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**Effect of Steroids and Sulfated Steroids on Growth
of the Human MG-63 Osteoblast-like Cell Line**

A Thesis
Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements
For the Degree of Master of Science

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Osteoblast-like Cell Line

Degree: Master of Science

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TABLE OF CONTENTS

Title	i
Signature Page	ii
Acknowledgments.....	iii
Table of Contents.....	iv
List of Figures.....	vi
Abstract	vii
Chapter 1-Background	1
Skeletal Functions	1
Components of Bone	1
Osteon Cells	2
Modeling and Remodeling of Bone	4
Bone Pathologies.....	5
Effect of Steroids.....	6
Local Production of Estrogen	8
The Role of Steroid Sulfatase in Intracrinology	9
Steroid Sulfatase.....	10
Regulation of Steroid Sulfatase.....	12
Steroid Sulfatase Inhibitors.....	13
Chapter 2-Proposed Research	14
Objective.....	14
Specific Aims	14
Model System	14
Chapter 3-Materials and Methods	15
MG-63 Cell Culture.....	15
MG-63 Whole Medium	15
Removal of Cells from 100mm Tissue Culture Dishes.....	16
Cell Counting	16
MG-63 Basal Medium.....	17
Charcoal-Stripped Fetal Bovine Serum.....	17
Standard Curve: 24-Well Plate	18
MTT Assay: 24-Well Plate	18
Standard Curve: 6-Well Plate	19
MTT Assay: 6-Well Plate.....	19
Growth in Basal and Whole Media	19
10 μ M Steroid Growth Assay	20
Proliferation Dose-Response	21
Growth Assay with 1 μ M DU-14 or EMATE	22
Growth Assay with 10 μ M DU-14.....	22
Growth Assay with 100nM ICI 182,780	23
Steroid Sulfatase Activity in Microsomes	24

Chapter 3-Materials and Methods Continued	
Microsome Preparation.....	25
Toluene Organic Extraction.....	25
BCA™ Protein Assay	26
Whole Cell Conversion Assay	26
Statistical Analysis	27
Chapter 4-Results.....	28
Chapter 5-Discussion	44
Appendix-Additional Figures	51
References.....	54

LIST OF FIGURES

Figure 1: Biosynthetic Pathway of Estrogens and Androgens	33
Figure 2: MG-63 Growth in Various Media.....	34
Figure 3: MG-63 Proliferation in the Presence of Steroid Treatments	35
Figure 4: MG-63 Proliferation in the Presence of Sulfated Steroids and Estradiol.....	36
Figure 5: MG-63 Proliferation Dose-Response to Estradiol and Estrone Sulfate	37
Figure 6: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and DU-14	38
Figure 7: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and DU-14	39
Figure 8: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and EMATE.....	40
Figure 9: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate, Estradiol, and ICI 182,780.....	41
Figure 10: Inhibition of Steroid Sulfatase Activity in MG-63 Microsomes by DU-14 and EMATE	42
Figure 11: Inhibition of Steroid Sulfatase Activity in MG-63 Whole Cells by DU-14 and EMATE	43
Figure A-1: MG-63 24-Well Plate Standard Curve.....	51
Figure A-2: MG-63 6-Well Plate Standard Curve.....	52
Figure A-3: BCA™ Protein Assay Standard Curve.....	53

ABSTRACT

The importance of estrogen in bone regulation is exemplified by the rapid loss of 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. While it is known that estrogens can stimulate the growth and maintenance of bone cells, it is not known if sulfated steroids can induce a similar response. The purpose of this study was to determine if sulfated steroids can induce proliferation of a human osteoblast-like cell line MG-63, and if steroid sulfatase inhibitors were capable of blocking that response. A growth assay was developed to assess proliferation of MG-63 cells in the presence of various steroids. The results of an initial experiment with a number of steroids indicated differences in growth, with estradiol, estrone sulfate, and dehydroepiandrosterone sulfate showing increased proliferation. A follow up experiment with estradiol, estrone sulfate, and dehydroepiandrosterone sulfate showed that proliferation was increased in the presence of estradiol and estrone sulfate. A dose-response testing proliferation of MG-63 cells to estradiol and estrone sulfate, resulted in estradiol stimulating growth above baseline at 10 μ M and 1 μ M, and estrone sulfate increasing growth only at 10 μ M. A steroid sulfatase inhibitor, DU-14, was able to block estrone sulfate-stimulated growth at 10 μ M, but not at 1 μ M. Another steroid sulfatase inhibitor, EMATE, actually stimulated growth at 1 μ M; however, this inhibitor is known to be estrogenic. The estrogen receptor antagonist ICI 182,780 inhibited estrone sulfate- and estradiol-stimulated growth at 100nM. Steroid sulfatase activity was assessed in MG-63 microsomes and whole cells in the presence of DU-14 and EMATE. It was found that steroid sulfatase activity in the presence of the inhibitors was virtually eliminated. These data demonstrate that growth in the human MG-63 osteoblast-like cells is stimulated by estrogens and

by sulfated estrogens. This supports the concept that sulfated steroids are important in maintaining bone density.

CHAPTER 1-BACKGROUND

Skeletal Functions

The human skeletal system is one of the largest organs of the body and provides many functions; among the most important is structural integrity (Walsh *et al.*, 2006). The skeletal system is the main reservoir of calcium and phosphate ions and has a pivotal role in the homeostasis of these minerals. Other important functions include: protecting vital internal organs, acting as an anchor for skeletal muscles, and supporting hematopoiesis (Cohen, 2006).

Components of Bone

The human skeletal system is a specialized type of connective tissue comprised of cells embedded in an extracellular matrix. The extracellular matrix accounts for 90% of bone volume and consists of an organic and inorganic component. Type I collagen is the main protein of the organic component (Downey and Siegel, 2006), while the inorganic component consists of a calcium-phosphate salt, hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) (Walsh *et al.*, 2006). During the process of mineralization hydroxyapatite crystals are deposited on and between the collagen fibrils producing hardened bone.

The remaining 10% of bone volume includes the blood vessels and cellular component. Four different types of osteon cells, which include osteoblasts, osteocytes, bone lining cells, and osteoclasts, can be found within the cellular network of bone (Downey and Siegel, 2006). Each of the cell types has a specific functional role in the formation and reabsorption of bone. However, the cell types are derived from different cell origins and respond to different signals for the processes of differentiation, proliferation, and activation or inhibition of activity (Robling *et al.*, 2006).

Osteon Cells

Osteoblasts are involved in the secretion of the extracellular matrix, the mineralization process, and in influencing bone reabsorption. Active osteoblasts form a monolayer of cells and secrete unmineralized matrix called osteoid, which then further matures and mineralizes under the influence of the 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). The process of differentiation takes between 25 to 30 days *in vitro*, during this time three phases have been defined. The initial proliferative phase occurs on days 0 to 4, the matrix deposition phase is seen between days 10 to 16, and maximal mineralization occurring on day 28 (Duplomb *et al.*, 2007).

The initial proliferative phase of osteoblast differentiation is characterized by proliferation of mesenchymal stem cells (Robling *et al.*, 2006). Differentiation of proliferating mesenchymal stem cells towards the osteoblastic lineage is accomplished through increased expression of runt-related transcription factor-2 (Runx2) (Robling *et al.*, 2006), the earliest known osteoblast-specific marker (Wagner and Karsenty, 2001). Newly committed preosteoblasts express the early phenotypic marker type I collagen. Expression of the transcription factor osterix (Osx) is necessary for preosteoblasts to continue the differentiation process towards mature bone forming cells (Robling *et al.*, 2006). One phenotypic marker of maturing preosteoblasts is the continued increase in expression of alkaline phosphatase. The late phenotypic marker typically used for identification of mature osteoblasts is osteocalcin, which is only expressed with the appearance of mineralized matrix (Malaval *et al.*, 1999).

During the process of mineralization, some osteoblasts become entrapped in the extracellular matrix secretions. The trapped cells differentiate into osteocytes, which make up

more than 90% of osteon cells in adults. As extracellular matrix is secreted, osteocytes become located deeper within the bone tissue and begin forming long cytoplasmic projections. These projections allow osteocytes to remain in contact with adjacent cells and the bone surface. The cellular network created by these projections is pivotal in sensing and responding to mechanical stimuli, communication, and exchange of nutrients within the bone microenvironment (Downey and Siegel, 2006).

The third type of osteon cells, bone lining cells, cover most of the bone surfaces in an adult skeleton. These cells are derived from the osteoblast lineage and are also referred to as resting osteoblasts or surface osteoblasts. The role of bone lining cells is relatively unknown. It has been speculated that these cells are involved in preparing the matrix for reabsorption, mineralization, and acting as a barrier between extracellular fluid and bone (Downey and Siegel, 2006).

Osteoclasts are the final type of osteon cells, which function in reabsorption of bone and subsequent release of calcium and phosphate ions into the bloodstream. These giant multinucleated cells are terminally differentiated and are derived from hematopoietic stem cells (Robling *et al.*, 2006). Mature osteoclasts are highly motile and only able to reabsorb bone by tightly adhering to peripheral bone surfaces (Robling *et al.*, 2006) and forming a specialized structure known as a sealing zone (Walsh *et al.*, 2006). Osteoclasts must first solubilize the mineralized matrix by pumping hydrogen ions across the characteristic ruffled border to acidify the isolated area. Once the mineralized matrix has been degraded, osteoclasts release lysosomal enzymes that continue the reabsorption of bone by further breakdown of the remaining organic component of the matrix. The amount of bone reabsorbed by osteoclasts is dependent on the lifespan of the osteoclasts (Walsh *et al.*, 2006). Osteoclasts work in tight coordination with the

other osteon cells, to model and remodel bones, during development and general maintenance of the skeleton.

Modeling and Remodeling of Bone

Bone is a dynamic structure that is constantly reabsorbed and formed. Each year 10% of bone is replaced and complete renewal of the skeleton occurs every 10 years (Cohen, 2006). Osteon cells work in a coordinated manner with one another in two distinct mechanisms, bone modeling and remodeling, which defines skeletal shape, maintains homeostatic levels of calcium and phosphate ions in the serum, and repairs any portion of 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, but not both within the same area. Activation of one or the other in a specific area allows for alteration of size, shape, and position of the bone within the growing tissue. Once the skeletal system has matured, bone modeling activity is reduced to a minimal level and bone remodeling takes over. However, in some bone diseases, bone modeling is reactivated, with serious consequences to the overall integrity of the affected bone(s) (Robling *et al.*, 2006).

In a mature skeleton, the process of bone remodeling dominates. During this process discrete packets of bone are removed and replaced with new bone. Bone remodeling is tightly regulated so that bone reabsorption and formation are coupled together in basic multicellular units. Coupling ensures that there is a balance between bone removal and new bone formation so that the reabsorbed area is completely filled in (Robling *et al.*, 2006). Many factors such as hormones, mechanical stresses, and growth factors influence the regulation and coordination of

the basic multicellular units (Harada and Rodan, 2003). Many bone diseases result from a disruption in the coupling process, which leads to an imbalance in either bone degradation or formation. The imbalance results in bones that become structurally compromised (Robling *et al.*, 2006).

Bone Pathologies

A loss in coupling of bone reabsorption and formation results in either an increase or a decrease of bone density. This alteration of bone density can result in various types of bone pathologies. Osteoporosis is one of the most prominent bone diseases, where there is a net loss of bone density. According to the National Osteoporosis Foundation, this disease is currently estimated to affect 10 million Americans, 8 million of which are women (NOF, 2007). It is projected by the year 2020, that one out of every two Americans over the age of 50 will have or be at risk for developing osteoporosis (Carmona, 2004). Fragility of bones due to the loss of bone density during in osteoporosis results in bones that are easily fractured; 1.5 million of the fractures seen annually are attributed to osteoporotic bones. Several risk factors for developing osteoporosis include: post-menopausal women, estrogen deficiencies, low levels of androgens, and prolonged use of certain medications such as corticosteroids (NOF, 2007).

Due to the observed clinical heterogeneity of osteoporosis, many ideas have been proposed as to the cause. The currently accepted cause is multiple pathogenic mechanisms converging on the skeleton, causing bone deterioration. Research indicates that skeletal deterioration is the result of complex interactions that regulate bone cell function, which includes both local and systemic regulators (Raisz, 2005).

The leading cause of osteoporosis is the estrogen decline at the onset of menopause, exemplifying the importance of estrogen in the maintenance and regulation of bone. Post-

menopausal osteoporosis is characterized by an increase in bone remodeling, where reabsorption far exceeds formation (Syed and Khosla, 2005). The increased bone remodeling is the result of an increase in formation and number of active bone multicellular units. Within these units, the reabsorptive phase is extended causing a larger pore in the bone that is too large for trailing osteoblasts to completely fill in. The overall result is an increase in the porosity of bone leading to a loss of bone density and strength (Riggs *et al.*, 2002).

The most prevalent form of secondary osteoporosis is glucocorticoid-induced. Attention to this type of osteoporosis has increased over the past several decades with the increased use of synthetic glucocorticoids in the treatment of inflammation (Mazziotti *et al.*, 2006). It is estimated that 30 million Americans are affected by diseases that are commonly treated with synthetic glucocorticoids (ACR, 2007). There are two phases of glucocorticoid-induced osteoporosis. The initial phase occurs early and is characterized by a rapid loss of bone density, possibly due to excessive bone reabsorption. The second phase is slower, characterized by a further progressive loss of bone density due to a decrease in new bone formation (Mazziotti *et al.*, 2006). The important role of steroids on bone maintenance is exemplified by both post-menopausal and glucocorticoid-induced osteoporosis.

Effect of Steroids

Sex steroids are known to play a major part in the development and maintenance of bone. The clinical observation of rapid bone loss at the onset of menopause exemplifies the role of estrogen in bone maintenance. Estrogen is able to exert its effects directly on osteon cells, through the presence of both nuclear estrogen receptors α and β (Braidman *et al.*, 1995; Kusec *et al.*, 1998; Braidman *et al.*, 2001). Estrogens function to increase bone formation by increasing osteoblast formation, differentiation, proliferation, and function (Riggs *et al.*, 2002). In

osteoclastic cells, 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 and an alteration in balance between the reabsorption and formation phases. The reabsorption phase is prolonged, the formation phase is shortened, and there is an increase in osteoclast recruitment that continues to prolong the reabsorptive phase, all contributing to an increase in reabsorption beyond what osteoblasts are able to fill in (Riggs *et al.*, 2002).

As with the estrogen receptors, the nuclear androgen receptor has also been detected in osteoblasts (Colvard *et al.*, 1989). One main action of testosterone is to enhance bone formation. Testosterone mediates this process by stimulating proliferation and inhibiting apoptosis of osteoblasts (Riggs *et al.*, 2002). Another main action of testosterone is to decrease bone reabsorption, the majority of this process is indirect due to aromatization of testosterone to 17 β -estradiol (Riggs *et al.*, 2002). However, it has been shown that 5 α -dihydrotestosterone interacts with osteoclasts to inhibit bone reabsorption directly (Pederson *et al.*, 1999).

Besides the sex steroids, glucocorticoids also exert their effects on osteon cells. In the presence of excessive amounts of glucocorticoids, rapid loss of bone density occurs. The rapid loss of bone density is due to increased osteoclastogenesis (Canalis *et al.*, 2004). Glucocorticoids increase osteoclastogenesis by the combination of increasing the expression of receptor activator of nuclear factor- κ B ligand, decreasing expression of receptor activator of nuclear factor- κ B ligand's decoy receptor, and increasing expression of colony-stimulating factor (Rubin *et al.*, 1998; Hofbauer *et al.*, 1999). 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) and also decreasing the number of osteoblasts and

osteocytes by inducing an apoptotic pathway (Ishida and Heersche, 1998). However, it has also been shown that when dexamethasone is added within a physiological concentration range to osteoblast-like cells lines in culture, differentiation into mature osteoblasts is promoted (Walsh *et al.*, 2001).

Local Production of Estrogen

In post-menopausal women most of the active sex steroids are synthesized by an intracrine mechanism in peripheral tissues (Labrie *et al.*, 2000). Peripheral target tissues capable of producing extragonadal estrogen include adipose tissue, osteoblasts, chondrocytes, vascular endothelial cells, aortic smooth muscle cells, and several areas within the brain. Estrogen produced by these tissues is more than likely to function in a paracrine or intracrine manner (Simpson *et al.*, 2000). The intracrine mechanism is a process that occurs in a specific target tissue or cell where inactive precursor hormone molecules are converted to active hormones and then utilized within that same tissue or cell, without being released into general circulation (Labrie *et al.*, 2000). The amount of active estrogen produced in this manner is relatively small, but at the local level is enough to invoke a significant biological response. However, peripheral tissues are unable to synthesize estrogen *de novo* and are dependent on circulating precursor molecules (Simpson *et al.*, 2000). In addition to the need of 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, 17 β -hydroxysteroid dehydrogenase, 3 β -hydroxysteroid dehydrogenase, and aromatase cytochrome p-450. One pathway for production of estrogen in peripheral tissues via these enzymes starts with conversion of estrone sulfate and dehydroepiandrosterone sulfate to estrone and dehydroepiandrosterone, respectively, by steroid sulfatase. Estrone is then oxidized to 17 β -

estradiol by 17 β -hydroxysteroid dehydrogenase. The pathway from dehydroepiandrosterone can synthesize 17 β -estradiol in several ways. Dehydroepiandrosterone can be converted to testosterone by 3 β -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase then metabolized by aromatase to 17 β -estradiol. Or testosterone can be converted by 17 β -hydroxysteroid dehydrogenase to androstenedione which can be converted to estrone by aromatase and then finally to 17 β -estradiol by 17 β -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). Once sexual maturity is reached, all of these enzymes are present, indicating a potentially important role of intracrinology in bone. The local production of estrogen by osteoblasts would influence local bone mass accumulation and maintenance at the onset of sexual maturity and continuing into adulthood (Van der Eerden *et al.*, 2004).

The Role of Steroid Sulfatase in Intracrinology

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 17 β -estradiol (Van der Eerden *et al.*, 2004). Estrone sulfate and dehydroepiandrosterone sulfate can be found in high circulating amounts (Pasqualini *et al.*, 1996; Labrie *et al.*, 2000); in fact, estrone sulfate in adults is found to be higher in concentration than the unconjugated form (Muir *et al.*, 2004). The enzymatic activity of steroid sulfatase is required for osteoblasts to be able to utilize sulfated precursors in the metabolism of estrogen (Muir *et al.*, 2004), by catalyzing the removal of the sulfate group from inactive sulfated precursors (Van der Eerden *et al.*, 2004).

Steroid Sulfatase

The sulfatase family of enzymes is highly conserved and functions to cleave sulfate ester bonds from a broad range of substrates. There are at least eight known human diseases that are associated with deficient sulfatases. The characteristic dark scaly skin of X-linked ichthyosis is caused by a deficiency in steroid sulfatase, resulting in an alteration of the cholesterol sulfate:cholesterol ratio 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 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 when purified to homogeneity from the microsomal fraction of human placenta yielded 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, with the polar catalytic domain buried in the “gill” of the “mushroom”, located near the membrane surface on the lumen side of the endoplasmic reticulum. Projecting out from the catalytic domain are two antiparallel helices of mainly of hydrophobic residues. 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 is 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 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). Kinetic analysis of purified steroid sulfatase indicates a K_m value of 72.75 μ M for estrone sulfate and K_m value of 9.59 μ M for dehydroepiandrosterone sulfate (Hernandez-Guzman *et al.*, 2001).

Steroid sulfatase was first identified in microsomes prepared from rat liver (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). Of all the known tissues containing steroid sulfatase activity, placenta has the highest activity (Pasqualini and Chetrite, 2005).

Steroid sulfatase is also suspected to have a role in supporting growth of hormone-dependent cancers, by providing a local supply of biologically active steroids. Estrogen levels found in breast tumors of post-menopausal women are 10 times higher than what is found in the plasma. Presumably, the high plasma concentration of circulating estrone sulfate is acting as an inactive steroid reservoir, which is activated by steroid sulfatase in the breast tumor and further metabolized to locally acting estrogen (Utsumi *et al.*, 1999). It has been suggested that steroid sulfatase could be used as an independent prognostic indicator of human breast carcinomas. Because, decreased expression of steroid sulfatase mRNA has been reported to correlate with relapse-free survival (Utsumi *et al.*, 1999; Utsumi *et al.*, 2000; Al Sarakbi *et al.*, 2006). This report further supports a role for steroid sulfatase in intracrinology.

It has also been found that there is no significant difference in the estrogen plasma concentration of post-menopausal women with osteoporotic fractures and those of a similar age without fractures. One explanation for the difference is possible *in situ* estrogen production by

bone cells (Reed *et al.*, 2005). Further support for this mechanism of estrogen production in bone has come from the detection of all the necessary enzymes in osteoblasts to produce estrogen from precursor steroids (Purohit *et al.*, 1992). 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). With the same cell line, another study showed that steroid sulfatase could utilize both estrone sulfate and dehydroepiandrosterone sulfate as substrates (Fujikawa *et al.*, 1997). The combined results of these two studies point to an important role of steroid sulfatase in local production of estrogen, from sulfated precursors, to support the maintenance of bone. The increasing realization of the importance of steroid sulfatase in both pathological and physiological mechanisms has increased attention to further understand the regulation of steroid sulfatase (Reed *et al.*, 2005).

Regulation of Steroid Sulfatase

Even with the importance of steroid sulfatase in pathological and physiological mechanisms very little is known about the regulation of expression or activity. Reed *et al.* indicates that in breast cancer cells, cytokines interleukin-6 and tumor necrosis factor- α have been shown to increase the level of steroid sulfatase activity (Reed *et al.*, 2005). The increase in activity can be due to either posttranslational modifications or by an increase in availability of substrates (Newman *et al.*, 2000). In endometrial stromal cells and vascular smooth muscle cells, inflammatory cytokine interleukin-1 β decreased both the activity and mRNA expression of steroid sulfatase (Matsuoka *et al.*, 2002; Nakamura *et al.*, 2003). In MCF-7 and MDA-MB-231 breast cancer cells, steroid sulfatase activity was increased by the presence of either basic fibroblast growth factor or insulin-like growth factor type-1 (Purohit *et al.*, 1992b). In addition to steroid sulfatase regulation by cytokines and growth factors, several steroids have also been

tested. Studies utilizing androgens and estrogens suggest that steroid sulfatase induction and regulation is in part controlled by these steroids (Lam and Polani, 1985; Moutaouakkil *et al.*, 1984). In promyelocytic cells, steroid sulfatase activity was induced and expression increased by either retinoids or 1,25-dihydroxyvitamin D₃ (Hughes *et al.*, 2001). The mechanisms of how these cytokines, growth factors, and steroids actually regulate steroid sulfatase activity and expression is yet to be determined (Reed *et al.*, 2005).

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. These compounds were made by replacing the sulfate group on estrone sulfate with some moiety that would mimic the sulfate group, but remain hydrolytically stable (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 irreversible inhibitor estrone-3-O-sulfamate, EMATE (Howarth *et al.*, 1994), was designed initially as a replacement estrone sulfate substrate however, it was found to be a potent site-directed irreversible inhibitor of steroid sulfatase. EMATE is not capable of being utilized as a therapeutic drug due to its estrogenicity. As a result, all subsequent irreversible inhibitors of steroid sulfatase have been designed as analogs of EMATE that are less estrogenic, but with a similar potency to the original parent compound (Li *et al.*, 1995; Selcer *et al.*, 1996; Selcer *et al.*, 1997; Kolli *et al.*, 1999; Reed *et al.*, 2005).

CHAPTER 2-PROPOSED RESEARCH

Objective

To determine the effect of sulfated steroids on growth of human osteoblast-like cells and to establish if steroid sulfatase is involved in mediating any response.

Specific Aims

1. To examine the role of sulfated steroids on growth of human osteoblast-like cells.
 - a. Hypothesis: The addition of sulfated steroids to basal culture medium will be sufficient to increase proliferation.
2. To examine the role of steroid sulfatase on growth of human osteoblast-like cells, in response to sulfated steroids.
 - a. Hypothesis: The response of human osteoblast-like cells to sulfated steroids is mediated by steroid sulfatase.

Model System

The model system utilized to carry out the specific aims was the human osteoblast-like MG-63 cell line. This particular cell line is a well established and well characterized cell line derived from a biopsy of a juxacortical osteosarcoma, in a fourteen-year-old Caucasian male (Heremans *et al.*, 1978). In culture, the MG-63 cell line grows morphologically uniform and rapidly, with a population doubling time of approximately 38 hours. As the cells reach a confluent state, no contact inhibition occurs and multiple layers can form on the culture dish surface. A disorganized hyperdiploid karotype of MG-63 cells was observed in 100% of the clones tested. Upon hormonal induction, the cells differentiate and exhibit characteristics of an osteoblast-like phenotype (Lajeunesse *et al.*, 1990) that closely resembles secretory, matrix producing osteoblasts (Cao *et al.*, 2003).

CHAPTER 3-MATERIALS AND METHODS

MG-63 Cell Culture

MG-63 cells (American Tissue Culture Collection-Manassas, VA) were cultured in 100mm tissue culture dishes with 10-12mL of sterile whole medium. Tissue culture plates were kept in a 37°C incubator with 5% carbon dioxide. Old medium was removed on a routine basis every 2-3 days and replaced with 10-12mL of fresh sterile whole medium.

MG-63 Whole Medium

Whole growth medium used for culturing the MG-63 cell line contained Hams F12 nutrient mixture (Invitrogen-Grand Island, NY), minimal essential medium (Invitrogen-Grand Island, NY), fetal bovine serum (Hyclone-Logan, UT), and a penicillin-streptomycin solution (Hyclone-Logan, UT).

The Hams F12 nutrient mixture was prepared in the following manner. 1.176g of sodium bicarbonate (Fisher Scientific-Fair Lawn, NJ) and one package of powdered Hams F12 nutrient mixture were mixed with 900mL of nanopure water. The pH of the solution was adjusted accordingly to fall within the pH range of 6.8-7.2 and then the total volume brought up to 1L.

To prepare minimal essential medium, 2.2g of sodium bicarbonate and one package of powdered minimal essential medium were dissolved in 900mL nanopure water. The pH was adjusted accordingly to fall within the pH range of 6.8-7.2 and then the final volume brought up to 1L.

The Hams F12 nutrient mixture and minimal essential medium were then combined together with 200mL of fetal bovine serum and 20mL of a penicillin-streptomycin solution and thoroughly mixed. The medium was filtered (0.2µM pore top filter) under the hood to sterilize and stored in the refrigerator.

Removal of Cells from 100mm Tissue Culture Dishes

To remove cells that have adhered to the bottom of a 100mm tissue culture dish, for either counting or re-plating, was done in the following manner. First, the old medium was removed and discarded. The 100mm dish was washed twice with 3mL of phosphate buffered saline (Sigma Chemical Company-St. Lewis, MO) and then enough trypsin-EDTA (Sigma Chemical Company-St. Lewis, MO) was added so that the bottom of the plate was completely covered. The trypsin-EDTA was allowed to remain on the cells for 15-30sec. Following removal of the trypsin-EDTA, the plate was left to sit for approximately 30sec to allow any residual trypsin-EDTA to continue lifting the cells from the plate. The trypsin-EDTA reaction was neutralized by addition of 3-4mL of whole medium. The sides of the plate were gently tapped to remove any cells that remained adhered. Once the cells were lifted from the bottom of the plate and suspended in whole medium, the 3-4mL of cell solution was transferred to a 15mL conical tube.

Cell Counting

To calculate the number of cells/mL, cells were first removed from a 100mm tissue culture dish. To a small test tube (12x75mm) the following was added 500 μ L of trypan blue (Sigma Chemical Company-St. Lewis, MO), 300 μ L of phosphate buffered saline, and 200 μ L of cell solution removed from the culture dish. The solution in the test tube was thoroughly mixed and viable cells counted using a hemacytometer. Viable cells were distinguished from dead cells which uptake trypan blue and appear bluish in color. The total number of viable cells was counted in a total of 10 squares on the hemacytometer grid. The total viable cell count was divided by 10 squares to determine the average number of cells/square, which was then

multiplied by the dilution factor 5 and by 10^4 to calculate the number of cells/mL in the cell solution removed from the tissue culture dish.

MG-63 Basal Medium

Basal medium that was utilized for the MG-63 cell line contained 0.5% charcoal-stripped fetal bovine serum, 49.25% Hams F12 nutrient mixture (Invitrogen-Grand Island, NY), 49.25% minimal essential medium (phenol-free) (Invitrogen-Grand Island, NY), and 1% penicillin-streptomycin solution (Hyclone-Logan, UT). After combining and thoroughly mixing the components, the medium was filtered (0.2 μ M pore top filter) under the hood to sterilize and stored in the refrigerator.

Charcoal-Stripped Fetal Bovine Serum

First, dextran-coated charcoal was prepared by combining 2mL of 2M Tris (Fisher Scientific-Fair Lawn, NJ) and 4mL of 0.1M EDTA (Fisher Scientific-Fair Lawn, NJ). The volume of the Tris and EDTA solution was brought up to 400mL with nanopure water and the pH adjusted to 7.5. Next, 0.2g of dextran (Sigma Chemical Company-St. Lewis, MO) was added and stirred on a stir plate for 1-2h at room temperature. Finally, 2g of Norit-A (activated, charcoal) (Sigma Chemical Company-St. Lewis, MO) was added and allowed to stir overnight at 4°C covered with foil. After stirring overnight, 25mL were removed and placed into 50mL conical tubes. These tubes were centrifuged at 2400rpm for 10min at 20°C (Fisher Scientific-Marathon 21K/BR) to pellet the dextran-coated charcoal. The supernatant was removed and fetal bovine serum poured over the pellet. The fetal bovine serum and dextran-coated charcoal were briefly mixed and placed in a 37°C water bath for 30min. Every 10min during the 30min incubation the tubes were removed and mixed. After the incubation, the tubes were centrifuged as described above and the supernatant poured onto another pellet of dextran-coated charcoal.

The conical tubes were mixed and incubated for another 30min as previously described. After the final incubation the tubes were re-centrifuged, the resulting supernatant poured into a clean 50mL conical tube, and stored in the freezer.

Standard Curve: 24-Well Plate

To assess cell proliferation in a 24-well plate a standard curve was generated. The curve was generated by seeding 256000, 128000, 64000, 32000, 16000, 8000, 4000, 2000, 1000, and 500cells/well in a 24-well plate by serial dilution. Cells were first removed from a pre-confluent 100mm culture dish and counted to determine the number of cells/mL. A stock solution of 512000cells/mL was prepared in whole medium and serially diluted to 500cells/well. Once all wells had been serially diluted, 500 μ L of whole medium was added to all wells to bring the total volume up to 1000 μ L. Plated along side of the serial dilution were two no cell control lanes that contained only 1000 μ L of whole medium. The 24-well plate was then placed into a 37°C incubator for 18h to allow the cells to adhere to the bottom of the plate. After the 18h incubation period, the medium was removed and a 24-well plate MTT assay ran. The average no cell absorbance was subtracted from the average absorbance and plotted against cell number to generate a 24-well plate standard curve.

MTT Assay: 24-Well Plate

After the incubation period of the assay, the medium was removed and replaced with 100 μ L of MTT (Dimethylthiazol tetrazolium bromide) (Sigma Chemical Company-St. Lewis, MO). Once all wells contained MTT the plate was returned to a 37°C incubator for 3h. Following the incubation the MTT was completely removed and replaced with 500 μ L of acidic isopropanol. The plate was placed onto a plate shaker for 10min to solubilize the converted product and then two 200 μ L samples from each well was removed and placed into a 96-well

microtiter plate. The 96-well plate was then read at an absorbance of 595nm using the BioRad Microplate reader (Model 3550).

Standard Curve: 6-Well Plate

A standard curve was generated to assess cell number in a 6-well plate. One 6-well plate was seeded in whole medium at the following cell densities 25000, 50000, 100000, 250000, 500000, and one million cells/well. Once the wells were seeded the volume of each was brought up to 2mL with whole medium. One 35mm tissue culture dish was plated with 2mL of whole medium only to serve as a no cell control. The plate was placed into a 37°C incubator overnight to allow the cells to adhere for 18h. After 18h, the plate was removed from the incubator and a 6-well plate MTT assay ran. The average no cell absorbance was subtracted from the average absorbance and plotted against cell number to generate a 6-well plate standard curve.

MTT Assay: 6-Well Plate

After the incubation period of an assay, the media was removed and replaced with 200µL of MTT. The plate was then returned to a 37°C incubator for 3h. Following the incubation, the plate was removed from the incubator and 1000µL acidic isopropanol was added to solubilize the converted product. The plate was then placed onto a plate shaker for 10min and then four 200µL samples were taken from each well and place into a 96-well microtiter plate. The 96-well plate was then read using the BioRad Microplate reader (Model 3550) set to read absorbance at 595nm.

Growth in Basal and Whole Media

Four 24-well plates were seeded at 25000cells/well with each plate representing a different time point (24, 48, 72, and 96h). Seeding of the 24-well plates was done in the following manner. First, cells were removed from a pre-confluent 100mm dish, counted, and a

stock solution of 25000cells/mL prepared in whole medium. 1mL of stock solution was placed into three lanes of wells on each of the four plates. Along side of the experimental lanes, were two wells containing 1mL of whole medium only to serve as no cell control wells. Once all plates were seeded, they were placed into a 37°C incubator overnight for 18h. After the incubation, the old medium was removed, and the wells washed with phosphate buffered saline. After the wash, 1mL of basal medium was added to all wells in the basal and addback lanes, and 1mL of whole medium was added to all wells in the growth lanes. The plates were then returned to the incubator for 48h. Following the 48h, the old medium was removed and replaced as follows, 1mL of basal medium was added to all wells in the basal lanes, and 1mL of whole medium was added to all wells in both the addback and growth lanes. Once the medium was replaced timing for the 24, 48, 72, and 96h time points began. Then a 24-well plate MTT assay was performed at each time point to assess cell number.

10µM Steroid Growth Assay

One 24-well plate was initially seeded at 25000cells/well by first removing cells from a pre-confluent 100mm dish, counting, and preparing a stock solution of 25000cells/mL in whole medium. 1mL of stock solution was then added to the appropriate number of lanes, and 1mL of whole medium only was added to two no cell control wells. Once the 24-well plate was seeded, it was placed into a 37°C incubator overnight for 18h. Following the 18h incubation, the old medium was removed, and all wells washed with phosphate buffered saline. After washing, the medium was replaced with 1mL basal medium, and returned to the incubator for a 48h starvation period. Following the starvation period, the old medium was removed, and steroid treatments added. The steroids tested included estradiol (Sigma Chemical Company-St. Lewis, MO), estrone sulfate (Steraloids-Newport, RI), dehydroepiandrosterone sulfate (Steraloids-Newport,

RI), progesterone (Sigma Chemical Company-St. Lewis, MO), testosterone (Nutritional Biochemicals-Cleveland, OH), dehydroepiandrosterone (Nutritional Biochemicals-Cleveland, OH), cortisol (Sigma Chemical Company-St. Lewis, MO), and dexamethasone (Sigma Chemical Company-St. Lewis, MO). 10 μ M steroid treatments were prepared in basal medium from 1mM steroid stock solutions (prepared in 100% ethanol). Basal lanes and no cell control lanes were prepared by adding 10 μ L/mL of 100% ethanol to basal medium, to control for the effect of ethanol on growth. Once the treatments had been added, the plates were returned to the incubator, and timing for the 72h time point began. Following the 72h incubation period, a 24-well plate MTT assay was performed to assess cell number.

Proliferation Dose-Response

One 24-well plate was seeded at 25000cells/well by first removing cells from a pre-confluent 100mm dish, counting, and preparing a 25000cells/mL stock solution in whole medium. 1mL of stock solution was then added to four lanes of wells, and 1mL of whole medium only was added to two no cell control wells. Once the 24-well plate was seeded, it was placed into a 37°C incubator for 18h. After the 18h incubation period, the old medium was removed, and all wells washed with phosphate buffered saline. Following the wash, 1mL of basal medium was added to the wells and returned to the incubator for a 48h starvation period. After 48h, the basal medium was removed and the treatments were added. Estradiol and estrone sulfate were prepared by serially diluting 1mM stock solutions (prepared in 100% ethanol) to concentrations of 10 μ M, 1 μ M, 0.1 μ M, and 0.01 μ M in basal medium. The concentration of ethanol within each dilution were taken into consideration, and adjusted for accordingly so that all dilutions contained an equal amount of ethanol. The dilutions were then added to the

appropriate lanes and returned to the incubator for 72h. A 24-well plate MTT assay was performed at 72h following the treatment addition to determine cell number.

Growth Assay with 1 μ M DU-14 or EMATE

The estrone sulfate growth assay with steroid sulfatase inhibitors followed the same set up as the 10 μ M steroid assay. Inhibitors used for the assays were either DU-14 or EMATE. Treatments for this assay were prepared in the following manner. No cell control wells and basal lanes contained 11 μ L/mL 100% ethanol added to basal medium. The basal+1 μ M inhibitor treatment was prepared in basal medium by adding 10 μ L/mL of 100% ethanol and 1 μ M inhibitor diluted from 1mM inhibitor stock solutions (prepared in 100% ethanol). The estrone sulfate treatment was prepared by adding 1 μ L/mL 100% ethanol to basal medium and diluting estrone sulfate to 10 μ M from a 1mM stock solution (prepared in 100% ethanol). The 10 μ M estrone sulfate+1 μ M inhibitor treatment was prepared by diluting a 1mM estrone sulfate stock solution to 10 μ M and also diluting a 1mM inhibitor stock solution to 1 μ M in basal medium. Once the treatments had been prepared and added to the appropriate lanes the plate was returned to the incubator and timing for the 72h time point began. Following 72h, a 24-well plate MTT assay was performed to assess cell number.

Growth Assay with 10 μ M DU-14

The estrone sulfate growth assay utilizing 10 μ M DU-14 was set up as the 1 μ M inhibitor assay. However, treatments for this assay were prepared as follows. No cell control wells and basal lanes contained 20 μ L/mL 100% ethanol added to basal medium. The basal+10 μ M DU-14 treatment was prepared in basal medium by adding 10 μ L/mL of 100% ethanol and 10 μ M DU-14 diluted from a 1mM stock solution (prepared in 100% ethanol). The estrone sulfate treatment was prepared by adding 10 μ L/mL 100% ethanol to basal medium and diluting estrone sulfate to

10 μ M from a 1mM stock solution (prepared in 100% ethanol). The 10 μ M estrone sulfate+10 μ M inhibitor treatment was prepared by diluting a 1mM estrone sulfate stock solution to 10 μ M and also diluting a 1mM inhibitor stock solution to 10 μ M in basal medium.

Growth Assay with 100nM ICI 182,780

The growth assay utilizing the estrogen receptor inhibitor ICI 182,780 (Tocris Bioscience-Ellisville, MO) was set up in the following manner. One 24-well plate was initially seeded at 25000cells/well, by removing cells from a pre-confluent 100mm tissue culture dish, counting, and preparing a 25000cells/mL stock solution in whole medium. 1mL of stock solution was then added to six lanes of wells, and 1mL of whole medium only was added to two no cell control wells. Once the plate was seeded, it was placed into a 37°C incubator overnight for 18h. After 18h, the old medium was removed, and the wells washed with phosphate buffered saline. 1mL of basal medium was then added to the wells, and the plates returned to the incubator for 48h. Following the 48h starvation period, the basal medium was removed, and replaced with the appropriate 1mL of treatment solution. The treatments were prepared in the following manner. No cell control wells and basal lanes contained 11 μ L/mL of 100% ethanol prepared in basal medium. The basal+inhibitor treatment was prepared by diluting a 1mM ICI 182,780 stock solution to 10 μ M (prepared in 100% ethanol) and then to 100nM in basal medium and adding 10 μ L/mL 100% of ethanol. For the estrone sulfate and 17 β -estradiol treatments 1mM stock solutions were diluted to 10 μ M in basal medium plus 1 μ L/mL of 100% ethanol was added. The estrone sulfate+ and 17 β -estradiol+ICI 182,780 treatments were prepared by diluting 1mM steroid stock solutions to 10 μ M and also diluting a 1mM stock ICI 182,780 solution to 10 μ M in ethanol and then to 100nM in basal medium. Once the treatments had been added the

plate was returned to a 37°C incubator for 72h. After the 72h incubation, a 24-well plate MTT assay performed to assess the resulting cell number.

Steroid Sulfatase Activity in Microsomes

Steroid sulfatase activity in MG-63 microsomes was assessed in duplicate 13x100mm test tubes, set up as follows. To the DU-14 and EMATE treatment tubes the following was added 100µL 6,7-³H(E1S) prepared at approximately 100,000dpm/100µL (PerkinElmer-Boston, MA), 100µL 1µM E1S (final concentration), and 100µL 1µM appropriate inhibitor (final concentration). To the no inhibitor treatment and no microsome control tubes 100µL 6,7-³H(E1S) prepared at approximately 100,000dpm/100µL, 100µL 1µM E1S (final concentration), and 100µL ethanol control (1µL/1mL 100% ethanol) was added. All dilutions of 6,7-³H(E1S), 1mM estrone sulfate stock, 1mM inhibitor stock solutions, and ethanol control were prepared in Tris-HCl buffer to yield the appropriate final concentration in all reaction tubes. Once all solutions were added, the test tubes were covered, and placed into a 37°C water bath, while the microsomes were prepared.

To prepare the microsomes, 2000µL of Tris-HCl buffer was added to a Beckman tube containing a microsomal pellet and then transferred to a beaker. The microsomes were then homogenized with the Tissue Tearor (Biospec-Model 985370) by four 30sec on, 15sec off intervals. Once homogenized, 200µL of the microsome solution was added to all test tubes, except for the no microsome control tubes, to which 200µL Tris-HCl buffer was added instead of the microsomes. The test tubes were then incubated in a 37°C water bath for 1h. At the end of the 1h incubation, an organic extraction was performed to assess steroid sulfatase activity. The resulting conversion rate was adjusted for total protein concentration determined by a BCA™ protein assay performed on the remaining microsome solution.

Microsome Preparation

To make microsomes five pre-confluent 100mm tissue culture plates were utilized. The medium was removed, and each plate washed with 3mL of phosphate buffered saline. Following the wash, 2.5mL of Hank's balanced salt solution (Cellgro-Herndon, VA) was added to each plate. The plates were scraped with a cell scraper, transferred to a 15mL conical, and centrifuged at 4°C for 10min at 1000rpm (Fisher Scientific-Marathon 21K/BR). After centrifugation the supernatant was removed, the pellet re-suspended in 5mL of Tris-Sucrose buffer, and transferred to a beaker. The re-suspended pellet was homogenized with the Tissue Tearor (Biospec-Model 985370) by four 30sec on, 15sec off intervals. The homogenized solution was then transferred back into the 15mL conical, and centrifuged at 4°C for 10min at 2400rpm. Following centrifugation, the resulting supernatant was transferred to a prechilled 11x60mm Beckman tube, and ultracentrifuged at 31200rpm for 1h at 4°C (Beckman-Ultracentrifuge XL-70). Finally, the supernatant was completely removed, 200µL Tris-HCl buffer added to the pellet, and stored at -80°C.

Toluene Organic Extraction

To assess conversion of 6,7-³H(E1S) to the unconjugated 6,7-³H(E1) form an organic toluene extraction was utilized. 3mL of toluene (Fisher Scientific-Fair Lawn, NJ) was added to the experimental 13x100mm test tubes, and vortex for 1min. The tubes were then centrifuged at 2500rpm for 10min at 24°C (Fisher Scientific-Marathon 21K/BR). From the resulting organic phase, duplicate 1mL samples were taken, and placed into scintillation vials. 5mL of Ultimate Gold scintillation fluid (PerkinElmer-Shelton, CT) was then added to each vial, and the amount of radioactivity assessed by scintillation counting (Packard-Liquid Scintillation Analyzer Tri-Carb 2100TR).

BCA™ Protein Assay

Protein concentration was determined by the Pierce Protein Assay Kit (Pierce Chemical Co., Rockford, IL). First, a standard curve of bovine serum albumin was generated. The following concentrations were prepared in duplicate from a 2mg/mL bovine serum albumin stock solution, 150, 100, 50, 25, 12.5, and 0µg/tube so that the final volume of each 13x100mm tube totaled 100µL. The microsome samples were prepared in duplicate by placing 50µL nanopure water and 50µL sample into 13x100mm test tubes. To all tubes 2mL of working reagent was added. Working reagent was prepared by mixing 50 parts Reagent A with 1 part Reagent B. The tubes were briefly vortex, covered, and then placed into a 37°C water bath for 30min. After the incubation, the tubes were removed from the water bath and allowed to cool to room temperature before reading at 562nm on the spectrophotometer (Thermo Spectronics-Genesys 20). The average 0µg/tube absorbance was subtracted from the average absorbance and plotted against concentration to generate a standard curve. The average 0µg/tube absorbance was also subtracted from the average absorbance of the samples, and protein concentration calculated from the standard curve.

Whole Cell Conversion Assay

Initially, one 6-well plate was seeded at 250000cells/well in whole medium. 2mL of whole medium only was also added to two 35mm culture plates to serve as no cell control wells. The plates were then placed into a 37°C incubator for 18h to allow the cells to adhere. After 18h, the plates were removed from the incubator, the old medium removed, and each well washed with phosphate buffered saline. The medium was then replaced with the appropriate treatment. Treatments were prepared as follows. The DU-14 and EMATE treatments were prepared in 2mL of 6,7-³H(E1S) 0.5% charcoal-stripped basal medium (approximately

100,000dpm/mL) that contained a final concentration of 1 μ M E1S and 1 μ M of the appropriate inhibitor, both diluted from 1mM stock solutions. The no inhibitor and no cell control treatments were prepared in 2mL of 6,7-³H(E1S) 0.5% charcoal-stripped basal medium (approximately 100,000dpm/mL) that contained a final concentration of 1 μ M E1S diluted from a 1mM steroid stock, and 1 μ L/1mL 100% ethanol. Once the treatments were added to the appropriate wells the plates were returned to the incubator for 24h. Following the incubation period, the medium was removed from each well and placed into 13x100mm test tubes. From each test tube two 500 μ L samples were removed and placed into separate 13x100mm test tubes, and an organic toluene extraction performed on the 500 μ L samples of medium. To the remaining 6-well plate and 35mm dishes a 6-well MTT assay was performed to adjust the resulting conversion rate for total cell number.

Statistical Analysis

All statistical analysis was performed using GraphPad-Prism4 software (San Diego, CA). Details of the statistical analysis performed for each experiment can be found in the results section.

CHAPTER 4-RESULTS

Validation of Growth Assay

In order to establish basal growth conditions for the MG-63 cell line, a series of preliminary experiments were run to determine the minimal amount of charcoal-stripped fetal bovine serum needed to maintain minimal growth (data not shown). It was determined that 0.5% charcoal-stripped fetal bovine serum maintained cells, while not promoting growth. Figure 2 shows the results of growth experiments conducted in basal, addback, and growth media. A two-way repeated measures ANOVA resulted in a significant interaction value ($p < 0.001$). Therefore, separate one-way repeated measures ANOVAs were utilized to determine differences among treatment groups at each time point. At all time points, addback and growth media resulted in significantly higher growth than basal medium, and growth medium resulted in higher growth than addback medium (24h: $F=41.24$; 2,8 df; $p < 0.001$; 48h: $F=87.83$; 2,8 df; $p < 0.001$; 72: $F=606.6$; 2,8 df; $p < 0.001$; 96h: $F=1704$; 2,8 df; $p < 0.001$ and Newman-Keuls Multiple Comparison Tests for each time point; $p < 0.05$). Therefore, 0.5% charcoal-stripped basal medium was established to be a good baseline for growth experiments with potential growth-causing agents.

Growth of MG-63 Cells in the Presence of Various Steroids

Figure 3 shows the results of MG-63 growth experiments using various steroids and sulfated steroids. One-way repeated measures ANOVA revealed significant differences among treatments ($F=6.475$; 8,26 df; $p < 0.001$). Growth in treatments with estrone sulfate and estradiol were significantly higher than with basal (Newman-Keuls Multiple Comparison Test; $p < 0.05$). However, growth in treatments with testosterone, dehydroepiandrosterone, dehydroepiandrosterone sulfate, dexamethasone, progesterone, and cortisol were not

significantly different from growth in basal medium. It should be noted that dehydroepiandrosterone sulfate, cortisol, and progesterone resulted in higher growth than basal, but not significantly so. The dexamethasone treatment was found to have a decrease in growth below basal, but this difference was also not significant.

Growth with Estradiol, Estrone Sulfate, and Dehydroepiandrosterone Sulfate

Because the previous experiment indicated that dehydroepiandrosterone sulfate, estrone sulfate, and estradiol might stimulate MG-63 growth, an experiment was run to test these compounds separately (Fig. 4). A one-way repeated measures ANOVA revealed differences in growth among treatments ($F=13.25$; 3,19 df; $p<0.001$). Significantly higher growth was found in estradiol and estrone sulfate treatments than in basal medium (Newman-Keuls Multiple Comparison Test; $p<0.05$). Treatment with estrone sulfate resulted in significantly higher growth than treatment with estradiol. Growth in dehydroepiandrosterone sulfate was found not to be significantly different from growth in basal medium.

Dose-Response to Estradiol and Estrone Sulfate

Since growth was significantly increased in the presence of estrone sulfate and estradiol in the previous experiment, proliferation dose-responses were performed for each steroid (Fig. 5). Treatment with estradiol showed growth above baseline at $10\mu\text{M}$ and $1\mu\text{M}$ (Fig. 5A). Estrone sulfate only showed significant growth at the highest concentration $10\mu\text{M}$ (Fig. 5B).

Effect of Steroid Sulfatase Inhibitors on Growth

Given that estrone sulfate was shown to stimulate growth of MG-63 cells, several experiments were performed utilizing steroid sulfatase inhibitors, in an attempt to determine if growth is mediated via steroid sulfatase.

Figure 6 shows the results of MG-63 growth experiments with estrone sulfate and DU-14 as treatments. Differences among treatment groups were found, using a repeated measures one-way ANOVA ($F=12.41$; 3,11 df; $p<0.01$). Significantly higher growth was found in estrone sulfate and estrone sulfate+DU-14 treatments, in comparison to basal and basal+DU-14 treatment (Newman-Keuls Multiple Comparison Test; $p<0.05$). Growth in basal and basal+DU-14 treatments were not significantly different from one another, and growth in estrone sulfate and estrone sulfate+DU-14 treatments were not significantly different from each other.

In the previous experiment, DU-14 did not inhibit growth at a low concentration ($1\mu\text{M}$). Figure 7 reveals the effect of an increased concentration ($10\mu\text{M}$) of DU-14 on MG-63 growth. Differences among means of the treatment groups were found by a repeated measures one-way ANOVA ($F = 14.10$; 3,27 df; $p<0.001$). Basal, estrone sulfate, and estrone sulfate+DU-14 treatments were found to be significantly higher in growth than the basal+DU-14 treatment (Newman-Keuls Multiple Comparison Test; $p<0.05$). The estrone sulfate treatment was significantly higher in growth than all other treatments; however, growths in basal and estrone sulfate+DU-14 treatments were not statistically different from one another.

MG-63 growth with estrone sulfate and EMATE was also tested (Fig. 8). A repeated measures one-way ANOVA revealed differences among treatment groups ($F=27.97$; 3,11 df; $p<0.001$). Estrone sulfate and estrone sulfate+EMATE treatments resulted in a significant increase in growth in comparison to basal and basal+EMATE treatments, and estrone sulfate+EMATE treatment was found to be significantly higher in growth than the estrone sulfate treatment alone (Newman-Keuls Multiple Comparison Test; $p<0.05$). Growths in basal and basal+EMATE treatments were not statistically different from one another.

Effect of an Estrogen Receptor Antagonist ICI 182,780 on Growth

Several estrogenic compounds from previous experiments indicated stimulation of MG-63 growth. The effect of the estrogen receptor antagonist, ICI 182,780, on MG-63 growth, in the presence or absence of estrone sulfate or estradiol were tested in order to determine if this effect was mediated by the estrogen receptor (Fig. 9). A repeated measures two-way ANOVA revealed differences among the treatment groups. Estradiol and estrone sulfate significantly increased growth over basal ($p < 0.05$) and this effect was eliminated with ICI 182,780.

Effect of Steroid Sulfatase Inhibitors on Steroid Sulfatase Activity

Steroid sulfatase activity was assessed with and without the addition of steroid sulfatase inhibitors, DU-14 and EMATE, in MG-63 microsomes and whole cell estrone sulfate conversion assays. Figure 10 represents the effect of steroid sulfatase inhibitors on steroid sulfatase activity in microsomes prepared from MG-63 cells. Differences among the treatments were determined by a one-way ANOVA ($F=99.45$; 2,8 df; $p < 0.001$). Steroid sulfatase activity in the presence of either inhibitor was found to be significantly decreased from steroid sulfatase activity found without an inhibitor (Newman-Keuls Multiple Comparison Test; $p < 0.05$). However, no statistical difference of steroid sulfatase activities was found between DU-14 and EMATE.

Figure 11 shows the effect of steroid sulfatase inhibitors on steroid sulfatase activity in MG-63 whole cells. A one-way ANOVA revealed differences among treatment groups ($F=39.59$; 2,8 df; $p < 0.001$). Steroid sulfatase activity in the presence of DU-14 or EMATE treatments were found to be significantly decreased in comparison to the steroid sulfatase activity found in the absence of an inhibitor (Newman-Keuls Multiple Comparison Test; $p < 0.05$). No significant difference of steroid sulfatase activities was found between DU-14 and EMATE.

Determination of Cell Number

A MG-63 24-well plate standard curve was developed to determine cell number at the end of growth assays. Figure A-1 is one representative MTT 24-well plate standard curve. The resulting equation of the best-fit nonlinear trendline for this graph was $y = 4e^{-6}x + 0.0092$, $r^2 = 0.9986$.

Figure A-2 is another representative MTT standard curve developed to determine cell number from a 6-well plate so that normalization of the whole cell estrone sulfate conversion assays could be performed. The resulting equation of the best-fit nonlinear trendline was $y = 2e^{-6}x + 0.0431$, $r^2 = 0.9997$.

Determination of Protein Concentration

To determine and adjust for total protein concentration in the microsomes used for the estrone sulfate conversion assays a BCA standard curve was generated (Fig. A-3). The resulting equation of the best-fit linear trendline was $y = 0.0134x + 0.1032$, $r^2 = 0.9896$.

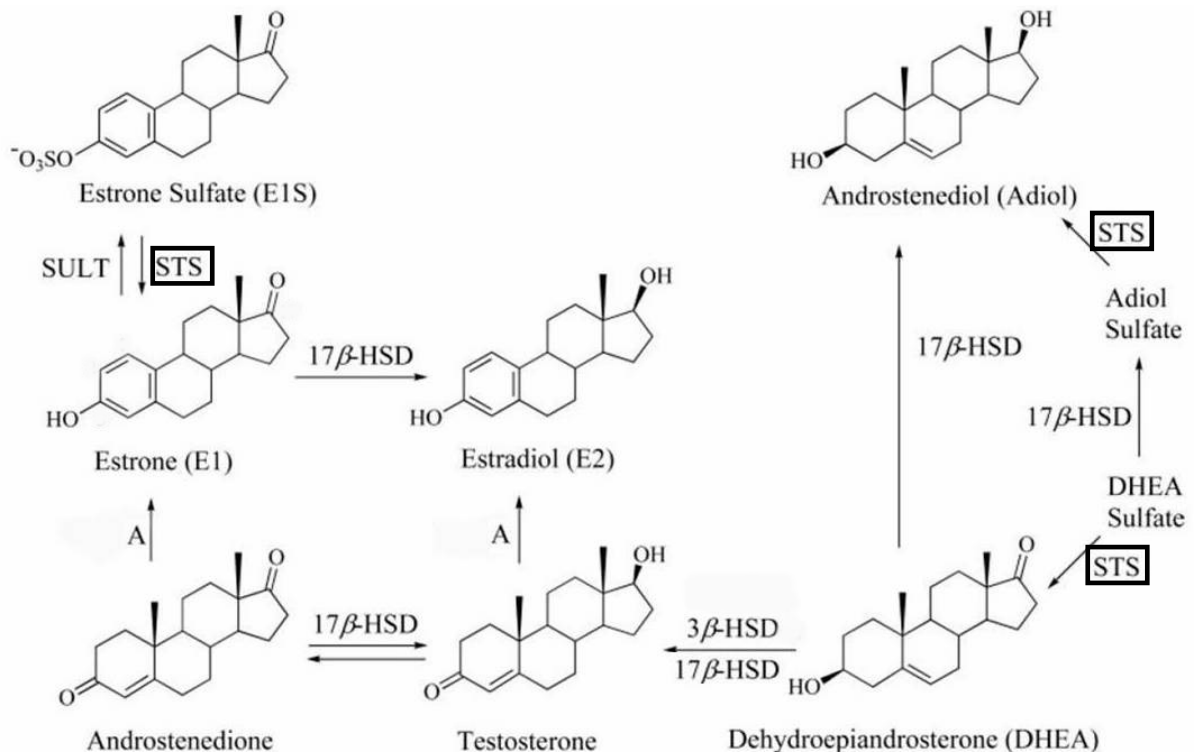


Figure 1: Biosynthetic Pathway of Estrogens and Androgens.

Abbreviations: SULT=Sulfotransferase, STS=Steroid Sulfatase, 17β-HSD=17β-Hydroxysteroid

Dehydrogenase, A=Aromatase Cytochrome p-450, and 3β-HSD=3β-Hydroxysteroid

Dehydrogenase. Modified from Reed *et al.* 2005.

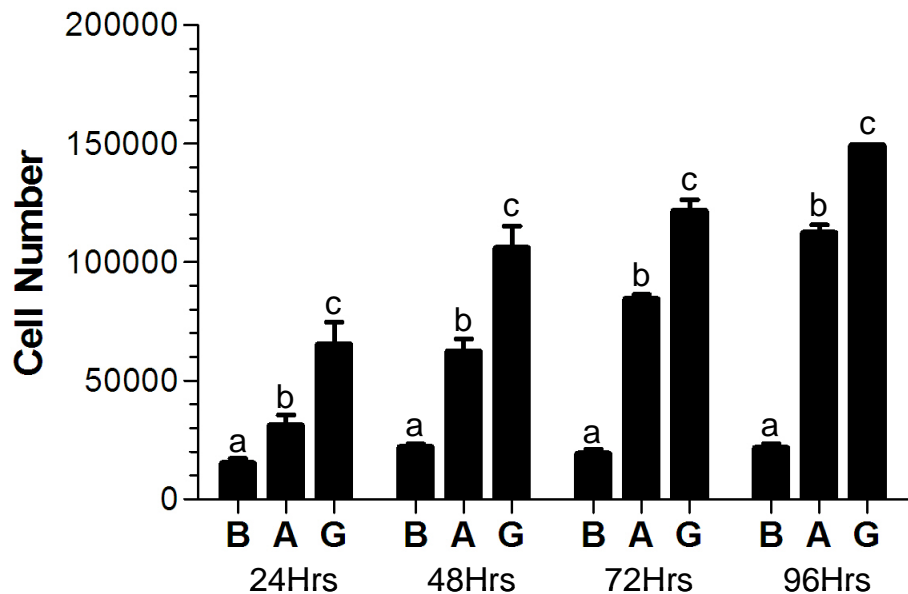


Figure 2: MG-63 Growth in Various Media.

Cells were initially seeded and allowed to adhere for 18h. Basal and addback wells were then starved for 48h in 0.5% charcoal-stripped basal medium, while growth wells were incubated in whole medium. After the starvation period, basal wells were treated with 0.5% charcoal-stripped basal medium, while addback and growth wells were treated with whole medium. At 24, 48, 72, and 96h following the treatment addition, a 24-well plate MTT assay was performed to determine cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means within each time point. Abbreviations: B = Basal, A = Addback, and G = Growth. Data represent three independent experiments, each run in quadruplicate.

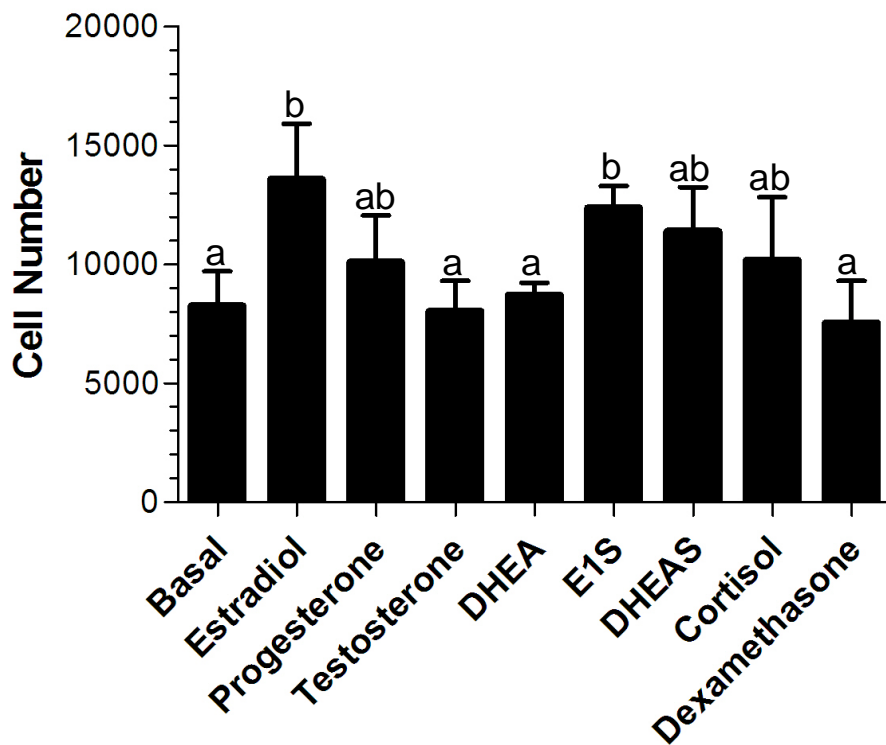


Figure 3: MG-63 Proliferation in the Presence of Steroid Treatments.

Cells were seeded and allowed to adhere for 18h. Then all treatment wells were starved for 48h in 0.5% charcoal-stripped basal medium. Following the starvation period, 10 μ M steroid treatments or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added and incubated with the cells for 72h. After the incubation period, a 24-well plate MTT assay was performed to assess cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means. Abbreviations: DHEA=Dehydroepiandrosterone, E1S=Estrone sulfate, and DHEAS=Dehydroepiandrosterone sulfate. Data represent three independent experiments, each run in duplicate.

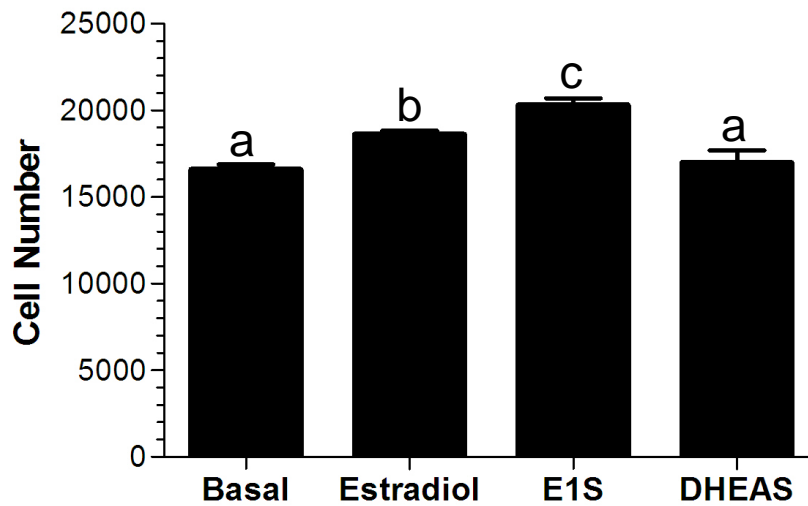


Figure 4: MG-63 Proliferation in the Presence of Sulfated Steroids and Estradiol.

Cells were allowed to adhere for 18h following initial seeding. After 18h the whole medium was replaced with 0.5% charcoal-stripped basal medium for 48h. Following the starvation period, the appropriate 10 μ M steroid treatment or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added to the cells and incubated for 72h. Then a 24-well plate MTT assay was performed to determine cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means. Abbreviations: E1S=Estrone sulfate and DHEAS=Dehydroepiandrosterone sulfate. Data represent five independent replicates, each run in quadruplicate.

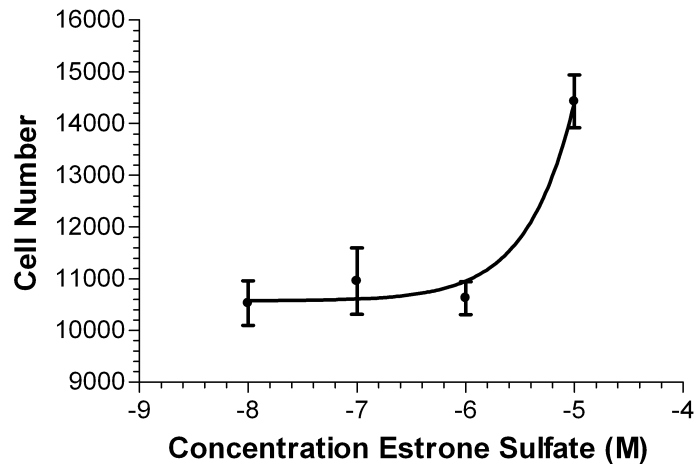
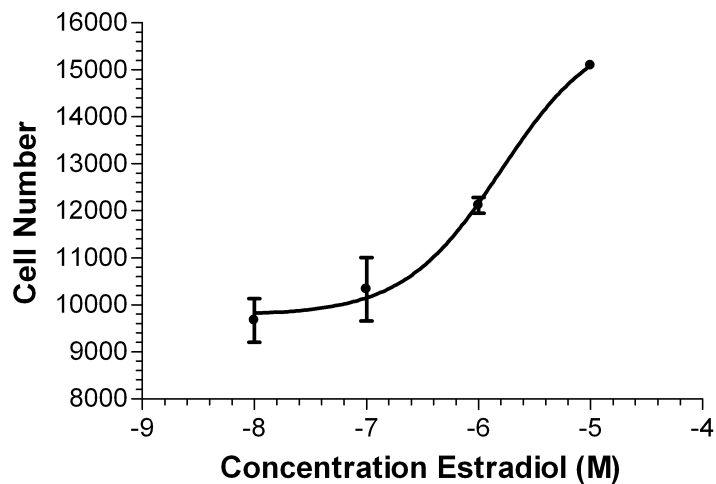


Figure 5: MG-63 Proliferation Dose-Response to Estradiol and Estrone Sulfate.

Cells were seeded and allowed to adhere for 18h. Once the cells had adhered, all treatment wells were starved for 48h in 0.5% charcoal-stripped basal medium. The various concentrations were prepared by serial dilution in 0.5% charcoal-stripped basal medium, added to the appropriate wells, and incubated for 72h. Then a 24-well plate MTT assay was performed to assess cell number. Points represent mean±SEM of for each steroid concentration. Data represent two independent replicates for estradiol and three independent replicates for estrone sulfate, each run in triplicate.

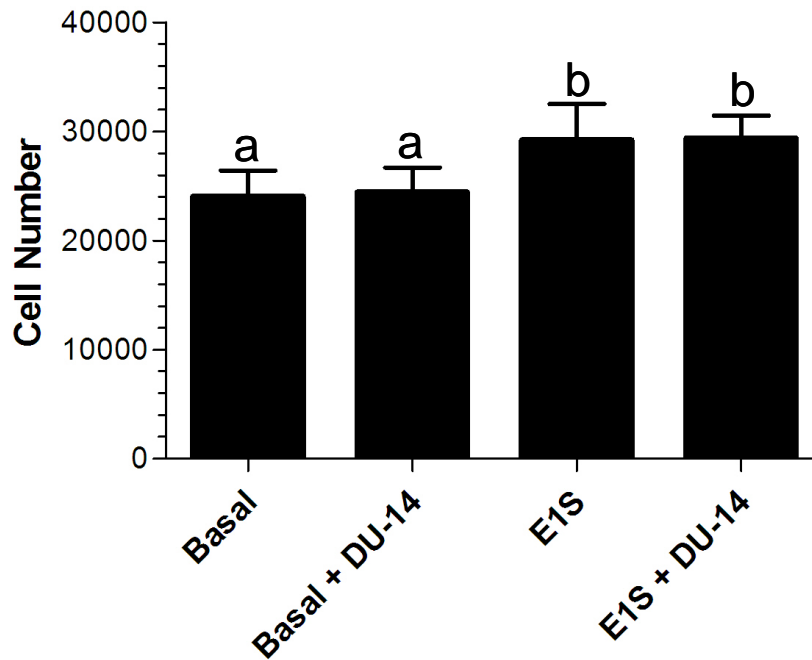


Figure 6: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and DU-14.

Cells were initially seeded and allowed to adhere for 18h. Treatment wells were then starved for 48h in 0.5% charcoal-stripped basal medium. Following the starvation period, the appropriate combination of 10 μ M estrone sulfate, 1 μ M DU-14, or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added to the treatment wells and incubated for 72h. After the incubation, a 24-well plate MTT assay was performed to determine cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means.

Abbreviations: E1S=Estrone sulfate. Data represent three independent experiments, each run in quadruplicate.

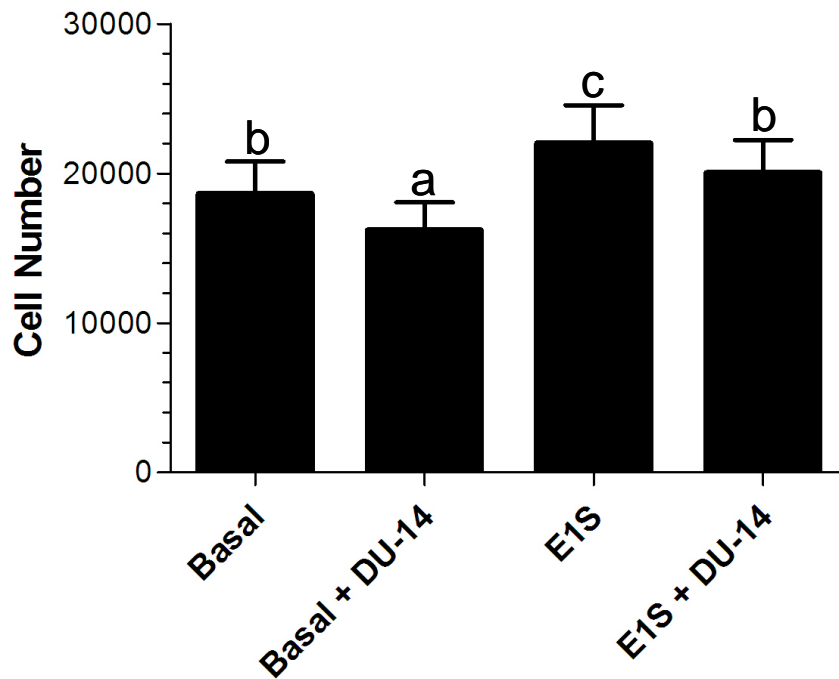


Figure 7: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and DU-14.

Cells were allowed to adhere for 18h following initial seeding. Treatment wells were then starved for 48h in 0.5% charcoal-stripped basal medium. Following the starvation period, the appropriate combination of 10 μ M estrone sulfate, 10 μ M DU-14, or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added to the treatment wells and incubated for 72h.

A 24-well plate MTT assay was then performed to determine cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means. Abbreviations:

E1S=Estrone sulfate. Data represent seven independent replicates, each run in quadruplicate.

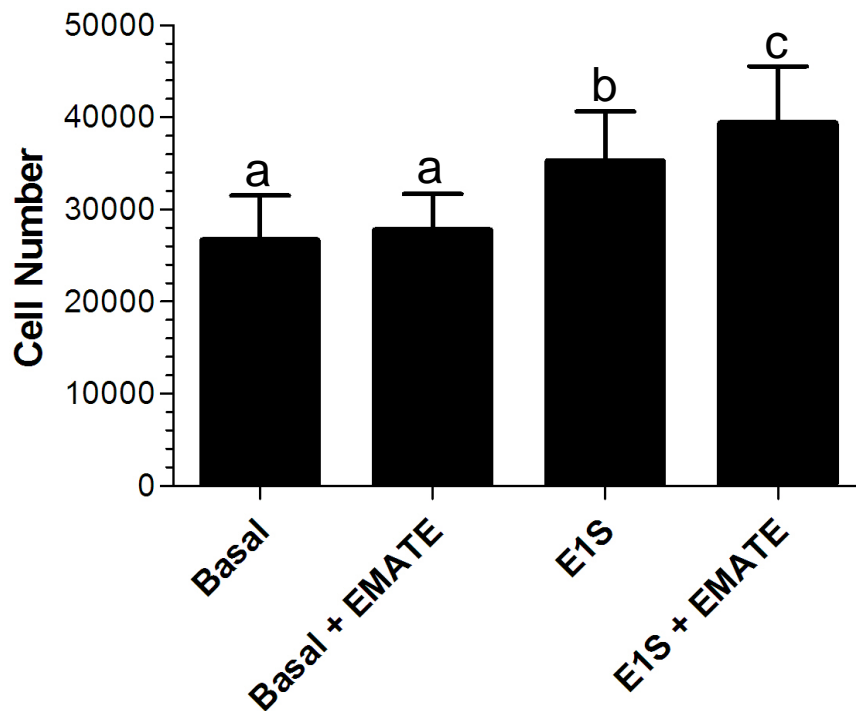


Figure 8: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate and EMATE.

Cells were seeded and allowed to adhere for 18h. After 18h, the whole medium was replaced with 0.5% charcoal-stripped basal medium for 48h. Following the starvation period, the appropriate combination of 10 μ M estrone sulfate, 1 μ M EMATE, or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added to the treatment wells and incubated for 72h. Then a 24-well plate MTT assay was performed to assess cell number. Bars represent the mean \pm SEM and different letters indicate significantly different means. Abbreviations: E1S=Estrone sulfate. Data represent three independent experiments, each run in quadruplicate.

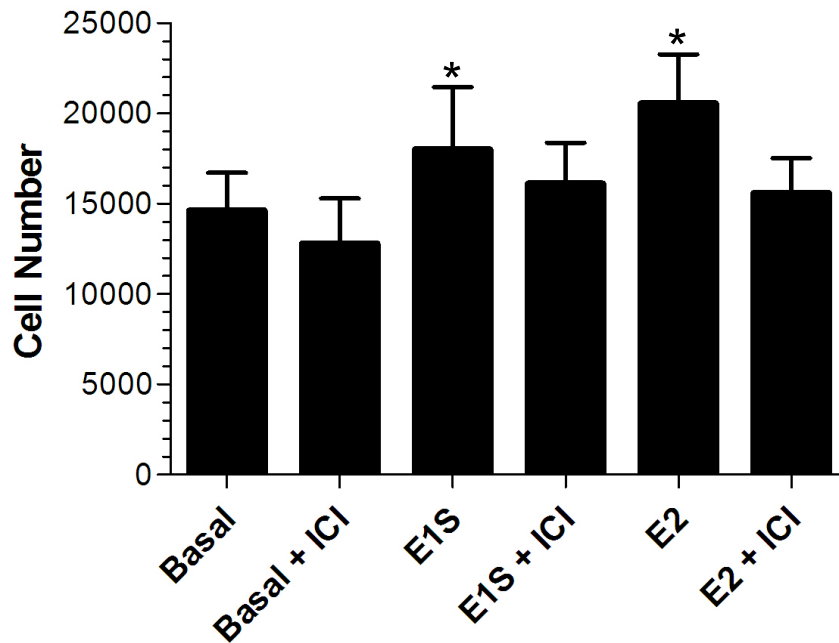


Figure 9: MG-63 Proliferation in the Presence or Absence of Estrone Sulfate, Estradiol, and ICI 182,780.

Cells were seeded and allowed to adhere for 18h. Treatment wells were then starved for 48h in 0.5% charcoal-stripped basal medium. Following the starvation period, the appropriate combination of 10 μ M estrone sulfate, 10 μ M estradiol, 100nM ICI 182,780, or ethanol control, prepared in 0.5% charcoal-stripped basal medium, were added to the treatment wells and incubated for 72h. Then a 24-well plate MTT assay was performed to assess cell number. Bars represent the mean \pm SEM and * indicates significantly different from basal. Abbreviations: ICI=ICI 182,780, E1S=Estrone sulfate, and E2=Estradiol. Data represent three independent replicates, each run in triplicate.

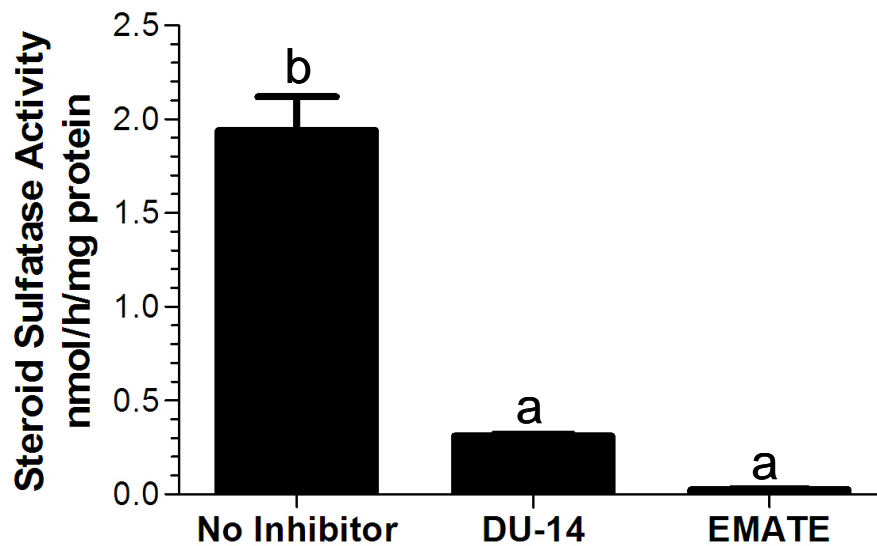


Figure 10: Inhibition of Steroid Sulfatase Activity in MG-63 Microsomes by DU-14 and EMATE.

MG-63 microsomes were incubated with 6,7-³H(E1S), 1 μ M estrone sulfate, and 1 μ M inhibitor or ethanol control for one hour. Following the incubation, a toluene organic extraction was performed to assess the amount of conversion from 6,7-³H(E1S) to 6,7-³H(E1). The conversion rate was adjusted for total protein concentration determined by a BCATM protein assay. Bars represent the mean \pm SEM and different letters indicate significantly different means. Data represent three independent experiments, each run in duplicate.

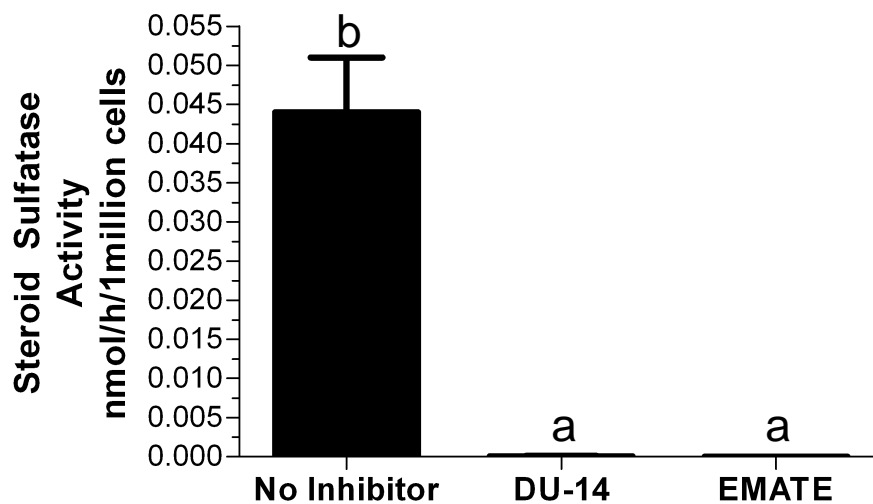


Figure 11: Inhibition of Steroid Sulfatase Activity in MG-63 Whole Cells by DU-14 and EMATE.

Cells were seeded and allowed to adhere for 18h. Cells were then treated with 6,7-³H(E1S) 0.5% charcoal-stripped basal media, 1μM estrone sulfate, and 1μM inhibitor or ethanol control and incubated for 24h. Following the incubation period, a toluene organic extraction was performed on the medium to assess the amount of conversion from 6,7-³H(E1S) to 6,7-³H(E1). The conversion rate was adjusted for total cell number determined by a 6-well plate MTT assay that was performed on the experimental plate, once the medium had been removed for analysis. Bars represent the mean ± SEM and different letters indicate significantly different means. Data represent three independent experiments, each run in duplicate.

CHAPTER 5-DISCUSSION

Growth of bone cells is known to be stimulated by estrogens, as exemplified by rapid loss of bone at the onset of menopause (Pouilles *et al.*, 1995; NOF, 2007). However, it is unknown if sulfated steroids can stimulate bone growth. The role of sulfated steroids in the growth of bone is important because post-menopausal women have high levels of sulfated steroids in their blood (Pasqualini *et al.*, 1996). This study tested the effect of sulfated steroids on growth of a bone cell line (MG-63) and found that estrone sulfate stimulated growth. Additionally, inhibitors of the enzyme that converts sulfated steroids into their active forms (steroid sulfatase), blocked estrone sulfate-stimulated growth. Thus, steroid sulfatase may be an important agent providing active steroids needed for bone growth in post-menopausal women.

In order to assess the effect of sulfated steroids on growth, an assay was needed that could test for growth stimulation by a given compound. A basal, addback, growth (BAG) assay was developed for this purpose. Criteria for a successful assay were selected to be as follows: 1) Basal growth should remain constant and at a low level over time, and 2) Addback growth should be higher than basal growth for the time points used, showing that growth is possible under the conditions of the experiment. Furthermore, as an internal control for the assay, growth medium is an indication of the maximal growth possible and therefore should be the highest among the groups. The test statistic for the developed assay is that growth in a particular treatment will be different from that in basal medium alone. A series of experiments were performed initially in (96-well plates) testing basal medium containing various concentrations of charcoal-stripped fetal bovine serum, including 10%, 5%, 2.5%, and 0.5%, over a 96h period. After testing of the four different concentrations of charcoal-stripped fetal bovine serum, it was found that 0.5% charcoal-stripped fetal bovine serum at all time points better met the established

criteria for basal, addback, and growth media treatments than the other three concentrations. The 0.5% charcoal-stripped fetal bovine serum basal medium sustained the cells, did not promote growth over time, and resulted in significantly lower growth than addback and growth media treatments. It was also found that at 72h there was a significant difference in growth between basal and addback treatments. This area of difference is the workable range for the growth assays; basal treatment indicates the minimal amount of growth, and addback represents the maximal amount of growth that can occur once the cells have been starved with basal medium. Therefore, it was important to find a range large enough to detect any growth differences that might occur with the potentially growth-stimulating treatments. From this assay it was determined that for all future experiments basal medium should contain 0.5% charcoal-stripped fetal bovine serum and exposure to the test compounds should last for 72h.

Various steroid treatments were tested to determine growth stimulating effects. The initial steroids tested included 17β -estradiol, progesterone, testosterone, dehydroepiandrosterone, estrone sulfate, dehydroepiandrosterone sulfate, cortisol, and dexamethasone. These steroids were chosen as treatments based on the following information. Sex steroids are known to have important roles in the development and maintenance of bone. The positive effects of estrogen on bone development and maintenance include increasing osteoblast formation, differentiation, proliferation, and function (Riggs *et al.*, 2002). Androgens also play a role in bone maintenance, mainly by affecting mature osteoblasts to enhance bone formation (Syed and Khosla, 2005). In contrast, little is known about the effect of progestins on bone metabolism (Compston, 2001), but we wanted to know if they have an effect on our system. The estrogen (Kusec *et al.*, 1998; Braidman *et al.*, 2001), androgen (Nakano *et al.*, 1994), and progestin (MacNamara *et al.*, 1995) receptors have all been detected in osteoblasts (Saito and Yanaihara, 1998), and therefore could

directly affect these cells. It has been demonstrated in the MG-63 cell line that 17β -estradiol (Fohr *et al.*, 2000; Luo and Liao, 2003) and progesterone (Liang *et al.*, 2003) increase proliferation, whereas testosterone was reported to have no effect on growth (Fohr *et al.*, 2000).

The effect of sulfated steroids on bone growth is relatively unknown. One study reported that dehydroepiandrosterone sulfate and its unconjugated active form, dehydroepiandrosterone, had no effect on proliferation of MG-63 cells. However, dehydroepiandrosterone sulfate was found to be more potent than dehydroepiandrosterone in enhancing 1,25-dihydroxyvitamin D₃-stimulation of MG-63 cells (Scheven and Milne, 1998).

Glucocorticoids were also tested in our study, due to rapid bone loss observed in glucocorticoid-induced osteoporosis (Canalis *et al.*, 2004). Excessive amounts of glucocorticoids are known to influence the number and function of osteoblasts by inhibiting differentiation and proliferation of cells in the osteoblastic lineage (Mazziotti *et al.*, 2006; Canalis *et al.*, 2004). It has been shown that excess glucocorticoids can decrease the number of osteoblasts and osteocytes by inducing an apoptotic pathway (Ishida and Heersche, 1998). However, there appears to be a dual role of glucocorticoids on osteoblast function. When dexamethasone is added within a physiological concentration range to cells in culture, differentiation into mature osteoblasts is promoted (Walsh *et al.*, 2001). As with the sex steroids and progestins, glucocorticoid receptors are also found in osteoblasts (Abu *et al.*, 2000). In the MG-63 cell line, proliferation in the presence of dexamethasone has been shown to decrease (Jones *et al.*, 2006).

In the present study, 17β -estradiol and progesterone treatments stimulated growth, no effect on growth was found in response to the testosterone treatment, and a decrease in growth was found with the dexamethasone treatment. These findings are generally consistent with

previously published literature. The finding that estrone sulfate treatment significantly stimulated growth over the basal treatment supports the hypothesis for the role of sulfated steroid in the maintenance of bone. There was also an indication of growth by other steroids progesterone, dehydroepiandrosterone sulfate, and cortisol that under the experimental design might not allow for the finding of significance.

A more focused experimental design was developed for testing using only 17β -estradiol and sulfated steroids. Again, it was found that 17β -estradiol and estrone sulfate significantly increased proliferation. This result is consistent with the previous results and further supports the role of sulfated steroids in mediating bone cell growth.

Given that sulfated steroids were shown to cause growth, we wanted to determine if this process is mediated by steroid sulfatase. If so, then inhibitors of steroid sulfatase should block estrone sulfate-stimulated growth. Due to the role of steroid sulfatase in supporting estrogen-dependent tumor growth, a number of inhibitors have been designed as potential treatments. One steroid sulfatase inhibitor estrone-3-O-sulfamate (EMATE) is a potent irreversible site-directed inhibitor it inhibits steroid sulfatase activity in placental microsomes with an IC_{50} of 80nM (Howarth *et al.*, 1994). Also, 99% of steroid sulfatase activity was inhibited in human placental microsomes at an EMATE concentration of 10 μ M. For the hormone-dependent breast cancer cell line MCF-7, proliferation was inhibited with a calculated IC_{50} =100nM (Selcer *et al.*, 1996). However, this inhibitor has been shown to be estrogenic and therefore unable to be utilized as a therapeutic agent (Elger *et al.*, 1995). As a result, attempts have been made to develop potent non-steroidal inhibitors of steroid sulfatase. One non-steroidal inhibitor (DU-14) was developed by our lab (Li *et al.*, 1995; Kolli *et al.*, 1999). DU-14 inhibits steroid sulfatase activity in placental microsomes at a calculated IC_{50} of 55.8nM and in a hormone-independent breast cancer

cell line (MDA-MB-231) at a calculated $IC_{50}=350nM$. Inhibition of proliferation of the hormone-dependent breast cancer cell line (MCF-7) was calculated to inhibit at an IC_{50} value of $38.7nM$ (Selcer *et al.*, 1997). Due to well-characterized inhibition of steroid sulfatase activity in placental microsomes and breast cancer cell lines, these two agents (EMATE and DU-14) were utilized in the experiments testing the role of steroid sulfatase in estrone sulfate-stimulated growth in the MG-63 bone cell line. It was found that DU-14 was capable of blocking estrone sulfate-stimulated growth at $10\mu M$, but not $1\mu M$. However, EMATE was shown to stimulate proliferation at $1\mu M$, probably due to its known estrogenicity. These data further support the hypothesis that sulfated steroid are important in bone maintenance and that this process is mediated by steroid sulfatase.

Given that MG-63 proliferation is stimulated by steroid sulfates (and nonsulfated steroids) as discussed previously, we wanted to determine if this process is mediated by the estrogen receptors. If so, then an estrogen receptor inhibitor should block this response. The inhibitor chosen for this set of experiments was ICI 182,780. ICI 182,780 is a pure antiestrogen that competes with 17β -estradiol for the estrogen receptor (Wakeling *et al.*, 1991) and binds with high affinity to both the α - and β -estrogen receptors (Sun *et al.*, 1999). MCF-7 hormone-dependent breast cancer cell proliferation has been shown to be inhibited with ICI 182,780, with an IC_{50} calculated to be $0.29nM$ (Wakeling *et al.*, 1991). In this study, estrone sulfate and 17β -estradiol were chosen as treatments for the MG-63 cells. Estrone sulfate and 17β -estradiol were previously shown to stimulate growth. It is known that of the estrogenic compounds tested in this experiment, 17β -estradiol is capable of binding to the estrogen receptor; however estrone sulfate is not able to bind (Kuiper *et al.*, 1997). As expected, ICI 182,780 inhibited estradiol-stimulated growth of MG-63 cells. Interestingly, estrone sulfate-stimulated growth was also

inhibited by ICI 182,780, indicating that estrone sulfate is also mediating growth through the estrogen receptor. These results further support the hypothesis that steroid sulfatase is mediating estrone sulfate-stimulated growth. It converts estrone sulfate, which unable to bind the estrogen receptor, to an active steroid that is capable of binding and therefore supports bone growth.

To demonstrate that the MG-63 cells indeed have steroid sulfatase and that the inhibitors work as expected, steroid sulfatase activity was assessed using estrone sulfate conversion assays in microsomes and whole cells. It has previously been demonstrated that the MG-63 cell line contains steroid sulfatase activity (Purohit *et al.*, 1992) and that it is capable of utilizing both estrone sulfate and dehydroepiandrosterone sulfate as substrates (Fujikawa *et al.*, 1997). The estrone sulfate conversion assay has been used extensively by our lab to demonstrate steroid sulfatase activity. The assay involves conversion of 6,7-³H(E1S) by steroid sulfatase to 6,7-³H(E1) which can be extracted and assessed by scintillation counting. The results of both the microsome and whole cell estrone sulfate conversion assays revealed that MG-63 cells do have steroid sulfatase, consistent with previous reports, and that the inhibitors DU-14 and EMATE do block steroid sulfatase activity in MG-63 microsomes and whole cells. These data provide further support for the hypothesis that steroid sulfatase is mediating estrone sulfate-stimulated growth.

Overall, this study tested the effect of sulfated steroids on growth of the MG-63 bone cell line and found that estrone sulfate was capable of stimulating growth. Both an inhibitor of steroid sulfatase and an estrogen receptor antagonist blocked estrone sulfate-stimulated growth. This block in growth demonstrated that estrone sulfate-stimulated growth is mediated by the estrogen receptor, indicating that estrone sulfate is being converted to an active form, since it is unable to bind the estrogen receptor. Finally, it was demonstrated that steroid sulfatase is present

in the MG-63 cells and is blocked by steroid sulfatase inhibitors. The combined results of this study support the hypothesis that sulfated steroids are capable of supporting bone growth, through a process that is mediated by steroid sulfatase.

The main conclusions to be drawn from the data in this study are that the sulfated steroid estrone sulfate is capable of stimulating growth of MG-63 osteoblast-like cells and that this is mediated by steroid sulfatase. These findings have implications regarding post-menopausal women. Post-menopausal women have low circulating unconjugated estrogens (particularly 17β -estradiol), but high circulating levels of estrone sulfate. 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. Why then do women still develop osteoporosis? One possibility is that there is insufficient steroid sulfatase in bone cells to generate sufficient estradiol to maintain bone. It should be noted that there is substantial variation among women in the incidence and onset of osteoporosis. It is plausible that steroid sulfatase levels are one factor contributing to this difference. Women with greater levels of bone steroid sulfatase may enjoy some level of protection from osteoporosis. If steroid sulfatase levels in bone are able to influence the incidence of osteoporosis, then increasing the level of steroid sulfatase in bone would be desirable. Unfortunately, little is known about regulation of steroid sulfatase.

APPENDIX-ADDITIONAL FIGURES

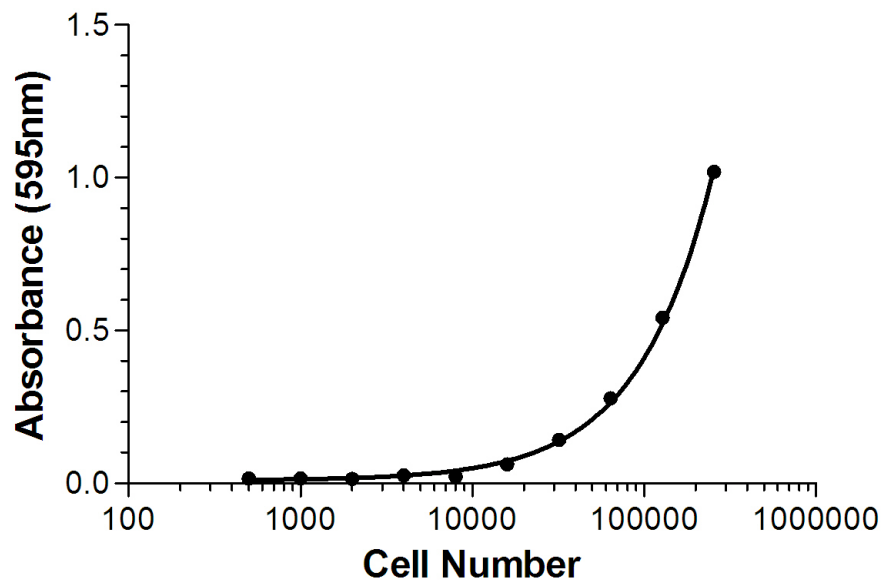


Figure A-1: MG-63 24-Well Plate Standard Curve.

Standard curve of cell number versus absorbance for MTT assay of MG-63 cells in a 24-well plate. The equation represents the best-fit nonlinear trendline for the data, $y=4e^{-6}x + 0.0092$, $r^2=0.9986$.

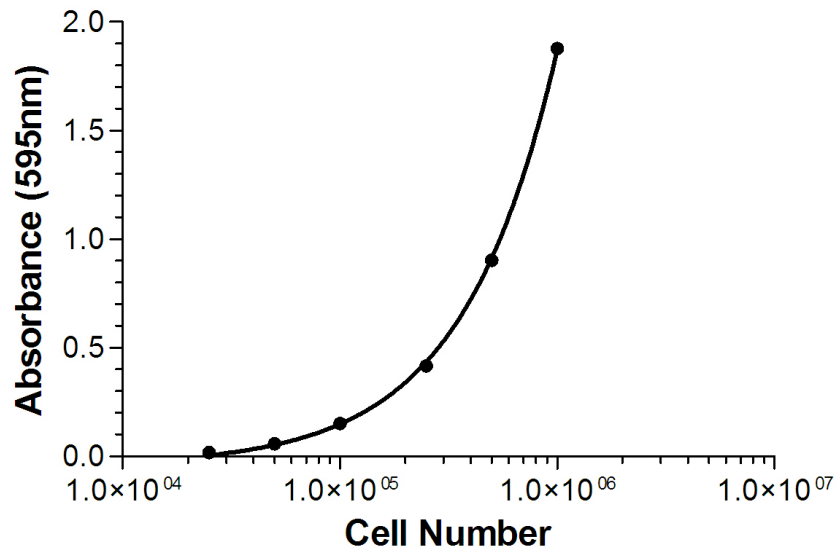


Figure A-2: MG-63 6-Well Plate Standard Curve.

Standard curve of cell number versus absorbance for MTT assay of MG-63 cells in a 6-well plate. The equation represents the best-fit nonlinear trendline for the data, $y=2e^{-6}x + 0.0431$, $r^2=0.9997$.

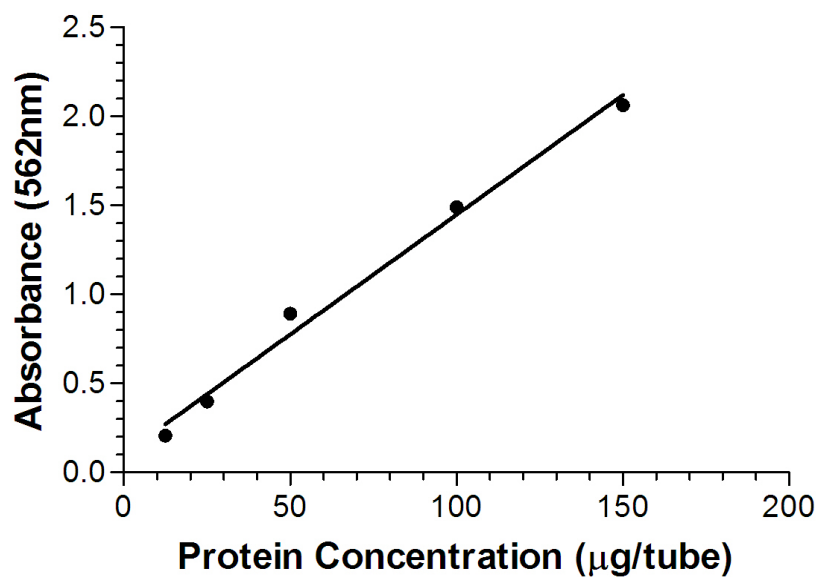


Figure A-3: BCA™ Protein Assay Standard Curve.

Standard curve of protein concentration versus absorbance generated from the Pierce Protein Assay Kit. Bovine serum albumin stock was diluted to the appropriate duplicate concentrations and incubated with the working reagent for 30min. The equation represents the best-fit linear trendline for the data, $y=0.0134x + 0.1032$, $r^2=0.9896$.

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