EFFECTS OF 20 (S)-HYDROXYCHOLESTEROL ON MODULATING THE MULTILINEAGE DIFFERENTIATION POTENTIAL OF CHICKEN COMPACT BONE-DERIVED MESENCHYMAL STEM CELLS AND DEVELOPING EMBRYOS OF BROILER CHICKEN

by

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ABSTRACT

Oxysterols are intermediates of large family 27-carbon cholesterol that are present in the circulation of human and animals. 20(S) –Hydroxycholesterol (20S) is the most potent naturally occurring oxysterol that has the capacity to promote osteogenesis and decrease adipogenesis in pluripotent mesenchymal stem cells (MSCs) derived from mammals. However, the effect of the 20S on chicken derived MSCs has not been studied yet. Understanding the effect of 20S oxysterol in multilineage differentiation potential of MSCs can identify 20S as a potential bioactive compound that can be useful for the poultry industry. MSCs isolation methodology was developed to isolate primary MSCs from the compact bone of day old broiler chicks. 20S oxysterol was subjected to treatment with isolated primary cells at passage 4 to understand the osteogenic, adipogenic, and myogenic differentiation of MSCs and pathway involved triggered by 20S in the process of differentiation. Further, *in ovo* injection was conducted in chicken embryo at the different time point of incubation to understand the effect of the 20s on

embryogenic development, the growth of chick, multilineage gene expression and hatchability *in vivo*. Compact bone derived MSCs showed all the characteristic such as multilineage differentiation potential, adherent to plastic, appropriate cell surface markers, and ability to form a colony to be considered as mesenchymal stem cells. 20S oxysterol induced pro-osteogenic, pro-myogenic and anti-adipogenic differentiation potential when subject to cBMSCs on confluency. 20S oxysterol exerted its osteoinductive and anti-adipogenic effects through activation of Hedgehog signaling pathway. 20S oxysterol also inducted Hedgehog signaling and osteoinductive genes at the different time point of harvest when injected into the embryo at 3, 7 and 18 day of incubation. However, hatchability, weight of hatched chicks, bone mineral density, bone mineral content was not different between the treatment groups. 20S could have a very important role in the regulation of lipid and osteogenic metabolism. Further studies need to be conducted to understand the details of the pathways involved in multilineage differentiation of MSCs exerted by 20S *in vivo* and to understand the possibility of using 20S as a bioactive compound *in ovo* for better chick quality and post hatch preformation efficiency.

INDEX WORDS: 20(S)-Hydroxycholesterol, Mesenchymal stem cells, *In ovo*, Embryo, Chicken

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DEDICATION

I dedicate this work to my parents, wife and my brother. Thank you for your unconditional love and support in every step. To my Mom, Rita Adhikari, for her encouragement, love, and support. To my dad, Janak Adhikari, who has always been an idol to me for hard work and determination to achieve what you dream. To my wife, Pratima Acharya Adhikari, for her unconditional love and being my best friend. Without her support, this work would have been simply impossible. To my brother, Rabin Adhikari, for his sincerity and always pushing me to the limits for getting things done.

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CHAPTER 1

INTRODUCTION

Oxysterols are intermediates of large family 27-carbon cholesterol that are present in the circulation of human and animals. Oxysterols may be derived from either enzymatic or non-enzymatic hydroxylase of cholesterol compound in mitochondria and endoplasmic reticulum or from dietary intake (Russell, 2000). The most abundant oxysterols found in human serum are 27, 24(s), 7β, 4α, 4β hydroxycholesterol (OHC). The concentration of oxysterol is very less compared to cholesterol but has significantly potent regulatory functions. Oxysterol has been reported as a potential bioactive compound in various physiological and pathological process such as cell differentiation, cholesterol efflux, lipoprotein metabolism, calcium uptake, apoptosis, and atherosclerosis (Kim, et al., 2007). Oxysterol is derived from the endogenous cellular cholesterol biosynthetic pathway and the same pathway is important in osteogenic differentiation of progenitor cells. Thus, oxysterol can have an important role in the regulation of lipid and osteogenic metabolism. 20(S) –Hydroxycholesterol (20S) is the most potent naturally occurring oxysterol that has the capacity to promote osteogenesis and decrease adipogenesis in pluripotent mesenchymal stem cells (MSCs) such as M2-104 and C3H10T1/2 embryonic fibroblast (Kha, et al., 2004). With the discovery of different pathway and receptors of 20S and its role to act as a potent regulator of lipid and osteogenic metabolism, novel interest in these compounds are evoked in biomedical research (Kha, et al., 2004). In 2006, Dwyer, et al., (2007), suggested that osteogenic effect of oxysterols in MSCs are exerted through activation of

Hedgehog signaling pathway(Hh). Consistently, it was reported that 20S oxysterol exerts antiadipogenic effects in MSCs through Hh signaling pathways (Kim, et al., 2007).

Hedgehog signaling pathway (Hh) play a key role in a variety of embryonic development, growth, and patterning of tissues, postnatal development and maintenance of tissue/ organ integrity and function, stem cell physiology, cancer and cardiovascular disease (Kim, et al., 2010). Hedgehog signaling begins with a hedgehog protein binding to a transmembrane protein Patched (Ptch) which removes the inhibitory effects on another transmembrane protein Smoothened (Smo) in Hh responsive cells. Smo activates the intracellular signaling cascade that results in activation of Gli transcription factors, which transcribe the Hh target genes (Gli-1 and Ptch). Previous experiments in mice reported that lacking Indian Hh resulted in the disorder of endochondral bone pattern and osteoblast formation and those mice lacking sonic hedgehog showed disorders in craniofacial bones, vertebral column and calcified ribs (Chiang et al., 2001; Chiang et al., 1996; St-Jacques et al., 1999). Studies have been conducted in human and mice MSCs to understand the effect of 20S on the adipogenic and osteogenic differentiation of MSCs. However, there is no study to understand the effect of oxysterol in osteogenic and adipogenic as well as embryonic development pattern in chicken MSCs.

Mesenchymal stem cells (MSCs) are pluripotent, plastic adherent non-hematopoietic cells initially derived from bone marrow which can differentiate into different cell types, for example, osteocytes, adipocytes, chondrocytes, and myocytes depending on the appropriate cellular cues. While bone marrow serves the most common supply of MSCs population, MSCs have also been isolated and characterized from several tissues including adipose tissue (Zuk, et al., 2001), blood vessels (Crisan, et al., 2008), bone marrow stem cells (Feyen, et al., 2016), skin (Toma et al., 2005), compact bone (Zhu, et al., 2010), dental tissues (Lei, et al., 2014), menstrual blood

(Ulrich, et al., 2013), amniotic fluid (Kim, et al., 2014), and cord blood. MSCs has been generated from bone marrow of different animal species such as pigs (Feyen, et al., 2016), mouse (Nadri and Soleimani, 2007), chickens (Bai, et al., 2013; Khatri, et al., 2009), rabbit (Wee, et al., 2013), humans (Kar, et al., 2016; Li, et al., 2016). One of the most important drawbacks described while isolating MSCs from bone marrow is the contamination of blood cells. To reduce blood cell contamination, some of the techniques were used such as centrifugation of bone marrow with the cell suspension, use of ficole to remove the contaminating blood cells, and use of the anti-CD34 antibody for positive selection of MSCs. All of these methods are employed to obtain a homologous population of bone marrow derived MSCs often times includes contamination of another cell type (blood cells) and also incur an extra cost and time to isolate the cells. Using cell sorting techniques reduces osteogenic potential and clonogenicity compared to unsorted cells (Van Vlasselaer et al., 1994). Exposure of MSCs to cytotoxic materials resulted in differentiation of failure into other cell lines (Falla et al., 1993). The positive selection method is providing a high proliferation rate, however, this method is not feasible in isolation of MSCs in all species due to a limitation in surface markers availability. We developed a protocol to isolate and grow MSCs from the compact bone of day old chicken. Our method of isolation of MSCs from compact bones reduces the changes of hematopoietic contamination. It is a reliable, user-friendly and a low-cost protocol for isolation of MSCs. The newly isolated MSCs were examined to meet the criteria as described by the Mesenchymal and Tissue Stem Cell Committee of the international Society for Cellular Therapy (ISCT) (Dominici et al., 2006). *In vitro* primary, MSCs derived from chicken compact bone can be used to understand the effect of 20S on the osteogenic, adipogenic, and myogenic differentiation in a chicken model.

Rapid growth and development of body weight and a decrease in market age, of modern day broiler chicken, has increased the importance of supplementing adequate nutrition even before hatch. Avian embryo relies on the nutrient deposited in the egg from the hen during her lay for successful embryogenesis and growth of embryo (Cherian, 2015). Nutritional deficiencies during formation of egg could have a significant impact on the development of the embryo and post hatch chick performance. One unique way of introducing nutrients to the incubating embryo is through *in ovo* injection. Moreover, supplementation of nutrient in early embryo development could offer a powerful tool to promote the health of hatched chicks and boost early growth and development. (Foye et al., 2006). *In ovo* feeding of incubating eggs with dietary nutrients such as amino acids, carbohydrate, minerals and vitamins, stimulants, and hormones has been reported to enhance the growth and development of chicken embryo, promotes gut health, boost immune response, and post-hatch development and performance (Lee, et al., 2014; Ohta, et al., 2001; Pruszynska-Oszmalek, et al., 2015; Tako, et al., 2004; Zhai, et al., 2011b). Use of 20S in developing embryo could help to understand the adipogenic, osteogenic and myogenic effect in developing embryos. *In ovo* feeding of 20S also could help to promote skeletal, muscular growth in broilers thus increasing hatchability, body composition, boost production efficiency and reducing skeletal issues post hatch.

Also, understanding the embryonic stages of development and timing of the injection is very important to maximize the effect of the injected compounds in the development of chick, increase hatchability, and chick quality post hatch. Ohta and Kidd (2001) demonstrated that *in ovo* injection of amino acids at day 7 of incubation in the yolk sac and extraembryonic coelom had higher hatchability compared to eggs injected in the chorioallantoic membrane and amniotic cavity. When amino acids were injected *in ovo* at day 7 in the yolk sac, hatchability was not

affected and body weight of the chicks was increased relative to the egg weight (Ohta et al., 1999). Embryos that received the amino acids at 14th day of incubation had higher hatchability and improved post-hatch growth (Kadam, et al., 2008). Furthermore, *in ovo* injection study at the different time point of the incubation can help to understand the effect of 20S in development and growth of the chicken embryo and post hatch. Understanding the differentiation potential and functional pathway of 20S using cBMSCs and understanding the developmental effects on chick embryo can help to access the potential use of 20S in avian species and reduce several welfare problems (skeletal and leg problems in broilers, tibial dyschondroplasia, angular bone,) and economic problems (woody breast in broilers, high fat deposition broilers and layers, leg and keel bone breakage in layers and broilers) in poultry industry.

The main objectives of this study were:

1. To establish a chicken compact bone-derived mesenchymal stem cell model that can be used to understand the effect of different bioactive compounds on chicken models.
2. To understand the effect of 20S on multilineage differentiation of chicken compact bone-derived mesenchymal stem cells and the mechanism through which 20S affects the differentiation.
3. To understand the effect of *in ovo* injection of 20S at different time points on skeletal embryogenesis, hatchability, and expression of multilineage differentiation genes at hatch.

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CHAPTER 2

LITERATURE REVIEW

Oxysterols are intermediates oxidized derivatives of large family 27-carbon cholesterol or by-products of the cholesterol biosynthetic process that are present in the circulation of human and animals with multiple biological activities. Oxysterols were first identified as autoxidation products of cholesterol and were perceived as a step of biotransformation of cholesterol for elimination (Figure 2.1). Later it was found that oxysterols were also intermediates of pregnenolone and steroid hormones synthesis and had potential effects in animals (Luu-The, 2013). Initially, oxysterols were thought of as inactive metabolic intermediates. However, the involvement of oxysterols in cholesterol homeostasis, their role as ligands to nuclear and G protein-coupled receptors and their potential as easily measured biomarkers of the disease has enhanced interest in their biosynthesis, metabolism, and measurement (Luu-The, 2013).

Oxysterols may be derived from either enzymatic or non-enzymatic hydroxylase of cholesterol compound in mitochondria and endoplasmic reticulum or from dietary intake (Russell, 2000). In general, biological oxysterols fall into two categories. Biologically available oxysterols are either non-enzymatically oxygenated on the sterol ring, mainly at the 7 position or are enzymatically oxygenated on the side chain such as 24S-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC) and 27-hydroxycholesterol (27HC), 20(S) hydroxycholesterol (20S), and 22(S) hydroxycholesterol (Figure 2.2). Non-enzymatic oxidation occurs by direct addition of hydroxyl or keto group on the C-7 carbon of the B ring of the sterol. Cholesterol hydroperoxides are formed in the early stage of non-enzymatic oxidation of cholesterols, which are further

processed to 7-oxygenated products by non-enzymatic lipid oxidation as well as enzymatic oxidation (Brown et al., 1997). In presence of transition metals, cholesterol hydroperoxides can further transit non-enzymatically to 7αβ-alkoxy radicals, which undergo further reactions to generate 7a/b-hydroxycholesterols and 7-ketocholesterol. 7a/b-hydroperoxycholesterol, 7-ketocholesterol, and 7a/b-hydroxycholesterol are the major non-enzymatically generated oxysterols present in the tissues (Lyons et al., 1999). Enzymatic side chain hydroxylation is generated mainly by separate enzymes such as sterol 27-hydroxylase (CYP27A1) and cholesterol 24-hydroxylase (CYP46A1), and cholesterol 25-hydroxylase (25-HC) (Russell et al., 2009). CYP27A1 is a mitochondrial P450 enzyme expressed highly in liver and macrophages. This enzyme acts as a catalyst in the first step of bile acid synthesis by addition of hydroxyl group to the side chain of cholesterol forming 27-hydroxycholesterol oxysterols (Bjorkhem, 2007; Gill et al., 2008; Javitt, 2002). CYP46A1 is also a P450 enzyme found in the endoplasmic reticulum and is highly expressed in brain and retina which catalyze the synthesis of 24 (S) hydroxycholesterol (Lund et al., 1998). 25HC belongs to a larger family of transmembrane enzymes that catalyze oxidations using a di-iron cofactor in the catalytic site instead of a heme iron center (Lund et al., 1998). It is expressed in very low amounts in membranes of endoplasmic reticulum and Golgi. This enzyme acts as the regulator of the sterol regulatory element binding protein pathway for cholesterol-dependent transcriptional regulation. 20(S) and 22(R)-hydroxycholesterol form a special case and no single hydroxylase produces either of them as a stable product. These oxysterols are produced as intermediates in the conversation of cholesterol to pregnenolone through the action of the cytochrome P450scc side chain cleavage enzyme (known as CYP11A1 in humans) in the adrenal glands (Gill et al., 2008; Javitt, 2002). 20(S) are described as one of the most potent oxysterols in the animal and is found in rat brains and placenta (Lin et al., 2003).

Oxysterols also arise *in vivo* or during food processing through enzymatic, lipid peroxide, or divalent cation-induced oxidative process, or cholesterol autoxidation. Most of the oxysterols produced through cholesterol auto-oxidation include 7-ketocholesterol and 7β oxysterols. The most abundant oxysterols found in human serum are 27, 24(S), 7β, 4α, 4β hydroxycholesterol (Olkkonen et al., 2012).

The concentration of oxysterol is relatively low compared to cholesterol but it has very potent regulatory functions. Oxysterol has been reported to be a potential bioactive compound in various physiological and pathological process such as cell differentiation, cholesterol efflux, lipoprotein metabolism, calcium uptake, apoptosis, and atherosclerosis (Kim et al., 2007; Lyons et al., 1999). Oxysterol is derived from the endogenous cellular cholesterol biosynthetic pathway and the same pathway is important in osteogenic differentiation of progenitor cells. Thus, oxysterols are believed to be novel endogenous regulators of gene expression in lipid and osteogenic differentiation with a key role in the regulation of metabolism, differentiation, and inflammation process in animals. With the discovery of different pathway and receptors of oxysterol and its role to act as a potent regulator of lipid and osteogenic metabolism, novel interest in these compounds has evoked biomedical research (Kha et al., 2004).

Effect of oxysterol in transcription control of Liver X receptors

Cholesterol and its oxysterol derivatives are important constituents of cell membrane function and act as an intermediate of several biosynthetic pathways. Endogenous oxysterols activate the transcription of nuclear receptor LXRα & β (Janowski et al., 1996). The nuclear receptors LXRα & β are the important regulators of genes involved in lipid metabolism (Fu et al., 2001). LXRα & β are mostly affected by 24S, 25, 24(S), 22(R), 20(S), and 27-Hydroxycholesterol (Fu et al., 2001; Janowski et al., 1996; Lehmann et al., 1997). LXRα is

highly expressed in liver, adipose tissue, and macrophages, whereas LXRβ is expressed in all the tissues examined. These proteins contain a zinc finger-like DNA-binding domain and ligand binding domain that accommodates specific small lipophilic molecules (Tontonoz and Mangelsdorf, 2003). LXRs has been reported to play a central role in sterol absorption in the intestine, bile acid synthesis, biliary neutral sterol secretion, hepatic lipogenesis, and synthesis of high-density lipoproteins, suppressors of inflammatory gene expression in macrophages and influence the immune regulations in macrophages (Joseph et al., 2003; Quinet et al., 2006). In an *in vitro* study conducted in LXRα-/- and LXRβ -/- mice, macrophages suggested an equivalent role for LXRα and LXRβ in the regulation of ABCA1 and SREBP-1c gene expression which derived apoA1-dependent cholesterol metabolism from macrophages (Quinet et al., 2006). Mice lacking the oxysterol receptor LXRα lost the ability to respond normally to dietary cholesterol and were unable to induce transcription of genes encoding the cholesterol 7 alpha-hydroxylase, the rate limiting enzyme in bile acids (Peet et al., 1998). Overexpression of oxysterol catabolic enzyme, cholesterol sulfotransferase, was able to inactivate LXR signaling in several cultured mammalian cell lines *in vitro* but did not alter the receptor response to the nonsterol agonist T0901317. Moreover, 24S-hydroxycholesterl, 25-hydroxycholesterol, and 27-hydroxycholesterol respond to dietary T090317 by inducing LXR target genes in the liver of the triple -knockout mice deficient in the biosynthesis of three oxysterol ligands of LXRs but showed impaired responses to dietary cholesterol (Chen et al., 2007). On the other hand, in a transgenic mouse model with human cholesterol 24-hydroxylase (the enzyme responsible for the formation of 24S hydroxycholesterol), production of 24S oxysterol was significantly increased in cerebral, plasma, biliary and fecal oxysterol but did not affect the activation of LXR target genes in the brain or liver. Despite the fact that 24S oxysterol is one of the most efficient endogenous LXR agonist

known, it did not show as a critical activator of target genes to the nuclear receptor *in vivo* in brain and liver (Shafaati et al., 2011).

Effect of oxysterol in transcription control of SREBP

Sterol regulatory element binding protein (SREBPs) have been established as lipid synthetic transcription factors for cholesterol, oxysterols, and fatty acid synthesis. SREBP activates the expression of numerous genes participating in the metabolism of mostly lipids and some glucose. SREBP family members, SREPB-1 and SREBP-2 are synthesized fat membrane protein in the endoplasmic reticulum. SREBP-1 is mostly abundant in the liver and adrenal glands whereas SREBP -2 is ubiquitously present. SREBP-2 is considered to be deeply involved in the regulation of cholesterol metabolism. Sterol metabolism is largely governed by SREBP-2 and certain metabolites, including cholesterol, 24, 25-dihydrolanosterol and oxysterols that are produced in the synthetic pathway which directly binds regulatory proteins, changing their activities and thereby regulating sterol metabolism (Sato, 2010). Both SREBP forms a complex with another endoplasmic reticulum membrane protein SREBP-cleavage-activating protein (SCAP) which regulates the intercellular localization and proteolytic maturation of SREBPs.

When there is a lower concentration of cholesterol, SREBP-SCAB complex are transported by a coat protein complex II-dependent mechanism to the Golgi, where SREBPs are proteolytically processed to release a basic helix-loop-helix leucine zipper transcription factor, which enters the nucleus and binds to sterol regulatory elements in the promoters of target genes. However, there is higher sterol in the cells, SCAP sensed the cholesterol in the endoplasmic reticulum membrane and interacts with Insulin -induced gene protein and the SREBP-SACP complex retains in the ER. Cholesterol and various oxysterols mainly 25-OH influence the transport of SREBP to Golgi complex. 25-HC is more potent that cholesterol in eliciting SCAP

binding to the Insigs, but 25-HC does not cause a detectable conformational change in the SCAP. 25-HC works directly through a putative 25-HC sensor protein to elicits SCAP-Insig binging (Adams et al., 2004).

Oxysterol in modulation of Hedge Hog signaling

Hh signaling

Hedgehog signaling pathway is a key regulatory signaling pathway in the development, cell proliferation, embryogenesis and stem cell maintenance (Gorojankina, 2016). Hh signaling is tightly regulated by various factors and is highly conserved. Elevated Hh signaling can cause various cancers including medulloblastoma and basal cell carcinoma whereas an insufficiency of Hh signaling in the animals can lead to development defects, such as cyclopia, holoprosencephaly (Taipale and Beachy, 2001; Xie et al., 1998). The first member of the Hh pathway was identified in a series of genetics screens on the fruit fly *Drosophila melanogaster* in 1908's by Christiane Nusslein-Volhard and Eric Wieschaus (Nusslein-Volhard and Wieschaus, 1980). During genetic screening that were based on chemically induced random mutations, they identified more than 50 genes that were involved in the embryonic development. One of the mutations in the gene that resulted in a distinct phenotype of short larva where ventral cuticular denticles looked like a dense lawn compared to some distinctly segmented bands in the wild type was named as Hh (Nusslein-Volhard and Wieschaus, 1980). The Hh gene is also highly conserved in vertebrates and three distinct Hh homologous were identified Indian Hedgehog, Desert Hedgehog, and Sonic Hedgehog (Echelard et al., 1993a). Other important players in Hh signaling pathway are Patched, Smoothened, and three mammalian Ci related proteins named as Glioma-associated oncogenes 1-3(Gli1, Gli2, and Gli3) (Ingham et al., 1991; Mimeault and Batra, 2010; Ogden et al., 2008; van den Heuvel and Ingham, 1996). Hh orthologous from

vertebrates -including *Mus Musculus, Danio rerio,* and *Gallus gallus* were cloned in 1993 and cloning of first human Hh genes were reported in 1994 (Echelard et al., 1993b; Krauss et al., 1993; Marigo et al., 1995; Riddle et al., 1993; Roelink et al., 1994). Auto processing and lipid modification of Hh protein as they enter the secretory pathway produce a signaling peptide. The Hh protein is duly modified at N and C terminal by palmitoyl and cholesterol adducts (Buglino and Resh, 2012). The primary role of cholesterol in the modification is Hh trafficking and movement and palmitoylation is Hh signaling (Buglino and Resh, 2012; Jeong and McMahon, 2002). In mammals, Sonic Hh is most broadly expressed such as node, notochord, left-right, dorso-ventral axes, limb bud patterning and later in development during organogenesis (Johnson et al., 1994; Meyer and Roelink, 2003; Pagan-Westphal and Tabin, 1998; Riddle et al., 1993), Indian hedgehog is primarily involved in bone differentiation (St-Jacques et al., 1998; Vortkamp et al., 1991) and Desert Hedgehog is involved in gonad differentiation (Bitgood et al., 1996; Wijgerde et al., 2005). Hh pathway is activated by stoichiometric binding of Hh ligand to Patched (Ptch), a 12 transmembrane protein receptor, which releases its antagonist action towards Smoothened (Smo), a 7 span transmembrane protein (Figure 2.3). Inactivation of Patch by Hh protein leads to activation of Smo, which results in the subsequent activation of cytoplasmic transactivation factors, the Ci protein in Drosophila, and the homologous Gli proteins in the mammals (Lum and Beachy, 2004; Taipale et al., 2002; Varjosalo et al., 2006; Varjosalo and Taipale, 2007).

Hh pathway in development of embryo

Hh proteins are key regulators in controlling multiple developmental processes from the development of a fertilized egg into an adult. Fertilized zygote is totipotent and has the capacity to develop into every type of cell to form an adult organism. Each cell type maintains a pattern of

gene expression and is regulated by transcription factors and signaling proteins. Pattern formation is the process of development of equivalent cells into more complex structures of cell fate and helps in determining the lineage of the cells to develop as an organ. Pattern formation is controlled by signaling of the adjacent cells which forms repetitive segments and gradients of secreted signaling ligands, called morphogens. The first insight of the role of Hh signaling was studied in development and involvement in the segmentation of patterns of fruit fly larva and in the development of limb pattern and fly appendages (Basler and Struhl, 1994; Burke and Basler, 1997). In *drosophila*, Hh is required for the development of the wings and legs and most importantly in the patterning of the embryonic segments (Ingham and McMahon, 2001) which helps in the formation of posterior/anterior and dorsal-ventral axis in the early embryo development (Basler and Struhl, 1994). Mammalian Hh proteins also have a critical role in development stages of the embryo and have an important function in the adult. Shh is most broadly expressed and is widely considered to play a role in patterning of several parts of embryo such as patterning of left–right and dorsal-ventral axis of the embryo, midline of node, notochord, floor plates, neural tube, development of limbs, gut and bone growth (Chiang et al., 2001; Chiang et al., 1996; Riddle et al., 1993; Varjosalo and Taipale, 2007). The role of Hh signaling in development has been shown in figure 2.4. Shh was highly expressed in the zone of polarizing activity (ZPA) of the limb bud during embryo development and was critically involved in the patterning of the distal limbs (Chang et al., 1994; Johnson et al., 1994; Riddle et al., 1993). Transplantation of the part of the tissue of ZPA from the posterior side to the anterior side of the limb bud triggered the digit development pattern from both side of the limb bud, resulting in mirror image duplication of the digits (Hill, 2007; Tickle, 1981; Tickle, 2006). Shh is also reported to be required for lungs formation and correct branching morphogenesis of the

lungs (Chuang and McMahon, 2003; Pepicelli et al., 1998). Deletion of *Shh* leads to cyclopia, and defects in ventral neural tube, somite, and foregut patterning severe distal limb malformation, absence of vertebrae and most of the ribs, and failure of lung branching (Chiang et al., 1996; Litingtung et al., 1998) (Taipale and Beachy, 2001).

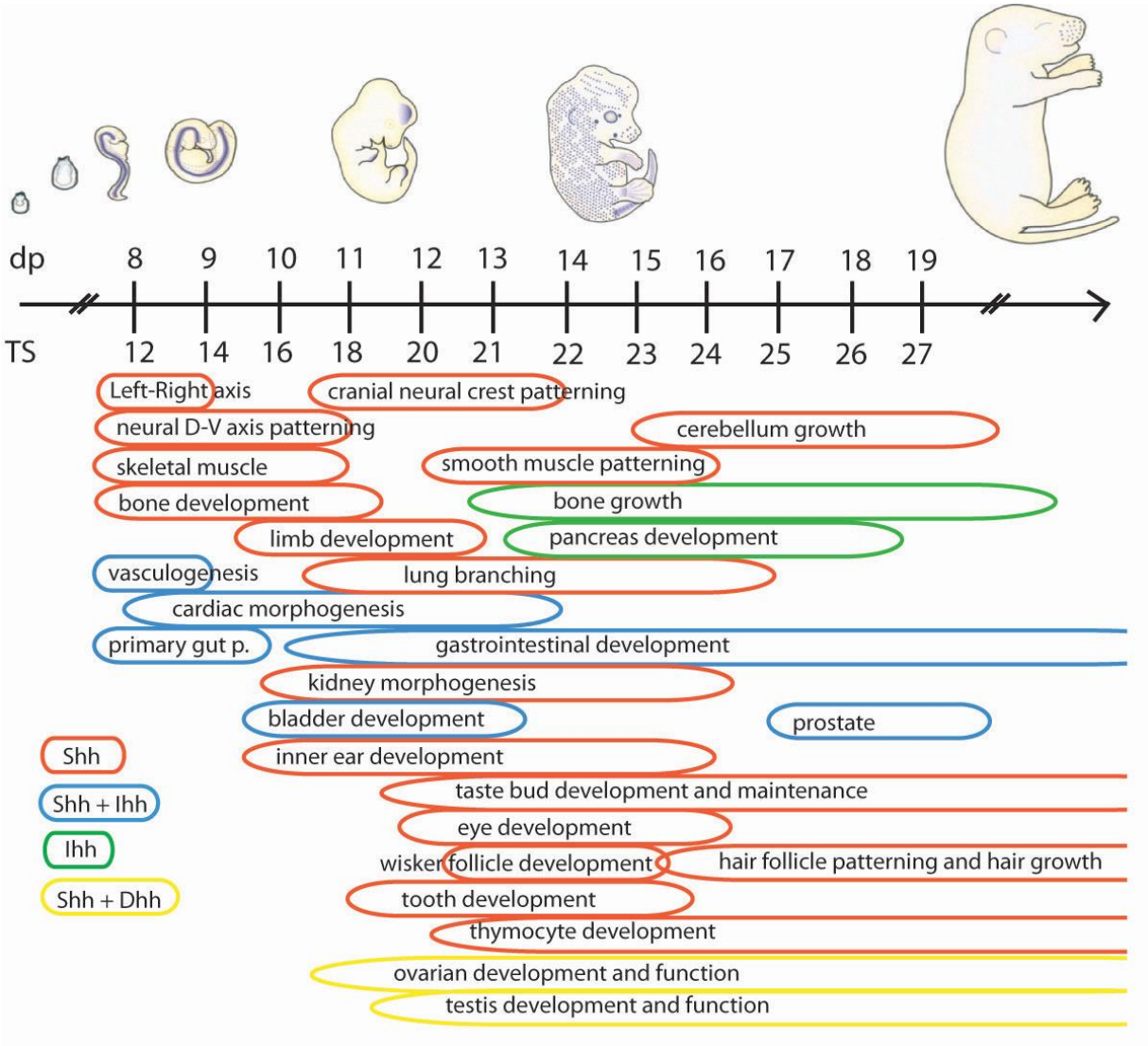

Figure 2.4: Control of mouse development by Shh signaling pathway. The approximate development stage is indicated by dp and Theiler stage (TS). Bars show the approximate embryonic stages and when the different Hh signaling controls the developmental process. Red is

for Shh, Blue for Shh+Ihh, green for Ihh and yellow for Shh and Dhh. Figure adopted from (Varjosalo and taipale, 2007).

The role of oxysterol in Hh pathway and MSCs:

Oxysterol has emerged as an important molecule that is capable of regulating the lineage-specific differentiation of mesenchymal stem cells. The study suggested that the products of cholesterol biosynthetic pathway are important in the proper development of MSCs into functional osteoblastic cells capable of forming a mineralized matrix (Parhami et al., 2002). Studies have indicated that oxysterols can promote the osteogenic, chondrogenic, and adipogenic differentiation in MSCS (Parhami et al., 2002) (Kha et al., 2004). In a study with M2-10B4 pluripotent marrow stromal cell line, MC3T3-E1 mouse preosteoblastic cell line, C3H-10T1/2 mouse pluripotent embryonic fibroblast cells, and primary marrow stromal cells, a combination of 22S and 20S hydroxycholesterols were able induce osteogenic differentiation including induced ALP activity and osteocalcin mRNA expression and mineralization (Kha et al., 2004). Individual or combination of both oxysterols decreased markers of adipogenic differentiation including lipoprotein lipase, FABP4 mRNA expression and adipocyte formation (Kha et al., 2004). 20S and 22S were reported to exert osteoinductive effects in MSCs through activation of Hh signaling pathway. This was demonstrated in M2-10B4, C3H10T1/2 cells and Smo-/- mouse embryonic fibroblast cells where oxysterol-induced expression of Hh target genes Gli-1 and Patched, oxysterol-induced activation of a luciferase reporter driven by a multimerized Gli-responsive element, osteogenic effects of oxysterol was inhibited by hedgehog signaling pathway inhibitor, cyclopamine and smoothened-/- mouse embryonic fibroblast was unresponsive to treatment of oxysterol (Dwyer et al., 2007). Furthermore, in the same study oxysterols did not compete with fluorescently labeled cyclopamine, BODIPY-cyclopamine, for direct binding to

Smoothened which demonstrate that oxysterols stimulate hedgehog pathway activity by indirectly activating the seven-transmembrane pathway component Smoothened (Dwyer et al., 2007). In another study with M2-10B4 MSCs line, 20S completely inhibited the adipocyte formation and PPARγ, and c/EBPα mRNA expression induced by troglitazone in M2-10B4 MSCs through Hh-dependent mechanism (Kim et al., 2007). Similarly, structural analogs of 20S, Oxy34 and Oxy49 induced osteogenic of bone marrow stromal cells (MSCs) through activation of Hedgehog signaling and induced expression of RunX2, Osterix, ALP, BSP, and osteocalcin mRNA expression. Treatment of MSCs with those oxysterols also inhibited adipogenic differentiation of bone marrow stromal cells and reduced mRNA expression of adipogenic genes such as PPARγ, LPL, FABP4, and inhibited the formation of adipocytes (Johnson et al., 2011). Such oxysterol also showed comparable osteogenic efficacy to BMP2/collagen implants in posterolateral intertransverse process rat spinal fusion model suggesting that they could show trabecular and cortical bone induction *in vivo*. Similar results have been reported with other structural analogs of oxysterol such as Oxy 133 which induced the osteogenic effect *in vitro* and *in vivo* (Montgomery et al., 2014). The Hh signaling pathway plays an important modulating role in the patterning of embryos, process, and regulatory functions during the development of vertebrate and invertebrate organisms as well as in modulating the stem cell differentiation process including osteogenic and adipogenic differentiation (McMahon et al., 2003; Plaisant et al., 2009). Oxysterols are the natural molecules that can modulate the activity of Smo allosterically by binding to the membrane protein (Nachtergaele et al., 2013; Nedelcu et al., 2013). In addition to activation of Hh signaling pathway, oxysterol-induced osteogenic differentiation in MSCs through a Wnt signaling -related, Dkk-1 inhibitable mechanism, along with selective induced expression of Wnt target genes through a non-canonical mechanism

(Amantea et al., 2008). Furthermore, in a study with M2-10B4 MSCs, 20S significantly induced HES-1, HEY-1, and HEY-2 mRNA expression compared to untreated cells, which was similar to the observation made when M2-10B4 MSCs cells were treated with Shh (Kim et al., 2010). In addition to the previous result, the specific Hh pathway inhibitor cyclopamine blocked the 20S-induced Notch target gene expression and HES-1 and HEY-1 siRNA transfection significantly inhibited 20S-induced osteogenic genes, suggesting the pro-osteogenic effects of 20S are partly regulated by HES-1 and HEY-1through non-canonical Notch signaling (Kim et al., 2010). These findings established a novel role of oxysterols as regulators of adipogenic and osteogenic differentiation of MSCs as well as in mammalian embryonic and adult development through Hh signaling pathway. Hh signaling has emerged as an important modulator of stem cell differentiation processes, including adipogenic differentiation, and has been shown to play crucial roles in the developmental processes of both vertebrates and invertebrates.

Mesenchymal Stem cells:

MSCs are pluripotent, plastic adherent non-hematopoietic cells which can be isolated from several species, including human, mouse, rat, dog, cat, sheep, and chickens. MSCs were first identified in the bone marrow indicating that the bone marrow contains heterogeneous population, including hematopoietic cells, reticular cells and endosteum elements which undergo differentiation after transplantation (Friedenstein et al., 1966). Caplan in 1991 introduced the term MSCs to designate the chick limb mesenchymal cells from which cartilage and bones were derived depending on specific cues (Caplan, 1991). While bone marrow serves the most common supply of MSCs population, MSCs have also been isolated and characterized from several tissues and adult organs (Meirelles et al., 2006), such as adipose tissue (Zuk et al., 2001), blood vessels (Crisan et al., 2008), bone marrow stem cells (Feyen et al., 2016), skin (Toma et al., 2005),

compact bone (Zhu et al., 2010), dental tissues (Lei et al., 2014), menstrual blood (Ulrich et al., 2013), amniotic fluid (Kim et al., 2014), and cord blood (Zhang et al., 2011). MSCs have also been isolated from different animal models such as canine (Baustian et al., 2015), cat (Martin et al., 2002), bovine (Sampaio et al., 2015), horse (Kisiday et al., 2013), poultry (Khatri et al., 2009), and mice (Liu et al., 2015) .

Due to various isolation techniques, difference in claims on expression of cell markers, variability in different animals/tissues, the mesenchymal and tissue stem cell committee of the international society for cellular therapy (ISCT) has defined these 3 minimal criteria for MSCs (Dominici et al., 2006): a) MSCs should have the capacity to adhere to plastic surface under standard culture medium. b) MSCs should have a multilineage property; i.e. able to differentiate into chondroblast, osteoblast, and adipocytes under their specific differentiating medium. c) Cells should maintain specific surface antigen, must have a positive expression of CD105, CD73, CD90 markers and lack expression of CD45, CD34, CD14, CD11b, and CD19 surface markers.

Easy isolation techniques, readily available, minimum manipulation, less ethical issues, and ease of expansion has made MSCs an attractive model for cellular therapy application. MSCs have proven to act in a broad range of immunomodulatory activities and displayed anti-inflammatory, tissue regeneration, proliferative and chemotactic properties with various *in vitro* and *in vivo* application. While initial studies using MSCs were focused on tissue repair and regenerative medicine, the discovery of immune –modulating mechanism of MSCs has prompted their use in immune disorders, cardiovascular diseases, graft-versus-host-disease, and chronic inflammatory autoimmune diseases. However, a concrete mode of action and defined cellular pathway has not been summarized yet. MSCs have been used as a potential treatment strategy for regeneration of damaged tissues of joints, cardiovascular diseases, injury in cartilage, bone,

nerves, and spinal cord. Injection to the location of injury and systemic infusion of MSCs has been of interest. However, effective strategy to implement MSCs therapy with high efficiency and engraftment has not been a particular success. Some of the questions regarding MSC trafficking are: Can a host MSCs travel and home to ischemic tissues through mobilizing in the blood? Will MSCs transport to sites where there are ischemic tissues and areas like joints and tendons? Do we have to place MSCs directly in the ischemic and inflammatory sites or will be able to migrate itself? If so what time and inflammatory stages, does the MSCs must be infused? Numerous reports have been published on method isolation techniques, primary organs of MSCs isolation, specific cell surface characteristic in different passage, proliferation and differentiation of cells, storage condition between different research groups, site and methods of treatment, timing of treatment on different disease condition, individual variation between diseased animals and its immunogenic property (Aliborzi et al., 2016; Casado-Diaz et al., 2016; Dominici et al., 2006b; Kumar et al., 2016; Lee et al., 2014; Zhang et al., 2004). This makes MSCs a highly promising research area for future therapeutic potential in regenerative medicine.

Using animal models for understanding differences in morphology, colony formation, growth rates, factors involved, specific markers and differentiation potential between cells of different model animals have always helped scientist to find a new direction for biomedical research. Murine, canine and porcine models have been widely used in research to evaluate the effect of MSCs on regenerative medicine. Chicken derived MSCs can be a good model to stem cell biologist because chicken derived MSCs can be easily used/injected/grafted in chicken embryos that are easy for manipulation and maintenance. Chicken can be a suitable model to understand patterns of molecular signaling after stem cells implantation, cell-cell interactions and cell-host interactions between the grafted and developing cells/tissues of developing embryo and

in the adult animal. Use of chicken MSCs shows promise in use of agriculture traits, biomedicine, and xenotransplantation. The avian industry itself also possess a huge potential in drug discovery and serving welfare needs of the poultry industry which is growing as the biggest meat/protein supplier in the world. Several reports have been published about isolation protocol and methods from different development stage and tissue of chickens, yet a thorough comparison of fundamental differences in isolation, characteristics and global molecular signature in chicken driven MSCs is lacking.

Source and culture conditions for isolation of chicken Mesenchymal Stem Cells (cMSCs)

Chicken MSCs were first isolated from the bone marrow of femur bones of 1-14 day old chickens (Khatri et al., 2009). Cells were also collected from the femur of 12-day old chicken embryo and the tibia of 30 to 60-day old chicken (Bai et al., 2013a; Bhuvanalakshmi et al., 2014). Since MSCs in the bone marrow represents a minor fraction of the cell population, one disadvantage reported in the isolation of MSCs from bone marrow is the possible contamination with the hematopoietic cells leading to a heterogeneous population of cells. Numerous techniques are reported to overcome and isolate the homogeneous population of cells from bone marrow. To reduce blood cell contamination and purify MSCs isolation various purification techniques have been reported such as, preferential attachment to culture plastic density gradient centrifugation (Yamamoto et al., 2015), use of ficole to filter the blood cells, antibody-based cells sorting (El-Sayed et al., 2015; Van Vlasselaer et al., 1994), low and high density culture technique (Eslaminejad and Nadri, 2009), negative selection technique (Baddoo et al., 2003; Dumas et al., 2008), frequent media change (Soleimani and Nadri, 2009), and positive selection method (Nadri and Soleimani, 2007). These methods are employed to obtain a homologous population of bone marrow derived MSCs. However, some purifying techniques could result in a

reduced capacity of MSCs to proliferate, may not be replicated in other strain or species (Phinney et al., 1999; Sun et al., 2003), and incurred extra cost and time to isolate the cells (Soleimani and Nadri, 2009). Osteogenic cells sorted out from the bone marrow of 5-fluorouracil treated mice lost their capacity to synthesize bone proteins and the ability to mineralized matrix upon subcultivation (Van Vlasselaer et al., 1994). Isolation of MSCs from compact bone could be an easy isolation technique which can avoid the use of other purification techniques and reduce the chances of hematopoietic cell contamination (Guo et al., 2006; Zhu et al., 2010).

MSCs have been isolated from several tissue sources of chickens, apart from bone marrow. MSCs were isolated from the lungs of 1 to 2 weeks old chicken (Khatri et al., 2010); from adipose tissue (sub cutaneous fat of abdomen and inguinal fat pads) of one-day-old broiler (Gong et al., 2011); liver of 7-day old embryo (Mu et al., 2013). Similarly, Dermis-derived MSCs was isolated from the 16d old embryo (Gao et al., 2013); from Wharton jelly of the 12-day embryo (Bai et al., 2013b) and yolk sac of the 4-day old embryo (Bai et al., 2013b; Gao et al., 2013). Cells isolated from another tissue source such as adipose tissue, dermis, Wharton jelly, and yolk sac were enzymatically digested with collagenase type IV. The resulting suspension was filtered through a mesh sieve, centrifuged and pellet so obtained was plated in petri dishes with cell culture medium.

Cells isolated from different tissue source of chickens are primarly cultured in a basal medium such as Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin and 0.292mg/mL of L-glutamine. Apart from DMEM, a combination of DMEM/F-12, fibroblast growth factor (FGF), and L-glutamine, b-27, the epidermal growth factor is also used as a basal medium to plate chicken

derived MSCs from various sources. (Gong et al., 2011); (Bai et al., 2013b; Gao et al., 2013). A summary of tissue source for isolation and supplemental media are reported in table 2.1.

Morphology and Growth curve of MSCs

MSCs isolated from bone marrow of human cells exhibited spindle-shaped cells. The growth of cells usually followed a few days of lag phase, proliferated rapidly in logarithmic phase and slowed down in plateau phase (Tuli et al., 2003). Similarly, MSCs derived from murine bone marrow were spindle-shaped and grew rapidly in cell culture plate but higher passage number required a longer time to reach confluency and the morphology gradually changed with passage number (Nadri and Soleimani, 2007). MSCs isolated from chickens have unique characteristics to adhere to the plastic and exhibit fibroblast-like morphology after about 3-5 days of initial culture, which forms colonies that grow to attend confluency. MSCs isolated from chicken after 2-4 passages were reported to be more homogeneous, exerted spindle-shaped cells with elongated ends (Khatri et al., 2010). Cultures from the different source were passaged and no difference in morphology and growth was observed between passage at least up to passage 16. However, cells derived from dermis were detached from plates when cultured for more than 16 passage (Gao et al., 2013). Growth curve of the cells showed sigmoidal shaped with 1-3 days of latency phase, logarithmic growth phase and plateau phase in about 7 days (Bai et al., 2013). MSCs isolated from a different source on chicken showed similar basic morphology, growth pattern and homogeneity.

MSCs have the capacity to proliferate and expand in *in vitro* conditions thus the can be regenerated and expanded for the long term. However, it has been reported that MSCs derived from a human with higher passage have a higher population doubling time, increased expression of senescence markers such as p16 and p53, increased senescence-associated β-gal activity, and

lose telomerase activity (Bonab et al., 2006; Rosland et al., 2009). Recent studies have been conducted to modify methodologies to culture human and mouse-derived MSCs in serum free media and low-oxygen environment to decrease population doubling time and delay senescence of MSCs (Brunner et al., 2010; Chen et al., 2014; Wang et al., 2014). However, such studies have not yet been conducted in chicken derived MSCs to understand the differentiation potential and biological characteristics of chicken-derived MSCs. Further studies need to be conducted to evaluate the effect of serum depletion on chicken derived MSCs growth, proliferation, and cellular aging in long-term culture, as well as in therapeutic and clinical application.

Expression of cell surface markers

Numerous papers have reported and extensive work has been conducted to identify the specific markers for selection, detection, and identification of MSCs from the heterogeneous population of cells. According to ISCT, MSCs should maintain specific surface antigen, must have a positive expression of CD105, CD73, CD90 markers and lack expression of CD45, CD34, CD14, CD11b, CD19 surface markers to be considered as MSCs (Dominici et al., 2006). MSCs express a large array of extracellular matrix proteins, cytokines, growth factor receptors, and cell surface antigens within the microenvironment that could differ between the source, passage, and species of origin. CD34 identified as a negative marker of MSCs is reported not necessarily as true nature of MSCs identification (Lin et al., 2012). Some reports express CD34 positive in supra-adventitial adipose tissue-derived MSCs at isolation (Zimmerlin et al., 2013). While some reported expression of CD34 positive cells in at isolation but lost expression in the further passage (Braun et al., 2013; Kaiser et al., 2007). Such difference in expression of markers indicates that markers could vary depending on the source and species of MSCs isolation (Pachon-Pena et al., 2011). Several additional markers have been reported and widely accepted

as additional MSCs markers. Some of the markers that are positive for MSCs identification and verification reported in humans MSCs are STRO-1, CD271/SUSD2, CD200, and Frizzled-9 (Sivasubramaniyan et al., 2013).

MSCs isolated from different organs of the chicken positively expressed CD105, CD73, CD90, CD44, and CD29 (Dominici et al., 2006). Studies have shown positive expression of β-integrin, CD39 in dermis derived MSCs (Gao et al., 2013); CD71 was expressed in bone marrow and adipose-derived MSCs (Bai et al., 2013a); CD105 and CD106 was reported positive in cartilage derived MSCs (Li et al., 2015); transcription factor PouV (homologue of mammalian Oct4) was expressed in lung-derived MSCs (Khatri et al., 2010); Oct4, NanoG, B integrin was positive in yolk sac-derived MSCs. The expression of CD31 and CD34 was reported negative in all source of MSCs, though the expression of CD34 regarded as a negative marker is still controversial (Lin et al., 2012). Other negative expression markers such as CD14, CD45, and CD19 was not explored in any of the primary isolations of cMSCs.

Osteogenic differentiation of chicken mesenchymal stem cells

MSCs are classically known to differentiate into bone, fat, and cartilage *in vitro*. A well-known method to differentiate MSCs into osteocytes *in vitro* involves culturing the MSCs population with media containing ascorbic acid, β-glycerophosphate, and dexamethasone for 2–3 weeks. Osteogenic induction of MSCs are indicated by mineral deposition and can be detected by alizarin red, alkaline phosphate (ALP) and Von Kossa staining. ALP is an indication of early osteogenesis and Alizarin red and Von Kossa are indicators of calcification. Key transcription factors that are described to paly roles are Runx2, Osterix, BMPs, Wnt family and B-Catenin. BMP-2 induces p300-mediated acetylation of Runx2, a master osteogenic gene, which enhances RunX2 transactivation capability and is an essential regulator of bone formation and osteogenic

differentiation of MSCs (Jeon et al., 2006). RunX2 directs MSCs to differentiate into pre-osteoblast and inhibits adipogenic and chondrogenic differentiation maintaining the supply of immature osteoblasts (Figure 2.4). Osteogenic specific genes such as alkaline phosphatase, osteopontin, collagen type I, and secreted phosphoprotein 1 (SPP1) were expressed in most of the MSCs isolated from chicken. Activation of Wnt signaling pathway in MSCs induces expression of osterix and suppress PPARγ. B cantine and osterix further help to direct pre-osteoblasts into immature osteoblasts thus commenting the cells to form mature osteoblasts (Komori, 2006). Immature osteoblasts express a high level of osteopontin. Osteocalcin, Col1 A1, BSP are measured in high levels when differentiated into mature osteoblasts. cMSCs differentiated into osteogenic lineage express positive ALP activity assay, Von Kossa stain and Alizarin Red assay *in vitro*.

Adipogenic differentiation of chicken mesenchymal stem cells

Exposure of MSCs to insulin, dexamethasone, and isobutyl-methyl-xanthine can trigger MSCs population to differentiate into adipogenic cells which result in accumulation of lipid in intracellular vacuoles (Hemmingsen et al., 2013). Dexamethasone helps to stimulate the differentiation of cells in a dose dependent manner. Insulin helps to mimic insulin-like growth – factor-1, activating mitogenic –activated protein kinase pathway. IBMX helps to raise intracellular cAMP and protein kinase A (Scott et al., 2011). PKA is required for transcriptional activation of PPARγ. After induction with adipocyte medium, MSCs isolated from all sources of an animal expressed higher expression of PPARγ and LPL *in vitro*. PPARγ is considered to be the main transcription factor that plays role in differentiation of MSCs into adipocytes (Zhuang et al., 2016). Upregulated expression of PPARγ along with C/EBP promotes the adipogenic differentiation of cells. Recent studies with miRNAs in animal models suggested MiR-143,

miRNA 378 are associated with regulation of adipocyte differentiation along with PPARγ (McGregor and Choi, 2011). FABP4, LPL, FAS genes are upregulated in the adipocyte differentiated cells (Moseti et al., 2016). Induction of adipocytes can be detected by oil red O staining of cells grown *in vitro* (Parhami et al., 1999). In human MSCs during pre-adipocyte phase cells shows similar morphology to fibroblast and cannot be distinguished from their MSCs precursors (Muruganandan et al., 2009). However, during their development MSCs become oblate shaped mature adipocytes and express adipocytes specific RNA on gene expression analysis (Rosen and MacDougald, 2006). A new combination of adipogenic supplements has been studied in other cell types isolated from chicken and have shown better results than the traditional adipogenic cocktail. The addition of oleic acid in combination with adipogenic cocktail increases adipogenesis in preadipocyte cells isolated from laying hen (Regassa and Kim, 2013) and in broiler preadipocytes cells (Matsubara et al., 2008; Matsubara et al., 2005).

Myogenic differentiation of chicken mesenchymal stem cells

Myogenic differentiation of MSCs was achieved when the cells were exposed to corticosteroid, horse serum, and demethylation agent 5 azacytidine (Jackson et al., 2007). Myogenic differentiation is also promoted by co-culturing MSCs with skeletal myocytes. Myogenic differentiation of MSCs occurs via activation of myogenic transcription factors, Pax3, MyoD, Myf-5 and Myogenin (Braun and Arnold, 1996; Gang et al., 2008). Pax 3 and Pax7 are a master regulator of myogenic differentiation and contribute to early striated muscles development during skeletal muscles differentiation (Yin et al., 2013). Insulin-like growth factor II (IGF-II) induces the myogenic differentiation through regulation of MyoD, whereas TNFα downregulates MyoD and inhibits myogenic differentiation (Meyer et al., 2015).

Another lineage-specific differentiation

Cells isolated from bone marrow of chicken induced endothelial differentiation in presence of VEGF, βFGF and IGF (Bai et al., 2013a). Chondrogenesis of cBMSCs derived from a different source was promoted *in vitro* by using chondrocyte-specific media, DMEM/F12 supplemented with FBS, ITS, L-proline, dexamethasone, sodium pyruvate, L-ascorbic acid, and TGF-β3 (Li et al., 2015). It has been reported that MSCs derived from Warton jelly, yolk sac, and chicken dermis could differentiate into neural-like cells when treated with neural differentiation media. Such derived cells expressed neural cell markers nestin, β-III tubulin, NF, and SYP and Map-2 (Gao et al., 2014; Gao et al., 2013).

Table 2:1: Method of Isolation, CD markers, differentiation potential and supplemental media required to trigger lineage differentiation in chicken mesenchymal stem cells

Source of MSCs	Method of isolation	Positive marker	Negative marker	Differentiation	Media supplement	References
Bone Marrow	-Density gradient, centrifugation	CD44 CD45 CD90 CD105	CD34 CD31	-Adipogenic -Osteogenic -Chondrogenic	-DMI -OM -Ascorbic acid + Insulin + TGF-β1	(Khatri et al., 2009)
Bone Marrow of 13d embryo		CD44, CD73, CD90, CD105 Oct4, Sox2, Nanog	CD34 CD45	-Osteogenic -Adipogenic -Chondrogenic -Hepatogenic -Islet -Neuronal -Cardiac	- OM - DMI - Dxa + amino acid + sodium pyruvate - bFGF + Dxa + ITS -Nicotinamide + mercaptoethanol + glucose -bFGF + Retinoic acid - 5-azacytidine,	(Bhuvanalakshmi et al., 2014)
Bone Marrow	Total blood adherent; Density gradient centrifugation	CD29 CD44 CD71 CD73	CD34 CD31	-Adipogenic -Osteogenic -Endothelial	-DMI -OM -VEGF+ bFGF+ IGF-1	(Bai et al., 2013a)
Bone Marrow	Density gradient centrifugation			Osteogenic	OM	(Pande et al., 2015)
Dermis of 16d old embryo	Enzymatic	CD44, CD71, CD73,B-integrin	CD34	-Adipogenic -Osteogenic -Neurogenic	-SM+DMI - SM+OM - SM+ All-trans-retionic acid + 2-mercaptoethanol	(Gao et al., 2013)
Wharton's Jelly	Enzymatic (collagenage IV)	CD29 CD44 CD71 CD73	CD34 CD31	-Adipogenic -Osteogenic -Neurogenic -Cardiomyocytes	-DMI -OM + hexadecadrol -20% FBS+ 2-mercaptoethanol + DMSO + butylated hydroxyanisole -5-Azacytidine	(Bai et al., 2013b)
Adipose Tissue	Enzymatic (collagenage I)	CD29 CD44 CD71 CD73	CD31	-Osteogenic -Adipogenic -Cardiomyocytes	-DMEM/F1/2 + OM -DMEM/F1/2 + DMI -DMEM/F1/2 + 5-Azacytidine	(Gong et al., 2011)
Yolk Sac	Enzymatic (collagenage I)	B-integrin Oct4 Nanog CD44 CD71 CD73	Cd34	-Neurogenic	M ATRA	(Gao et al., 2014)

Lungs	Enzymatic and density gradient	CD44 CD90 CD105 PouV		-Osteogenic -Adipogenic	-OM -DMI	(Khatri et al., 2010)
Cartilage from embryo day 20	Enzymatic (collagenage I)	CD29 CD44 CD105 CD166		-Osteogenic -Adipogenic -chondrogenic	-DMEM/F1/2 + OM -DMEM/F1/2 + DMI -DMEM/F12 + 5% FBS + ITS+ L-proline + Dxa + Sodium pyruvate + L-ascorbic acid + TGF-β3.	(Li et al., 2015)

All cells were grown in DMEM, 10% FBS, and Penicillin-streptomycin unless indicated. Only supplements that are additional or different are indicated in the table.

SM = (L-DMEM supplemented with epidermal growth factor, basic fibroblast growth factor, 10% FBS, L-glutamine, and sodium pyruvate)

DMI= dexamethasone, isobutyl-methylxanthine, and insulin

OM =β-glycerol phosphate, Ascorbic acid, Dexamethasone

DXA = Dexamethasone

ITS= Insulin transferrin selenium

Storage of MSCs

Cryopreservation is the only way to store primary MSCs for long term post isolation so that they can be used in the future for various therapeutic purpose and to replicate research studies. To have a higher number of cells available for clinical application a set of proper storage technique and a compatible cryoprotectant is required. Cryoprotectant is any chemical or agent that is added to the media which is used to freeze the cells, can help in better freezing, yield high post-thawing survival of the cells, has less toxicity to the cells, and does not alter morphological characteristics and differentiation potential of the cells post-thawing (Shivakumar et al., 2016). Different cell lines are successfully stored by defined cryopreservation methods for years and are further thawed and used for further research. There are several research reports to optimize freezing media composition, cooling device, the rate of cooling, storage conditions and developing of good cryopreservation technique to ensure that MSCs retain their therapeutic characteristics following cryopreservation and that they are safe for clinical use (Thirumala et al., 2009). Generally, cryoprotectant plays their role by two ways: first, by protecting the crystal formation known as surface protectants and permeable protectants, and the second by slowing down the freezing rate of MSCs.

Most common cryopreservant used for storing cells is DMSO. However, DMSO is reported to be toxic to both human and animals upon injection which could develop some cardiovascular and respiratory issues (Rowley et al., 1999; Zenhausern et al., 2000). 10% DMSO is used in standard cryopreservation technique, DMSO was reduced up to 5% (Akkok et al., 2008; Rowley et al., 1999) or 3.5% (Halle et al., 2001) to reduce its load in cells and served well as a cryoprotectant. Another method of reducing the harmful effect of DMSO is to wash the cells before treating. There are also reports of using glycerol, sucrose, and trehalose alone or in

combination with 2% DMSO which yield superior viability and generation of hematopoietic colonies of erythroid and myeloid lineage than 5% DMSO and sucrose and trehalose alone (Mantri et al., 2015). Use of DMSO catalyzed differentiation of MSCs characterized by rapid change in morphology, higher hepatic marker gene expression and increasing transcriptional level of CK18, HNF 4a, and HNF6. Use of DMSO as a cryoprotectant in MSCs could be advantageous to accelerate hepatogenic differentiation but could produce variable results due to inconsistent immune response *in vivo*.

Freezing rate is another important component to be considered in cryopreservation techniques which can affect the viability of cells following storage. The slow freezing rate of 1° C /min is the optimum rate for MSCs preservation (Thirumala et al., 2005). Slow freezing rate along with cryoprotectant helps to balance the intracellular and extracellular osmotic pressure thus preventing lysis of cells post-thaw and formation of crystals.

Beside basal medium, MSCs require additional growth factor to maintain their undifferentiated state. Fetal bovine serum is one of the most frequently used media supplement in cell culture practice. Other growth factors like LIF, βFGF are used for proliferation and self-renewal and for differentiation to a different lineage. Use of FBS in the culture condition can increase the risk of transferring infectious agent. FBS engulfed by patient cells can cause rejection on transplantation (Sundin et al., 2007). Use of serum free media or animal derived products is essential for vaccine production and several therapeutic potentials in poultry industry (Shittu et al., 2016).

Chicken embryo as a tool for developmental study

The chicken embryo model is one of the most used embryonic tools for studying embryonic development because of its surgical accessibility and easy manipulation. Chicken

embryos take 21 days to hatch and the development of the embryo is divided into different stages and the division takes very rapidly provided optimal environmental conditions (Hamburger and Hamilton, 1992). Chicken embryo has been used as a model to study various disease, developmental, pharmacological, and toxicological studies relating to different animals. It is readily available, cost efficient, and present an alternative approach for the treatment of pregnant mammals. Shh signaling along with, gene networks were Patched 1, Hoxd13, Sall1, BMP2 and Gli target genes in limb and cell cycle genes controls the integrated specification of digit pattern and growth in the chicken limb wing pattern and integrating digit specification in other mammals (Fisher et al., 2011). It has been reported that organogenesis and proper lining of important organs of the chicken embryo occur in the first week of incubation. First 7 doi are important for protuberance of limb buds, development of head and gastrointestinal organogenesis (Bowen et al., 1989). Development of ovary, skeletal patterning of digits, and formation of intestine takes place in 9 doi (Tickle, 2004). Due to these early and rapid changes, there have been studies to understand the development and embryogenesis by manipulation of the embryo as early as phase 10 (Bangs et al., 2010; Bortier and Vakaet, 1992; Ezin and Fraser, 2008; Welten et al., 2011). The role of retinoic acid in the endogenous zone of polarizing activity and development of skeletal pattern was examined by placing the beads soaked with retinoic acid near somite 15 and 20 of stage 14 embryos (Ohta et al., 2001). Such early manipulation requires an extensive opening of the embryo and may not be suitable for getting higher hatch percentage in industrial implication for commercial hatcheries. However, such understanding of early embryo development will give a better understanding of the pathways and molecular cascade that the nutrients will play during early embryogenesis.

Use of in ovo feeding methods to enhance chick health, hatchability and post hatch chick production efficiency

In the last decade, due to rapid genetic selection and nutrition, the broiler has evolved to be a fast-growing meat type bird (Knowles et al., 2008) and laying hens as a prolific layer that lay eggs in about every 24 hours continuously for more than a year (Whitehead and Fleming, 2000). Both of these action are predisposing factors for poor mineralization of bones that can lead to compromised leg development, tibial dyschondroplasia, loss of medullary and cortical bones, and osteoporosis in poultry (Knowles et al., 2008; Whitehead and Fleming, 2000). These bone problems in poultry are not only a major welfare issue of birds but also a major economic loss to the poultry industry. Several nutritional strategies have been developed to prevent leg problem in poultry, such as feeding *in ovo* minerals at 17-18 day of incubation (Yair and Uni, 2011; Zhai, et al., 2011), supplementation of Ca and P in the diet (Bar et al., 2002; Proszkowiec-Weglarz and Angel, 2013), supplementation of phytase enzyme (Perney et al., 1993), vitamin D (Driver et al., 2005; Garcia et al., 2013) in the diet. Above all, metabolic 1-25 -vitamin D is the prohormone that regulates Ca and P balance and absorption (Proszkowiec-Weglarz and Angel, 2013). Some success for prevention of bone problems is only attained when birds are supplemented externally at life (Ebrahimi et al., 2012; Keralapurath et al., 2010; Yair et al., 2013; Yair and Uni, 2011; Zhai et al., 2011a). *In ovo* supplementation of vitamin D has been shown to improve hatchability and higher mineralization and better mechanical properties of bones when injected into eggs (Yair et al., 2013; Zhai et al., 2011). *In ovo* feeding of nutrients would be a more effective option to supplement proper supplementation of nutrient in chicks before hatch as developed by Uni and Ferket (2003). Injection of Ross X Ross 708 broilers with 25-hydroxycholesterol at 18d of incubation increased hatchability but did not affect body weight

gain, tibia ash, breaking strength and ash concentration (Bello et al., 2015; Bello et al., 2013, 2014).

Nutritional deficiencies during formation of egg could have a significant impact on the development of the embryo and post hatch chick performance. Moreover, supplementation of nutrient in early embryo development could offer a powerful tool to promote the health of hatched chicks and boost early growth and development. (Foye et al., 2006). Injection of nutrients at late embryogenesis in the amniotic fluid are subsequently swallowed, digested, and absorbed by embryo thus helping in embryo development (Tako et al., 2004, 2005). *In ovo* feeding of incubating eggs with dietary nutrients such as amino acids, carbohydrate, minerals and vitamins, stimulants, and hormones has been reported to enhance the growth and development of chicken embryo, promotes gut health and immune status and post-hatch development and performance (Lee et al., 2014; Ohta et al., 2001; Pruszynska-Oszmalek et al., 2015; Tako et al., 2004; Zhai et al., 2011b).

In ovo feeding, however, has been successfully carried out as early as day 7 in the yolk of the incubated eggs. *In ovo* injection of amino acids solution in the yolk at day 7 increased amino acid contents of embryo, yolk, albumen, allantoic, and amnion fluids (Ohta et al., 2001). *In ovo* injection of vitamin E at day 14 of incubation increased hatchability, level of IgM and IgA relative to sham control at 42 days of age(Salary et al., 2014). *In ovo* injection of Threonine onto the yolk of 14d old embryo increase hatched chick body weight and increased humoral response to sheep red blood cells (Kadam et al., 2008). Injection of creatine monohydrate and glucose at day 17.5 doi provided a synergistic effect on improvement of the energy status of embryos and hatchlings, and increased glycogen and glucose in the liver and creatine and phosphocreatine in the pectoralis major muscles (Zhang et al., 2016). *In ovo* feeding of carbohydrate solution into

the amniotic fluid of Cobb embryos at day, 17.5 doi increased villus surface of the intestine, increased mucin mRNA expression and enhanced goblet cells development at 19doi, at hatch and 3day post hatch (Smirnov et al., 2006). Similarly, *in ovo* feeding of mannan oligosaccharides at 17 days of incubation increased intestinal villus height at hatch, and increased mRNA abundance of aminopeptidase, sucrose-isomaltase, TLR4 (Cheled-Shoval et al., 2011). Hatched chicks injected with mannan resulted in enhanced epithelial barrier and digestive and absorptive capacity at the day of hatch (Cheled-Shoval et al., 2011). Similarly, *in ovo* injection of prebiotic and symbiotic on day 12 of incubation reduced carcass yield percentage. However, abdominal fat, pH of the intestine, and cholesterol of the pectoral muscles were not affected by the treatments (Maiorano et al., 2012).

In ovo feeding of solutions containing protein, β-hydroxy-β-methylbutyrate (HMB), and carbohydrate increased percentage body weight at hatch, enhanced pectoral muscles glycogen and higher liver glycogen content than the control ones (Foye et al., 2006). *In ovo* injection of L-carnitine at 17d of incubation did not affect chick body weight and % hatch, yolk sac body weight and hatchability (Zhai et al., 2008). Similarly, injection of carbohydrate-electrolyte solution at 16 and 18 doi reduced embryo moisture loss but did not affect body weight and % hatch, yolk sac body weight nad hatchability (McGruder et al., 2011). *In ovo* injection of Se at 18day of incubation into amniotic cavity enhanced the immune system and antioxidant response in the hatched chickens exposed to necrotic enteritis causing pathogens (Lee et al., 2014). However, there has not been any study to conduct the effect of oxysterol in embryonic development and its effect on hatchability, chick quality and post hatch performance in broilers.

Several gaps sill persist in the understanding of gene expression patterns of developmental markers of early embryos. Learning the specific effects of oxysterol in

development osteogenic transcripts could help to understand the effect of oxysterol on early bone development. Supplementation of the novel osteogenic compound at early embryonic stage could help in the rapid development of osteogenic genes which could help to reduce osteogenic problems later in adult life.

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FIGURES

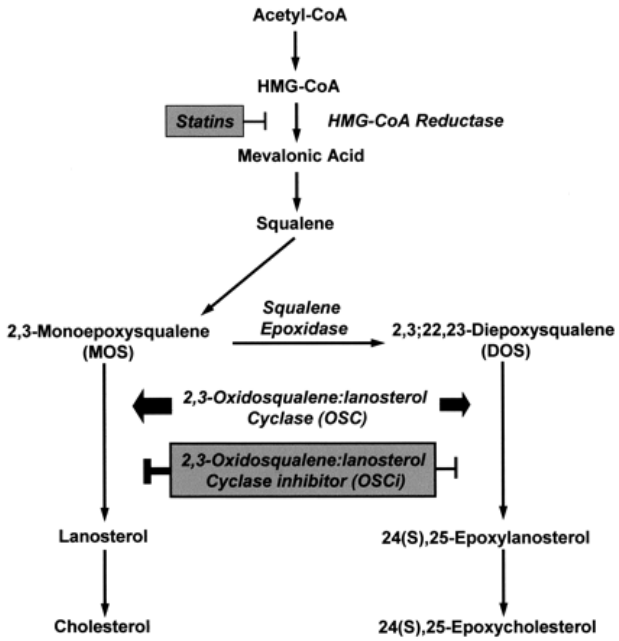

Figure 2.1: Cholesterol biosynthesis pathway and alternate pathway for oxysterol synthesis. Adopted from (Rowe et al., 2003)

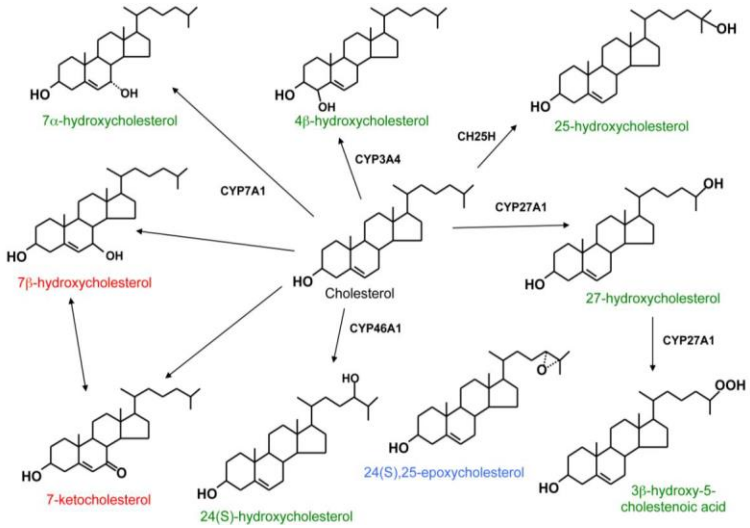

Figure 2.2: Structures of different types of oxysterols that are derived from a cholesterol compound. The enzymatically derived oxysterols are indicated in green, products of cholesterol autoxidation with red and species from a shunt of the cholesterol biosynthetic process is written in blue. This figure was adapted from (Olkkonen et al., 2012).

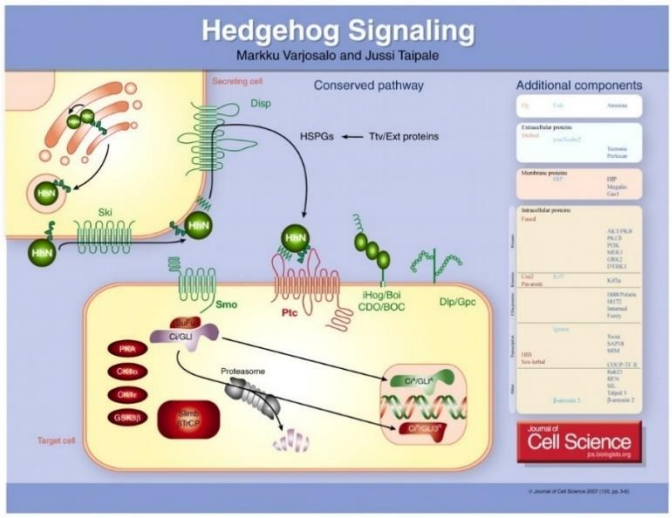

Figure 2.3: Multiple processing steps in Hh signaling. Figure adopted from (Varjosalo and Taipale, 2007)

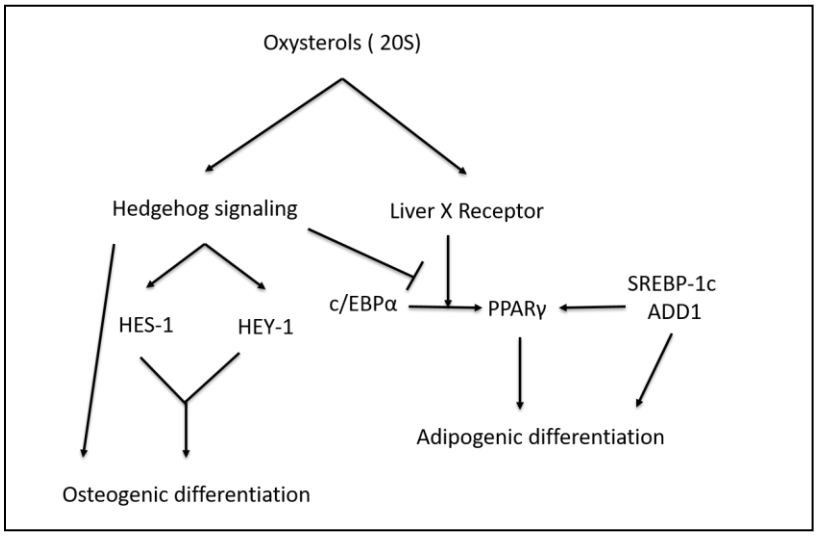

Figure 2.4: Regulation of osteogenic and adipogenic differentiation of MSCs by 20S. Figure is adopted and modified from (Kim et al., 2010; Kim et al., 2007). 20S induce and activates LXRs. Notch signaling, and Hh signaling pathway to induce osteogenesis and reduce adipogenic differentiation in MSCs

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STEM CELLS FROM BROILER COMPACT BONES[1]

[1] Roshan Adhikari and Woo Kyun Kim. To be submitted to *Cell Biology International*

ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells that are capable of differentiation into the different cell lineage and express specific cell surface markers. MSCs have been isolated from the various source of human, mouse, rabbit, other mammals and poultry with potential application for various therapeutic and clinical application. MSCs from chicken compact bone has not been isolated yet. MSCs like cells were isolated from compact bones of femur and tibia of day-old male broiler chicken to investigate the biological characteristics of the isolated cells. Isolated cells took 8-10 days to grow and define in tissue culture treated 100mm dish before they could be passaged further. Isolated cells were plastic adherent and remained as a monolayer. They were mainly spindle-shaped cells with both ends elongated and the cells proliferated rapidly like whirlpool or flamboyance. MSCs were able to differentiate into osteoblastic, adipocytic and myogenic pathways when induced with specific differentiation media. Cell surface markers CD such as CD 90, CD 105, CD73, CD44 were detected positive and CD31, CD34, and CD 45 cells were detected negative by PCR assay. The results suggest that MSCs can be isolated from broiler compact bones that possess similar biological characteristics as an MSCs isolated from another tissue source of species. MSCs from compact bones of broilers can be used as MSC culture model in avian species to provide an opportunity for better understanding of adipogenic and osteogenic pathways in avian species and to identify novel nutrients/bioactive molecules which can promote skeletal health and efficient feed utilization in poultry.

Key words

Mesenchymal stem cells, isolation, avian, biological characteristics. Pluripotency

INTRODUCTION

Mesenchymal stem cells (MSCs) are pluripotent, plastic adherent cells that have been isolated from several species, including human, mouse, rat, dog, cat, sheep, and chickens. The ability of MSCs to differentiate between different tissue types (Freeman et al., 2015; Pittenger et al., 1999), homing potential to injured sites (Sohni and Verfaillie, 2013), tissue repair (Melo et al., 2016) has generated increasing interest in utilizing MSCs in different research, clinical, therapeutic and regenerative settings. Fibroblast-like colony forming cells were first identified from bone marrow, which maintained differentiation potential and proliferation (Friedenstein et al., 1987). The Mesenchymal and Tissue Stem Cell Committee of the international Society for Cellular Therapy (ISCT) has defined these minimal criteria for MSCs: a) MSCs should have the capacity to adhere to plastic surface under standard culture media, b) MSCs should have a multilineage differentiation property and c) Cells should maintain specific surface antigens, must have positive expression of CD105, CD73, and CD90 markers and lack expression of CD45, CD34, CD14, CD11b, and CD19 surface markers (Dominici et al., 2006).

MSCs have also been isolated and characterized from several tissues including adipose tissue (Zuk et al., 2001), blood vessels (Crisan et al., 2008), bone marrow stem cells (Feyen et al., 2016), skin (Crigler et al., 2007), compact bone (Zhu et al., 2010), dental tissues (Lei et al., 2014), menstrual blood (Ulrich et al., 2013), amniotic fluid (Kim et al., 2014), and umbilical cord tissue cells (Kumar et al., 2016). MSCs have commonly been generated from bone marrow of different animal species such as pigs (Feyen et al., 2016), mouse (Nadri and Soleimani, 2007), chickens (Bai et al., 2013; Khatri et al., 2009), rabbit (Wee et al., 2013), and humans (Kar et al., 2016; Li et al., 2016).

Isolation of MSCs from bone marrow of chicken has been reported in previous studies (Khatri et al., 2009). However, there are no reports of MSCs isolated from compact bones of the

chickens. There are two main stem cell populations that are the main resident of bone marrow, hematopoietic stem cells and mesenchymal stem cells (Pittenger et al., 1999; Zhu et al., 2010). One of the drawbacks described while isolating MSCs from bone marrow could be the contamination of blood cells and hematopoietic stem cells. In order to reduce blood cell contamination and purify MSCs isolation, various purification techniques have been reported such as, preferential attachment to culture plastic density, gradient centrifugation (Yamamoto et al., 2015), use of ficole to filter the blood cells, antibody-based cells sorting (El-Sayed et al., 2015; Van Vlasselaer et al., 1994), low and high density culture techniques (Eslaminejad and Nadri, 2009), negative selection techniques (Baddoo et al., 2003; Dumas et al., 2008), frequent media change (Soleimani and Nadri, 2009), and positive selection method (Nadri and Soleimani, 2007). All of these methods are employed to obtain a homologous population of bone marrow-derived MSCs. However, sometime purifying techniques could result in a reduced capacity of MSCs to differentiate and grow to subsequent passages (Phinney et al., 1999; Sun et al., 2003), and also incur extra cost and time to isolate the cells (Soleimani and Nadri, 2009). Osteogenic cells sorted out from bone marrow of 5-fluorouracil treated mice lost their capacity to synthesize bone proteins and for mineralized matrix upon subcultivation (Van Vlasselaer et al., 1994). Isolation of MSCs from compact bone could be an easy and economic isolation technique which can avoid the use of other purification techniques and also reduce the chances of hematopoietic contamination (Guo et al., 2006; Zhu et al., 2010).

In this study, we present for the first time, an effective, simple, and economic method for isolation and characterization of MSCs from compact bones of day old chicken (cBMSCs). cBMSCs can be used as an MSC culture model in avian species to provide an opportunity for better understanding of osteogenic, adipogenic, and myogenic pathways in avian species and to

identify novel nutrients/bioactive molecules which can promote skeletal health, muscular growth, fat development, and efficient feed utilization in poultry

MATERIALS AND METHODS

Experimental Animal

All experiments were performed in according to the guidelines for the use of animal in research stated by the Animal care and use committee at the University of Georgia. Femurs and tibia bones from both legs were obtained from the day-old chicks after cervical dislocation.

Isolation of cBMSCs

cBMSCs were isolated by using a modified approach of the previously described methods in human trabecular and murine compact bones (Tuli et al., 2003; Zhu et al., 2010). The birds were soaked in alcohol for 2 min after cervical dislocation. Legs were removed from hip joint and metatarsals. Dissected legs were kept in Dulbecco's Modified Eagle's medium (DMEM) (Mediatech Inc.,VA, USA) containing 10% Fetal Bovine Serum (FBS) (Mediatech Inc.,VA, USA), 100U/mL penicillin, 100µg/mL streptomycin and 0.292mg/mL L-glutamine (Thermo Fisher Scientific, MA, USA) until connective tissues and muscles were completely removed. Muscles and connective tissues around tibia and femurs were removed immediately using a scalpel and micro-dissecting scissors in a bio-safety cabinet. Tibia and femurs were placed in washing buffer containing Phosphate-Buffer Saline (PBS) (Mediatech Inc., VA, USA) mixed with 2% FBS. The epiphysis of the bones was removed to expose the bone marrow cavity. Bone marrow inside the bone was flushed four times with washing buffer and a syringe to remove the bone marrow and hematopoietic cells adhered to the compact bones. The bones were cracked with a scalpel and washed three more times with washing buffer to make sure that all the

blood cells were washed. The bones looked whitish in color after the wash. The bones were transferred to new petri dishes with 5ml of digestion media (DMEM containing 100 IU/ml penicillin and 100 ug/ml streptomycin, 0.25% collagenase (Sigma-Aldrich, MO, USA), and 20 % FBS). The bones were chopped into smaller fragments of about 3 mm^3. Bone fragments were suspended in a 50 ml tube that contained digestion media. The bone fragments were digested in a shaking water bath for 60 min at 37 °C at 180 rpm. After digestion, the media containing bone fragments were filtered using a 40 µm sterile filter. Bone particles were washed with 5 ml of 10% DMEM in the filter. Filtered contents were centrifuged at 1,200 rpm for 10 min. The supernatant was discarded and the cell pellet was disrupted with 20 ml 10% DMEM, and cells were plated in two 100-mm petri dishes. Cultures were incubated at 37 °C in a humidified incubator containing 5% CO_2. Half of the medium was replaced by fresh media at 12 h, complete media were changed at 24 h to remove the non-adherent cells. After that, media were changed once every 2-3 days. These cells were labeled as P0. Once the cells reached 90-95% confluently, the cells were washed twice with 5ml DPBS, dissociated with 0.1% Trypsin-EDTA (Mediatech Inc., USA) for 2 min and subcultured at a ratio of 1:3 in 100-mm petri dishes. This passage was marked as P1, subsequent cultures were named as P2, P3, P4… Pn consecutively. P4 cells were used for cBMSCs differentiation experiments.

cBMSCs growth and Morphology observation

cBMSCs were observed daily to monitor their morphology and growth characteristics. Cells were observed under a microscope daily, and pictures of cells were taken at different time points. Cells were observed for adherence to plastic plates and its morphological features.

Colony forming unit-fibroblast (CFU-F) assay

The capacity of self-renewal and colony forming potential were tested by plating cells at low densities in 100-mm plates. Colonies formed from single cells were counted at P4 and P8. At both passage, 25 cells/cm^2, 50 cells/cm^2, and 75 cells/cm^2 were plated in 100-mm discs and incubated for 10 days. On day 10, cells were fixed and stained with 1% crystal violet (Sigma-Aldrich, MO, USA) in 100% methanol for 30 min. The colony was defined as more than 50 fibroblastic cells in a cluster. The number of colonies was scored, the capacity of cell proliferation was observed and microscopic pictures were obtained.

Growth Kinetics

Cells at P2, P4, and P8 were dissociated by 0.25% trypsin and plated in 24-well plates at a density of 1×10^4 cells /ml. The number of cells was counted by a viability detection method using a trypan blue exclusion test on 4-wells daily for 12 days. Each well was counted three times, and mean value was calculated. Growth curves were plotted for each passage. Population doubling time (PDT) was calculated by formula reported by (Aliborzi et al., 2016). PDT was calculated using the formula PDT=T ln2/ln (Xei/Xbi), where T means incubation time in hours, Xbi is the number of cells at the beginning of the incubation and Xei corresponds to the number of cells at the end of incubation.

Multilineage differentiation of cBMSCs in vitro

Osteogenic differentiation

cBMSCs at P4 were seeded at a density of 20,000 cells /cm^2 in 24-well plates for Alizarin Red, Alkaline Phosphate (ALP), and Von Kossa stain (VK) and in 6-well plates for measurement of osteogenic gene regulation. Cells were cultured in basal media containing DMEM, 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin until 90% confluent. On confluency, cells were

treated with osteogenic media (OM) containing DMEM with 10^{-7}M dexamethasone (DXA) (Sigma-Aldrich, MO, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, MO, USA), 50 µg/ml ascorbate (Sigma-Aldrich, MO, USA), and 5% FBS for osteogenic induction. Cells cultured in DMEM basal media with 10% FBS were used as negative control. Fresh media was replaced with the culture plate in every 2-3 days. The cells were stained with Alizarin Red and VK for detection of mineralization and ALP for detection of osteogenic differentiation on 7 and 14 days of treatment. Cells plated in 6-well plates were harvested at 72 h for osteogenic gene expression analysis using qRT-PCR. Alkaline Phosphatase (ALP), Runt-related transcription factor 2 (RUNX2), Bone Morphogenetic Protein (BMP2), Bone Sialo Protein (BSP), and Bone Gamma-Carboxyglutamate Protein (BGLAP) were analyzed to detect osteogenic differentiation of cBMSCs.

Alizarin Red

Alizarin Red test was used to detect mineralization of osteoblasts which gives bright orange-red color when exposed to Alizarin red solution (Sigma-Aldrich, MO USA). Alizarin red test was conducted following protocol defined in previous cell culture assay (Gregory et al., 2004). In brief, Alizarin Red Stain solution was prepared to dissolve 1 gm of Alizarin Red S in 50 ml distilled water and pH was adjusted to 4.1-4.3 with 0.1% NH_4OH solution. Cells were washed twice with PBS without Ca^{++}/Mg^{+}. Cells were fixed using 10% buffered formalin for 30 min and washed 4 times with distilled water. Cells were stained with Alizarin Red solution for 45 min in dark. Cells were washed to remove the excess dye, PBS was added after washing and the cells were observed under a microscope for mineralization.

Alkaline Phosphate Assay

Alkaline Phosphatase activity was tested by using BCIP/NBT solution following a protocol for osteogenic differentiation and analysis of MSCs following the protocol (PromoCell, Heidelberg, Germany). In brief, substrate solution with one SigmaFast^{TM} BCIP/NBT tablet (Sigma-Aldrich, MO, USA) was dissolved in 10 ml distilled water. Washing buffer was prepared by adding 0.05% Tween 20 in PBS. Cells were washed with PBS and fixed in 10% neutral buffered formalin (Sigma-Aldrich, MO, USA) for 60 sec. Cells were washed with washing buffer twice, and 1mL substrate solution was added to the wells. Cells were incubated at room temperature for 5-10 min. Cells were observed every 2-3 min to check if the purple stain was developed. After the stain was developed, cells were washed with washing buffer, and PBS was added to evaluate the results.

Von Kossa Stain

Von Kossa stain was used to detect the osteogenic differentiation and calcium deposition in differentiated cells. Matrix mineralization in cell monolayers was detected by silver nitrate staining as previously described (Parhami et al., 1997). In brief, cells were washed and treated with 0.1% glutaraldehyde and incubated for 15 min. Cells were washed with deionized water, and 5% silver nitrate was added to the cells. The cells were incubated in dark for 30 min and washed twice with double distilled water. The cells were air dried and exposed to bright light until black spots were developed in the area of calcification.

Adipocyte differentiation

cBMSCs at P4 were placed at a density of 20,000 cells /cm^2 in 24-well plates and 6-well plates. At confluency, cells were treated with adipogenic cocktail (DMI containing 500 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 20 mg/mL insulin) (Cedarlane, NC,

USA) + 300µM Oleic acid. The induced cells were observed in an inverted microscope for fat vacuole deposition. After 96h of treatment with adipogenic media, cells cultured in 24-well plates were harvested and stained with Oil Red O stain using a procedure described by (Kim et al., 2009). Cells in 6-well plates were harvested at 48 h for adipogenic gene expression using qRT-PCR. Key adipogenic gene, PPARγ, FABP2, c/EBPα, and c/EBPβ were measured for adipogenic differentiation of cBMSCs.

Myogenic differentiation

cBMSCs were cultured in 6-well plates at a density of 20,000 cells /cm^2 and treated with myogenic medium (MM) containing DMEM, 5% horse serum (Thermo Fisher Scientific, MA, USA), 50µM hydrocortisone (Sigma-Aldrich, MO, USA), and 0.1µM dexamethasone when confluent. Cells cultured at 6-well plates were harvested after 72 h for key myogenic gene expressions such as MyoD, Pax7, Myf5, and Myogenin were analysis using qRT-PCR

Quantative Reverse Transcription Polymerase Chain Reaction to detect multilineage gene expression

Total RNA was extracted from harvested cell with QIAzol Lysis reagent (Qiagen, MD, USA) following the manufacturer's protocol. Two microgram of RNA was reverse-transcribed to make cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA). Template, multiscribe reverse transcriptase enzyme, random primers, dNTPs, and buffers were subjected to thermocycling (25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min) in a veriti 96-well thermal cycler (Thermo Fisher Scientific, MA USA). Specific primers were used to quantify the osteogenic, myogenic, and adipogenic mRNA expression in harvested cells by qRT-PCR. iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, CA, USA), primers and cDNA templates were subjected to qRT-PCR at 95° C for 10 min, followed by 40 cycles of 95°

C for 15 sec, annealing temp according to primer for 20 sec, and 72° C for 15 sec, followed by 95° C for 15 sec and a melt curve stage in a StepOne™ Real-Time PCR machine (Thermo Fisher Scientific, MA, USA). All samples were prepared in duplicated wells in 96-well plates. Relative abundance of mRNA between the samples were analyzed by the ΔΔCT method. GAPDH was used as a housekeeping gene. The values were reported as fold changes of the mRNA expression of the target genes in differentiation group compared to control group. Primers for each gene were designed and checked for target identity using the National Centre for Biotechnology Information (NCBI). Primers used for qRT-PCR assays and their sequences are presented in Table 3.1.

PCR for cell surface markers

Cells were plated in 6-well plates and harvested when confluent at P2, 4, and 8 to analyze cell surface markers. RNA was isolated from the harvested cells, and cDNA was transcribed following the method described above. Synthesized cDNA was subjected to PCR amplification using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, MA, USA) following manufacturer's protocol. Samples were subjected to thermocycling at 95° C for 3min, followed by 40 cycles of 95° C for 30 sec, the annealing temperature of primer for 30 sec, and 72° C for 30 sec, followed by 72° C for 7 min. PCR products obtained were separated by 1.5% agarose gel electrophoresis to visualize the bands detected using SYBR Safe DNA gel stain (Thermo fisher Scientific, MA, USA). mRNA expression of cell surface markers, CD44, CD29, CD45, CD90, and CD34 was used to characterize cBMSCs population at P2, P4, and P8. GAPDH was used as an internal control. Primer sequences for cells surface markers such as CD29, CD34, CD44, CD71 were used to characterize cBMSCs as reported (Bai et al., 2013; Khatri et al., 2009). Primers for each gene were designed and checked for target identity using the National Centre

for Biotechnology Information (NCBI). Primers used for PCR assays and their sequences are presented in Table 3.1.

Statistical Analysis

Statistical analysis was performed using the ANOVA in GLM procedure of the Statistical Analysis System (SAS) Institute. Mean separation test was conducted using Tukey test and $P \leq 0.05$ was considered as statistically significant among the groups.

RESULTS

Characterization of cBMSCs

cBMSCs were isolated from one-day-old broiler compact bones of tibia and femur (Figure 3.1). cBMSCs at P0 were round for initial 2 days and appeared to be spindle-shaped after 3-4 days of culture (Figure 3.2a). Cells formed distinct colonies and were passed for P1 at 10-12 days. Cells were passaged at a ratio of 1:3 plates for subsequent passage once they got confluent (Figure 3.2c). Cells adhered to the plates, were spindle-shaped, divided rapidly like a whirlpool and reached confluent within 2-3 days. This rapid proliferation and spindle-shaped morphology were consistently observed in subsequent passage 4, 6, and 8 (Figure 3.2d, 3.2e, 3.2f). The passage was done up to P16 to observe the morphological characteristics. Cells at P4 were used for further analysis of multilineage capacity, proliferation potential, immune histochemical analysis, and PCR.

Colony forming Units Colony

As cBMSCs have the ability to grow and self-renew, a CFU assay was used to assess the ability of cells to proliferate and colony forming potential of isolated cBMSCs. cBMSCs formed distinct colonies of cells when seeded at lower densities, indicating that they have the potential to

self-renew (Figure 3.3). Cells plated at densities of 25cells/cm^2, 50cells/cm^2, and 75 cells/cm^2 formed 90, 156, and 209 colonies in P4 and 75, 106, and 144 colonies in P8 respectively.

Growth Kinetics

The number of cells was counted over 12 days by Trypan blue exclusion test using a cell hemocytometer at P2, 4, and 8. Growth and proliferation potential of cBMSCs were similar at P2, 4, and 8. Cells initially had a latent phase of 1-3 days, a logarithmic growth phase for 4 – 7 days, and reached a plateau phase in about 9 - 10 days in passage 2 and 4 and in about 9 days in passage 8 (Figure 3.4). PDT of the passage 2, 4, and 8 were 66, 76, and 74 h respectively. Comparing the growth curve and PDT, cBMSCs showed favorable proliferation rate at all passages.

Lineage specific differentiation of cBMSCs

Multipotent differentiation capacity of cBMSCs was examined by inducing cells with osteogenic, myogenic, and adipogenic media. cBMSC have shown differentiation potential into all 3 lineages when treated with specific differentiation media. Cells were treated with OM for 2 wk. At the end of wk1 and wk2, calcification deposits of cells treated with OM were detected by the positive stain on Alizarin Red and Von Kossa stain compared to control cells treated without differentiation media. Differentiated cells stained with Alizarin red were bright orange-red in color (Figure 3.5b), whereas undifferentiated cells were slight to non-reddish in color (Figure 3.5a). Differentiated cells stained with Von Kossa stain showed blackish brown mineralized spots which indicate increased mineralization of differentiated cells (Figure 3.5c, 3.5d). Cells treated with OM showed positive alkaline phosphatase activity compared to untreated cBMSCs which are indicative of early osteogenic marker (Figure 3.5e, 3.5f). Undifferentiated cBMSCs

were colorless or faintish blue (Figure 3.5e), whereas differentiated cBMSCs had dark blue-violet stain in the cells (Figure 3.5f). Cells treated with adipogenic cocktail expressed positive adipogenic differentiation by the appearance of lipid vacuole in the cytoplasm detected by oil red O stain. Cells treated with DMIOA expressed reddish lipid vacuole when treated with Oil Red O stain (Figure 3.6b) whereas control cells had no reddish couples (Figure 3.6a).

Quantitative gene expression of lineage differentiation of cBMSCs

cBMSCs treated with specific differentiation media in 6-well plates were harvested for osteogenic, adipogenic, and myogenic gene expression using qRT-PCR. Cells treated with OM expressed higher level of Runx2, BMP2, BSP, and BGLAP mRNA expression indicating osteogenic differentiation of cBMSCs (Figure3.7a). Cells treated with adipogenic media expressed a higher level of FABP4 and PPARγ mRNA expression in comparison to non-treated control cell at 48 h post treatment. However, c/EBPα and c/EBPβ were not significantly different between treatments at 48 h post treatment (Figure 3.7b). Cells subjected to MM expressed higher levels of Myogenin and MyoD mRNA expression which are early differentiation markers of myogenesis. However, Myf5 and Pax7 were not significantly different between the treated and control cells (Figure 3.7c). Cell surface markers of cBMSCs were performed at P2, P4, and P8. cBMSCs at all 3-passage revealed positive results for mRNA transcription of CD90, CD44, and CD29. CD 45 and CD34 mRNA were expressed negatively in cBMSCs at all 3 passage GAPDH was positive in all 3 passages which were used a housekeeping control (Figure 3.8).

DISCUSSION

In this study, we have for the first time characterized MSCs from compact bones of broilers. Isolation of cBMSCs was efficient because the unwanted hematopoietic and non-

mesenchymal cells could deplete by flushing with wash buffer. Our experimental approach was based on a slight modification of previously published protocol on mouse compact bones (Zhu et al., 2010). The result of this study provides the evidence that the cBMSCs showed all 3 characters of rapid proliferation, adherence to plastic and multilineage differentiation as described by ICST.

MSCs isolated from compact bones displayed spindle-shaped cells that proliferate rapidly and arrange in whirlpool shapes which is similar to MSCs isolated from murine compact bones (Short and Wagey, 2013; Zhu et al., 2010). Cells were able to form colonies from a single cell and demonstrated the ability of self-renewal and differentiation, which is a well-known characteristic of stem cell population (Dominici et al., 2006).

Cells had a latency phase of 1-3 days with a logarithmic phase and a plateau phase in about 7-8 days. The growth curve of the cBMSCs isolated from compact bones in this study is similar to the growth curve of the MSCs isolated from bone marrow of chicken (Bai et al., 2013; Khatri et al., 2009). In the present study, PDT of the cBMSCs was 66h, 76h, 74h for P2, P4 and P6 respectively, which was different from the PDT of MSCs isolated from the mouse which was 20h for primary culture and 80h for P3 (Nadri and Soleimani, 2007). Also, in the guinea pig, PDT of cells isolated from bone marrow was 62.9h, 65.6h and 91.4h at passage 2, 5, and 8 respectively (Aliborzi et al., 2016). The reports indicate that growth potential of the MSCs could vary depending on the passage rate as well as tissue source of MSCs isolation and species of origin.

Multilineage differentiation potential was tested by the ability of MSCs to proliferate into osteogenic, adipogenic or myogenic cells when specific differentiation media were introduced to the cells. Cell treated with OM promoted osteogenic differentiation of MSCs by increasing

osteogenic gene transcripts. Similarly, in previous reports, treatment of MSCs with OM resulted in osteogenic differentiation of MSCs isolated from bone marrow in poultry (Bhuvanalakshmi et al., 2014) and in human (Klepsch et al., 2013; Noth et al., 2002). Osteogenic differentiation of cells was characterized by staining the treated cells with Alizarin Red, Von Kossa, and ALP. These cytochemistry techniques are routinely used to characterize the osteogenic differentiation of MSCs (Berberi et al., 2016; Donato et al., 2016).

Alizarin Red and Von Kossa stain both indicate mineralization of MSCs through osteogenic differentiation whereas ALP is an early osteogenic differentiation marker (Gregory et al., 2004; Parhami et al., 1997). Differentiated osteoblasts show a high alkaline phosphatase activity compared to undifferentiated MSCs which show very weak activity. Undifferentiated MSCs do not have extracellular calcium deposit, whereas differentiated osteoblasts have extracellular calcium deposit. Calcium deposit is an excellent way to detect if there is osteogenic differentiation of MSCs when treated with OM (Bellows et al., 1986; Wang et al., 2006). Silver ions in Von Kossa stain react with anions (phosphates, sulfates, or carbonates) of calcium salts in the cells and the reduction of silver salts forms a brownish black staining. Only Von Kossa stain cannot justify the osteogenic differentiation of cells and calcium deposits as $AgNo_3$ could be displaced by SO4 ions in any metal (Bonewald et al., 2003). Another test done to confirm calcium deposits along with Von Kossa stain is Alizarin Red test. Alizarin Red reacts with calcium cations to form an orange-red chelate thus confirming the deposits of Ca in the cells (Wang et al., 2006). Further osteogenic differentiation was confirmed in cBMSCs by analyzing key osteogenic genes using qRT-PCR. BGLAP, BSP, BMP2, and ColA1 mRNA was highly expressed in cells treated with OM which indicates osteogenic differentiation capacity of MSCs

isolated in this study. Treatment with OM induced similar osteogenic gene expression and expressed positive ALP, Alizarin Red, and VK stain in MSCs derived from bone marrow of chicken (Bai et al., 2013), mouse (Nadri and Soleimani, 2007), bovine (Nogueira de Moraes et al., 2016), and human (Honda et al., 2013; Kulterer et al., 2007). In this study, differentiated cells transformed from elongated to shorter cuboidal cells and formed mineralized nodules, which was also reported in MSCs derived from bone marrow of chicken (Khatri et al., 2009).

Adipogenic differentiation of cBMSCs was induced by treating cells with adipogenic cocktail DMI and OA in this study. The characteristics and molecular mechanism of adipocyte differentiation in murine preadipocyte cell line 3T3 have been extensively studied (Ntambi and Young-Cheul, 2000). It is reported that preadipocytes after differentiation from MSCs stay in growth arrest stage, which reenters to cell cycle and undergoes mitotic division in response to an adipogenic cocktail which then terminally differentiates into adipocytes (Tang et al., 2003). However, in human adipose precursor cells derived from adipose tissue and bone marrow stem cells do not undergo division during differentiation (Entenmann and Hauner, 1996) (Lehmann et al., 1997). The exact mechanism of adipogenic differentiation by addition of DMI and AO in chicken MSCs is not known. In our study addition of DMI and OA induced adipogenic differentiation of cBMSCs which was detected by accumulation of lipid vacuole within the cells and increase in master regulator of adipogenesis, PPARγ as well as aP2. This is in agreement with the results on adipogenic differentiation of adipose-derived MSCs in humans when treated (Neubauer et al., 2004; Scott et al., 2011; Tontonoz et al., 1994), and bone marrow-derived MSCs in mouse (Scott et al., 2011) when treated with DMI, an adipogenic cocktail. Myogenic regulatory factors regulate the differentiation of MSCs into muscles cells. MyoD and myogenin are believed to be early differentiation markers of myogenic differentiation and Myf5 and MRF4

are believed to regulate terminal differentiation and cell fusion (Perez-Serrano et al., 2016). MyoD and Myogenin are main muscles specific transcription factors that are expressed during myogenic differentiation of MSCs (Gang et al., 2008; Gang et al., 2004). Similarly, in this study cBMSCs expressed higher MyoD and myogenin mRNA expression when subjected to myogenic media. This indicates that cBMSCs are capable of differentiating onto osteogenic, myogenic, and adipogenic lineage when subjected to appropriate differentiation media.

Availability of stem cell specific markers is limited in poultry thus have to rely on reports published in mammalian cells cell surface markers. Use of markers to verify MSCs identity is an important quality control step to reduce experimental variability and obtain a homogenous population of MSCs. Our study detected the presence of mesenchymal cell surface markers CD90, CD44, CD29, and GAPDH and lack of hematopoietic cell surface markers CD 45 and CD34 mRNA expression at all passage studies. Similar to our finding, previous studies have reported positive CD44, CD90, and CD105 and negative CD45 in MSCs derived from bone marrow of poultry (Khatri et al., 2009).

Despite significant progress in our understanding of the biology of MSCs derived from humans and murine during recent years, much of the information about the identity of MSCs derived from avian and its functional biology remains poorly defined. In the present study, we showed that mesenchymal stem cells can be isolated, cultured and characterized from the compact bone of day-old broilers. The adherence, morphology, differentiation potential, specific markers are comparable with the MSCs derived from other source and animals, making them a suitable model for various research applications. Establishment of avian primary MSCs culture can have a huge potential impact in therapeutics, developmental and regenerative medicine because of their easy availability. cBMSCs can be a cell culture model in avian species to

understand osteogenic, adipogenic, and myogenic differentiation mechanism exerted by different bioactive/nutrient compounds which can promote skeletal health, muscular growth, fat development, and efficient feed utilization in poultry. Chicken compact bone-derived MSCs could be used as an ideal stem cell source for various biological research and could be replacement model to avian embryo because of its easy purification, amplification, multipotency and maintenance. These cells can be a potential model to study discovery of vaccines, chimera chickens, gene manipulation experiment, RNA interference experiments, drug screening as well as storage of cells to make induced pluripotent cells for the conservation of genetically endangered avian species.

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FIGURES

Table 3:1: List of primers used in the study.

Gene Name	Primer sequence (5'---3')	Product length (bp)	Annealing temperature (°C)
GAPDH[a]	Fwd: GCTAAGGCTGTGGGGAAAGT	116	55
	Rev: TCAGCAGCAGCCTTCACTAC		
FABP4[a]	Fwd: TGCTGGGCATCTCAATCACA	106	57
	Rev: GCATTAGTCAGAACGGGCCT		
PPARγ[a]	Fwd: TGAATGTCGTGTGTGTGGGG	229	55
	Rev: GCATTCGCCCAAACCTGATG		
C/EBPα[a]	Fwd: CCTACGGCTACAGAGAGGCT	205	55
	Rev: GAAATCGAAATCCCCGGCCA		
C/EBPβ[a]	Fwd: CCGCTCCATGACCGAACTTA	204	55
	Rev: GCCGCTGCCTTTATAGTCCT		
Col1A2[e]	Fwd: AGAAAGGAATCCAGCCCAAT	238	58
	Rev: ACACCTGCCAGATTGATTCC		
BMP2	Fwd: TGCTGTTGCTCTCAAAGGCT	300	57
	Rev: CTGTGCTTTCTGCCTGGAAGT		
BSP	Fwd: GGAACAGGGAGTCAGCAAGG	156	57
	Rev: TGCAGGGTGAAATGAAGCTCT		
BGLAP[a]	Fwd: GACGGCTCGGATGCTCGCAG	226	55
	Rev: CAGACGGGGCCGTAGAAGCG		

MyoD [f]	Fwd: CAGCAGCTACTACACGGAATCA	102	57
	Rev: GGAAATCCTCTCCACAATGCTT		
Myogenin	Fwd: AGCAGCCTCAACCAGCAGGA	179	58
	Rev: TCTGCCTGGTCATCGCTCAG		
Pax7	Fwd: AGGCTGACTTCTCCATCTCTCCT	156	57
	Rev: TGTAACTGGTGGTGCTGTAGGTG		
Myf5	Fwd: GAGGAACGCCATCAGGTACATC	126	57
	Rev: ACATCGGAGCAGCTGGAGCT		
CD 29 [c]	Fwd: GAA CGG ACA GAT ATG CAA CGG	300	60
	Rev: TAGAACCAGCAGTCACCAACG		
CD34 [c]	Fwd: GTGCCACAACATCAAAGACG	239	60
	Rev: GGAGCACATCCGTAGCAGGA		
CD45 [b]	Fwd: CACTGGGAATCGAGAGGAAA	574	55
	Rev: CTGGTCTGGATGGCACTTTT		
CD90 [b]	Fwd: GGTCTACATGTGCGAGCTGA	471	56
	Rev: AAAGCTAAGGGGTGGGAGAA		
CD44 [c]	Fwd: CATCGTTGCTGCCCTCCT	134	55
	Rev: ACCGCTACACTCCACTCTTCAT		

[a] (Regassa and Kim, 2013)
[b] (Khatri et al., 2009)
[c] (Bai et al., 2013)
[d](Gabriel et al., 2003)
[e](Usui et al., 2008)
[f](Sławińska et al., 2013)

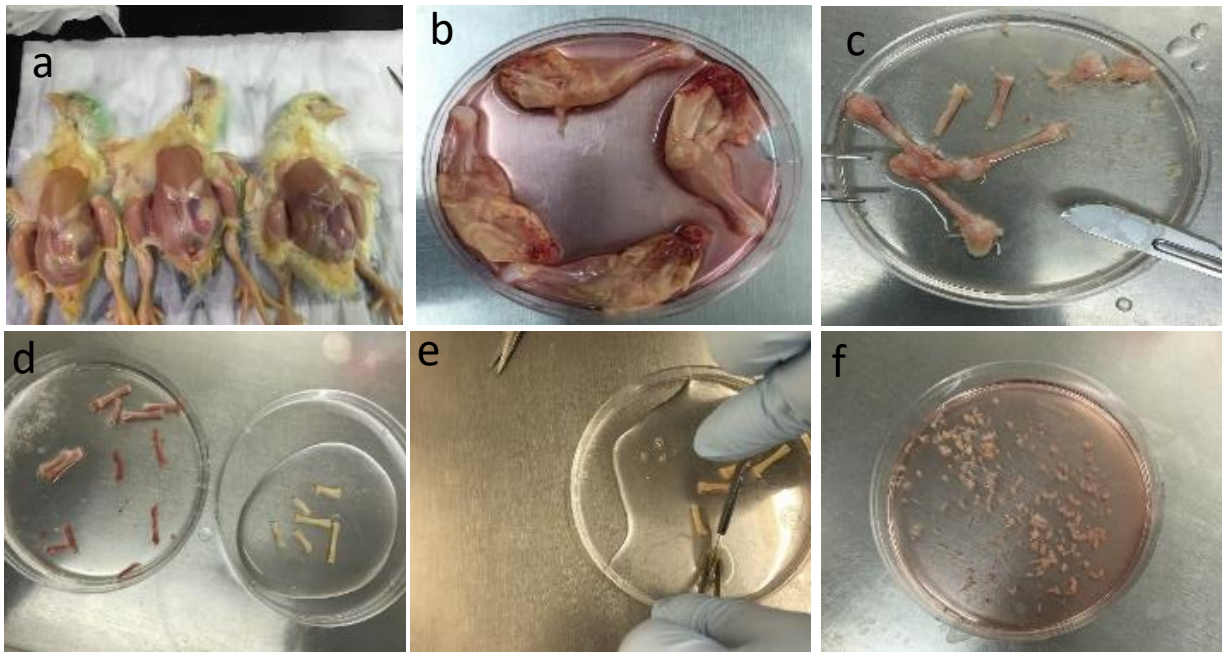


Figure 3.1: Isolation procedure of MSCs from compact bones of chick (cBMSCs). (a) Chick soaked in alcohol for leg dissections (b) legs dissected and soaked in DMEM with 10% FBS. b) Muscles were separated to obtain femur and tibia. (d) Epiphysis was dissected and bone marrow was flushed with PBS containing 2% FBS. (e) After washing, the bones appeared whitish in color. (f) Bones were chopped in 1-3 mm^3 and digested in digestion media.

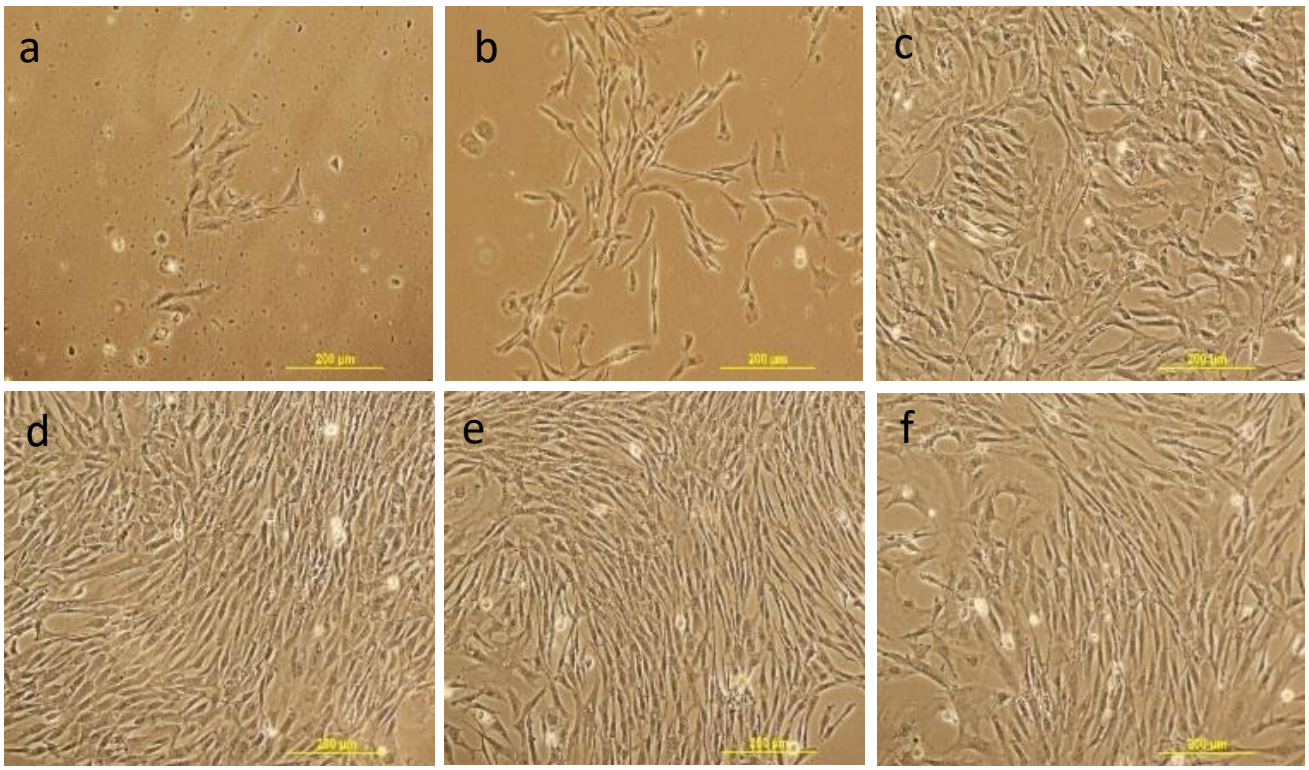


Figure 3.2: Representative diagram of cell morphology at different stages and passage of chicken. MSCs isolated from compact bones. (a) passage 0; day 4, (b) passage 0; day 8, (c) passage 2, (d) passage 4, (e) passage 6, and (f) passage 8.

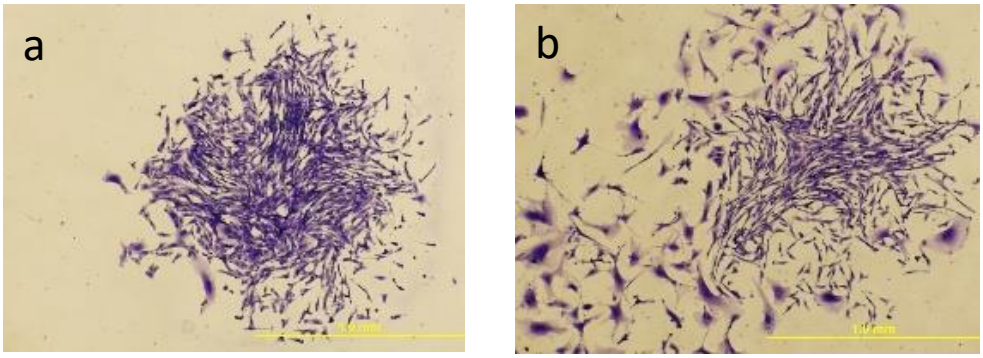


Figure 3.3: Colony forming assay. Formation of the colony from chicken compact bone-derived MSCs stained with 1% crystal violet in methanol.a) Passage 4, b) passage 8.

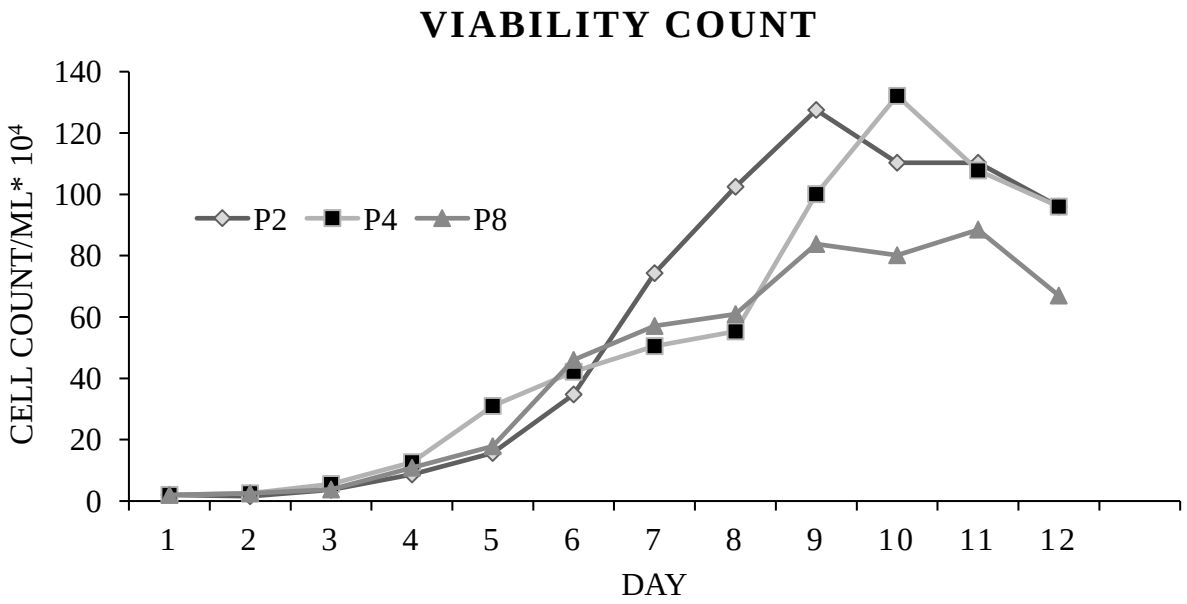


Figure 3.4: Growth curve of the isolated chicken BMSCs.Growth curve of P2, P4, and P8 were all sigmoidal in shape that showed latent phase, log phase, and a plateau phase

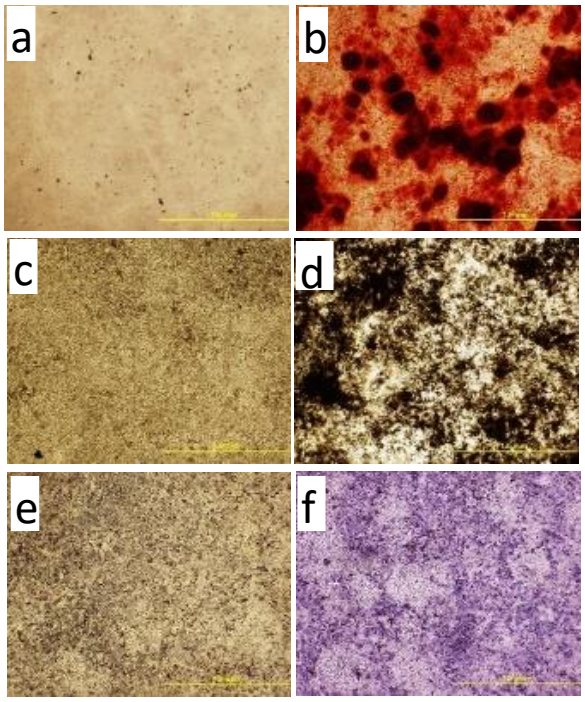


Figure 3.5: Multilineage differentiation potential of cBMSCs.On confluency, cBMSCs were treated with osteogenic media (OM) containing DMEM with 10-7M dexamethasone (DXA) (Sigma-Aldrich, MO, USA), 10 mM β-glycerophosphate, 50 µg/ml ascorbic acid, and 5% FBS for osteogenic induction. Cells cultured in DMEM basal media with 10% FBS were used as negative control. Both cells were stained with Alizarin red stain and Von Kossa stain at day 14. Alizarin Red stain in (a) control cells (b) OM treated cells. Von Kossa stain in (c) control cells and (d) OM treated cells. Alkaline Phosphate assay in (e) control cells and (f) OM treated cells.

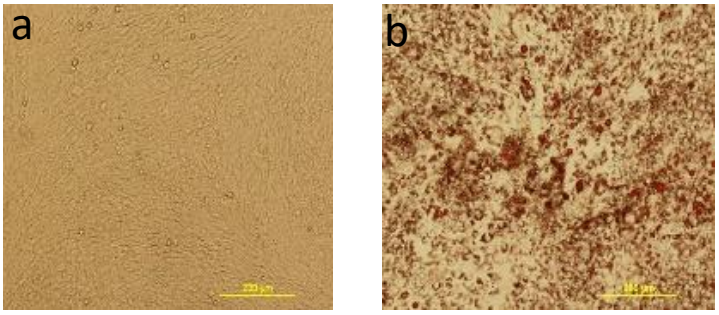
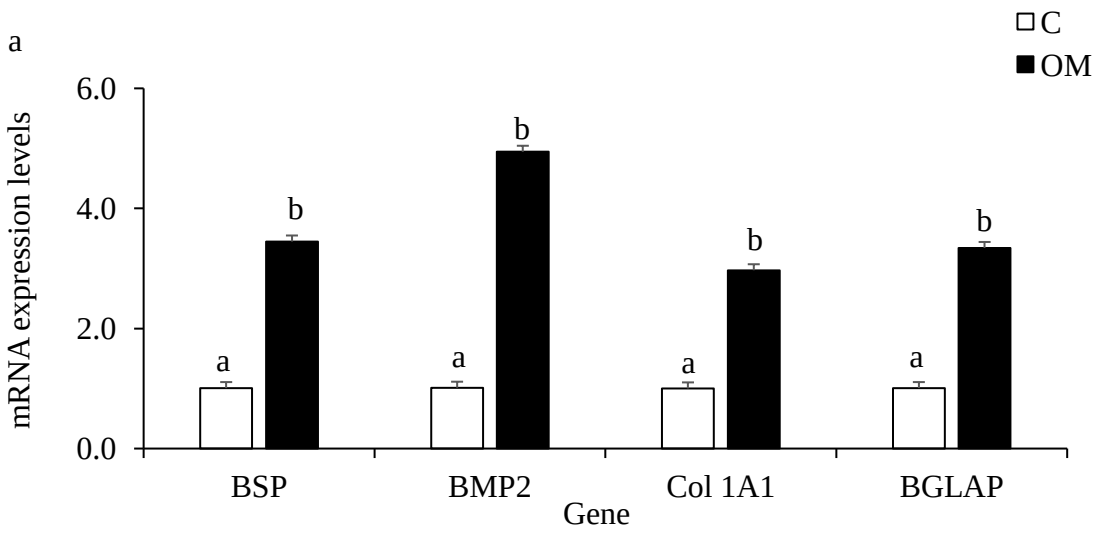


Figure 3.6: Adipogenic differentiation of cBMSCs.cBMSCs were treated with an adipogenic cocktail (DMI containing 500 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 20 mg/mL insulin) and 300µM of OA when cells were confluent. Cells cultured in 10% DMEM were used as negative control. Differentiation of adipocyte was detected by Oil Red O stain in (a) induced cells vs (b) control cells at 96 h.

a


C
OM
mRNA expression levels
6.0
4.0
2.0
0.0
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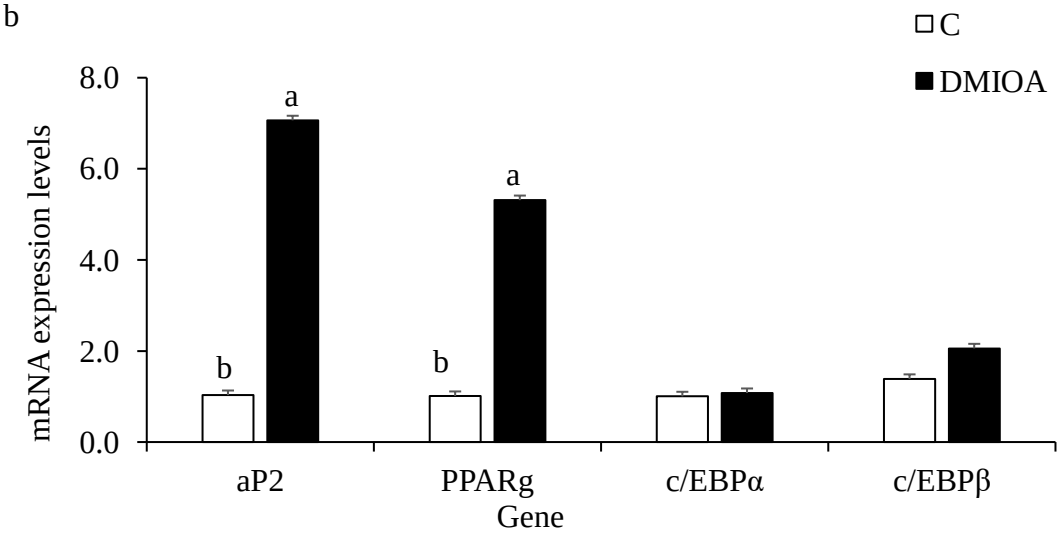
b
a
b
a
b
a
b
BSP
BMP2
Col 1A1
BGLAP
Gene

b


C
DMIOA
mRNA expression levels
8.0
6.0
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a
b
a
aP2
PPARg
c/EBPα
c/EBPβ
Gene

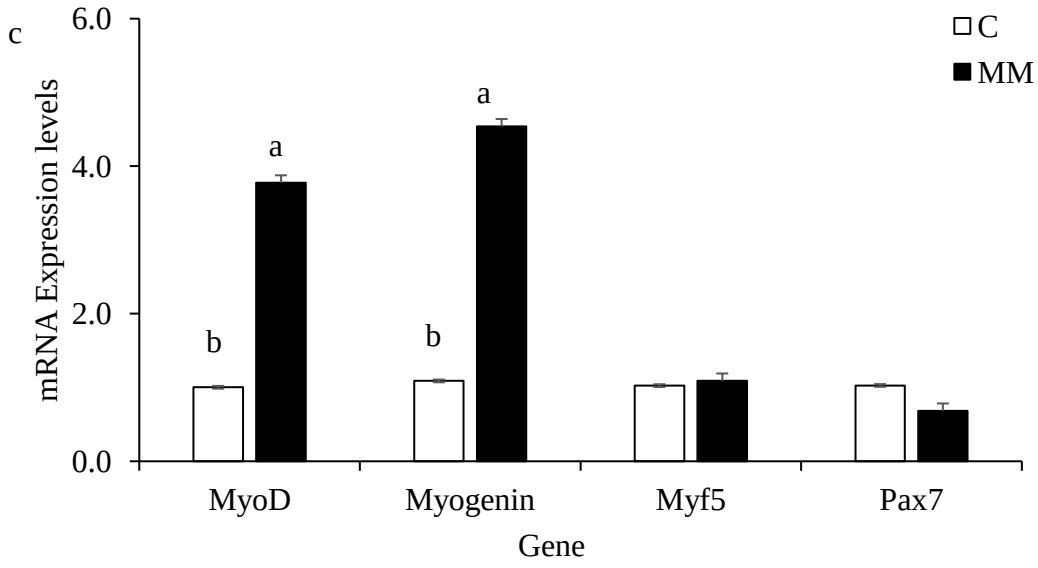

Figure 3.7: Comparative analysis of lineage differentiation specific gene expression in cBMSc.Relative gene expression of osteogenic, adipogenic and myogenic markers were analyzed between induced cells and control cells using qRT-PCR. (a) BSP, Collagen Type 1 Alpha 2 (Col 1A2), BMP2, and BGLAP mRNA expression were analyzed in cells treated with osteogenic media (OM) and control media (C) at 72h post treatment. (b) FABP2, PPARγ, c/EBPα, and c/EBPβ expression were analyzed in cells treated with adipogenic media and control media after 48h post treatment (c) MyoD, Myogenin, Myf5, and Pax7 mRNA expression were analyzed in cells treated with myogenic media and control media for myogenic differentiation at 72h post treatment. GAPDH was used as a housekeeping gene.

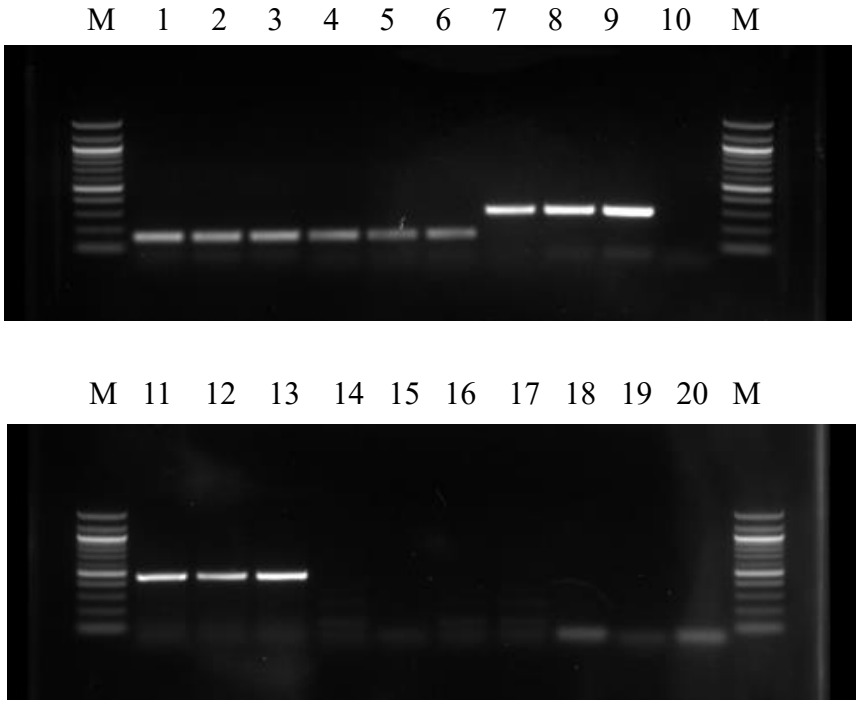

Figure 3.8: Qualitative expression of cell surface markers of cBMSCs were detected by RT-PCR in all three passage (P2, P4, and P8).High level of expression of cell surface markers CD90, CD105, CD44, CD29 and GAPDH as well as lack of expression of CD 45 and CD34 mRNA in MSCs indicates that the cells were homogenous population or mesenchymal stem cells. M- Marker; Lane 1- P2 GAPDH; Lane 2- P4 GAPDH; Lane 3- P8 GAPDH; Lane 4- P2 CD44; Lane 5- P4 CD44; Lane 6- P8 CD44; Lane 7- P2 CD29; Lane 8- P4 CD29; Lane 9- P8 CD29; Lane 10- Control; Lane 11- P2 CD90; Lane 12- P4 CD90; Lane 13- P8 CD90; Lane 14- P2 CD34; Lane 15- P4 CD34; Lane 16- P8 CD34; Lane 17- P2 CD45; Lane 18- P4 CD45; Lane 19- P8 CD45; and Lane 20- Control.

CHAPTER 4

EFFECT OF 20(S)-HYDROXYCHOLESTEROL ON MULTILINEAGE DIFFERENTIATION OF MESENCHYMAL STEM CELLS ISOLATED FROM COMPACT BONES OF CHICKEN2

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ABSTRACT

Mesenchymal stem cells (MSCs) are common progenitors of different lineages such as osteoblasts, adipocytes, and myocytes. Specific oxysterols have shown to be pro-osteogenic and anti-adipogenic in mouse and human MSCs. To determine the effect of 20(S)-hydroxycholesterol (20S) on osteogenic, adipogenic, and myogenic differentiation, mesenchymal stem cells isolated from compact bones of broilers (cBMSCs), were subjected to a various doses of 20S and markers of lineage-specific mRNA was analyzed using real-time PCR and cell cytochemistry. Further studies were conducted to evaluate the molecular mechanism involved in the lineage-specific differentiation pathway. Like human and mice, 20S oxysterol induced pro-osteogenic, pro-myogenic, and anti-adipogenic differentiation potential in cBMSCs. 20S oxysterol-induced markers of osteogenic genes and myogenic regulatory factors when cBMSCs were treated with differentiation medium. 20S oxysterol also suppressed the expression of adipogenic marker genes when cBMSCs were treated with OA, an adipogenic activator of cBMSCs. To elucidate the molecular mechanism by which 20S exerts its differentiation effects in all 3 cell lineages, we focused on the hedgehog signaling pathway. The hedgehog inhibitor, cyclopamine, completely reversed the effect of 20S induced osteogenic and anti-adipogenic mRNA expression. However, there was no change in mRNA expression of myogenic genes when cBMSCs were treated with cyclopamine. Results show that 20S oxysterol promotes osteogenic and myogenic differentiation and decrease adipocyte differentiation of cBMSCs. In addition, these studies show that induction of osteogenesis and inhibition of adipogenesis in cBMSCs by 20S is mediated through hedgehog signaling mechanism. These results indicated that 20(S) could play an important role in the differentiation of chicken-derived MSCs and could be used as an intervention strategy to regulate skeletal, myogenic, and adipogenic differentiation. The current results provide a rationale for

further study on the regulatory mechanisms of bioactive molecules on the differentiation of MSCs from broilers which could help address skeletal problems in poultry.

Key Words: adipogenesis, broilers, mesenchymal stem cells, myogenesis, osteogenesis, 20(s)-hydroxycholesterol, hedgehog signaling

INTRODUCTION

Modern Broiler meat type chicken have grown in its size tremendously compared to its ancestors in last 60 years. However, with advanced genetic selection and an increase in production efficiency, several welfare problems such as an increase in leg and skeletal disorder, woody breast, high fat mass are challenges for broiler industry (Bailey et al., 2015; Julian, 1998; Knowles et al., 2008). Genetic selection has provided an efficient increase in growth rate and meat yield every year; however, support system such as skeletal and cardiovascular system have not kept up with the increasing rate and body mass, making them susceptible to several welfare and economic problems in the poultry industry (Dibner et al., 2007). Similarly, laying hens and breeders suffer from osteoporosis, cage layer fatigue, keel bone damage, resorption of cortical bone, increase in medullary bone mass and high abdominal fat deposition during the course of egg production (Bain et al., 2016; Nasr et al., 2013). Modern laying hen strains have a negative calcium balance as soon as they enter laying phase (Neijat et al., 2011). There is a heavy demand of calcium during egg formation and rapid turnover of medullary bones during egg production (Kim et al., 2012). Such processes increases osteoporosis, brittle bone, breakage of keel bone, tibial dyschondroplasia and other skeletal problems in laying hens and breeders (Regmi et al., 2016; Whitehead and Fleming, 2000). Change of housing system and the introduction of the aviary and enriched cage system has increased skeletal problems such as keel bone fracture in modern laying hens (Heerkens et al., 2016; Nicol et al., 2006; Petrik et al., 2015). Bone problems are still among the most costly disorder to poultry industry both in meat and egg type birds as they result in reduced feed intake, decrease weight gain, lower egg production, decrease production efficiency, increase lameness and reduced paw quality, increase the frequency of downgrades and condemnations when affected birds are processed. Bone abnormality is reported to be initiated by developmental, degenerative, nutritional, environmental and infectious

problems and could be due to a combination of one or more of these cause (Dibner et al., 2007). Mesenchymal Stem cells derived from chicken compact bones (cBMSCs) are multipotent cells which can be a potential source/model to understand and address the skeletal and adipogenic health issues associated with broilers, laying hens and breeders. Osteogenic and adipogenic cells are reported to have opposite regulations. Reduction in the numbers of osteoblastic cells, increase in bone resorption by osteoclast, and shift of osteoblast towards adipogenic cells in aging and osteoporotic cells could have caused the shift in skeletal problems (Chan and Duque, 2002). It has been reported that the number of adipocyte cells increase with a decrease in osteoblasts and proliferation capacity of osteoprogenitor cells in various cases of osteoporosis (Nuttall and Gimble, 2000; Stenderup et al., 2003). It has been suggested that bone loss in age-related osteoporosis could be due to shifting of mesenchymal stem cells (MSCs) differentiation into adipogenic pathway rather than osteogenic pathway (Muruganandan et al., 2009; Takada et al., 2009).

MSCs are multipotent cells with high proliferation and differentiation capabilities that can differentiate into different tissue lineages such as adipocytes, osteoblasts, chondrocytes, myocytes, and fibroblast (Barry and Murphy, 2004; Bellotti et al., 2016; Pittenger et al., 1999; Sanjurjo-Rodriguez et al., 2017). MSCs are isolated from different tissue origin such as bone, adipose tissue, cord blood, placenta, and many other tissues (Fellows et al., 2016; Friedenstein et al., 1987; Hoffmann et al., 2016; Pittenger et al., 1999; Sanjurjo-Rodriguez et al., 2017; Weiss and Troyer, 2006). MSCs isolated from bone have been reported to differentiate into several different progenitor cells, of which osteoblast and adipocytes share a potential reciprocal relationship in differentiation (Kassem et al., 2008; Park et al., 1999). Increase in loss of bone formation and increase in adipogenesis in the bone marrow could lead to increase the risk of

osteoporosis and bone fracture (Jilka et al., 1996). Decrease in bone synthesis, alteration in bone turnover due to reduced osteoblast formation from progenitor cells, and increase in adipocytes formation at the expense of those osteoblasts due to age or disease could lead to fragile bone and fracture (Nuttall et al., 1998; Singh et al., 2016). Differentiation fate of MSCs population is determined by the interplay of complex extracellular mediators such as hormones, growth factors, nutrients, and pharmacological agents that affect the molecular mechanism and signaling pathways of lineage-specific transcription factors. Regulation of stem cell fate down these various lineages is essential for tissue development, homeostasis, and repair (Oh et al., 2014). MSCs derived from human and mouse has been used as a useful tool for the study of mineralization and differentiation potential of MSCs towards osteogenic lineage. MSCs has been isolated from chicken bone marrow, lungs, cartilage, adipose tissue in previous studies (Bai et al., 2013; Gong et al., 2011; Khatri et al., 2009; Khatri et al., 2010; Li et al., 2015b). However, there is a limited information to understand the multilineage differentiation potential and pathways of cBMSCs when subjected to possible osteogenic/adipogenic bioactive compounds.

Oxysterols are oxygenated derivatives of cholesterol oxidation, a 27-carbon molecule present in the circulation in human and animal tissue (Bjorkhem et al., 2002). Oxysterol is involved in many different physiological, biological, and pathological roles in the animal body such as cholesterol efflux, lipoprotein metabolism, calcium uptake, atherosclerosis, and apoptosis (Kim et al., 2007). It has been reported that oxysterol plays a possible role of regulating cellular differentiation of MSCs. Different oxysterol such as 20 (S)-hydroxycholesterol (20S), alone or in combination with 22 (S) or 22 (R)-hydroxycholesterol exerts pro-bone and anti-fat effects in M2-10B4 mouse bone marrow stromal cells (Kha et al., 2004). Further studies have described that 20S oxysterol stimulated differentiation of pluripotent bone marrow stem cells derived from

mouse and humans through activation of hedgehog signaling invitro (Kim et al., 2010). Some oxysterols are potential bioactive molecules and are capable of suppressing adipogenic differentiation in mouse and human MSCs (Moseti et al., 2016). Oxysterol are derivatives of endogenous cellular cholesterol biosynthesis pathway and this pathway are important in osteogenic differentiation of progenitor cells, thus oxysterol could act as an osteoinductive compound (Aghaloo et al., 2007; Parhami et al., 2002). Based on studies on mouse and human MSCs, studies can be conducted to understand the effect of oxysterol, a potential bioactive compound on multilineage differentiation of cBMSCs.

Research on the skeletal health of poultry has only focused on Ca, P, and vitamin D metabolism. There is a lack of research on understanding the combination of developmental, regenerative and nutritional aspect to address the skeletal health of poultry. Research on the effect of different potential bioactive compounds such as 20S in differentiation and the pathways involved in differentiation of cBMSCs can help to address the skeletal and adipogenic issues in poultry. The aim of the study was to understand the effect of 20S on osteogenic, adipogenic, and myogenic differentiation potential of cBMSCs and examine the possible role of the hedgehog signaling pathway in multilineage differentiation of cBMSCs.

MATERIAL AND METHODS

Isolation of Compact bone derived Mesenchymal stem cells

All experiments and procedures involving the birds were performed following the guidelines for the use of animal in research stated by the Animal Care and Use Committee at the University of Georgia. MSCs were obtained from the compact bones of the tibia and femur from day old broiler chicken following a modified protocol as described earlier in MSCs derived from mouse compact bone (Zhu et al., 2010). Two broiler chicks were aseptically dissected and the

legs were collected. Bone marrow was flushed with phosphate buffered saline (PBS) containing 2% FBS (Fisher Scientific, USA), and compact bone was chopped into small pieces. Bones were digested with 10 ml Dulbecco's Modified Eagle's Medium (DMEM), (Mediatech Inc, USA) digestion buffer containing 0.25% collagenase (Sigma-Aldrich, USA) and 20 % FBS for 55 min in a shaker at 180 rpm and 37°C. Contents were filtered in 100 mm sterile nylon meshes (Fisher scientific, USA) and centrifuged at 1,200 rpm for eight min. The supernatant was discarded, and cell pellets were suspended in 10 ml DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and L-glutamate (Mediatech, USA). Cells were then seeded into 100 mm petri plates (BD Bioscience, USA) and cultured at 37°C in a 5% CO_2 incubator. Half of the medium was replaced by fresh media at 12 h, complete media was changed at 24 h to remove the non-adherent cells. After that, media were replaced every three days until confluent.

Cell Culture

Cells were expanded in 10 ml DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and L-glutamate. Once cells were confluent, cells were washed twice with 5 ml PBS, trypsinized with 1X Trypsin Ethylenediaminetetraacetic acid (TE) (Mediatech, USA) at 37°C for two min. Eight ml of 10% DMEM media was added to the petri plate, cells were washed to remove any adhere cells and transferred to a centrifuge tube. The contents were centrifuged at 1,200 rpm for eight min, the supernatant was discarded, and cell pellets were plated at a rate of 20,000 cells/cm^2. All experiments were conducted between passage 4 to 5. Series of experiments were conducted to understand the effect of 20S in the osteogenic, myogenic, and adipogenic differentiation of cBMSCs isolated from day-old broiler compact

bones. Confluent cBMSCs were plated in 6-well plates and treated with different levels of assigned treatments when confluent. All experiments were repeated twice to validate the results.

Osteogenic Differentiation of cBMSCs

Two experiments were conducted to understand the effect of 20S on osteogenic differentiation of cBMSCs. In the first experiment, at passage four, cBMSCs once confluent were treated with five treatments; 1) Control (C), 2) osteogenic media (OM) containing DMEM with 10% FBS, 50ug/ml ascorbate, 0.5µM dexamethasone (DXA), and 10mM β-glycerophosphate, 3) OM + 2.5µM 20S, 4) OM + 5µM 20S, and 5) OM + 10µM 20S. Cells treated in 6-well plates were harvested at 24 h, 72 h, and 7 d to understand osteogenic mRNA transcripts using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cell cytochemistry (Von Kossa and Alizarin Red) were conducted in cells cultured in 24-well plate (Greiner bio-one, USA) at 7 d and 14 d to observe osteogenic differentiation of cells.

The second experiment was conducted to understand if Hh was involved in the molecular mechanism of osteogenic differentiation of cBMSc when treated with 20S. Cells when confluent were pre-treated with control vehicle (Veh) or with 4µM of Hedgehog (Hh) signaling pathway inhibitor cyclopamine (Cyc) for 2 h followed by four treatments for each pre-treated group; 1) C, 2) osteogenic media (OM) containing DMEM with 5% FBS, 50ug/ml ascorbate, 0.1µM DXA, and 10mM β-glycerophosphate, 3) OM + 10µM 20S, and 4) 10µM 20S alone, which makes a total of eight treatments. Cells treated with or without Cyc in 6-well plates were harvested at 24 h and 72 h to understand osteogenic mRNA through qRT-PCR.

Adipogenic Differentiation of cBMSCs

The third experiment was conducted to understand the dose effect of 20S in the adipogenic differentiation of cBMSCs. Cells confluent at passage 4 were treated with five treatments; 1) control (C), 2) 300µM Oleic Acid (OA), 3) OA + 2.5 µM 20S, 4) OA + 5µM 20S, and 5) OA + 10µM 20S for 12, 24, 48, and 96 h. Cells cultured in 6-well plates were harvested for adipogenic mRNA expression of adipogenic transcripts using qRT-PCR at 12 h, 24 h, 48 h, and 96 h. Cells were stained with Oil Red O staining to visualize lipid deposition at 48 h.

The fourth experiment was conducted to evaluate whether the anti-adipogenic effects of 20S are mediated through the hedgehog signaling pathway. Cells when confluent were pre-treated with Veh or Cyc for 2 h. Each pretreated group of cells was treated with C, 300µM OA, 10µM 20S, and 300µM OA + 10µM 20S for 24 h and 48 h. Samples in 6-well plates were harvested 24 h and 48 h to understand osteogenic mRNA transcripts using qRT-PCR.

Myogenic Differentiation of cBMSCS

The fifth experiment was conducted to understand the effect of the 20S in the adipogenic differentiation of cBMSCs. cBMSCs at passage four were treated with five treatments 1) C, 2) myogenic medium (MM) containing DMEM with 5% Horse Serum, 50µM Hydrocortisone, and 0.1µM DXA, 3) MM + 2.5 µM 20S, 4) MM+ 5µM 20S, and 5) MM + 10µM 20S. Cells in 6-well plates were harvested for adipogenic mRNA expression of adipogenic transcripts using quantitative reverse transcription polymerase chain reaction (qRT-PCR) at 24 h, 72 h, 7 d, and 14 d.

The sixth experiment was conducted to evaluate the involvement of Hh signaling pathway in myogenic differentiation of cBMSCs when treated with the 20S. Cells were pre-treated with Veh or with Cyc for 2h. Each pre-treated group of cells was treated with C, MM, 10µM 20S, and MM + 10µM 20S treatment for 24 h, and 72 h. Samples in 6-well plates were

harvested to analyze myogenic mRNA transcripts using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Oil Red O stain

Oil Red O stain was performed on harvested cells at 48 h to detect adipocytes differentiation following a previously described protocol (Kim et al., 2007; Parhami et al., 1999). In brief, cells were washed and rinsed with PBS and fixed in 60% isopropanol for two min. Cells were stained with 0.4% Oil Red O stain in 100% isopropanol for 30 min and rinsed with double distilled H2O. After staining, cells were visualized and photographed under a microscope for red lipid droplets.

Alizarin Red

Alizarin Red test was used to detect mineralization of osteoblasts which gives a bright orange-red color when exposed to the Alizarin red solution (Sigma-Aldrich, USA). The Alizarin red test was conducted following a modified protocol as previously described (Gregory et al., 2004). In brief, Alizarin Red Stain solution was prepared to dissolve 1 gm of Alizarin Red S in 50 ml distilled water and pH was adjusted to 4.1 - 4.3 with 0.1% NH_4OH solution. Cells were fixed using 10% buffered formalin for 30min and washed 4 times with distilled water. Cells were stained with Alizarin Red solution for 45min in dark. Cells were washed to remove the excess dye; PBS was added after washing and observed in a microscope for calcified orange/red staining.

Von Kossa Stain

Von Kossa stain was used to detect the osteogenic differentiation and calcium deposition in differentiated cells. Matrix mineralization in cell monolayers was detected by silver nitrate

staining as previously described (Parhami et al., 1997). In brief, cells were washed twice with PBS, treated with 1% glutaraldehyde, and incubated for 15 mins. Cells were washed with deionized water, and 5% silver nitrate was added to the cells. The cells were incubated in dark for 30 min, washed twice with double distilled water, air dried, and exposed to bright light until black spots are developed in the areas of calcification.

Quantification of mRNA Expression using qRT-PCR

At the specific time point of each experiment, cells were harvested and total RNA was isolated using Qiazol reagent (Qiagen, MD, USA) following manufacturer's protocol. Two µg of RNA was reverse-transcribed using high capacity cDNA reverse transcription synthesis kits (Applied Biosystem, USA) following the manufacturer's protocol. Primers for each gene were designed and checked for target identity using the National Centre for Biotechnology Information (NCBI). To understand the possible alteration in expression of specific transcripts in the harvested cells, the qRT-PCR analysis was performed using nuclease-free water, the forward and reverse primers of each specific target gene, template cDNA, and SYBR Green (Bio-Rad, USA) using StepOne^{TM} Real-Time PCR systems (Applied Biosystems, USA). Temperature cycles were as follows: 95 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds, annealing temp for 20 seconds, and extension 72 °C for one minute. All analyses were done in duplicate reaction, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. After 40 cycles of qRT-PCR, melt curves were examined to ensure primer specificity. Fold changes in gene expression were calculated using the -ΔΔCt method and reported as fold changes of the expression of the target genes in experimental groups compared to the control group. Details of primers sequences use for the experiment are presented in Table 4.1.

Statistical Analysis

Data obtained were analyzed using the general linear model procedure of the Statistics Analysis System (SAS) Institute. Tukey test was used to measure the mean separation when statistically significant. Results were analyzed and presented as means ± SEM. The level of significance used in all three studies was probability value of $P < 0.05$.

RESULTS

Oxysterol Induced Osteogenic Differentiation of cBMSCs

To elucidate the effects of 20S on the osteogenic differentiation of MSCs, we examined the effects of oxysterol on osteogenic differentiation of MSCs using cell cytochemistry and gene expression analysis. Cells treated with 20S induced higher proportion of Alizarin Red (Figure 4.1) and Von Kossa stain (Figure 4.2) which are mineralization markers compared to cells treated with OM and C. 10µM of 20S significantly increased Bone Sialoprotein (BSP), Bone morphogenic protein (BMP2), Collagen Type I Alpha 2 Chain (Col1A2), Sonic Hedgehog (Shh), and Runt-related transcription factor 2 (RunX2) expression compared to other treatment groups at 24 h (Figure 4.3A, B, D, G, H). The expression of Gli 1 was higher in cells treated with 10µM 20S compared to control cells and cells treated with osteogenic media at all time points of the study (Figure 4.3C). Significant increase in patched (Ptch) mRNA expression was observed in cells treated with OM and all levels of 20S at 24 h compared to control cells (Figure 4.3E). However, the inclusion of 20S did not increase Ptch expression compared to cells treated with OM at 24 h. At 72 h and 7 d, cells treated with 10µM of 20S significantly increase expression of Pathc mRNA compared to cells treated with other treatments (Figure 4.3E). Bone Gamma-carboxyglutamic acid-containing protein (BGLAP) mRNA expression was significantly increased in cells treated with 10µM of 20S at 7 d (Figure 4.3F). OM and all level of 20S

significantly increased mRNA expression of Shh at 7d. However, there was no significant difference between cells treated with OM only and 20S (Figure 4.3G). Results indicated that 10μM 20S could induce a stimulatory effect on the osteogenic differentiation of cBMSCs.

Role of Hh Signaling in Osteogenic differentiation of cBMSCs

To determine if Hh signaling molecular mechanism are involved in regulation of osteogenic differentiation in MSCs by oxysterol, 20S was added to the cells to understand osteogenic differentiation pretreated with vehicle only or with cyclopamine. Expression of markers of osteoblastic differentiation were examined after 24 h and 72 h of treatment with 20S. Cells treated with 20S + OM significantly increased mRNA expression of Hh target genes Gli, Shh, and Pthc at 24 h in Vec pre-treated cells (Figure 4.4 E, F, G). Other osteogenic genes RunX2, BMP2, BSP, and BGLAP were also significantly increased in cells treated with OM + 20S compared to control cells in vehicle pre-treated cells at both time points of the study (Figure 4.4 A, B, C, D). Cyclopamine completely reversed the effect of OM+20S on the expression of osteogenic differentiation marker genes, RunX2, BMP2, BSP, BGLAP, Gli1, Patch, and Shh at 24h post treatment (Figure 4.4 A1, B1, C1, D1, E1, F1, G1). Cyclopamine continued to block the expression of BSP, BGLAP, and Gli1 mRNA expression in cells treated with OM+20S up to 72 h of the study (Figure 4.4 C2, D2, E2).

Effects of 20S on adipogenic differentiation of cBMSCs

To understand the effect of oxysterol in the adipogenic differentiation of cBMSCs, we examined the effects of adipogenic genes mRNA expression on cells treated with a different dose of 20S in adipogenic media. Cells treated with OA increased adipocyte formation detected by oil red O staining whereas inclusion of 20S decreased the number of oil red O staining in the cells as

quantified by light microscopy (Figure 4.5). Cells treated with OA alone significantly increased Peroxisome Proliferator-Activated Receptor Gamma (PPARγ), Fatty Acid-Binding Protein (FABP4), Lipoprotein Lipase (LPL), and CCAAT/Enhancer Binding Protein beta (C/EBPβ) mRNA expression at the different time point of the study. OA induced expression of PPARγ expression at 12 h, 24 h and 48 h of the study (Figure 4.6A), FABP4 expression at 48 h and 96 h of the study (Figure 4.6B) and C/EBPβ (Figure 4.6D) at 12 h of the study compared to the control cells. The inclusion of oxysterol decreased expression of OA induced adipogenic mRNA in cBMSCs at different time points of the study. PPARγ mRNA expression was significantly decreased in cells treated with all three levels of 20S compared to OA treated cells at 12, 24, and 48 h of study (Figure 4.6A). Cells treated with 10μM of oxysterol significantly decreased mRNA expression of FABP4 compared to cells treated with OA at 48 and 96 h of treatment (Figure 4.6B). Cells treated with 10μM 20S significantly decreased mRNA expression of C/EBPβ at 12 h compared to cells treated with OA alone (Figure 4.6D). Results indicated that inclusion of 20S could reduce adipogenic differentiation of cBMSCs.

The role of Hh Signaling Mechanism in Adipogenic Signaling Pathway.

In response to the previous reports in mouse and human MSCs about the importance of hedgehog signaling mechanism of oxysterol in osteogenic differentiation and anti-adipogenic potential (Kha et al., 2004), we examined if the Hh signaling pathway played a role in anti-adipogenic effects of 20S in cBMSCs. OA treated cells significantly increased expression of FABP4 (Figure 4.7 A2), PPARγ (Figure 4.7 B1), CEBPβ (Figure 4.7 D1) at the different time point of the study. The inclusion of 20S blocked the expression of FABP4 (Figure 4.7 A2), PPARγ (Figure 4.7 B1), CEBPβ (Figure 4.7 D1) mRNA transcripts in the cells harvested at same time points. Cells pre-treated with cyclopamine significantly increased expression of FABP4

(Figure 4.7 A2), PPARγ (Figure 4.7 B1), CEBPβ (Figure 4.7 D1), and LPL (Figure 4.7E1) at 24 h and FABP4 (Figure 4.7 A2) at 48 h which was blocked by 20S treatment in Veh pre-treated group (Figure 4.7). This study indicates that cells treated with cyclopamine completely abolished the inhibitory effects of 20S and increased adipogenic mRNA gene expression of FABP4, c/EBPβ, PPARγ, and LPL at 24 h and FABP4, and PPARγ mRNA expression at 48 h of study.

Effect of 20S in myogenic differentiation of cBMSCs.

In addition to the effect of osteogenic and adipogenic differentiation on cBMSCs, we examined the effect of 20S on myogenic differentiation potential of cBMSCs. 5μM and 10μM of 20S increased MyoD mRNA expression at 72 h of treatment compared to control cells (Figure 4.8 A). Inclusion of 20S oxysterol increased myogenic factor 5 (Myf5) mRNA expression at 24 h and 72 h of treatment compared to the control cells (Figure 4.8 B). 2.5μM and 5μM of 20S increased Myogenin mRNA expression compared to control cells at 24h of the study. Cells treated with 5 μM and 10 μM of 20S significantly increased mRNA expression of paired box 7 (Pax7) compared to control cell at 72 h of the study. However, expression of myogenic genes treated with oxysterol were decreased to basal level by 7 d of study.

The role of Hh signaling mechanism in the myogenic signaling pathway.

To determine if Hh signaling pathway was involved in oxysterol-induced myogenic differentiation of cBMSCs, cells were pre-treated with Cyc or Veh for 2 h before subjected to myogenic media and 20S. In cells subjected to combination of myogenic media and oxysterol, there was no significant difference in expression of MyoD (Figure 4.9 A1, A2), Myf5 (Figure 4.9 B1, B2), Myogenin (Figure 4.9 C1, C2) and Pax7 (Figure 4. 9 D1, D2) mRNA expression between cells pre-treated with or without cyclopamine. However, pre-treatment with

cyclopamine increased expression of Pax7 mRNA in cells treated with MM alone and expression of Myf5 mRNA in cells treated with 20S alone at 24 h time points compared to the same group of cells pretreated with Veh. The inclusion of cyclopamine decreased expression of MyoD mRNA at 72h of harvest compared to Veh pretreated cells treated with myogenic media only. This study indicates that oxysterol-induced myogenic expression of cBMSCs is not mediated by Hh signaling pathway.

DISCUSSION

Primary cBMSCs has the multilineage capacity and can differentiate into osteogenic, adipogenic and myogenic lineage per their specific cues they are subjected to. In this study, osteogenic media, adipogenic media and myogenic media induced cBMSCs to osteogenic, adipogenic, and myogenic differentiation of cells respectively which indicates the primary cells cultured from the compact bone of chicken are capable of multilineage differentiation. MSCs isolated from mouse and human compact bones have similar multilineage differentiation capacity and adhered to the surface easily (Freeman et al., 2015; Pittenger et al., 1999). Multilineage differentiation potential and capacity to adhere to the plastic surface under standard culture medium are two of the three minimum criteria that The Mesenchymal and Tissue Stem Cell Committee of the international Society for Cellular Therapy (ISCT) has published for the cells to be defined as MSCs (Dominici et al., 2006).

The present study clearly demonstrates that 20S have potential to induce osteogenic and myogenic differentiation and reduce adipogenic differentiation of cBMSCs. This is the first study conducted to understand the ability of oxysterol to regulate osteogenic, adipogenic and myogenic differentiation of chicken compact bone derived stem cells. Our data strongly suggest that 10μM 20S can induce osteogenic differentiation of cBMSCs. Alizarin and VK assay not

only confirm the cellular commitment towards osteoblastic differentiation pathway but also provides a clear evidence of functional mature cell (Li et al., 2015a; Parhami et al., 1997). In our study, cells treated with 10µM of 20S showed higher deposition of calcium on Von Kossa and alizarin red assay compared to control cells and cells treated with OM alone. Mineral deposition begins during extracellular matrix maturation and is an indicator of the late stages of osteogenic differentiation of MSCs (Kim et al., 2010).

In the present study 10µM of 20S increased expression of osteogenic genes such as BSP, BMP2, col1A2, and RunX2. Run X2 is a key osteogenic gene that indicates the early commitment of MSCs to osteochondrogenic progenitor cells. RunX2 directs MSCs to differentiate into pre-osteoblast and inhibits adipogenic and chondrogenic differentiation maintaining the supply of immature osteoblast. (Jeon et al., 2006). Immature osteoblast express a high level of osteopontin whereas BGLAP, Col1a1, and BSP are measured in high levels when differentiated to mature osteoblasts. (Komori, 2006). These types of induced cells show positive Von Kossa stain and Alizarin Red assay invitro (Komori, 2006).

A combination of 2 naturally occurring oxysterols 20S and 22S induced osteoinductive effects in pluripotent mouse marrow stromal cell line M2-10B4 (Dwyer et al., 2007; Richardson et al., 2007). Similarly, oxysterol 133 was able to induce expression of RunX2, ALP, BSP, and BGLAP mRNA in C3H10T1/2 mouse embryonic fibroblasts and in M2-10B4 mouse marrow stromal cells (Montgomery et al., 2014). In the same study oxysterol 133 increased bilateral posterolateral lumbar spinal fusion of L4-L5 in Lewis rats whose transverse process were decorticated with high-speed burr. Similarly, oxysterol 49, a novel oxysterol analog induced osteogenic properties similar to those of BMP2 in rabbit bone marrow stromal cells (Hokugo et

al., 2013). These findings from the mineralized assay and osteogenic gene expression indicate that oxysterol is a potent bioactive molecule to induce osteogenic differentiation in cBMSCs.

Apart from the osteogenic property of oxysterol, it is also reported that oxysterol possesses anti-adipogenic effects in mouse and human pluripotent mesenchymal stem cells (Kha et al., 2004). In this study, OA significantly increased PPARγ and c/EBPβ as early as 12 h of the study, which then increased expression of FABP4 at 48 and 96h. OA has been reported to increase adipogenic differentiation in chicken derived MSCs rather than using DMI alone which is an adipogenic cocktail used in mammalian cells differentiation (Matsubara et al., 2008; Matsubara et al., 2005). In our study, the inclusion of 20S along with OA in the cBMSCs suppressed the expression of PPARγ, c/EBPβ, and aP2 mRNA expression that was induced by OA. This indicates that PPARγ and c/EBPβ could regulate the adipogenic differentiation of cBMSCs and use of oxysterol could have suppressed the adipogenic differentiation by suppressing the effect of PPARγ or c/EBPβ. It has been previously reported that Novel oxysterol compounds have potential to inhibit adipocyte differentiation and decrease expression of adipogenic marker genes such as PPARγ in mouse and human derived progenitor cells (Kha et al., 2004; Kim et al., 2007). PPARγ is previously described as a key regulator of adipogenic differentiation (Tontonoz et al., 1994). In early adipogenesis, expression of c/EBPβ and c/EBPδ induced the expression of PPARγ and c/EBPα which then regulates the positive feedback mechanism and induce other adipogenic genes for adipogenic differentiation. PPARγ is also thought to play a role in the regulation of bone metabolism. Embryonic stem cells derived from homozygous PPARγ -deficient mice did not show adipogenic differentiation when treated with troglitazone, whereas the expression of RunX2 and osteocalcin gene was greatly increased (Akune et al., 2004). Moreover, heterozygous PPARγ -deficient mice exhibited increased

osteogenesis of cortical and trabecular bone and decreased adipocyte compared to wild types *in-vivo* and in the bone marrow progenitors of those mouse *in-vitro* (Akune et al., 2004; Sun et al., 2013). However, when PPARγ expression construct was transfected into murine derived bone marrow stromal cells and activated with thiazolidiones, the cell lines increased adipocytes differentiation and decreased expression of RunX2 and osteocalcin gene expression (Lecka-Czernik et al., 1999). This indicates that PPARγ could be a key player in the adipogenesis of cBMSCs and use of oxysterol could have suppressed the expression of PPARγ thus suppressing the adipogenic differentiation of cBMSCs.

The result here demonstrates that 20S is a novel oxysterol compound that can influence the adipogenic and the osteogenic differentiation of cBMSCs. In previous studies, it has been reported that oxysterols are novel activators of the Hh signaling pathway that influences the osteogenic and the adipogenic differentiation of mouse and human derived MSCs (Dwyer et al., 2007). Because 20S has been found to increase osteogenic and decrease adipogenic markers genes in our study, we further investigated the molecular mechanism by which it exerts its function on cBMSCs. Our data further provides evidence that 20S could direct the osteoinductive and anti-adipogenic effects through activation of the hedgehog signaling pathway. This was demonstrated by an increase in Hh target mRNA expression such as Gli1, Ptch, and Shh when cBMSCs were treated with 20S along with OM. Another evidence is the reduction of osteogenic markers mRNA expression such as Gli1, Pthc, BSP, RunX2, BGLAP, and BMP2 mRNA expression in cBMSCs induced by 20S when subjected to hedgehog pathway inhibitor, cyclopamine pre-treatment. Pre-treatment of cyclopamine decreased the osteogenic mRNA expression of cBMSCs caused by 20S compared to the mRNA expression of cBMSCs pre-treated with Veh alone. Cyclopamine continued to suppress the expression of osteogenic

differentiation markers until 72 h of the study which suggests that the activation of Hh signaling pathway is prominent in the pro-osteogenic mechanism by which 20S regulates osteogenic differentiation of MSCs.

To elucidate the molecular mechanism by which 20S inhibits the expression of adipogenic genes that were expressed in cBMSCs when treated with OA, we focused on the Hh signaling pathway, which also has a role in osteogenic differentiation of cBMSCs. Treatment of cBMSCs with 20S completely inhibited adipocyte formation that was induced by OA. Our result showed that use of cyclopamine reversed the inhibitory effects of 20S and induced adipogenic gene expression of PPARγ, c/EBPβ, and aP2 mRNA expression in cells treated with OA and 20S. This indicates that 20S could block PPARγ promoter activity promoted by C/EBPβ through Hh signaling mechanism. Cyclopamine significantly blocked the inhibition of adipocyte formation and adipogenic mRNA expressions caused by 20S, which suggests that the 20S oxysterol regulates the anti adipogenic differentiation of cBMSCs through Hh signaling pathway. Consistent with our findings in cBMSCs, 20S has been reported to inhibit PPARγ expression and adipogenic differentiation of M2-10B4 murine pluripotent bone marrow stromal cell lines through Hh-dependent mechanism (Kim et al., 2007). Similarly, Shh signaling pathway was reported to be involved in reducing the adipogenic differentiation of C3H10T1/2 pre-adipocyte cells by reducing expression of c/EBPα and PPARγ transcription factors (Spinella-Jaegle et al., 2001).

The hedgehog signaling pathway plays a key role in a variety of embryonic development, growth, and patterning of tissues, postnatal development and maintenance of tissue/ organ integrity and function, stem cell physiology, cancer and cardiovascular disease(Kim et al., 2010). There are three members of Hh family Indian Hh, Sonic Hh and Desert Hh. Hh signaling

involves a complex and array of factors that affects and influence the function and distribution of Hh molecules. Hedgehog signaling begins with a hedgehog protein binding to a transmembrane protein Patch which removes the inhibitory effects on another transmembrane protein Smo in Hh responsive cells (Gupta et al., 2010). Smo activates the intracellular signaling cascade that results in activation of Gli transcription factors, which transcribe the Hh target genes, Gli-1 and Patch (Dwyer et al., 2007). This transcription regulation involves the Ci/Gli transcription factors and intricate interaction between membranes of a complex accessory molecule, including Fused, suppressor of fused and Rab 23 to regulate localization and stability of Gli (Gupta et al., 2010). Previous experiments in mice reported that lacking Indian Hh resulted in the disorder of endochondral bone patterning and osteoblast formation and those mice lacking sonic hedgehog showed disorders in craniofacial bones, vertebral column, and calcified ribs (Chiang et al., 1996; St-Jacques et al., 1999). Previous studies have also reported the lineage-specific osteogenic differentiation of MSCs is controlled by Hh signaling mechanism (Nakamura et al., 1997; Wang et al., 2017). Furthermore, there are several reports of other bioactive compounds that stimulate osteogenic differentiation of MSCs derived from human and mice which regulate osteogenic and adipogenic differentiation through Hh signaling (Lin et al., 2016; Yuasa et al., 2002).

Previous studies have reported that different forms of oxysterol such as oxy 49, oxy 133, 20S, and 22S induced osteogenic expression and reduced adipogenic expression potential of MSCs derived from mouse and human cells through Hh signaling mechanism (Dwyer et al., 2007; Hokugo et al., 2016; Johnson et al., 2011; Kim et al., 2010; Kim et al., 2007; Li et al., 2015a; Montgomery et al., 2014). Further studies in mouse and human MSCs has indicated that in addition to activating Hh signaling pathway, oxysterol could induce osteogenic differentiation of mouse and human stromal cells through a Wnt signaling related, Dkk-1-inhinitable and PI3-

kinase mechanism (Amantea et al., 2008), through activation of HES-1 and HEY-1 expression, and through LXR activation pathway (Kim et al., 2010). Studies in mouse NIH-3T3 fibroblast, C3H10T1/2 MSCs, and 3T3-L1 preadipocytes have shown that Hh signaling blocks the early step of adipogenesis, upstream of PPARγ, presumably after mitotic clonal expansion by regulating Gata expression (Suh et al., 2006). This indicates that the anti-adipogenic effect through Hh signaling could be at least in part by regulating Gata expression.

Several *in vitro* studies have demonstrated that MSCs are capable of interconversion between osteoblast and adipocyte phenotype *in vitro* (Song and Tuan, 2004). It has also been reported that mature osteoblasts could undergo adipogenesis under appropriately modified conditions (Nuttall et al., 1998). Also, mature adipocytes have been differentiated along osteoblastic pathway under appropriate culture condition. Several studies provide an inverse relation between osteoblast and adipocyte differentiation. TAZ increased the transcription of RunX2 dependent gene and decreased PPARγ thus promoting osteogenesis and reducing adipogenesis (Hong et al., 2005). Similarly, Dlk1 has been reported to be involved in regulatory effects of both osteoblast and adipocyte differentiation of human derived MSCs (Abdallah et al., 2004). It has also been described that oxysterols effects on cyclooxygenase and phospholipase A2 and also acts in synergy with bMP2 in inhibiting adipogenesis and inducing osteogenic differentiation (Kha et al., 2004). It has been previously described that 20S could induce a pro-osteogenic effect on MSCS by Hh signaling through activating Notch target genes Hes-1, HEY-1, and HEY-2 expression in bone marrow stem cells (Kim et al., 2010).

Notch target genes are involved in the various biological process including osteogenesis, adipogenesis, and myogenesis. We also studied if 20S has any significant effect on the myogenic transformation of MSCs through Hh signaling pathway. In our study, we demonstrated that 20S

oxysterol significantly increased myogenic expression of MyoD, Myogenin, MyF5, and Pax7 mRNA expression. The previous study in human umbilical cord-derived MSCs showed MyoD and Myogenin were expressed as early as 3 days after incubation with myogenic media but were not expressed after two weeks of culture (Gang et al., 2004). Myogenic differentiation of MSCs occurs via activation of myogenic transcription factors, PAX3, MyoD, Myf-5, and Myogenin (Braun and Arnold, 1996; Gang et al., 2008). Pax3 and Pax7 are a master regulator of myogenic differentiation and contributes to early striated muscles development during skeletal muscles differentiation (Yin et al., 2013). Cells pre-treated with cyclopamine did not decrease the expression of myogenic transcription factors indicating that Hh signaling does not play roles in controlling the myogenic regulation expressed by 20S oxysterol.

Skeletal myogenesis is the developmental cascade that involves the regulatory MyoD gene family that determines the progress of multipotent mesodermal stem/progenitor cells into myogenic lineage (Jalali Tehrani et al., 2014). Myogenesis is regulated by a family of transcription factor including MyoD, Myogenin, Myf5, and MRF4 (De Bari et al., 2003a; Sabourin and Rudnicki, 2000). Skeletal muscles stem cells are composed of multinucleated myofibers that are established during embryogenesis by fusion of myogenic cells also called satellite cells (Yablonka-Reuveni et al., 2008). Satellite cells are considered as precursors for muscles growth and repair. Typically, the myofiber nuclei are mitotically inactive but they are activated in response to muscles damage and provide progeny for myofiber repair and growth. Thus, satellite cells exhibit stem cell-like properties and are competent to form the basal origin of muscles regeneration (Collins et al., 2005). Myogenesis of satellite cells is regulated by muscles-specific regulatory factors such as MyoD, Myf5, and Myogenin. Mouse satellite cells and myogenic cell lines express the Myf5 protein in proliferating cells, whereas the protein was not

detected upon differentiation and fusion with myotubes(Lindon et al., 1998). Expression of MyoD is observed during satellite cell proliferation, whereas their differentiation is marked by expression of Myogenin expression with a decrease in PAx7 and Myf5 (Lindon et al., 1998; Zammit et al., 2006). Skeletal myogenesis is a developmental cascade, where MyoD family and myogenic regulatory factors directly regulate myocyte cell specification, differentiation and express at the early stage of myogenic differentiation (De Bari et al., 2003b; Gang et al., 2004). Mutant mice that lack MyoD and dystrophin displayed a significant increase in the severity of myopathy and premature death highlighting the role of MyoD in muscles regeneration (Yin et al., 2013). In previous study treatment of muscles, cells derived stem cells indicates that incubation of satellite cells with 1-25-D3 increased myogenic markers such as MyoD, Myogenin, MYH1 and muscles troponin (Lecka-Czernik et al., 1999).

In summary, this study shows that 20S are a novel oxysterol compound with pro-osteogenic, pro-myogenic and anti-adipogenic properties. Our study further provides evidence that 20S increased the osteogenic differentiation and decreased adipogenic differentiation of cBMSCs by activating Hh signaling pathway. Furthermore, 20S oxysterol also increased the myogenic differentiation of cBMSCs but did not exert its differentiation through Hh signaling pathway. These findings provide the evidence that oxysterols could induce the Hh signaling pathway and therefore could play an important role in osteogenesis, adipogenesis and another developmental process of chicken that are regulated by hedgehog signaling pathway. Further study needs to be conducted to improve understanding of detail molecular mechanism and downstream targets of oxysterol in cBMSCs which could lead to the development of novel oxysterol compounds and intervention therapies targeting MSCs regeneration/lineage

differentiation to tackle skeletal, adipose, and muscles derived economic and welfare related problems in poultry.

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FIGURES

Table 4:1:Primers sequences that were used for qRT-PCR gene expression assay.

Gene	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)	Accession #
GAPDH	Fwd: GCTAAGGCTGTGGGGAAAGT	161	55	(Regassa and Kim, 2013)
	Rev: TCAGCAGCAGCCTTCACTAC			
BSP	Fwd: CAGTGGGAGTACGAGGTGAC	141	55	NM_205162.1
	Rev: CAGTGGGAGTACGAGGTGAC			
Gli1	Fwd: GCACAGCTCCAACGACCGCT	205	57	NM_001305245.1
	Rev: GTTGCCGTCGGAAGCACCCA			
BMP2	Fwd: TCAGCTCAGGCCGTTGTTAG	163	57	NM_204358.1
	Rev: GTCATTCCACCCCACGTCAT			
BGLAP	Fwd: GACGGCTCGGATGCTCGCAG	226	56	(Regassa and Kim, 2013)
	Rev: CAGACGGGGCCGTAGAAGCG			
Ptch	Fwd: GGCGTTCGCGGTGGGACTAC	205	56	NM_204960.2
	Rev: GGTGCTGCCGGAGTGCTTCT			
Shh	Fwd: TGC TAG GGA TCG GTG GAT AG	197	56	NM_204821.1
	Rev: ACA AGT CAG CCC AGA GGA GA			
RunX2	Fwd: ACTTTGACAATAACTGTCCT	192	52	NM_204821.1

	Rev: GACCCCTACTCTCATACTGG			
FABP4	Fwd: TGCTGGGCATCTCAATCACA	106	57	(Regassa and Kim, 2013)
	Rev: GCATTAGTCAGAACGGGCCT			
PPARγ	Fwd: TGAATGTCGTGTGTGTGGGG	229	55	(Regassa and Kim, 2013)
	Rev: GCATTCGCCCAAACCTGATG			
C/EBPα	Fwd: CCTACGGCTACAGAGAGGCT	205	55	(Regassa and Kim, 2013)
	Rev: GAAATCGAAATCCCCGGCCA			
C/EBPβ	Fwd: CCGCTCCATGACCGAACTTA	204	55	(Regassa and Kim, 2013)
	Rev: GCCGCTGCCTTTATAGTCCT			
LPL	Fwd: TGCCCCTATCCGCCTCTCCC	297	57	(Regassa and Kim, 2013)
	Rev: GTTGCAGCGGTAGGCCATGCT			
Col1A2	Fwd: AGAAAGGAATCCAGCCCAAT	237	58	NM_204426.1
	Rev: ACACCTGCCAGATTGATTCC			
MyoD	Fwd: CAGCAGCTACTACACGGAATCA	102	57	(Sławińska et al., 2013)
	Rev: GGAAATCCTCTCCACAATGCTT			
Myogenin	Fwd: AGCAGCCTCAACCAGCAGGA	179	58	NM_204184.1
	Rev: TCTGCCTGGTCATCGCTCAG			
Pax7	Fwd: AGGCTGACTTCTCCATCTCTCCT	156	57	XM_015296832.1
	Rev: TGTAACTGGTGGTGCTGTAGGTG			
Myf5	Fwd: GAGGAACGCCATCAGGTACATC	126	57	NM_001030363.1
	Rev: ACATCGGAGCAGCTGGAGCT			

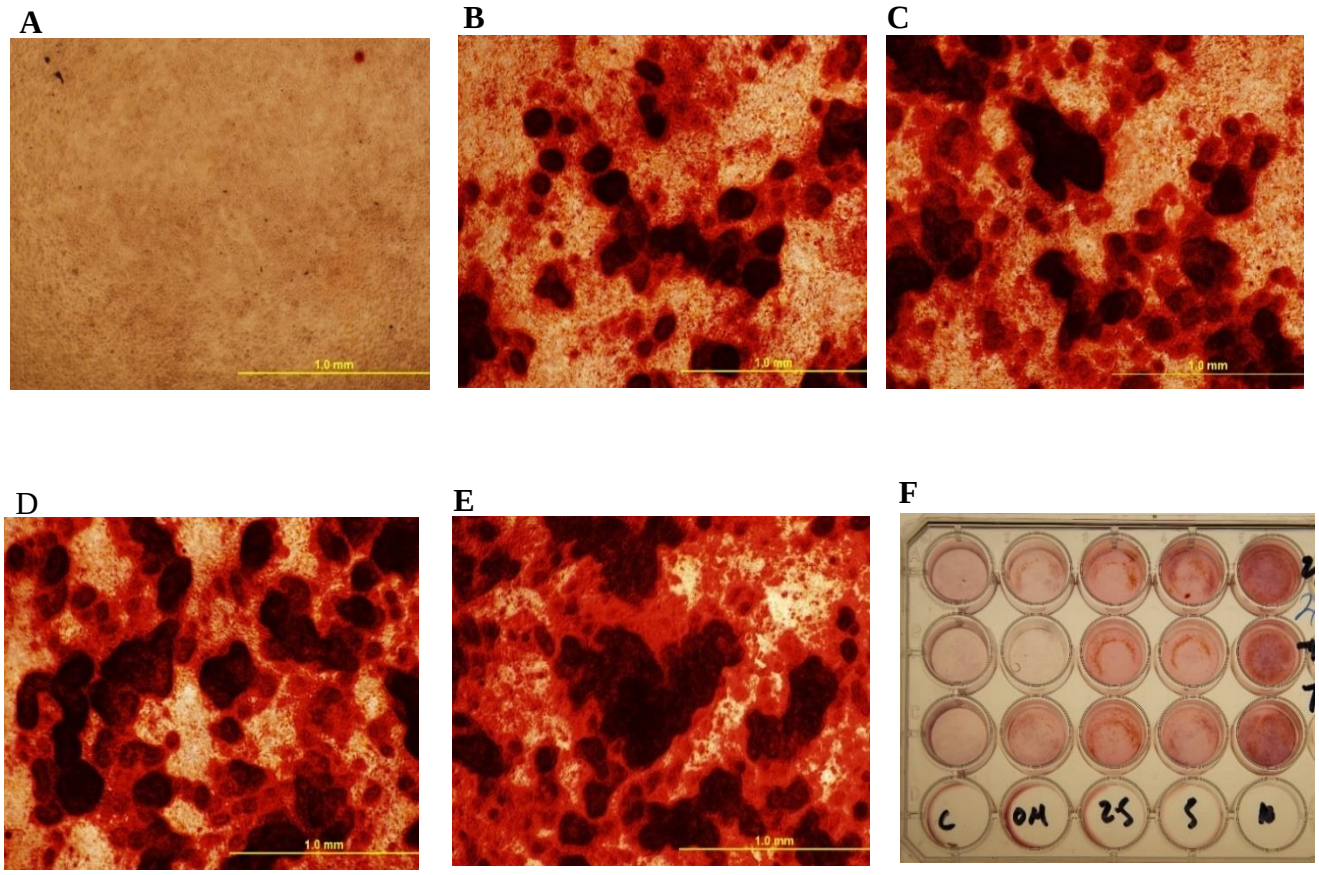


Figure 4.1: Mineral deposition in mesenchymal stem cells isolated from chicken compact bone stem cells treated with different levels of 20S oxysterol for 14 days detected by alizarin red stain. Calcium deposition was increase in cells treated with 20S oxysterol compared to control cells. Cells were treated with A) Control (C), B) osteogenic media (OM) containing DMEM with 10% FBS, 50µg/ml ascorbate, 0.5µM DXA, and 10mM β-glycerophosphate, C) OM + 2.5µM 20S, D) OM + 5µM 20S, E) OM + 10µM 20S,and F) Alizarin red stained plate with different treatments. Pictures were taken by Olympus DP70 at 4X. Scale bar was 1.0mm.

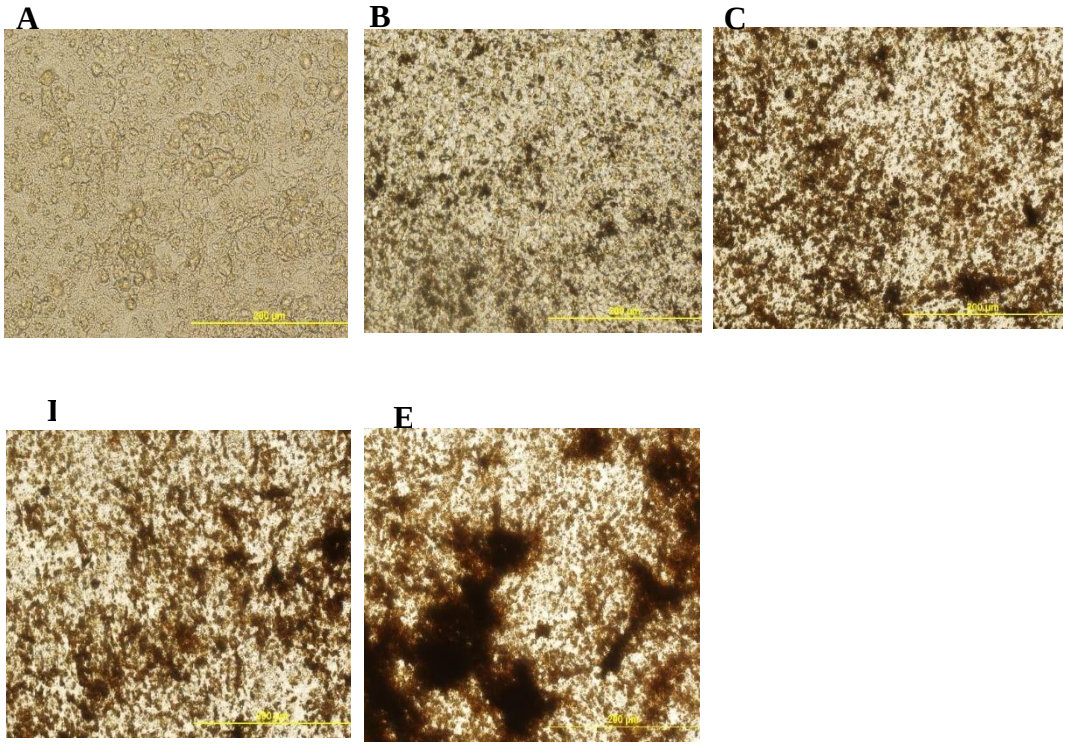
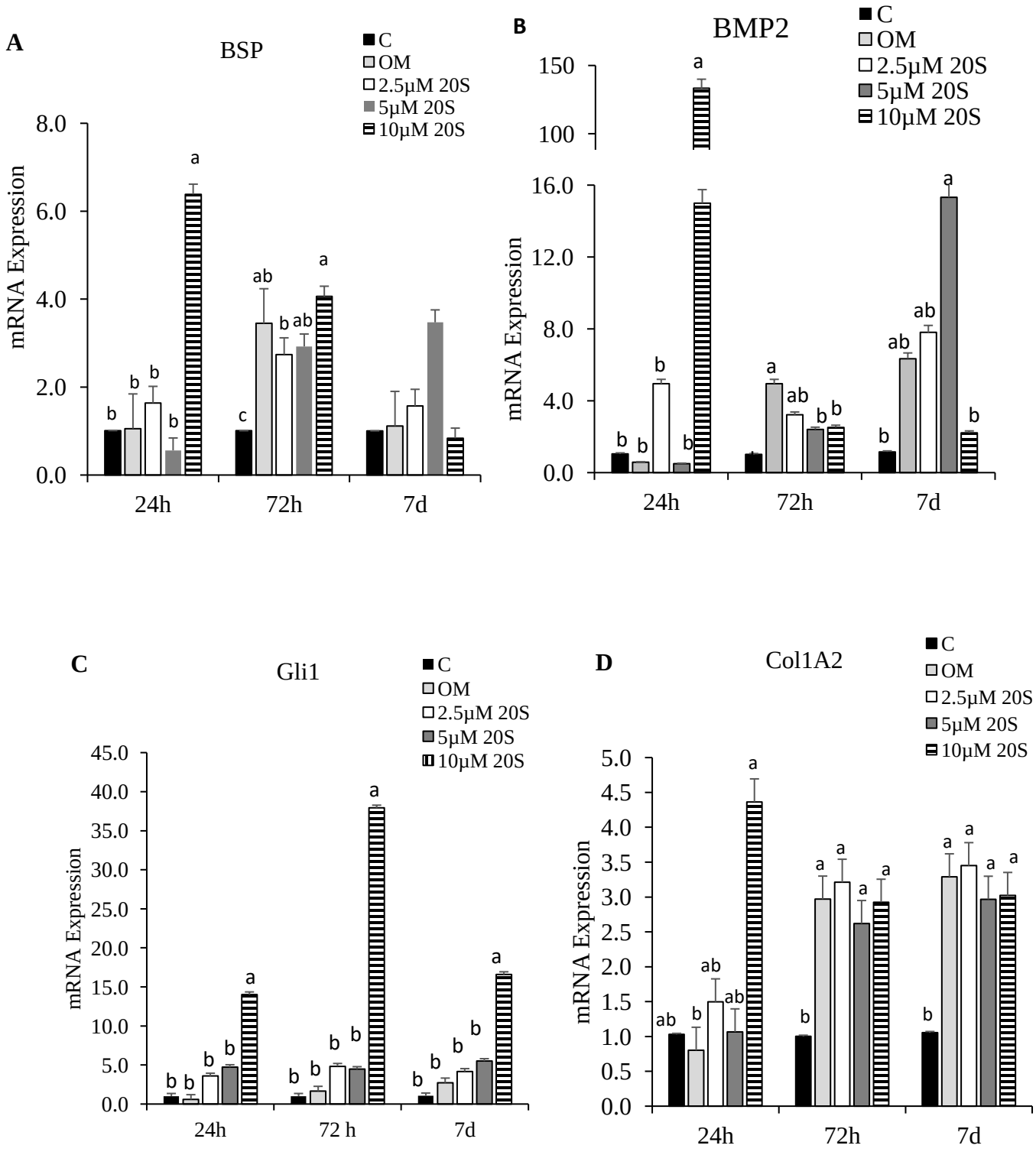
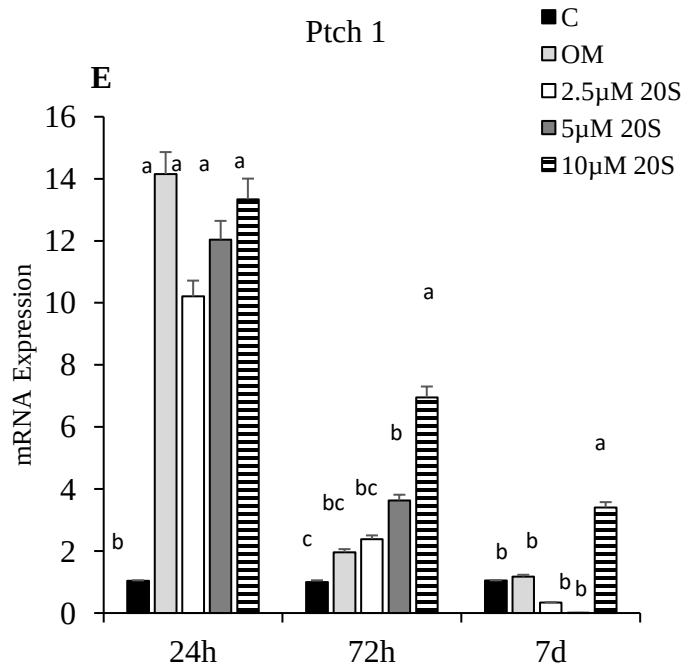
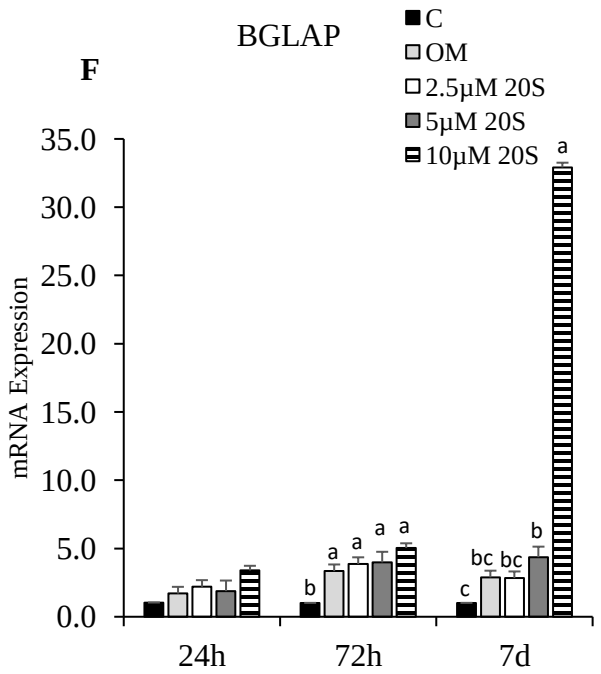
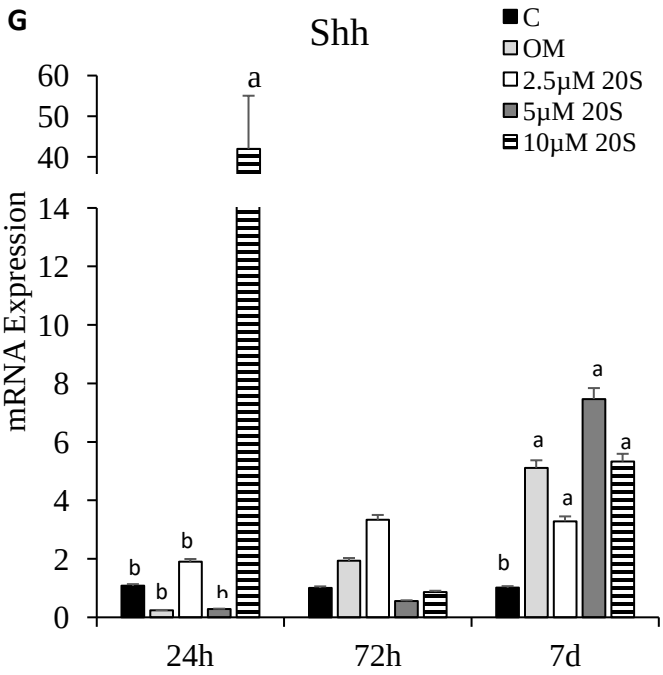


Figure 4.2:Mineral deposition on mesenchymal stem cells isolated chicken compact bone stem cells treated with different levels of 20S oxysterol for 14 days detected by Von Kossa stain. Calcium deposition was increase in cells treated with 20S oxysterol compared to control cells. Cells were treated with A) Control (C), B) osteogenic media (OM) containing DMEM with 10% FBS, 50µg/ml ascorbate, 0.5µM DXA, and 10mM β-glycerophosphate, C) OM + 2.5µM 20S, D) OM + 5µM 20S, and E) OM + 10µM 20S. Pictures were taken by Olympus DP70 at 20X. Scale bar was 1.0mm.


A
BSP
C
OM
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
8.0
6.0
4.0
2.0
0.0
24h
72h
7d
B
BMP2
150
100
16.0
12.0
8.0
4.0
0.0
C
Gli1
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
72 h
D
Col1A2
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5


E
Ptch 1
C
OM
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
16
14
12
10
8
6
4
2
0
24h
72h
7d


F
BGLAP
C
OM
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
24h
72h
7d


G
Shh
C
OM
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
60
50
40
14
12
10
8
6
4
2
0
24h
72h
7d

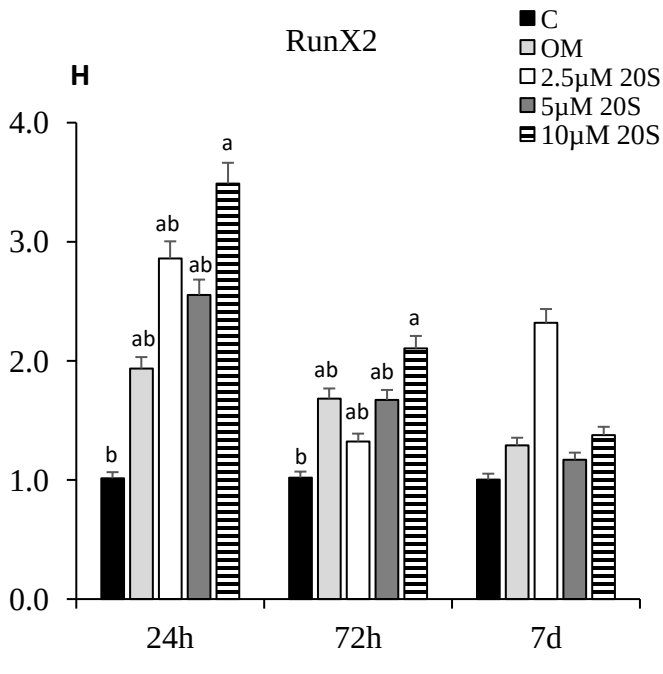
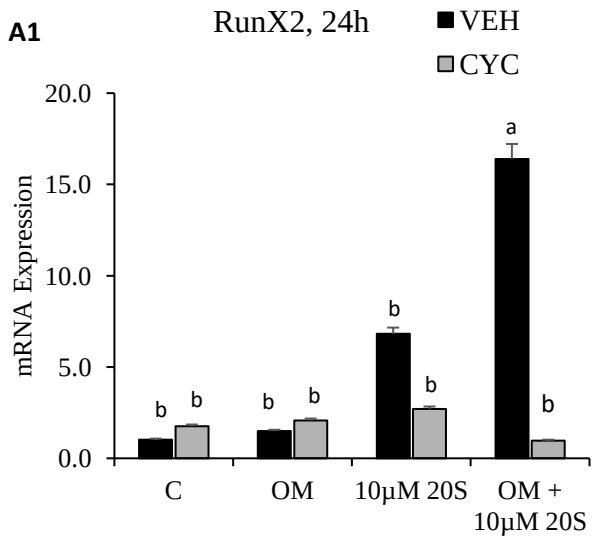
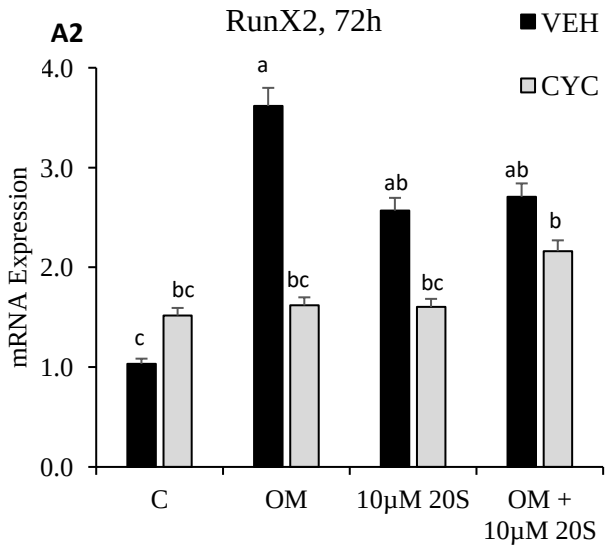
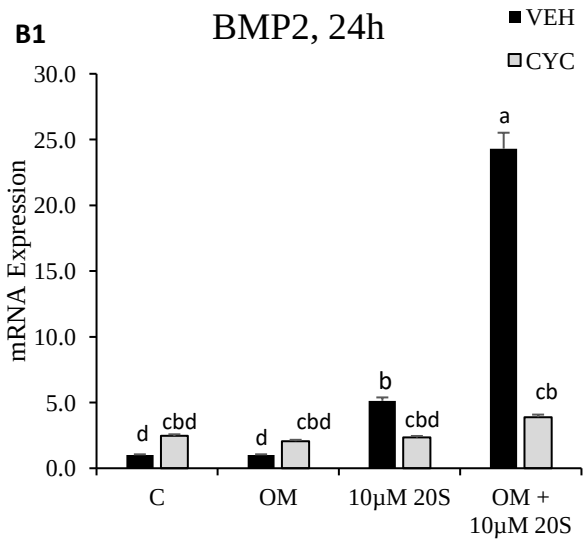
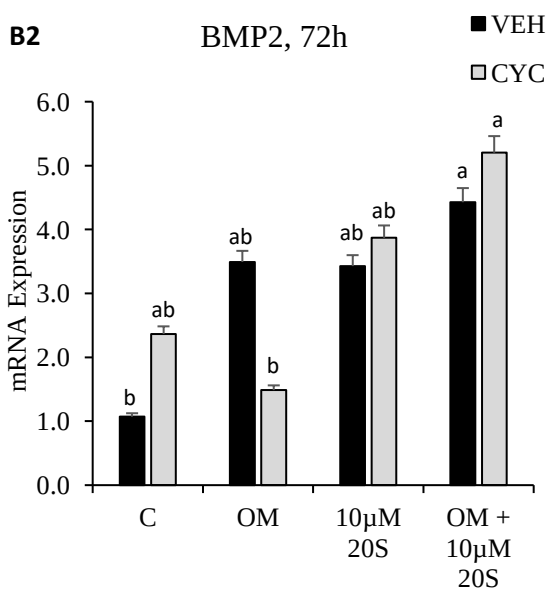

H
RunX2
C
OM
2.5μM 20S
5μM 20S
10μM 20S
4.0
3.0
2.0
1.0
0.0
24h
72h
7d

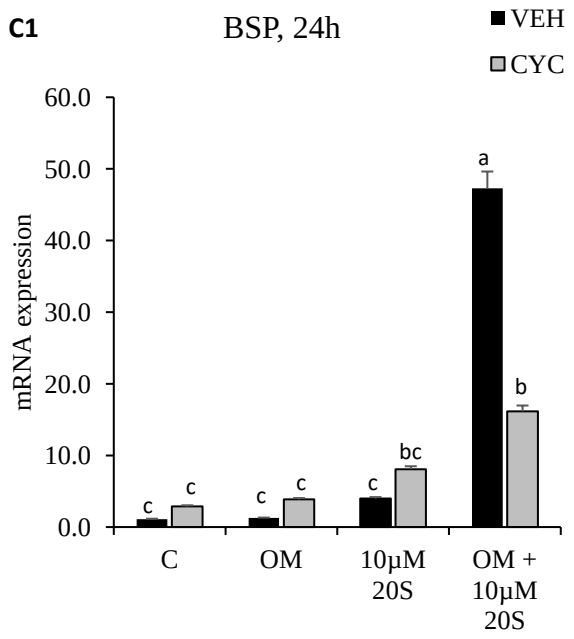
Figure 4.3:Osteogenic effects of 20S oxysterol in mesenchymal stem cells. Relative mRNA expression levels of osteogenic genes A) BSP, B) BMP2, C) Gli-1, D) Col1A2, E) Ptch, F) BGLAP, G) Shh, and H) RunX2 in chicken compact bone derived mesenchymal stem cells (cBMSCs) treated with different levels of oxysterol. cBMSCs were treated with 1) Control (C), 2) osteogenic media (OM) containing DMEM with 10% FBS, 50µg/ml ascorbate, 0.5µM DXA, and 10mM β-glycerophosphate, 3) OM + 2.5µM 20S, 4) OM + 5µM 20S, and 5) OM + 10µM 20S. Cells were harvested at 24h, 72h, and 7d after treatment. RNA was isolated, reverse transcribed to cDNA and qRT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the harvested period are significantly different ($P < 0.05$) when analyzed with Tukey's test.

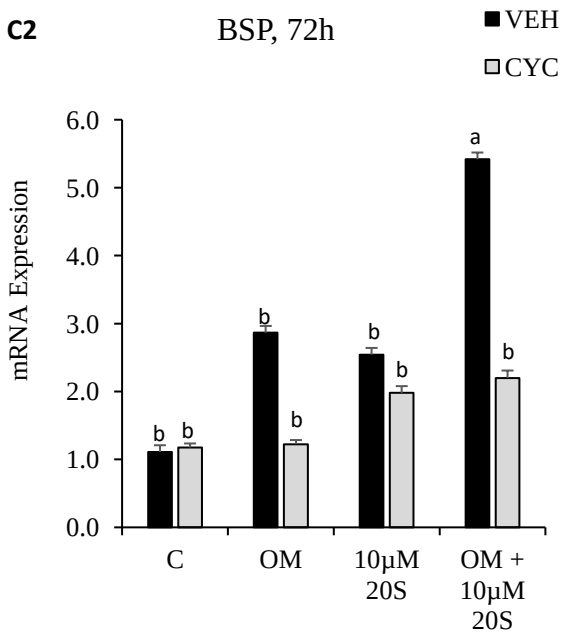

A1
RunX2, 24h
VEH
CYC
mRNA Expression
20.0
15.0
10.0
5.0
0.0
b b b b b a b
C
OM
10µM 20S
OM + 10µM 20S

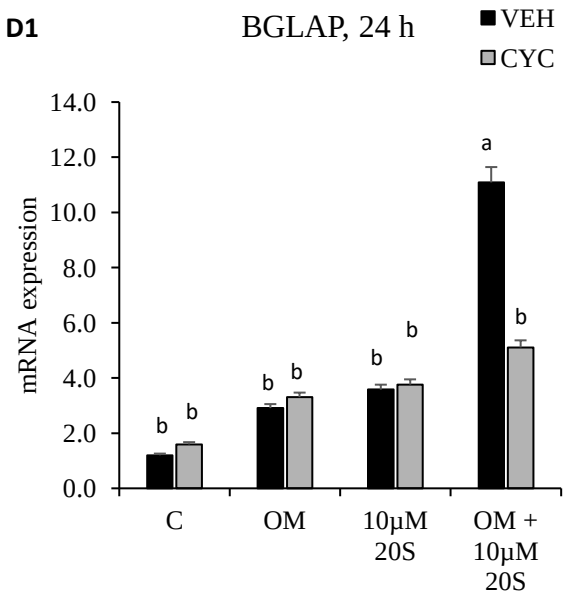

A2
RunX2, 72h
VEH
CYC
mRNA Expression
4.0
3.0
2.0
1.0
0.0
c bc a bc ab bc ab b
C
OM
10µM 20S
OM + 10µM 20S

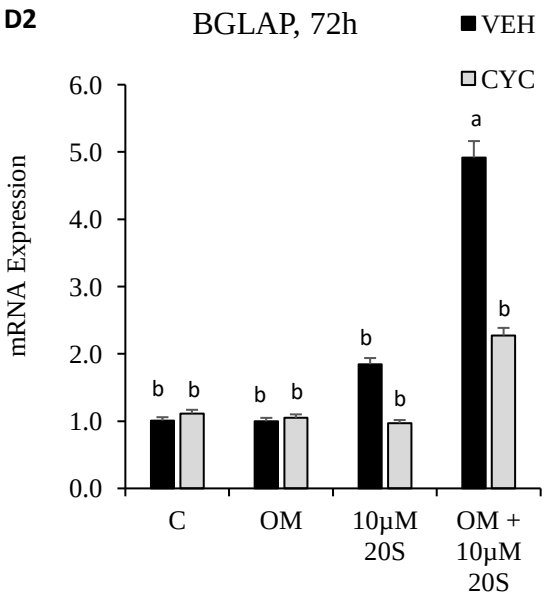

B1
BMP2, 24h
VEH
CYC
mRNA Expression
30.0
25.0
20.0
15.0
10.0
5.0
0.0
d cbd d cbd b cbd a cb
C
OM
10µM 20S
OM + 10µM 20S

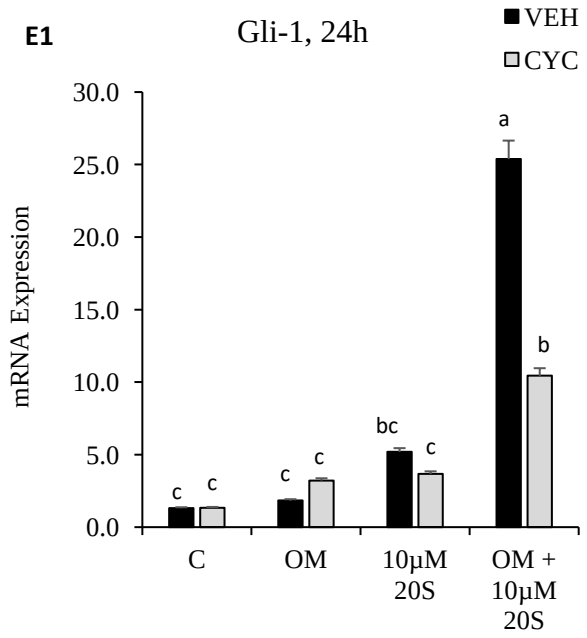

B2
BMP2, 72h
VEH
CYC
mRNA Expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
b ab ab b ab ab a a
C
OM
10µM 20S
OM + 10µM 20S

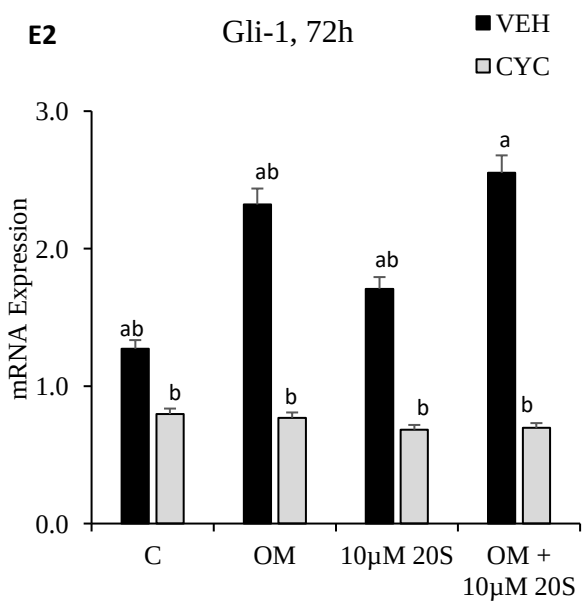

C1
BSP, 24h
VEH
CYC
mRNA expression
60.0
50.0
40.0
30.0
20.0
10.0
0.0
c
c
c
c
c
bc
a
b
C
OM
10µM 20S
OM + 10µM 20S

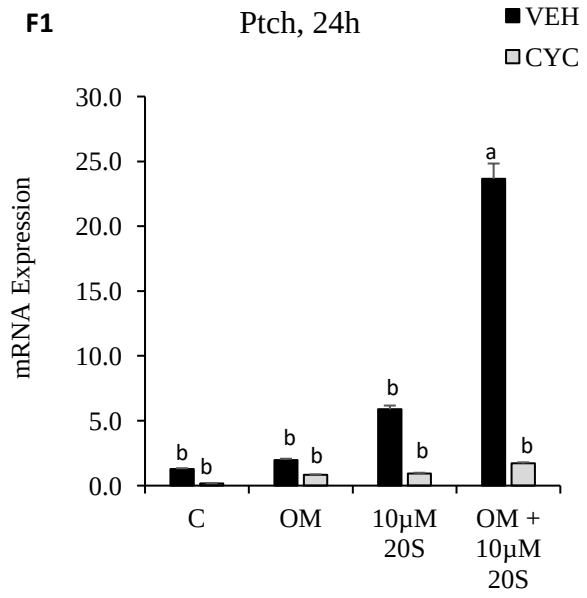

C2
BSP, 72h
VEH
CYC
mRNA Expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
b
b
b
b
b
b
a
b
C
OM
10µM 20S
OM + 10µM 20S

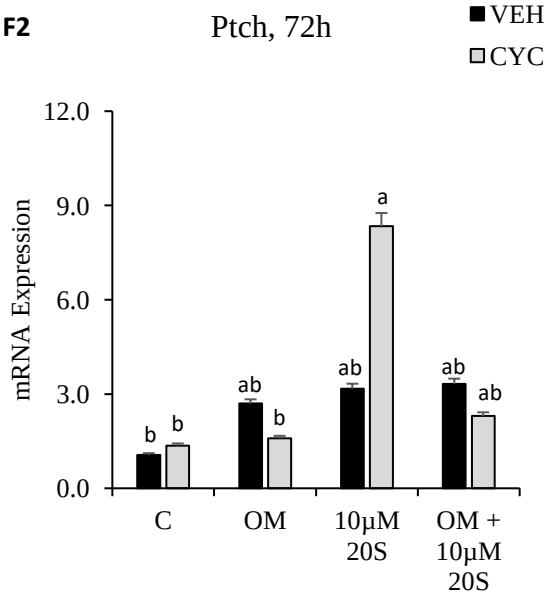

D1
BGLAP, 24 h
VEH
CYC
mRNA expression
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
b
b
b
b
b
b
a
b
C
OM
10µM 20S
OM + 10µM 20S


D2
BGLAP, 72h
VEH
CYC
mRNA Expression
6.0
5.0
4.0
3.0
2.0
1.0
0.0
b
b
b
b
b
b
a
b
C
OM
10µM 20S
OM + 10µM 20S


E1
Gli-1, 24h
VEH
CYC
mRNA Expression
30.0
25.0
20.0
15.0
10.0
5.0
0.0
c c
c c
bc c
a
b
C
OM
10µM 20S
OM + 10µM 20S


E2
Gli-1, 72h
VEH
CYC
mRNA Expression
3.0
2.0
1.0
0.0
ab b
ab b
ab b
a
b
C
OM
10µM 20S
OM + 10µM 20S


F1
Ptch, 24h
VEH
CYC
mRNA Expression
30.0
25.0
20.0
15.0
10.0
5.0
0.0
b b
b b
b b
a
b
C
OM
10µM 20S
OM + 10µM 20S


F2
Ptch, 72h
VEH
CYC
mRNA Expression
12.0
9.0
6.0
3.0
0.0
b b
ab b
ab a
ab ab
C
OM
10µM 20S
OM + 10µM 20S

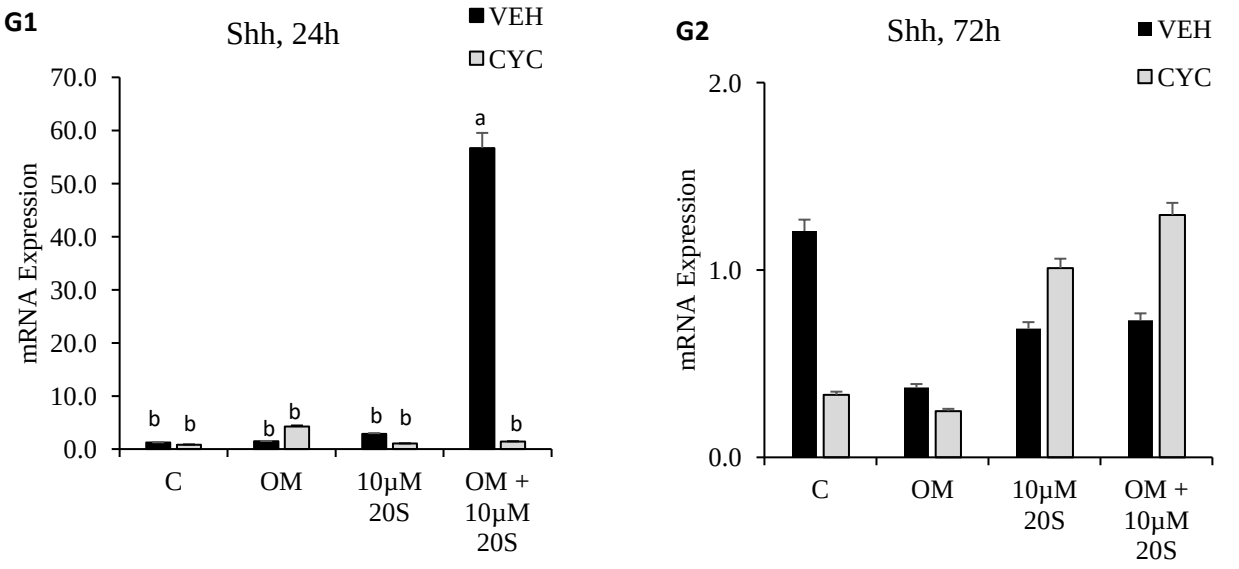


Figure 4.4:20S activates osteogenic genes through the Hedgehog signaling pathway. Chicken bone marrow stem cells (cBMSCs) when confluent was pretreated for 2h with control vehicle (VEH) or 4µM cyclopamine (CYC), followed by 24h and 72h of treatment with control vehicle (C), osteogenic media (OM) containing DMEM with 10% FBS, 50µg/ml ascorbate, 0.5µM DXA, and 10mM β-glycerophosphate, 10µM 20S, and OM + 10µM 20S. RNA was isolated, reverse transcribed to cDNA and qRT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. mRNA expression of A) RunX2, B) BMP2, C) BSP, D) BGLAP, E) Gli-1, F) Ptch, and G) Shh were significantly reduced in cell treated with OM+20S in Cyc pretreated group compared to VEH pre-treated group. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) are significantly different ($P < 0.05$) when analyzed with Tukey's test.

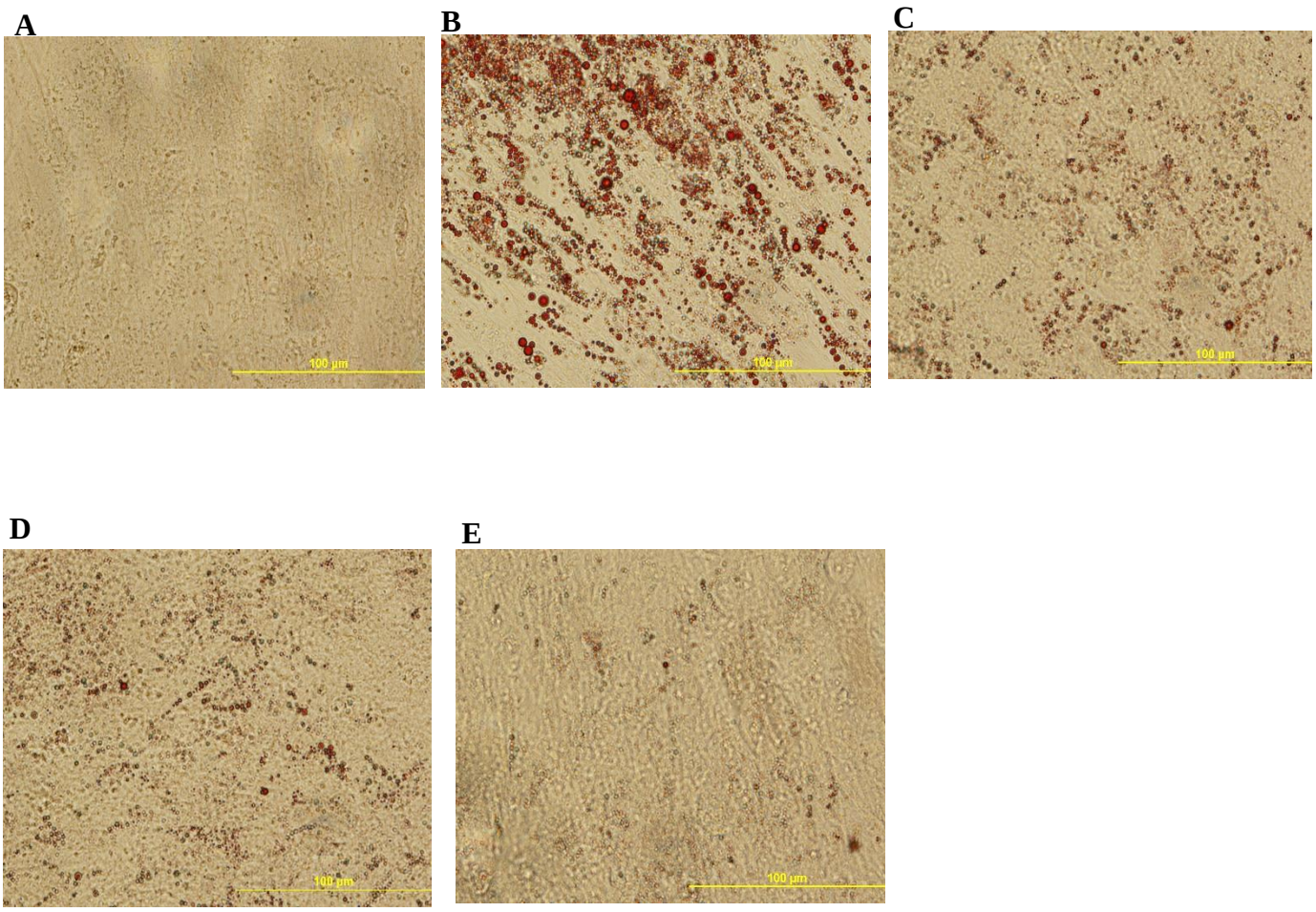
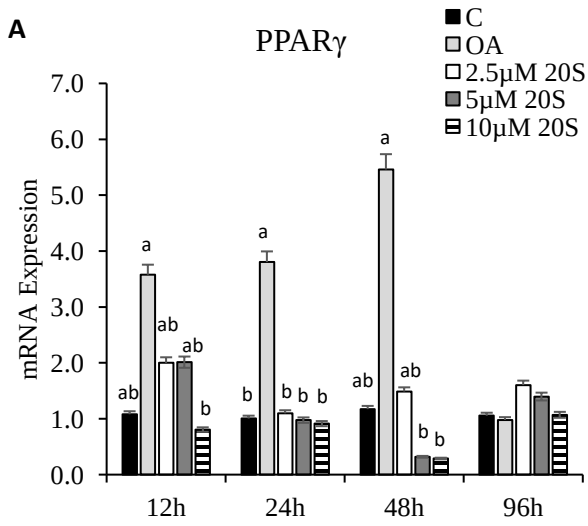
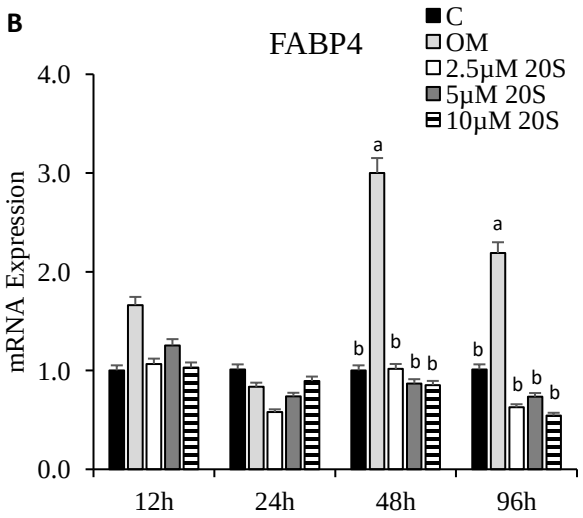


Figure 4.5: 20S inhibits adipogenic differentiation of mesenchymal stem cells isolated from compact bones of chicken. Cells were treated with A) Control, B) Oleic acid media (OA), C) OA + 2.5µM 20S, D) OA + 5µM 20S, and E) OA + 10µM 20S for 96h. Adipocyte formation was examined by Oil Red O staining procedure. Pictures were taken by Olympus DP70 at 20X. Scale bar was 100 µ m.


A
PPARγ
C
OA
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
12h
24h
48h
96h


B
FABP4
C
OM
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
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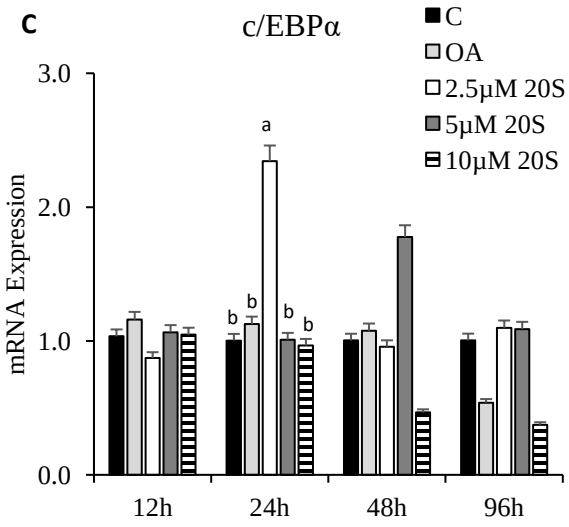
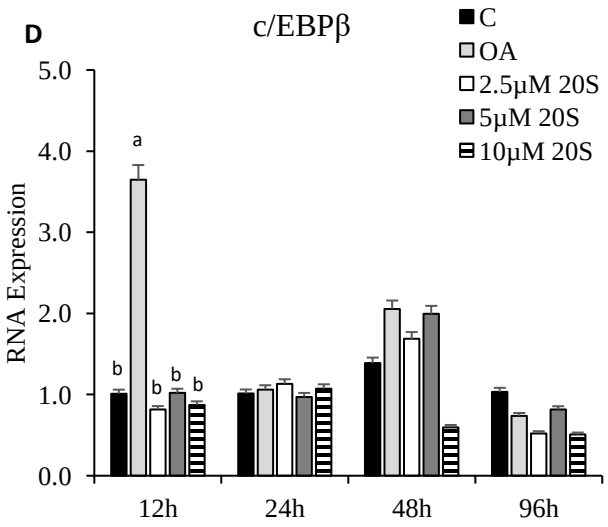
3.0
2.0
1.0
0.0
12h
24h
48h
96h


C
c/EBPα
C
OA
2.5μM 20S
5μM 20S
10μM 20S
mRNA Expression
3.0
2.0
1.0
0.0
12h
24h
48h
96h


D
c/EBPβ
C
OA
2.5μM 20S
5μM 20S
10μM 20S
RNA Expression
5.0
4.0
3.0
2.0
1.0
0.0
12h
24h
48h
96h

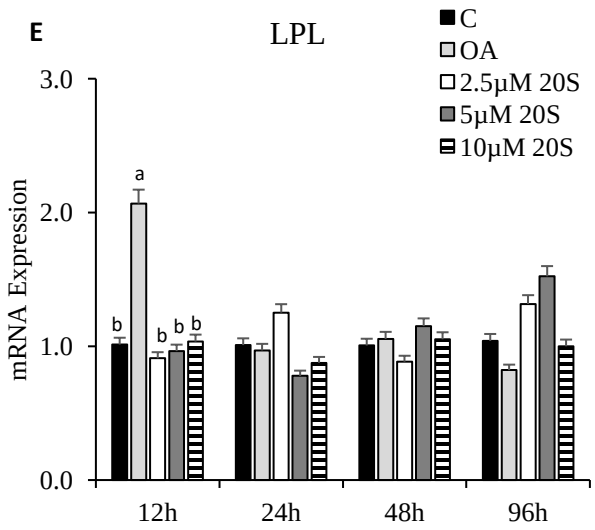
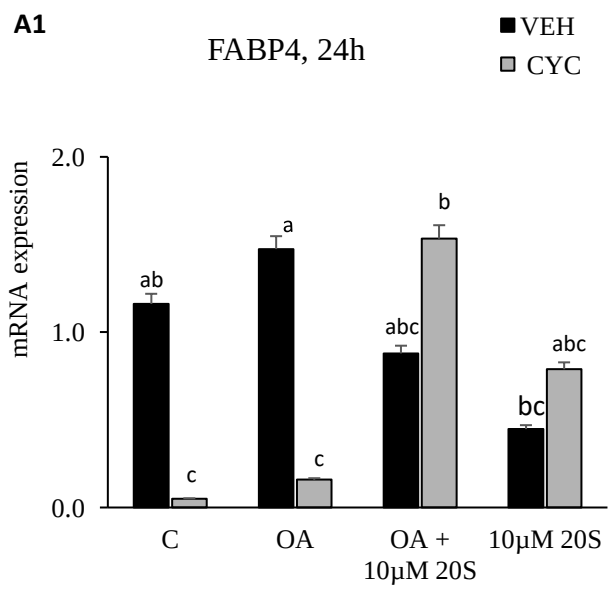


Figure 4.6 : Adipogenic effects of 20S oxysterol in mesenchymal stem cells. Relative mRNA expression levels of osteogenic genes A) PPARγ, B) FABP4, C) c/EBPα, D) c/EBPβ, and E) LPL in chicken compact bone derived mesenchymal stem cells (cBMSCs) treated with different levels of oxysterol. cBMSCs were treated with 1) Control (C), 2) Oleic acid media (OA), 3) OA + 2.5µM 20S, 4) OA + 5µM 20S, and 5) OA + 10µM 20S. Cells were harvested at 12h, 24h, 48h, and 96h after treatment. RNA was isolated, reverse transcribed to cDNA and qRT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the harvested period are significantly different (P < 0.05) when analyzed with Tukey's test.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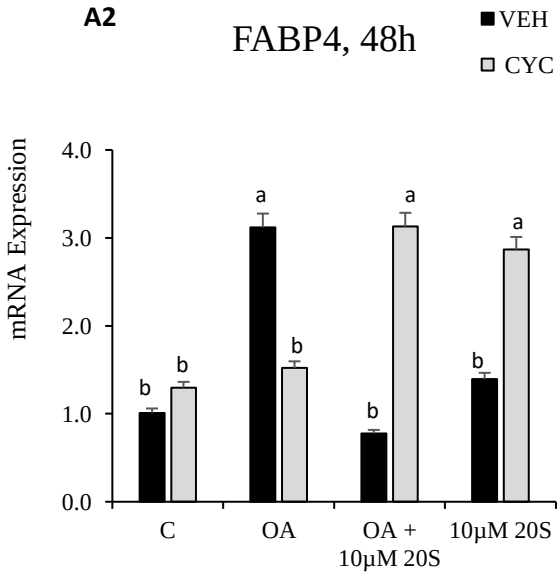
FABP4, 24h
VEH
CYC
mRNA expression
2.0
1.0
0.0
ab
c
a
c
abc
b
bc
abc
C
OA
OA + 10µM 20S
10µM 20S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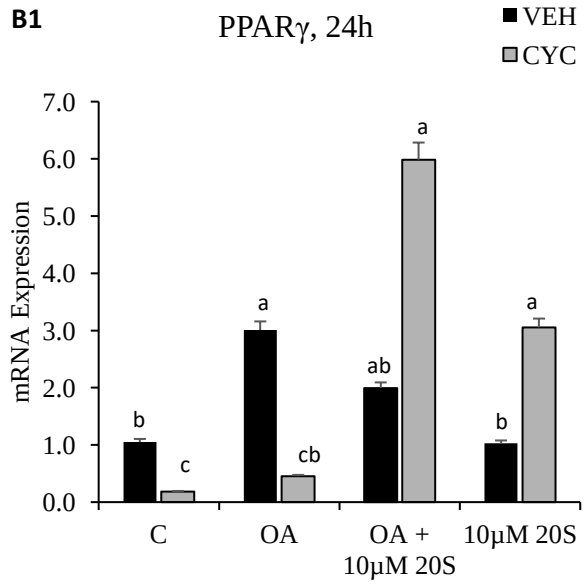
FABP4, 48h
VEH
CYC
mRNA Expression
4.0
3.0
2.0
1.0
0.0
b
b
a
b
b
a
b
a
C
OA
OA + 10µM 20S
10µM 20S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PPARγ, 24h
VEH
CYC
mRNA Expression
7.0
6.0
5.0
4.0
3.0
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1.0
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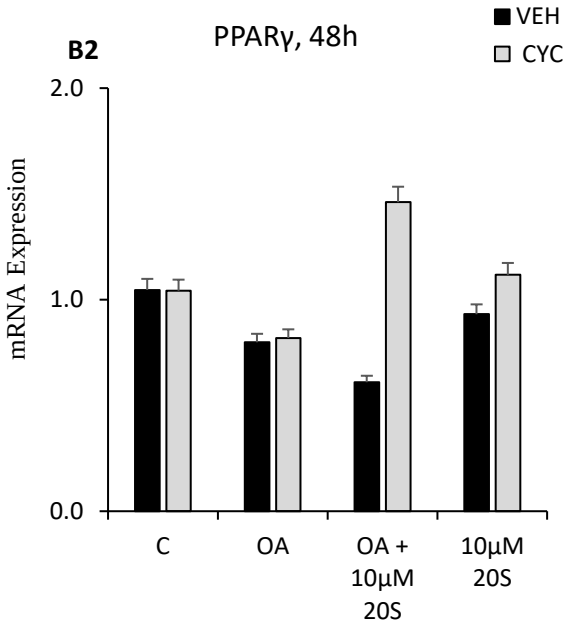
b
c
a
cb
ab
a
b
a
C
OA
OA + 10µM 20S
10µM 20S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PPARγ, 48h
VEH
CYC
mRNA Expression
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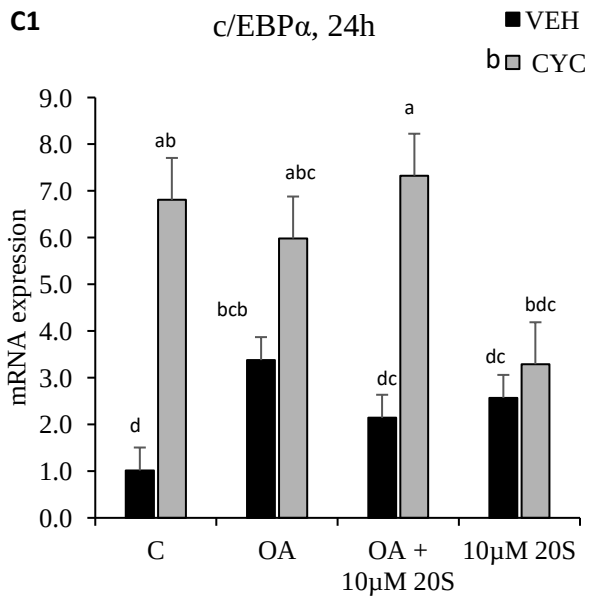
1.0
0.0
C
OA
OA + 10µM 20S
10µM 20S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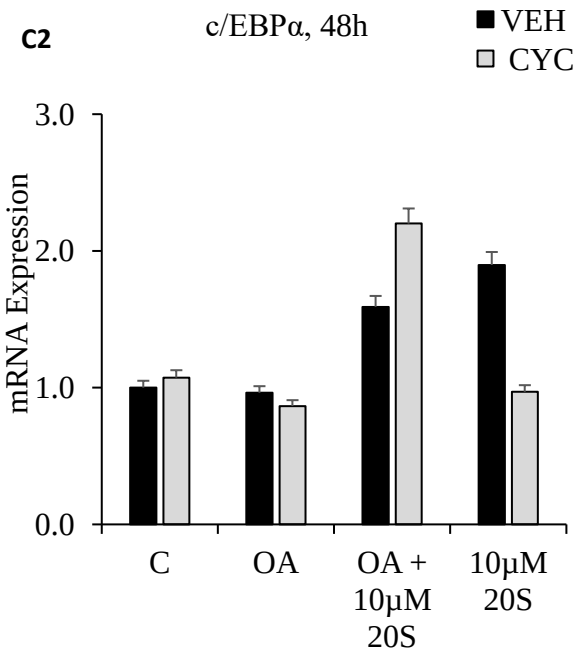
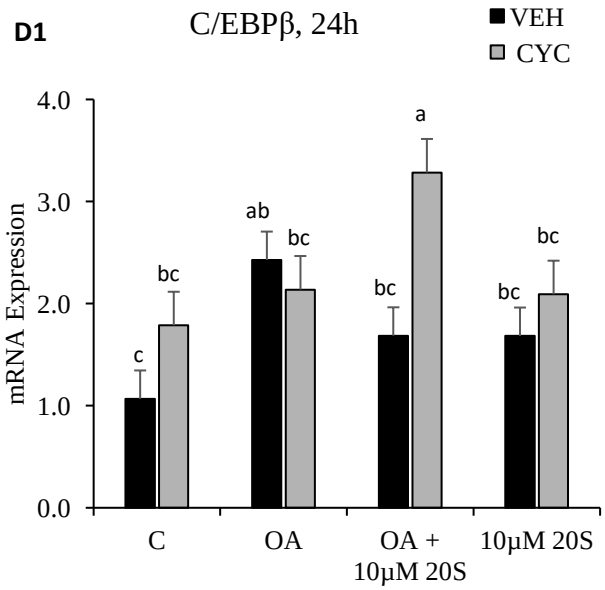
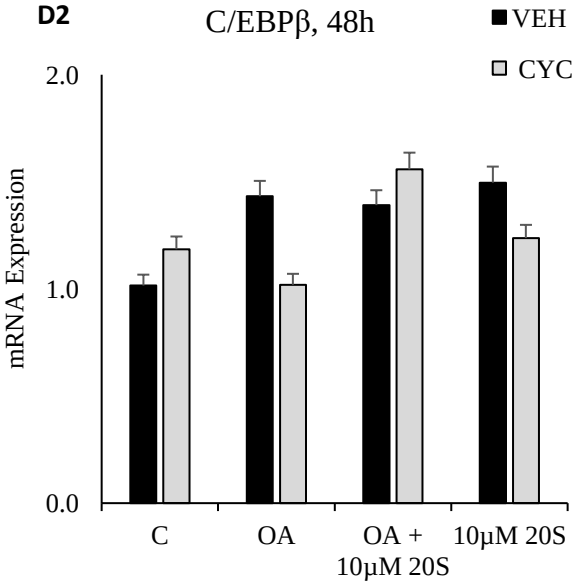
c/EBPα, 24h
VEH
CYC
mRNA expression
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
d
ab
bcb
abc
dc
a
dc
bdc
C
OA
OA + 10μM 20S
10μM 20S


C2
c/EBPα, 48h
VEH
CYC
mRNA Expression
3.0
2.0
1.0
0.0
C
OA
OA + 10μM 20S
10μM 20S


D1
C/EBPβ, 24h
VEH
CYC
mRNA Expression
4.0
3.0
2.0
1.0
0.0
c
bc
ab
bc
bc
a
bc
bc
C
OA
OA + 10μM 20S
10μM 20S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C/EBPβ, 48h
VEH
CYC
mRNA Expression
2.0
1.0
0.0
C
OA
OA + 10μM 20S
10μM 20S

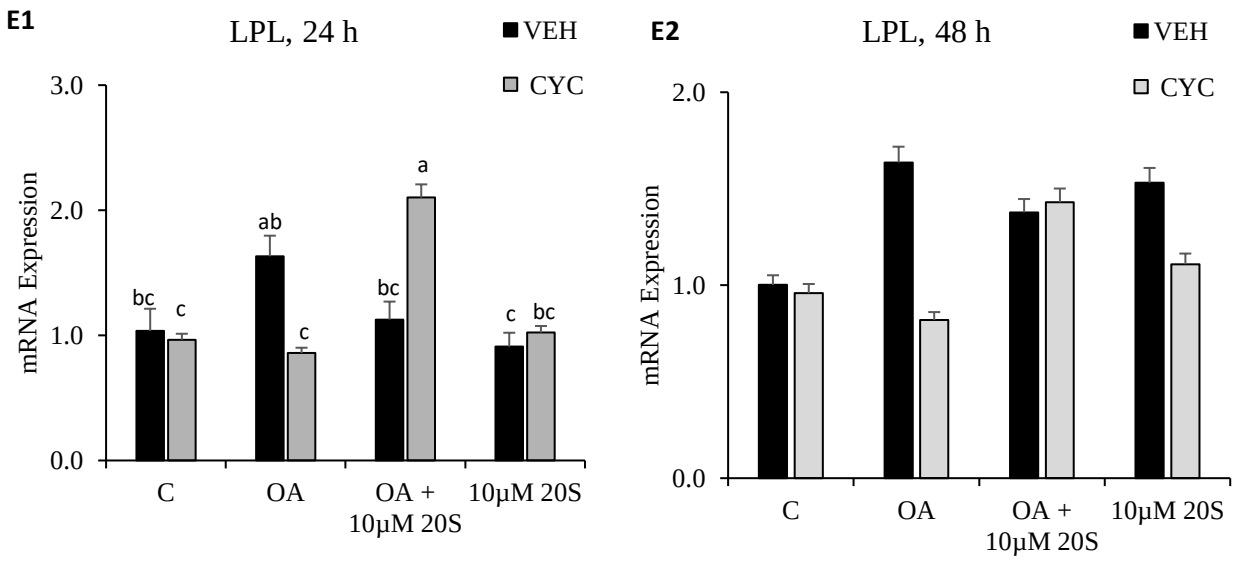


Figure 4.7 : Hedgehog signaling pathway inhibitor, Cyclopamine block the inhibitory effect of 20S on OA induced adipogenic differentiation of chicken bone marrow stem cells (cBMSCs). cBMSCs when confluent was pretreated for 2h with control vehicle (VEH) or 4µM cyclopamine (CYC), followed by 24h and 48h of treatment with control vehicle (C), Oleic acid (OA), OA + 10µM 20S, and 10µM 20S. RNA was isolated, reverse transcribed to cDNA and q RT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. mRNA expression of A) FABP4, B) PPARγ, C) c/EBPα, D) c/EBPβ, and E) LPL are presented above. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) are significantly different (P < 0.05) when analyzed with Tukey's test.

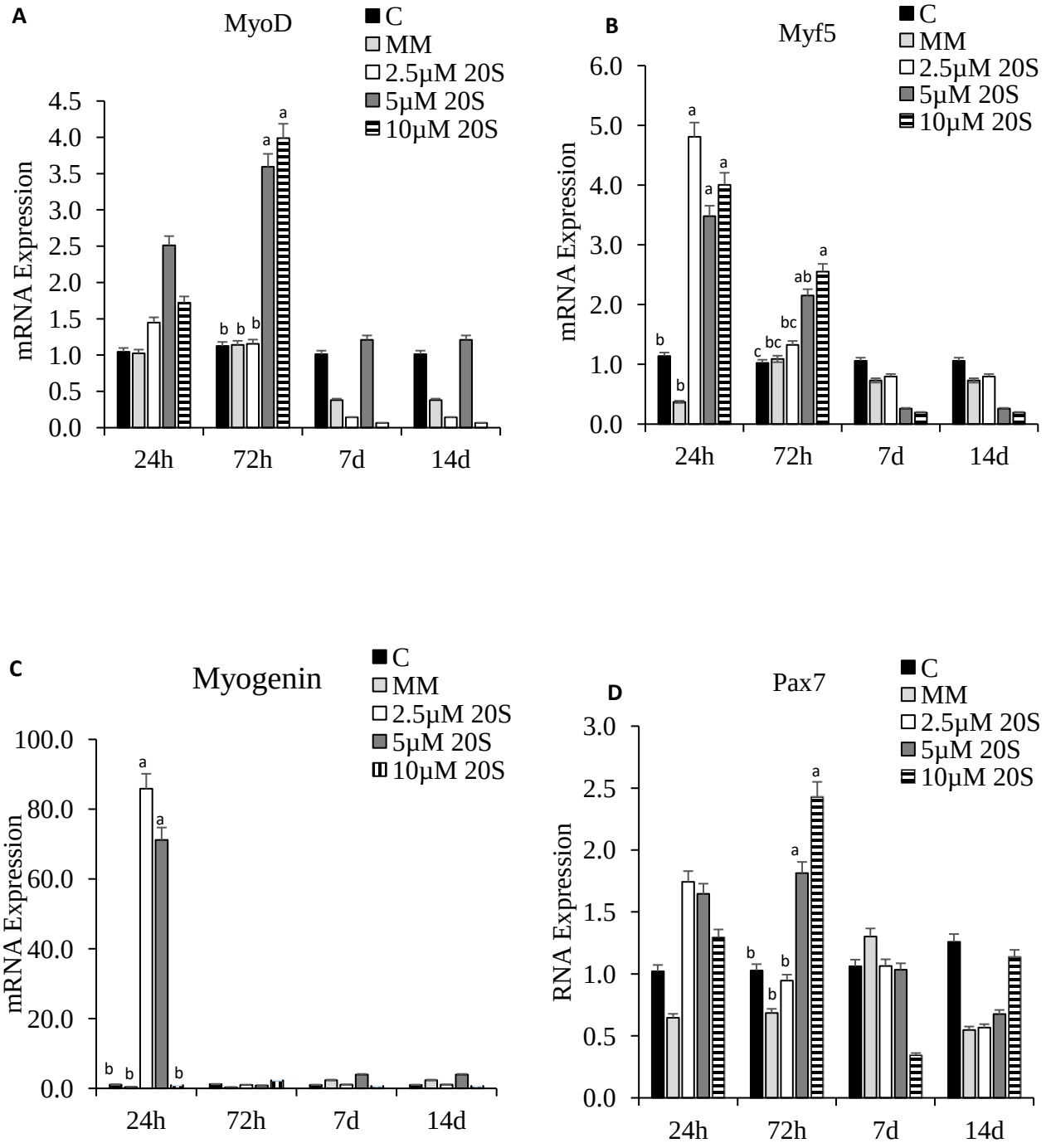
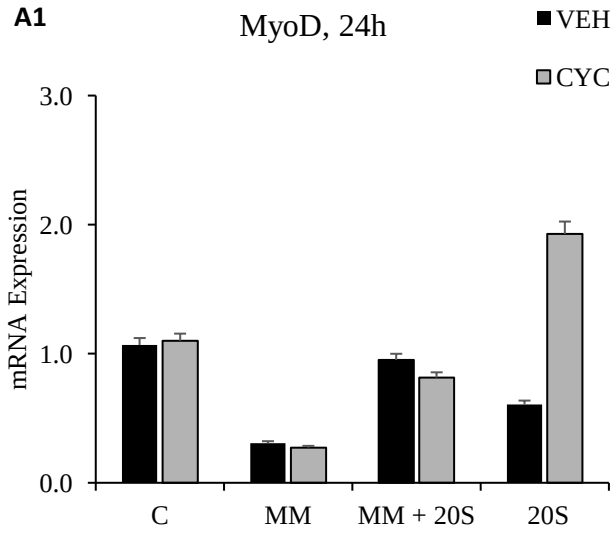


Figure 4.8: Myogenic effects of 20S oxysterol in mesenchymal stem cells. Relative mRNA expression levels of osteogenic genes A) MyoD, B) Myf5, C) Myogenin, and D) Pax7 in chicken compact bone derived mesenchymal stem cells (cBMSCs) treated with different levels of oxysterol. cBMSCs were treated with 1) Control (C), 2) myogenic media (MM) containing DMEM with 5% Horse Serum, 10µM

Hydrocortisone, and 0.1μM DXA, 3) MM + 2.5μM 20S, 4) MM + 5μM 20S, and 5) MM + 10μM 20S. Cells were harvested at 24h, 72h, and 7d after treatment. RNA was isolated, reverse transcribed to cDNA and qRT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping genes. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the harvested period are significantly different (P < 0.05) when analyzed with Tukey's test.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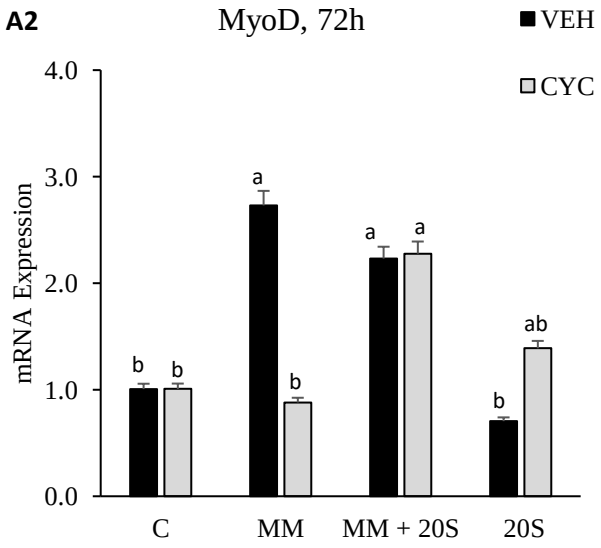
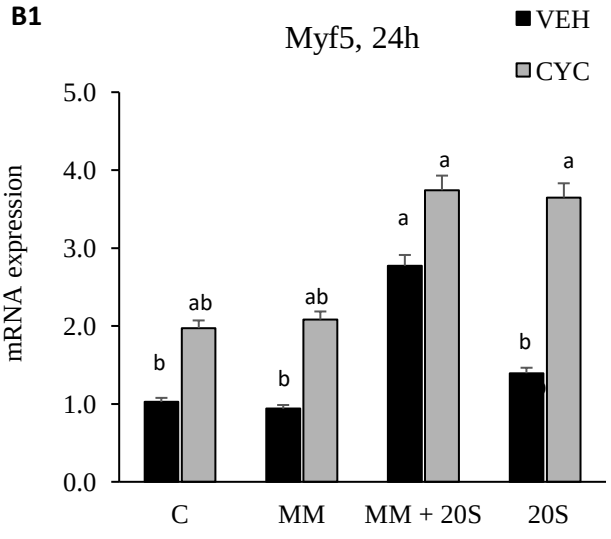
MyoD, 24h
VEH
CYC
mRNA Expression
3.0
2.0
1.0
0.0
C
MM
MM + 20S
20S


A2
MyoD, 72h
VEH
CYC
mRNA Expression
4.0
3.0
2.0
1.0
0.0
a
b
a
a
b
b
ab
C
MM
MM + 20S
20S


B1
Myf5, 24h
VEH
CYC
mRNA expression
5.0
4.0
3.0
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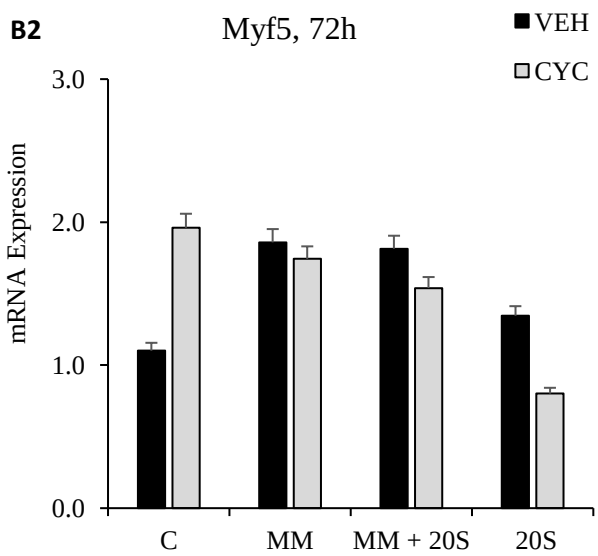
b
ab
b
ab
a
a
b
a
C
MM
MM + 20S
20S


B2
Myf5, 72h
VEH
CYC
mRNA Expression
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C
MM
MM + 20S
20S

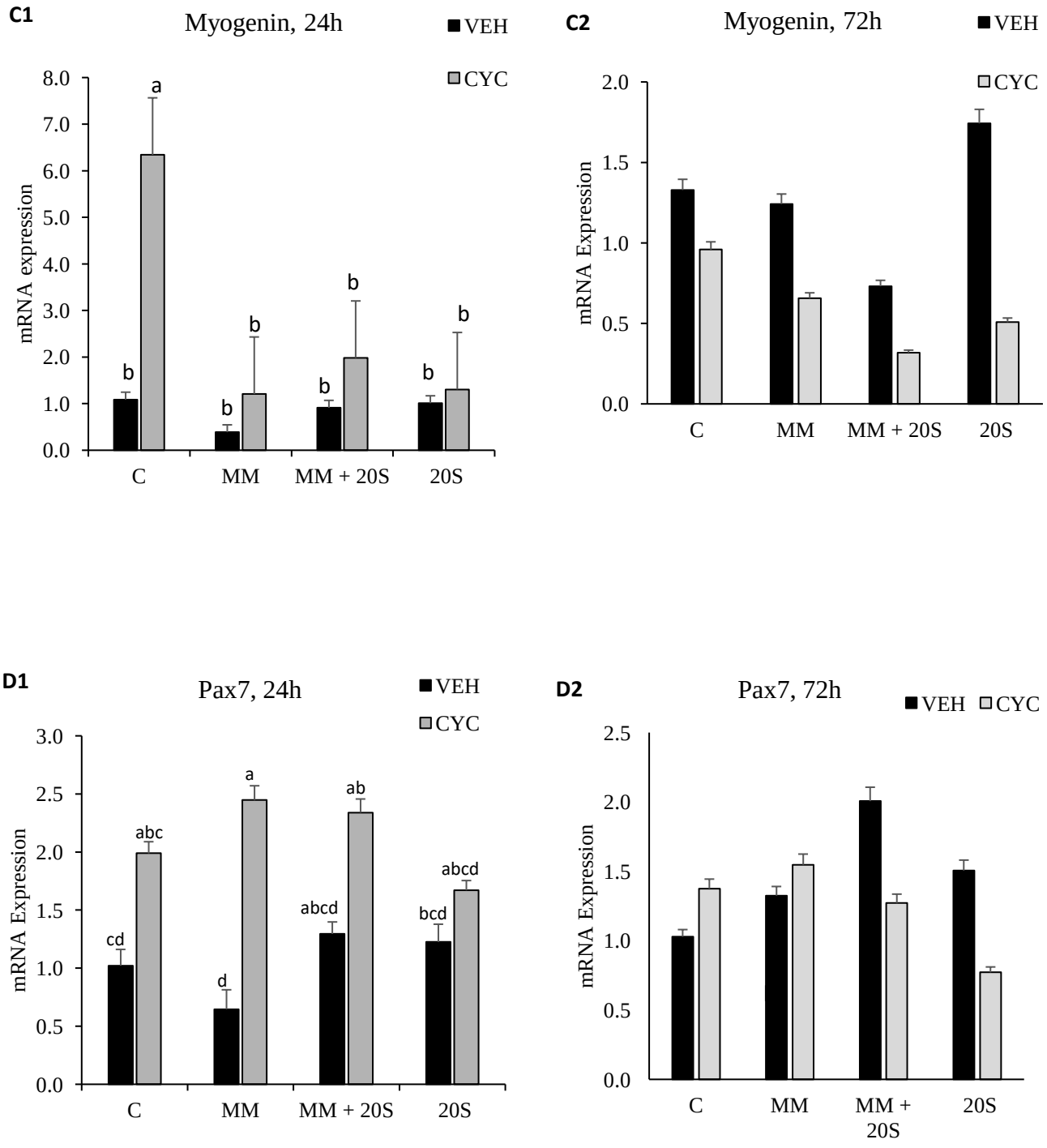

Figure 4.9: Hedgehog signaling pathway inhibitor, Cyclopamine did not block the inhibitory effect of 20S on myogenic differentiation of chicken bone marrow stem cells (cBMSCs). cBMSCs when confluent was pretreated for 2h with control vehicle (VEH) or 4μM cyclopamine (CYC), followed by 24h and 72h of treatment with 1) Control (C), 2) myogenic media (MM) containing DMEM with 5% Horse Serum, 10μM

Hydrocortisone, and 0.1µM DXA, 3) MM + 2.5µM 20S, 4) MM + 5µM 20S, and 5) MM + 10µM 20S. RNA was isolated, reverse transcribed to cDNA and q RT-PCR was conducted to analyze osteogenic gene expression. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. mRNA expression of A) MyoD, B) Myf5, C) Myogenin, and D) Pax7 are presented above. Data from the representative experiment are reported as mean of the triplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) are significantly different (P < 0.05) when analyzed with Tukey's test.

CHAPTER 5

EFFECT OF *IN OVO* INJECTION WITH 20(S)-HYDROXYCHOLESTEROL ON BROILER EMBRYOGENESIS[3]

[3] Roshan Adhikari, Robert Beckstead, and Woo Kyun Kim. To be submitted to *Poultry Science Journal*

ABSTRACT

The influence of *in ovo* administration of 20(S)-hydroxycholesterol (20S) injected at three different time points on hatchability of fertilized eggs, body weight at hatch, body composition, and expression pattern of osteogenic, myogenic and adipogenic genes of broiler chicks, hatched from eggs laid by 40-46 wks old broiler flock were investigated. Three separate experiments were carried out for injection of 20S at three different times of incubation. In the first experiment, 20S was injected *in ovo* (n=144) on 3 days of incubation (doi) into the vitelline vein of the broiler chicken embryo. Whole embryos were harvested at 1h, 3h, 12h, 24h post-injection for gene expression analysis. In the second experiment, 20S was injected *in ovo* (n=250) on 7 doi into the yolk of the broiler chicken embryo. Whole embryos were harvested at 6h, 12h, 24, 48h post injection and at hatch. In the third experiment, 20S was injected *in ovo* (n=180) on 18 doi into the yolk of the broiler chicken embryo. The embryo were harvested at 24h post injection and at hatch. Expression of hedgehog (Hh) signaling pathway and osteogenic gene expression were evaluated in the trunk of the embryo in the first experiment and in the hind limbs in the second experiment. Gene expression of the cortical tibia, abdominal fat, and pectorals major from harvested samples was conducted at 24h post-injection of the third experiment and at the hatch of both second and third experiment. Hatch body weight, hatchability, body composition of the hatched broilers was analyzed. There was no effect of 20S on hatchability, hatch body weight or bone mineral density, the bone mineral content of the birds at hatch. Embryo injected with the different levels of 20S at 3 doi and 7 doi significantly increased ($P<0.05$) expression of Hh signaling and osteogenic gene expressions of Gli-1, Patched, Shh, BMP2, RunX2, and BSP at the different time point of harvest. 20S *in ovo* injection at day 7 and day 18 significantly increased expression of osteogenic genes in bones, myogenic genes in pectoralis muscles and adipogenic genes in fat at harvest. *In ovo* injection of 20S

increased the adipogenic, myogenic and osteogenic gene expression, thus affecting the differentiation capacity of the developing embryo. *In ovo* supplementation of an appropriate dose of 20S might help in improving the growth rate of chicken embryos and development of chicks.

Key Words: 20(S)-hydroxycholesterol, DEXA, body weight, gene expression, *in ovo* injection

INTRODUCTION

Rapid growth and development with the decrease in market age of modern day broiler chicken have increased the importance of supplementing adequate nutrition even before hatch. Avian embryo development relies on the nutrient provided from the hen for successful embryogenesis and growth of embryo over one-third of its life (Cherian, 2015). Nutritional deficiencies during formation of egg could have a significant impact on the development of embryo and post hatch chick performance (Moran, 2007). Moreover, supplementation of nutrient in early embryo development could offer a powerful tool to promote the health of hatched chicks and boost early growth and development. (Foye et al., 2006). *In ovo* feeding of incubating eggs with dietary nutrients such as amino acids (AA), carbohydrate (CHO), minerals and vitamins, stimulants, and hormones has been reported to enhance the growth and development of chicken embryo, thus promoting gut health and immune status as well as post-hatch development and performance (Lee et al., 2014; Ohta et al., 2001; Pruszynska-Oszmalek et al., 2015; Tako et al., 2004; Zhai et al., 2011b).

Organogenesis and proper lining of important organs of the chicken embryo occur in the first week of incubation. First 7 days of incubation (doi) are important for protuberance of limb buds, development of head and gastrointestinal organogenesis (Bowen et al., 1989). Development of ovary, skeletal patterning of digits, and formation of intestine takes place in 9 doi (Tickle, 2004). Due to such early and rapid changes, there have been studies reported to understand the development and embryogenesis by manipulation of the embryo as early as stage 10 (Bangs et al., 2010; Bortier and Vakaet, 1992; Ezin and Fraser, 2008; Welten et al., 2011). The role of retinoic acid in the endogenous zone of polarizing activity and development of skeletal pattern was examined by placing the beads soaked with retinoic acid near somite 15 and 20 of stage 14 embryos (Ohta et al., 2001). Such early manipulation requires the extensive

opening of the embryo and may not be suitable for getting higher hatch percentage in industrial applicaiton for commercial hatcheries. Understanding of the early embryo development will give a better understanding of the pathways and molecular cascade that the nutrients will play during early embryogenesis. *In ovo* feeding, however, has been successfully carried out as early as 7 d in the yolk of the incubated eggs. *In ovo* injection of AA solution in the yolk at 7 doi increased amino acid contents of embryo, yolk, albumen, allantois, and amnion fluids (Ohta et al., 2001). A study reported an injection of the embryo at 17-18 doi could help to overcome the depletion of essential nutrients at the phase of rapid development and hatch (Foye et al 2006). Injection of nutrients at late embryogenesis in the amniotic fluid are subsequently digested, swallowed and absorbed by embryo thus helping in embryo development (Tako et al., 2004, 2005). Injection of creatine monohydrate and glucose at 17.5 doi provided a synergistic effect on improvement of the energy status of embryos and hatchlings, and increased glycogen and glucose in the liver and creatine and phosphocreatine in the pectoralis major muscles (Zhang et al., 2016). *In ovo* supplementation of selenium at 18 doi into the amniotic cavity showed a beneficial effect on immune and antioxidant response in post-hatch chick performance exposed to the *Eimeria* and C*lostridium Perfringens* (Lee et al., 2014). Studies have also been conducted to understand the effect of *in ovo* feeding of supplements such as carbohydrate (Zhai et al., 2011b), *Lactobacillus* (Pruszynska-Oszmalek et al., 2015), minerals (Lee et al., 2014), vitamins (Bello et al., 2013), AA (Tako et al., 2005), antibodies (Kim et al., 2006), on hatchability, chick organ development at hatch, post-natal growth performance, and nutritional status of chick and bird.

Oxysterol is a 27 carbon oxygenated products of cholesterol oxidation that are derived from diet or endogenous cellular biosynthesis (Bjorkhem and Diczfalusy, 2002). They are formed enzymatically during sterol metabolism and are intermediate in the formation of sterol

hormones, bile acids and 1,25-dihydroxyvitamin D_3 (Russell, 2003). Oxysterol has also been identified as a bioactive compound that is involved in various biological and pathological process such as lipoprotein metabolism, cholesterol efflux, regulate immune and inflammatory response, calcium uptake and cellular differentiation (Burgett et al., 2011; Olkkonen et al., 2012; Vaya et al., 2001; Venkateswaran et al., 2000). Specific oxysterols namely 20S, 22S, 133oxy have been reported to be potent osteoinductive properties when they are subjected to bone marrow stromal cells (Kha et al., 2004). Oxysterol increased genes related to osteoblastic differentiation such as ALP, RunX2, BSP, and ALP. Apart from osteoinductive properties, specific oxysterols such as 20S can inhibit the adipogenic differentiation of MSCs by suppressing the transcriptional activity of PPARγ through Hh signaling pathway-dependent mechanism (Kim et al., 2007). Oxysterol has been shown to induce bone growth and increased trabecular and cortical bone formation *in vivo* when applied to rat and rabbit spinal cord (Johnson et al., 2011a). Osteoinductive properties of oxysterol are mediated by Hh signaling pathway by increasing Gli1, Patch, and sonic hedgehog (Shh) gene expression, notch signaling pathway (Kim et al., 2010) and Wnt signaling pathway (Kim et al., 2007). As Hh signaling pathway functions an important role in embryo development and embryogenesis, we hypothesized that oxysterol could be a potential bioactive compound that would affect osteoinductive, adipogenic, and myogenic properties of broilers during embryonic development and post hatch. *In ovo* feeding of oxysterol could be a powerful bioactive compound for early diet manipulation to shift the stem cells differentiation and promote the health of hatchling in a natural way.

The objective of our study was to understand the effect of 20S in embryonic development, differentiation of osteogenic, adipogenic, and myogenic genes during

development, hatchability, and hatch chick quality when injected at the different time point of the incubation. Our first experiment studied the effects of 3 doi injection of 20S on Hh signaling pathway and osteogenic genes. The second experiment studied the effect of 20S injected in the yolk of the embryo at 7 doi on osteogenic gene expression and Hh signaling pathway genes immediately after injection and the gene expression of bone, fat and m/s at the day of hatch. The third experiment studied the effect of 20S on osteogenic, myogenic and adipogenic gene expression of bone, muscles, and fat 24h post injection and at hatch when injected into the amniotic cavity at 18 doi. The information derived from this study will expand our knowledge in use of oxysterol as a potential early nutritional bioactive compound that could be *in ovo* fed to influence the health of broiler.

MATERIALS AND METHODS

Study to estimate the site of injection

Three experiments were conducted to understand the livability and hatchability of the eggs when injected with control vehicle and oxysterol. Eggs for preliminary and main studies were obtained as a kind gift from Dr. Jeana Wilson's Cobb 500 breeder flock at 40 wks of age raised at the research farm of the University of Georgia. Two preliminary trials were conducted to understand the accuracy of the site of injection. All eggs used for the preliminary experiment and main experiment were from the same flock. Eggs were kept in the incubator a day after it was collected from the flock. Eggs were incubated in the hatchery following the normal procedure. All eggs were candled at 6 or 12 doi for the first and second experiment respectively and unfertilized eggs were discarded. For the first preliminary experiment, 50 eggs were injected with 25mm long 26G needle with India ink. Eggs were immediately broken after the injection to confirm the site of injection was egg yolk. *In ovo* injection at different sites of the embryo at 7

doi reported that yolk injection was the optimal site of delivery for a higher survival rate of the embryo compared to direct injection at the amniotic cavity (Ohta and Kidd, 2001). For the second experiment, 50 eggs were separated and injected with India ink following the procedure as previously described (Tako et al., 2004). Eggs were taken out of the incubator and were disinfected with 70% ethanol, a hole was punched with the help of punch hole in the center of the large end of each egg. India ink was delivered in the amniotic cavity at 18doi by handheld 1mL tuberculin injection with a 26G-25mm long needle. The eggs were broken immediately to determine the site and accuracy of injection.

Main Study

In ovo injection at 3 d

In the first experiment, eggs were obtained from Broiler breeder at 42 wks of age. Eggs were weighed individually and 64 ± 3.3 g eggs were randomly kept in egg tray in a horizontal position at room temperature for 4-5 h. The eggs were then kept in egg incubator at 99°F in horizontal position without rotating. After 70 h (stage19), 144 randomly selected eggs were injected allocating six eggs per treatment per time point of harvest. Treatment consist of 1) non-injected control eggs 2) No injection window open 3) Injection with 1X PBS 4) 0.08µg 20S 5) 0.8µg 20S 6) 1.6µg 20S. Eggs were microinjected into the vitelline vein with little modification of previously described protocol (Blank et al., 2007). In brief, the eggs were sprayed with alcohol and wiped with Kim-wipes. The individual egg was taken out from the incubator; 1 mL of albumin was withdrawn from the eggs using sterile 18G needle and syringe. The window was opened in the horizontal part of the egg to expose the embryo with the help of a dental drill and scissors. The egg shell and shell membrane were removed with the help of forceps. Once the

embryo was exposed, 1-2 drops of 10X pen-strep (Life technologies, USA) was kept in the egg to reduce contamination. The treatment solution was loaded to microinjection made by using borosilicate glass capillaries (Harvard Apparatus, 1.5 mm OD, 1.17 mm ID, 100 mm L). One µL of the treatment solution was injected into a vitelline artery of the embryo using picospritzer III microinjection dispense system (Parker Hanniffin Corporation). After the injection was performed few drops of pen-strep was added and the window was sealed back with a glue gun and cover slide. The eggs were kept back in the incubator without rotation. Livability of the embryo was checked and the whole embryo was collected after 1 h, 3 h, 12 h, and 24 h of injection for gene expression analysis. Control non-injected eggs were kept outside of the incubator for the same amount of time as other injected eggs during the treatment procedure. Head was separated from the trunk and the whole trunk was used to extract RNA.

In ovo injection at 7 d

In the second experiment, the effect of 20S *in ovo* injection in the yolk of 7 d chicken embryos was evaluated. Fertile eggs from Cobb 500broilers, 44 wks of age were obtained from the experiment. Eggs were weighed at d 0 and eggs weighing 64 ± 3.3 g were kept for the experiment. The eggs were incubated under the optimal condition (temp humidity rotation) in the hatchery at the research farm of the Department of Poultry Science at the University of Georgia. Eggs were candled at 7 doi to discard infertile eggs. The blunt end of the eggs was held against the candling lamp. A line was drawn on the shell at the edge of the air sac of fertile eggs. A cross mark was made approximately 5mm above the line avoiding the blood vessels as the site of injection. Eggs were wiped with alcohol and a hole was made with an egg shell punch. Eggs were observed in the candle to detect the embryo and injection was performed from the blunt side of the egg near to air shell border to target the yolk. The injection was performed by the use

of handheld 1mL tuberculin syringe coupled with 26 G needle 25 mm long. The hole was sealed with glue gun after the injection was completed and placed back into the incubator.

A total of 250 fertile eggs were randomly injected with one of the 5 treatments 1) Control, 2) Sham injection, 3) 5µg of 20S, 4) 10µg of 20S, and 5) 50µg of 20S. Each egg was injected with 0.1mL of the treatment solution. Ten eggs per treatment per tray level were equally distributed in 5 tray levels of the incubator after injection. Five eggs (one egg from each tray level per treatment) from each treatment were removed and whole embryos were collected at 6 h, 12 h, 24 h, and 48 h post injection for real-time PCR. Hind limb was separated from the harvested embryo for downstream analysis and immediately snap frozen in liquid nitrogen. The separated limbs were used to isolate RNA to understand the effect of 20S on osteogenic and Hedgehog signaling gene expression. On the day of hatch, hatchability and hatch chick body weight were determined from remaining 150 birds (30 birds/treatment), 5 birds per treatment were euthanized to collect bone, fat, and muscles to isolate RNA for downstream PCR analysis. Bone density and body mass composition were measured from 6 birds per treatment with the help of Dual Energy X-ray Absorptiometry (**DEXA**).

In ovo injection at 18 d

In the third experiment, eggs were obtained from Cobb 500 broiler breeder flock at 46 wk of age. Eggs were weighed and kept in the incubator as in previous 7 d injection study. Eggs were candled on 12 d to remove unfertilized eggs. On 18 doi, a total of 180 viable embryos were randomly divided into five treatment groups 1) Control, 2) Sham injection, 3) 5µg of 20S, 4) 10µg of 20S, and 5) 50µg of 20S. Eggs were taken out of the incubator and were disinfected with 70% ethanol, a hole was punched with the help of punch hole in the center of the large end of each egg. Respective treatments were delivered in the amniotic cavity at 18 doi by handheld 1mL

tuberculin injection with a 26G-25mm long needle. Detail of the injector needle was followed as a modified protocol previously described (Zhai et al., 2011a). The previous study in such injection procedure validation showed that all the injections were in the amnion (77.8%), intramuscular (11.5%), subcutaneous (9.9), in the yolk (0.8%), and in air cell or allantois (0%) (Bello et al., 2014a). Injection hole was sealed with the help of Glue gun. Injected eggs were kept back into the hatcher. On 19doi (24h after injection) 6 embryos from each treatment were euthanized to collect bone, muscles and fat. On the day of hatch, hatchability and hatch bird's body weight was determined, 6 birds per treatment were euthanized to collect bone, fat, and muscles to isolate RNA for downstream PCR analysis. Bone density and body mass composition were measured from 6 birds per treatment with the help of DEXA.

RNA extraction, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

At the specific time point of each experiment, samples were collected and snap frozen in liquid nitrogen and harvested at -80°C until the further procedure. Abdominal fat, muscles from pectoralis major, and femur were collected for RNA isolation. 60-80 mg of fat and muscle tissue were used to isolate total RNA using Qiazol reagent (Qiagen, USA) following manufacturer's protocol. RNA was isolated from bone following a slight modification of the two protocol as reported previously. (Carter et al., 2012; Kelly et al., 2014). In brief, flesh surrounding the bones were removed quickly and the bones were cut longitudinally in ice cold PBS to expose the marrow. Bones were centrifuged for 30s at 14,000g at 4°C in a nested microcentrifuge tube to remove any adhered marrow from the bones. Bones were immediately transferred to 1.7 mL centrifuge tubes that contained pre-chilled Qiazol and beads. Bones were chopped into small

pieces in the tube. Bone samples were kept in the Qiazol reagent chilled with liquid nitrogen until the bone tissues were disrupted with a tissue homogenizer.

Two µg of RNA was reverse-transcribed using high capacity cDNA reverse transcription synthesis kits (Applied Biosystem, USA) following the manufacturer's protocol. cDNA was synthesized at 20°C for 10 min, 37°C for 2h using a thermal cycler system (Applied Biosystem, USA). Primers for each gene were designed and checked for target identity using the National Centre for Biotechnology Information (NCBI). To understand the possible alteration in expression of specific transcripts in the harvested cells, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed using nuclease-free water, the forward and reverse primers of each specific target gene, template cDNA, and SYBR Green (Bio-Rad, USA) using StepOne^{TM} Real-Time PCR systems (Applied Biosystems, USA). Temperature cycles were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 15s, annealing temp for 20s, and extension 72°C for 1 min. All analysis was done in duplicate reaction, and GAPDH was used as a housekeeping gene. After 40 cycles of qRT-PCR, melt curves were examined to ensure primer specificity. Fold changes in gene expression were calculated using the -ΔΔCt method and reported as fold changes of the expression of the target genes in experimental groups compared to the control group. Details of primers sequences used for all three experiments are presented in Table 5.1.

Statistical analysis

Data obtained were analyzed by ANOVA using the general linear model procedure of the Statistics Analysis System (SAS) Institute. Tukey test was used to measure the mean separation when statistically significant difference was found. Results are presented as means ± SEM. The level of significance used in all three studies was probability value of $P < 0.05$.

RESULTS

Study to estimate the site of injection

In the first preliminary experiment, India ink was injected at 7 doi. Such injection showed that the injected India ink was found in the yolk (76%), amniotic vesicle (8%), chick (6%), allantoic cavity (6%), air sac (0%), and albumin (0%). In the second preliminary experiment, India ink was injected in the amnion at 18 doi. Such injection showed that the injected. India ink was found in the amnion (82%), yolk (2%), chick (16%), allantoic cavity (0%), air sac (0%), and albumin (0%).

Injection validation study, Hatchability, and Body composition

In preliminary injection study with India ink for validation of injection site, out of 50 embryos injected on 7 doi 38 (76%) were injection in the yolk, 4 (14%) were injected into the amniotic cavity, 3(6%) were injected into the allantoic cavity, 3 (6%) were injected in the chick, none of them were injected in the albumin or air sac. Out of 50 embryos injected with India ink on 18 doi, 41 (82%) were injected into the amniotic cavity, 8 (16%) were injected in the chick, none of them were injected in the allantoic cavity, albumin or air sac.

Eggs that were injected at day 7 and 18 were hatched to evaluate the hatchability, body composition and chick body weight at hatch. There was no effect of 20S in hatchability and weight of chicks at hatch among the treatments at both 7 d and 18 d *in ovo* injection (Table 5.2). BMD, BMC, Bone area, tissue (lb) of hatched chicks injected at 7 doi (Table 5.3) and 14 doi (Table 5.4) was not affected by 20S treatment. Tissue fat percentage of the hatched chicks significantly increased in the treatment group injected with 20S compared to the control non-injected treatment group. 50µg of 20S significantly increase tissue fat percentage compared to other treatments. Fat mass weight was significantly higher and lean mass weight was

significantly lower in the embryos treated with 50µg of 20S. Similarly, at 18 d of injection, 20S *in ovo* injected significantly increased the tissue fat percentage compared to control and sham injected group. Fat mass was significantly higher in chicks treated with 20S compared to control and sham injected chicks. This indicates that *in ovo* injection of 20S in 7 doi and 18 doi could affect the body fat and muscles composition in the chicks. However, there was no difference in bone mineral content and bone mineral density among the treatments.

In ovo injection at day 3

The effect of 20S on the expression of mRNA transcripts on harvested body of the embryo was analyzed using real-time PCR. The mRNA expression level of Patch was significantly increased in 0.08µg 20S injected groups compared to control and sham injected group at 3-hour post injection. However, the expression level was reduced to normal levels as control groups by 12h post injection. There was no difference of Pthc expression at 1 h, 12 h, and 24 h post injection (Figure 5.1B). Gli-1 expression was significantly increased in the group treated with 1.6µg of 20S at 1, 3, and 12 h post-harvest compared to control group. 0.8µg of 20S significantly increased expression of Gli-1 at 12 h post injection compared to other treatment groups. There was no effect of 20S on Gli mRNA expression at 24 h. The inclusion of 20S significantly increased expression of Shh mRNA expression at 1 h and 3 h post injection. 0.08µg of 20S significantly continued to increase Shh expression up to 12 h post injection compared to other 2 levels of 20S. There was no effect of 20S on Shh mRNA expression at 24 h post injection (Figure 5.1C). BMP2 expression was significantly increased in the group treated with 0.08 and 0.8µg of 20S at 3 h post injection, whereas there was no difference in mRNA expression at 1 h and 12 h. However, at 24 h post injection 0.8µg 20S significantly increased mRNA expression compared to other treatment groups (Figure 5.1D). There was no difference in

RunX2 among the treatment at 1 h, 3 h, and 12 h post injection. RunX2 mRNA was significantly increased in groups treated with 20S compared control groups (Figure 5.1E). 0.08µg and 0.8µg of 20S significantly increased expression of BSP compared to control group at 24 h post-injection. However, there was no effect on BSP expression at 1 h, 3 h, and 12 h post injection (Figure 5.1F). These cumulative results indicate that 20S could increase the Hh dependent gene and osteogenic gene expression during embryogenesis when injected at 3 doi. 0.08 µg of 20S could be the most potent dose to obtain the significant effect of 20S in the embryo development without causing negative effects on gene expression,

In ovo injection at day 7

Effect of 20S on osteogenic and developmental mRNA expression in harvested embryo limbs were studied in the second experiment. 5µg of 20S significantly increased Patched mRNA expression at 6h post-injection. 5µg and 50µg of 20S significantly increased expression of Patched at 12 h post injection whereas 50µg of 20S significantly increased Patched expression at 24h post injection. Interestingly, at 48 h inclusion of 20S at all 3 levels significantly decreased Patched expression (Figure 5.2B). *In ovo* feeding of 20S increased expression of Gli1 (Figure 5.2A), Shh (Figure 5.2C), and BMP2 (Figure 5.2D) expression at 12 h post injection. However, there was no difference in Gli1, Shh, and BSP expression among treatments at 6 h, 24 h, and 48 h post injection. 50µg of 20S significantly increased expression of BMP2 mRNA expression at 6h post-injection. 50µg of 20S significantly decreased mRNA expression of 20S at 6 h post injection whereas 10µg of 20S significantly increased RunX2 mRNA expression at 12 h post injection (Figure 5.2E). BSP was significantly increased by 5µg of 20S at 12 h post injection compared to other treatment groups (Figure 5.2F).

This experiment also examined the effect of 20S gene expression of marrow free compact bone, muscles, and fat at hatch. In mRNA isolated from the bone at hatch, all 3 levels of 20S significantly increased expression of Ptch mRNA compared to control (Figure 5.3A). 5µg of 20S significantly increased expression of BMP2 and BSP compared to other treatment groups (Figure 5.3B). There was no effect of 20S on RunX2, Gli1 and Shh mRNA expression in harvested bone samples among the treatments (Figure 5.3A and 5.3B). In harvested abdominal fat samples, PPARγ, LPL, FASn, c/EBPα and c/EBPβ mRNA was significantly increased in groups treated with 10µM and 50µM of 20S compared to control samples (Figure 5.3C and 5.3D). However, Inclusion of 10 and 50µg of 20S significantly decreased mRNA expression of FABP4 compared to control groups (Figure 5.3C). In muscles samples, Myogenin and MyoD mRNA was significantly increased in the group treated with 5µg of 20S compared to other groups (Figure 5.3E). There was no effect of 20S on Pax7 Myf5, and Pax3 mRNA expression (Figure 5.3F). This experiment supports that 20S has an immediate positive effect on expression of osteogenic genes and Hh signaling pathway genes in embryo injected in yolk at 7 d. *In ovo* injection of 5µg of 20S at 7 d in the yolk of the embryo has overall positive effect on osteogenic, adipogenic, and myogenic differentiation genes in bone, fat and muscles samples respectively.

In ovo injection at 18 d

The effect of 20S *in ovo* injection at day 18 on the expression of mRNA transcripts on harvested bone, muscles, and fat was analyzed using real time PCR. 10µg and 50µg of 20S significantly increased expression of Gli1, Shh and BMP2 at 24 h post injection compared to other treatments (Figure 5.4A and 5.4B). 50µg significantly increased mRNA expression of BSP compared to control samples at 24 h post injection (Figure 5.4B). No significant difference was observed in BGLAP and RunX2 mRNA expression at 24 h post injection. 50µg of 20S

significantly increased mRNA expression of Gli-1, Shh, BSP, and BMP2 in bones at harvest compared to the control samples (Figure 5.4C and 5.4D).

In abdominal fat samples harvested 24 h post injection, 5µg 20S significantly increased mRNA expression of FASn, PPARγ, FABP4, LPL, c/EBPβ and c/EBPα compared to other control treatment groups (Figure 5.5 A and B). FABP4 mRNA was also significantly increased in treatment groups treated with 10µg of 20S (Figure 5.5B). 5 µg and 10 µg of 20S significantly increased FABP4 mRNA expression compared to other treatments at 24h post injection whereas embryos treated with 50µg of 20S significantly reduced FABP4 expression compared to control samples (Figure 5.5A).

The inclusion of 20S significantly increased FASn, PPARγ, FABP4, and c/EBPβ mRNA expression in all three treatment groups compared to control group harvested at hatch (Figure 5.5C and 5.5D). 10 µg and 50µg of 20S significantly increased LPL mRNA expression compared to other treatment groups at hatch (Figure 5.4D). Overall 5µg of 20S caused significantly increased in most of the adipogenic mRNA transcripts examined in fat cells isolated from 24 h post injection embryo. However, at hatch, all 3 level of 20S increased expression of adipogenic genes in fat samples

Similarly, in muscles, Pax3 and FGF4 mRNA expression were significantly increased in embryo treated with 50µg 20S compared to other treatment groups at 24 h postinjection (Figure 5.6A). There was no significant different in MyoD, Myogenin, Myf5, and Pax7 mRNA expression at 24 h post injection among the treatments (Figure 5.6A and 5.6B). A significant difference was observed in MyF5 mRNA expression in groups treated with 10µg and 50µg 20S compared to other treatment groups at hatch (Figure 5.6C). All levels of 20S significantly increased MyoD mRNA expression at hatch compared to control samples. There was no

significant difference in Myogenin, Pax3, FGF4, and Pax7 mRNA expression at 24 h post injection among the treatments (Figure 5.6C and 5.6D). The cumulative results of the third experiment indicate that 10µg and 50µg of 20S could induce osteogenic, adipogenic and myogenic genes in bones, fat, and muscles respectively at the different time point of the study.

DISCUSSION

In our study, *in ovo* injection of 20S at 7 and 18 d had no effect on body weight, hatchability, bone mineral density and bone mineral content at the day of hatch. However, use of oxysterol significantly increased the tissue fat percentage and decreased the lean weight of the chicks at hatch. Similarly, there was no effect of 18 d *in ovo* injection of 25(OH)D3 on body weight or relative tibia weight of broiler chicks at the day of the hatch (Bello et al., 2014a; Bello et al., 2014b). *In ovo* injection of different levels of L-carnitine did not increase chick BW and % hatch in Ross X 708 broiler chicks (Zhai et al., 2008). *In ovo* injection of different carbohydrates at 18.5 d has a positive effect on body weight on the day of the hatch of Ross 708 broiler chicks (Zhai et al., 2011a). Similarly *in ovo* feeding of CHO, HMB, and CHO + HMB at 17.5d significantly increased body weight of Ross x Ross broiler at hatch (Tako et al., 2004). This indicates that CHO, AA could be directly used for chicken but circulating levels of hormone and its interaction could play a crucial role in regulating vitamins, prohormones and bioactive compounds (de Matos, 2008). As oxysterol is not a direct nutritional supplement like AA, CHO or minerals, *in ovo* feeding of oxysterol may not have direct effect on the growth of embryo but could increase or decrease the body composition and molecular mechanism of differentiation of certain cell types that could affect the growth of birds until it reached the market body weight. 20S and its oxysterol analogs are reported to induce differentiation of pluripotent mesenchymal

stem cells and inhibited adipogenic differentiation in mouse and human cells invitro and induce spinal fusion in rats and rabbits *in vivo* (Li et al., 2015).

Oxysterols are the derivatives of cholesterol oxidation which are found in circulation and tissue of animals. Oxysterols are reported to increase osteogenic differentiation of MSCs through Hh signaling pathway by indirectly activating smoothened expression (Dwyer et al., 2007). Hedgehog signaling is a key pathway that plays a significant role in normal patterning of the multicellular embryo during development and various function in postembryonic development and stem cell physiology of animals and humans (Dwyer et al., 2007; Zhang and Kalderon, 2001). Reduced Hh signaling pathway can cause various developmental defects (Mullor et al., 2002). Lack of Shh signaling result in defect of craniofacial bones and exhibit marked defects in development of vertebral column and stunted and disorganized limbs (Chiang et al., 1996; St-Jacques et al., 1999) As MSCs can differentiate into any cell type including osteoblast, adipocyte, and myocyte and use of oxysterol could direct the lineage differentiation of MSCs through hedgehog signaling pathway we were interested in understanding the effect of oxysterol on osteogenic genes, Hh signaling and developmental aspect of embryo in early stage.

In our study, *in ovo* injection of 20S at 3 and 7 d increased mRNA expression of Shh mRNA expression. Shh is expressed in posterior limb bud, notochord, floor plate of the neural tube and plays a significant role in development and patterning of the embryo (Weed et al., 1997). Shh can act as a cell-cell signaling factor to modulate cell fate over short and long distances. In the early stages of embryonic limb development, Shh is expressed in the posterior region of the limb bud (Wang et al., 2000). Shh acts as a major morphogen in patterning and development of anteroposterior axis of the limb buds, ossification and induction of osteoblast (Kicheva et al., 2012). Previous studies have reported that mutation of Hh can lead to abnormal

digit number or change in digit identity during development (Tickle and Barker, 2013; Towers et al., 2008). Thus, induction of Shh by oxysterol could play a positive role in early limb development and proper limb development in the chick embryo.

Patched, a 12-span transmembrane protein is the receptor of Shh which inhibits the function of HH signaling pathway (Barnfield et al., 2005). Hh ligand binds to its receptor Patched1 to release the suppression effect of Patched on Smoothened thus facilitating the downstream signaling cascade (Incardona et al., 2000). Smoothened, a 7-span transmembrane protein, in turn, abolishes PKA function and allows the movement of Kif7 and Gli2/3-SuFu complex (Incardona et al., 2000; Rohatgi and Scott, 2007). Kif7 then facilitates protein trafficking and dissociating binding between Gli and Sufu which helps in activation of Gli2/3 that relocates to the nucleus to activate the expression of the Hh target genes Ptch1 and Gli1 (Law et al., 2012; Li et al., 2012). Interestingly, Patch 1 also acts as a transcriptional target of Hh signaling thus forming a negative feedback system in Hh signaling (Ribes and Briscoe, 2009). In the present study, *in ovo* injection of 20S at 3 d, 7 d and 18 d significantly increased expression of Gli mRNA expression at different time points of the study. Similarly, *in ovo* injection of 20S at 3 d and 7 d d significantly increased mRNA expression of Patched at the different time point of the study. However, 20S did not increase mRNA expression of Patched mRNA at both time points of the harvest when injected at 18 d and were significantly decreased at 48 h post injection. In a previous study in mouse, embryonic fibroblast and other mouse derived MSCs, 20S significantly induced osteogenic differentiation of MSCs through Hh signaling pathway and activation the expression of Ptch and Gli1 gene (Dwyer et al., 2007). However, this is the first study to understand the gene expression related to Hh signaling pathway after *in ovo* injection of 20S in chicken embryo at the different time point of incubation. It has also been reported that

Shh must be proteolytically cleaved and modified by the addition of a cholesterol moiety to become active as a signaling molecule (Porter et al., 1995). Cholesterol is covalently attached to the C-terminal of Shh which serves as signaling transduction of Hh domain and acts as an intramolecular cholesterol transferase (Porter et al., 1996a; Porter et al., 1996b). This explains the important of cholesterol for Shh function in embryo development without which could cause birth defects, perturbation, and midline defects. As oxysterol is a family of 27-carbon derivative products of cholesterol present in circulation, oxysterol could act as a modifier of Hh signaling in chicken development.

Oxysterol has also been reported to increase osteogenic differentiation of mammalian MSCs *in vitro* (Kha et al., 2004). *In vivo* application of oxysterol on mouse vertebral column increased bone formation and trabecular growth (Johnson et al., 2011b). Treatment of M2010B4 mouse marrow stromal cells with specific oxysterol increased osteogenic differentiation genes such as osteocalcin, BSP, and RunX2 *in vitro* and enhanced bone formation in calvarial defect mice which identifies oxysterol as a potential agent for local and systemic enhancement of bone formation (Aghaloo et al., 2007). Similarly, in our current study, 20S significantly increased expression of bone-related genes BMP2, BSP, and RunX2 at the various time point of injection.

BMP2 is a member of the transforming growth factor-beta superfamily of proteins and plays a role in heart, neural, and cartilage development in the embryo and postnatal bone formation (Chen et al., 2004; Shu et al., 2011). Previous studies reported that oxysterol could increase bone regeneration in cranial defects of mature rabbits similar to bone growth induced by the exogenous BMP-2 application (Li et al., 2015). BMP2 is involved in Hh signaling pathway, TGFβ signaling pathway and cytokine-cytokine receptor interaction (Li et al., 2015). BMP2 are expressed in murine embryonal skeletogenesis and acts on the murine limb buds to promote

differentiation of osteoblast (Kaneko et al., 2000). Offspring from BMP2-cKO^{ob} mouse had thinner bones and smaller unfused vertebra with 5% decrease in length compared to control. At 4 months μCT analysis of vertebra and long bones shows 35% smaller vertebra, 20% decreased trabecular thickness and 30-40% decrease in bone volume of long bones in Bmp2-cKO^{ob} mouse compared to control ones (Yang et al., 2013). BMP2 has been BMP2 expressed in postnatal life serves to induce osteoblast differentiation and helps in commitment towards osteoblastic lineage. In our study, 20S increased BMP2 mRNA expression in harvested embryo as well as in cortical bones at all 3 injection time points.

BSP gene encodes a tissue-specific non-collagenous protein that is glycosylated, phosphorylated and sulfated and is a major structural protein of bone matrix (Chen et al., 1992). The regulation of BSP gene is important in differentiation of osteoblast and in bone matrix mineralization (Chen et al., 1992). In this study, BSP mRNA was significantly increased when the 20s was *in ovo* injected at all 3 injection time points. BSP-/- newborn mice expressed delayed membranous primary ossification, wider cranial structure, thinner femoral cortical bones and lower tissue mineral density measured through μCT analysis. (Kobayashi et al., 2000). Similarly, in mouse C3H10T1/2 mouse embryonic fibroblast and M2-10B4 mouse marrow stromal cells oxysterol 133 an analog like the 20s induced significant expression of BSP gene *in vivo* and increased bilateral spine fusion through endochondral ossification invitro in Lewis rats (Montgomery et al., 2014).

RunX2 belongs to a family of runt-related transcription factors, which regulates the commitment of mesenchymal stem cells to the osteoblastic lineages and positively influences the stages of osteoblast differentiation and are expressed in mesenchymal stem cells during early embryonic development (Liu and Lee, 2013). RunX2 is also required for both intramembranous

and endochondral bone development as well as regulation of chondrocyte proliferation and maturation (Komori et al., 1997). During osteogenic differentiation, RunX2 plays an important role in the regulation of the several osteoblast marker genes in osteoblast and induces several osteoblast marker genes such as osteocalcin, Col1A2, BSP in non-osteoblastic cells (Kobayashi et al., 2000). However, for further bone maturation, the RunX2 expression must be downregulated as RunX2 inhibits osteoblast maturation and mature bone formation (Kobayashi et al., 2000). In this study, at 7 d injection RunX2 was significantly increased in groups treated with the 20s at 12 h post injection. However, by 48 h post injection, RunX2 was significantly decreased compared to control samples. There was no effect of RunX2 in bone samples harvested at both time points in 18d injection or at the hatch in 7 d injection. Similarly, 20S analog Oxy34 and oxy49 induced expression of RunX2, Osterix, BSP in mouse multipotent bone marrow stromal cells *in vivo* and increased bone trabecular and cortical bone formation *in vivo* (Johnson et al., 2011a). During mouse embryogenesis, RunX2 mRNA expression was observed at E11.5 in limb buds and the condensation of the humerus (Inada et al., 1999; Kim et al., 1999). Thus, RunX2 plays an important role in the initiation of the expression of the bone matrix related genes during early stages of osteoblastic differentiation and chondrocyte hypertrophy, but RunX2 may not be essential for the maintenance of this gene expression during mature osteoblast stage (Komori, 2010).

Studies have been conducted to understand the effect of 20S on adipogenic differentiation of MSCs derived from mouse and human tissue, however, there is limited information in understanding the effect of oxysterol metabolism during normal embryonic development. The current study evaluated the effect of *in ovo* injection of 20S on adipogenic gene expression in fat tissues harvested at hatch. In the current study, 20S increased expression of adipogenic genes

such as PPARγ, LPL, c/EBPα, c/EBPβ, FASn, and FABP4 in fat tissue samples harvested at hatch from the chicks that were *in ovo* fed at 7 doi and 18 doi. Previous studies in M2-10B4 pluripotent marrow stromal cell, C3H-10T1/2 mouse pluripotent embryonic fibroblast and primary mouse stromal cells reported that oxysterol inhibits adipogenic differentiation of MSCS by decreasing PPARγ transcription (Kha et al., 2004). Another study in M2-10B4 murine MSC reports that 20S inhibit PPARγ promoter activity and adipogenic differentiation of MSCs through a Hh-dependent mechanism (Kim et al., 2007). However, our results indicate that oxysterol could increase mRNA expression of adipogenic genes in the adipose tissue. Total fat mass % of the chicks injected with 20S was also higher when measured with the help of DEXA. The previous study has not been conducted to understand the effect of oxysterol on embryogenic preadipocyte development. Previously, studies have been conducted to understand the role of selenium in adipogenesis *in vitro* and *in ovo*. Selenium supplementation increased lipid droplet accumulation in cultured CEFs isolated from 6 d old embryo but did not promote adipogenic differentiation of CEFs isolated from 9 to 12 d old embryos (Hassan et al., 2014). Also, *in ovo* injection of Se at 8 doi increased adipose tissue mass by 30% and caused adipocyte hypertrophy ion 17day old chicken embryos (Hassan et al., 2014). In contrast, in our study oxysterol inhibited the genes involved in adipocyte differentiation in chicken derived MSCSs *in vitro* but increased adipocyte differentiation genes when 20S was injected *in ovo* in 7 and 18 d old embryos. The expression exerted by oxysterol in MSCs derived from mammals and chickens *in vivo* and expression exerted in the adipocyte cells while embryonic development *in vivo* could be completely different due to several mechanisms related to adipogenesis. Further study needs to be conducted to understand the exact mechanism by which 20S effects the adipogenic differentiation of developing an embryo.

As modern broiler reach market age within 6-7 wks. of hatch it is important to have a positive influence in muscles development and growth. Myogenesis is the process of formation of muscle tissue and it differentiation during embryonic development and post-natal life through stem and progenitor cell maintenance, lineage specification and terminal differentiation (Saccone and Puri, 2010). Muscles fibers are usually formed by the fusion of myoblast to form a multinucleated fiber called myotubes. Serum receptor factors, androgen receptor factors, and Myogenic regulatory factors MyoD, Myf5, Myogenin are important factors in the regulation of myocytes (Francetic and Li, 2011). Pax3 and Pax7 are expressed specifically during early development of the muscular system (Jostes et al., 1990). MyoD and Myf5 both are the markers of the terminal specification to the muscles lineage (Pownall et al., 2002). Maternally transferred bioactive molecules and hormones such as androgen, testosterone and *in ovo* supplemented nutrients can influence differentiation and growth potential of the embryo and hatched chick post hatch. In our study, 20S did not influence the hatch body weight but increased the myogenic gene expression such as MyoD, Myogenin, Pax7, Pax3 and FGF4 in skeletal muscles harvested at 7 d and 18 d of injection. Previous studies have suggested that androgens in the eggs deposited from the maternal body are anabolic for muscles development and skeletal growth, and boost post-natal body mass gain (Lipar and Ketterson, 2000; Pilz et al., 2004; Schwabl, 1996). However, some other studies have failed to show such positive effect of androgens *in ovo* injection and some have even shown a negative effect on post-natal growth (Possenti et al., 2016; Rubolini et al., 2006). *In ovo* supplementation of testosterone prior to incubation did not affect the weight of 12, 16 or 20 d old male chicken embryo or on the characteristics of pectoralis superficial muscles. However, the weight of female embryos at 12 d and protein concentration of pectoralis muscles at 16 d were depressed.

In conclusion, different dose did not influence the body weight and composition of hatched embryo. *In ovo* injection at 3 doi immediately increased the function of hedgehog signaling pathway and increased the expression of osteogenic differentiation genes of the developing embryo. *In ovo* feeding at 7 doi and 18 doi increased osteogenic, myogenic and adipogenic marker genes in developing embryo and at hatch. Further research concerning the pathways involved *in ovo* injected oxysterol induced differentiation genes and its effect on postnatal growth, body weight gain, and body mass index should be investigated.

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FIGURES

Table 5:1: Primers sequences that were used for qRT-PCR gene expression assay.

Gene	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)	Accession #
GAPDH	Fwd: GCTAAGGCTGTGGGGAAAGT Rev: TCAGCAGCAGCCTTCACTAC	161	55	(Regassa and Kim, 2013)
BSP	Fwd: CAGTGGGAGTACGAGGTGAC Rev: CAGTGGGAGTACGAGGTGAC	141	55	NM_205162.1
Gli1	Fwd: GCACAGCTCCAACGACCGCT Rev: GTTGCCGTCGGAAGCACCCA	205	57	NM_001305245.1
BMP2	Fwd: TCAGCTCAGGCCGTTGTTAG Rev: GTCATTCCACCCCACGTCAT	163	57	NM_204358.1
Ptch	Fwd: GGCGTTCGCGGTGGGACTAC Rev: GGTGCTGCCGGAGTGCTTCT	205	56	NM_204960.2
Shh	Fwd: TGC TAG GGA TCG GTG GAT AG	197	56	NM_204821.1

	Rev: ACA AGT CAG CCC AGA GGA GA			
RunX2	Fwd: ACTTTGACAATAACTGTCCT	192	52	NM_204821.1
	Rev: GACCCCTACTCTCATACTGG			
FABP4	Fwd: TGCTGGGCATCTCAATCACA	106	57	(Regassa and Kim, 2013)
	Rev: GCATTAGTCAGAACGGGCCT			
PPARγ	Fwd: TGAATGTCGTGTGTGTGGGG	229	55	(Regassa and Kim, 2013)
	Rev: GCATTCGCCCAAACCTGATG			
C/EBPα	Fwd: CCTACGGCTACAGAGAGGCT	205	55	(Regassa and Kim, 2013)
	Rev: GAAATCGAAATCCCCGGCCA			
C/EBPβ	Fwd: CCGCTCCATGACCGAACTTA	204	55	(Regassa and Kim, 2013)
	Rev: GCCGCTGCCTTTATAGTCCT			
LPL	Fwd: TGCCCCTATCCGCCTCTCCC	297	57	(Regassa and Kim, 2013)
	Rev: GTTGCAGCGGTAGGCCATGCT			
FASn	Fwd: AGAGGCTTTGAAGCTCGGAC	127	57	NM_205255.2
	Rev: GGTGCCTGAATACTTGGGCT			
MyoD	Fwd: CAGCAGCTACTACACGGAATCA	102	57	(Sławińska et al., 2013)

	Rev: GGAAATCCTCTCCACAATGCTT			
Myogenin	Fwd: AGCAGCCTCAACCAGCAGGA	179	58	NM_204184.1
	Rev: TCTGCCTGGTCATCGCTCAG			
Pax7	Fwd: AGGCTGACTTCTCCATCTCTCCT	156	57	XM_015296832.1
	Rev: GTAACTGGTGGTGCTGTAGGTG			
Pax3	Fwd: GCACCAGGCATGGATTTTCC	184	59	NM_204269.1
	Rev: AGACCTCCAGTCAAAGGGGA			
Myf5	Fwd: GAGGAACGCCATCAGGTACATC	126	57	NM_001030363.1
	Rev: ACATCGGAGCAGCTGGAGCT			

Table 5:2: Effect of in ovo injection of 20S oxysterol at 7d and 14d on hatchability and chick weight of broilers at hatch.

	In ovo Injection					
	7d*			18d*		
Treatment	Hatchability %	Initial egg weight (g)	Chick BW (g)	Hatchability %	Initial egg weight (g)	Chick BW (g)
Control	93.33	63.45	45.98	96.67	66.30	47.94
Sham Injection	90.00	64.56	46.51	93.33	65.11	46.92
5µg Oxy	90.00	64.25	46.55	90.00	64.60	46.82
10µg Oxy	86.67	62.69	45.38	90.00	64.99	46.91
50µg Oxy	93.33	64.22	46.43	86.67	65.07	46.79
SEM		0.605	0.526		0.669	0.541
P value		0.183	0.478		0.458	0.517

n = 30 birds

*Eggs were injected in the yolk at day 7 of incubation and in the amniotic cavity at day 18 of incubation.

Table 5:3: Results of DEXA scan of the whole chick at the day of hatch that were in ovo fed with different levels of 20S at day 7 of incubation.

Treatment	BMD[3]	BMC[4]	Tissue Fat %	Tissue (lb)	Fat (lb)	Lean (lb)
Control[1]	0.03817	0.15	7.28333c	0.05	0.00383c	0.04617a
Sham Injection[2]	0.03133	0.11667	13.9b	0.04717	0.0065ab	0.041a
5µg Oxy	0.03088	0.11667	15.7167ab	0.05017	0.00783ab	0.04233a
10µg Oxy	0.0315	0.1	16.0833ab	0.05067	0.00817ab	0.04267a
50µg Oxy	0.029	0.11667	19.6a	0.04667	0.00933a	0.03717b
SEM[5]	0.00287	0.01633	1.25842	0.00185	0.00076	0.041
P value	0.2365	0.3157	<.0001	0.4166	0.0004	0.0062

[1]Control = Eggs not injected but kept outside of incubator for the same period as injected eggs during injection; [2]Sham injection= Injected with the vehicle in which oxysterol was dissolved (1X PBS).[3]BMD: Bone Mineral Density; [4]BMC; Bone Mineral Content; [5]SEM: standard error of the mean.

[a-c] Means within the same row without the same superscripts are significantly different ($P<0.05$).

Six birds per treatment were used for each treatment group. Eggs were injected in the yolk at day 7 of incubation

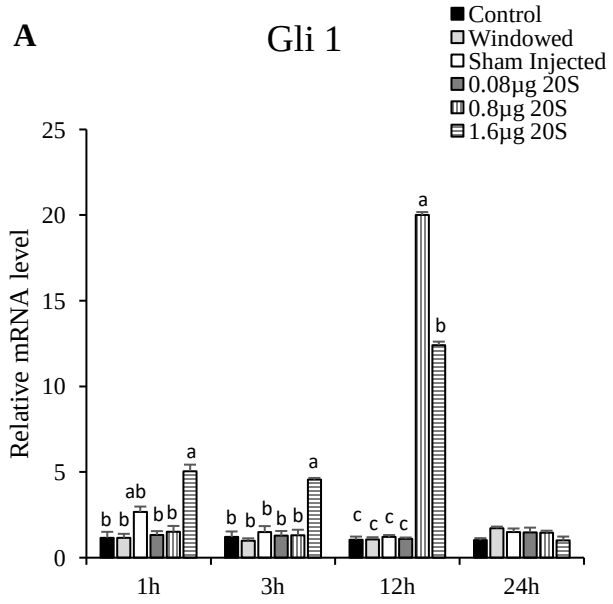
Table 5:4: Results of DEXA scan of the whole chick on the day of hatch that were in ovo fed with different levels of 20S at day 18 of incubation.

Treatment	BMD	BMC	Tissue Fat	Tissue (lb)	Fat (lb)	Lean (lb)
Control	0.0342	0.16	10.54b	0.0538	0.0058b	0.0482
Sham Injection	0.0315	0.11667	12.8833b	0.05233	0.007b	0.04567
5µg Oxy	0.03617	0.08333	18.7167a	0.05217	0.00983ab	0.04233
10µg Oxy	0.03383	0.13333	18.8a	0.05833	0.011a	0.0475
50µg Oxy	0.03267	0.08333	18.85a	0.05267	0.00983ab	0.04267
SEM	0.004	0.02421	1.26749	0.00269	0.00094	0.00206
Pvalue	0.9191[a]	0.1178	<.0001	0.3781	0.0017	0.1334

[1]Control = Eggs not injected but kept outside of incubator for the same period as injected eggs during injection; [2]Sham injection= Injected with the vehicle in which oxysterol was dissolved (1X PBS).[3]BMD: Bone Mineral Density; [4]BMC; Bone Mineral Content; [5]SEM: standard error of the mean.

[a-c] Means within the same row without the same superscripts are significantly different ($P<0.05$).

Six birds per treatment were used for each treatment group. Eggs were injected in the yolk at day 14 of incubation.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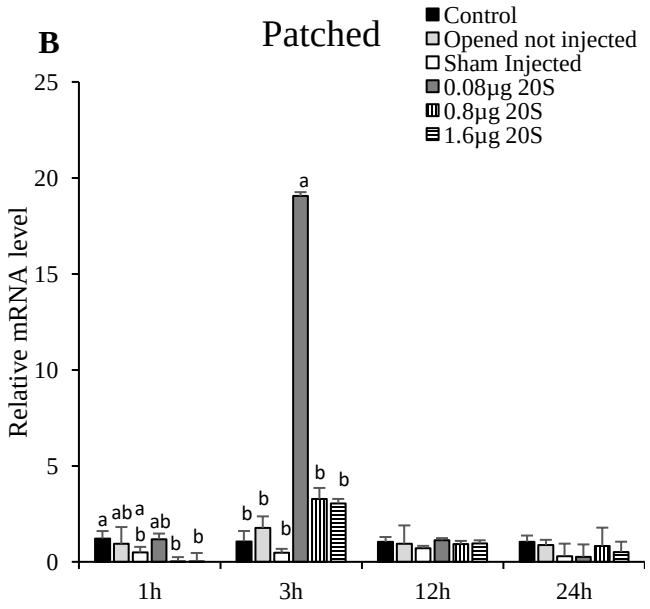
24h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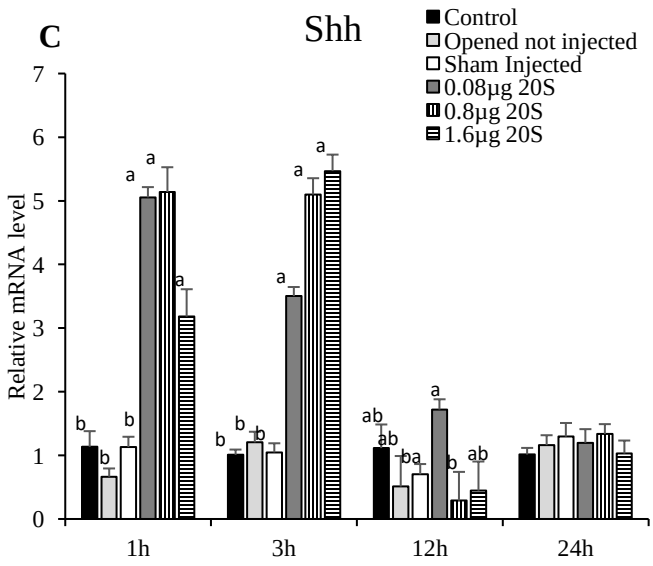
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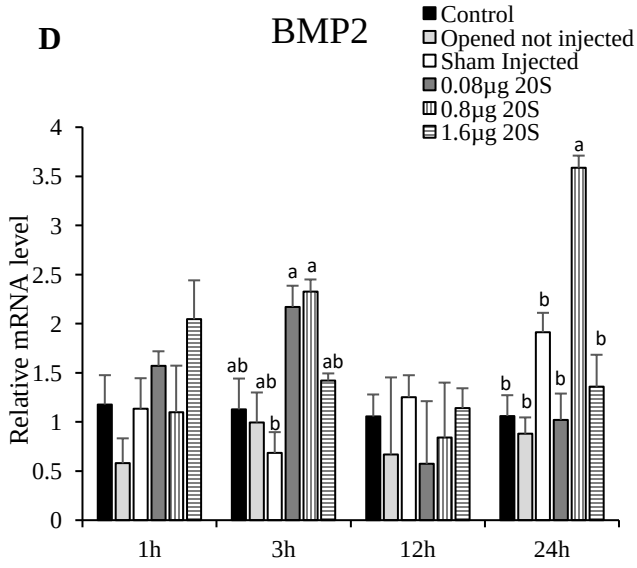
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24h

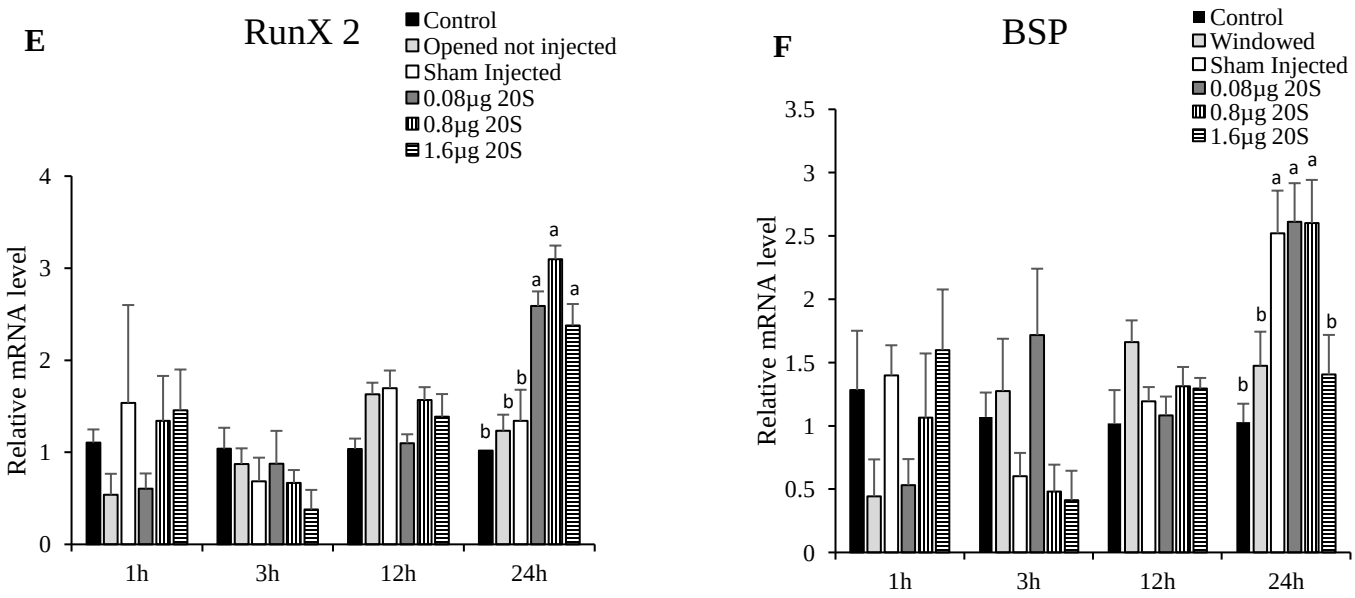
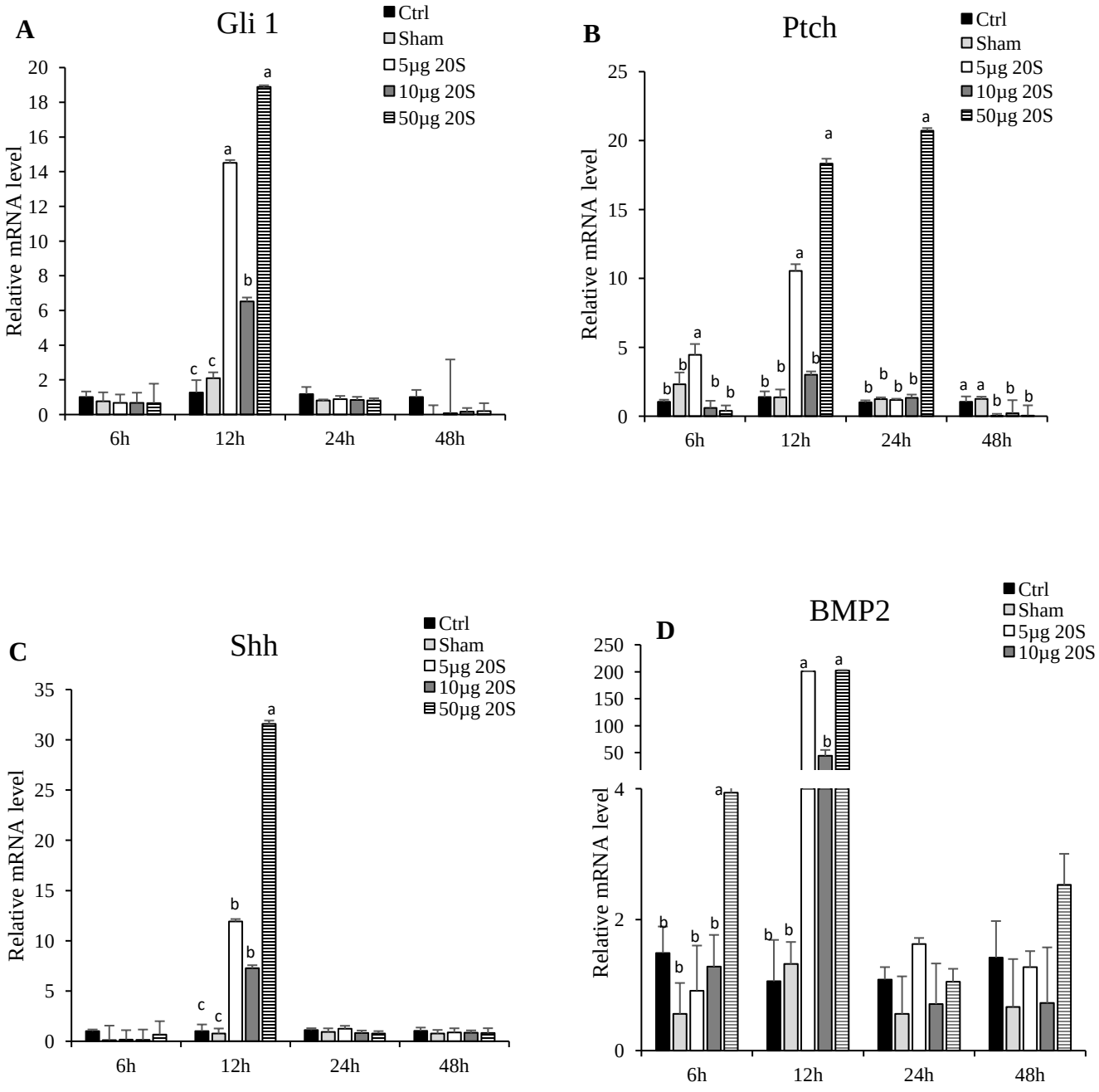


Figure 5.1: Effects of 20S oxysterol in osteogenic and hedgehog signaling mRNA expression in the trunk of the 3d embryo. Total RNA was extracted from the whole trunk of broiler embryos (n=4) at 1h, 3h, 12h, and 24h post-injection. cDNA was transcribed and relative expression pattern of each gene was examined using qRT-PCR. Relative mRNA expression levels of each genes A) Gli-1, B) Patched, C) Sonic Hedgehog (Shh), D) BMP2, E) RunX2, and F) BSP expressed at different harvest time points are presented. Embryo at day 3 was in ovo injected in vitelline vein with 1) Control, 2) Window open but not injected, 3) Sham injection, 4) 0.08µg 20S, 5) 0.8µg 20S, 6) 1.6µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter within the harvested period are significantly different (P < 0.05) when analyzed with Tukey's test.


A
Gli 1
Ctrl
Sham
5μg 20S
10μg 20S
50μg 20S
Relative mRNA level
6h
12h
24h
48h
B
Ptch
C
Shh
D
BMP2

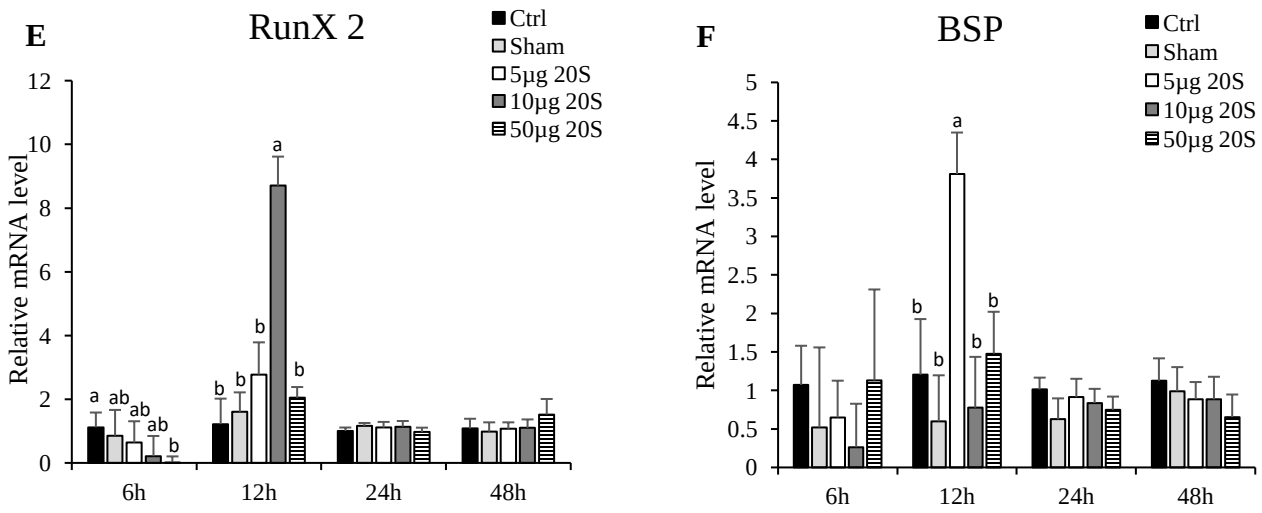
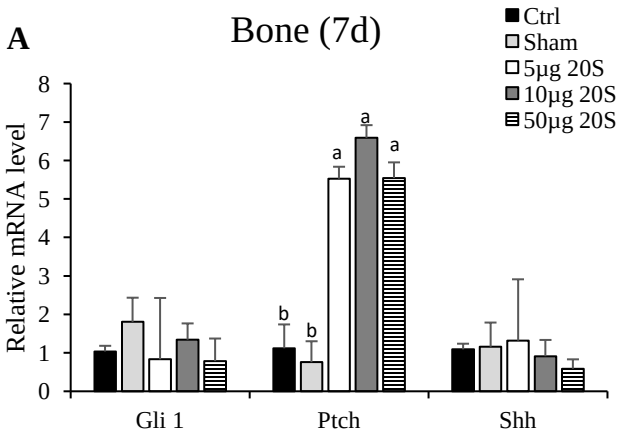
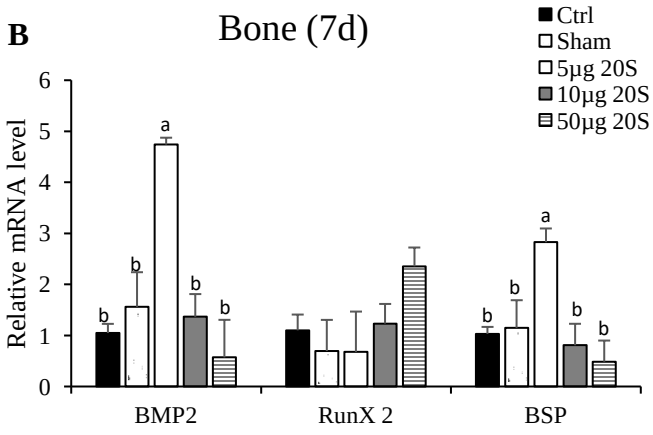
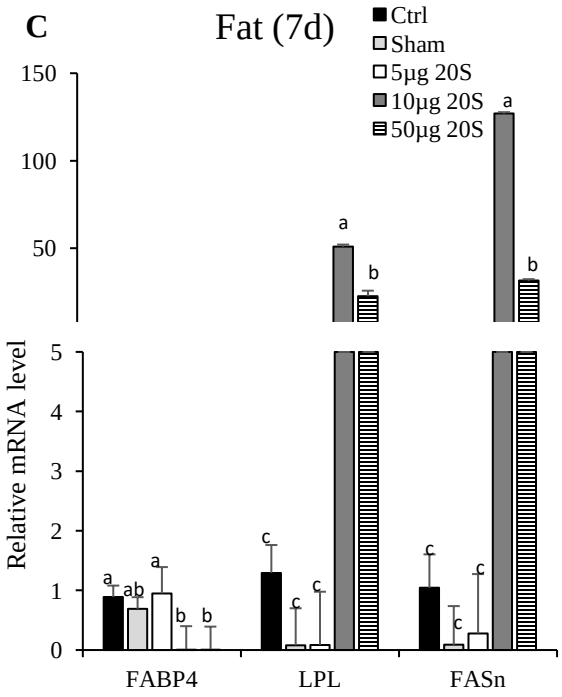
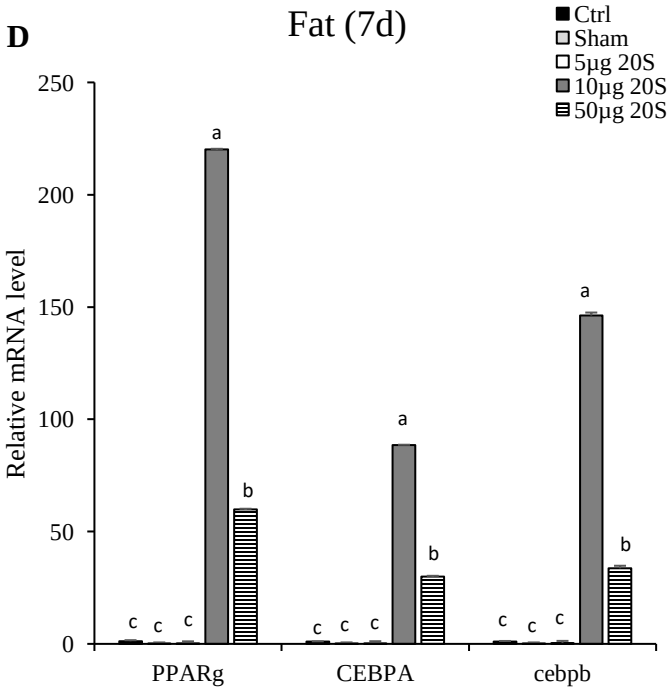


Figure 5.2: Effects of 20S oxysterol in osteogenic and hedgehog signaling mRNA expression in a hind limb of the 7d embryo. Total RNA was extracted from hind limb of harvested broiler embryos (n=5) at 6h, 12h, 24h, and 48h post-injection. cDNA was transcribed and relative expression pattern of each gene was examined using qRT-PCR. Relative mRNA expression levels of each genes A) Gli-1, B) Patched, C) Sonic Hedgehog (Shh), D) BMP2, E) RunX2, and F) BSP expressed at different harvest time points are presented. Embryo at day 7 was in ovo injected in yolk with 1) Control, 2) Sham injection, 3) 5µg 20S, 4) 10µg 20S, and 5) 50µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the harvested period are significantly different (P < 0.05) when analyzed with Tukey's test.


A
Bone (7d)
Ctrl
Sham
5μg 20S
10μg 20S
50μg 20S
Relative mRNA level
Gli 1
Ptch
Shh


B
Bone (7d)
Ctrl
Sham
5μg 20S
10μg 20S
50μg 20S
Relative mRNA level
BMP2
RunX 2
BSP


C
Fat (7d)
Ctrl
Sham
5μg 20S
10μg 20S
50μg 20S
Relative mRNA level
FABP4
LPL
FASn


D
Fat (7d)
Ctrl
Sham
5μg 20S
10μg 20S
50μg 20S
Relative mRNA level
PPARg
CEBPA
cebpb

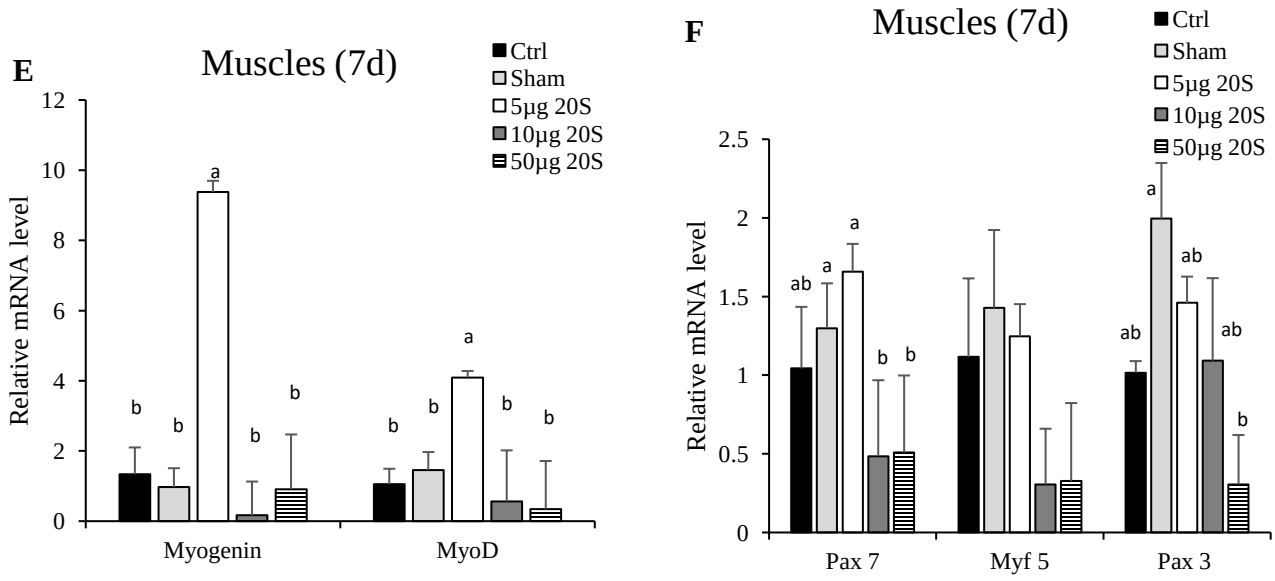


Figure 5.3: Effects of 20S oxysterol in mRNA expression of bone, muscle and fat harvested from a chick at hatch injected at 7d of incubation. Embryo at 7 days of incubation was injected with a different level of 20S in the yolk. Total RNA was extracted from cortical bone, pectoralis major muscles and abdominal fat of hatched chick. cDNA was transcribed and relative expression pattern of osteogenic, adipogenic and myogenic genes in bone, fat, and muscle respectively was examined using qRT-PCR. Relative mRNA expression levels of osteogenic genes in bone A) and B); adipogenic genes in fat C) and D), and myogenic gene expression in muscles E) and F) are presented. Embryo at day 7 was in ovo injected in yolk with 1) Control, 2) Sham injection, 3) 5µg 20S, 4) 10µg 20S, and 5) 50µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-c) within the harvested period are significantly different (P < 0.05) when analyzed with Tukey's test.

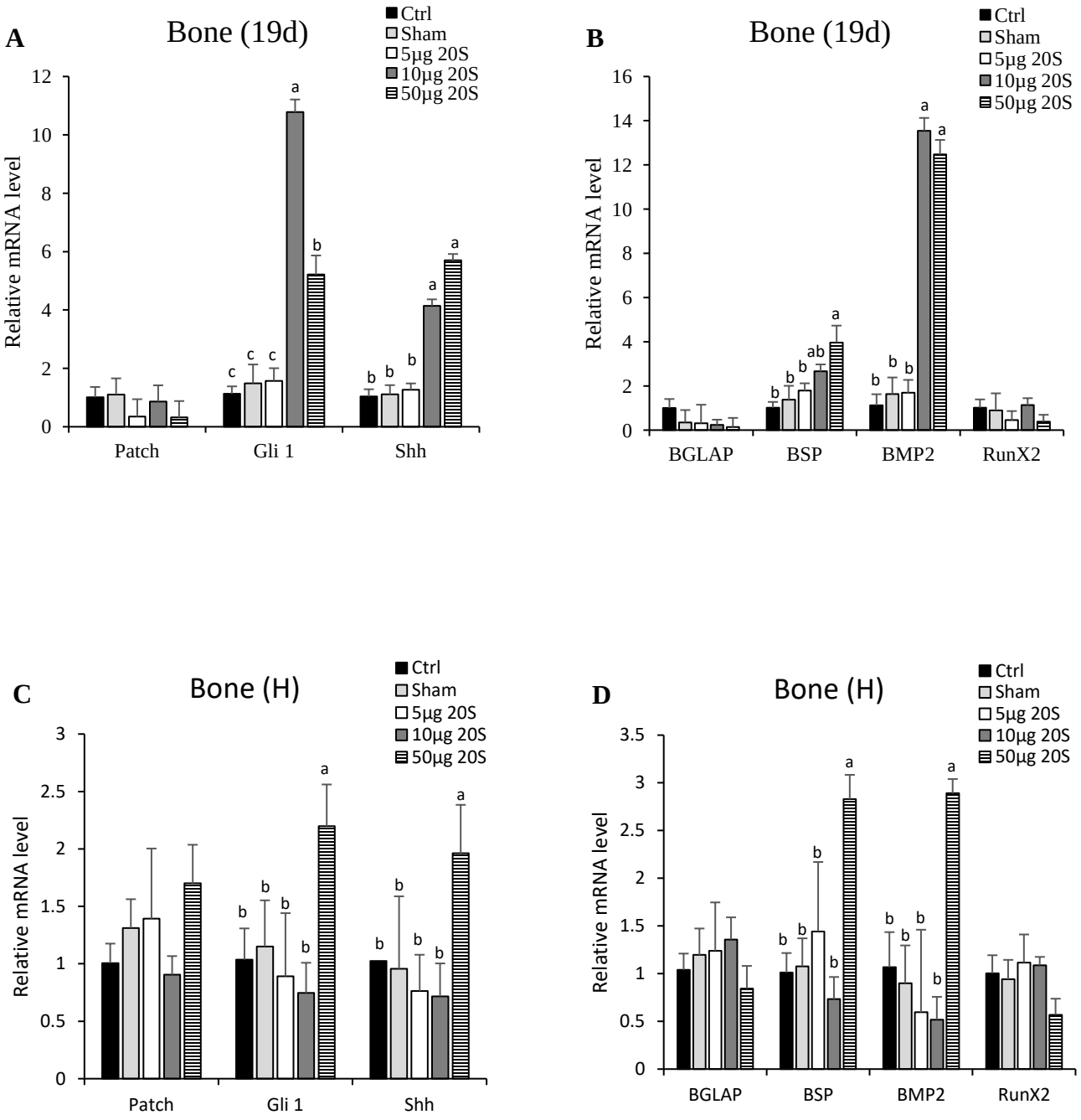


Figure 5.4: Effects of 20S oxysterol in mRNA expression of the cortical long bone of chick injected at 18 d of incubation and harvested at 24h post injection and at hatch. Embryo at 18 d of incubation was

injected with a different level of 20S in the amniotic cavity and bone sample was harvested 24 h post injection and at hatch. Total RNA was extracted from cortical bone of tibia of harvested sample. cDNA was transcribed and relative expression pattern of osteogenic genes was examined using qRT-PCR. Relative mRNA expression levels of osteogenic genes expressed in bones harvested 24 h post injection are presented in A) and B) and genes expressed at hatch are presented in fat C) and D. Embryo at day 18 was in ovo injected in amnion with 1) Control, 2) Sham injection, 3) 5µg 20S, 4) 10µg 20S, and 5) 50µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the same gene are significantly different (P < 0.05) when analyzed with Tukey's test.

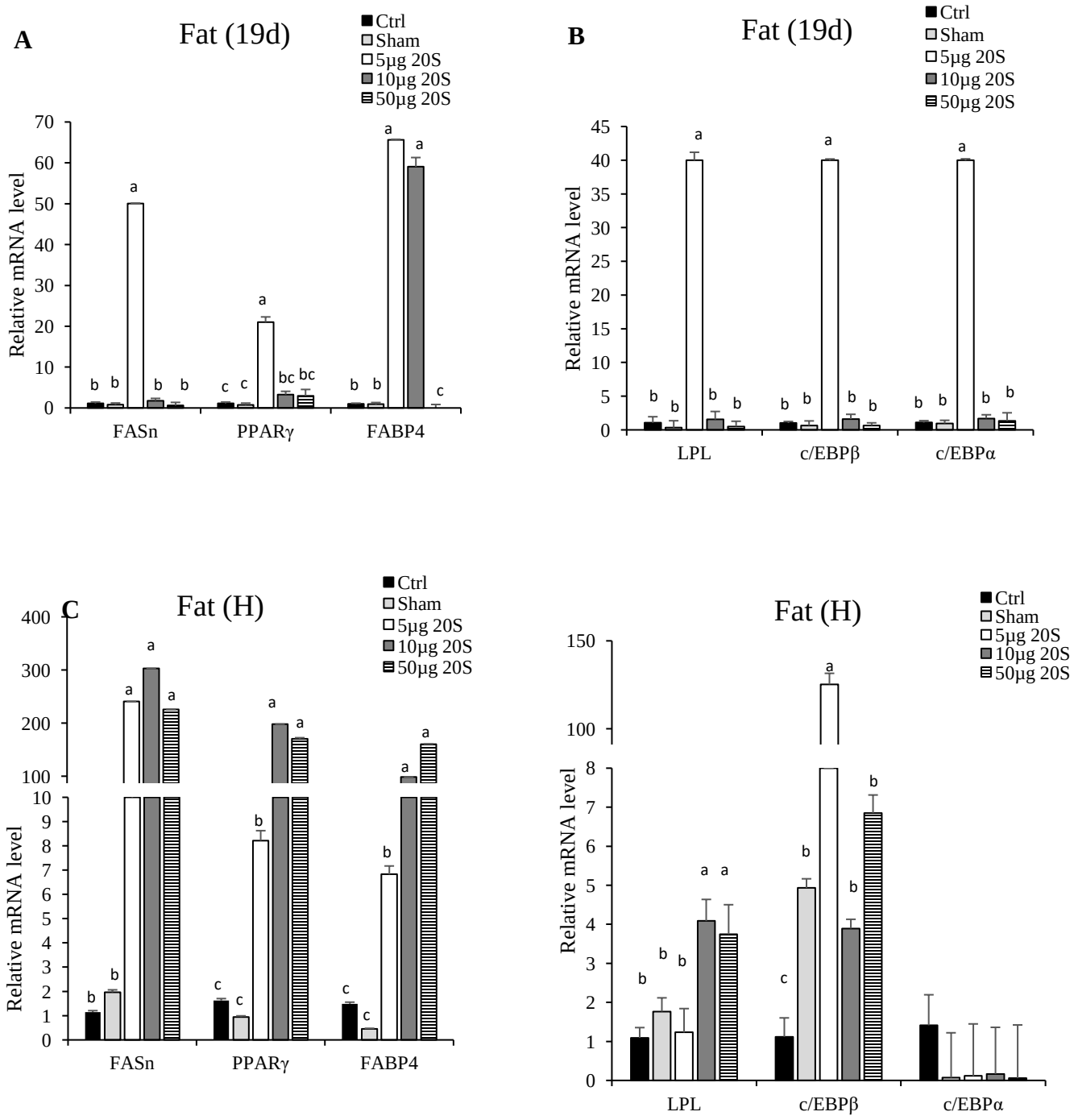
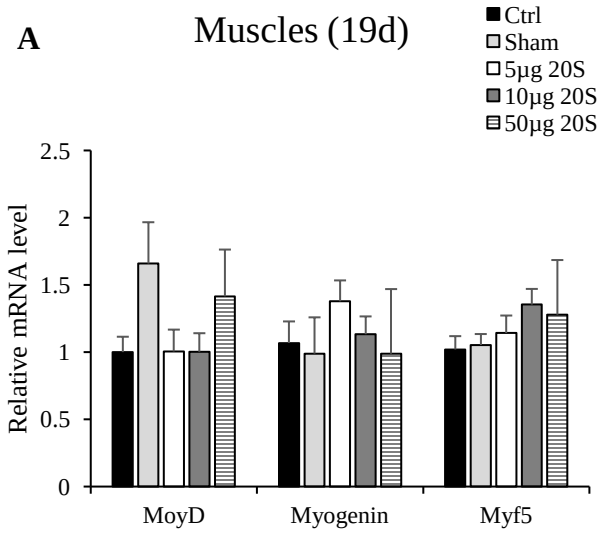
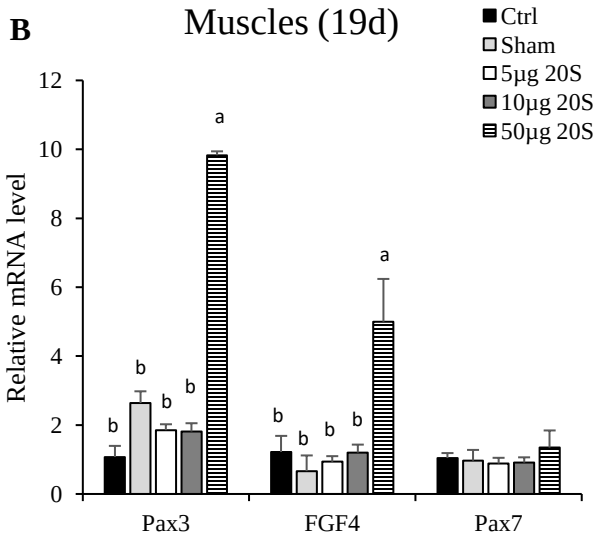
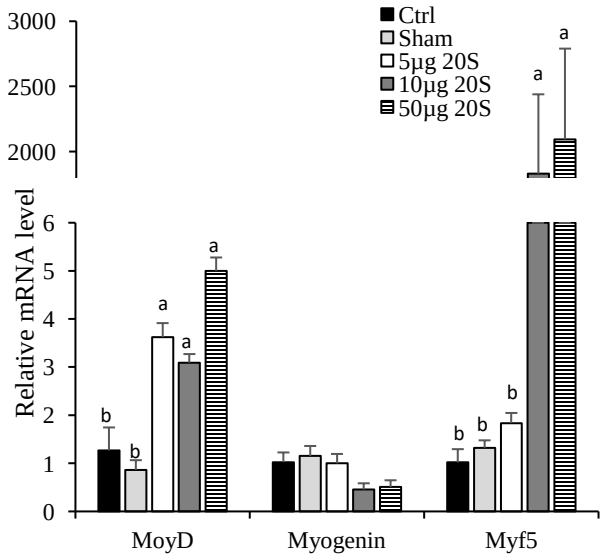


Figure 5.5: Effects of 20S oxysterol in mRNA expression of abdominal fat harvested from chick injected at 18 d of incubation and harvested at 24h post injection and at hatch. Embryo at 18 d of incubation was

injected with a different level of 20S in the amniotic cavity and abdominal fat sample was harvested 24 h post injection and at hatch. Total RNA was extracted from the harvested samples. cDNA was transcribed and relative expression pattern of adipogenic genes was examined using qRT-PCR. Relative mRNA expression levels of adipogenic genes expressed in bones harvested 24 h post injection are presented in (A) and (B) and genes expressed at hatch are presented in fat (C) and (D). Embryo at 18 d was in ovo *injected in the amnion with 1) Control, 2) Sham injection, 3) 5µg 20S, 4) 10µg 20S, and 5) 50µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-c) within the same gene are significantly different (P < 0.05) when analyzed with Tukey's test.*


A
Muscles (19d)
Ctrl
Sham
5µg 20S
10µg 20S
50µg 20S
Relative mRNA level
2.5
2
1.5
1
0.5
0
MoyD
Myogenin
Myf5


B
Muscles (19d)
Ctrl
Sham
5µg 20S
10µg 20S
50µg 20S
Relative mRNA level
12
10
8
6
4
2
0
a
b
a
b
b
b
b
b
b
Pax3
FGF4
Pax7


C
Muscles (H)
Ctrl
Sham
5µg 20S
10µg 20S
50µg 20S
Relative mRNA level
3000
2500
2000
6
5
4
3
2
1
0
a
a
a
a
b
b
b
b
b
b
MoyD
Myogenin
Myf5

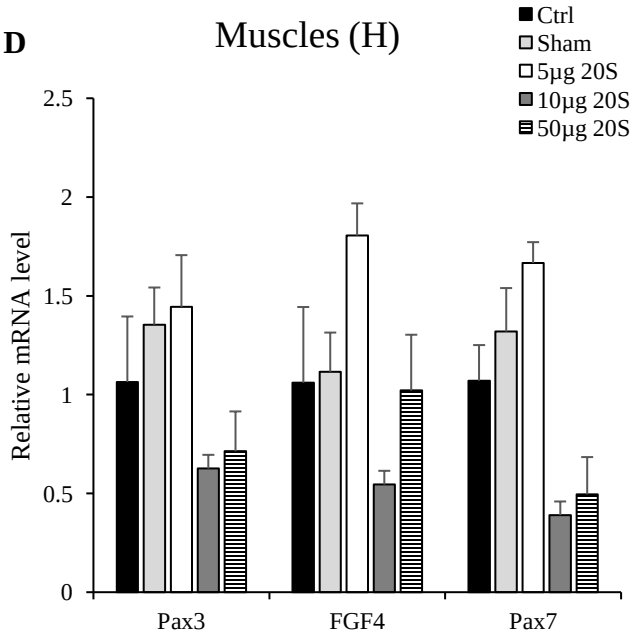

D
Muscles (H)
Ctrl
Sham
5µg 20S
10µg 20S
50µg 20S
Relative mRNA level
2.5
2
1.5
1
0.5
0
Pax3
FGF4
Pax7

Figure 5.6: Effects of 20S oxysterol in mRNA expression of muscles harvested from chick injected at 18 d of incubation and harvested at 24h post injection and at hatch. Embryo at 18 d of incubation was injected with a different level of 20S in the amnion and pectoralis muscle sample was harvested 24 h post injection and at hatch. Total RNA was extracted from the harvested samples. cDNA was transcribed and relative expression pattern of myogenic genes was examined using qRT-PCR. Relative mRNA expression levels of myogenic genes expressed in muscle sample harvested 24 h post injection are presented in (A) and (B) and genes expressed at hatch are presented in fat (C) and (D). Embryo at 18 d was in ovo *injected in the amnion with 1) Control, 2) Sham injection, 3) 5µg 20S, 4) 10µg 20S, and 5) 50µg 20S. GAPDH was used as housekeeping gene. Fold changes in gene expression relative to the control were calculated using the -ΔΔCt method. Data from the representative experiment are reported as the mean of the duplicate determination. Bars represent mean ± SEM. Bars with a different letter (a-b) within the same gene are significantly different (P < 0.05) when analyzed with Tukey's test.*

CHAPTER 6

CONCLUSION

MSCs are multipotent stromal cells that were originally isolated from bone marrow but have been isolated from other organs and tissues as well. As the MSCs can differentiate into multiple tissues and it has a unique ability to help in tissue repair, there is a rising interest in utilizing MSCs in a broad repertoire of cell-based therapies in experimental preclinical animal models. MSCs has been used as a cell culture model to understand the multilineage differentiation potential and understanding the molecular pathways of various bioactive compounds. In ordered to achieve consistent results in such *in vitro* and *in vivo* experiments a high-yield, homologous pool of MSCs is required. In this study, we established a reliable and a user-friendly, low-cost protocol for the isolation and culture of chicken compact bone-derived MSCs. cBMSCs could differentiate into adipogenic, osteoblast and myogenic cells and expressed lineage-specific mRNA expression when subjected to adipogenic, osteogenic and myogenic media respectively. cBMSCs could form colonies and were able to adhere to the plastic surface. cBMSCs at all 3-passage revealed positive results for mRNA transcription of CD90, CD105, CD73, CD44, and CD29. CD 45 and CD34 mRNA were expressed negatively in cBMSCs at all 3 passage. GAPDH was positive in all 3 passages which were used as a housekeeping control.

Oxysterols are oxygenated derivatives of cholesterol oxidation, a 27-carbon molecule present in the circulation in human and animal tissue. Oxysterol is involved in many different

physiological, biological, and pathological roles in the animal body such as cholesterol efflux, lipoprotein metabolism, calcium uptake, atherosclerosis, and apoptosis. It has been reported that oxysterol plays a possible role of regulating cellular differentiation of MSCs. Our study showed that 20S are a novel oxysterol compound with pro-osteogenic, pro-myogenic and anti-adipogenic properties. Our study further provides an evidence that 20S increased the osteogenic differentiation and decreased adipogenic differentiation of cBMSCs by activating Hh signaling pathway. Furthermore, 20S oxysterol also increased the myogenic differentiation of cBMSCs but did not exert its differentiation through Hh signaling pathway. These findings provide the evidence that oxysterols could induce the Hh signaling pathway and therefore could play an important role in osteogenesis, adipogenesis and other developmental process of chicken that are regulated by hedgehog signaling pathway.

We further studied the effect of 20S oxysterol on embryogenesis, multilineage gene expression and hatchability of chicken. Over the years, studies have been conducted on the experimental injection of small amounts of vaccines and nutrients into the egg during incubation. These early developments have led to increased research into *in ovo* techniques in poultry for improved starting weights, better feed utilization, faster growth and higher final weights. Chicken embryo model has also been used to understand the effect of different compounds/drugs on developmental transcripts, multiple gene expression patterns and signaling pathway to understanding various research questions relating to health and disease.

In our study, *in ovo* injection at day 3 of incubation immediately increased hedgehog signaling pathway and increased the expression of osteogenic differentiation genes of the developing embryo. *In ovo* feeding at day 7 and day 18 of incubation increased osteogenic, myogenic and adipogenic marker genes in developing embryo at different harvest time points

and at hatch. However, 20S did not affect hatchability, hatched chick body weight, bone mineral density, and bone mineral content of day old hatched chicks. Chicks injected with oxysterol at 7 doi in the yolk and at 18 doi in the amniotic cavity increased body fat mass of the hatched chick. Further research concerning the pathways involved *in ovo* injected oxysterol-induced differentiation genes and its effect on postnatal growth, body weight gain, and body mass index should be investigated.