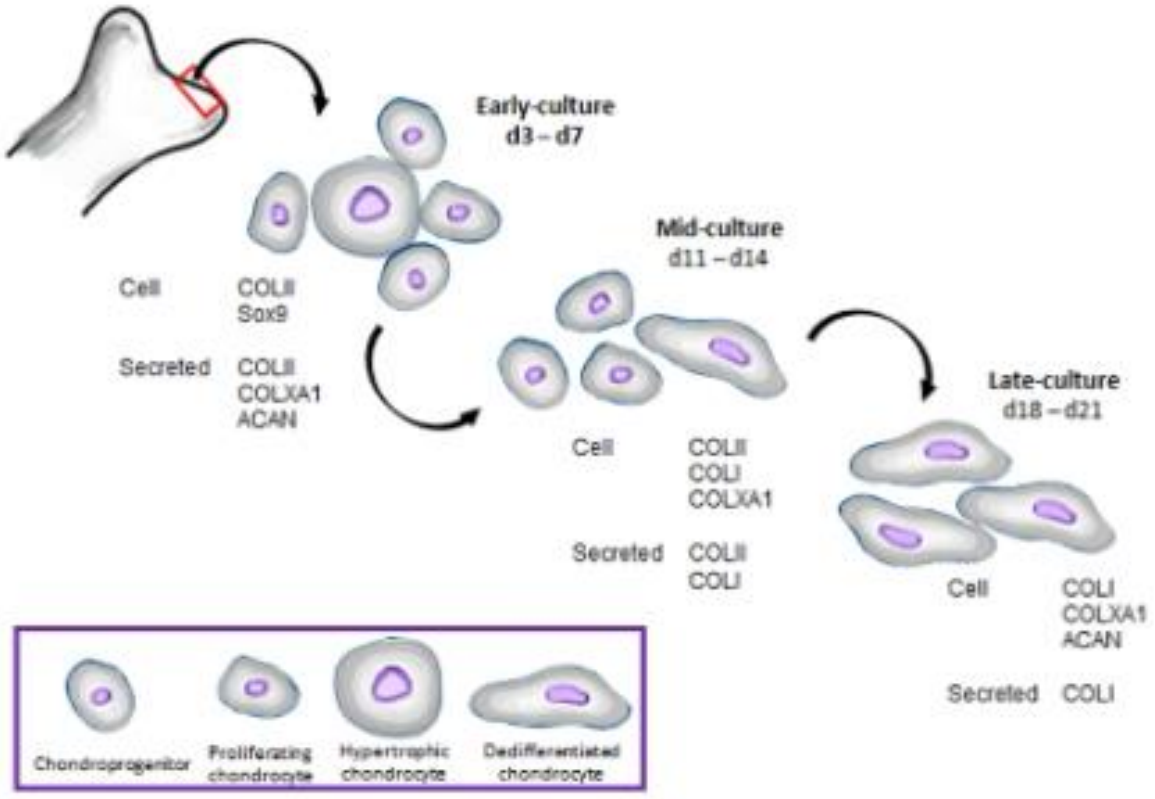

Figure 5. Summary of avian primary growth plate chondrocyte cell stages and phenotypes within culture

Chondrocytes isolated from 10-day-old chicks and cultured at a high-density (2 x 10^5 cells/cm^2) exhibit a transition of phenotype during early-, mid-, and late-culture indicative of changes in representative cell stages and a subsequent dedifferentiation of chondrocytes. These shifts can be visualized through the mRNA expression ad protein production and secretion of the cells. Chondrocyte markers are the most optimal for growth-plate *in vitro* models between d3 and d7 of culture.

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Chapter 9 – Perspectives

BCO has clear systemic effects and involves numerous cellular processes and pathways. Indeed, bone metabolism and homeostasis are linked to immune response as well as hormone and growth factors [1-3]. Bone density in particular can be affected by lipid metabolism with hyperlipidemia associated with weaker bone [4]. In humans with osteonecrosis of the femur head, hyperlipidemia has been identified as an associated factor and serum lipid levels are shown to be higher in affected patients [5]. Patients with non-traumatic necrosis of the femur head also showed increased total cholesterol, low-density lipoprotein with decreased high-density lipoprotein indicating abnormal lipid metabolism is a strong risk factor of necrosis of the femur head [6].

Recently, research analyzing the lipid profile of broilers with FHN showed hyperlipidemia along with increased infiltration of lipid within the bone coinciding with increased expression of lipid metabolism genes within the femur head [7]. A similar study also identified increased apoptosis and decreased bone metabolic markers in FHN in conjunction with increased fat within the liver of FHN-affected broilers [8]. These studies suggest a serious implication of fat metabolism in bone integrity and metabolism, which could contribute to BCO through increasing susceptibility to mechanical stressors or be the cause of a non-traumatic etiology [9].

A key factor in obesity and insulin resistance, which has been studied extensively in mammals, is visfatin. Visfatin is an adipokine that has been assigned numerous functions depending on species and physiological state [10]. It is known to be involved in metabolic disorders through its regulation of glucose-stimulated insulin secretion [11]. The liver is the main site of lipid metabolism (lipogenesis) in the broiler [12]. Therefore, the investigation into visfatin' s regulation within avian hepatic cells by hormones, cytokines, and other factors was undertaken

to first understand how visfatin is regulated within this primary organ for lipid metabolism. Visfatin levels were also measured in BCO affected bone and found to be significantly upregulated (Figure 1). Therefore, further research into the potential role of visfatin in the lipid metabolism seen in BCO is warranted and the current work in hepatic cells provides a foundation for future studies.

A marker for energy homeostasis and mitochondrial function is UCP [13]. In chapter 3, UCP was shown to be significantly upregulated in BCO indicating its potential involvement in mitochondrial dysfunction and ultimately energy homeostasis. The regulation of UCP in avian muscle cells, where mitochondria are abundant, was undertaken. This research provides deeper understanding into how UCP specifically is regulated under hormonal and cytokine factors. Cytokines, such as IL-1β, were shown to be activated in BCO conditions either systemically or locally in Chapter 7.

These studies provide a basis for future work and specific analysis of gene regulation under different physiological challenges or states. Given BCO's systemic effects and complexity, research into how these genes function within the BCO etiology is warranted.

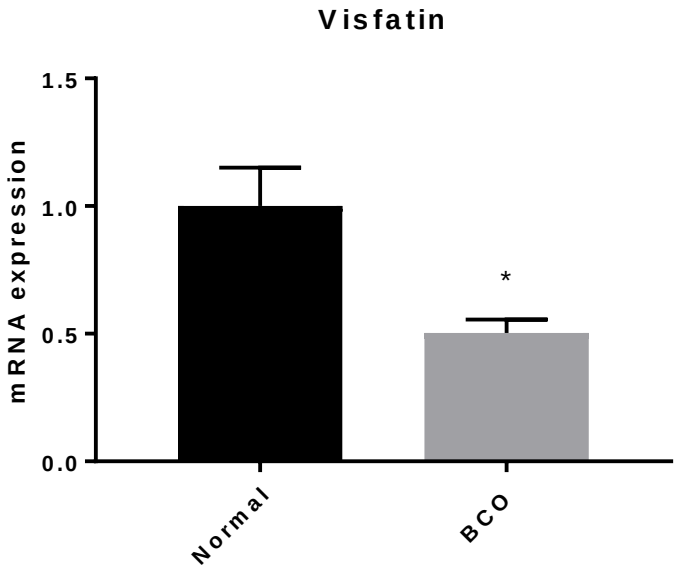

Figure 1. Visfatin mRNA expression form BCO-affected bone compared to normal
Visfatin was measured using rt-qPCR in the bone of BCO-affected and normal broilers using the same experimental conditions as previously reported [14]. * indicates significant difference in gene expression ($P < 0.05$).

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Chapter 10 –

Title: Hormonal regulation of visfatin gene in avian Leghorn male hepatoma (LMH) cells

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Abstract: Visfatin has been extensively studied in mammals and has been shown to play an important role in obesity and insulin resistance. However, there is a paucity of information on visfatin regulation in non-mammalian species. After characterization of chicken visfatin gene, we undertook this study to determine its hormonal regulation in avian (non-mammalian) liver cells. Addition of 5 ng/mL TNFα, 100 ng/mL leptin, 1, 3, 10 or 100 ng/mL T3 for24 h upregulated visfatin gene expression by 1.2, 1.8, 1.95, 1.75, 1.80, and 2.45 folds ($P < .05$), respectively, compared to untreated LMH cells. Administration of 10 ng/mL of orexin A significantly down regulated visfatin gene expression by 1.35 folds compared to control cells. In contrast, treatment with IL-6 or orexin B for 24 h did not influence visfatin mRNA abundance. These pro-inflammatory cytokines and obesity-related hormones modulate the expression of CRP, INSIG2, and nuclear orphan receptors. Hepatic CRP gene expression was significantly

upregulated by IL-6, TNFα, orexin B, and T3 and down regulated by leptin and orexin A. LXR mRNA abundances were increased by orexin A, decreased by orexin B, and T3, and did not affected by IL6, TNFα, or leptin. The expression of FXR gene was induced by IL-6, leptin, and T3, but it was not influenced by TNFα, orexin A or B. CXR gene expression was up regulated by TNFα, leptin, orexin B, and T3, down regulated by 5 ng/mL orexin A, and was not affected by IL-6. INSIG2 mRNA levels were increased by TNFα(5 ng/mL), leptin (100 ng/mL), and T3 (1, 3, 10, and 100 ng/mL), decreased by orexin A, and remained unchanged with IL-6 or orexin B treatment.

Together, this is the first report showing hormonal regulation of visfatin in avian hepatocyte cells and suggesting a potential role of CRP, INSIG2, and nuclear orphan receptor LXR, FXR, and CXR in mediating these hormonal effects.

Key words: LMH cells, avian species, visfatin, hormonal regulation

Introduction

Adipocytokines or adipokines are bioactive factors mainly produced and secreted by adipose tissues. Visfatin or Nampt is among these adipokines and it was originally identified as a growth factor for early β cells and thereby called pre-β-cell colony-enhancing factor (PBEF) [2,3]. Nampt has an intracellular (iNampt) and extracellular (eNampt) forms. iNampt is a nicotinamide phosphoribosyl transferase (Nampt), a cytosolic enzyme, involved in nicotinamide adenine dinucleotide (NAD) biosynthesis [4]. It regulates the activity of NAD-consuming enzymes such as sirtuins and affects several metabolic and stress processes [5]. Although the physiological roles of eNampt have been controversial and a matter of debate for decades, several studies have assigned to eNampt a cytokine function and thereby they named it “visfatin” which plays a key

role in metabolic disorders (diabetes and obesity) via regulating glucose-stimulated insulin secretion and signaling [6,7,8,9].

Visfatin was found to be expressed in several mammalian cell types and tissues including hepatocytes [10] and has been shown to be regulated by various cytokines, hormones, and factors [11,12,13,14,15]. Such studies are currently lacking in avian (non-mammalian) species.

Avian genetic selection for high growth rate and muscle enhancement has resulted in hyperphagic broilers that are prone to obesity. Modern broiler (meat-type) chickens consume over 4 kg of feed to achieve an average slaughter-weight of 2.8 kg in only 42 days. This body weight increase arises mainly from breast (*pectoralis*) muscle and abdominal fat [16,17]. Additionally, chickens are hyperglycemic compared to mammals, with their plasma glucose levels averaging three times that found in human [18]. They require insulin doses greater than four times that required in mammals to achieve hypoglycemia, and hence they are insulin resistant [19,20,21]. They are also lacking functional brown adipose tissue and glucose transporter GLUT4 [22]. Interestingly, the majority (more than 95%) of *de novo* fatty acid synthesis (lipogenesis) occurs in the liver in chickens [23,24]. As a follow up to our previous study where we have shown that visfatin is expressed in chicken liver and is regulated by nutritional status and leptin administration, we undertook this study to determine the effects of IL-6, TNFα, leptin, T3, and orexin A/B on the expression of visfatin gene in chicken hepatocytes (LMH) in culture.

Materials and Methods

LMH cell culture

LMH cells [25], purchased from American Type Culture Collection (ATCC® CRL-2117, Manassas, VA), were cultured in McCoy 5A medium supplemented with FBS (10%), chicken serum (1%), penicillin-streptomycin (100 µg/mL), and amphotericin B (100 µg/mL) at 37°C in a 5% CO_2/95% O_2 humidified incubator. The medium and reagents were purchased from ThermoFisher Scientific (Waltham, MA). At exponential phase of growth (~80%), the complete medium was removed and replaced by a serum-free medium or a culture medium supplemented with thyroidectomized FBS and chicken serum for the thyroid hormone study [26] overnight to synchronize the cells. Depletion of thyroid hormones from sera was confirmed by RIA as we previously described [27]. Cells were then treated with 5 and 10 ng/mL of recombinant human IL-6, TNFα (ImmunoTools, Friesoythe, Germany), orexin A or orexin B (Alpha Diagnostic International, San Antonio, TX), and 10 and 100 ng/mL of recombinant ovine leptin (Protein Laboratories Rehovot, PLR, Ltd, Rehovot, ISR) or triiodothyronine (T3, MyBioSource, San Diego, CA) for 24h. Untreated cells were used as controls. The doses and duration of treatments were chosen based on pilot and previous studies [28,29,30,31,32,33,34].

RNA isolation and RT-qPCR analysis

Total RNA was extracted from LMH cells using Trizol® reagent (ThermoFisher Scientific, Waltham, MA) according to manufacturer's recommendations. RNA integrity and quality was assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 Micro-Volume Plate using Synergy HT multi-mode micro plate reader (BioTek,Winooski, VT). The RNA samples were RQ1 RNase-free DNase treated

(Promega, WI) and 1 µg RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). The RT reaction was performed at 42°C for 30 min followed by an incubation at 85°C for 5 min. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) was performed using 5 µL of 10X diluted cDNA, 0.5 µM of each forward and reverse specific primer, and SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) in a total 20 µL reaction. Oligonucleotide primers used for chicken visfatin, C-reactive protein (CRP), chicken xenobiotic-sensing orphan nuclear receptor (CXR), liver X receptor (LXR), farnesoid X receptor (FXR), insulin-induced gene 2 (INSIG2) and r18S and beta-actin (ACTN) as housekeeping genes are summarized in Table 1. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by 2% agarose gel and showed only one specific band of the predicted size. For negative controls, no cDNA templates were used in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were normalized to the expression of 18S rRNA and calculated by the $2^{-\Delta\Delta Ct}$ method [35]. The untreated cells were used as a calibrator.

Statistical analysis

Data were subjected to one-way ANOVA with hormonal treatments (doses) as the fixed effects. If ANOVA revealed significant effects, the means were compared by Tukey multiple range test using the Graph Pad Prism version 6.00 for Windows (Graph Pad Software, La Jolla California, USA), and differences were considered significant at $P < 0.05$.

Results and Discussion

Visfatin is expressed in several peripheral tissues including visceral adipose tissue, muscle, and hepatocytes [36,37,38] and its plasma concentrations correlated strongly with the amount of human visceral fat indicating its potential implication in the development of obesity-associated insulin resistance and diabetes mellitus. Furthermore, a number of cytokines and adipokines have been reported to regulate visfatin, however the bulk of these accumulated data so far originated from mammalian species. Here we used avian (non-mammalian) hepatocyte (LMH) cell line where the majority (>95%) of *de novo* fatty acid synthesis occurs [23], and thereby constitutes a unique and an appropriate model to determine the effect of insulin resistance- and obesity-associated hormones on the expression of this adipokine. First, we showed that visfatin is highly expressed in LMH cells with a low Ct value compared to other cell lines (data not shown). Treatment with IL-6 or orexin B for 24h did not influence visfatin mRNA abundance (Fig. 1a and Fig. 5a). In contrast, addition of 5 ng/mL TNFα, 100 ng/mL leptin, 10 or 100 ng/mL T3 for 24h upregulated visfatin gene expression by 20%, 85%, 13%, and 11% ($P < 0.05$), respectively, compared to untreated cells (Fig. 2a, Fig. 3a, and Fig. 6a). Administration of 10 ng/mL of orexin-A significantly down regulated visfatin gene expression by 26 % compared to control cells (Fig. 4a). Due to the controversial results in the literature, our data corroborated several studies and disagreed with others. For instance, Kralisch and coworkers [39] have shown that TNFα administration decreased visfatin mRNA levels in 3T3-L1 adipocytes; however, Hector et al. [12] and Wang et al. [40] have reported that TNFα treatment increased visfatin gene expression in a time-dependent manner in human visceral adipose tissue and coronary arterial endothelial cells, respectively. Similarly, experimental studies have revealed controversial results, indicating that IL-6 treatment could increase visfatin gene expression in rheumatoid arthritic synovial

fibroblasts [41] and in human abdominal subcutaneous. However, Kralisch et al. [43] and Zhang et al. [11] have shown down regulation of visfatin gene expression by IL-6 in 3T3-L1 and BeWo cells, respectively. *In vivo* and *ex vivo* studies conducted by Tan et al. [13] supported leptin-induced visfatin expression and secretion in 3T3-L1 cells and humane/murine adipose tissue explants. Functional studies using pharmacological inhibitors and genetic manipulations showed that these effects of leptin were mediated by mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways [13].

Visfatin levels in hypo-and hyperthyroidism patients as well as the effects of T3 on the expression of visfatin system have been reported with controversial results. In patients with Graves' disease, circulating visfatin levels were found to be elevated and positively correlated with serum T3 [44]. On the other side, MacLaren et al. [15] demonstrated that T3 treatment down regulated visfatin gene expression in 3T3-L1 adipocytes. Han et al. [45] reported that both hyper- and hypothyroidism were associated with higher plasma visfatin levels compared to euthyroid subjects, and they showed that T3 administration induced a remarkable increase in visfatin mRNA abundance in 3T3-L1 adipocytes at low concentrations followed by a sharp decrease at higher concentrations.

Interestingly, only orexin A but not orexin B affected visfatin gene expression in our experimental conditions. This differential effect might be explained by the differential affinities of the orexin receptors. Indeed, orexin A triggers orexin receptor 1, while orexin receptor 2 is used by both orexins A and B [46]. Administration of orexin A has been reported to reduce plasma visfatin levels in obese and T2DM animals [47]. However, whether this effect of orexin in these animals is direct or indirect remains unclear.

The reported discrepancies between these studies might be due to differences in species, tissues, cell lines, hormone dosage, treatment duration, and/or methodological factors. Overall, chicken visfatin is expressed in hepatocyte cells and is directly regulated by pro-inflammatory cytokines (IL-6, TNFα), and obesity-related hormones (orexins, leptin, and T3).

As the pro-inflammatory cytokines and obesity-related hormones are associated with high levels of CRP and orphan receptors, which all have been shown to control visfatin regulation, we sought to determine the expression of CRP, LXR, FXR, CXR, and INSIG2 in this study. Treatment with IL6 (10 ng/mL), TNFα (5 and 10 ng/mL), or orexin B (10 ng/mL) significantly upregulated CRP gene expression compared to untreated LMH cells (Figs. 1b, 2b, and 5b), corroborating previous studies [48,49,50]. In contrast, administration of leptin (100 ng/mL), orexin A (5 and 10 ng/mL), or T3 (10 and 100 ng/mL) significantly down regulated CRP mRNA levels compared to control cells (Figs. 3b, 4b, and 6b). In mammals, T3 has been shown to reduce CRP expression via inhibition of hepatic IL-6 and STAT3 signaling [51]. In disaccord with our present results, plasma mammalian leptin and CRP were found to have a positive correlation in both normal weight and obese subjects [52,53]. Leptin has been shown to induce CRP expression in vascular endothelial (HCAEC) cells [54].

Administration of IL-6 did not elicit any change to the hepatic expression of LXR, CXR, or INSIG2; however, at the high dose of 10 ng/mL it induced the expression of FXR (Fig. 1c-f). TNFα did not influence LXR or FXR expression, but it up regulated the expression of CXR and INSIG2 (Fig. 2c-f). Leptin (100 ng/mL) treatment increased FXR, CXR and INSIG2 but not LXR mRNA abundances (Fig. 3c-f). Orexin A upregulated LXR gene expression and down regulated that of CXR and INSIG2 without affecting FXR (Fig. 4c-f). Orexin B, however, decreased the mRNA levels of LXR, and increased CXR gene expression without affecting FXR

and INSIG2 mRNA levels (Fig. 5c-f). T3 treatment down regulated LXR gene expression and increased FXR, CXR, and INSIG2 mRNA levels (Fig. 6b-e). Although it is not known whether chicken CRP and/or visfatin promoters harbor putative LXR-responsive elements, LXR ligands have been shown to be negative regulators of CRP and visfatin gene expression [55,56]. This suggest that the effects of orexin A and T3 on visfatin in our experimental conditions were probably mediated through LXR. Similarly, as FXR and CXR are probably presents in the visfatin promoter [57], the effects of obesity-associated hormones observed in our study might be mediated via these nuclear orphan receptors, however further functional studies are warranted to establish these interactions. It is also plausible that these hormones regulate visfatin gene expression via modulation of INSIG2 which blocks proteolytic activation of SREBPs by SREBP cleavage-activating protein (SCAP) [58,59].

In conclusion, this is the first report showing regulation of visfatin by pro-inflammatory cytokines and obesity-related hormones in avian (non-mammalian) hepatocytes and suggesting that these effects were mediated via nuclear orphan receptors (LXR, FXR, CXR), CRP, and INSIG2.

Acknowledgements

This work was supported by research grant from the Arkansas Bioscience Institute (ABI) to SD.

Table 1. Oligonucleotide real-time qPCR primers

Gene	Accession number[a]	Primer sequence (5' → 3')	Orientation	Product size (bp)
Visfatin	NM_001030728	GCTTCAGCCCATTTGGTGA	Forward	97
		ATCCCGGAACTGGATCTTTTG	Reverse	
CRP	NM_001039564	TCTCCTACGCCACCAAAGCT	Forward	102
		CGCGGAAGGTGACGTATTTC	Reverse	
LXR	AF492498	ACGATCAGGCACGGACAGTC	Forward	201
		GCCTTGCTTCGCGGTTATTAG	Reverse	
FXR	AF492497	AAAGCCTAGACTGGGCCACTC	Forward	190
		CATGTCCATCTCACAGTTGCC	Reverse	
CXR	NM_204702	GGTCATCAAATTCGCCAAGG	Forward	211
		TGGCTCCAGGTAGATCTGCTG	Reverse	
INSIG2	NM_001031261	CAGCGCTAAAGTGGATTTTGC	Forward	65
		CAATTGACAGGGCTGCTAACG	Reverse	
18S	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	
ACTB	NM_205518	CTGGCACCTAGCACAATGAA	Forward	123
		CTGCTTGCTGATCCACATCT	Reverse	

[a] Accession number refer to Genbank (NCBI).
ACTB, beta-actin; CRP, C-reactive protein; CXR, chicken xenobiotic-sensing orphan nuclear receptor; FXR, farnesoid X receptor; INSIG2, insulin-induced gene 2; LXR, liver X receptor.

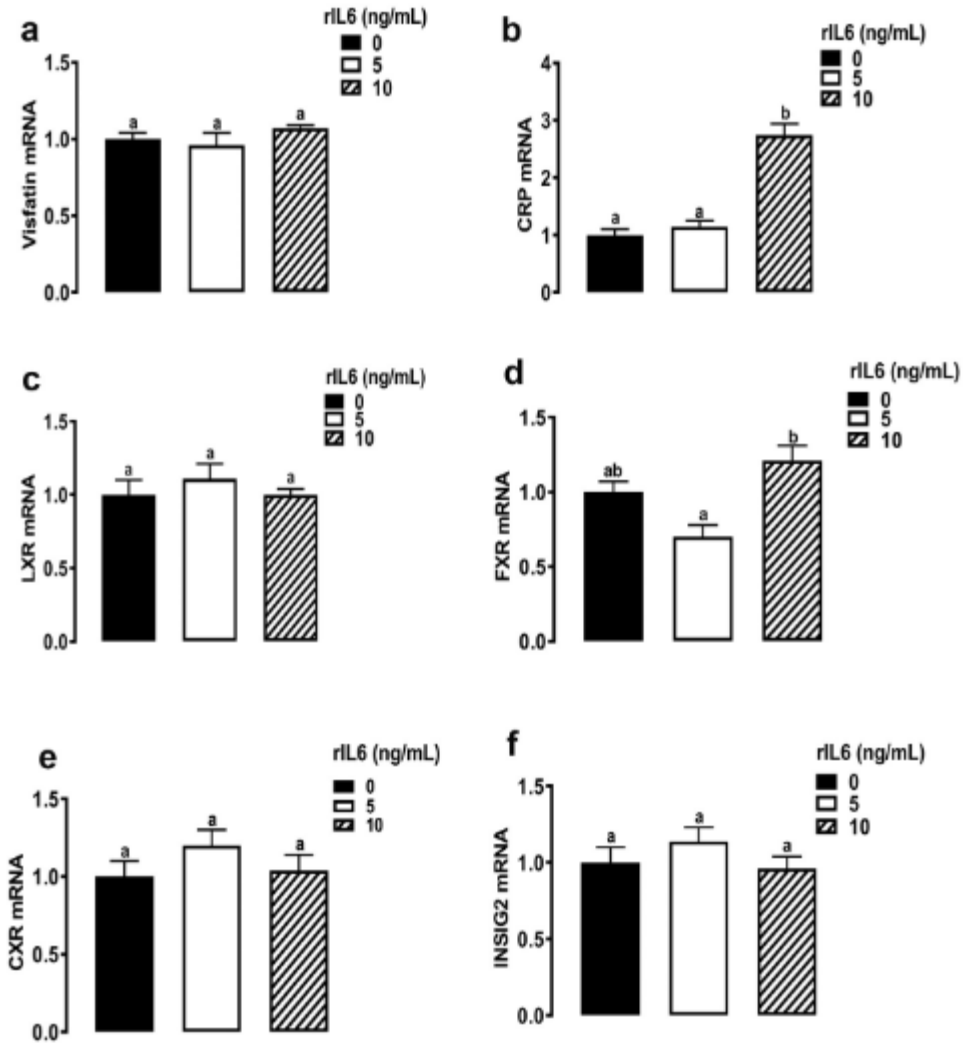

Figure 1. Effect of recombinant IL-6 on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 5 or 10 ng/mL of IL6 for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

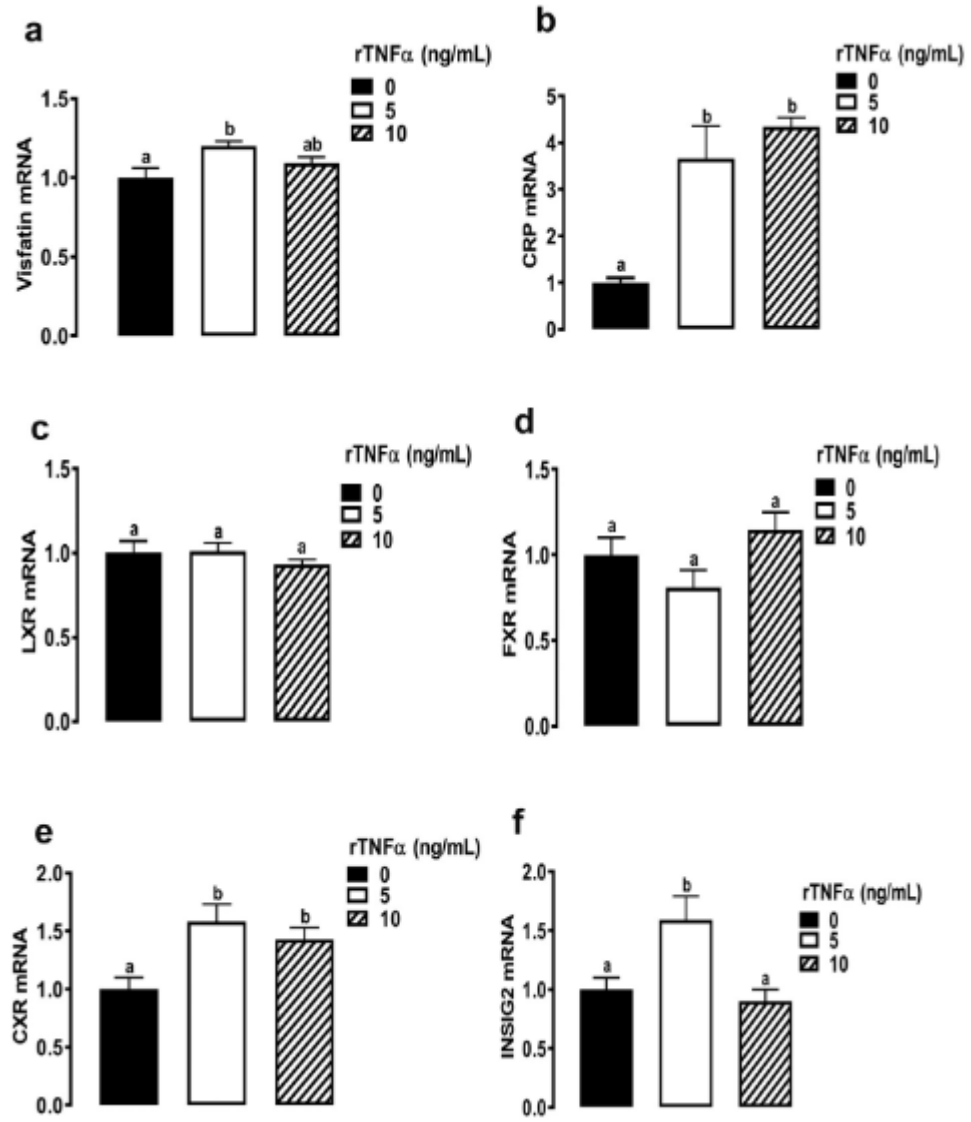

Figure 2. Effect of recombinant TNFα on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 5 or 10 ng/mL of TNFα for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

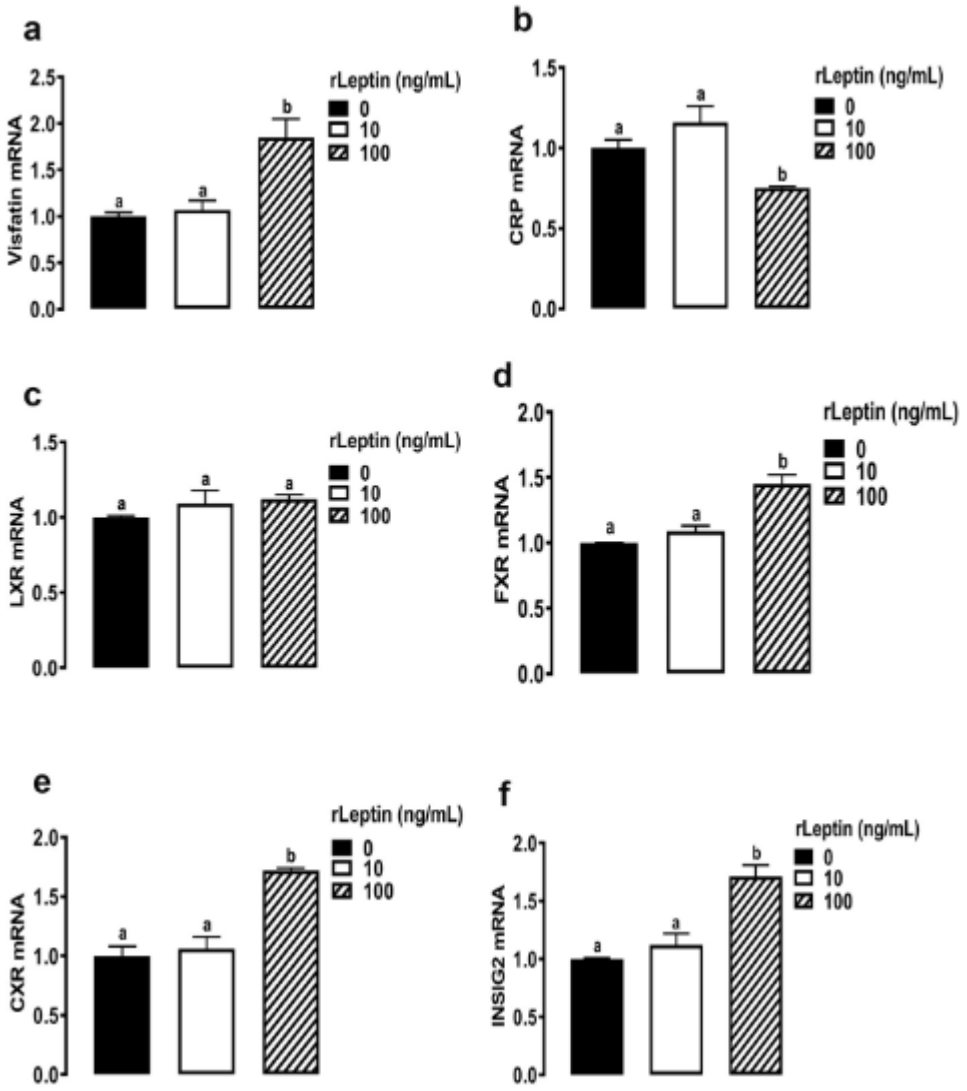

Figure 3. Effect of recombinant leptin on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 10 or 100 ng/mL of leptin for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

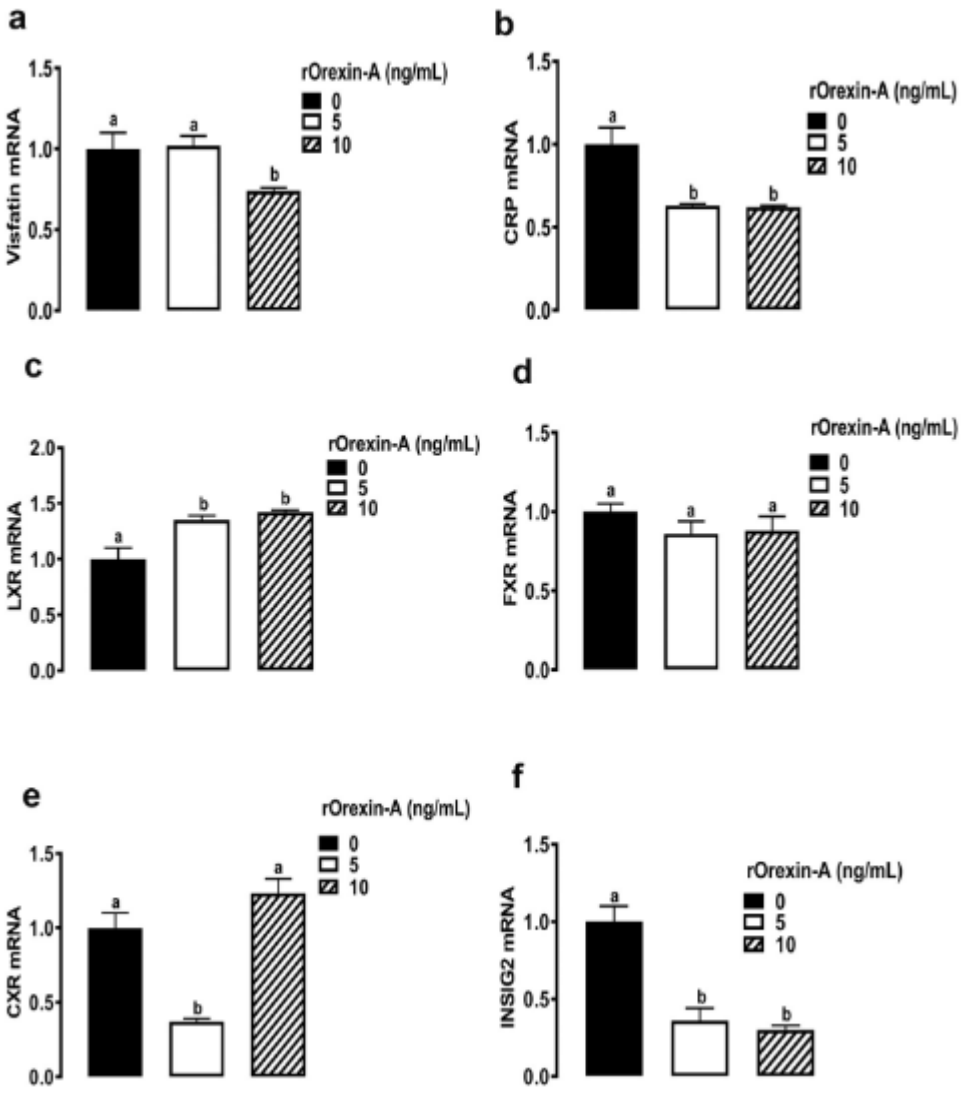

Figure 4. Effect of recombinant orexin-A on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 5 or 10 **ng**/mL of orexin A for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

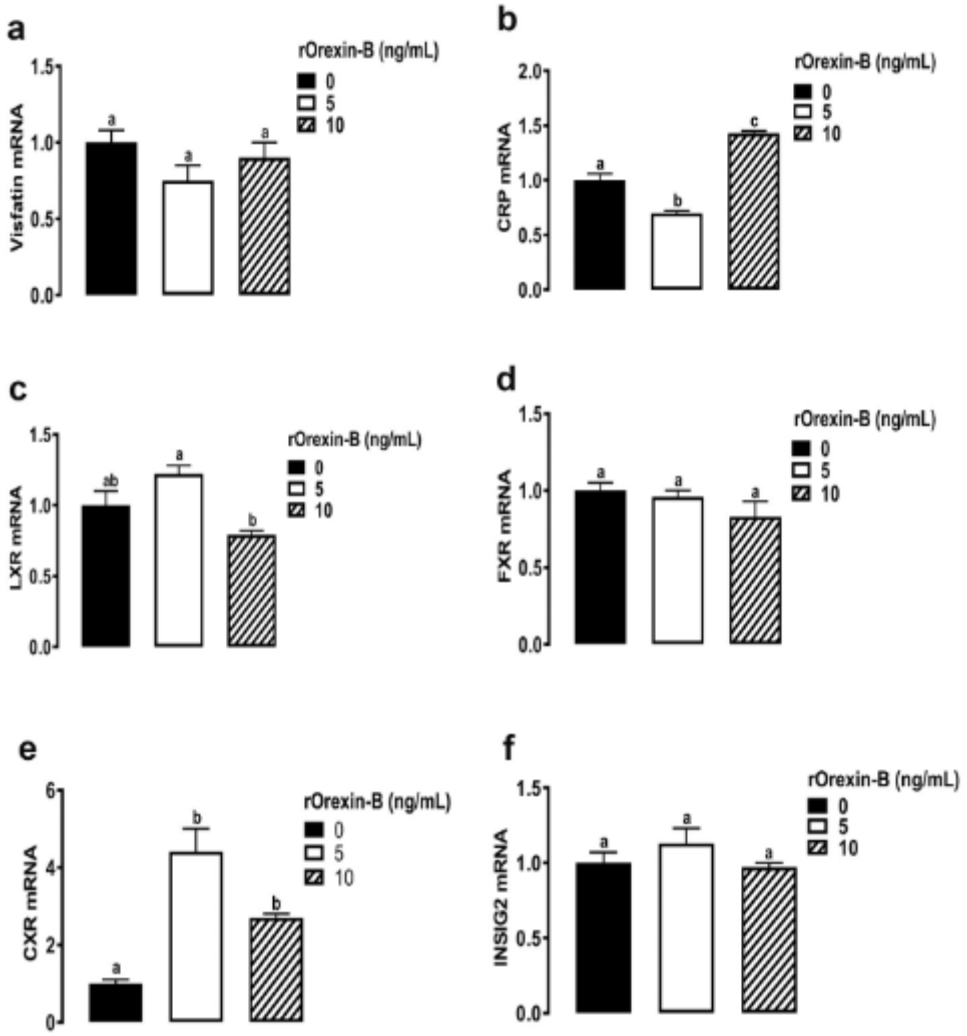

Figure 5. Effect of recombinant orexin-B on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 5 or 10 ng/mL of orexin B for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

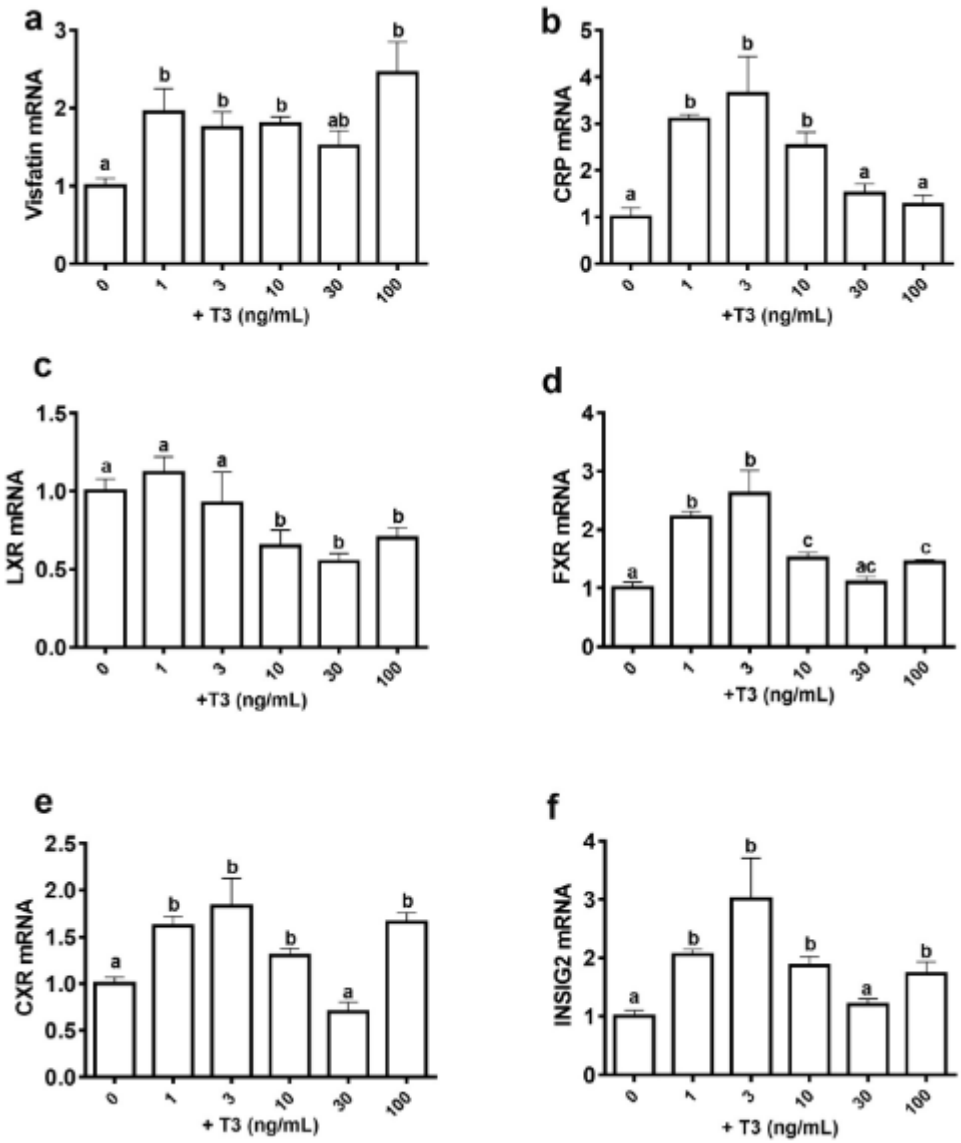

Figure 6. Effect of recombinant T3 on visfatin gene expression and its related regulators in LMH cells.

LMH cells were treated with 10 or 100 ng/mL of T3 for 24h. Untreated cells were used as controls. Relative expression of visfatin, CRP, LXR, FXR, CXR, and INSIG2 mRNA was determined by qPCR using $2^{-\Delta\Delta Ct}$ methods [35]. Data are presented as mean ± SEM (n=8). Different letters indicate a significant difference at $P < 0.05$.

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Chapter 11 –

Title: Regulation of avian uncoupling protein (av-UCP) expression by cytokines and hormonal signals in quail myoblast cells

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Abstract: Uncoupling proteins (UCPs), mitochondrial inner membrane transport proteins, have several functions including thermogenesis and fatty acid oxidation. There have been extensive studies of mammalian UCPs demonstrating their regulation by not only extreme metabolic states, but pro-inflammatory cytokines and energy homeostasis-related hormones. However, little is known regarding the regulation of avian-UCP (av-UCP), a homolog to mammalian UCP3. We undertook this study to determine av-UCP hormonal regulation in quail muscle (QM7) cells. Treatment of IL-6 (5 and 10 ng/mL), TNFα (5 and 10 ng/mL0, and T3 (3ng/mL) upregulated av-UCP expression compared to untreated QM7 cells. Administration of leptin (10 and 100 ng/mL) downregulated av-UCP expression compared to controls. Orexin-A and Orexin-B had no significant effect on av-UCP mRNA levels. Transcription factors PGC-1α, PPARα, and PPARγ

were upregulated by TNFα (5 ng/mL) and PPARα and PPARγ were both upregulated by IL-6 (5 ng/mL), TNFα (5 and 10 ng/mL), and T3 (1 ng/mL). PPARβ was upregulated by Orexin-B (5 ng/mL), however, Orexin-A (10 ng/mL) had no significant effect on all other transcription factors other than downregulation of PPARβ. Taken together, this report demonstrates hormonal regulation of av-UCP by both energy homeostasis-related hormones and pro-inflammatory cytokines with potential mediation of hormonal effects on av-UCP by peroxisome proliferator-activated receptor transcription factors.

Key words: QM7 cells, avian species, uncoupling protein gene, hormonal regulation

Introduction

Uncoupling proteins are transporter proteins found in the inner membrane of the mitochondria and are responsible for dissipating the proton gradient generated through the mitochondrial respiration pathway. The movement of protons by UCP is not coupled with ATP synthesis which prompts the uncoupling of respiration and thermogenesis. The uncoupling of respiration is stimulated by increased reactive oxidative species (ROS) and works to reduce their production while also maintaining redox balance by preventing over production of ATP which would inhibit respiration [1-3]. In addition to mitochondrial respiration, UCP homologs mediate and catalyze the export of fatty acids and fatty-acid peroxides. This increases the fatty acid oxidation rate and prevents lipotoxicity, as well as mediates insulin signaling and regulation of energy expenditure and intake in differing environmental and stress conditions [4-6].

The diverse functions of uncoupling proteins are partly due to the number of homologs, including, but not limited to, UCP1, UCP2, UCP3, UCP4, brain mitochondrial carrier protein-1 (BMCP-1), and avian UCP (av-UCP). UCP1 was first found in brown adipose tissue of

mammals and is the primary homolog responsible for thermogenesis through respiration uncoupling [7-10]. UCP2 and UCP3 have mild uncoupling capacity in the presence of activators, such as ROS, and differing tissue distribution in mammals with UCP2 being ubiquitous and UCP3 mainly in skeletal muscle [4, 11]. The physiological roles of UCP2 and UCP3 have been speculated to include lipid utilization for the purpose of energy homeostasis in muscle and evidence suggests their mild uncoupling reduces mitochondrial ROS production and protects against cellular damage [6, 12, 13]. UCP3 knock-out mice have impaired fatty acid oxidation and UCP3 is known to increase in fasting states for both mammals and avian species [14]. While UCP1-4 and BMCP-1 are mammalian homologs, avian species only have one homolog called av-UCP, which was cloned and characterized in 2001 [15]. av-UCP gene is a nuclear gene whose amino acid sequence is closer to that of mammalian UCP2 and UCP3 than UCP1 [16]. Its expression is primarily in skeletal muscle; however, levels have been found ubiquitously in 8-week-old-broilers and it has since been found in heart, liver, adipose tissue, spleen, brain, and lung [12, 17, 18]. Studies have shown up-regulation of av-UCP during fasting, cold-and heat exposure and metabolic stress [15, 19, 20]. In addition, av-UCP has been correlated with increased fatty-acid oxidation in skeletal muscle and shown to reduce mitochondrial superoxide production in chicken skeletal muscle during fasted states [21-23]. This suggests that av-UCP functions similarly to mammalian homologs, including fatty-acid oxidation, mild thermogenesis, and attenuation of ROS.

In chickens, hormonal regulation of av-UCP in differing metabolic states and ages has often conflicted with mammalian models [15, 18, 24, 25]. Mammalian models have shown hormonal regulation of UCPs by energy-homeostasis-regulating and obesity-related hormones such as leptin, triiodothyronine (T3), insulin, and glucagon [26-29]. A previous study demonstrated

upregulation of hepatic leptin and muscle UCP in both acute cold- and chronic heat- exposed broilers suggesting a potential mechanism between hormonal regulation of UCP to mediate energy expenditure and intake during extreme environmental conditions [19]. However, the mechanism behind the regulation of av-UCPs potential involvement in energy metabolism in differing metabolic conditions has yet to be uncovered. Additionally, there has yet to be a cell-model investigation into the regulation of av-UCP by hormones involved in energy homeostasis and stress-response pathways. The goal of the present study is to determine the effects of Interleukin-6 (IL-6), tumor necrosis factor- alpha (TNFα), leptin, T3, and orexin A/B on av-UCP in quail muscle (QM7) cells in culture.

Materials and Methods

QM7 cell culture

QM7 cells, purchased from American Type Culture Collection (ATCC® CRL-1962, Manassas, VA), were cultured in M199 medium supplemented with FBS (10%), penicillin-streptomycin (100 µg/mL), and tryptose phosphate broth (10%) at 37°C in a 5% CO_2/95% O_2 humidified incubator [30]. The medium and reagents were purchased from ThermoFisher Scientific (Waltham, MA). At exponential phase of growth (~80%), the complete medium was removed and replaced by a serum-free medium or a culture medium supplemented with thyroidectomized FBS and chicken serum for the thyroid hormone study overnight to synchronize the cells [31]. Depletion of thyroid hormones from sera was confirmed by RIA as was previously described [32]. Cells were then treated with 5 or 10 ng/mL of recombinant human IL-6, TNFα (ImmunoTools, Friesoythe, Germany), orexin A or orexin B (Alpha Diagnostic International, San Antonio, TX), or 10 or 100 ng/mL of recombinant ovine leptin (Protein Laboratories Rehovot, PLR, Ltd, Rehovot, ISR) or 1, 3, 10, 30, or 100 mg/mL of triiodothyronine (T3,

MyBioSource, San Diego, CA) for 24h. Untreated cells were used as controls. The doses and duration of treatments were chosen based on initial pilot and several previous studies [33-38].

RNA isolation and RT-qPCR analysis

Total RNA was extracted from QM7 cells using Trizol® reagent (ThermoFisher Scientific, Waltham, MA) according to manufacturer's recommendations. RNA integrity and quality were assessed using 1% agarose gel electrophoresis and RNA concentrations and purity were determined for each sample by Take 3 Micro-Volume Plate using Synergy HT multi-mode micro plate reader (BioTek,Winooski, VT). The RNA samples were RQ1 RNase-free DNase treated (Promega, WI) and 1 µg RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). The RT reaction was performed at 42°C for 30 min followed by an incubation at 85°C for 5 min. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR system) was performed using 5 µL of 10X diluted cDNA, 0.5 µM of each forward and reverse specific primer, and SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) in a total 20 µL reaction. Oligonucleotide primers used for chicken UCP, peroxisome proliferator-activated receptor gamma coactivator (PGC-1α), chicken peroxisome proliferator-activated receptor alpha (PPARα), peroxisome proliferator-activated receptor gamma (PPARγ), and peroxisome proliferator-activated receptor beta (PPARβ) and r18S and beta-actin (ACTN) as housekeeping genes are summarized in Table 1. The qPCR cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of a two-step amplification program (95°C for 15 s and 58°C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. The PCR products were also confirmed by 2% agarose gel and showed only one specific band of the predicted size. For

negative controls, no cDNA templates were used in the qPCR and verified by the absence of gel-detected bands. Relative expressions of target genes were normalized to the expression of 18S rRNA and calculated by the $2^{-\Delta\Delta Ct}$ method [39]. The untreated QM7 cells were used as a calibrator.

Statistical analysis

Data were subjected to one-way ANOVA with hormonal treatments (doses) as the fixed effects. If ANOVA revealed significant effects, means were compared by Tukey multiple range test using the Graph Pad Prism version 6.00 for Windows (Graph Pad Software, La Jolla California, USA), and differences were considered significant at $P < 0.05$.

Results and Discussion

UCP was first found in brown adipose tissue as a key contributor to non-shivering thermogenesis [9]. Its homologs, UCP2 and UCP3, are involved in fatty-acid oxidation and movement as well as insulin signaling. The avian homolog, av-UCP, is closer to mammalian UCP2 and UCP3 and is upregulated by not only acute-cold and chronic-heat stress, but differing metabolic states such as fasting and high fat diets [19, 21, 22, 40]. This points to the potential involvement of av-UCP in key metabolic pathways regulating energy homeostasis as well as environmental stress responses. Although mammalian models have shown hormonal regulation of UCP, there has yet to be a similar avian cell model of av-UCP. Avian skeletal muscle has high expression of av-UCP and is rich in mitochondria. It is also the site of lipid utilization for energy homeostasis in extreme conditions [18, 41]. For these reasons, QM7 cells constitute an appropriate model to determine the effects of energy-homeostasis-related-, and pro-inflammatory hormones on av-UCP.

Treatment with Orexin-B did not affect UCP mRNA expression. However, treatment with IL-6 (5 and 10 ng/mL), TNFα (5 and 10 ng/mL0, and T3 (3ng/mL) significantly increased UCP expression compared to untreated cells (Figures 3, 4, and 2). In contrast, treatment of leptin (10 and 100 ng/mL) significantly down regulated UCP mRNA expression when compared to untreated control (Figure 1). Interestingly, administration of 5 ng/mL of Orexin-A did not have a significant impact on UCP expression, while 10 ng/mL resulted in slight down-regulation. The data showed both consistencies and inconsistencies with other avian models. In mouse brown adipose tissue, leptin treatment increased UCP expression [42-44]. The inconsistency between results could be due to the different cell type and the fact that chicken lacks functional brown adipose tissue. However, the down regulation of av-UCP under leptin treatment also conflicts with a previous study showing increased hepatic leptin and UCP in response to acute cold and chronic heat stress in broilers [19]. This could be because both leptin and UCP are activated during these extreme environmental conditions, but leptin is not responsible or needed for UCP activation and another pathway during these stress conditions results in increased UCP expression.

Treatment with IL-6 resulted in increased UCP expression compared to the control. IL-6 has been shown to increase UCP1 expression in mammals and to be necessary for cold-induced UCP upregulation, but not always coincide with UCP protein levels [45, 46]. IL-6 is both a pro-inflammatory cytokine and anti-inflammatory myokine induced by exercise and in response to low glycogen levels or metabolic demand [47]. High levels of IL-6 result in increased lipolytic rate through the AMPK or PI3-kinase pathway in muscle and adipose tissue resulting in oxidation of fatty acids and increased energy supply [48, 49]. In addition to being obese, IL-6 knock-out mice had reduced levels of UCP-3, hypertriglyceridemia, and were glucose intolerant

by 9 months of age [50]. The results coincide with mammalian studies and suggest potential involvement of IL-6 in av-UCP expression for the purpose of mediating lipid utilization in muscle and potentially as part of both the IL-6 anti-inflammatory and pro-inflammatory pathway. Treatment of TNFα strongly up-regulated av-UCP. Tumor necrosis factor-α (TNFα) is a pro-inflammatory cytokine involved in inflammation and non-shivering thermogenesis in brown adipose tissue in mammals [51]. Mammalian models have shown differentially and tissue dependent up-regulation of UCP homologs after TNFα treatment, with UCP3 and UCP2 being upregulated in muscle [52, 53]. Upregulation of av-UCP by TNFα indicates a potential role of UCP in TNFα regulated inflammation or thermogenesis mechanism. Further studies are warranted to investigate connections involving the TNFα pathway. T3 has been shown to upregulate UCP3 in mammalian skeletal muscle and in mice without functional brown adipose tissue (BAT), UCP3 was upregulated by administration of T3 in skeletal muscle suggesting that in the absence of BAT thermogenesis, T3 actions in skeletal muscle are sufficient to rescue cold tolerance [43, 54, 55]. Chickens lack BAT and are prone to obesity due to fast growth [56]. Studies have shown physiological normal levels of T3 to be between 2 to 4 ng/mL and that thyroid status affects avian UCP levels [25, 57]. Analysis of the promoter region of av-UCP has shown potential binding sites for thyroid hormone receptors [58]. T3 dosage of 3 ng/mL resulted in significant increased UCP mRNA expression and further supports regulation of UCP by T3 for the both energy metabolism and thermogenic purposes. Orexins are neuropeptides which regulate energy homeostasis, wakefulness, lipid metabolism, neuroendocrine stress response, and feeding behaviors in mammals [59]. Orexin receptors have been found in avian muscle and orexins are known to regulate mitochondrial dynamics within avian species [38]. Orexin-A had slight but insignificant upregulation of av-UCP (5 ng/mL) but a slight, insignificant drop in av-

UCP at 10 ng/mL. Orexin-B did not have any significant impact on av-UCP expression. This conflicts with other avian studies that saw decreased av-UCP expression in QM7 cells treated with Orexin A and B, however the doses were different with 10 and 100 ng/mL in that study and 5 and 10 ng/mL in this study [60]. A slight but insignificant drop in av-UCP is visible at 10 ng/mL of Orexin-A.

The differences between these studies could be due to a number of factors such as, differences in tissues, species, cell lines, dosage of hormones, duration of treatments and/or methodological factors. Overall, av-UCP is expressed in muscle and directly regulated by energy-homeostasis related hormones (leptin and T3) as well as pro-inflammatory cytokines and thermogenesis related hormones (IL-6 and TNFα).

In the present study, we sought to determine the expression of PGC-1α, PPARα, PPARβ, and PPARγ as potential transcription factors regulating av-UCP expression. As a mediator of mitochondrial ROS and fatty acid metabolism in mammals and a known player in avian thermoregulation and lipid utilization, the beta-adrenergic system has been shown to regulate UCP expression in both adipose tissue and muscle cells [24, 58]. Analysis of the av-UCP promoter region suggested potential PPAR and thyroid hormone receptor binding sites [58]. PGC-1α was upregulated by treatment with leptin (10 and 100 ng/mL) and TNFα (5 ng/mL). Interestingly, treatment with 5 ng/mL of Orexin-A increased relative PGC-1α mRNA levels while 10 ng/mL resulted in downregulation. Orexin-B (10 ng/mL) and T3 (1, 2, 10, 30, and 100 ng/mL) also downregulated PGC-1α mRNA expression. IL-6 had no significant impact on PGC-1α. PGC-1α is a transcription factor that is induced by exercise as well as adrenergic stimulation and lower temperatures in mammalian skeletal muscle for the purpose of thermogenesis and mitochondrial biogenesis [61]. In cold-exposed chickens, PGC-1α expression preceded increases

in av-UCP and av-ANT within skeletal muscle [62]. PGC-1α knock-out mice showed decreased capacity for UCP1 upregulation in cold exposure [63]. These studies in conjunction with our results indicate PGC-1α as a potential regulator for av-UCP in muscle. As a transcription factor, PGC-1α targets other transcription factors involved in fatty acid oxidation and UCP1/GyK induction such as PPARα, PPARβ, and PPARγ [62]. PPARγ has been shown to increase UCP1 expression in adipose tissue through cold exposure and β-adrenergic agonist induced PGC-1α expression [64]. IL-6 (5ng/mL), TNFα (5 and 10 ng/mL) and T3 (1 ng/mL) increased both av-UCP and PPARγ mRNA expression relative to untreated controls. However, this increase was not always coupled with a simultaneous increase in PGC-1α mRNA expression except in the case of TNFα. This suggests the effects of TNFα, and possibly IL-6 and T3, on av-UCP seen in our experimental conditions could be mediated through PPARγ, and potentially PGC-1α further upstream. PPARα was also upregulated by TNFα (5 and 10 ng/mL), IL-6 (5 and 10 ng/mL), and T3 (1 ng/mL). PPARα is expressed in mammalian tissues with high oxidative energy demands that depend on mitochondrial fatty acid oxidation and in the heart of rodents, it has been shown to directly regulate UCP3 expression [65, 66]. These studies, along with our results support the potential mediation of UCP expression by the transcription factor PPARα. Interestingly, IL-6, TNFα, leptin, and orexin-B did not have a significant effect on PPARβ mRNA expression when compared to controls. A recent study demonstrated that activation of UCP3 by the PGC1-α/PPARβ axis was induced by fatty acid induced metabolic stress in skeletal muscle [67]. The observed lack of increased PPARβ mRNA expression could be due to differing stimuli necessary for that pathway, outside of hormonal regulation alone.

In conclusion, this is the first report of regulation of av-UCP by energy-homeostasis-related hormones and pro-inflammatory cytokines in avian muscle and mediation of av-UCP expression

through peroxisome proliferator-activated receptor transcription factors (PGC-1α, PPARα, PPARβ, and PPARγ).

Acknowledgements

This work was supported by research grant from the Arkansas Bioscience Institute (ABI) to SD.

Table 1
Oligonucleotide real-time qPCR primers.

Gene	Accession number[a]	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
Av-UCP	AB602049	TTCCCTATGGATGCAGCAAAA GCTCTGGTTGGCTTCAGACAA	Forward Reverse	65
PGC-1α	NM_001006457	GAGGATGGATTGCCTTCATTTG GCGTCATGTTCATTGGTCACA	Forward Reverse	62
PPARα	AF163809	CAAACCAACCATCCTGACGAT GGAGGTCAGCCATTTTTTGGA	Forward Reverse	64
PPARβ	AF163810	CAACGGGAATGGCTTTGTG AAGGGCTTGCGCAACGT	Forward Reverse	54
PPARγ	NM_001001460	CACTGCAGGAACAGAACAAAGAA TCCACAGAGCGAAACTGACATC	Forward Reverse	67
18S	AF173612	TCCCCTCCCGTTACTTGGAT GCGCTCGTCGGCATGTA	Forward Reverse	60

Av-UCP, avian uncoupling protein; PGC-1α, peroxisome proliferator-activator receptor gamma coactivator-1 alpha; PPAR, peroxisome proliferator-activator receptor.

[a] Accession number refer to Genbank (NCBI).

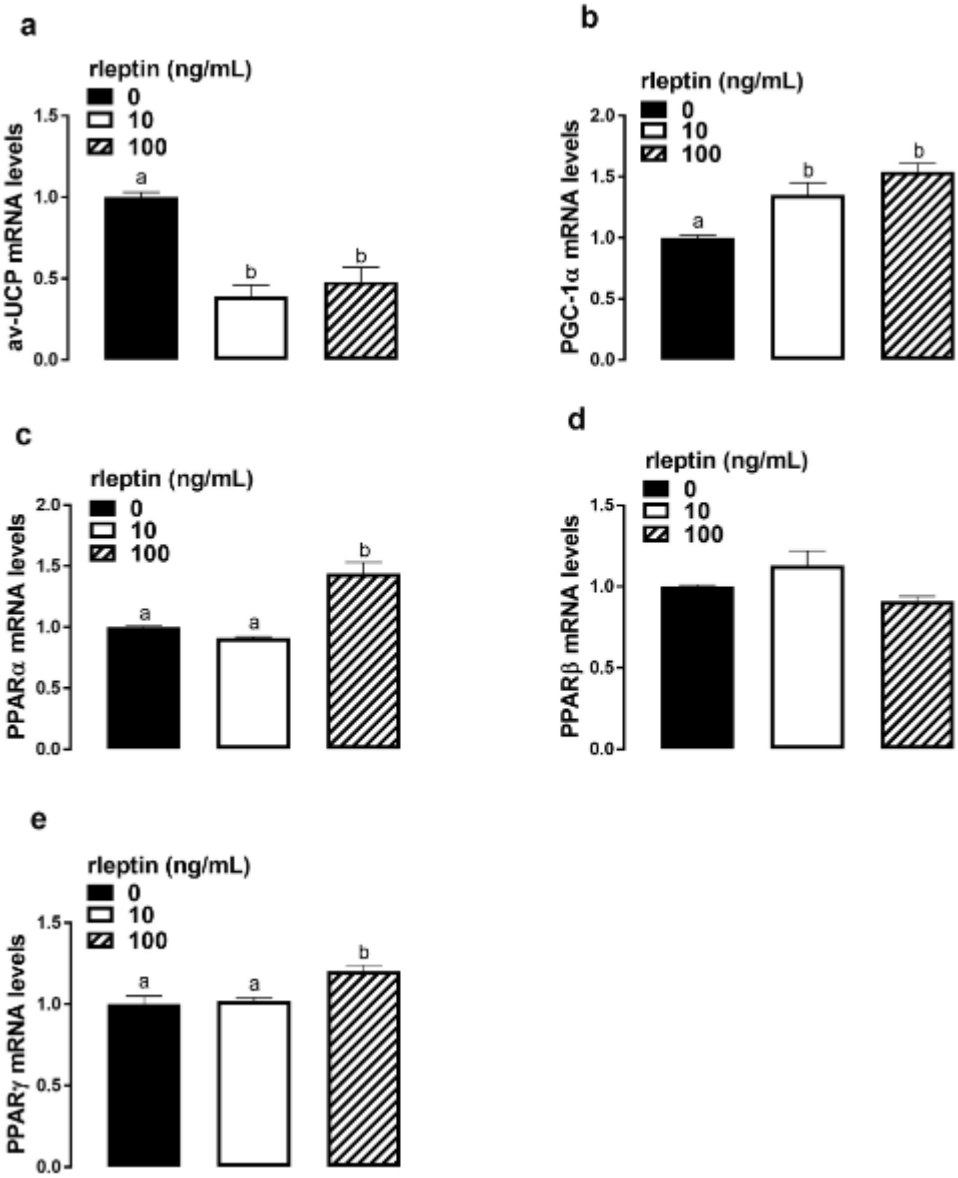

Figure 1. Effect of recombinant leptin on av-UCP gene expression and its related transcription factors **in QM7 cells**

QM7 cells were treated with 10 or 100 ng/mL of recombinant ovine leptin for 24 h. Untreated cells were used as controls. Relative expression of av-UCP (a), PGC-1α(b), PPARα(c), PPARβ(d), and PPARγ(e) mRNA was determined by qPCR using 2-ΔΔCtmethods. Data are presented as mean ± SEM (n= 9). Different letters indicate a significant difference at P< .05. av-

UCP, avian uncoupling protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PPAR, peroxisome proliferator-activated receptor; QM7, quail myoblast 7.

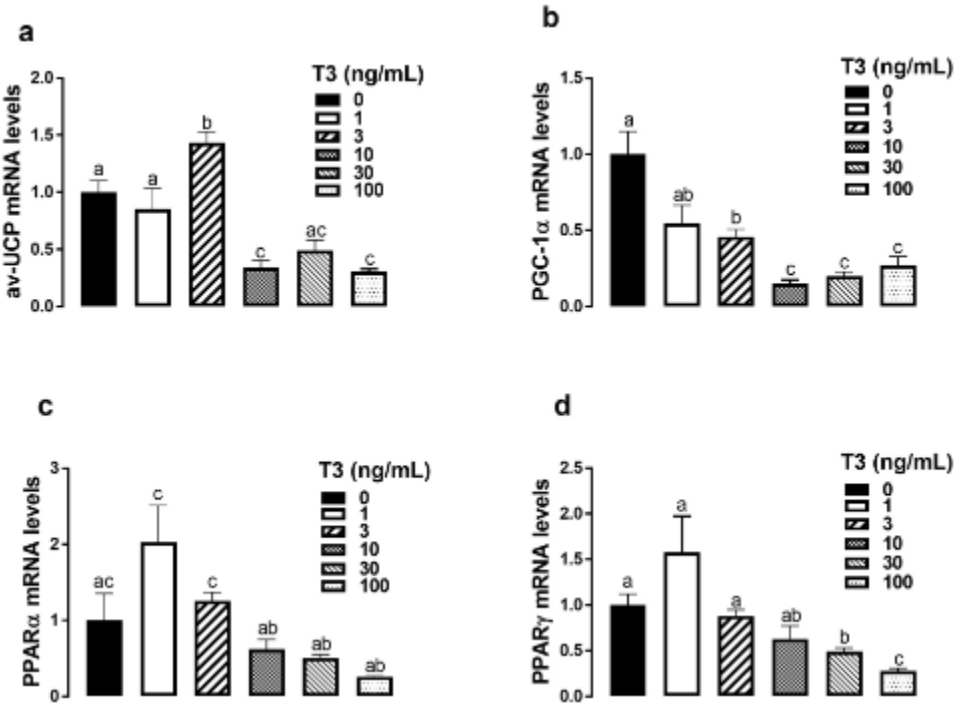

Figure 2. Effect of triiodothyronine (T3) on av-UCP gene expression and its related transcription factors **in QM7 cells**

QM7 cells were treated with different doses (1, 3,10, 30, and 100 ng/mL) of T3 for 24 h. Untreated cells were used as controls. Relative expression of av-UCP (a), PGC-1α(b), PPARα(c), and PPARγ(d) mRNA was determined by qPCR using 2-ΔΔCtmethods (Schmittgen and Livak, 2008). Data are presented as mean ± SEM (n = 9). Different letters indicate a significant difference at P< .05. av-UCP, avian uncoupling protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PPAR, peroxisome proliferator-activated receptor; QM7, quail myoblast 7.

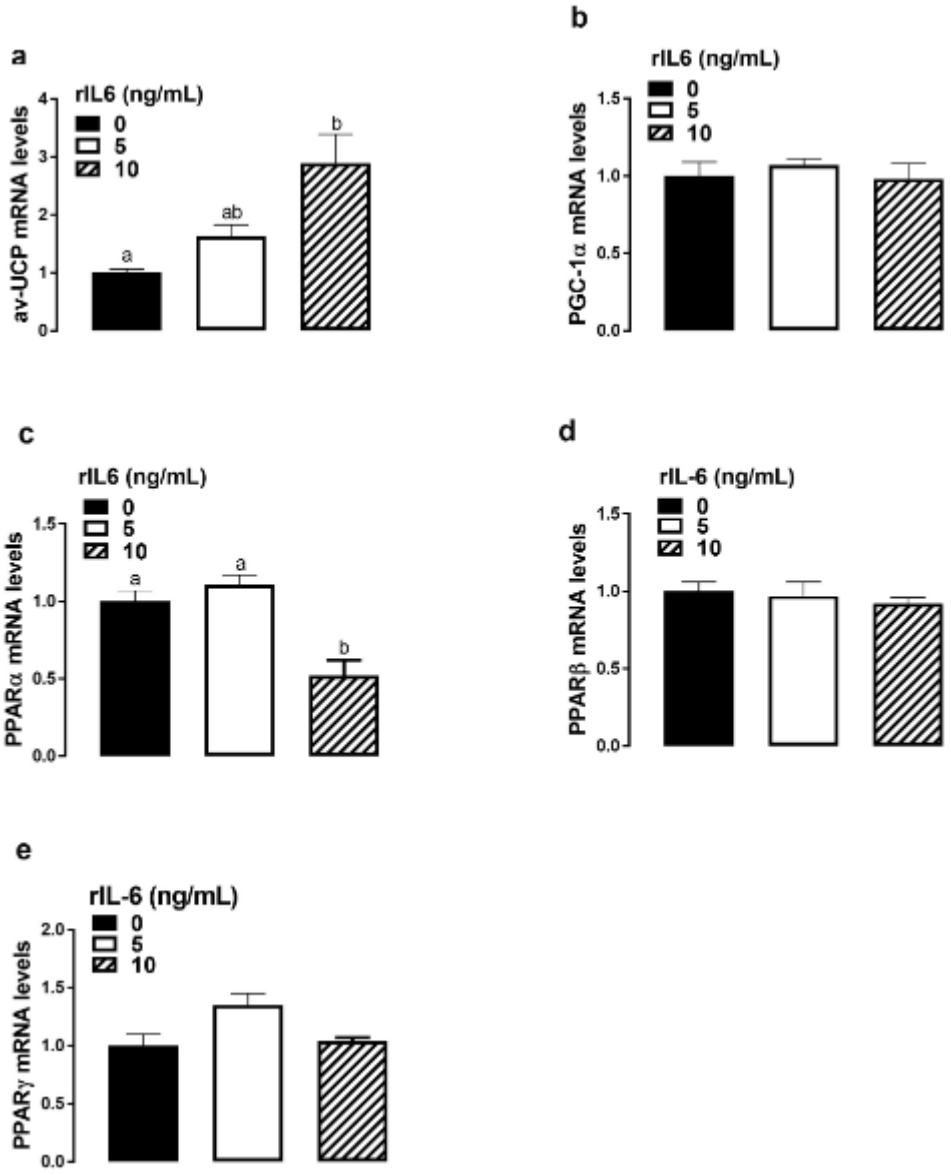

Figure 3. Effect of recombinant IL-6 on av-UCP gene expression and its related transcription factors in QM7 cells

QM7 cells were treated with 5 or 10 ng/mL) of IL-6 for24 h. Untreated cells were used as controls. Relative expression of av-UCP (a), PGC-1α(b), PPARα(c), PPARβ(d), and PPARγ(e) mRNA was determined by qPCR using 2-ΔΔCtmethods (Schmittgen and Livak, 2008). Data are presented as mean ± SEM (n = 9). Different letters indicate a significant difference at P< .05. av-

UCP, avian uncoupling protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PPAR, peroxisome proliferator-activated receptor;QM7, quail myoblast 7.

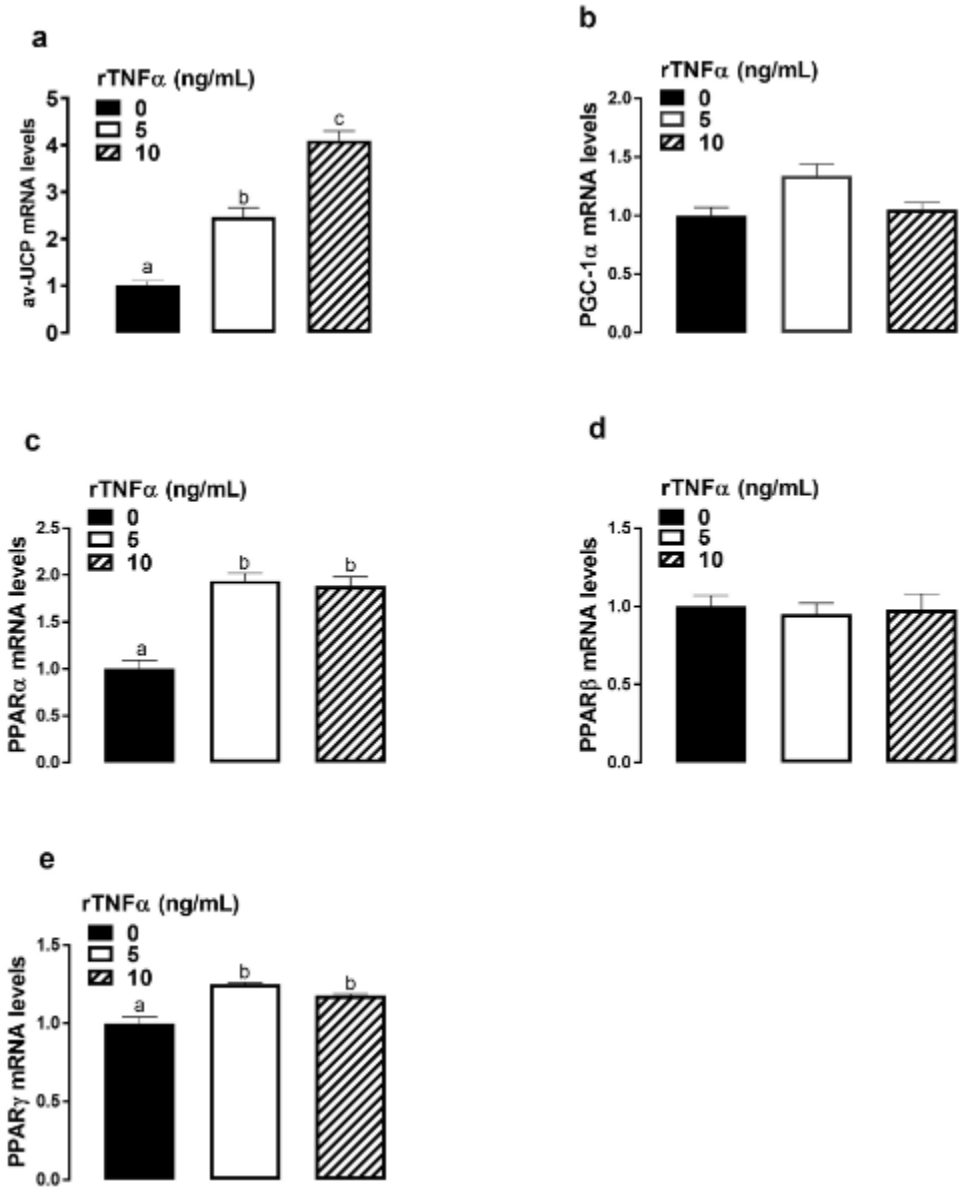

Figure 4. Effect of recombinant TNFα on av-UCP gene expression and its related regulators in QM7 cells

QM7 cells were treated with 5 or 10 ng/mL) of TNFαfor 24 h. Untreated cells were used as controls. Relative expression of av-UCP (a), PGC-1α(b), PPARα(c), PPARβ(d), and PPARγ(e) mRNA was determined by qPCR using 2-ΔΔCtmethods (Schmittgen and Livak, 2008). Data are presented as mean ± SEM (n = 9). Different letters indicate a significant difference atP< .05. av-UCP, avian uncoupling protein; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PPAR, peroxisome proliferator-activated receptor; QM7, quail myoblast 7.

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Chapter 12 – Overall Conclusion

BCO has proven to be a complex and multifaceted disorder, opening questions of the role bacteria play within a larger system and how cellular processes affect overall skeletal integrity and susceptibility. Overall, the modern broiler has proven to be an effective and high-yielding organism that, under the right conditions, can fall prey to BCO leading to bone attrition, inflammation, and bacterial infection. Mitochondrial dysfunction is evident based on gene expression of key mitochondrial regulators and machinery. Autophagy manipulation by known BCO isolates can affect the viability of bone cells and therefore alter cellular functions and potentially the balance of bone cell types within the growing bone. There is a clear, unique cyto(chemo)kine signature within the circulation and the local bone which points to pro-inflammatory pathways that can contribute to bone loss and have the ability to alter cellular viability. This profile also is the first to provide a potential non-invasive biomarker for BCO. Additionally, primary chondrocytes from modern broilers exhibit optimal phenotypes within a high-density culture around d3 to d7 and could offer a promising alternative to human cell line models currently used for *in vitro* modeling of BCO and other lameness issues. Visfatin and av-UCP are not only significantly upregulated in BCO-affected bone but are clearly hormonally regulated within avian cells. They provide a starting point to elucidate the mechanism behind abnormal lipid metabolism within BCO-affected bone. The molecular pathways involved in BCO etiology demonstrate the complex nature of skeletal homeostasis and its susceptibility to disease and infection. Through future investigations, the larger picture of systemic effects of or contributions to BCO could be defined leading to even more practical understandings of this animal welfare and production concern.