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Characterization and Regulation of Steroid Sulfatase in the Human MG-63 Pre-osteoblastic Cell Line

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CHARACTERIZATION AND REGULATION OF STEROID SULFATASE IN THE

HUMAN MG-63 PRE-OSTEOBLASTIC CELL LINE

A Dissertation

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In partial fulfillment of the requirements for

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By

Natasha J. Dias

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ABSTRACT

CHARACTERIZATION AND REGULATION OF STEROID SULFATASE IN THE

HUMAN MG-63 PRE-OSTEOBLASTIC CELL LINE

By

Natasha J. Dias

August 2015

Dissertation supervised by Dr. Kyle W. Selcer

The importance of estrogen in bone regulation is exemplified by the reduction in bone density at the onset of menopause. Post-menopausal women have low levels of estrogens, but high levels of inactive sulfated steroids. These can be converted to active steroids by steroid sulfatase (STS), which is a microsomal enzyme found in many tissues. STS desulfates common steroids such as dehydroepiandrosterone sulfate and estrone sulfate, the products of which can serve as precursors for active estrogens. We sought to characterize the activity and expression of STS in human bone cells with the idea that increasing STS expression in bone could offset low bone density.

STS activity was found to be relatively high in MG-63 pre-osteosarcoma cells, indicating that these cells can produce active estrogens from precursors. STS activity was

blocked by the known inhibitors EMATE and 667 Coumate. In addition, cell growth was stimulated by addition of sulfated steroids.

STS activity and expression were examined in pre-osteoblastic and differentiated MG-63 cells over a 21-day period. STS activity and expression were higher in pre-osteoblastic cells than in differentiated cells. The STS decline was found to be due to the presence of the glucocorticoid dexamethasone in the differentiation medium. Inhibition of the glucocorticoid receptor with RU-486 blocked the decline in STS activity.

Results from a collaborator suggested that NF-κB might regulate STS transcription. The NF-κB activators LPS and PMA increased STS expression, which was lowered in the presence of the NF-κB inhibitor BAY. Glucocorticoids and NF-κB are antagonistic to each other with regard to immune responses. Thus, steroid sulfatase appears to be regulated like an immune response protein in pre-osteoblastic cells. The significance of this for bone physiology is unclear.

Our data indicate that steroid sulfatase is present in bone cells and that it can influence bone cell growth by converting inactive sulfated steroids to estrogenic forms. Furthermore, STS expression is regulated by glucocorticoid and NF-κB pathways. Manipulation of STS expression via these pathways may lead to a potential treatment for osteoporosis.

DEDICATION

This thesis is dedicated to both my parents. My father, late Joaquim Dias, despite

several difficulties always supported my educational interests in the Biological Sciences.

My dad's dying words to me were, "Natasha, my girl, study well." He wanted me to

pursue my doctoral degree and it is unfortunate that he did not live long enough to see

this day of my life, but I am sure his blessings are upon me. My mother, Stephanie Dias,

has been a source of motivation and strength during moments of despair and

discouragement. She has always encouraged me to believe in myself and never give up

on my dreams. I am greatly indebted to their sacrifices and all they have done for me.

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Thanks to the faculty, staff, and fellow graduate students of the Biology Department. Your help and support has meant the world to me and I am grateful for having had the opportunity to share this experience with you. I would also like to thank all the undergraduates in Dr. Selcer's lab for their friendship and assistance throughout this project.

Last and most importantly, I would like to thank my family. I would not have embarked on this journey if it hadn't been for their persistence. I would like to thank my parents, Joaquim and Stephanie, and my sister, Ana. Words cannot begin to describe what you have meant to me in my life.

Without all of you, I would not have accomplished this goal!

ATTRIBUTIONS

Dr. Kyle Selcer is responsible for the development of the steroid sulfatase antibody, STS-

275, which was used throughout the project.

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LIST OF ABBREVIATIONS

ALP = alkaline phosphatase

BAY = (E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile

BMU = basic multicellular unit

DHEAS = dehydroepiandrosterone sulfate

DMSO = dimethyl sulfoxide

E_1S = estrone sulfate

EDTA = ethylenediaminetetraacetic acid

EMATE = estrone-3-O-sulfamate

FB = forward base primer

GC = glucocorticoid

GR = glucocorticoid receptor

HRT = hormone replacement therapy

LPS = lipopolysaccharides

MMTV = mouse mammary tumor virus

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-κB = nuclear factor-kappa B

OPG = osteoprotegerin

PBS = phosphate buffer saline

PCR = polymerase chain reaction

PKC = protein kinase C

PMA = phorbol 12-myristate 13-acetate

qRT-PCR = quantitative real time reverse transcription polymerase chain reaction

RANK = receptor activator of nuclear factor κB

RANKL = receptor activator of nuclear factor kappa-B ligand

RB = reverse base primer

RT-PCR = reverse transcription polymerase chain reaction

STS = steroid sulfatase

Chapter 1

Background

1.1 <u>Basic Bone Biology</u>:

Bone is a connective tissue that provides structural support for the body, protection for internal organs, and functions as the body's mineral and bone marrow storehouse (Compston, 2001; Walsh et al., 2006). The skeletal system acts as a reservoir for calcium and phosphate ions and plays an essential role in the homeostasis of these minerals. Other important functions of bone include locomotion, mechanical support of the diaphragm for breathing, attachment for muscles, ligaments, and tendons and hematopoiesis in the bone marrow (Cohen, 2006). Additionally, bone tissue has the unique ability to adapt its structure to mechanical stimuli and repair fractures and other defects through a remodeling process. Thus, the skeleton is not static, instead it is a highly metabolically active tissue (Lerner, 2006; Robling et al., 2006).

Almost eighty percent of the skeleton is cortical bone, which is a compact bone found in the shafts of long bones and surfaces of flat bones. The compact bone is very hard and dense, consisting of a Haversian system of cylindrical structures oriented parallel to the long axis of the bone (Compston, 2001). The Haversian system contains blood vessels, nerves, connective tissue, and lymphatic tissues. The compact bone is well organized and provides bone with maximal strength (Walsh et al., 2006). Furthermore, trabecular bone or cancellous bone is found mainly at the ends of long bones and in the inner parts of flat bones and consists of interconnecting plates and bars within which lies hematopoietic or fatty marrow (Compston, 2001).

Trabecular bone is thinner and less well organized than cortical bone. It is primarily found spanning the bone marrow space. A major function of trabecular bone is to provide a large surface area for metabolic processes. Additionally, bone turnover, consisting of bone resorption (removal) and its replacement with new bone, occurs much more frequently in trabecular bone than in cortical bone (Walsh et al., 2006).

The human skeletal system is a specialized type of connective tissue comprised of cells embedded in an extracellular matrix. Ninety percent of bone volume is extracellular matrix, while the remaining ten percent comprises cells and blood vessels. This extracellular matrix is composed of both an organic matrix and an inorganic component (Compston, 2001; Downey and Siegel, 2006). The organic matrix consists primarily of type I collagen synthesized by osteoblasts, secreted, and then assembled extracellularly (Downey and Siegel, 2006). Additional organic compounds include proteoglycans, and noncollagenous proteins including osteocalcin, bone sialoprotein, osteonectin, thrombospondin, and osteopontin (Compston, 2001). The inorganic phase of bone matrix is composed mainly of calcium-phosphate salt, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)(Compston, 2001; Walsh et al., 2006). Hydroxyapatite crystals are essential in providing the major portion of the tensile yield strength. During mineralization, hydroxyapatite crystals are deposited on and between the collagen fibrils, producing hardened bone (Downey and Siegel, 2006; Walsh et al., 2006). With regard to bone regulation, a large number of cytokines and growth factors that affect bone cell functions have been identified in bone matrix and these play an important regulatory role in bone remodeling

(Compston, 2001).

The cellular component of bone comprises four different types of osteon cells: bone lining cells, osteoblasts, osteocytes, and osteoclasts found within the cellular network of bone (Downey and Siegel, 2006). Each of the cell types has a specific functional role in bone formation and resorption. However, bone tissue osteon cells arise from different cell origins to undergo proliferation, differentiation, and activation or inhibition of cell activity in response to different cues within the bone tissue (Robling et al., 2006).

Osteoblasts are involved in the secretion of the extracellular matrix, the mineralization process, and in influencing bone resorption via a signaling axis that controls osteoclast generation and activity. Active osteoblasts form a monolayer of cells and secrete unmineralized matrix called osteoid, which then further matures and mineralizes under the influence of enzyme alkaline phosphatase. Osteoblasts are derived from undifferentiated bone marrow mesenchymal stem cells that can also give rise to other cell lineages, including adipocytes, myocytes and chondrocytes (Robling et al., 2006). As stated before, the major structural component of the osteoid matrix is the type I collagen. Osteoid is also composed of a number of other noncollagenous proteins that play pivotal roles in bone. Hydroxyapatite, the mineral crystal of bone, is a calcium-phosphate salt containing hydroxyl ions (Walsh et al., 2006). The mature osteoblasts expressing type I collagen (ColI) and osteocalcin (OC) also produce a key enzyme in the mineralization process, alkaline phosphatase. As a row of active osteoblasts secretes unmineralized osteoid matrix and advances away from the bone surface, a small number of cells fall behind and become incorporated

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into the matrix, inducing mineralization and matrix maturation, and in doing so influencing matrix calcification (Robling et al., 2006). These osteoblasts that become entrapped in the calcifying matrix, and persist as unique bone cells, are called osteocytes (Walsh et al., 2006).

Osteoblast maturation is characterized by the proliferation of mesenchymal stem cells. The signals involved in the formation of osteoblasts from mesenchymal progenitors are not fully understood. Nonetheless, several critical paracrine signals and transcription factors have been identified (Walsh et al., 2006). Differentiation of proliferating mesenchymal stems cells through the osteoblastic lineage is achieved by an increase in the expression of transcription factors Osterix (Osx) and runt-related transcription factor-2 (Runx2), the latter of which serves as the earliest known osteoblast-specific marker (Ducy et al., 1997; Robling et al., 2006). Preosteoblastic cells committed to the osteoblastic lineage express the early phenotypic marker type I collagen. Alkaline phosphatase continues to increase in expression as the pre-osteoblasts mature, thereby serving as another phenotypic marker for differentiation. Furthermore, osteocalcin is typically used in the identification of mature osteoblasts, as it is only expressed upon the appearance of mineralized matrix. Thus, osteocalcin serves as a late phenotypic marker (Malaval et al., 1999; Beck et al., 2001).

In mouse MC3T3-E1 bone cells, the process of differentiation takes place between 25 to 30 days *in vitro*, and is defined by three distinct phases (Fig. 13). The initial proliferation phase occurs from day 0 to day 4. Then, MC3T3-E1 cells enter a phase of bone matrix formation from day 10 to day 16, with ALP as the first gene expressed, followed by type 1 collagen and osteonectin expression. Thereafter, the

mineralization phase starts, with a maximum expression of osteocalcin mRNA at day 28 (Choi et al., 1996).

Osteocytes, are small, flattened cells found embedded within the bone matrix and are connected to one another and to osteoblastic cells on the bone surface via an extensive canalicular network containing bone extracellular fluid (Robling et al., 2006; Walsh et al., 2006). Osteocytes are believed to be mature osteoblastic cells that became entrapped in the extracellular matrix during the process of differentiation (Downey and Siegel, 2006; Robling et al., 2006). As extracellular matrix is secreted, osteocytes become embedded deeper within the bone tissue and begin to form long cytoplasmic projections, which allow the osteocytes to remain in contact with adjacent cells and the bone surface.

Bone lining cells are thin, long cells that cover most of the surfaces of mature skeleton. Compared to osteoblasts, they contain less cytoplasm and fewer organelles, reflecting their metabolically inactive condition and leading to their being referred to as 'resting osteoblasts' (Downey and Siegel, 2006). Bone lining cells play a significant role in bone resorption and formation. Mouse long bones and calvariae have bone lining cells surrounding collagen fibrils and, using metalloproteinases and serine proteinases, the bone lining cells digest the protruding collages fibrils from the Howship's lacunae that were produced by the osteoclasts (Everts et al., 2002). Subsequently, the bone lining cells deposit a thin collagenous matrix into the Howship's lacuna, along with an osteopontin-rich cement layer (Everts et al., 2002). Bone lining cells may be precursors for osteoblasts, and they may help regulate crystal growth in bone, and also possibly function as a barrier between the

extracellular fluid and bone. Furthermore, in the presence of parathyroid hormone, the bone lining cells secrete enzymes that remove the osteoid covering of the bone matrix preceding the removal of bone matrix by osteoclasts (Buckwalter et al., 1996). Moreover, bone lining cells play a role in the presence of multinucleated bone resorbing osteoclasts by attracting their precursor monocytes to the sites where resorption will occur (Perez-Amodio et al., 2004). The interaction between bone lining cells and osteoclast precursors results in altered gene expression and promote the formation of osteoclasts (Jansen et al., 2012).

Osteoclasts, the final type of osteon cells, are large, multinucleated cells derived from hematopoietic precursors of the myocyte/macrophage lineage, which can also give rise to macrophages and dendritic cells. They are rich in lysosomal enzymes, including collagenase and cathepsins, which degrade the bone matrix upon release. Osteoclasts, under the control of osteoblasts, are responsible for bone resorption and the subsequent release of calcium and phosphate ions into the bloodstream (Compston, 2001). Mature osteoclasts are highly motile and move across the bone surface to resorb relatively large areas of bone (Walsh et al., 2006). Osteoclasts attach themselves to bone surfaces to form a specialized structure called the sealing zone, which allows them to breakdown the extracellular matrix by pumping hydrogen ions, acidifying the resorption space and solubilizing the mineral component of bone. Once the mineralized matrix is degraded, osteoclasts release lysosomal enzymes that continue the resorption of bone by a further breakdown of the remaining organic component of the matrix. The extent of bone resorption by osteoclasts depends on the lifespan of the osteoclasts (Walsh et al., 2006).

Bone is a metabolically active tissue. As such, bone deposition and bone resorption are ongoing dynamic processes. About 10% of bone is replaced each year with complete renewal of the skeleton occurring every 10 years (Cohen, 2006). The coordinated action of the osteon cells within the bone tissue helps to maintain and develop the skeletal system via two main mechanisms, bone modeling and bone remodeling. These two processes define skeletal shape, maintain homeostatic levels of calcium and phosphate ions in the serum, and repair any portion of the bone that has become weakened or damaged (Robling et al., 2006).

Bone modeling is responsible for the growth and shaping of bones that occurs during development and earlier years of life. It can occur on any bone surface and requires activation of either osteoclasts or osteoblasts. Modeling is a process where bone resorption takes place in one site and bone formation at another. Thus formation of new bone is independent of removal of old bone (Lerner, 2006). Once skeletal maturity is achieved, bone modeling is reduced to a minimal level. However, in the adult skeleton, in some bone diseases with serious consequences to the overall integrity of affected bone, bone modeling is reactivated. In contrast, bone remodeling is a continuous process (Robling et al., 2006).

Bone remodeling dominates in the adult skeleton. This process involves a balance of osteoclast removal of small, isolated packets of bone and osteoblast replacement with new bone tissue. The two processes of resorption and formation are coupled to one another. The net amount of old bone removed and new bone restored in the remodeling cycle is called the bone balance. Coupling ensures that there is a balance between bone removal and new bone formation, so that the resorbed area is

filled in completely (Robling et al., 2006). Several local factors, such as cytokines, mechanical stresses and growth factors play an important role in the bone tissue as mediators of cell-to-cell and matrix-to-cell communication (de Vernejoul, 1996). Most bone diseases result from a disruption in the coupling process, which leads to an imbalance in either bone degradation or formation. This imbalance results in bones that become structurally compromised (Robling et al., 2006).

A balanced remodeling process involves continuous removal of discrete packets of old bone, replacement of these packets with newly synthesized proteinaceous matrix, and subsequent mineralization of the matrix to form new bone. This remodeling process to resorb old bone and form new bone prevents accumulation of bone microdamage and is modulated by the RANK/RANKL/OPG system (RANKL receptor activator of NK-κB ligand, RANK receptor activator of NF-κB ligand's decoy receptor and OPG osteoprotegerin), which has been recognized as the primary regulator of bone remodeling (Weitzmann and Pacifici, 2006). This osteoimmunological signaling system, which is regulated by the sex hormones called estrogens, determines the success or failure of bone homeostasis (McCormick, 2007).

Estrogens play a direct role in the bone remodeling process via the RANK/RANKL/OPG signaling axis. During normal bone remodeling, osteoblasts produce RANKL, which binds to the transmembrane receptor RANK on osteoclast precursors and activates osteoclast resorption. In response to estrogen, osteoblasts also produce osteoprotegerin (OPG), a soluble decoy receptor that blocks RANKL to maintain control of the remodeling process (McCormick, 2007). Additionally, during bone remodeling, estrogen exhibits its nuclear regulatory effects by inhibiting IL-6

activation. Activated estrogen receptors (via estrogen binding) can bind to the transcription factor NF-κB and inhibit activation of IL-6. IL-6 in turn regulates expression of RANKL (Boyle et al., 2003). Thus, IL-6, indirectly regulated by estrogen, regulates osteoclastogenesis (McCormick, 2007).

Bone resorption and bone formation do not occur randomly in the skeleton, but take place in distinct bone multicellular units (BMU) (Lerner, 2006). The primary region of the BMU is lined with osteoclasts that carry out bone resorption. Following closely behind the osteoclasts is a group of mononuclear cells that smooth off the uneven periphery of the resorptive areas. Behind the mononuclear cells, rows of osteoblasts adhere and deposit layers of unmineralized bone matrix to form new bone tissue (Robling et al., 2006).

1.2 <u>Effect of Sex Steroids on Bone Tissue</u>:

Sex steroids, estrogens and androgens, are known to play a central role in the maintenance of bone microarchitecture (Janssen et al., 1999; Raisz, 1999; Compston, 2001). The clinical observation of rapid bone loss at the onset of menopause exemplifies the role of estrogen in bone maintenance. Estrogens are able to act directly on bone to support normal bone physiology (Raisz, 1999; Heshmati et al., 2002; Riggs et al., 2002; Lerner, 2006). Within bone, the aromatase enzyme, which converts androgens to estrogens, appears to be a major source of estrogen responsible for maintaining bone mineralization (Simpson and Davis, 2001; Simpson, 2003). Increased resorption rates, without a comparable increase in bone formation, have been attributed to a loss of sex steroids, either estrogens in women or androgens in

men. Moreover, estrogen deficiency associated with menopause causes roughly a doubling in the bone loss rate, while increasing the risk of osteoporosis development (Raisz et al., 2005).

Estrogen is able to exert its effect directly on osteon cells, through the presence of both nuclear estrogen receptors, ER-α and ER-β (Braidman et al., 1995; Matthews and Gustafsson, 2003). Estrogens diffuse in and out of all cells, but are retained with high affinity and specificity in target cells by estrogen receptors (ER), which are ligand operated transcription factors. Once bound by estrogens, the ER undergoes a conformational change, allowing the receptor to bind with high affinity to chromatin and to modulate transcription of target genes (Murdoch and Gorski, 1991). Using expression vectors, estrogen signaling was found to occur via two receptors, ER-α and ER-β (Kuiper et al., 1997). Functional estrogen receptors have been found to be present in osteoblasts, osteoclasts and most recently in osteocytes, although differential expression patterns of the estrogen receptor types are seen. ER-α is the predominant isoform in human cortical bone, while ER-β is the principal isoform in human trabecular bone (Matthews and Gustafsson, 2003).

Estrogens function to increase bone formation by increasing osteoblast formation, differentiation, proliferation, and function (Riggs et al., 2002). In osteoclasts, estrogen causes a decrease in formation and activation and also induces apoptosis (Hughes et al., 1996). During estrogen deficiency, there is an increase in formation of new basic multicellular units leading to an alteration in balance between the resorptive and formation phases. Under low estrogen levels, the resorption phase is prolonged and the formation phased is shortened, resulting in an increase in

osteoclast recruitment that continues to prolong the resorptive phase. These all

contribute to an increase in resorption beyond what osteoblasts are able to refill

(Riggs et al., 2002). Thus, the balance between bone formation and bone resorption is

through the direct action of estrogen on bone (Janssen et al., 1999; Raisz, 1999;

Heshmati et al., 2002; Riggs et al., 2002).

Nuclear androgen receptors, along with estrogen receptors, are present in

normal human bone cells of the osteoblast lineage (Colvard et al., 1989). Testosterone

mediates bone remodeling by stimulating proliferation and inhibiting apoptosis of

osteoblasts (Riggs et al., 2002). Another function of testosterone is to decrease bone

resorption indirectly, through aromatization to 17β-estradiol (Riggs et al., 2002).

Furthermore, it has been shown that 5β-dihydrotestosterone interacts with osteoclasts

to inhibit bone resorption directly (Pederson et al., 1999). While testosterone is the

predominant circulating sex steroid in men, and estradiol and estrone are the

predominant circulating sex steroids in premenopausal women, androgens and

estrogens circulate in both sexes. Presence of androgen and estrogen receptors in

bone cells suggests that both sex steroids contribute to the maintenance of bone mass

in both sexes (Colvard et al., 1989). This might be particularly true in postmenopausal

women, whose serum estrogens fall to low levels, but whose serum testosterone

declines only slightly (Meldrum et al., 1981).

Glucocorticoids, a class of steroid hormones, have adverse effects on bone

cell replication, differentiation, and function (Canalis and Delany, 2002). Excessive

amounts of glucocorticoids increase bone resorption by stimulating

osteoclastogenesis, leading to a rapid loss of bone density (Canalis and Delany,

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2002). Glucocorticoids increase osteoclastogenesis by the combination of increasing the expression of receptor activator of NK-κB ligand (RANKL), receptor activator of NF-κB ligand's decoy receptor (RANK) and osteoprotegerin (OPG) (Swanson et al., 2006) along with an increase in expression of colony-stimulating factors (Rubin et al., 1998). Thus glucocorticoid induced bone resorption is associated with increased differentiation of osteoclasts and regulation of the RANKL-RANK-OPG system. Excessive amounts of glucocorticoids also affect the number and function of osteoblasts by inhibiting differentiation and proliferation of cells in the osteoblastic lineage (Mazziotti et al., 2006; Swanson et al., 2006) and also by decreasing the number of osteoblasts and osteocytes via induction of an apoptotic pathway (Ishida and Heersche, 1998). However, it has also been shown that when dexamethasone (a glucocorticoid) is added within a physiological concentration range to osteoblast-like cells lines in culture, it promotes the recruitment and further maturation of human cells of the osteoblast lineage (Walsh et al., 2001). Glucocorticoid receptors are expressed in osteoblasts (Liesegang et al., 1994) where they regulate gene expression by binding to glucocorticoid responsive elements in the regulatory regions of target genes, including osteocalcin, collagen I and transforming growth factor β (Heinrichs et al., 1993; Parrelli et al., 1998; Peterkofsky et al., 1999). While the exact role of glucocorticoids in regulating bone formation is unclear, the effect on bone is dependent on treatment duration and concentration of glucocorticoid treatment as well as stage of differentiation of both osteoblasts and osteoclasts (Pockwinse et al., 1995; Ishida and Heersche, 1998; Hirayama et al., 2002).

1.3 <u>Local Production of Estrogen</u>:

In postmenopausal women, and in men, the continuous formation of estrogen predominantly occurs by an intracrine mechanism involving converting precursors in peripheral tissues (Labrie et al., 2000). The primary tissues that are fully capable of producing extragonadal estrogen include adipose tissue, osteoblasts, chondrocytes, vascular endothelial cells, aortic smooth muscle cells, and several areas within the brain (Labrie et al., 2000; Simpson et al., 2000; Simpson and Davis, 2001). The estrogen produced in these tissues functions locally in a paracrine or even intracrine manner (Simpson and Davis, 2001). In peripheral tissues or specific target cells, the intracrine mechanism is the synthesis of active steroids from inactive precursors, where the steroid action is exerted within the same cells where synthesis takes place, and without release of the active hormones in the extracellular space or in the general circulation (Labrie et al., 2000). Because peripheral tissues are unable to synthesize estrogen *de novo*, they are dependent on conversion of circulating precursor molecules for their estrogen (Simpson et al., 2000). It is believed that estrogen and androgen bone-sustaining effects are a result of a local metabolic pathway in which inactive steroid precursors are converted to their active forms (Janssen et al., 1999; Simpson et al., 2000; Simpson and Davis, 2001; Reed et al., 2005).

In addition to the need for circulating precursor steroids, cells of the peripheral tissues must possess the necessary enzymes for estrogen metabolism. The enzymes responsible for estrogen synthesis in peripheral tissues include steroid sulfatase, 17β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase, and aromatase cytochrome p-450. One pathway for production of estrogen in peripheral

tissues by the enzymes steroid sulfatase involves conversion of estrone sulfate and dehydroepiandrosterone sulfate to estrone and dehydroepiandrosterone, respectively. Estrone is then oxidized to 17β-estradiol by 17β-hydroxysteroid dehydrogenase. The conversion of dehydroepiandrosterone to 17β-estradiol can occur in several ways. Dehydroepiandrosterone can be converted to testosterone by 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase. Testosterone is then metabolized by aromatase to 17β-estradiol. Similarly, testosterone can be converted by 17β-hydroxysteroid dehydrogenase to androstenedione which can be converted to estrone by aromatase, and then finally to 17β-estradiol by 17β-hydroxysteroid dehydrogenase (Fig. 1) (Janssen et al., 1999; Reed et al., 2005). It has been demonstrated that human osteoblasts possess all of the enzymes required for estrogen metabolism from the sulfated precursors (Purohit et al., 1992; Kuwano et al., 1997; Janssen et al., 1999).

1.4 <u>Steroid Sulfatase</u>:

  Steroid sulfatase, a microsomal enzyme found widely distributed in mammalian tissues, functions to cleave sulfate ester bonds from a broad range of substrates. The functional importance of steroid sulfatase is exemplified by dry scaly skin in X-linked ichthyosis, an inherited skin disorder that is associated with the deficiency of steroid sulfatase (Stein et al., 1989). In majority of patients with X-linked ichthyosis, the steroid sulfatase coding sequence, located on the short arm of the X-chromosome at Xp22.3, is mostly deleted (Stein et al., 1989). The dry, scaly

skin is the result of an altered ratio between cholesterol sulfate and cholesterol in the skin (Diez-Roux and Ballabio, 2005).

Steroid sulfatase, also known as aryl sulfatase C (EC 3.1.6.2), is a transmembrane protein located in the endoplasmic reticulum and capable of hydrolyzing 3β-hydroxysteroid sulfates, including estrone sulfate, dehydroepiandrosterone sulfate, pregnenolone sulfate, deoxycorticosterone sulfate, cholesterol sulfate, and *p*-nitrophenyl-sulfate (Reed et al., 2005). Steroid sulfatase, purified from the microsomal fraction of human placenta, is a 65 kilodalton hydrophobic protein of 562 amino acids with two membrane spanning domains (Hernandez-Guzman et al., 2001). From the crystal structure, steroid sulfatase appears to be "mushroom-like" in shape. The location of the transmembrane domain is such that the opening to the active site, buried deep in a cavity of the "gill" of the "mushroom," rests near the membrane surface on the luminal side of the endoplasmic reticulum, thereby suggesting a role of the lipid bilayer in catalysis. Projecting out from the catalytic domain are two glycosylated helices separated by a hydrophobic domain spanning the membrane twice in opposite directions (Stein et al., 1989). The helices traverse the membrane, interacting with one another creating a hydrophobic "tunnel" that leads to the catalytic domain and anchors steroid sulfatase to the membrane (Hernandez-Guzman et al., 2003). One proposed mechanism of entry of sulfated steroids into the active site occurs along the hydrophobic "tunnel" created by the transmembrane domains. However, the sulfate moiety would need to be shielded to pass through the membrane. Once in the polar catalytic site, the sulfate moiety undergoes nucleophilic attack by a water molecule, covalently linking it to the

formylglycine side chain and releasing the unconjugated steroid into the lumen of the endoplasmic reticulum (Hernandez-Guzman et al., 2003). Lineweaver–Burke enzyme kinetics gave a K_m of 9.59 μM for dehydroepiandrosterone sulfate as substrate and a K_m of 72.75 μM for estrone sulfate as substrate for steroid sulfatase from human placenta (Hernandez-Guzman et al., 2001).

Steroid sulfatase activity was first identified in microsomes prepared from rat liver and was found to be absent in the nucleus (Dodgson et al., 1954). Since the first identification of steroid sulfatase, activity has also been found in the testis, ovary, adrenal glands, placenta, prostate, skin, brain, fetal lung, viscera, endometrium, peripheral blood lymphocytes, aorta, kidney, and bone (Reed et al., 2005). However, the tissue distribution and activity varies considerably (Selcer et al., 2007). In comparison with all the known tissues containing steroid sulfatase activity, placenta has the highest activity (Pasqualini and Chetrite, 2005).

Steroid sulfatase has also been shown to play a significant role in supporting growth of hormone dependent cancers, by providing a local supply of biologically active steroids. The estrogen levels found in breast tumors of post-menopausal women are 10 times higher than what is found in the plasma. This high plasma concentration of circulating estrone sulfate is expected to act as an inactive steroid reservoir, which is activated by steroid sulfatase in the breast tumor and further metabolized to locally active estrogen (Utsumi et al., 1999). Steroid sulfatase expression to determine human breast carcinoma occurrence is therefore a useful prognostic marker for the identification of high- and low-risk patients. Similarly, decreased expression of steroid sulfatase mRNA has been reported to correlate with

relapse-free survival (Utsumi et al., 1999). This report further supports a role for steroid sulfatase in intracrinology of breast cancer (local conversion and action of active hormones from precursors).

1.5 <u>Steroid Sulfatase Intracrinology in Bone Tissue</u>:

The intracrine production of estrogen by osteoblasts influences local bone mass accumulation and maintenance starting from sexual maturation and continuing thereafter, thus influencing the rate of bone turnover (van der Eerden et al., 2004). Considering steroid sulfatase has the ability to convert biologically inactive steroids into active ones, it has received an increasing amount of attention as a potential source of estrogenic steroids necessary for maintaining the integrity of bone. Within the peripheral circulation of post-menopausal women, steroid precursors are found in abundance including both estrone sulfate and dehydroepiandrosterone sulfate (Purohit et al., 1992; Fujikawa et al., 1997; Janssen et al., 1999; Muir et al., 2004). For this reason, these two precursors may serve as a reservoir for the estrogenic steroids needed to sustain bone density. Production of estrogen locally is thought to be essential for maintaining bone mineralization and prevention of osteoporosis in men and women (Simpson et al., 2000). Accordingly, local conversion of these precursors to active estrogens may provide the estrogen needed for maintenance of bone density (Purohit et al., 1992; Fujikawa et al., 1997; Muir et al., 2004). This illustrates the importance of steroid sulfatase enzyme function in regulating bone turnover in post-menopausal women. It has also been found that there is no significant difference in the plasma estrogen concentration of post-menopausal women with osteoporotic

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fractures and those of a similar age without fractures. One explanation for this is the possibility of *in situ* estrogen production by bone cells (Reed et al., 2005).

Estrogen metabolism depends on the presence of metabolizing enzymes present in a given tissue. Since it has been shown that osteoblasts contain all of the necessary enzymes for estrogen metabolism, they would be capable of fully metabolizing estrone sulfate and dehydroepiandrosterone sulfate to 17β-estradiol (van der Eerden et al., 2004). In post-menopausal women, despite the low levels of circulating estrogens, estrone sulfate and dehydroepiandrosterone sulfate can be found in high circulating amounts (Pasqualini et al., 1996). Furthermore, the tissue concentrations of estrone sulfate levels in postmenopausal and premenopausal women are several-fold higher than those of unconjugated estrogens in plasma, and the same is true for men (Muir et al., 2004). The enzymatic activity of steroid sulfatase therefore may be essential for osteoblasts to be able to utilize sulfated precursors in the metabolism of estrogen, by catalyzing the removal of the sulfate group from inactive sulfated conjugates (van der Eerden et al., 2004).

Steroid sulfatase has been shown to be present in both human and rat bone and in bone cell lines (Fujikawa et al., 1997; Muir et al., 2004; van der Eerden et al., 2004; Raobaikady et al., 2005). Estrone to 17β-estradiol conversion by steroid sulfatase has been demonstrated in human hOB cell lines and human and rat OS cell lines (Muir et al., 2004). Additionally, previous studies have shown the conversion of estrone sulfate to estrone via steroid sulfatase in the human osteoblast cell lines, hOS, MG-63, and U20S (Purohit et al., 1992). Furthermore, hOS, human fetal osteosarcoma cells (HFO) and MG-63 cell lines have shown to have the ability to use

estrone sulfate as a substrate for steroid sulfatase activity (Fujikawa et al., 1997; Janssen et al., 1999; Reed et al., 2005). Interestingly, steroid sulfatase activity in an osteoblast-like cell line (MG-63) was found to be 1000-fold higher than aromatase activity (Purohit et al., 1992). Previous research on MG-63 showed steroid sulfatase to be able to utilize both estrone sulfate and dehydroepiandrosterone sulfate as substrates (Fujikawa et al., 1997) illustrating the potential for steroid sulfatase in local production of estrogen from sulfated precursors to support the maintenance of bone.

1.6 Clinical Implications:

    In premenopausal and nonpregnant women, the principal source of systemic estrogen is the ovaries. Yet, other sites of estrogen biosynthesis exist throughout the body. At the onset of menopause in women, these extragonadal sites of estrogen metabolism are thought to be the major sources of estrogen. Bone tissue, specifically osteoblasts, can serve as a site of non-ovarian estrogen biosynthesis. While the local estrogen production in bone maintains bone density, any disruption in estrogen metabolism can be harmful to skeletal health (Simpson et al., 2000; Simpson and Davis, 2001; Riggs et al., 2002; Raisz, 2005; Cohen, 2006). The importance of estrogen is profound when clinical observations of bone loss are seen at the onset of menopause. As mentioned before, estrogen decline leads to an increase in bone multicellular units, increase in bone resorption and a lapse in bone formation. Furthermore, the resorption is prolonged by an increase in osteoclast recruitment that continues to extend the resorptive phase and thereby shorten the formation phase. All these events lead to an increased resorption beyond what bone lining cells or

osteoblasts are able to fill in (Riggs et al., 2002; Raisz, 2005; Syed and Khosla, 2005; Cohen, 2006), resulting in bone loss.

A loss of coordination between osteon cells in the bone remodeling process can cause detrimental results. Altered coordination can result in either an increase in bone formation or a decrease in bone density. Osteopetrosis is a disease that occurs due to failure of osteoclast function leading to excessive bone production, in contrast osteoporosis is one of the most prominent bone pathologies that can occur as a result of an increase in osteoclast resorption function (Cohen, 2006). Characterized by the deterioration of bone, osteoporosis arises as a result of a net loss in bone density (Syed and Khosla, 2005; McCormick, 2007). The deficiency of estrogen associated with menopause has been implicated as a major factor in the development of this disease (Muir et al., 2004; Raisz, 2005; Reed et al., 2005).

It is estimated that a total of 54 million Americans age 50 and older are affected by either osteoporosis or low bone mass. Of the 54 million affected people 10.2 million have osteoporosis, of which, 8.2 million are women and 2 million are men (NOF, 2014). Moreover, the National Osteoporosis Foundation has projected that by the year 2020, the number of adults over age 50 with osteoporosis or low bone mass will grow from approximately 54 million to 64.4 million and by 2030, the number will increase to 71.2 million (a 29% increase from 2010), i.e. one out of every two Americans will either have osteoporosis or be at risk for developing this disease.

There is an increasing body of evidence that demonstrates that bone density loss is directly related to the decline of estrogen levels in aging women (Raisz, 1999; Muir et al., 2004). Moreover, a decline in ovarian estrogen levels have been shown to

both accelerate bone loss and increase susceptibility to fractures by disrupting the balance between bone formation and bone resorption (Gruber et al., 2002; Heshmati et al., 2002; Raisz, 2005). Therefore, the accelerated bone loss and increases in osteoporotic fractures associated with post-menopausal estrogen deficiency illustrate the importance of estrogens in bone. Some of the current treatments recommend the administration of adequate calcium, vitamin D, and anti-osteoporotic medication such as bisphosphonates (Lin and Lane, 2003), parathyroid hormone (PTH), selective estrogen receptor modulators (SERMs) like raloxifene, and estrogen hormonal replacement therapy (HRT) (Nayak et al., 2011). However, continuous usage of HRT could have side effects like continuation of regular menses, breast tenderness, or breast cancer (Pfeilschifter, 2001). While SERMs have been approved for treatment of postmenopausal osteoporosis and can rescue the bone mass with less side effect compared to HRT, the precise mechanisms of this effect is still ambiguous (Imai and Kato, 2010). Therefore, the need for a novel therapy to treat osteoporosis is vital. In post-menopausal women, one rational for the local production of estrogens is through conversion of inactive estrogen conjugates to active estrogen by the intracrine mechanism of steroid sulfatase enzyme. Therefore, steroid sulfatase enzyme may represent an important local pathway for maintaining the structural integrity of bone, as it may provide the estrogenic steroids needed to sustain bone density, especially in postmenopausal women (Labrie et al., 2000; Simpson et al., 2000; Simpson and Davis, 2001). The increasing realization of the importance of steroid sulfatase in pathological and physiological mechanisms has increased the attention to further understand the regulation of steroid sulfatase (Reed et al., 2005)

1.7 <u>Regulation of Steroid Sulfatase</u>:

Despite the potential importance of steroid sulfatase in pathological and physiological mechanisms very little is known about its regulation. In breast cancer cells, basic fibroblast growth factors, insulin-like growth factor-1 and the cytokines interleukin-6 and tumor necrosis factor-α have been shown to increase the level of steroid sulfatase activity (Purohit, A et al., 1992; Reed et al., 2005). Another study on breast cancer cells showed steroid sulfatase activation to occur independently of clearly defined promoter and enhancer elements, suggesting steroid sulfatase upregulation via a post-translational modification of the enzyme or by increasing substrate availability (Newman et al., 2000). In endometrial stromal cells and vascular smooth muscle cells, inflammatory cytokine interleukin-1β decreased both the activity and mRNA expression of steroid sulfatase (Matsuoka et al., 2002; Nakamura et al., 2003). In addition to steroid sulfatase regulation by cytokines and growth factors, other chemical compounds and metabolites have also been tested. In promyelocytic cells, steroid sulfatase activity was induced and expression increased by either retinoids or 1,25-dihydroxyvitamin D$_3$ (Hughes et al., 2001). The mechanisms of how these cytokines, growth factors, and steroids actually regulate steroid sulfatase activity and expression has yet to be determined (Reed et al., 2005).

Recent studies on human prostate cancer cells suggest that insulin-like growth factor II increases steroid sulfatase expression via a PI3-kinase/Akt-NF-κB signaling pathway, leading an increase in local estrogen production (Sung et al., 2013). Another study on activating the PI3-kinase signaling pathway, in human keratinocyte cell

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lines, by the cytokine interferon gamma also showed induction of STS gene expression through activation of NF-κB (Hattori et al., 2012). These preliminary results suggest steroid sulfatase expression may be transcriptionally controlled by the PI3-kinase-Akt-NF-κB pathway. With regard to bone, the effect of activation of NF-κB on steroid sulfatase expression and activity has yet to explored. If steroid sulfatase is shown to be transcriptionally upregulated by NF-κB in bone cells, resulting in increased formation of estradiol, it could have profound implications on the design of therapies for prevention of osteoporosis.

1.8 Steroid Sulfatase Inhibitors:

The role of steroid sulfatase in supporting estrogen-dependent tumor growth has led to the generation of steroid sulfatase inhibitors. These inhibitors can be classified into reversible and irreversible inhibitors. The initial search for steroid sulfatase inhibitors began by designing replacement substrates for steroid sulfatase that would compete with estrone sulfate for the active site. Both steroidal and non-steroidal inhibitors have been developed for possible therapeutic use in the treatment of hormone-dependent breast cancers (Woo et al., 1998; Nussbaumer and Billich, 2004). These compounds were made by replacing the sulfate group on estrone sulfate with some moiety that would mimic the sulfate group (Nussbaumer and Billich, 2004). The majority of steroid sulfatase inhibitors fall into the classification of irreversible inhibitors, which tend to be more potent.

The first generation of steroid sulfatase inhibitors include danazol (Nussbaumer and Billich, 2004); estrone-3-O-methylthiophosphonate (3-MTP), and

estrone-3-O sulfamate, EMATE (Howarth et al., 1994). Of these, EMATE proved to be a highly potent irreversible inhibitor of steroid sulfatase (Howarth et al., 1994). However, EMATE is not capable of being utilized as a therapeutic drug for breast cancer, due to its estrogenicity (Kolli et al., 1999; Purohit et al., 2001). Because first generation inhibitors were based on a steroid backbone, these compounds and their metabolites can have unwanted activities that make them undesirable for the treatment of hormone-dependent breast cancers (Kolli et al., 1999). Thereafter, a second generation of inhibitors was subsequently developed. A tricyclic coumarin-based compound ,667 COUMATE (STX64), was created that irreversibly binds steroid sulfatase and inhibits its activity (Purohit et al., 2000). While it is less potent in inhibiting steroid sulfatase than EMATE *in vitro* and *in vivo*, it does have the advantage of being nonestrogenic (Woo et al., 1998). 667 Coumate is now in Phase II clinical trials (Purohit and Foster, 2012).

Other non-steroidal compounds have been developed and shown to be effective in the inhibition of steroid sulfatase. Our laboratory has designed and generated one compound (DU-14), which was found to be a potent, non-steroidal inhibitor of steroid sulfatase (Kolli et al., 1999). This compound has been patented (Patent Number US 6,433,000B1), and is under investigation for therapeutic potential in breast cancer treatment.
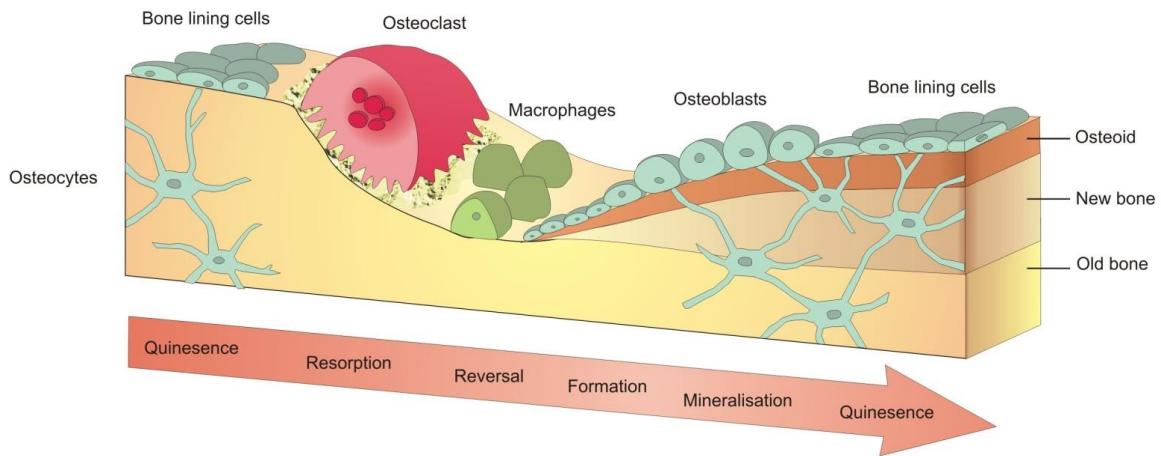
Fig. 1. Types of bone cells and bone remodeling process. The bone specialized connective tissue has four types of osteon cells, the bone lining cells, osteoblasts, osteocytes and osteoclasts. At discrete sites in the skeleton, bone is continuously remodeled. In the remodeling process, osteoclasts are activated that resorb old bone. Bone lining cells then recruit osteoblast precursors, which proliferate and differentiate into mature osteoblasts that secrete new bone matrix. Osteocytes are the mature osteoblastic cells that get entrapped in the matrix. The matrix then mineralizes to generate new bone to complete the remodeling process. Copyright BTR®. Image obtained from the website of Biomedical Research Group, The University of York, UK.
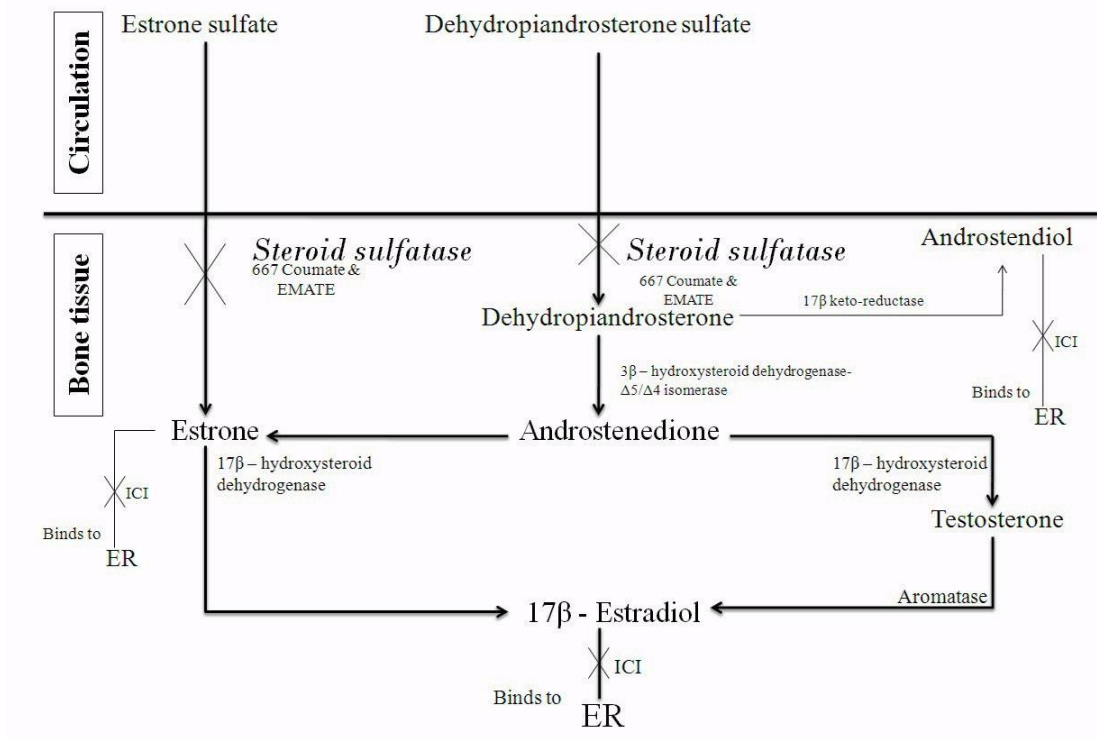
Fig. 2. Role of steroid sulfatase in metabolism of estrogen in bone.

Abbreviations are: ER = estrogen receptor, EMATE = estrone- 3-O-sulfamate; X = blockade by inhibitors. ICI = ICI 182,780, an ER antagonist.

Chapter 2

Research Projects

2.1 <u>Project I. Characterization of Steroid Sulfatase in MG-63 cells, a Human</u>

<u>Pre Osteoblastic Cell Line</u>

2.11 <u>Hypothesis and Objectives</u>

Model: MG-63 human pre-osteosarcoma fibroblast cell line derived from an osteosarcoma of a 14-year-old male.

Rationale:

While the ovaries are the principal source of systemic estrogen in premenopausal women, other sites of estrogen biosynthesis are present throughout the body, and these become important sources of estrogen after menopause. Bone osteoblasts are important for normal bone physiology and also may contribute estrogen to bone. Steroid sulfatase provides a mechanism for the production of estrogen at the local tissue level in bone. The role of steroid sulfatase in bone osteogenesis has not been clearly elucidated before. MG-63 cells are a human pre-osteoblast that has been used to study various types of bone disorders. We sought to characterize the activity and expression of steroid sulfatase in this cell line.

Hypothesis:

The steroid sulfatase provides a pathway for the conversion of inactive conjugated steroid precursors to active estrogens that are needed for bone maintenance. Therefore, this enzyme will be present in MG-63 cells. *In vitro* studies will show an increase in MG-63 cell proliferation when exposed to sulfated steroid as their only source of estrogen, and this proliferation will be steroid sulfatase dependent.

Objective:

1. To demonstrate the presence of steroid sulfatase protein in the MG-63 cell line using immunological methods

   Experiments to address Objective 1:

   a. Western blot for steroid sulfatase using MG-63 microsomes and cytosol

   b. Immunocytochemistry using polyclonal steroid sulfatase antibodies STS-275 and PA5-28259 with ER lumen protein BiP protein and cytoskeletal microtubules as controls.

2. To demonstrate activity of steroid sulfatase in MG-63 cells using radioactive ^{3}H-E$_1$S conversion assay

   Experiments to address Objective 2:

   a. Whole cell steroid sulfatase conversion assay

   b. Steroid sulfatase conversion assay using MG-63 microsomes in the presence and absence of a known steroid sulfatase inhibitor, EMATE

3. To demonstrate the presence of steroid sulfatase mRNA in MG-63 cells

   Experiments to address Objective 3:

   a. RT-PCR using primers specific for the human steroid sulfatase encoding gene

4. To demonstrate the effect of steroids, conjugated steroids and inhibitors on MG-63 proliferation

   Experiments to address Objective 4:

   a. Growth response to steroids by MTT colorimetric cell counting assay

   b. Growth response to steroid sulfatase inhibitors

5. To demonstrate the presence of estrogen receptors in MG-63 cells and to study the effect of estrogen receptor inhibition on MG-63 proliferation

   Experiments to address Objective 5:

   a. MTT colorimetric assay to measure growth response of MG-63 cells to estrogen receptor blocker

   b. RT-PCR using primers specific for the human ER-α and ER-β encoding genes

2.12 Project I Abstract

Estrogen plays an important role in maintaining bone density. Postmenopausal women have low plasma estrogen, but have high levels of conjugated steroids, particularly estrone sulfate (E_1S) and dehydroepiandrosterone sulfate (DHEAS). Conversion of these precursors to active estrogens may help maintain bone density in postmenopausal women. The enzyme steroid sulfatase (STS) converts sulfated steroids into active forms in peripheral tissues. STS occurs in bone, but little is known about its role in bone function. In this study, we investigated STS activity and expression in the human MG-63 pre-osteoblastic cell line. We also tested whether sulfated steroids can stimulate growth of these cells. MG-63 cells and microsomes both possessed STS activity, which was blocked by the STS inhibitors EMATE and 667 Coumate. Further evidence for STS in these cells was provided by RT-PCR, using STS specific primers, which resulted in cDNA products of the predicted size. We then tested for growth of MG-63 cells in the presence of estradiol-17β, E_1S and DHEAS. All three steroids stimulated MG-63 cell growth in a steroid-free basal medium. We also tested whether the cell

growth induced by sulfated steroids could be blocked using a STS inhibitor (667

Coumate) or using an estrogen receptor blocker (ICI 182,780). Both compounds inhibited

E_1S induced cell growth, indicating that E_1S stimulates MG-63 cell growth through a

mechanism involving both STS and the estrogen receptor. Finally, we demonstrated

using RT-PCR that MG-63 cells contain mRNA for both estrogen receptor alpha and

estrogen receptor beta. Our data reveal that STS is present in human pre-osteoblastic

bone cells and that it can influence bone cell growth by converting inactive sulfated

steroids to estrogenic forms that act via estrogen receptor alpha or beta.

2.13 <u>Introduction</u>

   Bone is constantly being remodeled in the adult skeleton by an integrated

mechanism involving bone resorbing osteoclasts and bone forming osteoblasts. Together

they maintain the structural integrity of the skeletal system throughout an individual's

lifetime. Estrogenic steroid hormones (primarily estradiol-17β) act directly on bone tissue

by decreasing bone turnover and by limiting osteoclast activity (Compston, 2001; Riggs

et al., 2002; Vanderschueren et al., 2004). However, at the onset of menopause, decreased

estrogen levels lead to increased osteoclast activity, paving the way for increased

resorption of bone and decreased bone mass. This facilitates the development of

osteoporosis and leads to an increased risk of bone fractures (Pouilles et al., 1995).

   Post-menopausal women have low levels of circulating estrogens, but have high

blood levels of inactive sulfated steroids, which can serve as precursors to more active

estrogens by removal of the sulfate group. Desulfonation is catalyzed by the enzyme

steroid sulfatase (STS), which converts sulfo-conjugated steroids into unsulfated forms

(Pasqualini et al., 1996). Two such sulfated steroids, estrone sulfate (E_1S) and dehydroepiandrosterone sulfate (DHEAS), are present at considerable levels in peripheral circulation of postmenopausal women (Reed et al., 2005) and these can be transformed to active estrogens in osteoblast cells (Purohit et al., 1992; Fujikawa et al., 1997; Muir et al., 2004) This involves the hydrolysis of E_1S and DHEAS to estrone and dehydroepiandrosterone, respectively, via STS. Subsequently, several enzymes known to be present in bone can catalyze transformation of estrone and dehydroepiandrosterone to the potent estrogen 17β-estradiol and other active estrogens (Ernst et al., 1988; Purohit et al., 1992; Lardy et al., 2005).

STS has been shown to be present in bone tissues (Muir et al., 2004; van der Eerden et al., 2004) and bone cell lines (Purohit et al., 1992; Saito and Yanaihara, 1998; Reed et al., 2005; Selcer and Difrancesca, 2012). Furthermore, several osteoblast-like bone cell lines (HOS, U2OS and MG-63) have been shown to possess the other enzymes, aromatase and 17-beta-hydroxysteroid dehydrogenase, that are necessary to produce active estrogens from circulating androgens (Purohit et al., 1992; Saito and Yanaihara, 1998; Janssen et al., 1999). Also, STS present in MG-63 cells has been shown to use both E_1S and DHEAS as substrates (Fujikawa et al., 1997), demonstrating the potential of the STS enzyme to convert these two prevalent circulating steroid precursors into active estrogenic steroid hormones in bone cells.

Little is known about the regulation of STS in bone. However, if a regulatory mechanism were deciphered, it could conceivably be used to increase STS activity in mature bone, increasing the local production of estrogen from circulating precursors. This could be a potential therapy for osteoporosis in post-menopausal women.

In this study, we examined STS activity and mRNA expression in the MG-63 pre-osteoblastic cell line using radioactive sulfated steroid conversion assays and RT-PCR. We also examined the effect of sulfated steroids on growth of MG-63 pre-osteoblastic cells, and the role of STS in this process, using steroid sulfatase inhibitors and an estrogen receptor blocker.

2.14 Materials and Methods

Chemicals and reagents

[6,7-^{3}H] estrone-sulfate (49 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). Radioinert steroids and 667 Coumate (STX 64) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT, USA). The steroid sulfatase inhibitor estrone 3-O-sulfamate (EMATE) was obtained from Dr. Pui-Kai Li (Ohio State University School of Pharmacy).

Cell culture methods

Human preosteoblastic MG-63 cells were purchased from ATCC (Rockville, MD). Cells were cultured in 100 mm tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ, USA) at 37 °C in a 5% CO_2 infrared humidified incubator (Thermo Scientific, Pittsburgh, PA) in 10–12 mL growth media containing 47.5% (v/v) Hams F12 nutrient mixture, 47.5% (v/v) Minimal Essential Medium (MEM) from Invitrogen (Grand Island, NY, USA), 5% (v/v) heat-inactivated fetal bovine serum from HyClone (Logan, UT, USA), and 10 mg/ml penicillin/ streptomycin solution from HyClone. Media added to plates was changed every 2 or 3 days to ensure continuous nutrient supply. Basal media,

used for cell growth assays, was 49.25% (v/v) Ham's F12 nutrient mixture, 49.25% (v/v)

Minimal Essential Medium, 0.5% (v/v) charcoal-stripped, heat-inactivated fetal bovine

serum (HyClone), and 10 mg/ml penicillin/streptomycin solution from HyClone.

Estimating presence of steroid sulfatase protein in MG-63 cells

Western blotting

Western blotting was performed on MG-63 microsomes and cytosol using an

antibody generated against a peptide representing a conserved region of mammalian

steroid sulfatase. The peptide was identified using a multiple alignment of mouse, rat and

human steroid sulfatase amino acid sequences, and the antibody against this peptide has

been shown to recognize steroid sulfatase protein in all three species. MG-63 microsomes

were dissolved in 500 μl Tris–HCl Buffer (pH 7.5). Proteins from MG-63 microsomes

and cytosol were then separated by SDS–PAGE, using a BioRad Mini Protean II

electrophoresis system. Gels were 4–15% Tris–glycine gradient gels (Bio-Rad).

Microsomes were diluted appropriately and mixed 1:1 with 2 X Laemmli sample buffer

(BioRad) containing 10% β-mercaptoethanol and boiled for 4 min. Samples (10 μl) were

loaded into wells, and proteins were electrophoretically separated at 30 mA per gel until

the dye front reached the bottom of the gel. After electrophoresis, one of the gels were

stained with Coomassie blue dye, or the proteins were transferred electrophoretically for

2 h at 70 V to a PVDF membrane (BioRad) in transfer buffer (25 mM Tris, 192 mM

Glycine, 20% Methanol). After transfer, the PVDF membrane was washed three times in

Tris–saline buffer (50 mM Tris–HCl, 154 mM NaCl, pH 7.5) for 10 min at room

temperature, and then blocked for 1 h with blocking reagent (5% nonfat dry milk in Tris–

Saline buffer). Membrane was then incubated with steroid sulfatase antibody (1:400

dilution in blocking reagent) overnight in a sealed plastic bag at room temperature. The membrane was washed once for 10 min in Tris–Tween buffer (Tris–saline buffer + 0.05% Tween 20) and two times 10 min washes in Tris-Saline buffer, then incubated with secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase, BioRad) at a 1:1000 dilution in blocking buffer, for 2 h. The membranes were washed in Tris–Tween and Tris-Saline buffer as before, then developed by incubating for 10 min in substrate reagent (Sigma Fast 3,3'-diaminobenzidine tablet set, Sigma Chemical Co.). After the bands appeared to the desired intensity, washing the membrane in deionized water for 10 min stopped the reaction.

Immunocytochemical assay for STS protein expression

Antibodies and reagents

Primary antibodies

To determine steroid sulfatase protein expression in MG-63 cells two antibodies were used, a commercially available steryl-sulfatase polyclonal antibody raised in rabbit (PA5-28259, Thermo Fisher Scientific) at 1:100 dilution and the second steroid sulfatase antibody was generated in rabbit against a peptide representing a conserved region of mammalian steroid sulfatase. This peptide was identified using a multiple alignment of mouse, rat and human steroid sulfatase amino acid sequence, STS-275 (Selcer et al., 2007).

Other primary antibodies used as control proteins were microtubules monomers α-tubulin monoclonal antibody raised in mouse (A-11126, Life Technologies) at 1:250 dilution and endoplasmic reticulum lumen proteins GRP78 BiP polyclonal antibody raised in rabbit (AB21685, Abcam Cambridge, MA) at 1:500 dilution. The proteins α-

tubulin of microtubules and ER lumen BiP chaperone protein, served as controls to help

to determine the localization of steroid sulfatase within the MG-63 cells.

Secondary antibodies

Alexa Fluor® 488 Goat Anti-Rabbit IgG labeled with green fluorescent dye was

used as secondary antibody to react against sulfatase antibody (Life Technologies) at

1:200 dilution. Alexa Fluor® 555 Donkey Anti-Rabbit IgG labeled with red fluorescent

dye was used as secondary antibody to react against BiP (Life Technologies) at 1:200

dilution. Alexa Fluor® 546 Goat Anti-Mouse IgG labeled with red fluorescent dye was

used as secondary antibody to react against tubulin (Life Technologies).

ProLong® Gold antifade mountant with DAPI (4,6-diamidino-2-phenylindole)

that stains nuclei blue was used as mounting media (Life Technologies)

Cell treatments and fixing

Poly-D-lysine coated coverslips (Becton Dickinson) were placed into Falcon 24-

well plates (Becton Dickinson). MG-63 cells were seeded at a density of 50,000

cells/well and incubated in 500 µl of growth medium for 48 h to allow them to adhere.

After 48 h incubation, the media was removed and wells were rinsed with 500 µl of

phosphate buffered saline. Cells were fixed in 4% paraformaldehyde for 15 min at 4 °C.

After fixation, cells were treated with 0.25% Triton X-100 in PBS without calcium and

magnesium for 10 min. MG-63 cells were washed in PBS and blocked with 1% bovine

serum albumin (Thermo Fisher, Pittsburgh, PA) for 30 minutes. Incubation with primary

antibody combinations was done overnight at 4 °C (steroid sulfatase STS-275 and α-

tubulin A-11126; steroid sulfatase STS-275 and GRP78 BiP; steroid sulfatase PA5-28259

and GRP78 BiP respectively). After being washed with PBS, cells were incubated with

secondary antibodies for 1 h at 1:200 dilution (Alexa Fluor® 488 and Alexa Fluor® 546; Alexa Fluor® 488 and Alexa Fluor® 555; Alexa Fluor® 488 and Alexa Fluor® 555 respectively). Cells were washed with PBS to remove excessive fluor and mounted with a drop of DAPI on Superfrost® Plus Gold slides (Thermo Fisher, Pittsburgh, PA).

For sulfatase and tubulin expression the dilutions of primary antibodies were STS-275 at 1:100 dilution and tubulin at 1:250 dilution. For sulfatase and BiP expression, both antibodies were raised in rabbit and hence a sequential staining was done to prevent cross-reactivity of the secondary antibodies. First steroid sulfatase primary antibody (1:200 STS-275 and 1:100 PA5-28259) treatment was done overnight followed by 1 h secondary green fluorescent labeling for sulfatase. Upon thorough washing with phosphate buffer saline BiP antibody (1:500) treatment was done overnight followed by 1 h secondary red fluorescent labeling for BiP.

Stained cells were examined with a Zeiss epifluorescent microscope at 40 X magnification (Zeiss AxioObserver Z1 inverted microscope, bright-field Xenon white light wide-field epifluorescence observation) and a confocal microscope at 60 X magnification (Zeiss AOTF, acousto optic tunable filter, 488/568/647nm ArgonKrypton laser lines fed into a Yokagawa QLC100 spinning disk confocal head capable of analyzing 360 frames/sec) and imaged with a digital camera.

Measurement of sulfatase activity of intact cells

MG-63 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson and Co.) at a density of approximately 250,000 cells/well and incubated in growth medium overnight to allow them to adhere. After incubation, the medium was replaced with 2 ml of growth media containing ^{3}H-estrone sulfate (100,000 dpm/ml) and

radioinert estrone sulfate (1 μM) in the presence or absence of specific inhibitors, estrone 3-O-sulfamate (EMATE, 1 μM) or 667 Coumate (1 μM). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13 x 100 borosilicate glass tubes. Three milliliters of toluene was added to each tube for extraction of unconjugated steroids. The mixture was vortexed for 1 min and then centrifuged for 10 min to separate the aqueous and organic phases. Duplicate aliquots of 1 ml were removed from the organic phase (containing the unconjugated steroids) and transferred to scintillation vials, after which 5 ml of scintillation cocktail was added. Steroid sulfatase activity was measured using the method of Selcer and DiFrancesca, 2012. Radioactivity was counted in a Packard Tri-carb scintillation counter at 50% efficiency for ^{3}H. The conversion values obtained for all treatments were adjusted for spontaneous product formation by subtracting the value obtained for wells containing medium and ^{3}H-estrone sulfate with no cells (18 h incubation). The experiment was repeated three times, with seven wells per experiment (two control wells, two EMATE treatment wells, two 667 Coumate treatment wells, and one 35 mm dish with media only).

Preparation of MG-63 microsomes

MG-63 microsomes were prepared using the methods of Selcer and DiFrancesca, 2012. Pre-confluent MG-63 cells were scraped off the surface of the dishes and the cells were pelleted by centrifugation (1000 g for 10 min). Pellets were resuspended in 1:5 w:v in ice-cold Tris-sucrose buffer (50 mM Tris–HCl, 25 mM sucrose, pH 7.5) and homogenized using three 30 s bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). The nuclear fraction was pelleted by centrifugation at 2500 g for 10 min at 4 °C. The resulting supernatant was removed to Beckman

ultracentrifuge tubes (11 x 60 mm) (Beckman Coulter Inc., Fullerton, CA) and centrifuged at 107,000 g for 1 h at 4 °C to yield the microsomal fraction. The resulting pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5) at 1:2 original w:v. Protein concentrations of microsomes were determined by BCA assay (Pierce Chemical Co., Rockford, IL).

Measurement of sulfatase activity of MG-63 microsomes

^{3}H-estrone sulfate was diluted in Tris–HCl buffer (50 mM, pH7.5) and 100 µl (100,000 dpm/ml) was added to the assay tubes. Radioinert estrone sulfate was dissolved in ethanol and then diluted into Tris–HCl buffer such that 100 µl would yield a concentration of 10 µM in the final assay volume. Estrone 3-O-sulfamate (EMATE) and 667 Coumate were also dissolved in Tris–HCl buffer. 100 µl of these solutions were added to the respective assay tubes to achieve the appropriate final concentration of inhibitor (1 µM). MG-63 microsomes were diluted with Tris–HCl buffer to achieve the final desired concentration of membranes (25–200 µg) in 100 µl of buffer (500 µl final volume). The assay tubes were preincubated for 5 min at 37 °C in a water bath. The assay was initiated by addition of the microsomes (100 µl) to the tubes containing the compounds. Control tubes with no inhibitor, tubes without microsomes (to control for spontaneous hydrolysis), and tubes with inhibitors at 1 µM concentrations were incubated simultaneously. After 30 min of incubation at 37 °C, 3 ml of toluene was added for extraction of unconjugated steroids. The samples were vortexed for 1 min and centrifuged at 2500 g for 10 min at 24 °C. Duplicate 1 ml aliquots were removed from the organic phase of the samples and added to 5 ml of scintillation cocktail. The aliquots were counted in a liquid scintillation counter (Packard Instrument Co.) for determination

of product formation. The experiments were run three times, with duplicate tubes for each experiment.

BCA protein assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared with 50 mM Tris–HCl as the diluent. Duplicate concentrations (µg/tube) were 200, 150, 100, 75, 50, 25, 12.5 and 2.5. Duplicate tubes of MG-63 microsomal samples were prepared using 100 µl of microsomal suspension. Absorbances were read at 562 nm using a spectrophotometer (Thermo Fisher, Genesys 20; Pittsburgh, PA, USA).

RNA isolation

Total RNA from MG-63 cells was isolated using TRIzol® Reagent (Invitrogen Life Technologies) according to manufacturer's instructions. Pre-confluent MG-63 cells from 100 mm culture dishes were lifted off the surface of the dishes and the cells were pelleted by centrifugation (1000 g for 10 min). Pellets were resuspended in 500 µl of TRIzol® reagent and incubated at room temperature for 5 min. 160 µl of chloroform was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was transferred, to which, 500 µl of isopropyl alcohol and 1 µl of glycogen was added. After a 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The resulting RNA pellet was washed with 500 µl of 75% ethanol, centrifuged at 7000 g for 10 min at RT, air-dried, and resuspended in 30 µl of diethylpyrocarbonate-treated water. Following a 10 min incubation at 60 °C, RNA was quantified using a spectrophotometer (Thermo Fisher, Genesys 8, Pittsburgh, PA, USA). The amount of RNA extracted was determined by

ultraviolet light absorption at 260 nm. RNA samples were stored at − 80 °C until use.

Reverse transcription polymerase chain reactions (RT-PCR)

Using the GoScript® Reverse Transcription System (Promega), according to manufacturer's instructions, 1 μg of template RNA, 1 μl of Oligo (dT) primer and diethylpyrocarbonate-treated water were added to make a total volume of 9 μl, heated to 70 °C for 5 min, and then chilled on ice for 5 min. In a separate tube, reverse transcription mix was prepared from 4 μl of 5x reaction buffer, 4 μl $MgCl_2$, 1 μl dNTPs and 0.5 μl of reverse transcriptase. 12 μl of reaction mix was added to RNA/primer mix. First-strand cDNA synthesis was carried out in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) with 5 min annealing at 25 °C. Next, the cDNA was allowed to extend for 1 h at 42 °C. The reverse transcriptase enzyme was thermally inactivated at 70 °C for 15 min after which the reaction tubes were held at 4 °C.

PCR primers

PCR primers were selected from the complete coding sequence for human steroid sulfatase (GenBank Accession No. M16505.1). The forward primer start position is 1402 and has the sequence (5'–3') TGA TGA GCC CAC TAG CAA CAT GGA (FB). The reverse primer start position is 1588 and has the sequence (5'–3') GTG CCA GCG CAC AGC ATT TAA GTA (RB). The expected RT-PCR product from this primer pair is 187 base pairs. To determine the mRNA expression of estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β), two new sets of primers were designed. For human ER-α (GenBank Accession No. NM000125.3), the forward primer start position is at 709 and has the sequence (5'–3') CAG GGT GGC AGA GAA AGA TT (FB). The reverse primer start position is at 908 with the sequence (5'–3') TTG GTG GCT GGA CAC ATA TAG

(RB), with an expected product of 200 base pairs. For human ER-β (GenBank Accession No. AB006590.1), the forward primer start position is at 1460 with the sequence (5'–3') CCT GGC TAA CCT CCT GAT GC (FB) for a product size of 167 base pairs. The reverse primer start position is at 1626 and has the sequence (5'–3') ACC CCG TGA TGG AGG ACT T (RB) for an expected product size of 167 base pairs. Primers were synthesized by Integrated DNA Technologies, Inc., (Coralville, IA).

PCR reagents and conditions

PCR reactions were performed on the cDNA templates using a Taq PCR Kit (New England Biolabs, Ipswich MA) according to manufacturer's instructions. 2 μl template cDNA, 2.6 μl 10X PCR buffer, 0.4 μl dNTP mix, 1 μl of 10 μM sense primer, 1 μl of 10 μM antisense primer, 0.2 μl Taq Polymerase, and DEPC-treated water were added to make total volume 20 μl. PCR reactions were then placed in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA). The reaction had a 4 min hold at 94 °C. For steroid sulfatase determination, the cDNA product was amplified for 35 cycles of: 45sec 94 °C, 45 s 58 °C, 90 s 72 °C. For ER-α, ER-β and osteocalcin amplification, the cDNA products were amplified at 35 cycles of: 5 s 94 °C, 45 s 52 °C, 90 s 72 °C. The PCR reaction was terminated with a final extension of 5 min at 72 °C. The PCR products were then separated in a 2% agarose gel containing 5 μl of 1 mg/ml ethidium bromide solution. The gel was run in 1X TAE running buffer (40 mM Tris–acetate, 2 mM Na$_2$-EDTA-2H$_2$O) for 2 h at 70 V. 10 μl of PCR product were mixed with 2 μl 6X cyanol loading dye (0.25% w/v xylene cyanol, 15% Ficoll in water). Quick load 2- log DNA ladder (New England Biolabs, Ipswich MA) was used as the standard.

Cell growth assays

MG-63 cells were seeded into Falcon 24-well plates (Becton Dickinson) at a density of 25,000 cells/well and incubated in 1 ml of growth medium for 18 h to allow them to adhere. After incubation, the medium was removed, the cells were washed once with phosphate buffered saline (Sigma–Aldrich Co., St. Louis, MO, USA), and basal medium was added to starve the cells. After 48 h, medium was changed to either basal medium alone (basal) or basal medium containing treatments.

In the first growth experiment, the treatments were estradiol-17β, estrone sulfate or dehydroepiandrosterone sulfate, all at 10 μM. Cells maintained in growth medium alone (not starved in basal medium) were used as a positive control (growth). In the second growth experiment, cells after starvation were treated with E_1S (10 μM), 667 Coumate (1 μM) or both. In a third experiment, cells after starvation were treated with E_2 or E_1S (10 μM) in the presence or absence of ICI 182,780 (100 nM). ICI 182,780 was also used alone.

In all experiments, cells were then allowed to grow in their treatment media for a 3-day period. Media were then removed and 200 μl MTT (dimethylthiazol tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) was added to the cells, after which they were incubated at 37 °C for 3 h. Following incubation, the MTT was completely removed and replaced with 500 μl of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the membranes and then two 200 μl samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595 nm using the BioRad Microplate reader (Model 3550). Absorbances were compared with a standard curve derived from plates with known numbers of cells (range of 500–256,000 cells). The first experiment compared cell growth in the presence of estradiol-17β,

estrone sulfate, or dehydroepiandrosterone sulfate (10 μM). The second experiment

compared cell growth in the presence of estradiol-17β or estrone sulfate, in combination

with the presence or absence of the estrogen receptor blocker ICI 182,780 (100 nM) and

steroid sulfatase inhibitor EMATE (100 nM). The third experiment compared cell growth

in the presence of estrone sulfate in combination with the presence or absence of the

other sulfatase inhibitor 667 Coumate (STX 64). All treatments were run in triplicates

and each experiment was repeated three times.

Statistical analyses

Statistical analyses were carried out using Prism Version 6.0 for Macintosh

(GraphPad Software, San Diego, CA). Probabilities of $P < 0.05$ were considered

significant.

2.15 <u>Results</u>

Western blotting

In order to determine if steroid sulfatase was expressed in MG-63 cells, Western

blot was performed using microsomal and cytosolic fractions of MG-63 cells (Fig. 3). In

the microsome fractions, specific steroid sulfatase antibody showed the greatest cross-

reactivity with three protein bands with estimated molecular weights of 63 kDa, 54.7

kDa, and 52.2 kDa (Fig. 3). In the cytosolic fractions steroid sulfatase antibody showed

cross-reactivity of two protein bands at 63 kDa and 52.2 kDa. Some minor reactivity was

also present for several other bands.

Immunocytochemical cell localization of STS

In order to determine the cellular localization of STS within a cell, MG-63 cells were fixed and treatments of specific primary antibodies and fluor conjugated secondary antibodies were done as mentioned in the methods section. Tubulin red staining revealed the cytoskeletal framework that maintains the shape of the cell (Fig. 4) while BiP chaperone (red) was found to be localized surrounding the nucleus suggesting its presence in the ER lumen (Fig. 5). Steroid sulfatase (green) was found to be distributed throughout the cell, but appeared to be more concentrated in the endoplasmic reticulum and Golgi regions (Fig. 4 and Fig. 5).

MG-63 cells contain STS activity

In order to demonstrate functional expression of STS, MG-63 cells were incubated overnight (20 h) in the presence of ^{3}H-estrone sulfate and in the presence or absence of two irreversible steroid sulfatase inhibitors: estrone 3-O-sulfamate (EMATE) and 667 Coumate (Fig. 6). Steroid sulfatase activity of MG-63 cells was found to be approximately 0.676 fmol/million cells/h. STS activity was virtually eliminated (<99%) in the presence of the inhibitors EMATE and 667 Coumate.

STS activity is localized to microsomes

In order to determine functional expression of STS in ER, an ^{3}H-estrone sulfate conversion assay, using microsomal preparations from MG-63 cells, steroid sulfatase activity was measured in the presence or absence of two irreversible steroid sulfatase inhibitors: estrone 3-O-sulfamate (EMATE) and 667 Coumate (Fig. 7). Sulfatase conversion activity in the absence of inhibitors was estimated to be 28 pmol/mg protein/h. STS activity of the microsomes was essentially eliminated (93%) in the presence of the inhibitors EMATE and 667 Coumate.

MG-63 cells contain STS transcripts

In order to demonstrate that STS is expressed in bone cells, reverse transcription PCR was performed using mRNA extracted from MG-63 cells (Fig. 8), using primers designed based on the known sequence of human steroid sulfatase. RT-PCR of MG-63 mRNA resulted in a single cDNA band when resolved on a 2% agarose gel and stained with ethidium bromide. This band had the expected size (187 base pairs) for this primer pair.

Cell growth assays

Cell growth response to media types and steroids

In order to determine how MG-63 cells respond to substrate and products of STS, proliferation was measured in cells incubated for 72 h in normal growth medium or basal medium, after a 24 h starvation period in basal medium (Fig. 9). Growth was also measured in cells incubated for 72 h with estrone sulfate, estradiol-17β, or DHEAS in basal medium, after the starvation period. Cells incubated in growth medium showed the highest levels of growth and cells incubated in basal medium showed the lowest levels. The steroid treatments were intermediate between these, with estradiol and estrone sulfate having significantly higher levels of growth as compared to the basal media.

Cell growth response to steroid sulfatase inhibitors and estrogen receptor blocker

In order to determine the role of STS in modulating MG-63 cell proliferation, MG-63 cells, treated with estradiol or estrone sulfate, were evaluated in the presence of the STS inhibitors, EMATE or 667 Coumate (1 μM). Cell proliferation was significantly stimulated by the addition of 10 μM estrone sulfate, and this growth was significantly inhibited in the presence of 1 μM 667 Coumate (Fig. 10).

Cell growth response to estrogen receptor blocker ICI 182,780

In order to determine the role of estrogen receptor in regulating MG-63 cell proliferation, MG-63 cells, treated with estradiol-17β (E_2) and estrone sulfate (E_1S), both at 10 μM) were also evaluated in the presence of the estrogen receptor antagonist ICI 182,780 (100 nM). E_2 and E_1S both significantly stimulated growth of MG-63 cells. Growth in the presence of each compound was significantly inhibited by ICI 182,780 (Fig. 11).

MG-63 cells contain estrogen receptor alpha (ER- α) and estrogen receptor beta (ER-β)

To determine if ERs were expressed in MG-63 cells, mRNA levels were analyzed using RT-PCR. MG-63 cells in culture showed the presence of mRNA for ER- α and ER-β, as determined by RT-PCR using human specific primers that we derived (Fig. 12). The ER- α DNA product band was 200 bp, as predicted, and the ER- β DNA product band was 167 bp, as predicted. The intensities of the ER- α and the ER- β bands appeared to be similar.

2.16 Discussion

In the present study, we have confirmed that human pre-osteoblastic MG-63 cells contain the steroid sulfatase (STS) enzyme, based on assays of STS activity and mRNA expression. Furthermore, we have demonstrated the ability of the sulfated steroids E_1S and DHEAS to stimulate growth of MG-63 cells, and we have shown that this growth is blocked by steroid sulfatase inhibitors and an estrogen receptor antagonist. These data support the idea that STS may provide estrogenic steroids to support osteoblast function.

The Western immunoblot of MG-63 cell fractions, using rabbit polyclonal

antiserum (STS-275) generated against conserved regions of the steroid sulfatase

transmembrane protein, showed two strong immunoreactive bands in cytosol at about 63

kDa, and 53kDa, and three bands in microsomes, with the additional band at 55 kDa.

Steroid sulfatase is a 65kDa protein based on amino acid sequence alone. The size and

migration rate of the mature protein could be reduced by removal of the signal peptide

and could be increased by glycosylation. Steroid sulfatase has four potential

glycosylation sites, of which two appear to be glycosylated (Stein et al., 1989). The two

glycosylations add 7 to 8 kDa to the protein. Thus, the larger band of 63 kDa likely

represents the mature, glycosylated steroid sulfatase protein and the smaller bands (55 or

53 kDa represent the unglycosylated or single glycosylated form. Similar results of a 68

kDa and 60 kDa sulfatase protein bands were previously observed (Mortaud et al., 1995)

using murine liver microsomes and a murine steroid sulfatase rabbit polyclonal antibody.

Their SDS-gel also revealed a third smaller band of about 43 kDa, possibly a degradation

product of the larger polypeptides. Interestingly, there was a faint a band of this size in

the cytosol of the MG-63 cells. Previously in our lab, MC3T3-E1 mouse pre-osteoblast

microsomes showed a similar three band pattern of 79, 65 and 50 kDa (Selcer and

Difrancesca, 2012).

Immunocytochemical analyses showed steroid sulfatase immunoreactivity to be

greatest in the endoplasmic reticulum and Golgi regions, with lighter staining throughout

the cytoplasm. Studies on purification and analysis of steroid sulfatase have focused on

the endoplasmic reticulum as the primary location of this enzyme (Kauffman et al., 1998;

Hernandez-Guzman et al., 2001), and indeed, detection of steroid sulfatase using

immune-gold labeling has confirmed that most protein is localized with the endoplasmic

reticulum (Willemsen et al., 1988; Stein et al., 1989). Willemsen et al., (1988) found that fibroblasts showed steroid sulfatase immunoreactivity primarily in the rough ER and Golgi, and not in the lysosomes, but there was also steroid sulfatase present in the multivesicular endosomes. Stein et al., (1989) found steroid sulfatase to be present in these same regions in BHK-21 kidney cells, but also in dense lysosomes and in the plasma membrane. They calculated that as much as 10% of the steroid sulfatase protein may reside in the lysosomes. Our data are consistent with those of Stein et al., in that there appeared to be some level of steroid sulfatase immunoreactivity in regions away from the perinuclear/rough endoplasmic reticulum/Golgi space. Determination of whether this represents lysosomal or cytoplasmic steroid sulfatase could be assessed by use of a lysosomal marker along with steroid sulfatase immunostaining.

MG-63 whole cells and microsomes both possessed STS activity, as determined by ^{3}H-E$_1$S conversion assays. The levels of steroid sulfatase activity in cultured MG-63 cells (0.676 fmol/million cells/h) are in the general range shown previously for this cell line and for other human and rat pre-osteoblastic cells lines (Purohit et al., 1992; Muir et al., 2004). STS activity (28 pmol/mg/h) was also found in microsomes prepared by differential centrifugation. STS activity had previously been reported in MG-63 cell-free homogenates (Fujikawa et al., 1997), but not microsomes. The presence of STS in microsomes of MG-63 cells, and MC3T3-E1 mouse pre-osteoblast cells (Selcer and Difrancesca, 2012) supports the expected localization of this enzyme to the endoplasmic reticulum, which is a major component of the microsomes.

STS activity was blocked by the STS inhibitor EMATE, in both intact cultured cells and microsomes. EMATE has previously been shown to block STS in MG-63, HOS

TE-85 and MC3T3-E1 osteoblastic cell lines (de Gooyer et al., 2001; Selcer and Difrancesca, 2012) and in MC3T3-E1 microsomes (Selcer and Difrancesca, 2012). EMATE was the first highly potent STS inhibitor developed (Howarth et al., 1994; Reed et al., 2005); however, this compound has limited therapeutic potential because it is estrogenic and would stimulate breast cancer growth rather than inhibit it (Elger et al., 1995; Li et al., 1998). Therefore we also tested a non-estrogenic, STS inhibitor, 667 Coumate (Malini et al., 2000). This compound also inhibited STS activity of MG-63 cells in both whole cells and microsomes, as determined by the conversion assays. 667 Coumate has not previously been tested on bone cells. The findings on EMATE and 667 Coumate inhibition of STS activity provide additional support that it is genuine STS that we are detecting in the conversion assays.

RT-PCR was performed in order to confirm that the particular clone of MG-63 cells used in this study, recently obtained from ATCC, possess STS mRNA. RT-PCR demonstrated the presence of the correct size product (187 bp) for STS, using newly designed primers. This confirms the presence of mRNA in MG-63 cells, as was shown previously by Fujikawa et al. (Fujikawa et al., 1997) and Muir et al. (Muir et al., 2004), using different primer pairs. Previous studies have shown STS mRNA in other bone cells lines (Fujikawa et al., 1997; Janssen et al., 1999; Muir et al., 2004) and bone tissues (Muir et al., 2004), indicating that STS mRNA is typically present in bone tissues, and has a function in bone. The ability to measure mRNA in MG-63 cells will facilitate studies of changes in STS mRNA expression during bone cell differentiation.

We developed a repeatable procedure for assessing MG-63 cell growth in the presence of steroids and sulfated steroids. The basal, addback, and growth assay (BAG

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assay) is a modification of the one we used previously for studying growth of mouse MC3T3-E1 preosteoblasts (Selcer and Difrancesca, 2012). The assay uses a basal medium for starvation of the cells (48 h) and into which treatments are added during the growth assessment phase (72 h). Basal medium has very little serum (0.5%), and the small amount of serum present is charcoal-stripped and therefore should be devoid of steroids. Basal medium alone provides a negative growth control, as growth is minimal in this medium. The addback treatments (labeled growth in Fig. 9) use growth medium in place of basal medium during the treatment phase, and provides an assessment of maximal growth for the experiment. Growth medium alone, without starvation, provides a determination of cell growth without starvation (not shown) and allows for assessment of cell growth between experiments.

Using the BAG assay, MG-63 cells showed enhanced growth compared to basal medium in the presence of E_2, E_1S and DHEAS, demonstrating that estrogens and conjugated estrogens stimulate pre-osteoblastic cell growth. Growth stimulation of MG-63 cells by estradiol-17β and estriol has been previously demonstrated (Fohr et al., 2000) (Luo and Liao, 2003; Dohi et al., 2008), but this is the first report of growth stimulation by the sulfated estrogens E_1S and DHEAS in this cell line. Luo and Liao, 2003 demonstrated estradiol-17β to promote higher MG-63 cell proliferation when compared to estriol. Furthermore, estriol showed no effect on alkaline phosphatase activity and osteocalcin production suggesting that, while estriol stimulates osteoblastic MG-63 cells proliferation it has no effect on promoting MG-63 cell differentiation (Luo and Liao, 2003). These data, along with a previous report from the mouse osteoblastic cell line MC3T3-E1 (Selcer and Difrancesca, 2012) support the concept that sulfated steroids can

be utilized by osteoblasts for growth. Also, MG-63 cells are known to possess all of the

enzymes necessary to convert estrone to estradiol (17-beta hydroxysteroid

dehydrogenase) and androgens to estrogens (aromatase) (Jakob et al., 1997; Dong et al.,

1998; Feix et al., 2001).

The assertion that STS was involved in the growth stimulation by E_1S and

DHEAS was supported by the inhibitor studies. MG-63 cells grown in the presence of

E_1S as the only steroid were inhibited by the STS inhibitor 667 Coumate, confirming the

role of STS in the growth process. Furthermore, E_1S induced growth was also inhibited

by the estrogen receptor blocker ICI 182,780, demonstrating that growth stimulation by

this sulfated estrogen involves the estrogen receptor. Similar findings on ICI 182,780

inhibition of E_1S-stimilated growth have been reported for MC3T3-E1 cells (Selcer and

Difrancesca, 2012) and for E_2-stimulated growth of MG-63 cells (Luo and Liao, 2003;

Dohi et al., 2008). Moreover, we have shown using RT-PCR that the MG-63 cells

possess mRNAs for both estrogen receptor alpha and estrogen receptor beta (Fig. 12),

confirming that estrogen receptor is available to bind any estrogen produced from STS

action on estrone sulfate.

Estrogen receptor alpha and beta have previously been detected by RT-PCR in

MG-63 cells (Dohi et al., 2008), with estrogen receptor beta slightly higher. Both

receptors have also been shown in other osteoblast cell lines (Monroe et al., 2005;

Solakidi et al., 2005). Taken together, the growth and growth inhibition studies indicate

that sulfated steroids are converted to more active estrogens by steroid sulfatase and that

these compounds stimulate cell growth via the estrogen receptor. Previous studies have

shown that bone cells possess all of the enzymes necessary to produce active estrogenic

steroids (e.g., estrone, estradiol-17β and androstenediol) from E_1S and DHEAS (Ernst et al., 1988; Purohit et al., 1992; Fujikawa et al., 1997; Saito and Yanaihara, 1998; Janssen et al., 1999; Muir et al., 2004; van der Eerden et al., 2004; Lardy et al., 2005; Reed et al., 2005). Our data reveal that osteoblast cell proliferation is one result of the estrogens produced by conversion of sulfated steroids.

In summary, the present data on STS enzyme activity, RT-PCR, and stimulation of cell proliferation indicate that MG-63 preosteoblast cells have steroid sulfatase, and that this enzyme is capable of converting inactive sulfated steroids into biologically active steroids that promote cell growth. These findings are consistent with previous studies that have demonstrated the presence and activity of STS in osteoblasts of several mammalian species (Ernst et al., 1988; Purohit et al., 1992; Fujikawa et al., 1997; Saito and Yanaihara, 1998; Janssen et al., 1999; de Gooyer et al., 2001; Muir et al., 2004; van der Eerden et al., 2004; Lardy et al., 2005; Selcer and Difrancesca, 2012). Increased understanding of the presence and activity of STS will enhance our understanding of the physiological role of this enzyme in bone growth and maintenance and could provide for future strategies for managing osteoporosis by regulating STS.

K M2 M1 C2 C1   C1 C2 M1 M2 K

101 kDa

79 kDa

63

54.7

52.2

35 kDa

28 kDa
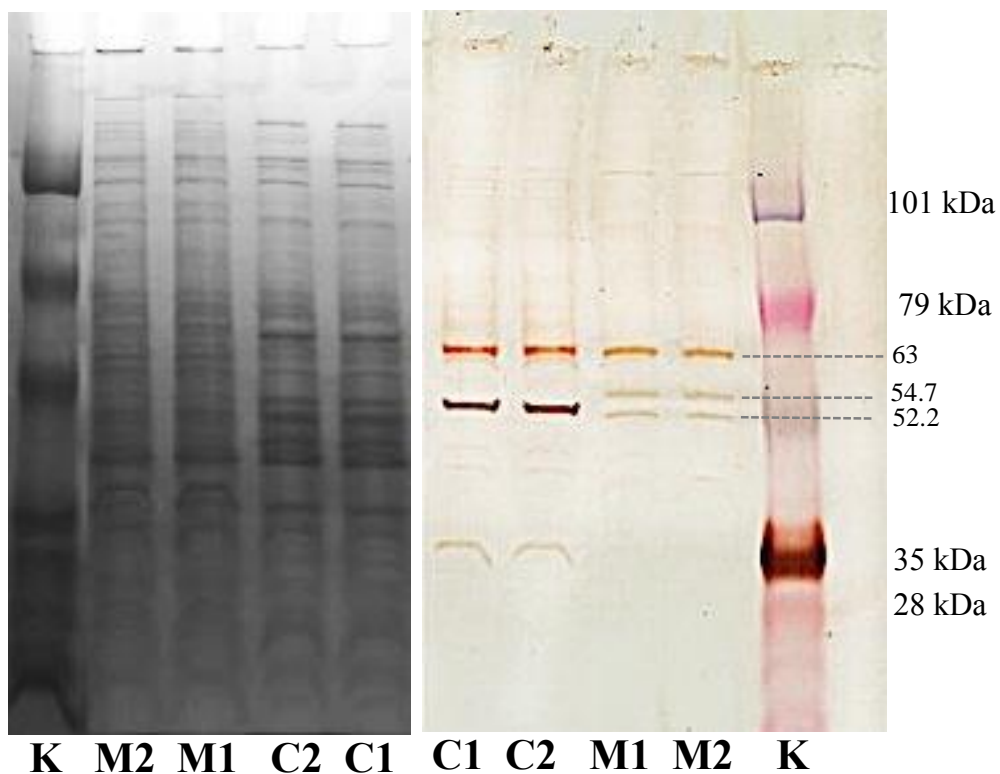
Fig. 3. Western blot showing immunoreactivity of a steroid sulfatase antibody with microsomal and cytosolic proteins from MG-63 cells. Microsomal and cytosolic proteins were separated by SDS–PAGE gel (left) and transferred to a PVDF membrane for immunostaining (right). Western blotting was performed as indicated in Methods. Antibody dilution was 1:400. Microsomal protein concentration used in the SDS–PAGE was 5 μg per lane and cytosol protein concentration was 50 μg per lane. Abbreviations are: K = molecular weight markers, M1 and M2 are duplicate lanes of microsomal proteins and C1 and C2 are duplicate lanes of cytosolic proteins.
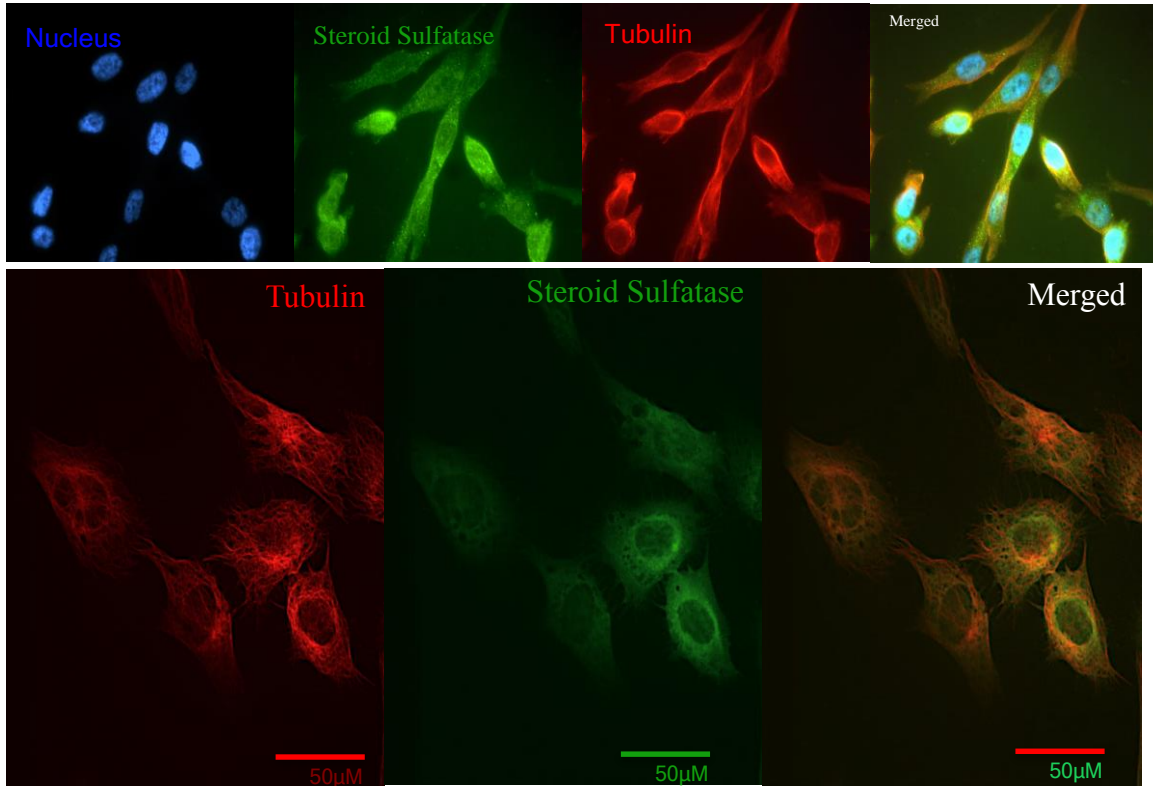
Fig. 4. Immunocytochemical analysis of steroid sulfatase expression in MG-63 cells.
Double immunofluorescence of MG-63 cells fixed with paraformaldehyde. Cells were stained with both antibodies against α-tubulin (red) and steroid sulfatase STS-275 (green). Steroid sulfatase is seen distributed throughout the cytoplasm but appear to be more concentrated in the endoplasmic reticulum and Golgi regions. Tubulin (red) serves as a marker for cytoskeleton and DAPI (blue) serves as a nuclear stain. Upper panel is epifluorescent imaging and lower is confocal imaging.
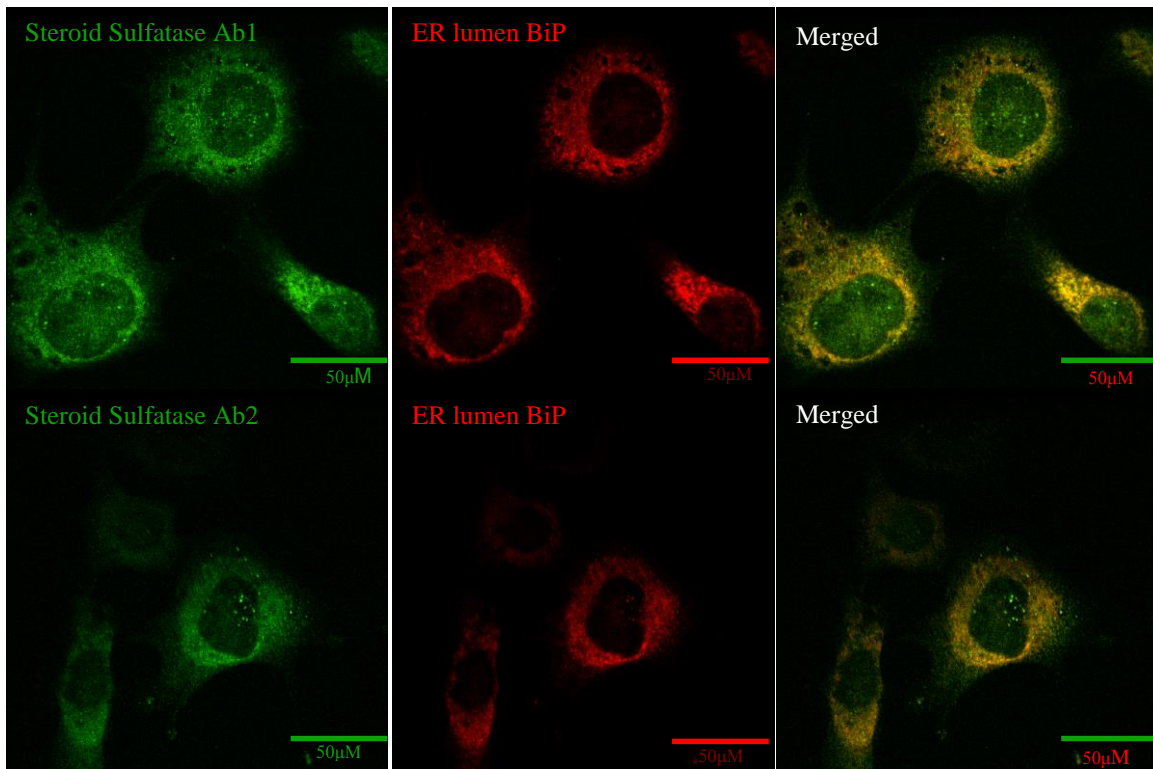
Fig. 5. Steroid sulfatase immunocytochemical expression in MG-63 cells. Sequential

staining of fixed MG-63 cells was performed. Cells were stained for sulfatase (green)

with two different antibodies, steroid sulfatase Ab1 (PA5-28259) and steroid sulfatase

Ab2 STS-275. ER lumen protein BiP (red) serves an ER marker. Steroid sulfatase is seen

to be distributed throughout the cytoplasm, but appears to be concentrated in the

endoplasmic reticulum and Golgi regions. Images taken at 60X magnification using

confocal imaging.
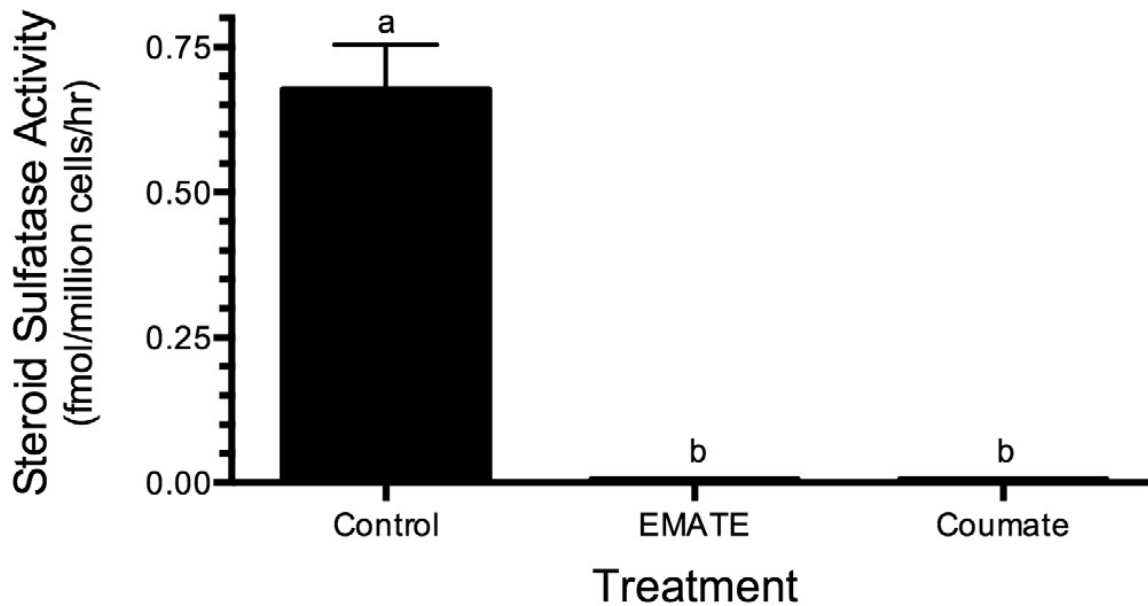
Fig. 6. Steroid sulfatase (STS) activity in MG-63 cells, as determined by ³H-estrone sulfate conversion assay. Cells were incubated overnight in the presence or absence of the STS inhibitors (1 μM) estrone-3-O-sulfamate (EMATE) and 667 Coumate. Whole cell conversion assays were performed. Bars represent the mean ± 1 S.E.M. STS activity levels varied significantly among treatment groups (one-way analysis of variance). Different letters represent differences among means (Newman–Keuls post hoc test, $P < 0.0001$).
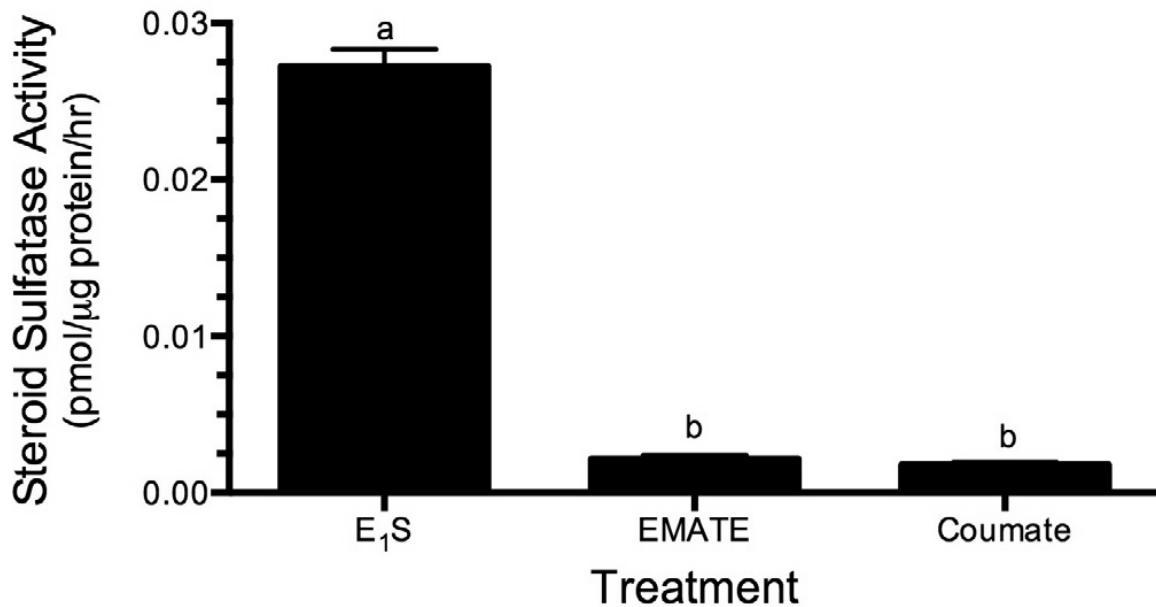
Fig. 7. Steroid sulfatase (STS) conversion assay for MG-63 microsomes incubated in the presence of ^{3}H-E$_1$S, with and without the STS inhibitors (1 µM) estrone-3-O-sulfamate (EMATE) and 667 Coumate. Bars represent the mean ± 1 SEM STS activity levels varied significantly among treatment groups (one-way analysis of variance). Different letters represent differences among means (Newman–Keuls post hoc test, $P < 0.0001$).
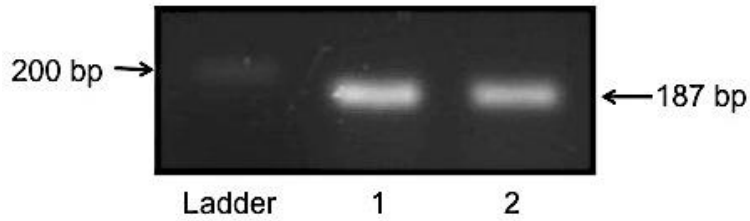
Fig. 8. Reverse transcriptase polymerase chain reaction of MG-63 cells using human-specific steroid sulfatase (STS) primers. Gel was a 2% agarose gel stained with ethidium bromide. Primer sequences, preparation of cDNA and reverse transcriptase polymerase chain reaction were performed. Lane assignments: (1) bp ladder, (2) MG-63 RNA sample #1, and (3) MG-63 RNA sample #2. 200 = marker on the ladder, 187 = expected size of the cDNA product for STS, with the primer pair used.
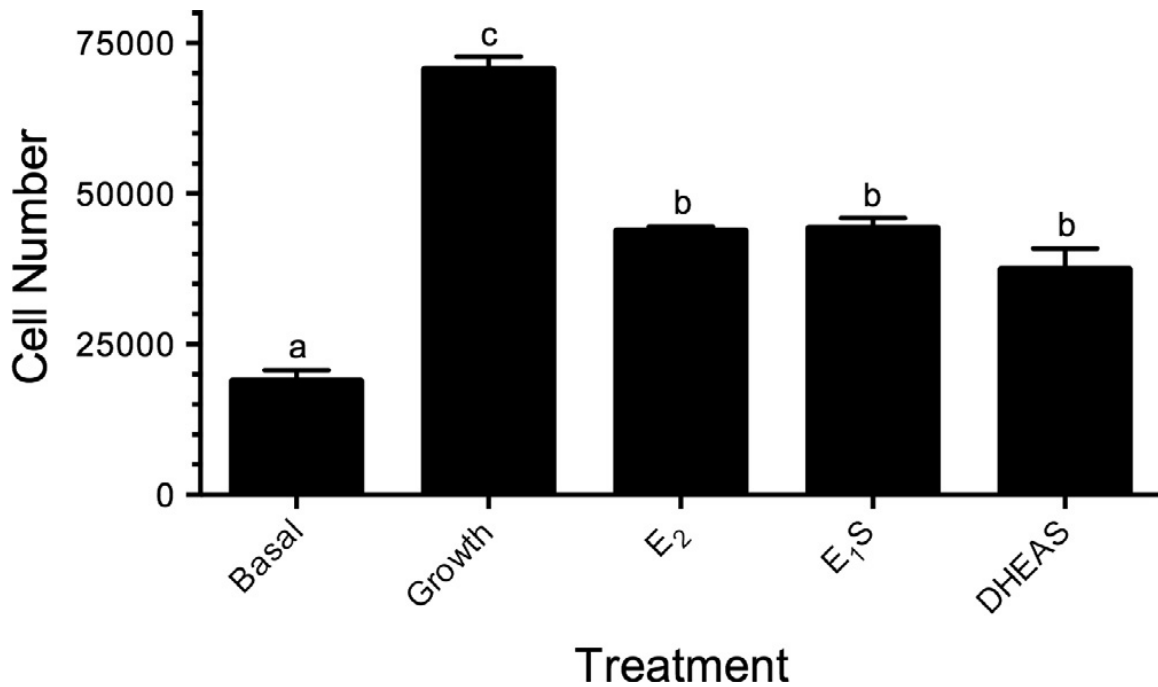
Fig. 9. Growth of MG-63 cells in basal medium with or without addition of 10 μM estradiol-17β (E₂), estrone sulfate (E₁S) or dehydroepiandrosterone sulfate (DHEAS), or growth medium (growth). Bars represent mean cell number ± 1 SEM. Cell growth differed significantly among groups (one-way analysis of variance; $F = 88.09$; 6, 21 df; $P < 0.0001$). Different letters represent differences among means (Newman– Keuls post hoc test, $P < 0.05$).
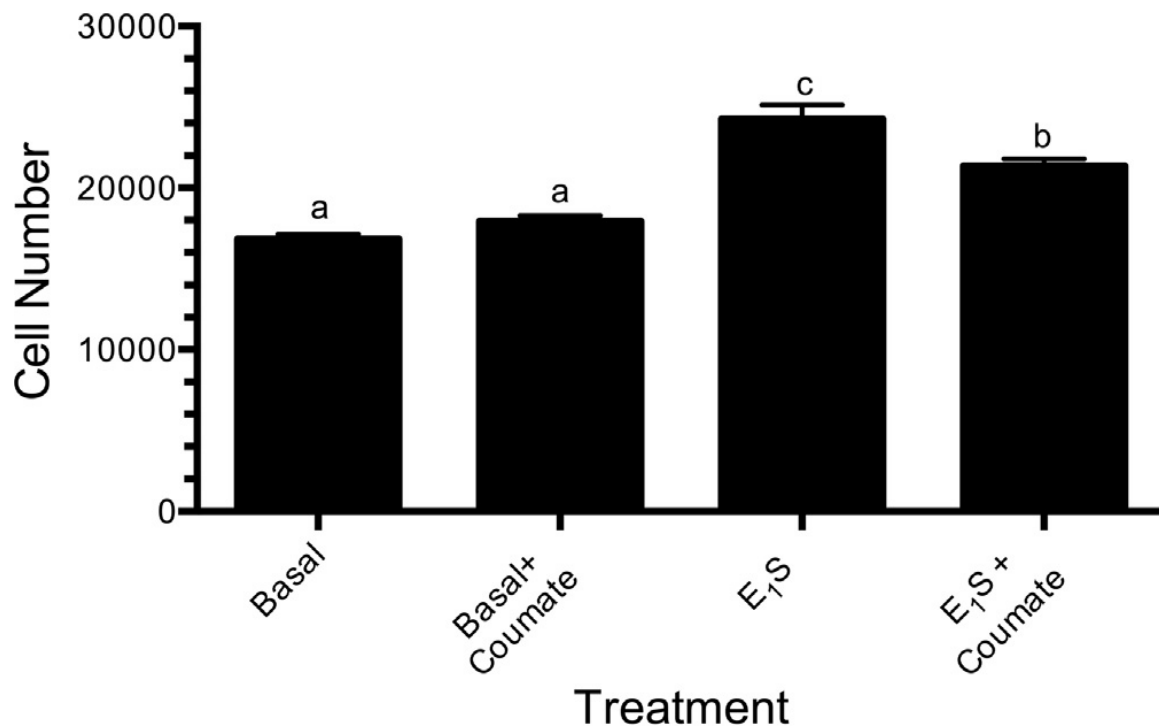
Fig. 10. Growth of MG-63 cells in basal medium with or without addition of estrone sulfate (10 µM) in the presence or absence of 667 Coumate (1 µM). Cell growth varied significantly among treatment groups (one-way analysis of variance; $F = 39.59$; 4, 31 df; $P < 0.0001$). Different letters represent differences among means (Newman–Keuls post hoc test, $P < 0.05$).
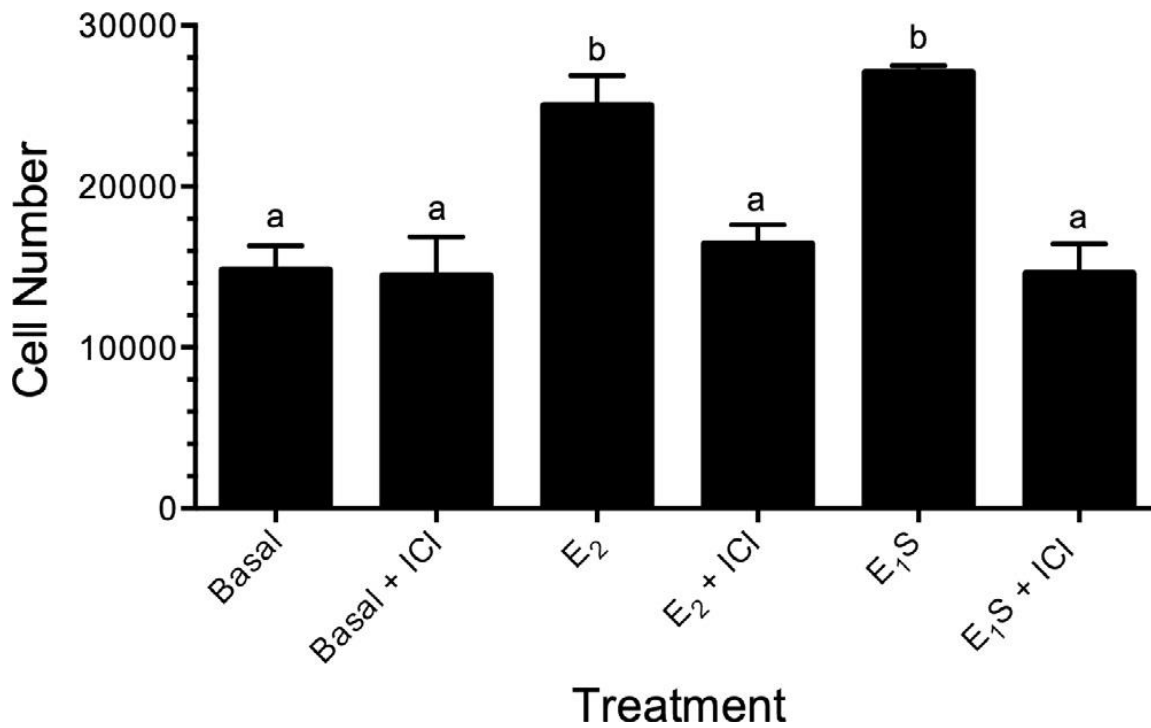
Fig. 11. Growth of MG-63 cells in basal medium, with or without addition of estradiol-17 β or estrone sulfate (10 µM), and in the presence or absence of ICI 182,780 (100 nM). Cell growth differed significantly among groups (one-way analysis of variance; F = 3.521; 11, 15 df; P < 0.05). Different letters represent differences among means (Newman–Keuls post hoc test, P < 0.05).
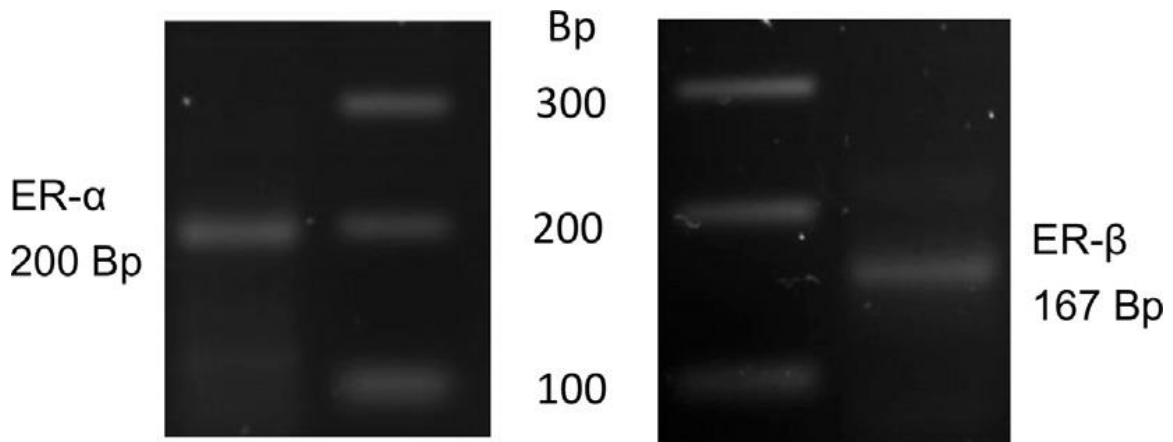
Fig. 12. Reverse transcriptase polymerase chain reaction of MG-63 cells using human-specific estrogen receptor alpha (ER- α) and estrogen receptor beta (ER- β) primers. Gel was a 2% agarose gel stained with ethidium bromide. Primer sequences, preparation of cDNA and reverse transcriptase polymerase chain reaction methods were performed.

2.2 <u>Project II. Activity and Expression of Steroid Sulfatase During Differentiation, in the Human MG-63 Pre-Osteoblast Cell Line and Effect of Glucocorticoids</u>

2.21 <u>Hypothesis and Objectives</u>

Model: MG-63 human pre-osteosarcoma fibroblast cell line derived from an osteosarcoma of a 14-year-old male.

Rationale:

MG-63 cells progress through various stages of bone cell differentiation under the influence of estrogen and osteogenic supplement. Steroid sulfatase has been shown to be present in cartilage of early developing mouse embryos suggesting it may participate in the differentiation of the skeletal system (Compagnone et al., 1997). However, there is no additional information on steroid sulfatase expression during differentiation of human bone cells, including MG-63 cells.

Hypothesis:

Differentiation of osteoblasts leads to bone formation, which is required to maintain structural integrity. Because steroid sulfatase provides a source of bioavailable 17β-estradiol, this enzyme is required during the osteoblastic differentiation process. Thus, expression and activity of steroid sulfatase will be present throughout the process. Proliferating cells show higher metabolic activities than differentiated cells, hence steroid sulfatase levels are expected to be lower in osteogenic supplement treated differentiated cells.

Objective:

1. Develop an assay to monitor osteoblast differentiation in MG-63 cells

    Experiments to address Objective 1:

    a. Measure growth in the presence or absence of osteogenic supplement.

b. Measure alkaline phosphatase expression during the 21-day process in the presence or absence of osteogenic supplement.

c. Measure osteocalcin mRNA expression during the 21-day process in the presence or absence of osteogenic supplement.

2. Measure steroid sulfatase activity and steroid sulfatase mRNA levels throughout the differentiation of MG-63 cells.

Experiments to address Objective 2:

a. Measure whole-cell steroid sulfatase conversion in the presence or absence of osteogenic supplement.

b. Conduct a quantitative real-time PCR using primers specific for the human steroid sulfatase-encoding gene in the presence or absence of osteogenic supplement.

3. To examine estrogen receptor expression throughout the differentiation of MG-63 cells

Experiments to address Objective 3:

a. Measure whole-cell steroid sulfatase conversion in the presence and absence of osteogenic supplement in response to steroid sulfatase inhibitor EMATE and estrogen receptor inhibitor ICI 182,780.

b. Determine ER-α and ER-β mRNA expression during differentiation with and without osteogenic supplement.

4. To study effect of osteogenic supplement on MG-63 cell differentiation

Experiments to address Objective 4:

a. Measure growth of MG-63 cells with different combinations of osteogenic supplement components.

b. Measure whole-cell steroid sulfatase conversion in response to glucocorticoids dexamethasone, cortisol and prednisolone.

5. To study dexamethasone effect on regulating steroid sulfatase activity levels during MG-63 cell differentiation

Experiments to address Objective 5:

a. Measure whole-cell steroid sulfatase conversion in response to glucocorticoid dexamethasone and in response to glucocorticoid receptor antagonist RU-486 in undifferentiated cells.

b. Measure whole-cell steroid sulfatase conversion in response to glucocorticoid dexamethasone and in response to glucocorticoid receptor antagonist RU-486 on Day 12 of OS- and OS+ treated MG-63 cells.

c. Measure fold change of steroid sulfatase mRNA expression in response to glucocorticoid dexamethasone and glucocorticoid receptor antagonist RU-486 by real time quantitative PCR.

2.22 Project II Abstract

Osteoblasts proceed through a well-defined process of proliferation, matrix maturation, and extracellular mineralization. Estrogens have been shown to be important in osteoblast physiology. Steroid sulfatase (STS) converts sulfated steroids into their active forms. STS occurs in bone, but little is known about its regulation. We are investigating the activity and expression of STS in the human preosteoblastic cell line

MG-63. MG-63 cells differentiate under the influence of an osteogenic supplement, leading to extracellular mineralization. We studied STS in cells grown in medium alone (OS-) or grown with osteogenic supplement (OS+) over 20 days. Cell proliferation was nearly exponential in OS- cells, but slowed in OS+ cells. STS activity and STS gene expression increased substantially over time in OS- cells, but showed only a small increase in OS+ cells. Bone cell differentiation was assessed by alkaline phosphatase activity and osteocalcin gene expression. Alkaline phosphatase activity increased substantially in the OS+ cells and only slightly in the OS- cells. Osteocalcin gene expression was high in OS- cells throughout the time course, but was high only during Day 2 - Day 14 in OS+ cells. STS inhibitor EMATE virtually eliminated steroid sulfatase activity throughout differentiation in OS- and OS+ cells. The estrogen receptor blocker ICI 182,780 had little effect on steroid sulfatase activity during differentiation in either OS- or OS+ cells, despite the observation that mRNAs for both estrogen receptor alpha and beta were more highly expressed in OS- cells. The effect of individual components of osteogenic supplement on cell proliferation revealed that the glucocorticoid dexamethasone is the component that stimulates cell differentiation. Dexamethasone decreased steroid sulfatase activity in undifferentiated cells. When RU486, an antagonist of the glucocorticoid receptor, was added to dexamethasone-treated cells, steroid sulfatase activity was upregulated compared to dexamethasone-treated cells. Similarly, Day 12 long term proliferating OS- cells and differentiated OS+ cells both showed a decline of steroid sulfatase levels upon dexamethasone treatment. Our data indicate that activity and expression of steroid sulfatase is higher during the early stages of bone differentiation and that glucocorticoid inhibits steroid sulfatase expression and activity.

The inhibition of steroid sulfatase in bone by glucocorticoids, leading to decreased active estrogen levels, may be one cause of glucocorticoid-induced osteoporosis. Also, blocking of endogenous glucocorticoids in bone may be an effective way to increase steroid sulfatase expression and activity that would release increase active estrogen and retain bone robustness, thereby preventing or offsetting osteoporosis.

2.23 Introduction

The bone remodeling system characterized by resorption of old bone tissue by osteoclast cells and subsequent ossification by osteoblast cells maintains the integrity of human adult skeletal tissue. The circulating hormone estrogen displays inhibitory effects on the bone-resorbing cytokine pathway and can decrease bone resorption. Estrogen can also stimulate growth factors to induce bone formation (Raisz, 1999); thus, the overall effect of estrogen is to decrease bone turnover. In post-menopausal women, estrogen deficiency promotes bone resorption, which leads to destruction of local bone architecture and induces osteoporosis (Vaananen and Harkonen, 1996).

While estrogen levels are low in post-menopausal women, estrone sulfate (E_1S), a major circulating estrogen precursor, is found in abundance in the blood of men, nonpregnant women, and postmenopausal women (Ruder et al., 1972). E_1S levels in premenopausal and postmenopausal women are several-fold higher than those of unconjugated estrogens, and can be transformed to active estrogens by steroid sulfatase (STS) in human bone (Muir et al., 2004). Steroid sulfatase is an aryl sulfatase C enzyme that cleaves the sulfate group from sulfate esters of phenol-or 3β-hydroxysteroids (Reed et al., 2005) converting inactive conjugated E_1S steroid to active estrogen. Thus, STS

could be an important enzyme in the local estrogen metabolic pathway required for normal bone physiology.

STS gene expression and protein activity have been shown to be present in rat and human pre-osteoblast cells (Fujikawa et al., 1997) as well as rat bone tissue (van der Eerden et al., 2004), but information on presence and expression of steroid sulfatase during osteoblast differentiation is lacking. Osteoblast differentiation proceeds through the three stages of cell proliferation, matrix maturation and matrix mineralization (Stein and Lian, 1993). Matrix formation is accompanied by expression of specific genes like alkaline phosphatase, osteopontin, and osteocalcin. STS could be involved in any of these stages, providing estrogen from circulating precursors.

In view of STS's potential beneficial effects on maintaining the structural integrity of bone, we investigated its presence, activity and gene expression levels throughout cell differentiation in the human pre-osteoblast MG-63 cells. Osteogenic supplement was used to transform cells, and alkaline phosphatase activity, osteocalcin gene expression and cell morphology were used to markers to confirm cell differentiation. We also examined the role of estrogen receptors on sulfatase activity levels by using estrogen receptor inhibitor ICI 182,780.

Frequently used protocols to bring about *in vitro* differentiation of bone cells use growth medium supplemented with ascorbic acid, β-glycerophosphate and dexamethasone (collectively called osteogenic supplement). Ascorbic acid increases secretion of collagen, β-glycerophosphate serves as a phosphate source for bone mineralization and dexamethasone induces differentiation (Langenbach and Handschel, 2013). The synthetic glucocorticoid dexamethasone has anti-inflammatory and

immunosuppressant effects and is used to treat patients with arthritis (Islander et al., 2011). However, synthetic glucocorticoids such as dexamethasone and prednisolone are known to cause or exacerbate osteoporosis when used chronically. While dexamethasone promotes bone cell differentiation, not much is known on how it regulates other signaling molecules to prevent proliferation and promote differentiation. Herein, we also studied the effect of dexamethasone on regulation of steroid sulfatase expression and activity during MG-63 cell differentiation.

2.24 <u>Materials and Methods</u>

Chemicals and reagents

[6,7-^{3}H] Estrone-sulfate (49 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, USA). Radioinert steroid dexamethasone was obtained from Sigma Chemical Co., (St. Louis, MO, USA). ICI 182,780 was purchased from Tocris Cookson Ltd. (Ellisville, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT, USA). The steroid sulfatase inhibitor estrone 3-O-sulfamate (EMATE) was obtained from Dr. Pui-Kai Li (Ohio State University School of Pharmacy).

Cell culture methods

Human preosteoblastic MG-63 cells were purchased from ATCC (Rockville, MD). Cell culture media and reagents were obtained from Invitrogen (Grand Island, NY, USA). Growth medium, used for routine growth of MG-63 cells, was 47.5% (v/v) Hams F12 nutrient mixture, 47.5% (v/v) Minimal Essential Medium (MEM) containing 5% (v/v) heat-inactivated fetal bovine serum from HyClone (Logan, UT, USA), and 10 mg/ml penicillin/ streptomycin solution from HyClone. Cells were cultured in 100 mm

tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ, USA) in 10 mL growth medium. Media added to plates was changed every 2 or 3 days to ensure continuous nutrient supply. To initiate pre-osteoblast cell differentiation, osteogenic supplement media was used. Osteogenic supplement media was 47.5% (v/v) Hams F12 nutrient mixture, 47.5% (v/v) Minimal Essential Medium (MEM), 5% (v/v) heat-inactivated fetal bovine serum and 10 mg/ml penicillin/ streptomycin solution supplemented with 50 µg/ml ascorbic acid (Sigma-Aldrich Co., St.Louis, MO), 10 µM β-glycerophosphate (Sigma), and 100nM dexamethasone (Sigma).

Steroid sulfatase activity, MTT cell viability, BCA protein levels, alkaline phosphatase activity, osteocalcin and sulfatase mRNA expression levels were measured on Days 2, 5, 8, 11, 14, 17, and 20 for cells treated in growth medium alone and for cells treated in growth medium with osteogenic supplement.

Cell growth assays

Growth in response to absence and presence of osteogenic supplement

MG-63 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson, NJ, USA) at a starting density of 100,000 cells/well and incubated in growth medium overnight to allow them to adhere. After incubation, the medium was removed, the cells were washed once with phosphate buffered saline (Sigma-Aldrich Co., St.Louis, MO), and 2 ml of either growth medium or growth medium containing osteogenic supplement was added to respective wells. Over a period of 21 days of experimental assays the media was changed every third day. Cell density was measured on Days 2, 5, 8, 11, 14, 17, and 20. One 6-well plate of cells harvested in growth medium alone and another 6-well plate of cells harvested in growth medium with osteogenic supplement

was used for each day of measurement. Medium was removed and 200 µl MTT (Dimethylthiazol tetrazolium bromide) (Sigma-Aldrich Co., St.Louis, MO) in growth medium was added to each well and the plates were incubated at 37 °C for 3 h. Following incubation, the MTT was completely removed and replaced with 1 ml of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the formazan crystals within the cells and then four 200 µl samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595 nm using the BioRad Microplate reader (Model 3550). Absorbances were compared with a standard curve derived from plates with known number of cells (31,250 – 500,000 cells). Experiment was run in duplicate and repeated three times.

Growth in response to absence and presence of ascorbic acid (Vit. C at 50 µg/ml), β-glycerophosphate (G-PO$_4$ at 10 µM) and dexamethasone (Dex at 100nM)

MG-63 cells were seeded into Falcon 24-well tissue culture plates (Beckton Dickinson and Co.) at a starting density of 20,000 cells/well and incubated in growth medium overnight to allow them to adhere. After incubation, the medium was removed, the cells were washed once with phosphate buffered saline (Sigma-Aldrich Co., St.Louis, MO), and 1 ml of respective treatments (Dex+G-PO$_4$, G-PO$_4$+Vit.C, Dex+Vit.C and Dex+G-PO$_4$+Vit.C) in growth medium was added to each treatment column. 1ml growth medium alone was used as control. Over a period of 21 days of experimental assay the respective supplemental media was changed every third day. Cell density was measured on Days 2, 5, 8, 11, 14, 17, and 20. One 24-well plate of treated cells was used for each day of measurement. Medium was removed and 100 µl MTT (Dimethylthiazol tetrazolium bromide) (Sigma-Aldrich Co., St.Louis, MO) in growth medium was added

to each well and the plates were incubated at 37 °C for 3 h. Following incubation, the MTT was completely removed and replaced with 500 μl of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the formazan crystals within the cells and then two 200 μl samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595 nm using the BioRad Microplate reader (Model 3550). Absorbances were compared with a standard curve derived from plates with known number of cells (500 – 256,000 cells).

Measurement of sulfatase activity of intact cells

Time course STS activity in response to absence and presence of osteogenic supplement and in response to presence and absence of inhibitors EMATE and ICI 182,780

MG-63 cells seeded in 6-well tissue culture plates (Beckton Dickinson and Co., NJ, USA) at a starting density of 100,000 cells/well in growth medium or growth medium containing osteogenic supplement at each experimental time point was considered. Twenty-four hours prior to assay, media was removed, washed and replaced with 2 ml of respective medium containing ^{3}H-estrone sulfate (100,000 dpm/ml) in the absence or presence of specific inhibitors, estrone 3-O-sullfamate (EMATE, 1 μM) or ICI 182, 780 (1 μM). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13 x 100 borosilicate glass tubes to measure radioactivity levels.

STS activity in response to absence and presence of glucocorticoids (dexamethasone, hydrocortisone, prednisolone)

MG-63 cells seeded in 6-well tissue culture plates (Beckton Dickinson and Co., NJ, USA) at a starting density of 100,000 cells/well in growth medium overnight for cells to adhere. Medium was removed and replaced with basal medium to starve the cells.

Twenty-four hours later starvation media was removed, washed and replaced with 2 ml of respective medium containing 1 μM dexamethasone, 1 μM hydrocortisone or 1 μM prednisolone in basal medium. Two ml of growth medium alone served as positive control while 2 ml of basal medium alone served as negative control. 48 h later treatment media was replaced with medium containing ^{3}H-estrone sulfate (100,000 dpm/ml). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13 x 100 borosilicate glass tubes to measure radioactivity levels.

STS activity in response to absence and presence of glucocorticoid dexamethasone and antiglucocorticoid RU486 in proliferating cells (short term assay) and differentiating cells (long term assay).

MG-63 cells seeded in 6-well tissue culture plates (Beckton Dickinson and Co., NJ, USA) at a starting density of 300,000 cells/well in growth medium in the short-term assay. After overnight adhering of cells, growth medium was removed and cells were starved in basal medium for 24 h. For the long-term assay MG-63 cells were plated at a starting density of 100,000 cells/well and then grown in the absence or presence of osteogenic supplement for 12 days. After the 12 day growth period cells were starved in basal medium for 24 h. After starvation, both short-term and long-term plate set-up was treated with either 100 nM RU486 or 1 μM Dex or 1 μM Dex with 100 nM RU486 in basal medium for 24 h. Basal medium alone served as control. Following incubation media was replaced with basal medium containing ^{3}H-estrone sulfate (100,000 dpm/ml). After 18 h of incubation, 0.5 ml of medium was aliquoted into each of two 13x100 borosilicate glass tubes to measure radioactivity levels.

Three milliliters of toluene was added to each tube for extraction of unconjugated

steroids. The mixture was vortexed for 1 min and then centrifuged for 10 min to separate the aqueous and organic phases. Duplicate aliquots of 1 ml were removed from the organic phase (containing the unconjugated steroids) and transferred to scintillation vials, after which 5 ml of scintillation cocktail was added. Radioactivity was counted in a Packard Tri-carb scintillation counter at 50% efficiency for ^{3}H. The conversion values obtained for all treatments were adjusted for spontaneous product formation by subtracting the value obtained for wells containing respective medium and ^{3}H-estrone sulfate with no cells.

Preparation of MG-63 crude homogenates

Medium was removed and cells were washed 2 times with phosphate buffered saline (Sigma-Aldrich Co., St Louis, MO). Plate was covered in trypsin EDTA (Sigma-Aldrich Co., St Louis, MO, St. Louis, MO) for approximately 2 min. Trypsin was removed and 2 ml of growth medium was added to make a cell suspension. The suspension was divided into two 15 ml conical tubes, 1 ml in each tube. Three ml of fresh growth medium was added to the conical tubes. Samples were centrifuged at 2000 g for 10 min to obtain a loose pellet. Samples were immediately placed on ice. The supernatant was removed and the pellets were resuspended in 2 ml of 50 mM Tris-HCl. The cell mixture was then placed in a pre-chilled beaker and homogenized using three 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). Samples were immediately stored at -80 °C.

BCA assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared

with 50mM Tris-HCl as the diluent. Duplicate concentrations (μg/tube) were 100, 50, 25, 12.5, 6.25, and 3.125. Duplicate tubes of MG-63 crude homogenate samples were prepared using 75 μl of homogenate suspension. Absorbances were read at 562 nm using a spectrophotometer (Thermospectronic, Genesys 20; Waltham, MA, USA).

Alkaline phosphatase activity assay

Crude homogenates were thawed at room temperature and 50 μl homogenates in duplicate were incubated in the presence of ABC buffer consisting of equal parts 1.55 M 2-amino-2-methyl propanol, 20 mM p-nitrophenol phosphate and 10 mM $MgCl_2$ (Sigma-Aldrich Co., St. Louis, MO) for 1 h, after which absorbance readings were taken at 400 nm on a microplate reader (BioRad Model 3550).

RNA isolation

Total RNA from MG-63 cells were isolated using TRIzol[®] Reagent (Invitrogen Life Technologies) according to manufacturer's instructions. 500 μl of TRIzol[®] reagent was added to one well of a 6-welled plate for each time point, cells were resuspended, transferred to a microcentrifuge tube and incubated at room temperature for 5 min. 160 μl of chloroform was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was transferred, to which, 500 μl of isopropyl alcohol and 1 μl of glycogen was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The resulting RNA pellet was washed with 500 μl of 75% ethanol, centrifuged at 7000 g for 10 min at RT, air-dried, and resuspended in 30 μl of diethylpyrocarbonate-treated water. Following 10 min incubation at 60 °C, RNA was quantified using a spectrophotometer (Thermo Fisher, Genesys 8, Pittsburgh, PA, USA). The amount of RNA extracted was

determined by ultraviolet light absorption at 260 nm. RNA samples were stored at − 80 °C until use.

Reverse transcription polymerase chain reactions (RT-PCR)

Reverse transcription

Using the GoScript® Reverse Transcription System (Promega), according to manufacturer's instructions, 1 g of template RNA, 1 μl of Oligo (dT) primer and diethylpyrocarbonate-treated water were added to make a total volume of 9 μl, heated to 70 °C for 5 min, and then chilled on ice for 5 min. In a separate tube, reverse transcription mix was prepared from 4 μl of 5X reaction buffer, 4 μl MgCl$_2$, 1 μl dNTPs and 0.5 μl of reverse transcriptase.12 μl of reaction mix was added to RNA/primer mix. First-strand cDNA synthesis was carried out in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) with 5 min annealing at 25 °C. Next, the cDNA was allowed to extend for 1 h at 42 °C. The reverse transcriptase enzyme was thermally inactivated at 70 °C for 15 min after which the reaction tubes were held at 4 °C.

PCR primers

Differentiation was determined by studying the expression of human osteocalcin gene (GenBank Accession No. NM_199173.4), the forward primer start position is at 165 and has the sequence (5'–3') CAA AGG TGC AGC CTT TGT GTC (FB). The reverse primer start position is at 314 with the sequence (5'–3') TCA CAG TCC GGA TTG AGC TCA (RB), with an expected product of 150 base pairs. Steroid sulfatase primers were selected from the complete coding sequence for human steroid sulfatase (GenBank Accession No. M16505.1). The forward primer start position is 1402 and has the sequence (5'–3') TGA TGA GCC CAC TAG CAA CAT GGA (FB). The reverse primer

start position is 1588 and has the sequence (5'–3') GTG CCA GCG CAC AGC ATT

TAA GTA (RB). The expected RT-PCR product from this primer pair is 187 base pairs.

To determine the mRNA expression of estrogen receptor alpha (ER-α) and estrogen

receptor beta (ER-β), two new sets of primers were designed. For human ER-α (GenBank

Accession No. NM_000125.3), the forward primer start position is at 709 and has the

sequence (5'–3') CAG GGT GGC AGA GAA AGA TT (FB). The reverse primer start

position is at 908 with the sequence (5'–3') TTG GTG GCT GGA CAC ATA TAG (RB),

with an expected product of 200 base pairs. For human ER-β (GenBank Accession No.

AB006590.1), the forward primer start position is at 1460 with the sequence (5'–3') CCT

GGC TAA CCT CCT GAT GC (FB). The reverse primer start position is at 1626 and has

the sequence (5'–3') ACC CCG TGA TGG AGG ACT T (RB) for an expected product

size of 167 base pairs. Primers were synthesized by Integrated DNA Technologies, Inc.,

(Coralville, IA).

RT-PCR reagents and conditions

PCR reactions were performed on the cDNA templates using a Taq PCR Kit

(New England Biolabs, Ipswich MA) according to manufacturer's instructions. 2 μl

template cDNA, 2.6 μl 10X PCR buffer, 0.4 μl dNTP mix, 1 μl of 10 μM sense primer, 1

μl of 10 μM antisense primer, 0.2 μl Taq Polymerase, and DEPC-treated water were

added to make total volume 20 μl. PCR reactions were then placed in a thermocycler

(PTC-100, MJ Research, Inc., Watertown, MA). The reaction had a 4 min hold at 94 °C.

For steroid sulfatase determination, the cDNA product was amplified for 35 cycles of:

45sec 94 °C, 45 s 58 °C, 90 s 72 °C. For ER-α and ER-β amplification, the cDNA

products were amplified at 35 cycles of: 45 s 94 °C, 45 s 52 °C, 90 s 72 °C. The PCR

reaction was terminated with a final extension of 5 min at 72 °C. The PCR products were then separated in a 2% agarose gel containing 5 µl of 1 mg/ml ethidium bromide solution. The gel was run in 1X TAE running buffer (40 mM Tris–acetate, 2 mM Na_2-EDTA-$2H_2O$) for 2 h at 70 V. 10 µl of PCR product were mixed with 2 µl 6X cyanol loading dye (0.25% w/v xylene cyanol, 15% Ficoll in water). Quick load 2-log DNA ladder (New England Biolabs, Ipswich MA) was used as the standard.

qRT-PCR reagents and conditions

Primers designed for RT-PCR of steroid sulfatase mRNA expression and osteocalcin expression was used. The real-time PCR assay was carried out by Maxima SYBR Green qPCR Master Mix (Thermo Fisher, Pittsburgh PA). Through the time course relative expression of target gene mRNAs for steroid sulfatase and osteocalcin was quantified by measuring the cycle threshold (Ct) and normalized by the housekeeping gene 18S ribosomal RNA. Relative mRNA expression levels for steroid sulfatase and osteocalcin were determined by the $1/\Delta Ct$ method. Steroid sulfatase expression in response to dexamethasone and RU486 was quantified by measuring the cycle threshold (Ct) and normalized by the housekeeping gene 18S ribosomal RNA; relative mRNA expression levels over basal control were determined by the $1/\Delta Ct$ method.

Statistical analyses

Statistical analyses were carried out using Prism 6.0 for Macintosh (GraphPad Software, San Diego, CA). Probabilities of $P < 0.05$ were considered significant.

2.25 Results

Cell growth response to absence or presence of osteogenic supplement

In order to establish or identify the growth pattern of MG-63 cells undergoing differentiation into osteoblasts, growth of MG-63 cells treated with osteogenic supplement was evaluated during cell differentiation over 21 days. Cell growth was significantly reduced in the presence of osteogenic supplement (OS+), starting at Day 8 and continuing through Day 20, (Fig. 14A). Cell number was 50% higher in OS- cells than in OS+ cells at Day 20.

Alkaline phosphatase assay in response to absence or presence of osteogenic supplement

In order to assess the extent of MG-63 differentiation into osteoblasts, alkaline phosphatase activity levels were measured in MG-63 crude homogenate preparations of cells at each time point for OS- and OS+ treated cells. Alkaline phosphatase levels were adjusted according to the total protein concentration in each sample. In OS- cells, alkaline phosphatase activity showed little change over 21 days (Fig. 14B). In contrast, OS+ cells increased in alkaline phosphatase from Day 1 to Day 17, at which time it decreased somewhat. Alkaline phosphatase was significantly higher in OS+ cells on all days from Day 11 to Day 20.

RT-PCR and qRT-PCR of osteocalcin mRNA expression in response to absence or presence of osteogenic supplement

In order to assess mRNA expression of osteocalcin in MG-63 cells differentiated into osteoblasts, MG-63 cells were grown in OS- or OS+ medium for 21 days. Reverse transcriptase PCR was performed on RNA extracted from cells, using primers based on the known sequence of human osteocalcin gene. mRNA was extracted at each time point

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for OS- and OS+ treated MG-63 cells. Single bands of 150 base pairs (Fig. 14C lower panel), corresponding to the expected product size, were evident on all days in OS-treated cells, with bands more intense from Days 2 – 11 than later days. For OS+ cells, bands were faint on Days 2 – 8, and very faint thereafter. Two-step qRT-PCR of changes in osteocalcin gene expression were calculated as relative mRNA expression for each time point for OS- and OS+ treated cells (Fig. 14C upper panel). This analysis demonstrated that osteocalcin expression was higher for OS- on all days, and that the difference in osteocalcin expression increased during the later stages of differentiation.

Whole cell sulfatase enzyme assay in response to absence or presence of osteogenic supplement

In order to assess STS activity pattern during the course of MG-63 differentiation into osteoblasts, MG-63 cells were incubated overnight (18 h) in the presence of ^{3}H-estrone sulfate at each time point of the differentiation experiment in order to measure steroid sulfatase activity. In cells incubated in growth medium (OS-), steroid sulfatase activity of MG-63 cells incrementally increased over the 20-day time course to 0.14 pmol/million cells/18h (Fig. 15A). In contrast, for cells treated with osteogenic supplement, steroid sulfatase activity showed no discernable trend, fluctuating around about 0.035 pmol/million cells/18h. Thus, steroid sulfatase activity is inhibited in differentiating cells compared with proliferating cells.

RT-PCR and qRT-PCR of STS mRNA expression in response to absence or presence of osteogenic supplement

In order to assess STS mRNA expression pattern during the course of MG-63 differentiation into osteoblasts, reverse transcriptase PCR was performed on the mRNA

extracted at each time point from OS- and OS+ treated MG-63 cells. Using primers based on the known sequence of human steroid sulfatase gene, single bands of 187 base pairs (Fig. 15B lower panel), corresponding to the expected product size, were evident on all days in both treatments. For any given day, cDNA bands were more intense for OS- compared to OS+. Relative STS mRNA expression as determined by two-step qRT-PCR, for each time point, in OS- and OS+ treated cells revealed STS mRNA expression to be higher in OS- growth medium treated cells (Fig. 15B upper panel). OS+ treated cells showed comparatively lower STS mRNA expression for each time point. Statistically significant increase in OS- treated cells was seen from Day 2 – Day 8 and Day 14 – Day 20.

Steroid sulfatase activity in response to inhibitors

In order to determine the role of STS and estrogen receptor in OS+ induced MG-63 differentiation into osteoblasts, MG-63 cells were incubated overnight (18 h) in the presence of ^{3}H-E$_1$S and in the presence or absence of steroid sulfatase inhibitor EMATE (estrone 3-o-sulfamate, 1 μM) and estrogen receptor inhibitor ICI 182,780 (1 μM) for each time point and medium treatment represented in the differentiation experiment. As shown previously, data showed a comparatively higher increase in STS activity of 0.37 pmol/18 h on Day 20 for cells treated in growth medium alone relative to 0.25 pmol/18 h on Day 20 for cells treated with osteogenic supplement (Fig. 16A). STS activity was virtually eliminated in the presence of EMATE for both types of cell treatment throughout differentiation. In contrast, for OS- and OS+ treated cells containing the ER blocker ICI 182,780 (1 μM), there was neither a statistical difference nor an obvious trend in steroid sulfatase activity over the 21 days as compared to the control sulfatase

levels.

RT-PCR of ER-α and ER-β mRNA expression

In order to assess the expression patterns of ER-α and ER-β during MG-63 differentiation into osteoblasts, reverse transcriptase PCR was performed on the mRNAs extracted at each time point of OS- and OS+ treated MG-63 cells for human ER-α and ER-β genes. Using primers based on the known sequences of the ER-α, single bands of 200 base pairs (Fig. 16B upper panel) corresponding to the expected product size, were evident on all days in OS- and OS+ treated cells. Bands of 167 base pairs representing ER-β transcripts appeared to remain constant during differentiation in OS- treated cells (Fig. 16B lower panel). However, low ER-β mRNA expression was observed for OS+ treated cells.

Cell growth response to components of osteogenic supplement medium

In order to determine the role of each ascorbic acid, β-glycerophosphate and dexamethasone in MG-63 cell differentiation into osteoblasts, MG-63 cells were treated in absence and presence of different combinations of osteogenic supplement components: ascorbic acid (50 μg/ml), β-glycerophosphate (10 μM) and dexamethasone (100 nM). MTT cell viability assay (Fig. 17A upper panel) and inverted phase contrast microscopy imaging (Fig. 17A lower panel) were performed. On Day 17, cells proliferated to about 1000,000 cells in the absence of any osteogenic supplement while cells proliferated to about 680,000 cells in the presence of β-glycerophosphate and ascorbic acid. In the presence of dexamethasone, with or without other components, cell growth was limited to about 300,000 cells. Microscopy images showed cells treated with the glucocorticoid dexamethasone to have an elongated cell morphology, as compared to spherical

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multilayered MG-63 cells in the absence of dexamethasone. Similar trend of cell morphology and proliferation levels was seen at each time point from Day 8 to Day 20 (Appendix Fig. A4).

STS activity in response to glucocorticoids (1 μM)

In order to determine the effect of glucocorticoids on STS activity in MG-63 cells, the cells were treated in absence and presence of one of these glucocorticoids, 1 μM dexamethasone, 1 μM hydrocortisone or 1 μM prednisolone. ^{3}H-E$_1$S radioactive conversion showed STS activity to be 1.78 fmol/million cells/h in positive control (growth media treated) cells after starvation (Fig. 17B). Basal medium treated control cells displayed activity levels of 1.24 fmol/million cells/h, while glucocorticoid treated cells declined in STS activity to 0.77 fmol/million cells/h for dexamethasone, 0.87 fmol/million cells/h for hydrocortisone and 0.86 fmol/million cells/h for prednisolone. Thus, glucocorticoids lower STS activity.

Whole cell conversion assay in proliferating MG-63 cells in response to glucocorticoid dexamethasone and antiglucocorticoid RU486

In order to determine the role of glucocorticoid receptor in MG-63 cell growth, MG-63 cells in basal medium plus the antiglucocorticoid RU486 increased STS activity, while those in dexamethasone decreased (Fig. 18A). Addition of RU486 with dexamethasone resulted in intermediate STS activity, between dexamethasone and RU486 alone.

Whole cell STS assay in proliferating and differentiated cells in response to glucocorticoid dexamethasone and antiglucocorticoid RU486

The previous experiment was repeated on MG-63 cells treated with or without

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osteogenic supplement for 12 days. Day 12 OS- cells showed lower STS activity levels

when treated with Dex (3.23 pmol/million cells/18h) and a statistically significant

increase in STS activity when cells were treated with Dex and RU486 (3.73 pmol/million

cells/18h) (Fig. 18B). Day 12 OS+ grown cells similarly showed lower STS activity

levels when treated with Dex (0.67 pmol/million cells/18h) which increased when Dex

treated cells were also provided 100 nM RU486 (1.06 pmol/million cells/18h).

Antiglucocorticoid RU486 alone increased STS activity in OS- and OS+ treated cells

when compared to basal control.

qRT-PCR of STS mRNA expression upon Dex and RU486 treatment

To assess the effect of glucocorticoids on STS mRNA expression in proliferating

MG-63 cells, two-step qRT-PCR of changes in STS gene expression with 1 uM

dexamethasone (Dex) or 100 nM RU486 or 1 uM Dex and 100 nM RU486 treated cells

were calculated as fold change relative to basal medium control cells using the $\Delta\Delta Ct$

method (Fig. 18C). A 1.13-fold increase of STS mRNA expression upon treatment with

RU486 was observed, while dexamethasone reduced STS mRNA expression to 0.5-fold

of basal control. Addition of RU486 to Dex treated cells increased STS mRNA

expression to 0.91-fold over basal control.

2.26 Discussion:

In humans, bone osteogenesis involves proliferation of osteoblasts, maturation of

cells to osteocytes and subsequent maintaining of bone architecture by osteoclast-

osteoblast remodeling. In our previous studies, we have shown steroid sulfatase to

mediate MG-63 cell proliferation by converting inactive sulfated steroids to active

estrogen (Dias and Selcer, 2014). In this project we have addressed presence and regulation of steroid sulfatase throughout differentiation of MG-63 cells. Through our experiments we have demonstrated for the first-time steroid sulfatase activity during osteoblast differentiation, and the effect of glucocorticoids on STS activity and expression.

To demonstrate the feasibility of the cell differentiation, we showed that we could induce differentiation by comparing proliferation of cells incubated in growth medium compared to cells treated with osteogenic supplement medium. The cells incubated in growth medium proliferated almost linearly, whereas the proliferation rate slowed in the cells treated with osteogenic supplement, suggesting that the cells were in the differentiation phase. We confirmed differentiation using alkaline phosphatase protein expression and osteocalcin mRNA expression, as well as cell morphology. Alkaline phosphatase and osteocalcin can be used to validate differentiation, as both are phenotypic markers of osteoblast differentiation (Beck et al., 2001). The activity of alkaline phosphatase (ALP) activity increased over the course of MG-63 differentiation process in those cells treated with osteogenic supplemental media until Day 14, following which ALP levels declined. This is consistent with other studies that have shown brief increase in ALP through Day 15, followed by decline to basal levels in osteogenic supplement treated MG-63 cells (Lian and Stein, 1992; Kumarasuriyar et al., 2009). Alkaline phosphate activity did increase over time in the cells incubated in growth media, albeit slowly. This is likely due to the fact that MG-63 cells slowly undergo differentiation even without the addition of osteogenic supplement. However, the differentiation process in cells not treated with the osteogenic supplement media occurred

at a much slower rate, consistent with previous studies (Kim et al., 2009). In the same way, mRNA expression of osteocalcin is indicative of MG-63 osteoblastic differentiation. Osteocalcin mRNA was found to be present throughout the entire differentiation process. Osteocalcin is solely secreted by osteoblasts and is expressed as the matrix begins the mineralization process (Lian and Stein, 1992). As shown in figure 13, cells cultured *in vitro* undergo proliferation, matrix maturation and matrix mineralization when grown for 30 days. Osteocalcin marker gene is found to increase in matrix mineralization phase of growth medium treated cells. Hence, expression levels increased over time. Osteogenic supplement treated cells undergo differentiation earlier than growth medium treated cells and thus, osteocalcin mRNA expression was found to be higher in the early stages of differentiation. It is unclear why osteocalcin mRNA expression declined over time in OS+ treated cells. Thus, as demonstrated by both alkaline phosphatase activity and osteocalcin mRNA expression, cells moved towards the osteoblast state more quickly upon treatment with osteogenic supplement media (Langenbach and Handschel, 2013). This was also supported by bone morphology, where the cells in osteogenic supplement showed a differentiated morphology compared with untreated cells.

Steroid sulfatase activity was found to steadily increase over time in OS- medium treated cells but was found to increase very slowly in OS+ treated cells. In previous studies, using the human fetal osteoblast cell line SV-HFO, steroid sulfatase activity and mRNA expression showed no significant changes over a 21-day differentiation period in intact cells or cell extracts upon treatment with osteogenic supplement (Janssen et al., 1999). Our results are similar to the findings of Janssen et al., who used a human fetal osteoblast cell line to semi-quantitate steroid sulfatase message levels. Nonetheless,

Janssen et al., did not compare steroid sulfatase activity or mRNA levels between cells undergoing differentiation and cells in active proliferation. Steroid sulfatase mRNA was found to be present throughout the differentiation process in the MG-63 cells. RT-PCR amplification showed a qualitative increase in steroid sulfatase mRNA expression in OS-treated cells over OS+ treated cells. Using qRT-PCR amplification, steroid sulfatase transcript levels were quantitatively found to be much higher in proliferating OS- treated cells over differentiated OS+ treated cells.

Steroid sulfatase activity in OS- and OS+ treated cells was eliminated in response to steroid sulfatase inhibitor EMATE (estrone- 3-O-sulfamate) for the four time-points tested (Day 2, Day 8, Day 14 and Day 20). EMATE has previously exhibited similar inhibitory effects on sulfatase activity in MG-63, HOS TE-85 and MC-3T3-E1 osteoblast cell lines (de Gooyer et al., 2001; Selcer and Difrancesca, 2012; Dias and Selcer, 2014) and in MG-63 and MC-3T3-E1 microsomes (Selcer and Difrancesca, 2012; Dias and Selcer, 2014). The data on EMATE inhibition of STS activity validates the radioactive conversion assays to be measuring levels of actual steroid sulfatase active enzyme.

No apparent decline in steroid sulfatase activity levels was detected in OS- and OS+ cells upon treatment with the estrogen blocker, ICI 182,780 throughout differentiation. ICI 182, 780 is known to block the ability of 17β-estradiol to bind to the estrogen receptor (Wakeling et al., 1991). The role of estrogen in differentiation of bone is not clear. One study has shown 17β-estradiol to enhance differentiation of osteoblasts in mouse bone marrow culture (Qu et al., 1998) in cells treated in OS- medium alone. From our results, it does not appear that 17β-estradiol is necessary for differentiation under our test conditions, indicating that estrogen-receptor-mediated processes may not

be required for human osteoblasts to undergo differentiation. Additionally, our results indicate that ICI 182, 780 does not affect steroid sulfatase activity during MG-63 differentiation. From our findings, it appears that estrogen acting through an estrogen receptor mechanism is not involved in steroid sulfatase regulation. Our results indicate that differentiation of MG-63 cells progress even when the estrogen receptor is blocked, as the ICI 182,780 data suggests. Thus, the effects of estrogen on bone differentiation, if any, are unclear.

Estrogen exerts its effects on target cells by specifically interacting with either of the two estrogen receptors, ER-α or ER-β. Both receptors have almost identical DNA-binding domains, and have similar affinities for estrogenic compounds (Kuiper et al., 1997). But estrogen receptors ER-α and ER-β have different amino acid sequences in their N- and C-terminal trans-activating regions (Paech et al., 1997). Hence, transcriptional activation of ER-α might differ from ER-β. During the 21-day culture period, the strength of expression of ER-α was observed to steadily increase in OS- and OS+ treated cells. However, ER-β mRNA expression was found to be present at low levels at every time point of OS- treated cells and levels declined over time course in OS+ treated cells. No previous studies have been conducted to show ER-α and ER-β expression throughout differentiation in OS- and OS+ treated cells. While our data are qualitative, they are consistent with a previous study on human primary osteoblast-like cells (hOB cells) obtained from the upper femur of female patients undergoing bipolar endoprosthesis arthroplasty osteoblasts (OB) from postmenopausal women (Chen et al., 2004). Their data reveal a higher expression of ER-α over a 25-day culture period and a very low level of ER-β expression. The differential strength of expression of ER-α and

ER-β in OS- and OS+ treated cells through the course of differentiation suggest expression of estrogen receptor isoform may be related to age and bone cell condition. Further investigation is needed to test if these differences in isoform expression of ERs play a major role in estrogen regulation of bone metabolism.

Classical *in vitro* differentiation is obtained through growth medium containing ascorbic acid, β-glycerophosphate and dexamethasone. To understand how each component of the osteogenic supplement medium effects cell growth, the medium components were tested in different supplement combinations. Cell proliferation was higher in the absence of dexamethasone. Morphology was more spherical with shorter cytoplasmic extensions in the absence of dexamethasone and more elongated and stellate in the presence of dexamethasone. This suggests that the glucocorticoid (GC) dexamethasone plays a crucial role in promoting differentiation in the osteogenic supplement medium. Investigators studying the effect of osteogenic supplement medium have emphasized dexamethasone as an inducer of differentiation through upregulation of Runx2 transcription factor (Langenbach and Handschel, 2013). With regard to the other components of osteogenic supplement, ascorbic acid influences secretion of collagen matrix protein and β-glycerophosphate serves as a source of phosphate for hydroxyapatite (Langenbach and Handschel, 2013).

STS activity was expression were decreased in differentiating cells in the presence of osteogenic supplement. Given that the glucocorticoid dexamethasone was the major stimulant of differentiation, we investigated effect of three glucocorticoids (dexamethasone, hydrocortisone and prednisolone) on STS activity and expression. For the first time, we have found that all three glucocorticoids down regulated STS activity

levels in comparison to basal control, suggesting that these glucocorticoids are inhibitors of STS activity levels.

To further investigate role of glucocorticoids on STS activity, we tested the effect of RU486, an antagonist of the glucocorticoid receptor. Our data revealed that RU486 significantly enhanced STS activity, which was higher than in basal control cells. This suggests that RU486 effect as a GR antagonist is blocking glucocorticoids from repressing STS expression. Dexamethasone alone significantly decreased STS activity, but the inhibitory effect of dexamethasone was reduced when RU486 was added.

Further analysis of dexamethasone-mediated STS inhibition after 12 days of growth in OS- medium showed similarly lowered STS activity levels with the addition of dexamethasone. This inhibitory effect was lessened when dexamethasone was supplemented with RU486. Day 12 OS+ cells upon dexamethasone treatment did not significantly inhibit STS activity further compared to control and can be attributed to the fact that OS+ media contains dexamethasone and additional dexamethasone had no further inhibitory effect. However, dexamethasone and RU486 combined treatment significantly increased sulfatase activity compared to dexamethasone treatment alone.

Quantitative real-time PCR showed an increase in STS mRNA expression of cells treated with dexamethasone and RU486 compared to dexamethasone treatment alone. This indicates that the dexamethasone effect on sulfatase expression is transcriptionally regulated. These results suggest that blocking dexamethasone may potentially enhance MG-63 cell proliferative capabilities during *in vitro* expansion. Effects of RU486 on proliferation and differentiation capabilities of human bone marrow mesenchymal stromal cells support these findings (Yu et al., 2012). Although the mechanisms

underlying the effects of RU486 on enhancing MG-63 proliferation need to be further investigated, RU486 upregulation of STS indicates that blocking endogenous glucocorticoids could function to maintain the growth of preosteoblastic cells and to provide them with a potential source of estrogen.

All of our data combined suggest that steroid sulfatase could be a key regulatory enzyme in promoting bone cell growth. Through our attempts of understanding STS expression during differentiation, we have shown that glucocorticoids inhibit sulfatase expression and activity levels. These data are suggestive of *in vivo* correlation situations wherein in post-menopausal women estrogen levels decline and endogenous glucocorticoid levels are known to be elevated. Also, the use of anti-inflammatory glucocorticoid drugs to relieve pain are higher in older women and the effects of these drugs are associated with bone loss. It has been shown that glucocorticoids decrease circulating osteoprotegerin (OPG), which suppresses bone formation and enhances bone resorption (Sasaki et al., 2001). The decline in osteoprotegerin leads to an increase in expression of receptor activator of nuclear factor-κB ligand (RANKL), which bind to RANK receptors to activate osteoclast bone resorption (Swanson et al., 2006). While it is known that glucocorticoids can induce osteoporosis (GIOP) in human bone, the mechanism of glucocorticoid action on bone turnover is complex and has not been elucidated completely (Mazzantini and Di Munno, 2014). Here, we have shown here for the first time that glucocorticoids suppress steroid sulfatase mRNA expression and protein activity. Inhibiting glucocorticoids in bone would therefore lead to an increase in STS expression in differentiated cells. Increase in STS expression could convert inactive

estrogen precursor conjugates to active estrogen, which that could in turn improve bone

remodeling.
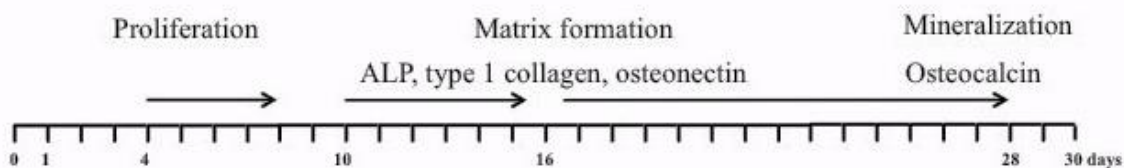
Fig. 13. Expression pattern of bone related proteins in mouse MC3T3-E1 bone cell line. *In vitro* mouse pre-osteoblastic MC3T3-E1 cells show typical phases of proliferation (days 4-10), bone matrix formation/maturation (days 10-16), and bone mineralization (days 16-28). Alkaline phosphatase, type I collagen, fibronectin, TGF- β1 and osteonectin proteins are expressed in bone matrix formation phase. Osteocalcin mRNA expression is higher in the bone mineralization phase (Choi et al., 1996). Our studies on MG-63 pre-osteoblastic cell line followed a similar time-line for 21 days to study expression of steroid sulfatase during the course of MG-63 cell differentiation.
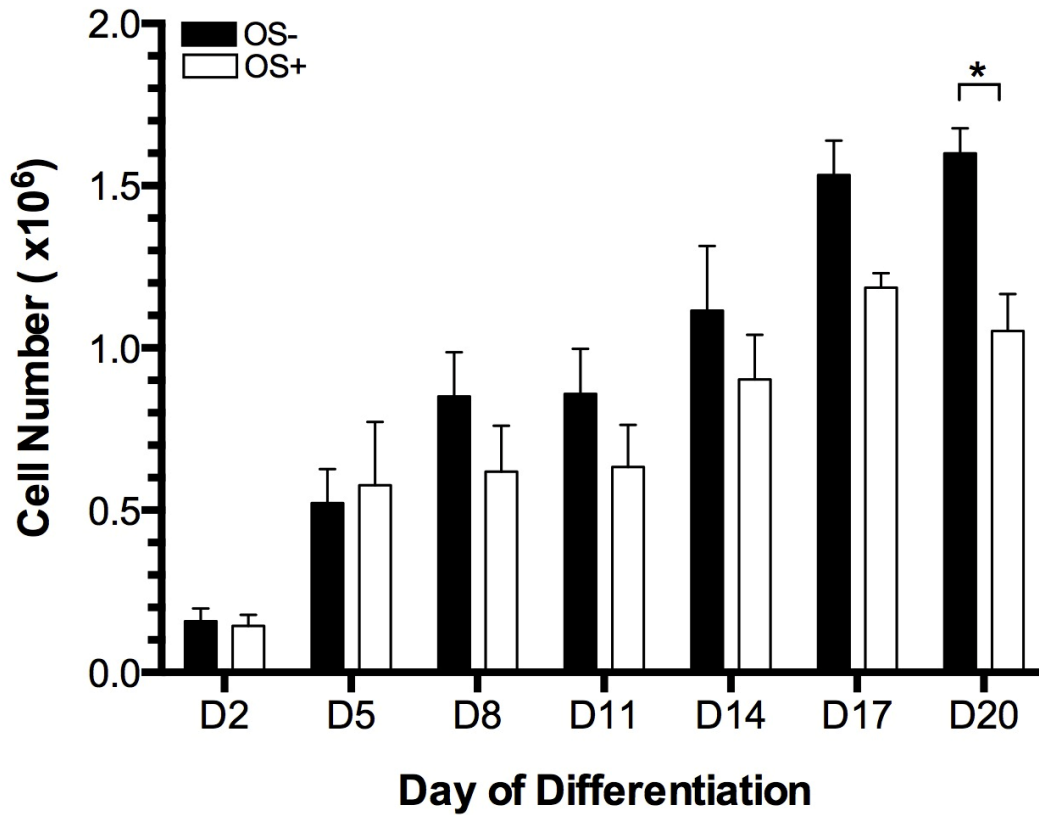
Fig. 14A. MG-63 cell proliferation response to osteogenic supplement. Growth of MG-63 cells in growth medium and growth medium containing osteogenic supplement determined by MTT assay. Data represent three independent experiments, run in duplicate. Two-way analysis of variance revealed significant differences between day ($F=23.95$; 6, 42 df; $P<0.0001$) and treatment ($F=10.69$; 1, 42 df; $P<0.005$) with interaction ($F=1.23$; 6, 42 df; $P=0.2788$) between day and treatment. Bars represent $\pm$ S.E.M. Asterisks indicate significant difference between means (Sidak's multiple comparison test: $*$ = $P<0.05$). Abbreviations are: OS- = growth medium alone; OS+ = growth medium with osteogenic supplement.
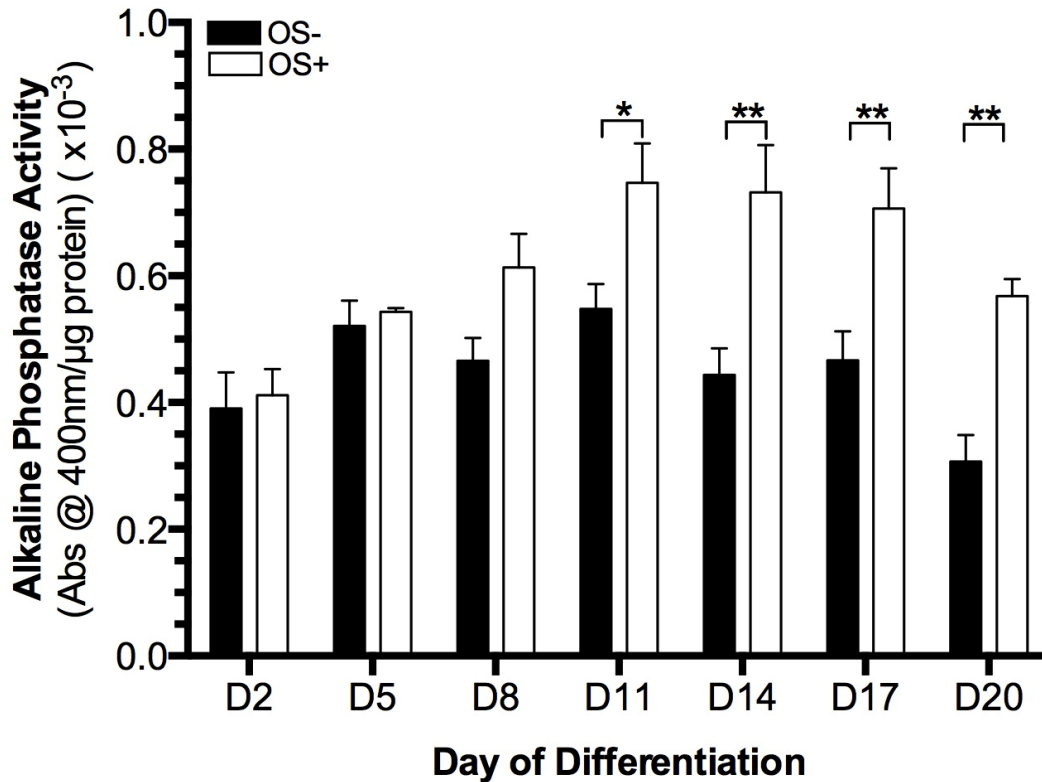
Fig. 14B. MG-63 alkaline phosphatase activity in response to osteogenic supplement.

Time course of alkaline phosphatase activity in cells treated with growth medium and growth medium containing osteogenic supplement as determined by p-nitrophenol phosphate hydrolysis. A BCA assay was performed to determine protein concentrations for each cell homogenate sample. Data represent three independent experiments. Two-way analysis of variance revealed significant differences between day (F=6.63; 6, 28 df; P=0.0002) and treatment (F=43.38; 1, 28 df; P<0.0001) with significant interaction (F=2.64; 6, 28 df; P=0.037) between day and treatment. Bars represent ± S.E.M. Asterisks indicate significant difference between means (Sidak's multiple comparison test: * = P<0.05, ** = P<0.01). Abbreviations are: OS- = growth medium alone; OS+ = growth medium with osteogenic supplement.
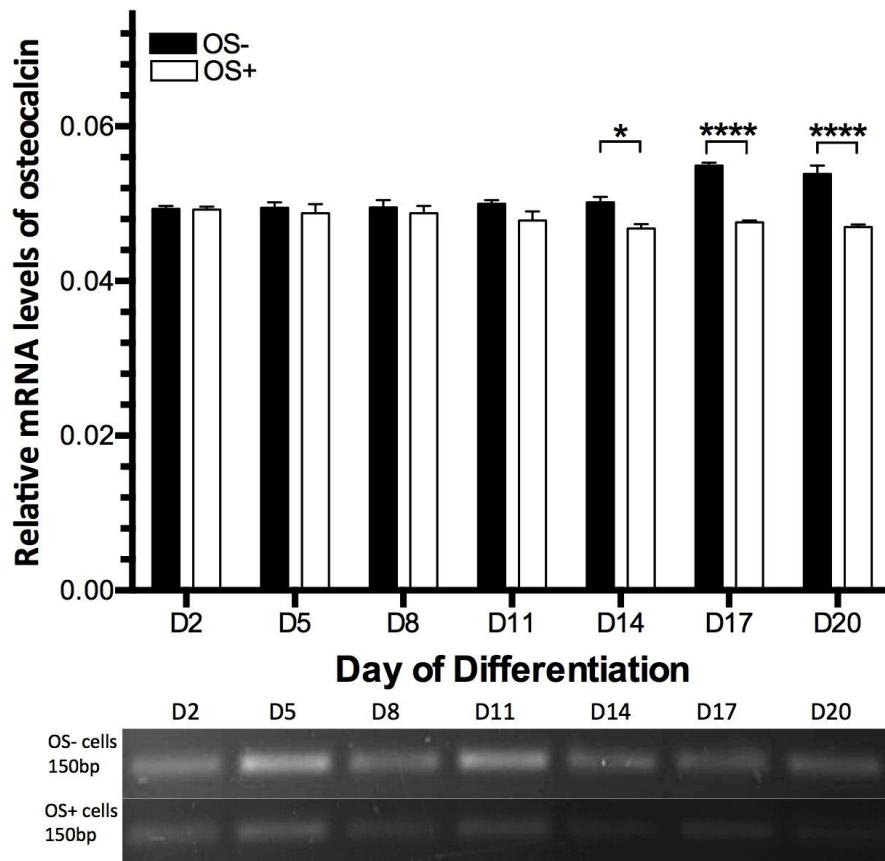
Fig. 14C. MG-63 cell osteocalcin expression in response to osteogenic supplement.

Time course osteocalcin mRNA levels in OS- and OS+ cells. Through real-time qRT-PCR, changes in osteocalcin mRNA expression were quantified by 1/ΔCt relative mRNA expression levels. Two-way analysis of variance revealed differences between day (F=3.28; 6, 28 df; P=0.014) and treatment (F=57.05; 1, 28 df; P<0.0001) with significant interaction (F=7.85; 6, 28 df; P<0.0001) between day and treatment. . Bars represent ± S.E.M from three separate experiments. Asterisks indicate significant difference between means (Sidak's multiple comparison test: * = P<0.05, **** = P<0.0001). Lower panel shows single bands of 150 bp RT-PCR amplified products on a 2% agarose gel with ethidium bromide staining.

Fig. 15A. Steroid sulfatase activity in MG-63 cells, as determined by ³H-estrone sulfate conversion assay. Data represent three independent experiments, run in duplicate. Bars represent ± S.E.M. Two-way analysis of variance revealed significant differences between day (F=178.2; 6, 70 df; P<0.0001) and treatment (F=913.8; 1, 70 df; P<0.0001) with significant interaction (F=68.06; 6, 70 df; P<0.0001) between day and treatment. Asterisks indicate significant difference between means (Sidak's multiple comparison test: ** = P<0.01, **** = P<0.0001).
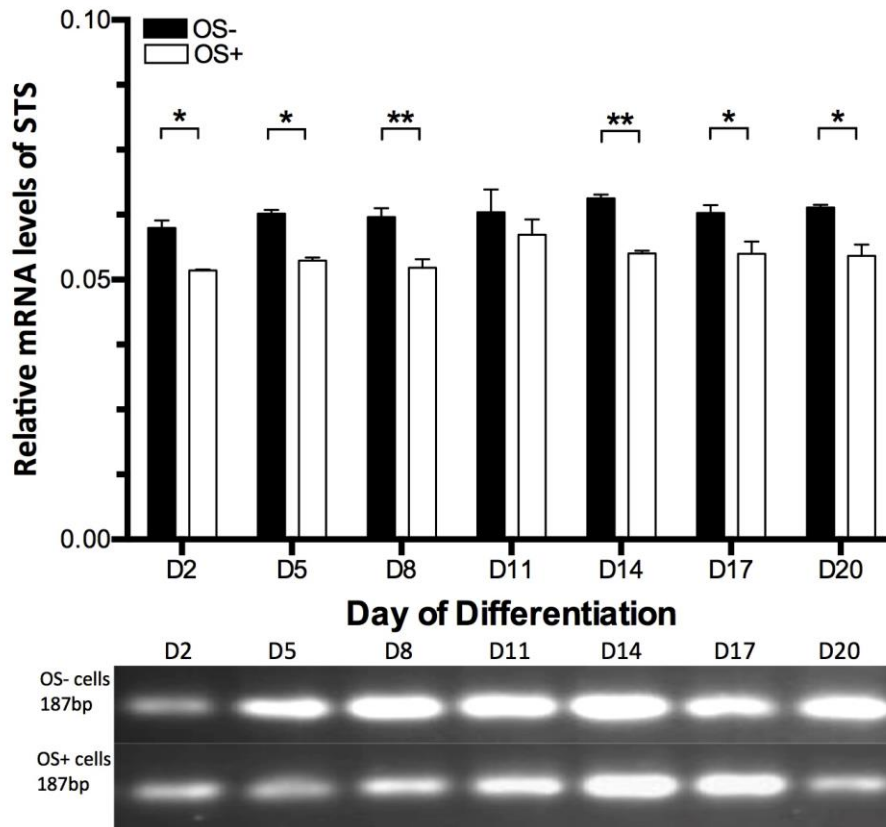
Fig. 15B. Steroid sulfatase expression in OS- and OS+ treated cells. By qRT-PCR method, steroid sulfatase mRNA expression levels were quantified in cells treated with growth medium (OS-) and growth medium containing osteogenic supplement (OS+). Two-way analysis of variance revealed differences between day (F=1.71; 6, 28 df; P=0.115) and treatment (F=69.87; 1, 28 df; P<0.0001) with interaction (F=0.58; 6, 28 df; P=0.074) bet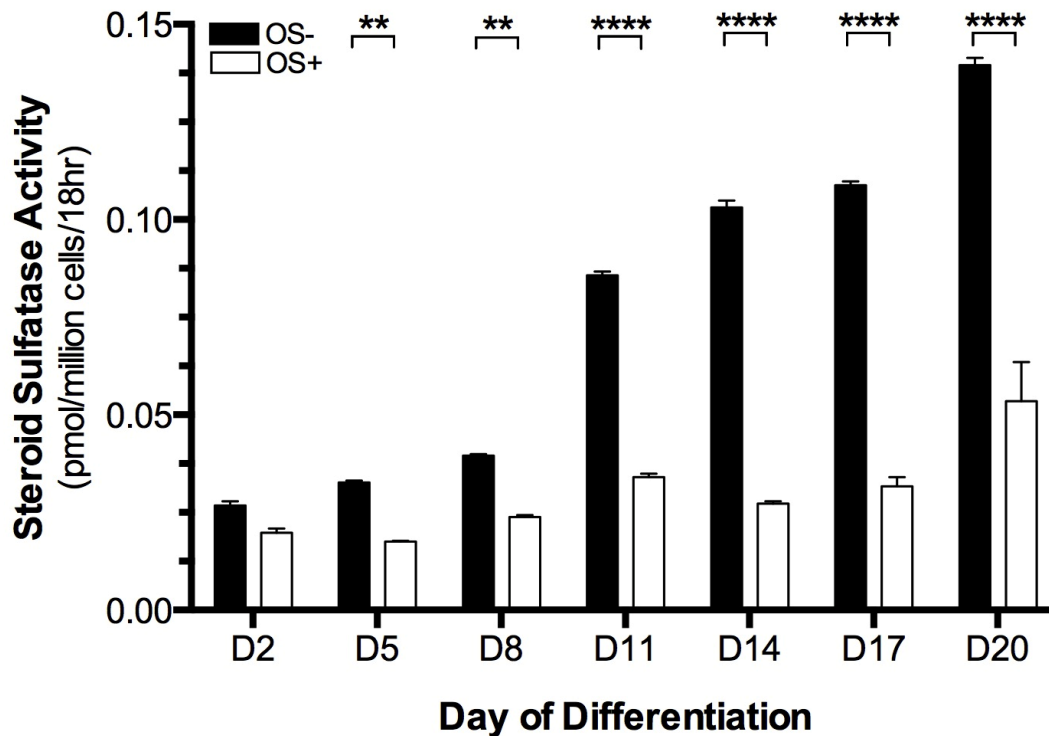ween day and treatment. Bars represent ± S.E.M from three separate experiments. Asterisks indicate significant difference between means (Sidak's multiple comparison test: * = P<0.05, ** = P<0.01). Lower panel shows RT-PCR products on a 2% agarose gel with ethidium bromide staining. A single band of 187bp amplified product was found for each time point with a higher expression of the gene seen in OS- treated cells.
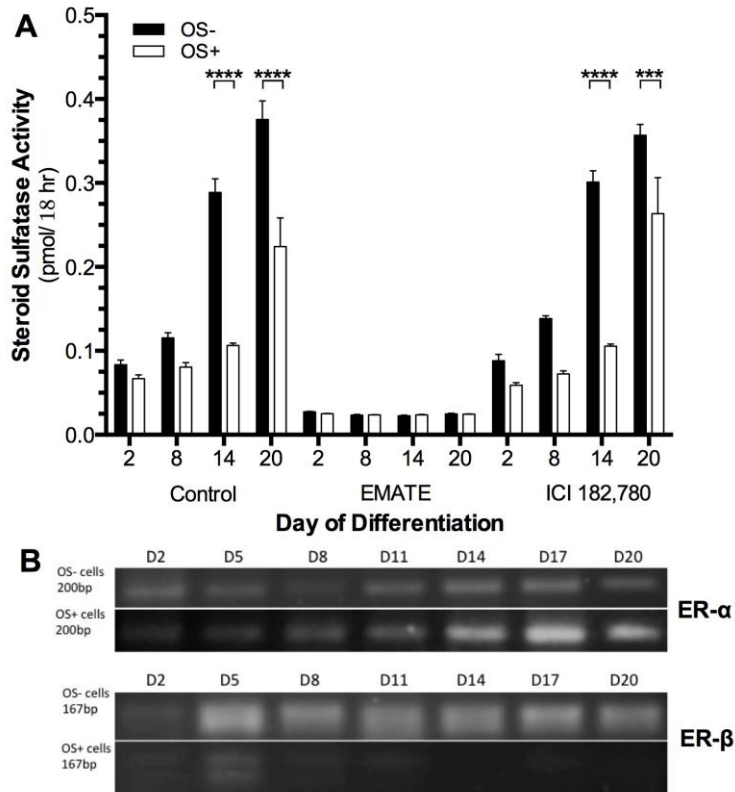
Fig. 16. Steroid sulfatase activity in the presence and absence of inhibitors and Estrogen receptor expression in OS- and OS+ treated cells. (A) STS activity in response to steroid sulfatase inhibitor EMATE (estrone 3-o-sulfamate, 1μM) and estrogen receptor inhibitor ICI 182,780 (1μM). Two-way analysis of variance revealed significant differences between day (F=161.4; 3, 72 df; P<0.0001) and treatment (F=166.8; 5, 72 df; P<0.0001) with interaction (F=22.96; 15, 72 df; P<0.0001) between day and treatment. Bars represent ± S.E.M. Asterisks indicate significant difference between means (Sidak's multiple comparison test: **** = P<0.0001, *** = P<0.0005). **(B)** RT-PCR was performed using primers based on the known human ER-α and ER-β sequences. Single bands of similar intensity for ER-α (200bp, consistent with the expected product) was found for each OS- and OS+ time point. ER-β RT-PCR products of 167 bp were observed in OS- treated cells but expression was reduced in OS+ time points.
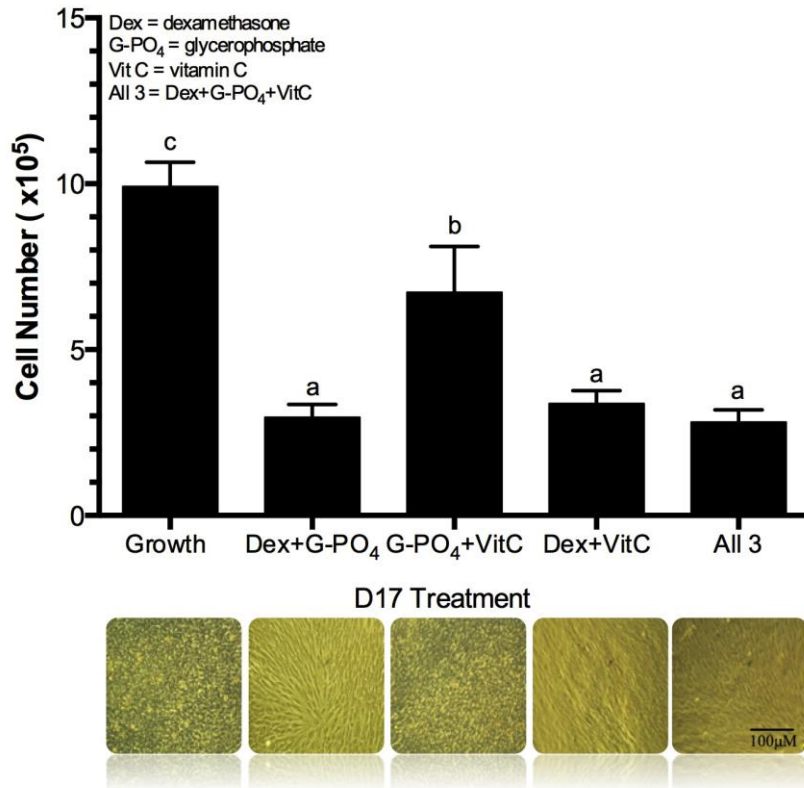
Fig. 17A. Effect of osteogenic supplement components on MG-63 cells. Growth of MG-63 cells in growth medium alone and growth medium with different combinations of osteogenic supplement (OS+) components, as determined by MTT assay. Bars represent mean cell number $\pm$ 1 SEM from three independent experiments, run in triplicate (one-way analysis of variance; F = 16.74; 4, 15 df; P < 0.0001). Different letters represent differences among means (Newman–Keuls post hoc test, P < 0.05). Lower panel displays morphological differences in cell growth in response to constituents of osteogenic supplement.
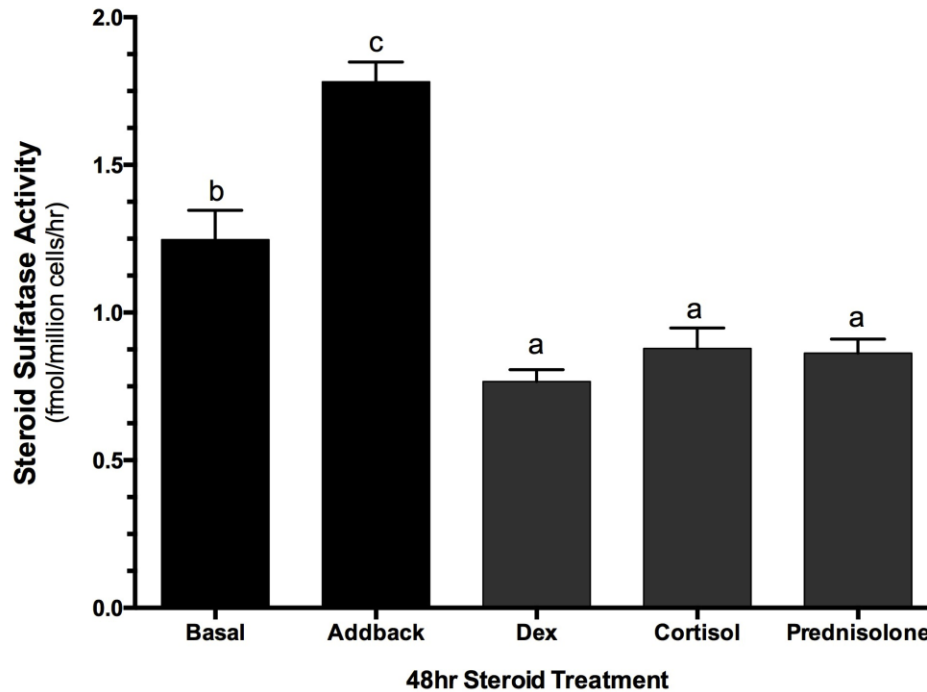
Fig. 17B. Effect of glucocorticoids on MG-63 cells. Steroid sulfatase (STS) activity in

MG-63 cells, as determined by ^{3}H-estrone sulfate conversion assay. Cells were incubated

for 48h in the presence or absence of 1μM glucocorticoids dexamethasone,

hydrocortisone or prednisolone. Whole cell conversion assays were performed as

indicated in Section 2. Bars represent the mean ± 1 SEM. STS activity levels varied

significantly between control and treatment groups (one-way analysis of variance, F =

38.13; 4, 15 df; P < 0.0001). Different letters represent differences among means

(Newman–Keuls post hoc test, P < 0.05).
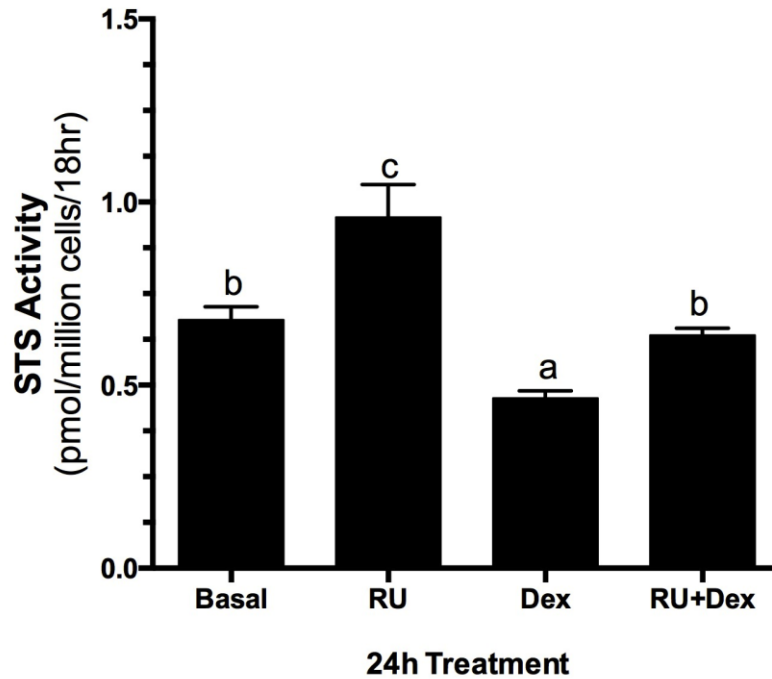
Fig. 18A. Dexamethasone regulation of STS activity. Steroid sulfatase (STS) activity in MG-63 cells, as determined by ^{3}H-estrone sulfate conversion assay. Cells were incubated for 24h in the presence or absence of 1μM Dex or 100nM RU486, or both. Whole cell steroid conversion assay showed significant variation among treatment groups (one-way analysis of variance, F= 17.11; 3, 212 df; P < 0.0001). Different letters represent differences among means (Newman–Keuls post hoc test, P < 0.05).
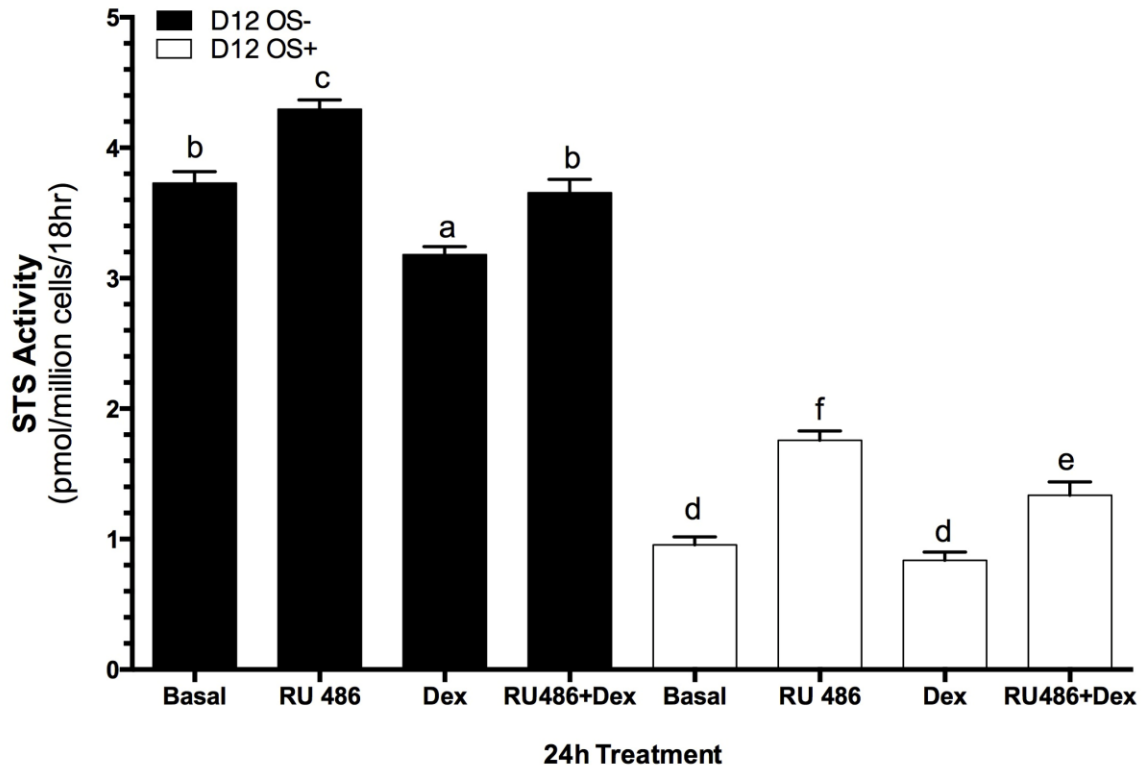
Fig. 18B. Dexamethasone regulation of STS activity in OS- and OS+ treated cells.

Steroid sulfatase (STS) activity in MG-63 cells, as determined by ^{3}H-estrone sulfate conversion assay in response to dexamethasone inhibitor RU-486 on Day 12 MG-63 cells grown in growth medium (OS-) or growth medium containing osteogenic supplement (OS+). D12 OS- and OS+ treated cells were incubated for 24h in the presence or absence of 1μM Dex or 100nM RU486 or both. Whole cell steroid conversion assay showed significant variation among treatment groups (one-way analysis of variance, F= 305.7; 7, 152 df; P < 0.0001). Different letters represent differences among means (Newman–Keuls post hoc test, P < 0.05).
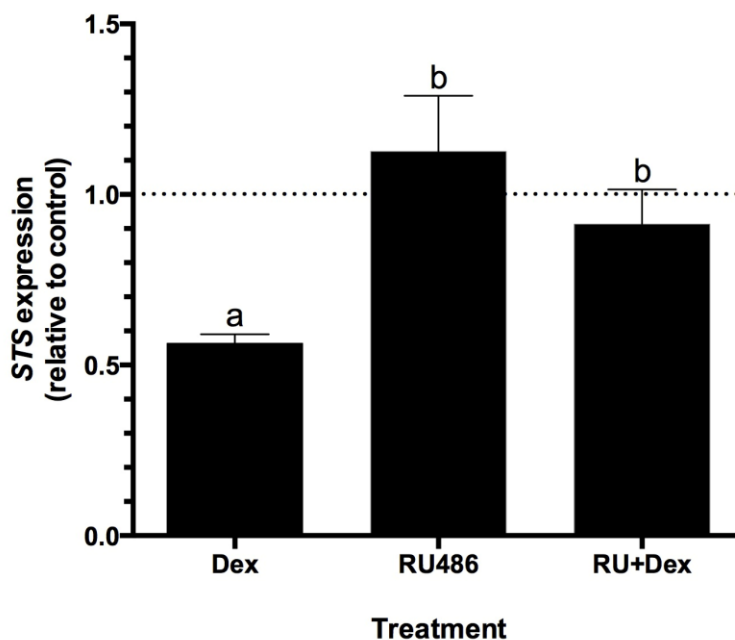
Fig. 18C. Dexamethasone regulation of STS mRNA expression. qRT-PCR comparing steroid sulfatase mRNA expression levels in MG-63 cells in response to dexamethasone, RU-486 or both. Cells treated with 1μM Dex or 100nM RU486 or both. Using the ΔΔCt method, changes in steroid sulfatase mRNA expression were quantified using real-time PCR. Bars represent STS expression relative to basal control (----- dotted line) ± 1 SEM. STS expression levels over basal control varied between control and treatment groups (one-way analysis of variance, $F = 6.41$; 2, 21; $P = 0.0067$). Different letters represent differences among means (Newman–Keuls post hoc test, $P < 0.05$).

2.3 <u>Project III. Distribution of Steroid Sulfatase mRNA in Various C57BL/6J Mouse Tissues</u>

2.31 <u>Hypothesis and Objectives</u>

Model:

- Wild type female and male mouse tissues obtained from C57BL/6J strain, juvenile and adult

Rationale:

Our ultimate goal is to understand how steroid sulfatase functions in a physiological system, mouse bone. Mouse physiology can be useful for understanding human physiology and mouse models are widely available for genetic and physiological manipulation. Steroid sulfatase expression in adult mouse bone and marrow has not been described before. Before mice can be used as a physiological model for steroid sulfatase regulation, we must first determine the presence of steroid sulfatase in male and female tissues especially in bone and marrow.

Hypothesis:

Steroid sulfatase is a fairly ubiquitous enzyme, therefore its mRNA expression will be present in varying amounts in all of the mouse tissues to be evaluated.

Objectives:

1. To examine presence of steroid sulfatase mRNA in various tissues of juvenile wild type C57BL/6J male and female mice

   Experiments to address Objective 1:

   a. RT-PCR with primers specific for the mouse steroid sulfatase encoding gene

2. To examine presence of steroid sulfatase, estrogen receptor variants ER-α and ER-β mRNA expression in bone and marrow of adult wild type C57BL/6J mice using liver expression of steroid sulfatase and estrogen receptor variants as control.

   a. RT-PCR with primers specific for the mouse steroid sulfatase, ER-α and ER-β encoding genes

2.32 <u>Abstract</u>

There is little information on the role of steroid sulfatase in various tissues under physiological conditions. The mouse is an ideal organism for such studies, due to the large amount of information on endocrinology of this species, and due to the availability of many strains and transgenic constructs. Unfortunately, little is even known about steroid sulfatase tissue distribution in the mouse. Thus, we sought to determine the expression of steroid sulfatase mRNA in a variety of mouse tissues that have been shown in other species to possess this enzyme (liver, lung, kidney, muscle, ovary and uterus). Information of presence of ER-α and ER-β were also collected for some tissues. Steroid sulfatase mRNA expression was found to be present in the juvenile male and female C57BL/6J mouse heart, liver, small intestine, skeletal muscle, ovary, testes, bone and bone marrow. In general, our data are consistent with reports from other species that steroid sulfatase is widely distributed and highly variable among tissues. Our results indicate that aged mice express lower levels of steroid sulfatase in bone and bone marrow. However, the juvenile mice tissues (heart, liver, small intestine, skeletal muscle, ovary, testes, bone and marrow) all have the ability to convert sulfated steroids, such as

estrone sulfate, into active17β-estradiol. ER-α and ER-β were present in both bone and bone marrow, indicating that any estrogen formed could result in estrogen mediated biological responses.

2.33 <u>Introduction</u>

The enzyme steroid sulfatase (EC 3.1.6.2) removes the sulfate group from 3β-hydroxysteroid sulfates, including the two abundant circulating conjugated steroids, dehydroepiandrosterone sulfate and estrone sulfate (Pasqualini et al., 1996; Pasqualini and Chetrite, 2005; Reed et al., 2005). Steroid sulfatase is best known for its role in estrogen production via the fetal adrenal-placental pathway during human pregnancy (Pasqualini and Chetrite, 2005); however, it also has important functions in other physiological and pathological steroid pathways (Pasqualini and Chetrite, 2005; Reed et al., 2005). Deficiency of steroid sulfatase in humans results in a condition known as X-linked ichthyosis, a genetic disorder caused by a deletion in the steroid sulfatase gene that results in scaly skin (DiGiovanna and Robinson-Bostom, 2003). Steroid sulfatase has also been implicated in stimulation of hormone-dependent cancers, particularly breast cancer, by converting inactive conjugated steroids to their active forms (Pasqualini and Chetrite, 2005; Reed et al., 2005). Steroid sulfatase is also believed to be important in the local production of steroids in bone (Reed et al., 2005).

Steroid sulfatase is widely distributed among human tissues. Steroid sulfatase expression has been shown during embryogenesis in placenta, skin and cartilage (Compagnone et al., 1997). Steroid sulfatase presence has been well determined in both placenta and breast, and this enzyme has also been identified in many other human

tissues, including skin, liver, lung, ovary, and adrenal gland (Pasqualini and Chetrite, 2005; Reed et al., 2005; Selcer et al., 2007). Levels of steroid sulfatase have been shown to vary within certain tissues under different physiological conditions, possibly reflecting this enzyme's physiological functions (Salido et al., 1990; Dibbelt et al., 1994). While steroid sulfatase has been reasonably well studied in humans, there is little information about the distribution and abundance of this enzyme in rodents. Steroid sulfatase purified from mouse liver has been partially characterized (Mortaud et al., 1995) and the gene encoding murine steroid sulfatase has also been identified (Salido et al., 1996; Van Der Eerden et al., 2002; van der Eerden et al., 2004)

Van der Eerden et al., demonstrated that steroid sulfatase mRNA was present during bone maturation in male and female rats; thereby, suggesting that this enzyme may have a role in development and maintenance of bone (van der Eerden et al., 2004). As mentioned before, within the peripheral tissues steroid sulfatase leads to the local production of estrogen (Nakamura et al., 2003). Estrogen responses could vary depending on the presence of the receptor isoforms ER-α or ER-β. Estrogen receptor beta variant protein expression has been shown to be present in human and murine bone (Vidal et al., 1999). In neonatal rib bones ER-α and ER-β protein expression was found to be differentially expressed with cancellous bone showing expression of ER-β and mineralized bone showing expression of ER-α (Bord et al., 2001). However, mRNA expression of ER-α or ER-β receptors in adult human or mice bone and bone marrow tissues is limited.

Given the known and potential physiological and pathological roles of steroid sulfatase and estrogen receptors, it is important to have more information about the

regulation of these genes in rodents, because rats and mice serve as the major physiological models for study of endocrine pathways. Therefore, the objective of this study was to investigate the distribution of mRNA for steroid sulfatase and estrogen receptor variants in various mouse bone and marrow tissues using the RT-PCR technique.

2.34 <u>Materials and Methods</u>

Animals

The model used for studying the presence of steroid sulfatase in different tissues were wild type young male and female 6-week-old C57BL/6J mice. For expression of ER-α, ER-β and steroid sulfatase in adult mice, wild type 9-month-old female C57BL/6J mice were used. Mice were euthanized (1μl/4g Euthasol® intraperitoneal injections), after which respective tissues (heart, liver, small intestine, skeletal muscle, ovary, testes, long bones) were collected and immediately frozen in liquid nitrogen.

Bone and Marrow Sample Preparation

The skeletal muscles surrounding the long bones were removed completely. The ends of each femur bone were clipped to expose the marrow. The bones were inserted into adapted centrifuge tubes and centrifuged for 2 minutes at 13,200 rpm to collect the marrow pelleted out (Peister et al., 2004). The bones were then immediately rinsed in sterile, RNase-free phosphate-buffered saline (PBS; pH 7.4) to remove traces of blood (Reno et al., 1997). Using a mortar and pestle the bone samples were pulverized in liquid nitrogen to form a powder of the tissue to be used for RNA isolation.

RNA Isolation

The tissues used for RNA isolation weighed between $30 - 35$ mgs for each sample. Total RNA from the tissues were isolated using TRIzol® Reagent (Invitrogen Life Technologies) according to manufacturer's instructions. 500 µl of TRIzol® reagent was added to each sample in 12 x 75 borosilicate glass tubes. The samples were then placed in an ice bucket and homogenized using three 20 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). The suspended tissues were transferred to microcentrifuge tubes and incubated at room temperature for 5 min. 160 µl of chloroform was added for phase separation of nuclear and protein fractions. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was transferred, to which, 500 µl of isopropyl alcohol and 1 µl of glycogen was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The resulting RNA pellet was washed with 500 µl of 75% ethanol, centrifuged at 7000 g for 10 min at RT, air-dried, and resuspended in 30 µl of diethylpyrocarbonate-treated water. Following 10 min incubation at 60 °C, RNA was quantified using a spectrophotometer (Thermo Fisher, Genesys 8, Pittsburgh, PA, USA). The amount of RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at $- 80$ °C until use.

Reverse transcription polymerase chain reactions (RT-PCR)

Reverse transcription

Using the GoScript® Reverse Transcription System (Promega), according to manufacturer's instructions, 1 g of template RNA, 1 µl of Oligo (dT) primer and diethylpyrocarbonate-treated water were added to make a total volume of 9 µl, heated to

70 °C for 5 min, and then chilled on ice for 5 min. In a separate tube, reverse

transcription mix was prepared from 4 µl of 5X reaction buffer, 4 µl MgCl$_2$, 1 µl dNTPs

and 0.5 µl of reverse transcriptase.12 µl of reaction mix was added to RNA/primer mix.

First-strand cDNA synthesis was carried out in a thermocycler (PTC-100, MJ Research,

Inc., Watertown, MA) with 5 min annealing at 25 °C. Next, the cDNA was allowed to

extend for 1 h at 42 °C. The reverse transcriptase enzyme was thermally inactivated at 70

°C for 15 min after which the reaction tubes were held at 4 °C.

PCR primers

Several steroid sulfatase primers were designed to determine the presence of

steroid sulfatase in mice. For the comparative tissue analysis primers were designed from

Mus musculus steroid sulfatase mRNA (GenBank Accession No. NM_009293.1). The

forward primer start position is 580 and has the sequence (5'– 3') GTG TAC CTG TTC

ACG GCG TC (FB). The reverse primer start position is 690 and has the sequence (5'–

3') CAG GTG CCA CTT CCC AAT GA (RB). The expected RT-PCR product from this

primer pair is 111 base pairs. Another steroid sulfatase primer to determine steroid

sulfatase expression in adult C57BL/6J mice bone and bone marrow was designed from

the complete coding sequence for *Mus musculus* steroid sulfatase (GenBank accession

no. U37545). The forward primer start position is 1592 and has the sequence (5'– 3')

AGC ACG AGT TCC TGT TCC AC (FB). The reverse primer start position is 1693 and

has the sequence (5'– 3') CGA AGT TGG GCG TGA AGT AGA (RB). The expected

RT-PCR product from this primer pair is 102 base pairs. To determine the mRNA

expression of estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β), two new

sets of primers were designed from the complete coding sequence for each gene. For

mice ER-α (GenBank Accession No. AB560752.1), the forward primer start position is at 656 and has the sequence (5'–3') CCT CCC GCC TTC TAC AGG T (FB). The reverse primer start position is at 783 with the sequence (5'–3') CAC ACG GCA CAG TAG CGA G (RB), with an expected product of 128 base pairs. For mice ER-β (GenBank Accession No. U81451.1), the forward primer start position is at 62 with the sequence (5'–3') CGG TAA CCT GGA AGG TGG GCC T (FB). The reverse primer start position is at 201 and has the sequence (5'–3') CAC ACC AAG GAC TCT TTT GAG GTT C (RB) for an expected product size of 140 base pairs. Primers were synthesized by Integrated DNA Technologies, Inc., (Coralville, IA).

PCR reagents and conditions

PCR reactions were performed on the cDNA templates using a Taq PCR Kit (New England Biolabs, Ipswich MA) according to manufacturer's instructions. 2 μl template cDNA, 2.6 μl 10X PCR buffer, 0.4 μl dNTP mix, 1 μl of 10 μM sense primer, 1 μl of 10 μM antisense primer, 0.2 μl Taq Polymerase, and DEPC-treated water were added to make total volume 20 μl. PCR reactions were then placed in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA). The reaction had a 4 min hold at 94 °C. For steroid sulfatase determination in comparative tissues of juvenile male and female mice, the cDNA product was amplified for 35 cycles of: 45sec 94 °C, 45 s 53 °C, 90 s 72 °C. For ER-α, ER-β and steroid sulfatase amplification, the cDNA products were amplified at 35 cycles of: 5 s 94 °C, 45 s 54 °C, 90 s 72 °C. The PCR reaction was terminated with a final extension of 5 min at 72 °C. The PCR products were then separated in a 2% agarose gel containing 5 μl of 1 mg/ml ethidium bromide solution. The gel was run in 1X TAE running buffer (40 mM Tris–acetate, 2 mM Na_2-EDTA-2H_2O)

for 2 h at 70 V. 10 μl of PCR product were mixed with 2 μl 6X cyanol loading dye (0.25% w/v xylene cyanol, 15% Ficoll in water). Quick load 2- log DNA ladder (New England Biolabs, Ipswich MA) was used as the standard.

2.35 <u>Results</u>

Steroid sulfatase mRNA is present in young male and female mouse tissues

Reverse transcriptase polymerase chain reaction, using primers based on the mouse steroid sulfatase encoding gene, resulted in a single product for all tissues in juvenile male and female mice, when resolved using agarose gel electrophoresis (Fig 19). The size of this band was identical to the expected size of the product (111 bp) for the primer pair used. Thus, in all of the tissues represented, steroid sulfatase mRNA was present.

ER-α, ER-β and steroid sulfatase mRNA expression in adult wild type C57BL/6J female liver, mice bone and bone marrow

Reverse transcriptase polymerase chain reaction, using primers for the mouse ER-α and ER-β encoding genes in the wild type C57BL/6J bone and marrow tissues resulted in single products for bone and marrow, when resolved using agarose gel electrophoresis (Fig 20). The size of this band was identical to the expected size of the products of 128 base pairs for ER-α and 140 base pairs for ER-β for the primer pairs used. The 100 base pair product of steroid sulfatase mRNA was not expressed in adult bone and marrow tissues of wild type C57BL/6J mice. C57BL/6J female liver sample showed expression of ER-α, ER-β and steroid sulfatase genes.

2.36 <u>Discussion</u>

In this study we demonstrated the presence of steroid sulfatase mRNA expression in a variety of mouse tissues at two different ages. Tissues were chosen based on STS levels in human (Selcer et al., 2007). We used reverse transcriptase polymerase chain reaction (RT-PCR) to assess whether or not steroid sulfatase mRNA was present in the various mouse tissues, using primers we designed based on the known mouse steroid sulfatase mRNA (GenBank accession no. NM_009293.1). On an agarose gel, a single prominent cDNA band resulted from the RT-PCR in the tissues of heart, liver, small intestine, skeletal muscle, bone, marrow, gonads in both male and female mice, and this band had the expected size for the primer pair used (111 bp). Steroid sulfatase was present at differing levels in all tissues, but no attempt was made to quantify these differences, as this type of RT-PCR is mostly qualitative. Our results indicate that these mouse tissues possess steroid sulfatase mRNA and therefore likely protein. Thus, these tissues may have the ability to convert sulfated steroids, such as estrone sulfate, into biologically potent steroids, such as 17β-estradiol. Such conversions may be important in the in situ synthesis of active steroids in peripheral tissues.

Compared to other tissues in the body steroid sulfatase activity has been shown to be present in higher levels in human liver (Munroe and Chang, 1987; Daniel and Chang, 1990; Shankaran et al., 1991) as well as in rat and mouse liver (Iwamori, 2005). Previous studies in our lab on human tissues have found high levels of hepatic steroid sulfatase using immunohistochemistry (Selcer et al., 2007). The immunohistochemical data on steroid sulfatase of human tissues (Selcer et al., 2007) also found intermediate levels of steroid sulfatase in kidney, uterus, skeletal muscle, and lower, but clearly detectable,

levels in ovary. Steroid sulfatase activity has been reported previously in rat ovary (Clemens et al., 2000).

Adult female C57BL/6J mice did not express steroid sulfatase mRNA in bone and bone marrow tissues (GenBank accession no. U37545). However, liver showed expression of steroid sulfatase. ER-α and ER-β variant mRNA expressions in bone and marrow of female adult wild type C57BL/6J mice were seen indicating adult mice to express both ER-α and ER-β receptors in compact mineralized bone. In a previous study on human neo-natal rib bones, protein expression of ER-α was found to be higher than ER-β in mineralized bone (Bord et al., 2001). Quantitative real-time PCR would need to be carried out to determine if there are differences in level of gene expression in the adult bone.

In conclusion, steroid sulfatase mRNA expression is present in higher levels in juvenile male and female tissues. In adult female mice the presence of steroid sulfatase declines significantly in bone and bone marrow tissues.
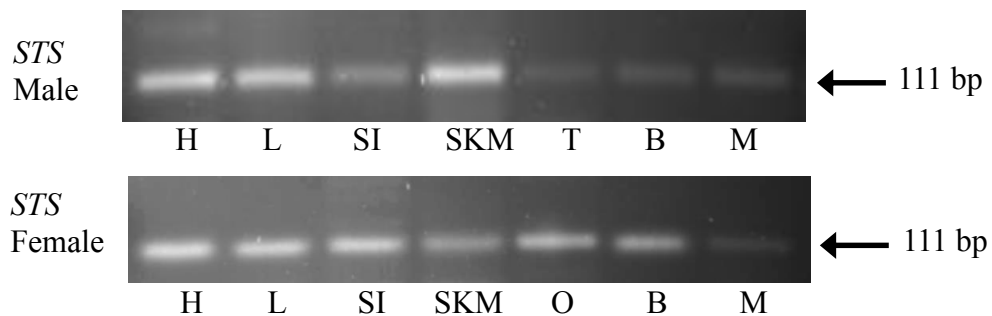
Fig. 19. Reverse transcriptase polymerase chain reaction of juvenile C57BL/6J male and female mouse tissues using mouse-specific steroid sulfatase primers. cDNAs were separated on a 2% agarose gel and stained with ethidium bromide. Primer sequences and RT-PCR methods were performed as indicated in Materials and Methods. Lane assignments: H = Heart, L = Liver, SI = Small Intestine, SKM = Skeletal Muscle, T = Testes, O = Ovary, B = Bone, M = Marrow

Female
C57BL/6J

[-----*STS* 102bp -----] [-------*ER-α* 128bp----] [------*ER-β* 140bp-----]
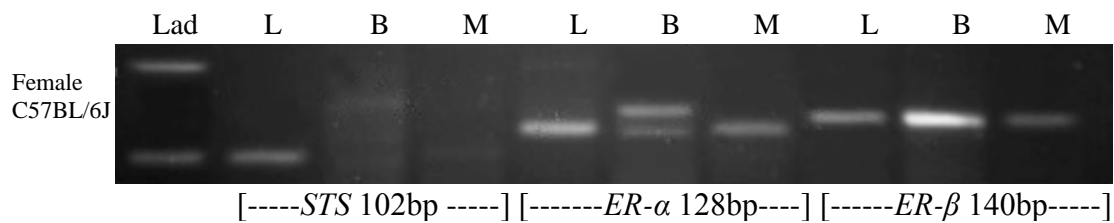
Fig. 20. Reverse transcriptase polymerase chain reaction of adult female wild type C57BL/6J mice liver, bone and marrow using mouse-specific steroid sulfatase, ER-α and ER-β primers. cDNAs were separated on a 2% agarose gel and stained with ethidium bromide. Primer sequences and RT-PCR methods were performed as indicated in Materials and Methods. C57BL/6J female mice liver was used as reference control. Abbreviations: STS = Steroid Sulfatase, ER-β = Estrogen Receptor-β, ER-α = Estrogen Receptor-α. Lane assignments: Lad = Ladder, L = Liver; B = Bone, M= Marrow

2.4 <u>Project IV. Role of the NF-κB Pathway in Regulation of Steroid Sulfatase Gene</u>

<u>Expression in the Human MG-63 Pre-Osteoblast Cell Line</u>

2.41 <u>Hypothesis and Objectives</u>

Model: MG-63 human pre-osteosarcoma fibroblast cell line derived from an osteosarcoma of a 14-year-old male.

Rationale:

Steroid sulfatase appears to be an essential enzyme converting inactive steroids to active estrogen and helping to maintain bone integrity in the adult skeleton. Therefore, it would be useful an understanding of how steroid sulfatase expression is transcriptionally regulated. To date, little information exists on the transcriptional regulation of steroid sulfatase, especially in bone cells. Recent reports in other cell types have shown that steroid sulfatase might be regulated by an inflammatory pathway involving NF-κB. Thus, we sought to determine if steroid sulfatase gene expression and activity in MG-63 cells was influenced by the NF-κB signaling pathway.

Hypothesis:

In the presence of NF-κB upregulating compounds LPS and PMA, MG-63 cell proliferation will increase and steroid sulfatase activity and gene expression will be upregulated. Alternatively, in the presence of BAY, an NF-κB inhibitor, cell proliferation and steroid sulfatase expression levels will decline.

Objectives:

1. To study the effects of LPS, PMA, and BAY on MG-63 cell proliferation.
   Experiments to address Objective 1:

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a. MTT colorimetric assay to measure growth response of MG-63 cells to LPS, PMA and BAY

2. To demonstrate steroid sulfatase activity in MG-63 cell homogenates in response to LPS, PMA and BAY

   Experiments to address Objective 2:

   a. Steroid sulfatase conversion assay using MG-63 cell homogenates in the presence and absence of a known NF-κB inhibitor, BAY

3. To compare steroid sulfatase mRNA expression levels in response to LPS, PMA and BAY in MG-63 cells

   Experiments to address Objective 3:

   a. RT-PCR using primers specific for the human steroid sulfatase encoding gene

   b. qRT-PCR to compare levels of sulfatase expression using primers specific for the human steroid sulfatase encoding gene

4. To compare steroid sulfatase immunoreactivity in response to LPS, PMA and BAY treatments in MG-63 cells

   a. Immunocytochemistry on fixed MG-63 using polyclonal steroid sulfatase antibody STS-275.

2.42 <u>Abstract</u>

Steroid sulfatase is responsible for the conversion of sulfated steroids to active forms. It has been suggested that this enzyme could be an important source of estrogen for maintaining bone formation in post-menopausal women. Little is known about the regulation of steroid sulfatase gene expression. Recently the NF-κB pathway has been

implicated in STS upregulation in some tissues. We sought to examine steroid sulfatase regulation, and cell growth, in the presence of compounds known to activate or inhibit the NF-κB pathway (Phorbol 12-myristate 13-acetate (PMA), bacterial lipopolysaccharides (LPS) and BAY((E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile), in human preosteoblastic MG-63 cells. Treatment with NF-κB activators LPS and PMA stimulated cell proliferation and increased steroid sulfatase activity levels. The NFκB inhibitor BAY blocked the LPS- and PMA-stimulated increases in steroid sulfatase expression and cell proliferation in MG-63 cells. These data suggest that LPS and PMA induce steroid sulfatase expression via an NF-κB signaling pathway in MG-63 cells. The activation of NF-κB pathway also results in increased cell proliferation in MG-63 cells, as it does in many other cell types.

2.43 Introduction

In an adult skeleton the bone connective tissue consists of an extracellular matrix into which are embedded the various bone cells; osteoblasts, osteocytes and osteoclasts. Under control by various signals, discrete packets of bone are removed by osteoclasts and subsequently filled in with new bone tissue secreted by the osteoblasts (Downey and Siegel, 2006). Estrogens, in particular, influence bone remodeling, maintaining a balance between bone formation and bone resorption (Rochira et al., 2001; Heshmati et al., 2002). In post-menopausal women the decline in gonadal secretion of estrogen disrupts the balance of bone remodeling, leading to an increase in osteoclast activity that reduces bone mass and disrupt bone architecture. This results in increased bone fragility and an increased fracture risk that characterizes osteoporosis.

In premenopausal women, estrogen from the gonads supports healthy bone, but this source of estrogen is lost upon menopause, increasing the probability of osteoporosis. In post-menopausal women, sulfated steroids, particularly estrone sulfate (E_1S) and dehydroepiandrosterone sulfate (DHEAS) are present at considerable levels in peripheral circulation (Reed et al., 2005). Conversion of these precursors to active estrogens may provide the estrogen needed for maintenance of bone density (Purohit et al., 1992; Fujikawa et al., 1997; Muir et al., 2004). Steroid sulfatase is an enzyme that can convert circulating sulfated steroids to active forms in peripheral tissues. Steroid sulfatase in the bone tissue can cleave the sulfate group from sulfate esters to release free estrogen that could curb osteoporosis (Reed et al., 2005). Although the importance of steroid sulfatase in the local production of estrogen has been demonstrated for some tissues (e.g. breast), little is known about steroid sulfatase activity and regulation in bone.

Steroid sulfatase regulation is poorly understood. One report indicated that tumor necrosis factor (TNF)- α and interleukin 6 (IL-6) may increase STS enzyme activity via a post-translational modification or by increasing substrate availability in MCF-7 cells. It was indicated that STS activation could occur independently of STS gene transcription (Newman et al., 2000). In another study, treatment of human promyeloid cells with retinoids or 1,25-dihydroxyvitamin D3 increased STS mRNA and activity in myeloid leukemia cells via the PI3K/Akt dependent activation of NF-κB (Hughes et al., 2001; Hughes et al., 2008). A report on LPS induction of steroid sulfatase in prostate cancer cells showed steroid sulfatase mRNA and protein to increase upon lipopolysaccharides (LPS) treatment through IL-6 signaling pathway (Im et al., 2012). LPS is a cell wall constituent of gram-negative bacteria and is a potent inflammatory stimulus, inducing the

release of a wide range of cytokines and growth factors from immune cells (Li et al., 2012). Additionally, LPS can alter cytokine levels by stimulating inflammatory cells in tumor microenvironment to induce tumor cell proliferation, promote angiogenesis and facilitate metastasis (Schetter et al., 2010), mostly by activation of the NF-κB pathway. Thus, it is possible that NF-κB is a regulator of steroid sulfatase.

The interaction between transcription factor proteins and DNA is elementary to the regulation of transcription. Therefore, the ability to predict and identify transcription factor binding sites is integral to understanding the details of steroid sulfatase gene regulation. NF-κB is a nuclear transcription factor that regulates expression of a large number of genes that are critical for the regulation of apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases (Haefner, 2002). The activation of NF-κB is by a variety of stimuli that include growth factors, cytokines, lymphokines, UV, pharmacological agents, and stress (Gilmore, 2006). In its inactive form, NF-κB is present as one of several dimers. The dimers are held in the cytoplasm, bound by members of the IkB family of inhibitor proteins. The various stimuli that activate NF-κB cause phosphorylation of IkB, which is followed by its ubiquitination and subsequent degradation (Li and Stark, 2002). This results in the exposure of the nuclear localization signals (NLS) on NF-κB subunits and the subsequent translocation of the activated NF-κB molecule to the nucleus. In the nucleus, NF-κB binds to a consensus DNA sequence (5'-GGGACTTTCC-3') in the promoter regions of various genes, and thus activates their transcription (Pierce et al., 1988).

Previous experiments of ChIP-Seq (Chromatin immunoprecipitation for high-throughput sequencing) by TNF-α stimulation of B-lymphocytes to study the promoter

regulatory regions of steroid sulfatase revealed several NF-κB binding sites within the gene (UCSC Genome Browser for steroid sulfatase, chrX:7,118,707-7,249,706). At least 3 NF-κB consensus sequences for the RELA/p50 form of NF-κB are present within the steroid sulfatase gene, upstream from the promoter region near exon 1. These findings provide the potential for NF-κB regulation of steroid sulfatase gene expression. However, NF-κB modulated activation of the steroid sulfatase has not been well elucidated for any tissue, and there is no data on NF-κB regulation of steroid sulfatase in bone tissue.

In this study, we explored the effect of known activators and inhibitors of the NF-κB pathway (LPS, PMA and BAY) on steroid sulfatase expression in MG-63 cells in order to elucidate whether this pathway is involved in regulation of steroid sulfatase. Three compounds were chosen to study the NF-κB pathway, LPS, PMA and BAY. LPS, as described above, is a potent activator of the NF-κB pathway. PMA (phorbol 12-myristate 13-acetate), is a DAG analog that activates NF-κB-dependent transcription by activating protein kinase C isoforms (Catley et al., 2004). PMA has been shown to activate NF-κB and increase cell proliferation in certain tissues (Hellweg et al., 2006). BAY (E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile) is an inhibitor of κB kinase (IKK) and has shown to significantly block NF-κB activity by blocking phosphorylation of the NF-κB complex, reducing translocation of the active p65 major subunit (RELA) of NF-κB into the nucleus (Garcia et al., 2005). Using these compounds, we tested cell proliferation, steroid sulfatase activity, and steroid sulfatase mRNA levels of MG-63 human preosteoblastic cells with the intention of determining the ability of NF-κB to regulate of steroid sulfatase.

2.44 <u>Materials and Methods</u>

Chemicals and reagents

[6,7-^{3}H] Estrone-sulfate (49 Ci/mmol) was purchased from New England Nuclear

Corp. (Boston, MA, USA). Liquid scintillation cocktail was Ultima Gold (Packard

Instrument Co., Meriden, CT, USA). PMA (Phorbol 12-myristate 13-acetate) powder was

obtained from Sigma Chemical Co., (St. Louis, MO, USA) and dissolved in DMSO.

Lipopolysaccharides (LPS) from *Escherichia coli* and BAY 11-7082 ((E)3-[(4-

methylphenyl)-sulfonyl]-2-propenenitrile), an inhibitor of cytokine-induced IκB-α

phosphorylation, were also obtained from Sigma Chemical Co., (St. Louis, MO, USA).

Cell culture methods

Human preosteoblastic MG-63 cells were purchased from ATCC (Rockville,

MD). Cell culture media and reagents were obtained from Invitrogen (Grand Island, NY,

USA). Growth medium, used for routine growth of MG-63 cells, was 47.5% (v/v) Hams

F12 nutrient mixture, 47.5% (v/v) Minimal Essential Medium (MEM) containing 5%

(v/v) heat-inactivated fetal bovine serum from HyClone (Logan, UT, USA), and 10

mg/ml penicillin/ streptomycin solution from HyClone. Media added to plates was

changed every 2 or 3 days to ensure continuous nutrient supply. Cells were cultured in

100 mm tissue culture dishes (Beckton Dickinson, Franklin Lakes, NJ, USA) in 10 mL

growth medium. Media added to plates was changed every 2 or 3 days to ensure

continuous nutrient supply. Basal media, used for cell growth assays, was 49.25% (v/v)

Ham's F12 nutrient mixture, 49.25% (v/v) Minimal Essential Medium, 0.5% (v/v)

charcoal-stripped, heat-inactivated fetal bovine serum (HyClone), and 10 mg/ml

penicillin/streptomycin solution from HyClone.

Cell growth response to LPS, PMA and BAY treatment

MG-63 cells were seeded into Falcon 24-well plates (Becton Dickinson and Co., NJ, USA) at a density of 25,000 cells/well and incubated in 1 ml of growth medium for 18 h to allow them to adhere. After incubation, the medium was removed, the cells were washed once with phosphate buffered saline (Sigma–Aldrich Co., St. Louis, MO, USA), and basal medium was added to starve the cells. After 24 h, medium was changed to either basal medium alone (basal control) or basal medium containing treatments. The treatments were LPS (1µg/ml), PMA (50ng/ml) and (BAY 30µM/ml). Also LPS and BAY or PMA and BAY was examined for which regulatory effects of BAY require a 30 min pretreatment before stimulating the cells with LPS and PMA (Lee et al., 2012; Rauert-Wunderlich et al., 2013). The control was adjusted with DMSO (dimethyl sulfoxide) as BAY and PMA compounds were dissolved in DMSO. Cells were allowed to grow in their treatment media for 24 h. Media was then removed and 200 µl MTT (dimethylthiazol tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) was added to the cells, after which they were incubated at 37 °C for 3 h. Following incubation, the MTT was completely removed and replaced with 500 µl of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the membranes and then two 200 µl samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595 nm using the BioRad Microplate reader (Model 3550). Absorbances were compared with a standard curve derived from plates with known numbers of cells (range of 500–256,000 cells). The experiment was run in quadruplets and the experiment was repeated four times.

Measuring effect of LPS, PMA, BAY on STS enzyme activity

Treatment of cells and preparation of cell homogenates

MG-63 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson) at a density of approximately 750,000 cells/well and incubated in growth medium overnight to allow them to adhere. After incubation, the medium was removed and cells were washed once with phosphate buffered saline (Sigma-Aldrich Co., St.Louis, MO). Two ml of basal medium was added to starve the cells. After 24 h starvation, the medium was removed and cells washed again with phosphate buffered saline. Cells receiving BAY treatment were pretreated for 30 min and further incubated with LPS or PMA accordingly. At 3 h and 6 h time points, treatment media was removed, wells washed with 500 μl phosphate buffered saline. Using a cell-scraper cells were lifted-off in 1 ml of phosphate buffered saline, transferred to 12 x 75 borosilicate glass test tubes and then placed in a pre-chilled beaker and homogenized using three 10 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK).

Steroid sulfatase activity in MG-63 cell homogenates

[3]H-estrone sulfate was diluted in Tris–HCl buffer (50 mM, pH 7.5) and 100 μl (100,000 dpm/ml) was added to 13 x 100 borosilicate assay tubes. Radioinert estrone sulfate was dissolved in ethanol and then diluted into Tris–HCl buffer such that 100 μl would yield a concentration of 5 μM in the final assay volume. A final volume of 500 μl per tube was obtained using Tris–HCl buffer (50 mM, pH 7.5). The tubes were incubated in a water bath. The assay tubes were preincubated for 5 min at 37 °C in a water bath. The assay was initiated by addition of the 100 μl MG-63 cell homogenates prepared above to the respectively labeled tubes. After 60 min of incubation at 37 °C, 3 ml of

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toluene was added for extraction of unconjugated steroids. The samples were vortexed for 1 min and centrifuged at 2500 g for 10 min at 24 °C. Duplicate 1 ml aliquots were removed from the organic phase of the samples and added to 5 ml of scintillation cocktail. The aliquots were counted in a liquid scintillation counter (Packard Instrument Co.) for determination of product formation. The experiments were run three times, with duplicate tubes for each experiment.

BCA protein assay

The Pierce (Rockville, IL) BCA Protein Assay was used according to the manufacturer's instructions. A standard curve of bovine serum albumin was prepared with 50 mM Tris–HCl as the diluent. Duplicate concentrations (µg/tube) were 200, 150, 100, 75, 50, 25, 12.5 and 2.5. Duplicate tubes of 100 µl MG-63 homogenate samples prepared above were used. Absorbances were read at 562 nm using a spectrophotometer (Thermo Fisher, Genesys 20; Pittsburgh, PA, USA).

STS mRNA expression in response to LPS, PMA, BAY treatment

RNA isolation

Total RNA from MG-63 cells was isolated using TRIzol® Reagent (Invitrogen Life Technologies) according to manufacturer's instructions. MG-63 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson and Co., NJ) at a density of approximately 750,000 cells/well and incubated in growth medium overnight to allow them to adhere. After 24 h starvation MG-63 cells were treated in basal media with LPS (1µg/ml), PMA (50ng/ml), (BAY 30µM/ml) or LPS and BAY or PMA and BAY or BAY alone. Vehicle control was MG-63 cells treated with DMSO. After a 6 h treatment old media was removed, well washed with phosphate buffered saline and using a cell scraper

cell were lifted off the surface of the dishes and the cells were pelleted by centrifugation (1000 g for 10 min). Pellets were resuspended in 500 μl of TRIzol® reagent and incubated at room temperature for 5 min. 160 μl of chloroform was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was transferred, to which, 500 μl of isopropyl alcohol and 1 μl of glycogen was added. After 10 min incubation at room temperature the tubes were centrifuged at 12,000 g for 15 min at 4 °C. The resulting RNA pellet was washed with 500 μl of 75% ethanol, centrifuged at 7000 g for 10 min at RT, air-dried, and resuspended in 30 μl of diethylpyrocarbonate-treated water. Following 10 min incubation at 60 °C, RNA was quantified using a spectrophotometer (Thermo Fisher, Genesys 8, Pittsburgh, PA, USA). The amount of RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at − 80 °C until use.

Reverse transcription polymerase chain reactions (RT-PCR)

Reverse transcription

Using the GoScript® Reverse Transcription System (Promega), according to manufacturer's instructions, 1 μg of template RNA, 1 μl of Oligo (dT) primer and diethylpyrocarbonate-treated water were added to make a total volume of 9 μl, heated to 70 °C for 5 min, and then chilled on ice for 5 min. In a separate tube, reverse transcription mix was prepared from 4 μl of 5X reaction buffer, 4 μl MgCl₂, 1 μl dNTPs and 0.5 μl of reverse transcriptase.12 μl of reaction mix was added to RNA/primer mix. First-strand cDNA synthesis was carried out in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) with 5 min annealing at 25 °C. Next, the cDNA was allowed to extend for 1 h at 42 °C. The reverse transcriptase enzyme was thermally inactivated at 70

°C for 15 min after which the reaction tubes were held at 4 °C.

PCR primers

PCR primers were selected from the complete coding sequence for human steroid sulfatase (GenBank Accession No. M16505.1). The forward primer start position is 1402 and has the sequence (5'–3') TGA TGA GCC CAC TAG CAA CAT GGA (FB). The reverse primer start position is 1588 and has the sequence (5'–3') GTG CCA GCG CAC AGC ATT TAA GTA (RB). The expected RT-PCR product from this primer pair is 187 base pairs. Primers were synthesized by Integrated DNA Technologies, Inc., (Coralville, IA).

PCR reagents and conditions

PCR reactions were performed on the cDNA templates using a Taq PCR Kit (New England Biolabs, Ipswich MA) according to manufacturer's instructions. 2 μl template cDNA, 2.6 μl 10 X PCR buffer, 0.4 μl dNTP mix, 1 μl of 10 μM sense primer, 1 μl of 10 μM antisense primer, 0.2 μl Taq Polymerase, and DEPC-treated water were added to make total volume 20 μl. PCR reactions were then placed in a thermocycler (PTC-100, MJ Research, Inc., Watertown, MA). The reaction had a 4 min hold at 94 °C. For steroid sulfatase determination, the cDNA product was amplified for 35 cycles of: 45sec 94 °C, 45 s 58 °C, 90 s 72 °C. The PCR reaction was terminated with a final extension of 5 min at 72 °C. The PCR products were then separated in a 2% agarose gel containing 5 μl of 1 mg/ml ethidium bromide solution. The gel was run in 1 X TAE running buffer (40 mM Tris–acetate, 2 mM Na2-EDTA-2H2O) for 2 h at 70 V. 10 μl of PCR product were mixed with 2 μl 6 X cyanol loading dye (0.25% w/v xylene cyanol, 15% Ficoll in water). Quick load 2- log DNA ladder (New England Biolabs, Ipswich

MA) was used as the standard.

qRT-PCR reagents and conditions

Primers designed for RT-PCR of steroid sulfatase mRNA expression were used. The real-time PCR assay was carried out by Maxima SYBR Green qPCR Master Mix (Thermo Fisher, Pittsburgh PA). Through the different treatments expression of target gene mRNAs for steroid sulfatase was quantified by measuring the cycle threshold (Ct) and normalized by the housekeeping gene 18S ribosomal RNA. Relative gene expression was determined as treatment over vehicle control.

Immunocytochemical assay for STS protein expression

Antibodies and reagents

Primary antibody

To determine steroid sulfatase protein expression in MG-63 cells an antibody was generated in rabbit against a peptide representing a conserved region of mammalian steroid sulfatase. This peptide was identified using a multiple alignment of mouse, rat and human steroid sulfatase amino acid sequence (STS-275).

Secondary antibody

Alexa Fluor® 488 Goat Anti-Rabbit IgG labeled with green fluorescent dye was used as secondary antibody to react against sulfatase antibody (Life Technologies).

ProLong® Gold antifade mountant with DAPI that stains nuclei blue was used as mounting media (Life Technologies)

Cell treatments and fixing

Poly-D-lysine coated coverslips (Becton Dickinson) were placed into Falcon 24-well plates (Becton Dickinson). MG-63 cells were seeded at a density of 50,000

cells/well and incubated in 500 µl of growth medium for 48 h to allow them to adhere.

After 48 h incubation MG-63 cells were treated in basal media with LPS (1µg/ml), PMA

(50ng/ml), (BAY 30µM/ml) or LPS and BAY or PMA and BAY or BAY alone. Vehicle

control was MG-63 cells treated with DMSO. Following a 6 h treatment, the media was

removed and wells were rinsed with 500 µl of phosphate buffered saline. Cells were fixed

in 4% paraformaldehyde for 15 min at 4 °C. After fixation, cells were treated with 0.25%

Triton X-100 in PBS without calcium and magnesium for 10 min. MG-63 cells were

washed in PBS and blocked with 1% bovine serum albumin (Thermo Fisher, Pittsburgh,

PA) for 30 minutes. Incubation with steroid sulfatase primary antibody was done

overnight at 4 °C. After being washed with PBS, cells were incubated with Alexa Fluor®

488 Goat Anti-Rabbit IgG for 1 h. Cells were washed with PBS to remove excessive

fluor and mounted with a drop of DAPI on Superfrost® Plus Gold slides (Thermo Fisher,

Pittsburgh, PA). Stained cells were examined with a Nikon epifluorescent microscope

and imaged with a digital camera at 40 X magnification.

Statistical analyses

Statistical analyses were carried out using Prism 6.0 for Macintosh (GraphPad

Software, San Diego, CA). Probabilities of $P < 0.05$ were considered significant.

2.45 <u>Results</u>

Cell growth response to LPS, PMA and BAY treatments

To assess the role of inflammatory mediators and NF-κB in MG-63 cell

proliferation, MG-63 cells treated with lipopolysaccharide (LPS), and phorbol-myristate-

acetate (PMA) was evaluated in the presence or absence of an inhibitor of NF-κB

activation BAY (30μM/ml). Cell growth was significantly stimulated by the addition of

1μg/ml LPS and 50ng/ml PMA, and this growth was significantly inhibited in the

presence of 30μM/ml BAY (Fig. 21)

Steroid sulfatase enzyme activity in response to LPS, PMA and BAY treatments

To assess the role of inflammatory mediators and NF-κB on STS activity in MG-

63 cells, the cells were treated for 3 h and 6 h in the presence or absence of LPS, PMA

and BAY, an NF-κB transcription factor inhibitor. Cell homogenates of the treatments

were prepared and an *in vitro* conversion assay was performed using ^{3}H-E$_1$S (Fig. 22).

Steroid sulfatase (STS) activity significantly increased for the 3 h treatment of LPS

(0.357 fmol/hr/μg of protein) and PMA (0.348 fmol/hr/μg of protein) and the increase

was inhibited upon BAY treatment (0.193 fmol/hr/μg of protein). Similarly, STS activity

increased upon 6 h treatment of LPS (0.444 fmol/hr/μg of protein) and PMA (0.475

fmol/hr/μg of protein) and this increase in STS activity were significantly inhibited upon

treatment with BAY.

RT-PCR and qRT-PCR of STS mRNA expression

To assess the effect of inflammatory mediators and NF-κB on STS mRNA

expression, reverse transcriptase polymerase chain reaction was performed on the cDNAs

prepared from the mRNA extracted from each treatment of MG-63 cells, using primers

based on the known sequence of human steroid sulfatase gene. Single bands of 187 base

pairs (Fig. 23, lower panel), corresponding to the expected product size were evident for

all treatments. Using qualitative RT-PCR, band intensity was observed to be higher in

LPS and PMA treatments, with lower band intensity observed in controls and BAY-

treated MG-63 cells. Two-step qRT-PCR of changes in sulfatase gene expression gave an

increase in STS mRNA expression upon LPS treatment in MG-63 cells compared to vehicle control (Fig. 23, upper panel). Similarly, STS mRNA expression in response to PMA treatment was significantly higher compared to vehicle control. For both LPS and PMA treatments, addition of BAY decreased STS mRNA levels. BAY alone had no effect compared to controls.

Immunocytochemistry of MG-63 cells upon LPS, PMA and BAY treatment

Immunocytochemical analysis was employed to examine the expression of steroid sulfatase in cultured MG-63 cells. As shown in Fig. 24, immunocytochemical staining confirmed the presence of steroid sulfatase protein in MG-63 cells for all treatments. Steroid sulfatase signal intensities were stronger upon LPS and PMA treatment, compared to control and BAY treatments. Additionally, BAY-treated MG-63 cells in the presence of LPS and PMA showed a weaker signal compared to LPS and PMA alone.

2.46 <u>Discussion</u>

Although steroid sulfatase (STS) is known to play an important role in the formation of biologically active steroids that can stimulate tumor growth, little is known about the regulation of STS gene expression or activity in general, and even less is known in bone (Reed et al., 2005). In PC-3 human prostate cancer cells, it has been reported that lipipolysaccharides (LPS) can stimulate STS protein and mRNA expression through an IL-6 STAT3 signaling/pathway (Im et al., 2012). Previous studies have reported STS expression by insulin-like growth factors (IGF-I and IGF-II) to be transcriptionally controlled, and that this mechanism may be regulated by PI3-kinase-Akt-NF-κB pathway in human PC-3 prostate cells (Suh et al., 2011; Sung et al., 2013). Thus there is an

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indication that the NF-κB pathway may regulate STS. Consequently, we sought to determine if the NF-κB pathway affected MG-63 cell proliferation and STS activity and STS mRNA expression.

We developed a repeatable procedure for assessing MG-63 cell proliferation in the presence of steroids, and adapted this for testing LPS, PMA and BAY (Selcer and Difrancesca, 2012; Dias and Selcer, 2014). The assay uses a basal medium for starvation of cells (24 h) and into which treatments are added (24 h) to assess growth response. Basal medium has very little serum (0.5%), and the small amount of serum present is charcoal-stripped and should be largely devoid of steroids. Basal medium alone served as negative growth control (adjusting with DMSO, since PMA and BAY compounds were dissolved in DMSO). Treatment of LPS and PMA (24 h) increased cell proliferation significantly (Fig. 19). The NF-κB pathway inhibitor BAY showed no effect on its own, but inhibited growth of cells treated with LPS or PMA. Taken together these data suggest that stimulation of the NF-κB pathway (LPS or PMA) increased cell proliferation while inhibition of NF-κB pathways (BAY) blocked this response. The NF-κB pathway is known to stimulate cell proliferation in certain cell lines, including fibroblasts (Guttridge et al., 1999). NF-κB-stimulated cell proliferation is typically accompanied by inhibition of differentiation, mediated by the NF-κB subunit RELA (Guttridge et al., 1999; Dahlman et al., 2009). Our data are consistent with the known proliferative effects of an activated NF-κB pathway, as indicated by LPS and PMA-stimulated cell growth, and inhibition of this growth by BAY.

Having demonstrated that the activators of NF-κB, LPS and PMA, stimulated proliferation of MG-63 cells, and that an inhibitor of this pathway (BAY) blocked cell

proliferation by LPS and PMA, we next focused on the effect of these compounds on STS activity and expression. Similar to the cell proliferation data, the activators of NF-κB increased STS activity (significant at 3h and more so at 6h) and the inhibitor BAY blocked the STS increase. The increase in STS protein upon LPS and PMA treatment is supported by the immunocytochemical analysis of STS. Qualitatively higher fluorescence of steroid sulfatase antibody immunoreactivity was seen upon LPS and PMA treatment, and lower signals were seen upon BAY treatment. Again, the data on STS activity and protein suggests that the NF-κB pathway is responsible for the observed increases in STS protein activity. Protein expression of steroid sulfatase has been previously shown, in a qualitative Western blot, to incrementally increase in prostate cancer cells, from 0 h to 48 h upon LPS treatment (Im et al., 2012). Our data confirm and extend the observation that STS activity is regulated through the NF-κB pathway.

To determine if the increase in STS activity and protein was due to transcriptional regulation, we used RT-PCR and qRT-PCR analysis of STS mRNA levels under various treatments of MG-63 cells. The RT-PCR data clearly show that 6h treatments of LPS and PMA induce STS mRNA expression, and that BAY-treated cells showed lower mRNA levels. Thus, the observed changes in STS activity and protein are mirrored by changes in STS mRNA, indicating that the protein changes are attributable changes in transcriptional regulation of the STS gene. Our data are supported by a previous study showing LPS induction of steroid sulftase expression, through the STS promoter in a luciferase assay in PC-3 cells (Im et al., 2012).

A recent analysis of the STS gene by Menxi Jiang in Wen Xie's laboratory at the University of Pittsburgh School of Pharmacy (Jiang et al., 2015, submitted manuscript)

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revealed three NF-κB consensus sequences in the promotor region near exon 1 of the steroid sulfatase gene. They found that two of these sequences were capable of binding to RELA (p65) and that they each initiated activation of the human STS promoter in the presence of p65, as determined by a luciferase assay using the hepatic cell line HEK 293. Alteration of these NF-κB concensus sequences resulted in a lack of activation of the STS promotor in the presence of p65. Thus, the STS promotor can be activated by the NF-κB pathway. This information provides a mechanism for the observed changes in STS and STS mRNA shown in our study, in that they reveal that the NF-κB pathway, involving RelA, can activate the STS promotor and can result in transcription of the STS gene.

While LPS and PMA are known to activate NF-κB signaling, there are several proteins from other pathways that are known to regulate NF-κB signaling negatively by inhibiting NF-κB mediated transcription, notably the glucocorticoid receptor and IL-4 responsive STAT6 (Nelson et al., 2003). Moreover, in several cell lines (HeLa cells, human leukemia T-cells, mouse microglial cells) it has been shown that dexamethasone activation of endogenous glucocorticoid receptor is sufficient to block activation of NF-κB at the levels of both DNA binding and transcriptional activation (Scheinman et al., 1995; Ramdas and Harmon, 1998; Garside et al., 2004; Chantong et al., 2012). This information provides context to our previous data on glucocorticoid effects on cell proliferation, STS activity and mRNA levels, all of which declined under dexamethasone treatment. The decreases in cell proliferation, STS activity and mRNA levels under dexamethasone treatment (including osteogenic supplement treatment) could be explained by dexamethasone inhibition of the NF-κB pathway. NF-κB is known to stimulate cell proliferation and to inhibit cell differentiation. Dexamethasone inhibition of

NF-κB would therefore be expected to inhibit cell proliferation and to promote cell differentiation, exactly what is observed with for the OS+ cells. Furthermore, NF-κB stimulated STS gene expression. Therefore, dexamethasone addition would be expected to lower STS mRNA levels, STS protein, and STS activity, exactly what was observed for the OS+ cells. The various experiments with dexamethasone addition to basal medium also fully support the conclusion that dexamethasone acts on MG-63 cells by inhibiting NF-κB signaling.

Our combined results on cell proliferation, STS activity, immunocytochemical analysis, RT-PCR and qRT-PCR thus strongly indicate that MG-63 pre-osteoblast cells have steroid sulfatase, and the steroid sulfatase gene is transcriptionally regulated by the NF-κB pathway (see Fig. A5 for a model). Furthermore, we have provided evidence that glucocorticoids act on MG-63 cells to promote differentiation by inhibiting the NF-κB pathway. These results could have important implication for design of therapies to increase STS activity in bone for treatment of osteoporosis.

Putting our STS data into a large context, it appears that STS is regulated in the manner of an inflammatory response protein. STS transcription and protein levels are upregulated by an NF-κB pathway mediated by the RELA subunit and this response is inhibited by glucocorticoids. What then, might be the role of steroid sulfate in an inflammatory response? Recently, several groups have proposed that estrogen influences the immune process (Jiang et al., 2014; Monteiro et al., 2014). The prevailing hypothesis is that estrogen responds to metabolic dysfunctions that result in moderate inflammation and alleviates them to some extent. Estrogen's actions related to metabolic inflammation include: 1) opposing excessive body fat (obesity is viewed as a type of modest

inflammation) through a variety of mechanisms and 2) limiting glucocorticoid action during chronic glucocorticoid excess resulting from metabolic inflammation. Types of metabolic dysfunctions that might be aided by estrogen are varied and include: visceral obesity, insulin resistance, dyslipidemia, inflammation, non-alcoholic fatty liver disease and oxidative stress (Jiang et al., 2014; Monteiro et al., 2014).

The relevance of estrogen influences on the immune response to STS regulation is that STS is one mechanism of locally increasing estrogen levels, and by extension, one method for responding to inflammation. The scenario is as follows (Fig. 25): 1) metabolic dysfunction stimulates a moderate immune response through production of immune-stimulating signals (metabolic by-products, reactive oxygen species, cytokines, etc., 2) various cell types (including bone cells, and likely adipose tissue) respond to the immune stimuli by activating the NF-κB pathway, 3) the NF-κB pathway stimulates transcription of STS, increasing STS mRNA and subsequently increasing STS protein and activity, 4) STS converts inactive steroid precursors, such as E_1S and DHEAS, into physiologically active estrogens, particularly estradiol-17β, 5) the estrogens acts locally, through intracrine, autocrine and paracrine signaling to counterbalance the immune response, providing amelioration of some of the adverse effects of an overactive immune response.

This modulation of the immune response by estrogen is likely through a variety of mechanisms, including inhibition of the NF-κB pathway and inhibition of glucocorticoid action. Note that the STS response to immune activity is complex and likely dependent on the interplay of multiple factors. For example, glucocorticoids are also increased in response to metabolic stress and inflammation, and these inhibit STS transcription,

production and action. The inhibition of STS by glucocorticoids could be indirect through inhibition of the NF-κB pathway (DeBosscher et al., 2003), or could be the result of direct inhibition of STS gene transcription by the activated glucocorticoid receptor.

Thus, our data provide evidence for a novel role of steroid sulfatase, and for circulating sulfated steroids, in the modulation of metabolic inflammation. The widespread tissue distribution of STS would allow this enzyme to be either broadly or locally responsive to a particular immune challenge, depending on the particular situation. It remains to be seen if the observations on regulation of STS in bone cells can be generalized to STS in other tissues throughout the body. The hypothesis that STS is upregulated in response to inflammation in bone is entirely consistent with the new broader concept of metabolic inflammation and its complex interaction with bone and other tissues that has led to such terms as "metaflammation" (Medzhitov, 2008) and "osteoimmunology" (Walsh et al., 2006). Information on regulation of STS in other tissues will help determine if the STS response to inflammation is a general one.
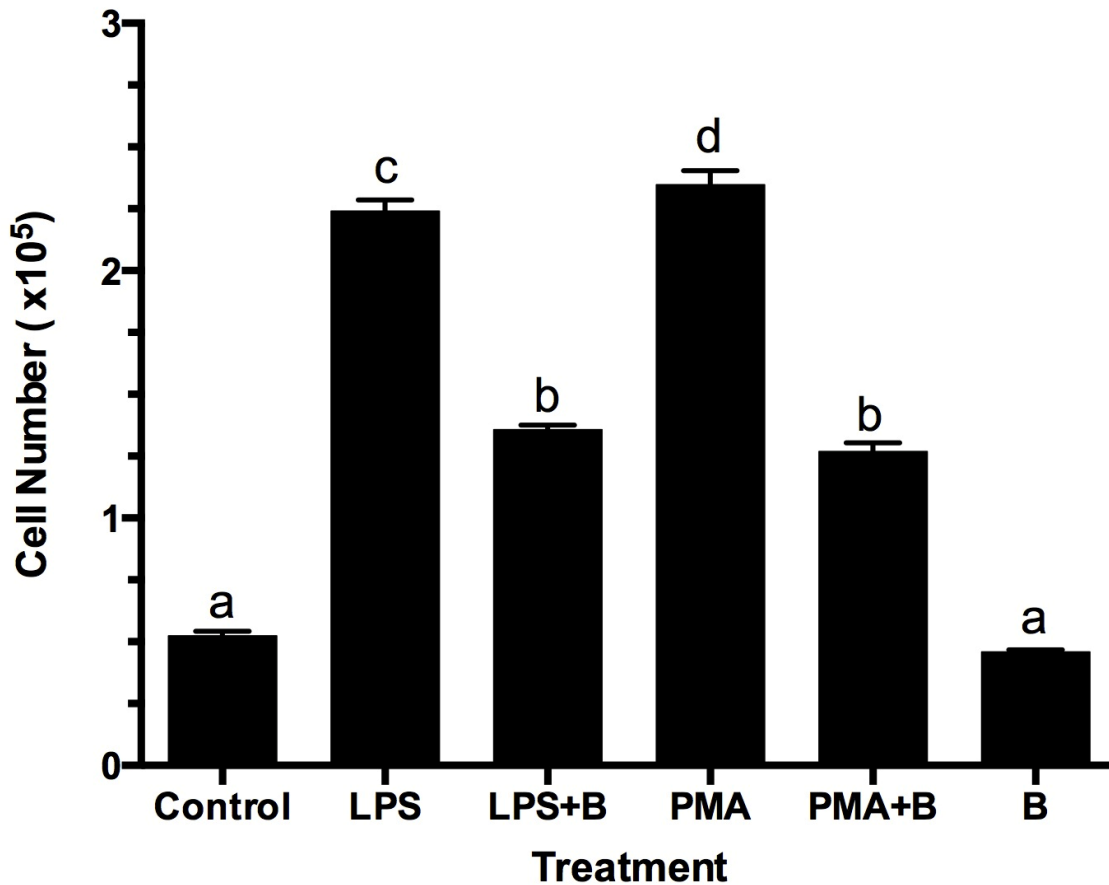
Fig. 21. Growth of MG-63 cells in basal medium with or without addition of 1µg/ml LPS, 50ng/ml PMA and 30µM/ml BAY inhibitor. MG-63 cells were plated overnight for adhering in growth medium followed by 24 h starvation in basal medium. 24 h treatments in the absence or presence of LPS, PMA and BAY in basal medium proliferated MG-63 cells. Bars represent mean cell number ± 1 SEM. Cell growth differed significantly among groups (one-way analysis of variance; F = 539.5; 5, 186 df; $P < 0.0001$). Different letters represent significant differences among means (Newman–Keuls *post hoc* test, $P < 0.05$). Abbreviations are: LPS = lipopolysaccharide, PMA = Phorbol Myristate Acetate, B = BAY ((E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile)
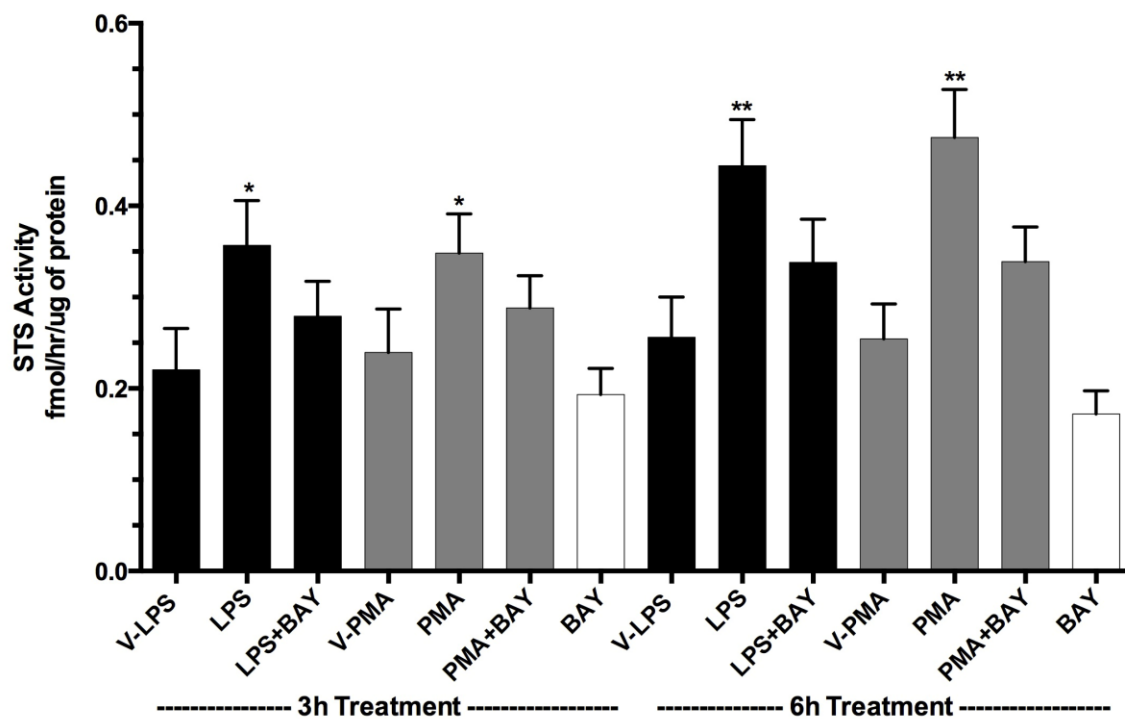
Fig. 22. Steroid sulfatase activity in MG-63 cells treated with 1μg/ml LPS, 50ng/ml PMA and 30μM/ml BAY inhibitor. Steroid sulfatase activity in MG-63 cell homogenates after 3h and 6h treatments of steroids. Data represent three independent experiments, run in duplicate. Bars represent the mean ± S.E.M., * = Significantly different from vehicle control (Newman-Keuls *post hoc*, $P<0.05$) for 3h treatment (one-way analysis of variance; $F = 2.33$; 6, 65 df), ** = Significantly different from vehicle control (Newman-Keuls *post hoc,* $P<0.0001$) for 6h treatment (one-way analysis of variance; $F = 6.07$; 6, 65 df). Abbreviations are: LPS = lipopolysaccharide, PMA = Phorbol Myristate Acetate, BAY = ((E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile), V-LPS = Vehicle control for LPS, V-PMA = Vehicle control for PMA.
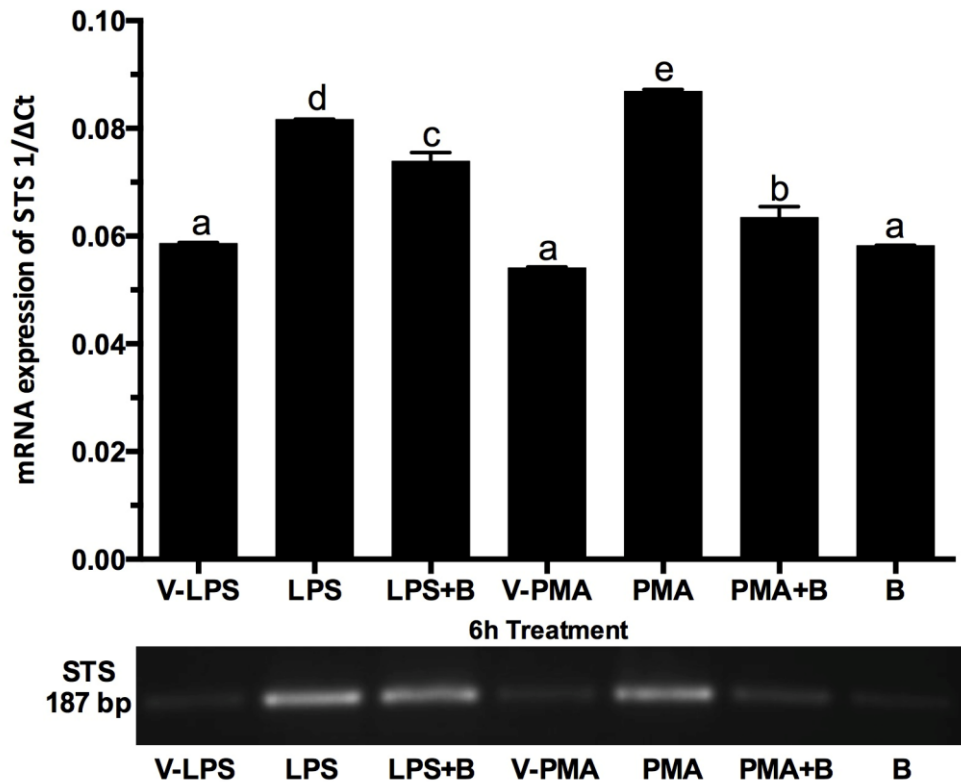
Fig. 23. Steroid sulfatase mRNA expression in MG-63 cells in response to 1μg/ml LPS, 50ng/ml PMA and 30μM/ml BAY inhibitor 6h treatment. Through real-time qRT-PCR, changes in STS mRNA expression were quantified by relative mRNA expression in response to treatment. Data represents an experiment, run in triplicate. Bars represent the mean ± SD. STS mRNA expression differed significantly among groups (one-way analysis of variance; F = 329.4; 6,7 df; P < 0.0001). Different letters represent differences among means (Newman – Keuls post hoc test, P < 0.05). Lower panel shows single bands of 187 bp RT-PCR amplified products on a 2% agarose gel with ethidium bromide staining. Abbreviations are: LPS = lipopolysaccharide, PMA = Phorbol Myristate Acetate, BAY = ((E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile), V-LPS = Vehicle control for LPS, V-PMA = Vehicle control for PMA.
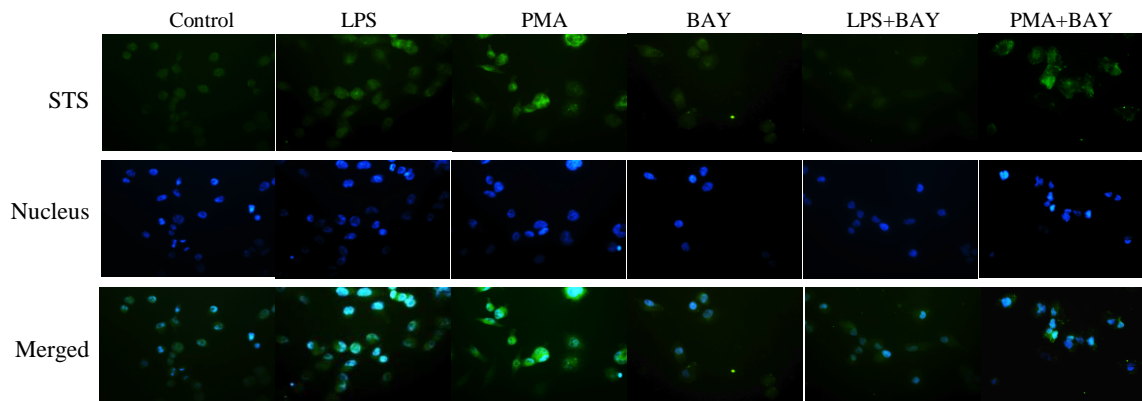
Fig. 24. Immunocytochemical analysis of steroid sulfatase in MG-63 cells in response to 1µg/ml LPS, 50ng/ml PMA and 30µM/ml BAY inhibitor 6h treatment.
See Materials and Methods for exact procedure. Cells were treated in the presence or absence of LPS (1µg/ml), PMA (50ng/ml) and BAY (30µM/ml), fixed and immunostained with a specific steroid sulfatase antibody at 1:250 dilution. ProLong®️ Gold antifade mountant with DAPI, that stains nuclei, was used as mounting medium. Steroid sulfatase stained green (top), nuclei stained blue (middle), the bottom panel is then a merged image of both. Images were captured using epifluorescent microscopy at 40 X magnification. Abbreviations are: LPS = lipopolysaccharide, PMA = Phorbol Myristate Acetate, BAY = ((E)3-[(4-methylphenyl)-sulfonyl]-2-propenenitrile).
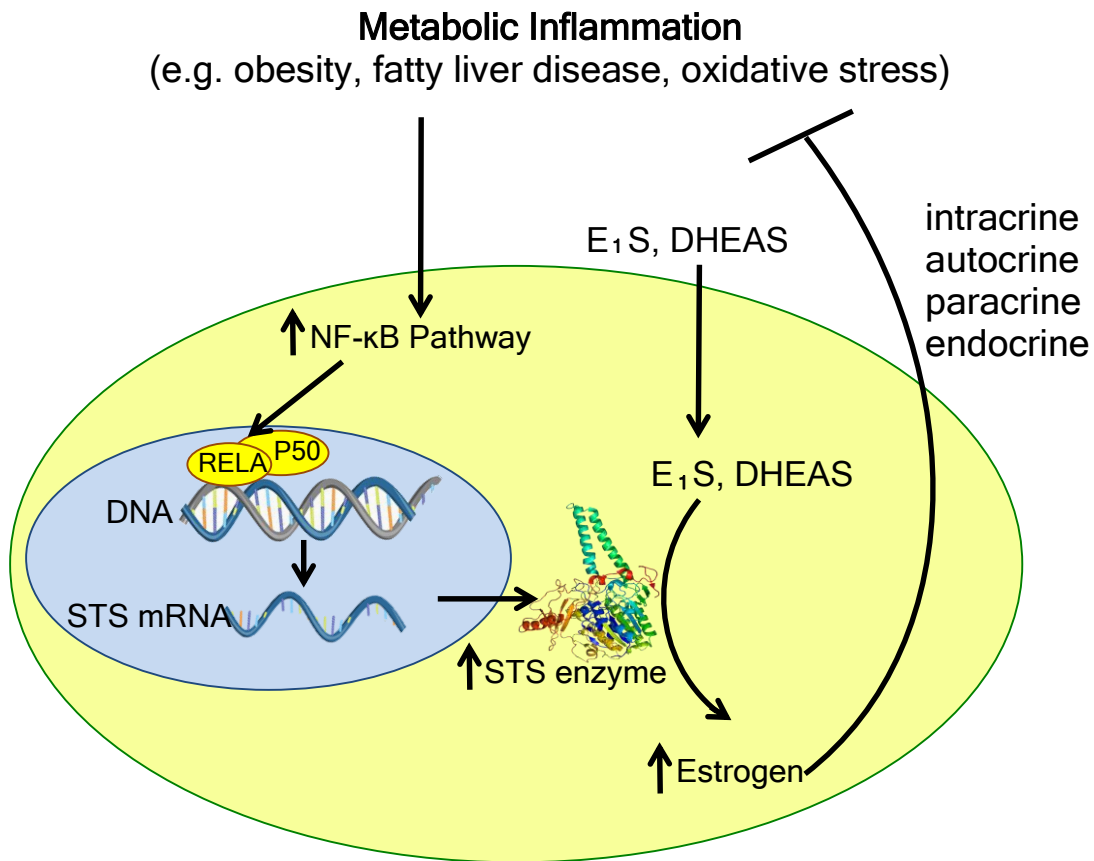
Fig. 25. A model depicting steroid sulfatase as an inflammatory response protein.

In response to metabolic disorders of obesity, fatty liver disease, oxidative stress, etc., several inflammatory signaling molecules like cytokines and reactive oxygen species are released. These ligands activate the NF-κB transcription factors, which in turn bind at the promoter upstream of the STS coding region to increase transcription of STS mRNA. Increased STS mRNA subsequently increases STS protein that converts inactive steroid precursors, such as E_1S and DHEAS to release active estrogen. Estrogen acts locally or globally through intracrine, autocrine, paracrine and endocrine signaling to modulate the inflammatory response.

Chapter 3

Summary

Project I. Characterization of Steroid Sulfatase (STS) in MG-63, a Human Pre-Osteoblastic Cell Line

We have provided three lines of evidence for the activity and presence of the STS enzyme and the presence of STS mRNA in the MG-63 human pre-osteoblastic cells. The data generated from Western blotting, immunocytochemical assays, enzyme activity assays and RT-PCR indicate that this cell line is capable of converting inactive steroids into biologically potent steroids using steroid sulfatase. These findings are consistent with previous studies that have demonstrated the presence and activity of STS in osteoblasts derived from several other mammalian species, including mice and rats.

Project II. Activity and Expression of Steroid Sulfatase (STS) During Differentiation, in the Human MG-63 Pre-Osteoblast Cell Line and Effect of Glucocorticoids

We developed a 21-Day protocol for the study of proliferation and differentiation in the MG-63 cell line. We found that OS+ media induces differentiation at the expense of proliferation. Furthermore, a time course of STS activity and mRNA expression levels showed that STS levels were decreased during the later stages of differentiation compared with controls. The glucocorticoid dexamethasone was found to induce differentiation and inhibit STS activity and mRNA expression. Other glucocorticoids, hydrocortisone and prednisolone, showed inhibitory effects on STS. Blocking of dexamethasone effect with RU486 upregulated STS activity and mRNA expression.

Project III. Distribution of Steroid Sulfatase (STS) mRNA in Various C57BL/6J Mouse Tissues

Our results demonstrated that STS mRNA is expressed in young male and female mice tissues of heart, liver, small intestine, skeletal muscle, bone, marrow, ovary and testes that we examined. These findings are consistent with reports on STS distribution and activity in other mammalian species. In adult female mice while liver expressed STS, bone and marrow showed very little STS expression. Our findings suggest STS to be present in bone and marrow, with expression levels declining with age.

Project IV. Role of NF-κB Pathway in Transcriptional Regulation of Steroid Sulfatase (STS) Gene Expression in the Human MG-63 Pre-Osteoblastic Cell Line

We have demonstrated that NF-κB activators LPS and PMA increased cell proliferation and STS mRNA and protein in MG-63cells, and that the NF-κB inhibitor BAY blocks these increases. These data suggest that induction of STS expression is transcriptionally controlled and that this mechanism may be regulated by NF-κB pathway in human MG-63 cells.

Conclusions

Post-menopausal women have low circulating unconjugated estrogens (particularly17β-estradiol), but high circulating levels of sulfated steroid estrone sulfate. Estrone sulfate is capable of supporting bone growth, through a process that is mediated by STS. These findings have implications regarding post-menopausal women. Many post-menopausal women suffer from osteoporosis, presumably due to the low estradiol levels. Our findings support that the estrone sulfate in circulation has potential for maintaining bone density via steroid sulfatase. We have seen that differentiated human MG-63 cells and adult bone and marrow of mice have lower expression levels of STS. In

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adult bone tissue, these low levels of STS expression are probably unable to generate sufficient estradiol to maintain bone. In post-menopausal women it is plausible that these lower steroid sulfatase levels are one factor contributing to the occurrence of osteoporosis. Women with greater levels of bone STS may enjoy some level of protection from osteoporosis. If STS levels in bone were able to influence the incidence of osteoporosis, then increasing the level of STS expression in bone would be desirable. Increased understanding of the presence, activity and regulation of STS will enhance our understanding of the physiological role of this enzyme in bone growth and maintenance and could lead to new therapies to treat osteoporosis.

The data from this project have provided results that support a new and novel hypothesis for a generalized role of STS as an inflammatory response protein. The scenario is as follows: 1) disease, pathogen or injury causes a local immune response, 2) local cells (including bone cells) respond to the immune stimuli by activating the NF-κB pathway, 3) the NF-κB pathway stimulates transcription of STS, resulting in increased STS protein and activity, 4) STS converts inactive steroid precursors, such as E_1S and DHEAS into active steroids, particularly estradiol-17β, 5) the estrogen acts locally or globally, through intracrine, autocrine, paracrine and endocrine signaling to counterbalance the immune response, providing amelioration of some of the adverse effects of an overactive immune response. The broad tissue distribution of STS would allow for this mechanism to be employed in a variety of tissues. It remains to be seen if this scenario holds true in other tissues besides osteoblasts.

Future Studies of STS Regulation and Function in Bone

The purpose of the proposed future research projects is to examine the regulation of steroid sulfatase reactivity, steroid sulfatase protein levels, and steroid sulfatase mRNA expression in mouse and human bone preosteoblastic cell lines and in mouse and human mesenchymal stem cells directed towards a bone lineage, with the ultimate goal of discovering a mechanism to increase steroid sulfatase enzyme activity, and therefore active estrogen, in mature bone cells. In addition, a transgenic mouse model will be constructed that overexpresses steroid sulfatase in bone tissues, under the negative control of a regulator (Dox). This model will help determine if increasing steroid sulfatase levels in bone at various stages of development can affect bone parameters relevant to osteoporosis, such as bone density.

Project #1

Examination of steroid sulfatase activity, protein levels and mRNA expression in mouse preosteoblastic cells after treatment with potential regulators of steroid sulfatase.

This is an extension of the MG-63 work done for this current project. A mouse cell line will be used for these experiments, which has been used extensively in the Selcer laboratory: the mouse preosteoblastic cell line MC-3T3-E1. The rationale for using a mouse and cell line is that the mouse offers the potential for future physiological experiments and transgenic model development.

Known regulators (activators and inhibitors) of NF-κB will be used, as well as various glucocorticoids and glucocorticoid inhibitors. Cells will be tested before, and after differentiation to osteoblasts.

Project #2

<u>Examination of steroid sulfatase activity, protein levels and mRNA expression in human and mouse mesenchymal stem cells after treatment with potential regulators of steroid sulfatase.</u>

This will take the work done on human and mouse cell lines and extend it to bone stem cells and transformed stem cells in both mouse and human models. Known regulators (activators and inhibitors) of NF-κB will be used, as well as various glucocorticoids and glucocorticoid inhibitors. Cells will be tested before and after transformation to osteoblasts.

Mesenchymal stems cells are pluripotent in that they can be directed to become several different types of tissues. Transformation of mesenchymal stem cells from several sources (e.g., bone marrow, umbilical cords (Wharton's jelly) and adipose tissue) into osteoblasts has been achieved using 'osteogenic supplements'. These are typically some combination of dexamethasone, vitamin C, and glycerophosphate, often with other components. Osteoblasts derived from mesenchymal stem cells are presumed to be physiologically more like normal bone cells than the commonly used preosteoblastic cells lines, which have evolved in culture to be different from their ancestral bone cells. Thus, experiments performed on transformed mesenchymal osteoblasts might provide stronger evidence for a hypothesis than those on cell lines. At the very least, they provide a separate line of evidence for a given set of experiments. This project would investigate steroid sulfatase regulation in mesenchymal stem cells and stem cells transformed into osteoblasts.

Project #3

Construction of a tet-off steroid sulfatase transgenic mouse to be used to examine the
effect of elevated steroid sulfatase levels on bone cell density at different stages of bone
development.

       This project would take the study of STS to the in vivo expression level using a
modification of an existing transgenic model. Existing TRE-STS transgenic mice, from a
collaborator, will be cross-bred with a commercially available SP7-tTA transgenic mouse
line to form a TRE-STS/SP7-tTA mouse line that should overexpress STS in bone cells
under the negative control of the regulator DOX. This line will first be tested to
determine if STS is indeed overexpressed in bone tissues under DOX regulation. If so,
experiments will be run to determine if various bone parameters (e.g., bone density) are
altered by the overexpression of STS.

       The overarching hypothesis of these proposed studies on steroid sulfatase is that
increasing the activity of this enzyme in mature bone tissue might represent a potential
therapy or prophylactic for osteoporosis. Supporting evidence for this hypothesis might
be provided using a transgenic mouse overexpressing steroid sulfatase in bone tissue.
This specific aim is for the generation and testing of such a transgenic mouse (the TRE-
STS/SP7-tTA mouse). The system is a tet-off transgenic, so that the overexpression of
STS can be regulated (turned off) by the regulatory molecule DOX. This will allow
manipulation of the timing of the overexpression, and therefore will facilitate testing
mouse bone parameters at different times of bone development and different stages of
maturity.

The Sp7-tTA-TRE-STS mice will be used, with and without Dox, to determine the effect of overexpression of steroid sulfatase on various bone parameters, including bone density. Both genders will be used for analysis. Additionally, the timing of overexpression will be altered to test for the effect of overexpression on mouse bone at different stages of development and at different ages. This will include testing the effect of overexpression of steroid sulfatase from birth to five weeks, fifteen weeks, and 30 weeks, compared with normal mice and with Dox-treated transgenic mice at these same time points. These time points roughly correspond with puberty, pre-menopause and post-menopause for the mice. Additionally, transgenic mice will be placed on Dox at birth and the Dox treatment will be removed at specified time points. This will include Dox treatment from birth to five weeks, then Dox removal until fifteen and thirty weeks, as well as Dox from birth to fifteen weeks and Dox removal until thirty weeks. The primary endpoint will be femur bone density, but other endpoints may be employed.

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APPENDIX I

List of Statistical Analyses

Statistical analyses were carried out using Prism 6.0 for Macintosh (GraphPad Software, San Diego, CA). Following are the statistical procedure carried out:

1. Analysis of Variance

    a) One-Way Analysis of Variance (ANOVA)

        i.   Newman-Keuls post-hoc, P<0.05

    b) Two-Way Analysis of Variance (Two way ANOVA)

        i.   Sidak's multiple comparison test, * = 0.05, ** = 0.01, *** = 0.005,

            **** = 0.0001

2. Regression

    a) Linear Regression with 95% Confidence Interval, $R^2 < 0.99$
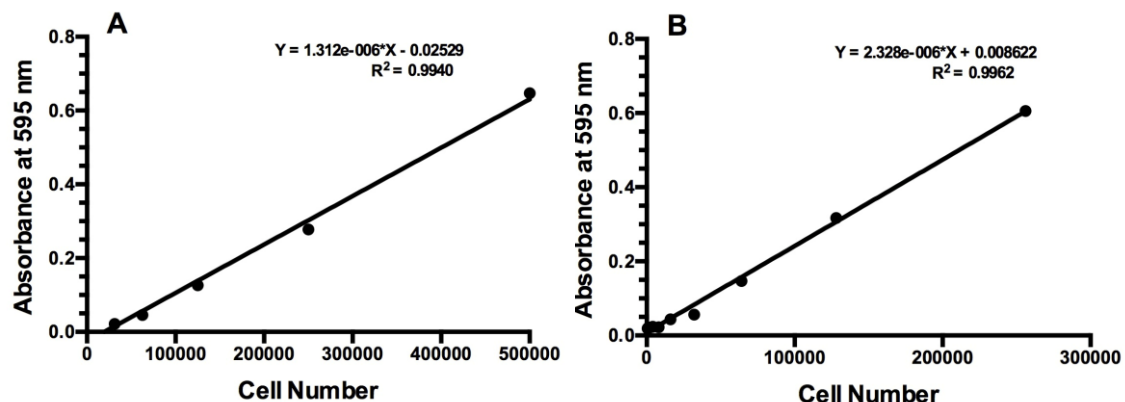
Fig. A1. Cell number standard curve for MG-63 cell by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MG-63 cells were seeded onto Falcon 6-well plates **(A)** and Falcon 24-well plates **(B)** at a range of densities and incubated for 24 hours prior to analysis. MTT (5 mg/ml) was added to each well and incubated for 3 h at 37 °C. Cells were lysed and the formazan derivative of MTT was solubilized using acidic isopropanol. Absorbances of the supernatant read at 595 nm showed an increase in absorbance with increasing cell-seeding density. Linear regression trend lines are shown. Data from 2 sample replicates are shown (n = 2, mean $\pm$ SEM) for (A) and (B) standard curves.
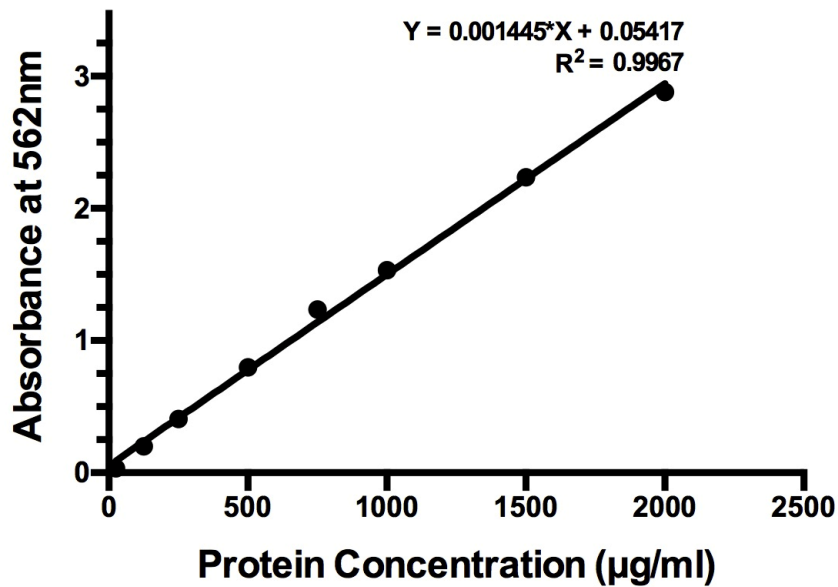
Fig. A2. BCA Protein Standard Curve. Standard curve using Pierce® (Rockville, IL) bicinchoninic acid (BCA) protein assay kit was generated from pure bovine serum albumin (2mg/ml) diluted in 50mM Tris-HCl. Protein concentrations were 2000, 1500, 1000, 750, 500, 250, 125 and 25 µg/ml. Absorbances were measured at 562 nm. Using the equation of the line, y = 0.0014x + 0.05417, protein concentrations of test samples were calculated.
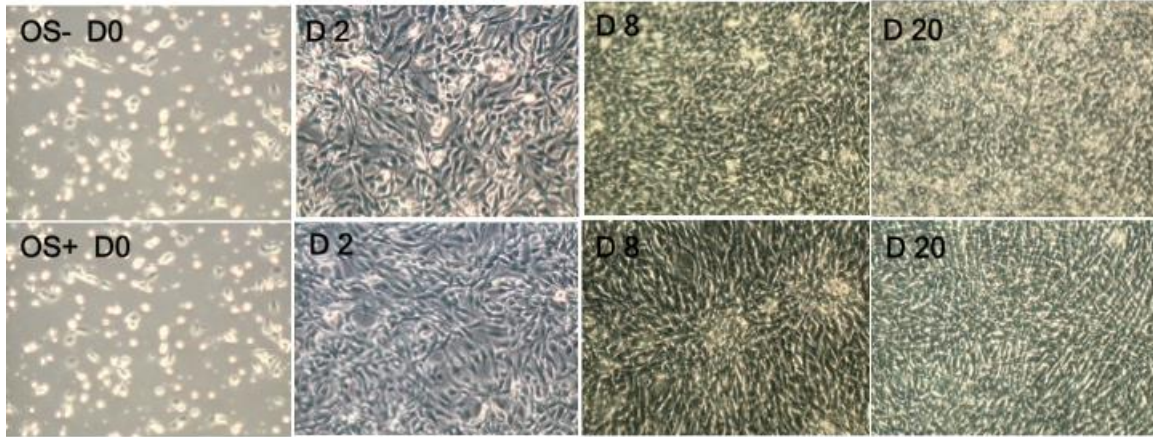
Fig. A3. Morphological features of MG-63 cells captured during the differentiation process. Images are representative of morphological changes seen in MG-63 cells treated with growth medium alone (OS-) or growth medium with osteogenic supplement (OS+) throughout differentiation. Images were captured throughout the time course using a 10X objective with 100X total magnification. Cells treated in growth medium alone were more spherical throughout differentiation, while cells treated with osteogenic supplement medium were more elongated and stellate displaying *in vitro* cytoplasmic extensions at the later stages of differentiation. Abbreviations are: OS- = growth medium alone; OS+ = growth medium with osteogenic supplement; D = Day.
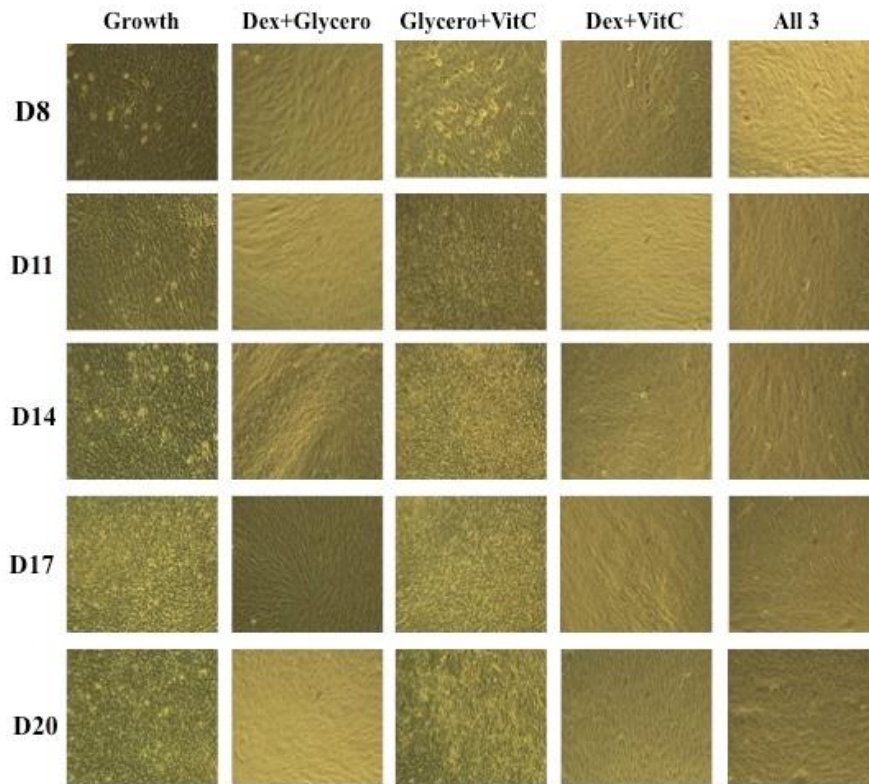
Fig. A4. Morphological effects of osteogenic supplement medium components on MG-63 cells captured during the differentiation process. Images are representative of morphological changes seen throughout differentiation in MG-63 cells treated with growth medium alone or growth medium with combinations of dexamethasone and β-glycerophosphate; β-glycerophosphate and ascorbic acid; dexamethasone and ascorbic acid; dexamethasone, β-glycerophosphate and ascorbic acid. Images were captured throughout the time course using a 10X objective with 100X total magnification. Cells treated in the absence of dexamethasone were more spherical throughout differentiation while cells treated with dexamethasone were more elongated and stellate. Abbreviations are: D = Day; Dex = dexamethasone; Glycero = β-glycerophosphate; VitC = ascorbic acid.

NF-κB Signaling Pathway for STS Gene Upregulation
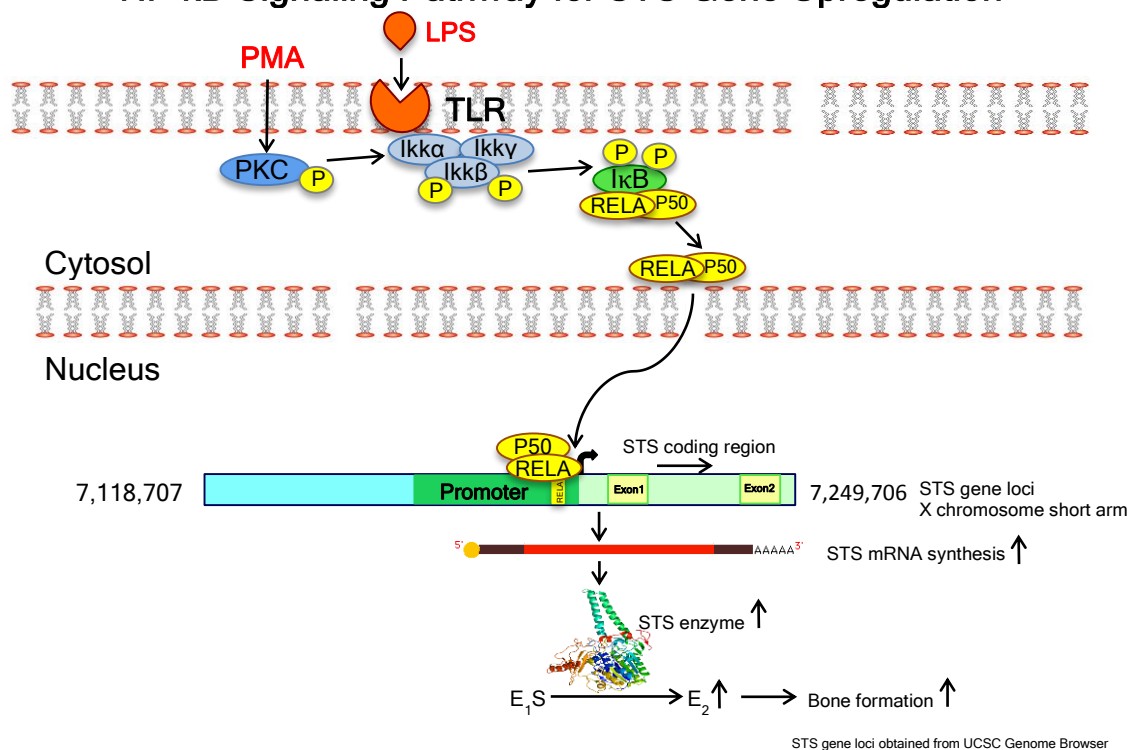
Fig. A5: A model of the NF-κB signaling pathway for STS gene regulation.

LPS binds to a membrane receptor to activate IKK (IκB kinase complex of IKK-α, IKK-β, IKK-γ subunits). Similarly, PMA activates PKC, which also activates IKK kinase complex. Activated IKK phosphorylates the IκB inhibitor. The IκB inhibitor undergoes proteasome degradation and the RELA/p50 NF-κB complex is free to enter the nucleus to bind to RELA binding site within the promoter region located upstream from the start of transcription site for the STS coding region. STS mRNA synthesis is increased, which leads to increased STS protein. Increased STS protein would cause an increase in estrogen synthesis from sulfated steroid precursors, such as E_1S, to promote bone formation. Abbreviations: PMA = phorbol 12-myristate 13-acetate; PKC = protein kinase C; LPS = lipopolysaccharides; NF-κB = nuclear factor-kappa B; E_1S = estrone sulfate; E_2 = 17β-estradiol.

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APPENDIX II

Expansion of Chapter 2.3: Distribution of Steroid Sulfatase in MMTV-neu mouse bone
and marrow of HRT treated and untreated mice

Objectives:

- To examine steroid sulfatase mRNA expression in bone and marrow of adult female control and HRT treated transgenic MMTV-neu mice

Model:

- Adult female mice bone and marrow tissues of transgenic mice MMTV-neu were of 9.9 months and 11.13 months of age for untreated mice. HRT treated mice were 11.1 and 11.6 months old. The HRT treated mice received an acute 30-day exposure of 0.5 mg estradiol and 100 mg progesterone before being sacrificed.

A2.1 Introduction:

The experiments conducted in chapter 2.3 on C57BL/6J juvenile and adult mice tissues revealed steroid sulfatase mRNA expression to be at higher levels in juvenile male and female bone and marrow tissues. In adult female mice the presence of steroid sulfatase declined significantly in bone and bone marrow. In chapter 2.4 we addressed steroid sulfatase gene upregulation by activating the NF-κB signaling pathway. Binding of estrogens or androgens to their receptors in the nucleus stimulates transcription of target genes resulting from direct interactions of the receptor proteins with DNA or from interactions with other transcription factor (Tsai and O'Malley, 1994). As an extension to project 2.3 we wanted to investigate if estrogens and androgens have the ability to upregulate steroid sulfatase expression in bone and bone marrow by examining STS mRNA expression in HRT treated and untreated adult female mice.

A2.2 Methods:

PCR primers

Primers for RT-PCR were selected from the complete coding sequence for *Mus musculus* steroid sulfatase (GenBank accession no. U37545). The forward primer start position is 1592 and has the sequence (5'– 3') AGC ACG AGT TCC TGT TCC ACT ACT (FB). The reverse primer start position is 1691 and has the sequence (5'– 3') AAG TTG GGC GTG AAG TAG AAG GC (RB). The expected RT-PCR product from this primer pair is 100 base pairs. An endogenous control gene *Mus musculus* peptidylprolyl isomerase A (cyclophilin A) primer was designed from the *Mus musculus* cyclophilin A mRNA (GenBank Accession No. NM_008907). The forward primer start position is 380 and has the sequence (5'– 3') TAT CTG CAC TGC CAA GAC TG (FB). The reverse primer start position is 524 and has the sequence (5'– 3') ACA GTC GGA AAT GGT GAT CT (RB). The expected RT-PCR product from this primer pair is 145 base pairs. Primers were synthesized by Integrated DNA Technologies, Inc., (Coralville, IA).

Bone and marrow sample preparation, RNA isolation, cDNA synthesis, PCR reagents and conditions, RT-PCR

As indicated in methods section of chapter 2.34

A2.3 Results

Steroid sulfatase mRNA expression in adult transgenic MMTV-neu mouse bone and marrow

In order to explore STS mRNA expression in HRT treated and untreated adult female bone and bone marrow tissues, reverse transcriptase PCR was performed using primers based on the known sequence of mice steroid sulfatase gene in control and HRT

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treated transgenic mice. Single bands of 100 base pairs corresponding to the expected product size was seen in bone and marrow of HRT treated mice but was found to be absent in the untreated control samples (Fig. A6). 145 base pair expected product bands of Cylophilin A endogenous control was expressed in all samples.

A2.4 Discussion:

Hormone replacement therapy of combined estrogen and progesterone treatment administered to post-menopausal women in the largest sample size three-year trial study has shown sustained increase in bone mineral density in the spine and hip (PEPI, 1996) when compared to placebo treatments. Other clinical trials with fracture outcomes confirm the use of estrogen and progesterone as hormonal supplements for bone maintenance in post-menopausal women (Nachtigall et al., 1979; Lindsay et al., 1980; Lufkin et al., 1992; Komulainen et al., 1998). Additionally, estrogen has shown to increase bone mass and strength in ovariectomized female mice and orchidectomized males (Kousteni et al., 2002). However, effect of estrogen and progesterone on steroid sulfatase expression has not been studied before.

To determine the effect of these hormones on steroid sulfatase mRNA expression in adult female mice we used reverse transcriptase polymerase chain reaction (RT-PCR) to assess whether or not steroid sulfatase mRNA was present in the control and HRT treated transgenic female mice. Using primers based on the known mouse steroid sulfatase sequence and known Cyclophilin A enzyme as endogenous control RT-PCR was carried out on isolates of control and HRT bone and bone marrow tissues. On an agarose gel, steroid sulfatase mRNA expression was seen in HRT treated bone and marrow but was absent in control tissues. Preliminary data indicate that untreated adult

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mice have low detectable steroid sulfatase but HRT treated mice have substantially

higher steroid sulfatase mRNA expression suggesting estrogens and progesterone to

upregulate steroid sulfatase in adult long bone.
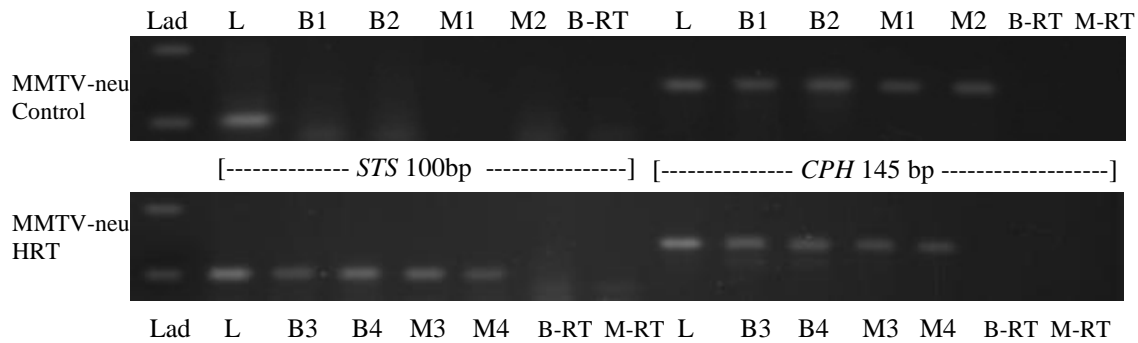
Fig. A6. Reverse transcriptase polymerase chain reaction of MMTV-neu control and HRT treated female mice bone and marrow using mouse-specific steroid sulfatase primers on a 2% agarose gel, stained with ethidium bromide. RT-PCR performed using mouse specific STS primers and cyclophilin primers used as control. STS mRNA expression was seen in HRT treated bone and marrow adult female mice tissues but expression levels were absent in control adult bone and marrow tissues. Adult C57BL/6J wild type female mice liver tissues were used as control. Lane assignments: Lad = Ladder, L = Liver; B1, B2 = Control Bones; M1, M2 = Control Marrows; B3, B4 = HRT Bones; M3, M4 = HRT Marrows; B-RT = Bone with no reverse transcriptase enzyme; M-RT = Marrow with no reverse transcriptase enzyme; CPH = Cyclophilin.

To summarize, in untreated adult female mice the presence of steroid sulfatase

declined significantly in bone and marrow tissues. Similarly, as seen in chapter 2.2,

differentiated human MG-63 pre-osteoblast cells showed lower sulfatase expression and

activity compared to proliferating MG-63 cells, due to glucocorticoid inhibitory effect on STS expression in differentiated cells. In an attempt to study upregulation of steroid sulfatase mRNA expression, we found the NF-κB pathway to increase steroid sulfatase expression and activity (chapter 2.4). Our preliminary results on adult HRT treated bone and marrow female tissues show steroid sulfatase mRNA expression to be upregulated by progesterone and estrogen. Further investigation would need to be done to understand how estrogen signaling upregulates steroid sulfatase gene expression in bone and bone marrow tissues of adult female mouse.