Characterization of Steroid Sulfatase in Human and Mouse Tissues and in the Mouse MC3T3-E1 Pre-Osteoblastic Cell Line

Heidi DiFrancesca

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CHARACTERIZATION OF STEROID SULFATASE IN HUMAN AND MOUSE TISSUES AND IN THE MOUSE MC3T3-E1 PRE-OSTEOBLASTIC CELL LINE

A Dissertation
Submitted 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 Doctor of Philosophy in Biological Sciences

By:
Heidi M. DiFrancesca

November 2007
CHARACTERIZATION OF STEROID SULFATASE IN HUMAN AND MOUSE TISSUES AND IN THE MOUSE MC3T3-E1 PRE-OSTEOBLASTIC CELL LINE

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ABSTRACT

CHARACTERIZATION OF STEROID SULFATASE IN HUMAN AND MOUSE TISSUES AND IN THE MOUSE MC3T3-E1 PRE-OSTEOBLASTIC CELL LINE

By

Heidi M. DiFrancesca

November 2007

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Steroid sulfatase cleaves the sulfate group from 3β-hydroxysteroid sulfates. This enzyme is widely distributed among mammalian tissues, and it has been suggested to play a role in the in situ conversion of inactive steroids to active forms in a variety of tissues. Two tissues in which steroid sulfatase may be particularly important are breast cancer and bone. Our laboratory is interested in the role of steroid sulfatase; consequently, I worked on four projects characterizing steroid sulfatase in human and mouse tissues and investigating the role of this enzyme in mouse bone cells. Recent evidence indicates that steroid sulfatase could be a prognostic indicator in breast cancer. Our lab generated a steroid sulfatase antibody to examine the presence of steroid sulfatase in a variety of tissues, including breast carcinomas. I performed immunohistochemical analyses on human tissue slides and a human tissue microarray. The findings confirm previous reports that steroid sulfatase is higher in hormone-
dependent breast cancers, suggesting that this enzyme could serve as a useful prognostic indicator of breast cancer. I next sought to determine activity and presence of steroid sulfatase and the presence of steroid sulfatase mRNA in a variety of mouse tissues. Using enzyme assays, Western blotting and RT-PCR, it was determined to each of six mouse tissues (liver, lung, kidney, muscle, ovary and uterus) contained steroid sulfatase protein and mRNA. Our results show that mouse tissues have the ability to convert inactive steroids into active steroids. The mouse model may prove useful in future studies of steroid sulfatase physiology. Steroid sulfatase may provide a local mechanism for 17β-estradiol needed to maintain bone health, as estrogens play a central role in the regulation of bone density. I characterized steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line, as these cells are a model for bone differentiation. I then developed a 21-day protocol to measure steroid sulfatase throughout differentiation. Steroid sulfatase showed changes in activity during development, depending on the treatment; however steroid sulfatase mRNA was constant throughout development. These data provide a baseline for future studies of the role of steroid sulfatase in bone development.
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Dr. Kyle Selcer is responsible for the development of the steroid sulfatase antibody, STS-275, which was used throughout the project.

Suman Barua, Dan Finnegan, Jessica Geiser, Shelby Hott, Jamie Skoloda, and Laura Vollmer assisted with the scoring of the human tissue slides in the immunohistochemistry project.
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Chapter 1

Background on Steroid Sulfatase

1.1 Introduction

Steroid sulfatase is an enzyme that converts inactive steroid hormone precursors into active molecules. In one particular important pathway, steroid sulfatase catalyzes the removal of the sulfate group from inactive estrone sulfate rendering estrone, which can then be reduced to the more potent 17β-estradiol. Accordingly, this enzyme is thought to be important in the local metabolism of estrogen in several tissues, including breast and bone. It is believed that local conversion of inactive estrogens into their active forms is necessary to maintain healthy breast tissue. However, overexposure to estrogen in breast tissue can lead to breast cancer. Steroid sulfatase is thought to be overexpressed in breast cancer and is considered a culprit for increased levels of estrogen in these cancers. Conversely, steroid sulfatase is thought to aid in the maintenance of bone density, as estrogens are required for normal bone physiology. Although steroid sulfatase provides a mechanism for the local estrogen metabolic pathway, not much is known about this enzyme in bone tissue, particularly in mouse bone. Even less is known about its role in the proliferation and differentiation of mouse osteoblasts. Moreover, information concerning steroid sulfatase mRNA levels, and protein expression and activity in various mouse tissues is lacking. Therefore, the purpose of this project was to try to determine the
prognostic value of steroid sulfatase in breast cancer, to explore its role in mouse bone tissue, and to describe its activity and expression in a variety of mouse tissues.

1.2 Steroid Sulfatase

Steroid sulfatase belongs to the highly conserved sulfatase family of enzymes (Reed et al., 2005). Sulfatases are enzymes that catalyze the hydrolysis of sulfate bonds from a broad range of physiological substrates. Steroid sulfatase is classified as an aryl sulfatase C (EC 3.1.6.2.) because of its ability to cleave the sulfate group of phenol- or 3β-hydroxysteroids. As such, steroid sulfatase is able to hydrolyze a variety of sulfated steroids, including estrone sulfate, dehydroepiandrosterone sulfate, pregnenolone sulfate, deoxycorticosterone sulfate, cholesterol sulfate, and p-nitrophenyl-sulfate (Nussbaumer and Billick, 2004; Reed et al. 2005).

Steroid sulfatase is a glycosylated, integral membrane protein, largely associated with the endoplasmic reticulum. Smaller fractions of the enzyme are found in the Golgi, nuclear envelopes and at the cell surface (Hernandez-Guzman et al., 2003; Nussbaumer and Billich, 2004). When the enzyme was purified to homogeneity from the microsomal fraction of human placenta, steroid sulfatase was found to be a 65 kDa hydrophobic monomer of 562 amino acids with two membrane spanning domains (Hernandez-Guzman et al., 2001). In other studies, the molecular weight of the monomer has been reported to range in size from 63-73 kDa (Nussbaumer and Billick, 2004). The crystalline structure of steroid sulfatase revealed a “mushroom-like” appearance, with the polar catalytic domain buried in the “gill” of the “mushroom”, located near the membrane surface on the lumenal side of the endoplasmic reticulum. Two antiparallel helices,
predominately made up of hydrophobic residues, project out from the catalytic domain (Hernandez-Guzman et al., 2003). Biochemical studies have suggested that the active site of steroid sulfatase is near or within the endoplasmic reticulum (Nussbaumer and Billick, 2004). Thus, the helices traverse the membrane, and in so doing interact with one another to create a hydrophobic “tunnel” leading to the catalytic domain. These hydrophobic helices presumably function to anchor steroid sulfatase to the membrane (Hernandez-Guzman et al., 2003).

Both estrone sulfate and dehydroepiandrosterone sulfate serve as substrates for steroid sulfatase. Kinetic analysis of purified placental steroid sulfatase indicated a $K_m$ value of 72.75µM for estrone sulfate and $K_m$ value of 9.59µM for dehydroepiandrosterone sulfate (Hernandez-Guzman et al., 2001).

Steroid sulfatase was first identified in rat liver microsomal preparations (Dodgson et al., 1954). It is a fairly ubiquitous enzyme (Nussbaumer and Billich, 2004; Pasqualini and Chetrite, 2005; Reed et al., 2005), as the presence and activity of steroid sulfatase have been demonstrated in the testis, ovary, adrenal glands, placenta, prostate, skin, brain, fetal lung, viscera, endometrium, peripheral blood lymphocytes, aorta, kidney, and bone (Pasqualini and Chetrite, 2005; Reed et al., 2005). The tissue distribution and activity, however, vary considerably (Dibbelt et al., 1994; Salido et al., 1996). Of all the known tissues containing steroid sulfatase, placenta has shown the highest activity levels (Pasqualini and Chetrite, 2005).
1.3 Relationship of Steroid Sulfatase and Breast Cancer

1.3.1 Breast Cancer

Breast cancer is the most commonly occurring cancer among women, and is the second leading cause of death in women in the United States. It is the major cause of death in women between the ages of 40 and 55. Nearly 180,000 new cases and over 40,000 deaths will occur in the U.S. this year alone (American Cancer Society, 2007). One out of eight women in North America will experience breast cancer at some time in their life (Marshall, 1993).

Breast cancer is classified as either hormone-independent (estrogen receptor negative) or hormone-dependent (estrogen receptor positive). This classification is mainly due to the presence or absence of estrogen receptors (ER). Hormone-independent cancers proliferate without the influence of hormones; whereas, hormone-dependent cancers express estrogen receptors, and thus require estrogen for their growth. Consequently, hormone-dependent cancers are treated with endocrine therapy, while hormone independent cancers are typically treated with other types of therapy, as they do not respond to hormones.

Approximately 40% of breast cancers are hormone-dependent, and the frequency of occurrence is even greater in post-menopausal women (Henderson and Canellos, 1990; Pasqualini et al., 1995; Stanway et al., 2007). Increased estrogen exposure appears to be adversely associated with hormone-dependent breast tumors (Dhingra, 1999); thus, the production of estrogens is considered to be responsible for the stimulation of hormone-dependent breast cancer development and growth (Stanway et al., 2007). Indeed, estrogens, 17β-estradiol in particular, contribute greatly to the growth and development
of breast cancer cells, and some of the cancers actually require estrogen for continued growth, particularly in post-menopausal women. Following menopause, estrogens are produced predominately through conversion of androgens in peripheral tissues. These tissues include skin, muscle, fat, and bone. Conversion of androgens to estrogens occurs via the aromatase enzyme, and this conversion process occurs mostly in the adipose tissue (Pasqualini and Chetrite, 2005; Reed et al., 2005; Sasano et al., 2006). Thus, elevated serum levels of estrogen may be a result of increased peripheral conversion of androgens to estrogens (Sasano et al., 2006), which provides a pool of estrogen precursors that can be metabolized intratumorally to potent estrogens.

Circulating estrogen is primarily in the sulfated form, estrone sulfate. Estrogen sulfotransferase sulfonates estrogens to their biologically inactive forms and steroid sulfatase catalyzes the removal of the sulfate group. As a result, the estrogen sulfotransferase and steroid sulfate enzyme systems play important roles in maintaining biologically active estrogens in peripheral tissues (Sasano et al., 2006). Upon conversion of estrone sulfate to estrone via steroid sulfatase, 17β-hydroxysteroid dehydrogenase type 1 is able to reduce estrone to 17β-estradiol. 17β-hydroxysteroid dehydrogenase type 1 is widely distributed in peripheral tissues (Chetrite et al., 2000; Pasqualini and Chetrite, 2005; Reed et al., 2005; Sasano et al., 2006).

Breast cancer tissues possess all of the enzymes necessary for the bioformation of 17β-estradiol (Fig.1) (Chetrite et al., 2000; Pasqualini and Chetrite, 2005; Reed et al., 2005; Sasano et al., 2006). Additionally, high concentrations of estrogen in malignant breast tissue in both pre- and post-menopausal women have been demonstrated. Because the ovary has ceased to produce estrogens in post-menopausal women, these findings
indicate that estrogen can be produced in the same tissues that it exerts its biological response (Nguyen et al., 1995; Chetrite et al., 2000); thereby confirming in situ estrogen production in malignant breast tissue (Reed et al., 2005).

Labrie et al., (2000) has coined the term “intracrine” activity to refer to the local production of estrogen in peripheral tissues. Intracrine activity accounts for estrogen biosynthesis that occurs without release into circulation and, therefore, is not necessarily dependent on serum concentrations of compounds. Malignant breast tissue has the ability to produce 17β-estradiol locally. Moreover, in situ synthesis of estrogen has been shown to predominate over estrone or 17β-estradiol uptake from plasma as a way of maintaining post-menopausal breast tissue estradiol concentrations. These findings demonstrate the biological significance of elevated intratumoral estrogen concentrations resulting from local estrogen production after menopause. This locally formed estrogen could invoke a growth advantage to tumoral tissues, regardless of the serum concentrations of estrogens (Sasano et al., 2006).

1.2.1 Current Treatment Methods

Prior to selecting an approach for clinical treatment, it is important to determine the cancer’s tumoral classification, as treatment methods differ depending to what the specific cancer will respond. In post-menopausal women, estrogens continue to be produced, albeit at low levels. To date, there are two key treatment options available for the therapy of hormone-dependent breast cancer. These options include blockade of hormone receptors and inhibition of hormone synthesis (Nakata et al., 2003). At a
minimum, one of the two strategies is frequently employed in the clinical treatment of hormone-dependent tumors (Jordan, 1997).

1.33 Hormone Receptor Blockade

The action of $17\beta$-estradiol is mediated by estrogen receptors (Gruber et al., 2002; Matthews and Gustafsson, 2006). Hormone-dependent breast tumor cells express estrogen receptors (Bentrem and Jordan, 1999; Gruvberger et al., 2001; Matthews and Gustafsson, 2006). Blocking the estrogen receptors using specific antagonists is one strategy that can be used for therapeutic treatment of hormone-dependent breast cancers. The purpose of the antagonist is to inhibit the transcriptional activity of the estrogen receptor, thereby inhibiting growth of cancerous cells (Bentrem and Jordan, 1999).

Presently, two strategies are used in the blockade of hormone receptors. Tamoxifen, the standard drug used as an endocrine treatment for breast cancer for over 20 years, is one method. Tamoxifen is a non-steroidal, competitive inhibitor of estrogens and is the most widely used therapeutic agent in hormone-dependent breast cancer treatment to date (Dhingra, 1999; Stanway et al., 2007). Although it is an anti-estrogen and classified as an endocrine therapy, tamoxifen has been used as an approach for treatment in both hormone-dependent and hormone-independent breast cancers. However, the success rate is much higher for the hormone-dependent tumors (Jordan, 1997). Nonetheless, the antiestrogenic effects of this drug are central to retarding the growth of breast cancer cells. Yet, it can have estrogenic effects in other tissues. While some of the estrogenic effects are beneficial, such as impeding osteoporosis in bone tissue and lowering cholesterol levels, other estrogen-like effects are detrimental.
Tamoxifen has been shown to stimulate growth of the stromal component of the endometrium, increasing the risk of endometrial cancer (Bentrem and Jordan, 1999; Dhingra, 1999). Other side effects associated with tamoxifen use include hot flashes, vaginal dryness, irregular periods, weight gain, and an increased risk of developing cataracts (Jordan and Morrow, 1999).

The issues related to tamoxifen therapy have provided a motive for development of other antiestrogenic compounds that would aid in the treatment of hormone-dependent breast cancer via the blockade of hormone receptors. As a result, a second method for blocking hormone receptors was developed, which involves the use of selective estrogen receptor modulators (SERMs). Ideally, SERMs would provide a desirable combination of tissue-selective estrogen agonistic and antagonistic activities (Dhingra, 1999; O’Regan and Jordan, 2001). The prototypical SERM would retain estrogenic effects on bone, while maintaining anti-estrogenic antagonistic effects in the breast. Raloxifene is one example of a SERM that is used in for the treatment of osteoporosis. This compound prevents bone loss and appears to lack estrogenic effects on the endometrium. Moreover, raloxifene has been found to have anti-tumor activity similar to tamoxifen (O’Regan and Jordan, 2001; Seeman, 2001).

1.34 Hormone Synthesis Inhibition

An alternative strategy for the treatment of hormone-dependent breast cancer is the inhibition of steroid hormone synthesis. Two possible mechanisms account for the origin of estrogens in breast tumors: 1) uptake of circulating estrogens, or 2) local formation from estrogen precursors (Reed et al., 2005) (See Appendix Fig.1). Two main
pathways account for the local metabolism of estrogen in breast cancer. One is the aromatase pathway, while the other is the steroid sulfatase pathway (Nakata et al., 2003). Thus, inhibitors for both pathways have been developed.

1.35 Aromatase Inhibitors

Because aromatase plays a fundamental role in the formation of estrogens from androgens, aromatase inhibitors have been utilized as a means of blocking estrogen synthesis (Purohit, et al., 2001). Androstenedione is converted from DHEA to estrone via aromatase, which can then be further converted to 17β-estradiol. Additionally, testosterone is aromatized to 17β-estradiol (Simpson 2003; Reed et al., 2005). Several lines of evidence have indicated that aromatase is an important mechanism of estrogen synthesis in post-menopausal women (Santen and Harvey, 1999; Sasano et al., 2006; Simpson 2003; Reed et al., 2005; Stanway et al., 2007). Consequently, inhibitors of aromatase provide a way of treating hormone-dependent breast cancer (Santen and Harvey, 1999). Indeed, aromatase inhibitors have been shown to repress plasma estrogen levels in post-menopausal women (Geisler, 2003).

Aminoglutethimide and testolactone comprise the first generation of aromatase inhibitors. While aminogluthethimide was found to be a potent inhibitor, it had considerable side effects. Second generation inhibitors include steroidal 4-hydroxyandrostenedione (4-OHA), non-steroidal fadrozole, and steroidal exemestane. Both 4-OHA and fadrozole are fairly potent with few side effects. Exemestane, an irreversible inhibitor, has been also found to be potent with minimal side effects. Anastrozole, letrozole, and vorozole are the third generation, non-steroidal aromatase
inhibitors. Third generation inhibitors were found to be potent, with the ability to decrease the levels of circulating estrogens to a greater extent than first and second generation inhibitors. In fact, anastrozole, the first aromatase inhibitor to be approved in the U.S. (Santen and Harvey, 1999), was found to effectively decrease high concentrations of estrogen in breast cancer tissue (Geisler, 2003). However, recent research overwhelming indicates that steroid sulfatase activity is much higher than aromatase activity in breast tumors (Santer et al., 1984; Simpson 2003; Nussbaumer and Billich, 2004; Pasqualini and Chetrite 2005; Stanway et al., 2007). Thus, steroid sulfatase may provide another route by which estrogen can be synthesized locally via aromatase-independent pathways. Indeed, several studies have determined that the steroid sulfatase pathway is quantitatively more important than the aromatase pathway (Chetrite et al., 2000; Reed et al., 2005; Pasqualini and Chetrite et al., 2005; and Stanway et al., 2007).

1.36 Steroid Sulfatase Inhibitors

Steroid sulfatase provides two potential pathways for the local conversion of inactive hormones into active estrogens. Steroid sulfatase is found in a larger percentage of breast cancers when compared with aromatase; therefore, the steroid sulfatase pathways appear to have greater importance than the aromatase pathways in the local production of estrogens in cancer cells (Pasqualini et al., 1996; Chetrite et al., 2000; Nussbaumer and Billich, 2004; Reed et al., 2005; Pasqualini and Chetrite 2005; Stanway et al., 2007).
Estrone sulfate and dehydroepiandrosterone sulfate can stimulate breast cancer cell growth via aromatase-independent metabolic pathways involving steroid sulfatase, and, because steroid sulfatase is believed to be a source for local estrogen production in breast tissue, inhibitors that block the action of steroid sulfatase have therapeutic potential in the treatment of both estrone sulfate-stimulated and dehydroepiandrosterone sulfate-stimulated breast tumor proliferation (Kolli et al., 1999; Nussbaumer and Billich, 2004; Reed et al., 2005). This has lead to the generation of steroid sulfatase inhibitors. Indeed, inhibitors of steroid sulfatase have been shown to block estrone sulfatase-dependent breast cancer cell proliferation in vitro (Selcer et al., 1997; Raobaikady et al., 2003; Nussbaumer and Billich, 2004). Similarly, tumor models in rodents have been used to show that numerous inhibitors of steroid sulfatase block in vivo activity (Purohit et al., 2001; Nussbaumer and Billich, 2004).

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). The majority of steroid sulfatase inhibitors fall into the classification of irreversible inhibitors, which tend to be more potent. Nonetheless, a wide variety of compounds with potential to block steroid sulfatase activity were explored; yet only a few of them demonstrated promise as therapeutic agents (Nussbaumer and Billich, 2004).

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, it is not capable of being utilized as a therapeutic drug 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). A second generation of inhibitors was subsequently developed. Purohit et al., (2000) developed tricyclic coumarin-based 667 COUMATE (STX64), which irreversibly binds steroid sulfatase, thereby inhibiting its activity. While it is less potent than EMATE, it does have the advantage of being nonestrogenic (Woo et al., 1998). This particular compound is now in Phase 1 clinical trial (Stanway et al., 2007). This same group developed another inhibitor, STX213, which also appears to have potential as a novel treatment for hormone-dependent breast cancer (Foster et al., 2006).

Other non-steroidal compounds have been developed and shown to be effective in the inhibition of steroid sulfatase. Our laboratory recently designed and generated DU-14, which was found be a potent, non-steroidal inhibitor of steroid sulfatase (Kolli et al., 1999). As a result, this compound has been patented (Patent Number US 6,433,000B1), and is under investigation for therapeutic potential in breast cancer treatment.

1.37 Diagnosis for Treatment

Diagnostic markers are widely used in breast cancer evaluation and treatment. Traditional diagnostic tools include estrogen receptor status, progesterone receptors status, and HER-2, human epithelial growth factor receptor 2. These traditional markers
remain the most useful prognostic indicators. In the treatment of breast cancer, accurate assessment of ER, PR, and HER-2 is essential (Sneige, 2004). Additional markers are used to enhance diagnosis and, thereby, determine the best treatment method for the cancer on an individual basis.

According to the College of American Pathologists, prognostic indicators have been divided into three groups. Category I indicators include tumor size, lymph node status, histological grade, mitotic figure count, and hormone receptor status (Fitzgibbons et al., 2000). This grouping has the highest prognostic importance, and is typically used in patient management (Sneige, 2004). Category II indicators include HER-2 and p53 (Fitzgibbons et al., 2000). Although these markers have been studied significantly, their biological and clinical importance remains to be validated in statistically robust studies (Sneige, 2004). Category III indicators include cathepsin D and angiogenesis (Fitzgibbons et al., 2000). This grouping of markers do not meet the criteria for category I or II (Sneige, 2004), and are, therefore, not necessarily considered independent markers (Fitzgibbons et al., 2000).

1.38 Steroid Sulfatase in Malignant Tissue

For several years, it has been know that steroid sulfatase activity was detectable in most breast tumors evaluated (Stanway et al., 2007). Newman et al. (2000), found steroid sulfatase activity to be higher in both malignant and benign tumors when compared to adjacent normal tissue. In addition, steroid sulfatase is found in a larger percentage of breast cancers when compared with aromatase, and the activity of steroid sulfatase is 100 to 500 times greater than aromatase activity (Santer et al., 1984; Simpson
2003; Nussbaumer and Billich, 2004; Pasqualini and Chetrite, 2005; Stanway et al., 2007). Only 40% to 60% of tumors displayed aromatase activity (Chetrite et al, 2000; Pasqualini and Chetrite, 2005; Reed et al., 2005). Furthermore, Utsumi et al., (2000) measured steroid sulfatase mRNA and aromatase mRNA in breast tumors and determined that steroid sulfatase levels are much higher than those of aromatase.

Estrone sulfate, one of the substrates for steroid sulfatase, appears to be a major source of estrogens in malignant breast tissues (Nussbaumer and Billich, 2004; Pasqualini and Chetrite, 2005; Reed et al., 2005), and does not require aromatase to be converted into a locally biologically active estrogen (Pasqualini and Chetrite, 2005; Reed et al., 2005). Concentrations of estrone sulfate, estrone, and 17β-estradiol in malignant breast cancer tissues are substantially greater than circulating levels. Additionally, plasma concentrations of estrogen sulfate are substantially higher than unconjugated estrogens (Chetrite et al., 2000; Pasqualini and Chetrite, 2005; Reed et al., 2005; Stanway et al., 2007). Plasma concentrations of estrone sulfate are 10-20 times greater when compared with those of estrone and estradiol (Stanway et al., 2007). Furthermore, both unconjugated estrogens have shorter half-lives (20-30 minutes) than does estrone sulfate, whose half-life is between 10-12 hours. Consequently, estrone sulfate is believed to serve as a reservoir for the conversion of locally active estrogens due to steroid sulfatase action (Pasqualini and Chetrite, 2005; Stanway et al., 2007). While estrone sulfate is biologically inactive, meaning that it is unable to bind the estrogen receptor, it can be converted to estrone by removal of the sulfate group. Estrone can then be reduced to 17β-estradiol by 17β-hydroxysteroid dehydrogenase. Estrone’s affinity for estrogen receptors is lower than that of 17β-estradiol; however, both are considered biologically
active estrogens (Nussbaumer and Billich, 2004; Reed et al., 2005). Nonetheless, 17β-
estriadiol, because of its high affinity for estrogen receptors, is considered a potent
estrogen.

Steroid sulfatase also acts on dehydroepiandrosterone sulfate. This inactive
steroid is secreted in large amounts (up to 20 mg per day) by the adrenal cortex (Reed et
al., 2005; Stanway et al., 2007). Upon cleavage of the sulfate group,
dehydroepiandrosterone can be either converted into androstenedione by 3β-
dehydroxysteroid dehydrogenase or converted to androstenediol by 17β-keto-reductase.
After androstenedione is aromatized to estrone, sulfotransferases, which are widely
distributed throughout the body, mediate the rapid conversion of much of the estrone to
estrone sulfate by adding a sulfate group (Stanway et al., 2007). Androstenediol is
estrogenic; it is able to bind to estrogen receptors and elicit a growth response in
hormone-dependent breast cancer cells in vitro (Lardy et al., 2005; Reed et al., 2005) and
carcinogen-induced mammary tumors in rodents (Reed et al., 2005; Stanway et al.,
2007). Because androstenediol has much higher plasma and tissue concentrations when
compared to 17β-estradiol, researchers have suggested that it may be equipotent to 17β-
estriadiol in post-menopausal women, regardless of its lower affinity for the estrogen
receptor (Reed et al., 2005; Stanway et al., 2007).

1.39 Steroid Sulfatase and Prognosis

Several studies provide support for the role of steroid sulfatase as an indicator of
prognosis in breast cancer. Suzuki et al. (2003b), detected greater levels of the enzyme at
both the protein and mRNA levels in breast tumor tissue when compared to normal breast
tissue. Recent reports have also revealed that over 80% of breast tumors express steroid sulfatase (Nakata et al., 2003). Utsumi et al., (1999b) determined that steroid sulfatase mRNA levels in tumors appeared to predict shorter relapse-free survival in breast cancer. From their findings, they concluded that high steroid sulfatase transcript levels could serve as an independent prognostic factor in predicting relapse-free survival (Utsumi et al., 1999b) and for identifying both high and low risk patients (Utsumi et al., 1999a). These results were later confirmed by Suzuki et al. (2003a), who found that 74% of breast cancers demonstrated higher levels of steroid sulfatase immunoreactivity, and that the immunoreactivity level was significantly correlated with tumor size and worsened prognosis, as well as increased risk of recurrence.

In the study performed by Miyoshi et al., (2003) steroid sulfatase mRNA levels were significantly correlated with lymph node metastasis, histological grade III, and poor prognosis. However, the correlation with steroid sulfatase activity and poor prognosis was only found in patients with estrogen-receptor positive tumors, but not in patients with estrogen-receptor negative tumors. As a result, the group concluded that steroid sulfatase mRNA levels would serve as a significant, independent prognostic marker in estrogen-receptor positive tumors. Yoshimura et al. (2004) reported an association between high steroid sulfatase mRNA levels and shorter disease-free survival. They too concluded that steroid sulfatase mRNA levels could serve as an independent prognostic indicator of estrogen-dependent breast cancers.

Taken together, these data suggest that steroid sulfatase may be useful as an independent prognostic indicator in hormone-dependent breast cancers. Currently, various prognostic indicators are used clinically to aid in treatments, such as tumor size,
lymph node involvement, distant metastasis, and presence of estrogen and progesterone receptors. Use of additional indicators, such as status of steroid sulfatase, may augment both the assessment and treatment of breast cancers. Thus, it would be prudent to evaluate various breast cancers for the expression of steroid sulfatase to determine its usefulness as a marker for prognosis.

1.4 Relationship of Steroid Sulfatase and Bone

1.41 Basic Bone Biology

Bone tissue provides structural support for the body, protection for internal organs, and functions as a storehouse for the bone marrow (Compston, 2001; Lerner, 2006; Walsh et al., 2006). The skeletal system plays a crucial role in the homeostasis of calcium and phosphate ions and acts as a reservoir for these minerals (Compston, 2001; Cohen, 2006; Lerner, 2006; Walsh et al., 2006). Moreover, bone is the site of hematopoiesis (Walsh et al., 2006). Additionally, bone tissue has the unique ability to adapt its structure to mechanical stimuli and repair structural damage through the remodeling process. As such, bone is not static, but rather a highly metabolically active tissue (Lerner, 2006; Robling et al., 2006).

Roughly eighty percent of the skeleton is cortical bone. Cortical bone is composed of compact bone and found largely in the shafts of long bones and surfaces of flat bones (Compston, 2001, Walsh et al., 2006). This compact bone is a well-organized pattern of collagen fibrils aligned along stress lines providing bone with maximal strength (Walsh et al., 2006). Cortical bone is concentrically laid down around central canals called Haversian systems. Haversian systems contain blood vessels, nerves, connective
tissue, and lymphatic tissues. The ends of long bones and the inner portions of flat bones, on the other hand, consist largely of trabecular, or cancellous bone (Compston, 2001).

Trabecular bone is thinner than cortical bone (Walsh et al., 2006) and is characterized by interconnecting plates and bars that contain hematopoetic or fatty marrow (Compston, 2001). Additionally, trabecular bone is primarily found traversing bone marrow space. This particular bone tissue functions to provide a large surface area for metabolic processes (Walsh et al., 2006). In fact, bone turnover occurs more readily in trabecular bone than in cortical bone (Lerner, 2006; Walsh et al., 2006).

The skeletal system is specialized connective tissue made up of cells embedded in an extracellular matrix. Ninety percent of bone volume is comprised of extracellular matrix; whereas, the remaining ten percent is composed of blood vessels and cells (Downey and Siegel, 2006). The extracellular matrix is comprised of two distinct phases, an organic phase and an inorganic phase (Compston, 2001; Downey and Siegel, 2006). The organic phase consists of type I collagen (Downey and Siegel, 2006), proteoglycans, and noncollagenous proteins, which include osteocalcin, bone sialoprotein, osteonectin, thromborespondin, and osteopontin (Compston, 2001), while calcium hydroxyapatite is the major component of the inorganic phase of bone tissue (Compston, 2001; Walsh et al., 2006). Hydroxyapatite crystals are important during the mineralization process, as they are deposited on and between the collagen fibrils, thereby producing hard bone (Walsh et al., 2006).

Four different types of osteon cells can be found within the bone cellular network. These cell types include osteoblasts, osteocytes, bone lining cells, and osteoclasts (Downey and Siegel, 2006). Osteon cells arise from disparate cell origins and undergo
proliferation, differentiation, and activation or inhibition of activity in response to
different cues within bone tissue. For this reason, each cell type has a specific functional
role in the formation and resorption of bone (Robling et al., 2006).

Osteoblasts, derived from pluripotent mesenchymal stem cells, are responsible for
both bone formation and mineralization (Compston, 2001; Robling et al., 2006). These
particular osteon cells influence bone resorption using a signaling axis that controls
osteoclast generation and activity (Lerner, 2006; Robling et al., 2006). Active osteoblasts
form a monolayer of cells and secrete an osteoid matrix, which is then mineralized
extracellularly (Lerner, 2006; Robling et al., 2006; Walsh et al., 2006). As stated above,
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). Alkaline phosphatase, produced via
osteoblasts, induces mineralization and matrix maturation, and in so doing, influences
matrix calcification (Robling et al., 2006, Walsh et al., 2006). A portion of the
osteoblasts then become entrapped in the calcifying matrix, and persist as unique bone
cells called osteocytes (Walsh et al., 2006).

Osteoblast maturation is characterized by the proliferation of mesenchymal stem
cells. 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 down the osteoblastic lineage is achieved through an increase in
the expression of runt-related transcription factor-2 (Runx2), which serves as the earliest-
known osteoblast-specific marker (Wagner and Karsenty, 2001; Walsh et al., 2006). Pre-osteoblastic cells committed to the osteoblastic lineage express the early phenotypic marker type I collagen. In order for pre-osteoblast cells to continue differentiating towards mature bone forming cells, expression of the transcription factor osterix (Osx) is required (Robling et al., 2006; Walsh et al., 2006). Moreover, 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 human bone cells, the differentiation process takes place between 25 to 30 days in vitro, and is defined by three distinct phases. The initial proliferative phase occurs on days 0 to 4, the matrix-deposition phase is between days 10 to 16, and maximal mineralization happens by day 28 (Duplomb et al., 2007).

As stated previously, 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). These cells 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 trapped 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.
Most of the bone surfaces of an adult skeleton is covered by bone lining cells. Derived from the osteoblast lineage, these cells are referred to as resting osteoblasts or surface osteoblasts (Downey and Seigel, 2006; Lerner, 2006). The role of surface osteoblasts is relatively unknown; however, it has thought that they are involved in matrix preparation for resorption and mineralization. Surface osteoblasts are also thought to function as a barrier between extracellular fluid and bone (Downey and Siegel, 2006).

Osteoclasts, on the other hand, are large, multinucleated cells derived from hematopoetic precursors from the monocyte/macrophage lineage. Osteoclasts, under the control of osteoblasts, are responsible for bone resorption (Compston, 2001; Robling et al., 2006) and subsequent release of calcium and phosphate ions into the bloodstream. Osteoclasts are highly motile, moving across the bone surface to resorb bone (Walsh et al., 2006). By tightly adhering to peripheral bone surfaces (Robling et al., 2006) osteoclasts are able to participate in the bone resorption process by forming a specialized structure known as a sealing zone. Osteoclasts create a resorption space isolated from the extracellular matrix (Walsh et al., 2006) that enables them to breakdown extracellular matrix (Robling et al., 2006) by solubilizing the bone mineral content (Walsh et al., 2006).

Bone is a metabolically active tissue (Robling et al., 2006). As such, bone undergoes a variety of processes to support its health and viability (Lerner, 2006; Robling et al., 2006). Processes to develop and maintain the skeletal system are accomplished through the coordinated action of the osteon cells that populate bone tissue. The skeletal system is developed and maintained via two main processes, bone modeling and bone remodeling. During skeletal development, both the bone modeling and remodeling
processes work in conjunction to define the skeletal shape, maintain appropriate serum ion levels, and repair regions of bone that have been structurally compromised.

Modeling is a process whereby resorption occurs at one site and formation at another. This process takes place at any bone surface, and involves either osteoclast activation followed by bone resorption or osteoclast activation followed by bone formation (Lerner, 2006; Robling et al., 2006). Formation of new bone is independent of removal of old bone (Lerner, 2006). However, once skeletal maturity is achieved, bone modeling lessens to a minor level (Robling et al., 2006). Conversely, bone remodeling, is a continuous process (Bland, 2000; Robling et al., 2006).

Normal bone architecture is maintained by the process of bone remodeling, which predominates as skeletal maturity is reached. This process involves a precise balance between osteoblast and osteoclast action, which act to sustain a normal bone microenvironment (Bland, 2000; Robling et al., 2006). The bone remodeling process always follows an activation resorption formation sequence, and is responsible for the removal and replacement of discrete packets of bone (Robling et al., 2006). The balance between osteoblastic formation and osteoclastic degradation is achieved via a tight coupling of the two tasks (Bland, 2000; Compton, 2001; Robling et al., 2006; Walsh et al., 2006). Coupling of the two cellular activities is a carefully controlled process, ensuring that new bone will be replaced where old bone was removed (Robling et al., 2006).

A balanced remodeling process ensures continual replacement of old bone with new bone, thereby maintaining bone health. This delicate balance involving osteoclastic bone resorption followed by osteoblastic bone formation is modulated by the
RANK/RANKL/OPG system, which is now being recognized as the primary regulator of bone remodeling (Weitzmann and Pacifici, 2006). This signaling system, which is regulated by estrogen (Weitzmann and Pacifici, 2006), 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; they also play an indirect role in bone turnover. 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 inhibits activation of IL-6. IL-6 in turn regulates expression of RANKL (Boyle et al., 2003). Thus, IL-6, indirectly regulated via estrogen, directly regulates osteoclastogenesis (McCormick, 2007).

Bone resorption and bone formation do not occur randomly, but rather take place in discrete bone multi-cellular units (BMU) (Lerner, 2006). Osteoblasts and osteoclasts arrange themselves into these temporary anatomical structures in order to carry out the bone remodeling process. Within the BMUs, osteoclasts first resorb bone; then, osteoblasts deposit layers of new bone in the locations where old bone was removed (Robling et al., 2006).

1.42 Effect of Sex Steroids on Bone Tissue

Estrogens and androgens appear to play a central role in the maintenance of bone microarchitecture (Janssen et al., 1999; Compston, 2001; Riggs et al., 2002; Raisz et al., 2005). Estrogens are able to act directly on bone to support normal bone physiology (Heshmati et al., 2002; Riggs et al., 2002; Raisz et al., 2005; Lerner 2006; Zallone, 2006).
Local aromatase expression in bone, which functions to convert androgens to estrogens, appears to be a source of estrogen responsible for maintaining bone mineralization (Simpson and Davis, 2001; Simpson, 2003). Increased resorption rates, devoid of a comparable increase in bone formation, have been attributed to a loss of either estrogen in women or androgen 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 effects directly on osteon cells, through the presence of both nuclear estrogen receptors α and β (Braidman et al., 1995; Matthews and Gustafson, 2006). Using expression vectors, Kuiper et al., 1998 determined that estrogen signaling occurs via two receptors, ERα and ERβ. Functional estrogen receptors have been demonstrated in osteoblasts, osteoclasts and most recently in osteocytes, although differential expression patterns of the estrogen receptor types are seen. ERα is the predominant isoform in human cortical bone, while ERβ is the principal isoform in human trabecular bone (Matthews and Gustafson, 2006).

Estrogen has been found to increase bone density by increasing osteoblast formation, differentiation, proliferation, and function (Riggs et al., 2002). Additionally, estrogen causes a decrease in formation and activation of osteoclastic cells (Hughes et al., 1996; Weitzmann and Pacifi, 2006; McCormick, 2007). Estrogen also induces apoptosis in osteoclasts (Hughes et al., 1996). Thus, the balance between bone formation and bone resorption is through the direct action of estrogen on bone (Janssen et al., 1999; Heshmati et al., 2002; Riggs et al., 2002; Raisz et al., 2005). Likewise, androgens have been shown to preserve bone health by sustaining the bone remodeling process.
(Compston, 2001; Riggs et al., 2002; Simpson et al., 2003; Vanderschueren et al., 2004).

Yet, the mechanisms by which these two sex steroids act to maintain bone mass are not fully established (Compston, 2001; Muir et al., 2004).

1.43 Local Production of Estrogens

Production of estrogen by peripheral tissues more than likely functions in either a paracrine or intracrine manner. These target tissues, which 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).

Because peripheral tissues are unable to synthesize estrogen \textit{de novo}, they are dependent on circulating precursor molecules. It is believed that estrogen and androgen bone-sustaining effects are as 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). The amount of active estrogen produced locally is relatively small; however, it is enough to invoke a significant biological response. Therefore, extragonadal estrogen biosynthesis plays a central role in bone physiology (Simpson et al., 2000; Simpson and Davis, 2001; Raisz, 2005; Syed and Khosla, 2005). Steroid sulfatase is an enzyme that converts inactive androgens and estrogens to active ones, and may, therefore, represent an important localized pathway for maintaining bone density. Utilizing this local metabolic pathway, bone cells are able to convert estrone sulfate and dehydroepiandrosterone sulfate into their biologically active forms for local use.
Local estrogen production may influence the rate of bone turnover (Janssen et al., 1999; Simpson et al., 2000; Simpson and Davis, 2001; Simpson, 2003; Reed et al., 2005). Because 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. In post-menopausal women, both estrone sulfate and dehydroepiandrosterone sulfate are abundantly circulating steroid precursors (Purohit et al., 1992; Fujikawa et al., 1997; Janssen et al., 1999; Muir et al., 2004). Accordingly, these two precursors may serve as a reservoir for the estrogenic steroids needed to sustain bone density. Production of estrogen locally is essential for maintaining bone mineralization and prevention of osteoporosis in men and women (Simpson et al., 2000). As a consequence, 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). It therefore seems likely that the steroid sulfatase enzyme system is of importance in osteoblast function, particularly in post-menopausal women.

1.44 Steroid Sulfatase Intracrinology In Bone Tissue

Metabolism of estrogen is dependent upon the profile of metabolizing enzymes present in a given tissue. Thus, estrogen metabolism in osteoblasts requires the coordinated action of the enzymes involved in its metabolic pathway. All of the enzymes found in the estrogen metabolic pathway (Fig. 14) are present in osteoblastic cells (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; Reed et al., 2005).
Dehydroepiandrosterone sulfate and estrone sulfate, both abundantly circulating steroid precursors, are converted to dehydroepiandrosterone and estrone, respectively, via steroid sulfatase. Dehydroepiandrosterone can then be converted to androstenedione by 3β-hydroxysteroid dehydrogenase-D5/D4 isomerase (3β-HSD). Androstenedione can be converted to estrone by aromatase or to testosterone by 17β-hydroxysteroid dehydrogenase. Dehydroepiandrosterone can also be converted to androstenediol by 17β-keto-reductase. Androstenediol is an androgen with estrogenic properties (Lardy et al., 2005). Estrone can be converted to the more potent 17β-estradiol by the enzyme 17β-hydroxysteroid dehydrogenase and testosterone can be converted to 17β-estradiol by aromatase. Thus, using pathways beginning with steroid sulfatase, bone cells have the ability to metabolize abundant steroid precursors, particularly dehydroepiandrosterone sulfate and estrone sulfate, to biologically potent estrogens.

Steroid sulfatase has been shown to be present in both human and rat bone (Fujikawa et al., 1997; van der Erden et al., 2002; van der Erden et al., 2004; Muir et al., 2004; Raobaikady et al., 2005). Muir et al. (2004) demonstrated the conversion of estrone to 17β-estradiol from steroid sulfatase in human hOB cell lines and human and rat OS cell lines. Additionally, Purohit et al. (1992) reported the conversion of estrone sulfate to estrone via steroid sulfatase in the human osteoblast cell lines, hOS, MG-63, and U20S. Moreover, hOS, human fetal osteosarcoma cells (HFO) and MG-63 cell lines were 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).

Because the enzymes required for estrogen metabolism have been demonstrated in osteoblasts, they would be fully able to metabolize estrone sulfate and
dehydroepiandrosterone sulfate to 17β-estradiol (van der Eerden et al., 2004). Estrone sulfate and dehydroepiandrosterone sulfate are found in high circulating amounts in adults (Pasqualini et al., 1996; Labrie et al., 2000; Muir et al., 2004; Pasqualini and Chetrite, 2005; Reed et al., 2005). The enzymatic activity of steroid sulfatase is required for osteoblasts to be capable of utilizing sulfated precursors in the metabolism of estrogen (Muir et al., 2004), because it catalyzes the removal of the sulfate group from inactive sulfated precursors (van der Eerden et al., 2004).

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). Within the same cell line, another study showed that steroid sulfatase could utilize both estrone sulfate and dehydroepiandrosterone sulfate as substrates (Fujikawa et al., 1997). Taken together, these two studies demonstrate the significance of steroid sulfatase in local production of estrogen from sulfated precursors to support the maintenance of bone.

1.45 Clinical Implications

In pre-menopausal and non-pregnant women, the principal source of systemic estrogen is the ovaries. Yet, other sites of estrogen biosynthesis exist throughout the body. These extragonadal sites of estrogen metabolism become the major source of estrogen in women after the onset of menopause. Bone tissue, specifically osteoblasts, serves as site of non-ovarian estrogen biosynthesis. Local estrogen production in bone maintains bone density (Simpson et al., 2000; Simpson and Davis, 2001; Riggs et al., 2002; Simpson, 2003; Raisz, 2005; Syed and Khosla, 2005; Cohen, 2006). However, any disruption in estrogen metabolism can be harmful to skeletal health. Accordingly, the
role of estrogen in bone maintenance is exemplified by the clinical observation of rapid bone loss at the onset of menopause. Estrogen deficiency is characterized by an increase in formation of new bone multicellular units and an alteration in the balance between the resorption and formation phases. Additionally, the resorption phase is prolonged by an increase in osteoclast recruitment that continues to extend the resorptive phase and thereby shorten the formation phase. Combined, these events act to increase resorption beyond what osteoblasts are able to fill in (Riggs et al., 2002; Raisz, 2005; Syed and Khosla, 2005; Cohen, 2006).

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. Osteoporosis is one of the most prominent bone pathologies that can occur as a result of a loss of coordination between osteon cells in the bone remodeling process (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). 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).

Osteoporosis affects approximately ten million Americans, eight million of which are women (NOF, 2007). Moreover, the National Osteoporosis Foundation has projected that by the year 2020, 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 (Muir et al., 2004;
Raisz, 2005). The decline in estrogen levels related to menopause results in an increase in the bone-remodeling rate (Syed and Khosla, 2005; Cohen, 2006; McCormick, 2007). Moreover, a decline in ovarian estrogen levels have been shown to both accelerate bone loss and increase susceptibility to fractures by disrupting the delicate balance between bone formation and bone resorption (Gruber et al., 2002; Heshmati et al., 2002; Raisz, 2005). Therefore, accelerated bone loss and increases in osteoporotic fractures associated with post-menopausal estrogen deficiency illustrate the importance of estrogens in bone. In post-menopausal women, active estrogens are synthesized by an intracrine mechanism in peripheral tissues, including bone (Labrie et al., 2000; Simpson et al., 2000; Simpson and Davis, 2001; Simpson et al., 2003). Consequently, the 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 post-menopausal women.
Chapter 2

Research Projects

2.1 Project I. Immunohistochemical Analysis Of Steroid Sulfatase In Human Tissues

2.11 Hypothesis And Objectives

Model: Human: normal tissues and both malignant and non-malignant breast tissues

Rationale:

Levels of steroid sulfatase protein and mRNA in malignant breast tissues have been shown to be high relative to those in non-malignant breast tissues. These data were derived from several studies using tissue samples of Japanese women to ascertain the prognostic value of steroid sulfatase in breast cancer evaluation. However, no such studies have been undertaken using samples from the United States population. We believe the presence of steroid sulfatase to be higher in breast cancer tissues when compared to normal breast tissues in samples from the US demographic. In order to determine the prognostic value of steroid sulfatase, we first need a way to assess the protein in tissue samples. Thus, the objective of this study was to determine if the steroid sulfatase antibody (STS-275) that was recently developed in our laboratory could be used in the evaluation of steroid sulfatase in human tissue samples, including breast cancer tissues.
Hypothesis:

Steroid sulfatase plays an important role in physiological and pathological processes in a variety of tissues. Comparison of steroid sulfatase among tissues could be useful in understanding these processes. Additionally, the presence of the steroid sulfatase protein could also serve as a prognostic indicator of breast cancer.

Objectives:

1. Validate the newly developed steroid sulfatase antibody, STS-275 for use in visualizing the distribution of the steroid sulfatase protein in various tissues.

Experiments to address Objective 1:

a. Western blotting of placenta and rat liver microsomes, and MDA-MB-231 and MCF-7 breast cancer cell microsomes.

2. To develop an immunohistochemical (IHC) assay to analyze the presence of the steroid sulfatase protein in various human tissues using the steroid sulfatase antibody (STS-275) developed in our laboratory.

Experiments to address Objective 2:

a. IHC assay on normal human single tissue samples using STS-275
b. IHC assay on normal human organ tissue microarray using STS-275
c. IHC assay normal and malignant single breast tissue samples using STS-275

2.12 Project I Abstract

Steroid sulfatase (E.C. 3.1.6.2) is an enzyme that removes the sulfate group from 3β-hydroxysteroid sulfates. This enzyme is best known for its role in estrogen production via
the fetal adrenal-placental pathway during pregnancy; however, it also has important
functions in other physiological and pathological steroid pathways. The objective of this
study was to examine the distribution of steroid sulfatase in normal human tissues and in
breast cancers using immunohistochemistry, employing a newly developed steroid
sulfatase antibody. A rabbit polyclonal antiserum (STS-275) was generated against a
peptide representing a conserved region of the steroid sulfatase protein. In Western
blotting experiments using human placental microsomes, this antiserum cross-reacted
with a 65 kDa protein, the reported size of steroid sulfatase. The antiserum also cross-
reacted with single protein bands in Western blots of microsomes from two human breast
cancer cell lines (MDA-MB-231 and MCF-7) and from rat liver; however, there were
some size differences in the immunoreactive bands among tissues. The steroid sulfatase
antibody, STS-275, was used in immunohistochemical analyses of individual human
tissue slides as well as a human tissue microarray. For single tissues, human placenta and
liver showed strong positive staining against STS-275. ER+/PR+ breast cancers also
showed relatively strong levels of steroid sulfatase immunoreactivity. Normal human
breast showed moderate levels of steroid sulfatase immunoreactivity, while ER-/PR-
breast cancer showed weak immunoreactivity. This confirms previous reports that steroid
sulfatase is higher in hormone-dependent breast cancers. For the tissue microarray, most
tissues showed some detectable level of steroid sulfatase immunoreactivity, but there
were considerable differences among tissues, with skin and lymph nodes having the
highest immunoreactivity and brain tissues having the lowest. These data reveal the
utility of immunohistochemistry in evaluation of steroid sulfatase activity among tissues. The newly developed antibody, STS-275, should be useful in studies of both humans and rats.

2.13 Introduction

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 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 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. This disorder is characterized by scaly skin. It also may be associated with corneal opacities, which do not affect vision (DiGiovanna and Robinson-Bostrom, 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 widely distributed among human tissues. Its presence has been well established in both placenta and breast, and this enzyme has also been documented to occur in many other tissues, including skin, liver, lung, ovary, and adrenal gland (Pasqualini and Chetrite, 2005; Reed et al., 2005). 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). Furthermore, steroid sulfatase levels vary between normal and cancerous breast tissues (Utsumi et al., 1999a; Utsumi et al., 1999b; Chetrite et al., 2000; Utsumi et al., 2000; Miyoshi et al., 2003; Suzuki et al., 2003a; Yoshimura et al., 2004), supporting the suggestion that this enzyme is involved in growth of hormone-dependent cancers. Recently, it has been suggested that steroid sulfatase levels might be a prognostic indicator for breast cancer, as the presence of steroid sulfatase and its mRNA are negatively correlated with survival in women (Utsumi et al., 2000; Suzuki et al., 2003a; Suzuki et al., 2003b).

Given the known and potential physiological and pathological roles of steroid sulfatase, it is important to have more information about the distribution of this enzyme in normal and cancerous tissues. Therefore, the objective of this study was to use a newly developed steroid sulfatase antibody, STS-275, in immunohistochemical analyses of the presence and distribution of steroid sulfatase in various human tissues, as well as in different types of breast cancers.

2.14 Materials and Methods

*Steroid sulfatase peptide and antibody production*

A rabbit polyclonal antiserum was made against a peptide representing a conserved region of the steroid sulfatase protein, based on a multiple alignment of human (GenBank accession no. M16505), rat (GenBank accession no. U37138) and mouse (GenBank accession no. U37545) cDNA sequences and their corresponding amino acid sequences.
The specific peptide sequence was selected based on several factors: 1) a high degree of homology between the three species, 2) a lack of homology to any protein other than steroid sulfatase, 3) high antigenicity, as determined from indices of hydrophobicity and hydrophilicity, and 4) location away from the sulfatase active site and the membrane spanning region. The 17 amino acid sequence chosen was: KGEIHGSNGIYKGGKA.

A peptide with this sequence was synthesized, coupled to keyhole limpet hemocyanin (KLH), and used as antigen in rabbits. The resulting antisera were screened against the peptide antigen using an ELISA assay (not shown) and then tested against potential steroid sulfatase-containing tissues using Western blotting.

*Antibody purification*

Affi-Gel Protein A Agarose (BioRad, Hercules, CA) chromatography was used for the purification of the steroid sulfatase antibody. The steroid sulfatase antiserum was thawed and sodium azide was added to a final concentration of 0.05%. The antiserum (2.5ml) was clarified by centrifugation at 15,000xg for five min at 4°C. The remaining clarified antiserum was passed through the column (1.0cm x 10cm column containing 5.0 ml of Protein A Agarose) twice at the rate of two ml per min. The column was then washed with TBS (50mM Tris-HCl, pH 7.4; 150mM NaCl; 0.05% sodium azide) at 10-fold the volume of antiserum. Centrifuge tubes (1.5ml) for collection of eluted sample were filled with 100μl of neutralization buffer (1M Tris-HCl, pH 8.0; 1.5M NaCl; 1mM EDTA; 0.5% sodium azide). The antibody was eluted (1 ml in each centrifuge tube) first using 15ml of elution buffer (50mM glycine-HCl, pH 2.7) and later using 10ml of elution buffer (50mM glycine-HCl, pH 1.9) at room temperature. A Coomassie protein assay was performed and the appropriate protein-containing fractions were pooled. The purified
antibody was stored at 2-8°C. The same procedure was used to purify the preimmune sera.

Microsome preparation

Pre-confluent cultures of MCF-7 and MDA-MB-231 breast cancer cells were scraped off the surface of the dishes and the cells were pelleted by centrifugation (1000xg 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 sec bursts of a Tissue Tearor homogenizer. The nuclear fraction was pelleted by centrifugation at 2500xg for 10 min at 4°C. The resulting supernatant was removed to ultracentrifuge tubes and centrifuged at 107,000xg for 1h at 4°C to yield the microsomal fraction. The resulting pellet was resuspended in 50mM Tris-HCl buffer (pH 7.5) at 1:2 original w:v. Protein concentration of the microsomal suspension was determined by BCA assay (Pierce Chemical Co., Rockford, IL).

Western Blotting

Proteins from placental, MDA-MB-231, MCF-7 and rat liver microsomes were 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 2x Laemmlli sample buffer (BioRad) containing 10% β-mercaptoethanol and boiled for 4 min. Samples (10µl) 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, the gels were either stained with Coomassie blue dye, or the proteins were transferred electrophoretically for two hours at 70 volts to a
PVDF membrane (BioRad) in transfer buffer (25mM Tris, 192mM glycine, 20% methanol).

After transfer, the PVDF membranes were washed three times in Tris-saline buffer (50mM Tris-HCl, 154mM NaCl, pH 7.5) for 10 min at room temperature, and then blocked for 30 min with blocking reagent (5% nonfat dry milk in Tris-saline buffer). Membranes were then incubated with steroid sulfatase antibody (1:500 dilution in blocking reagent) overnight in a sealed plastic bag at room temperature. The membranes were washed again three times for 10 min in Tris-Tween buffer (Tris saline buffer + 0.05% Tween 20), then incubated with secondary antibody (goat anti-rabbit IgG-HRP; 1:1000 dilution in blocking buffer; BioRad) for two h. The membranes were washed in Tris-Tween buffer as before, then developed by incubating for 10 min in substrate reagent (Sigma Fast 3,3’-diaminobenzidine tablet set, Sigma Chemical Co., St. Louis, MO). After the bands appeared to the desired intensity, the reaction was stopped by washing the membrane in water for 10 min.

In one experiment, the steroid sulfatase antibody was preincubated (16 h) in the presence of the peptide antigen before its use in the Western blot. For this experiment, placental microsomal protein was loaded at 25 and 5 µg per lane.

**Immunohistochemistry**

Immunohistochemical analysis was performed on various human tissues using the steroid sulfatase antibody developed in our laboratory. Tissues were obtained from Spring Bioscience (Fremont, CA). Five different individual human tissues were screened for steroid sulfatase presence, including placenta, liver, normal breast, breast carcinoma (ER+/PR+), and breast carcinoma (ER-/PR-). In addition, a tissue microarray (Imgenex
IMH-301 (AA)), San Diego, CA) containing various normal human tissues was examined. Fifty-nine tissue samples were present on each slide, with a total of forty-four distinct tissue types represented.

Tissues were deparaffinized in xylenes for five min, two times. This was followed by incubating in dilutions of ethanol (100%, 95%, 70%, 50% and 30%) for two min each. The slides were then immersed in tap water for five min. Antigen retrieval was accomplished by immersing the slides in 10mM citrate buffer, pH 6.0, followed by heating in a microwave at high, medium and low power for five min each. The slides were then allowed to cool to room temperature. This was followed by quenching of endogenous peroxidase by incubating the slides in 3% hydrogen peroxide for six min. The slides were then washed with PBS three times for five min followed by incubation in blocking solution (Vectastain Elite ABC kit, Vector laboratories, CA) for 30 min. Two serial slides were used for each tissue type. One slide was incubated with purified primary antibody (1:400) and the other with purified preimmune serum antibodies (1:400) at room temperature for one hour. After that, slides were washed with PBS three times for five min each. Slides were then incubated in secondary antibody (Vectastain biotinylated antirabbit IgG) for 30 min. This was followed by incubation in Vectastain Elite ABC Reagent for 30 min. After that slides were washed in PBS three times, for five min each. Slides were then incubated in DAB reagent for two min. The reaction was stopped by washing with tap water. Slides were then counterstained with Hematoxylin QS (Vector laboratories, CA) for 30 s and washed with tap water to get rid of excess stain. Slides were later dehydrated in a 75%, 80%, 95% and 100% ethanol series for two
min each. They were cleared by immersing in xylene twice for five min each. Slides were mounted in Permount (Fisher Scientific, NJ).

**Evaluation of immunostained slides**

After immunohistochemistry, slides were scored for steroid sulfatase status. A scale of 0 to 3 was used, with zero representing no steroid sulfatase immunoreactivity and three representing the highest immunoreactivity. Three pairs of slides, run on separate days, (control antibody and sulfatase antibody) for each tissue and were read by three independent individuals, in a blind fashion. On a particular slide, four different regions were read and scored from 0 to 3. An average of the readings was calculated and a final steroid sulfatase score was assigned to each tissue. Images of the tissues were also taken and permanently stored.

**Statistical analysis**

All statistics were run using the program Prism4 (Graph Pad Software, Inc., San Diego, CA). Comparisons among single tissue samples were made using the Kruskal-Wallis test. Dunn’s Multiple Comparison Test was used for post hoc analysis. Probabilities of less than 0.05 were considered significant.

**2.15 Results**

**Western blotting**

In Western blotting experiments using microsomal preparations from human placenta, the steroid sulfatase antibody, STS-275, cross-reacted with a single protein band of approximately 65 kDa. (Fig. 2). Cross-reactivity to this 65 kDa band was eliminated
when the steroid sulfatase antibody was exposed to the peptide fragment used to generate the antiserum (not shown).

In Western blotting experiments using microsomal preparations from various other tissues and cell lines (rat liver, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells), STS-275 also cross-reacted primarily with a single protein band (Fig. 3). There were some apparent differences in the size of the cross-reactive protein bands among tissues and cell lines. Rat liver microsomes and MDA-MB-231 microsomes had cross-reactive bands approximating 70 kDa. The MDA microsomes Western blot also had a less prominent cross-reactive band at 50 kDa. MCF-7 cells had a single cross-reactive band at approximately 85 kDa.

**Immunohistochemistry on single tissues**

Immunohistochemical analysis was carried out on various human tissues, using single tissue slides (Fig. 4). Slides of placenta, liver, and breast carcinoma (ER+/ PR+) showed high immunoreactivity for steroid sulfatase. Normal breast showed moderate steroid sulfatase immunoreactivity, whereas, breast carcinoma (ER-/ PR-) had very low steroid sulfatase immunoreactivity. Tissues were scored for steroid sulfatase immunoreactivity on a scale of 0 to 3, with zero representing no steroid sulfatase immunoreactivity and three representing the highest steroid sulfatase immunoreactivity (Fig. 5). There were significant differences in immunoreactivity among tissues, as indicated by Kruskal-Wallis test (H=24.97, P < 0.0001). Immunoreactivity for liver, placenta and estrogen-receptor positive breast cancer were all significantly greater than that for estrogen-receptor negative breast cancer (Dunn’s Multiple Comparison Test, P < 0.05).
Immunohistochemistry on tissue microarrays

Immunohistochemical analysis was carried out on 44 different human tissues using a tissue microarray (Fig. 6). Tissues were scored for steroid sulfatase immunoreactivity on a scale of 0 to 3, with zero representing no steroid sulfatase immunoreactivity and three representing the highest steroid sulfatase immunoreactivity (Fig. 7). Lymph node, liver, skin, nasal mucosa and stomach body averaged the highest levels of immunoreactivity (≈2.5) while placenta, breast, esophagus, small bowel, colon and prostate averaged intermediate values (2.0-2.2). Brain tissues were consistently very low (≤1.0).

2.16 Discussion

Most steroid sulfatase antibodies have been developed by partially purifying the enzyme from placental microsomes and then using the resulting protein preparation as antigen in rabbits or mice (Dibbelt et al., 1989; Okuda et al., 2001; Yanaihara et al., 2001; Suzuki et al., 2003a; Suzuki et al., 2003b; Utsunomiya et al., 2004; Nakamura et al., 2005). In contrast, Kawano et al. (1989) generated a monoclonal antibody against steroid sulfatase purified from rat liver microsomes. This antibody was subsequently utilized in the examination of steroid sulfatase in human tissues (Hoffman et al., 2001).

We used a different approach for steroid sulfatase antibody preparation, employing an antigen derived from a peptide representing a conserved region of the steroid sulfatase protein. The peptide sequence was chosen based on a multiple alignment of steroid sulfatase amino acid sequences of human, rat and mouse that were determined based on cDNA sequences. The sequence was also chosen so as not to include the steroid sulfatase active site or the membrane-spanning region. For the human steroid sulfatase protein,
this peptide represents amino acids 353 to 369 from the N-terminus. This sequence contains two potential N-myristoylation sites and a casein kinase II phosphorylation site. Furthermore, the specific peptide chosen is not similar to any sequence found in other ARS proteins, as determined by an exhaustive set of BLAST searches (Altschul et al., 1990). Therefore, the antiserum resulting from this peptide should not cross-react with any other sulfatases.

In Western blotting experiments using microsomal preparations from human placenta, the new steroid sulfatase antibody (STS-275) cross-reacted with a protein estimated to be approximately 65 kDa. This is similar to the reported size of steroid sulfatase from placenta. The monomeric molecular weight calculated from the primary sequence is 63 kDa (Stein et al., 1989). SDS-PAGE analysis of purified placental steroid sulfatase has produced values of 65 kDa (Hernandez-Guzman et al., 2001) and 73 kDa (Suzuki et al., 1992). Thus, STS-275 recognizes a protein band of the correct size for placental steroid sulfatase.

The 65 kDa immunoreactive band observed in the Western blots of human placental microsomes was eliminated when STS-275 was incubated prior to blotting with the peptide fragment used to generate the antiserum. This reveals that STS-275 recognizes the correct amino acid sequence on the steroid sulfatase protein and further suggests that the 65 kDa protein band is authentic steroid sulfatase.

Cross-reactivity of the steroid sulfatase antibody, STS-275, was observed for microsomal preparations of human breast cancer cell lines MDA-MB-231 and MCF-7. Each of these cell lines has been previously shown to have steroid sulfatase activity, as determined by tritiated estrone sulfate conversion assays (Purohit et al., 1992; Pasqualini
et al., 1995; Selcer et al., 1997; Kolli et al., 1999). STS-275 also cross-reacted with microsomal preparations of rat liver, as well as rat granulosa cells and rat ovary (not shown). Again, these tissues have been previously shown to possess steroid sulfatase activity (Chu et al., 1999; Clemens et al., 2000). It should be noted that the size of the immunoreactive protein differed somewhat among tissues, with placental microsomes resulting in a 65 kDa band, rat liver and MDA-MB-231 cell microsomes producing a 70 kDa band and MCF-7 cell microsomes resulting in an 85 kDa band. The source of this size variation is unclear. It may represent different levels of glycosylation of the protein in the different tissues, as steroid sulfatase is known to be a glycoprotein (Stein et al., 1989; Reed et al., 2005). Alternatively, it may represent different size proteins. Overall, the Western blotting data suggest that the new steroid sulfatase antiserum recognizes authentic steroid sulfatase in various tissues from both humans and rats. STS-275 may therefore be useful in studies of steroid sulfatase distribution or regulation for either species.

We used immunohistochemistry to investigate the distribution of steroid sulfatase among various human tissues. Two different formats of prepared human tissues were used, slides containing individual tissues and slides containing a microarray of tissues. For each format, we employed a scoring system based on a blind evaluation (see Materials and Methods) of each tissue that had been immunostained using the new steroid sulfatase antibody, STS-275, relative to a control slide that had been processed simultaneously with pre-immune antiserum. A semi-quantitative scoring system of 0 (no immunoreactivity) to 3 (high immunoreactivity) was used, and a single score was assigned to each tissue sections after first scoring them in four random locations on three
different occasions. This approach was used for the following reasons: 1) it provided sufficient resolution to determine which tissues contained high, medium, or low levels of steroid sulfatase immunoreactivity, which was the primary purpose of the study, and 2) it had relatively high throughput, allowing a number of tissues to be screened in a reasonable amount of time.

One disadvantage of the steroid sulfatase immunoreactivity scoring system employed in this study is that it does not take into account cell heterogeneity within tissues. The score given to any tissue represents the average immunoreactivity across all cell types present. For example, a tissue with a small number of highly positive cells dispersed with a large number of negative cells will receive a relatively low score. For this reason, a tissue receiving a low score should not be assumed to be devoid of steroid sulfatase. However, it should be noted that any procedure involving processing of bulk tissue samples, such as those used for assessing enzyme activity or mRNA levels, suffers from this same limitation.

Immunohistochemical analysis of the individual human tissue slides revealed relatively high levels of steroid sulfatase immunoreactivity in both placenta and liver. This is consistent with what is historically known about steroid sulfatase distribution. Placenta, particularly the syncytiotrophoblast region (Dibbelt et al., 1989), has been documented to contain high levels of steroid sulfatase activity (Dibbelt and Kuss. 1991; Shankaran et al., 1991; Hernandez-Guzman et al., 2001; Suzuki et al., 2003a; Pasqualini, 2005). The placental steroid sulfatase functions in the fetal adrenal-placental endocrine axis during pregnancy (Pasqualini, 2005). Liver also has been documented to possess steroid sulfatase activity (Munroe and Chang, 1987; Daniel et al., 1990; Shankaran et al.,
1991; Iwamori et al., 2005) and our results confirm the presence of this enzyme in liver. The physiological role of hepatic steroid sulfatase is not well understood, although it presumably functions in maintaining the appropriate balance of free and conjugated steroids in the blood (Iwamori et al., 2005).

Immunohistochemical analysis of the tissue microarrays indicated the presence of steroid sulfatase in most tissues, although at very different levels. Lymph node, liver, skin nasal mucosa and stomach body averaged the highest levels of immunoreactivity. Of these, skin is well documented to possess steroid sulfatase (Rabe et al., 1984; Milewich et al., 1988; Piraud et al., 1989). Indeed, the skin condition known as X-linked ichthyosis results from an absence of steroid sulfatase activity in skin fibroblasts (Piraud et al., 1989; DiGiovanna and Robinson-Bostrom, 2003), due to a deletion in the steroid sulfatase gene.

Analysis of the tissue microarrays further indicated that placenta, breast, esophagus, small bowel, colon and prostate all averaged intermediate values for steroid sulfatase immunoreactivity. As indicated above, placenta is known to have significant steroid sulfatase activity and our data are consistent with the presence of this enzyme in these tissues. Prostate also has been shown to have both steroid sulfatase activity (Selcer et al., 2002) and immunoreactivity (Nakamura et al., 2006). The exact role of steroid sulfatase in the prostate remains to be determined, but it may be involved in local production of bioactive steroid hormones (Platia et al., 1984; Selcer et al., 2002).

Brain tissues (cerebellum, white matter and gray matter) were consistently very low in steroid sulfatase immunoreactivity. However, several reports have shown steroid sulfatase activity and mRNA levels in certain brain tissues (Platia et al., 1984;
Steckelbroeck et al., 2004). It is likely that steroid sulfatase in brain is highly localized, and therefore may not be revealed in immunohistochemical analysis of entire brain sections.

The immunohistochemical assay on the normal human organ microarray using the steroid sulfatase antibody, STS-275, proved to be useful for demonstrating the general distribution of steroid sulfatase in a variety of tissues; however, the value of the data are limited by the small amount of information provided for each tissue type represented on this microarray (age, sex, and primary cancer of the individual), and the small sample size (Fig. 7). A more robust sampling of tissues with complete background data would allow us to address questions concerning the possible role of steroid sulfatase in tissues. Based on the present data, it can be stated that steroid sulfatase is widely distributed suggesting a role in a variety of tissues. The only known function of steroid sulfatase at this time is conversion of sulfated steroids to unconjugated forms.

Recently, Miki et al. (2002) measured steroid sulfatase immunoreactivity, enzyme activity and steroid sulfatase mRNA of various human adult and fetal tissues. In that study, they failed to find immunoreactivity to any tissue except placenta. It is probable that methodological differences account for the discrepancy between their data and ours. This group used a monoclonal steroid sulfatase antibody that was raised against the enzyme purified from human placenta, and recognizes the steroid sulfatase peptide corresponding to amino acids 420-428. As indicated above, there is a substantial body of evidence that steroid sulfatase is present in many tissues (also see Pasqualalini and Chetrite, 2005; Reed et al., 2005), and our data are consistent with these observations.
In the present study, ER+/ PR+ human breast carcinoma had high steroid sulfatase immunoreactivity whereas ER-/ PR- human breast carcinoma had minimal steroid sulfatase immunoreactivity. Normal breast tissue was intermediate. These data support previously published information concerning the distribution of steroid sulfatase in different types of breast cancer tissues. Suzuki et al. (2003a, 2003b) studied steroid sulfatase immunoreactivity in human breast cancers and found steroid sulfatase present only in carcinoma cells. Miyoshi et al. (2003) showed that steroid sulfatase mRNA levels are elevated in ER positive breast cancer, but not in ER negative breast cancers. These findings, as well as ours, suggest that steroid sulfatase activity is elevated in estrogen receptor positive breast cancer cells. Interestingly, Chetrite et al. (2000) found higher levels of steroid sulfatase activity in and around the breast tumors as compared to adjacent tissues. More recently, Irahara et al. (2006) demonstrated that steroid sulfatase mRNA is elevated in metastatic breast cancers as compared to primary cancers. Taken together, the evidence indicates that steroid sulfatase is somehow involved in human breast cancer, as has been suggested previously (Pasqualini and Chetrite, 2005; Reed et al., 2005). This idea is further supported by several reports that steroid sulfatase mRNA levels are adversely correlated with prognosis of survival and positively correlated with risk of recurrence from breast cancer (Utsumi et al., 1999a; Utsumi et al., 1999b; Utsumi et al., 2000; Miyoshi et al., 2003; Suzuki et al., 2003a; Suzuki et al., 2003b).

Because steroid sulfatase protein expression has been demonstrated to be adversely correlated with worsened prognosis in some ER+ breast cancers (Utsumi et al., 1999; Utsumi et al., 2000; Suzuki et al., 2003; Myoshi et al., 2004), the steroid sulfatase antibody, STS-275 could be utilized in the clinical diagnosis and subsequent treatment of
ER+ breast cancers. Physicians readily use immunohistochemistry on patient breast
cancer specimens to screen for the presence of the estrogen receptor in order to classify
breast cancer as either ER+ or ER- (Sneige, 2004). STS-275 could be used to determine
steroid sulfatase protein expression in these same samples. This would enhance the
evaluation of malignant breast tissue by providing the physician with another tool for
determining the approach to clinical treatment.

In summary, we have generated a new antibody against steroid sulfatase and used this
antibody in immunohistochemical analyses of steroid sulfatase distribution among human
tissues. We have shown that the immunoreactivity of the steroid sulfatase enzyme varies
significantly among tissues. We have also demonstrated that steroid sulfatase activity
varies among different types of breast cancer tissues, with ER+/PR+ breast tumors having
the highest levels. The new antibody, STS-275, should be useful in studies of steroid
sulfatase distribution, function and regulation.
Figure 1. Estrogen metabolism pathway in peripheral tissues.
Figure 2. Western blot showing immunoreactivity of steroid sulfatase antiserum with human placental microsomes. Microsomal proteins were separated by SDS-PAGE and transferred to a PVDF membrane for immuno-staining. Western blotting was performed as indicated in Materials and Methods. Antiserum dilution as 1:500. Units represent µg of protein. Numbers below lanes indicate the amount of placental microsomal protein loaded per lane. MW: molecular weight markers.
Figure 3. Western blot showing immunoreactivity of steroid sulfatase antiserum with microsomes prepared from various tissues and cell lines. Microsomal proteins were separated by SDS-PAGE and transferred to a PVDF membrane for immuno-staining. Western blotting was performed as indicated in Materials and Methods. Antiserum dilution as 1:500. Abbreviations are: MW: molecular weight markers; MCF-7: human breast cancer cell line; RL: rat liver; and MDA: human breast cancer cell line MDA-MB-231.
Figure 4. Immunohistochemical analysis of steroid sulfatase for single tissue slides of various human tissues. See Materials and Methods for exact procedure. For each tissue type, serial slides were used. One slide was treated with purified steroid sulfatase antiserum (left side of each pair) and the other slide was treated with purified preimmune serum (right side of each pair). Slides were counterstained with hematoxylin. Slides were scored blindly for immunoreactivity on a scale of 0-3 (see Materials and Methods). Numbers after tissue name represent the score assigned to the individual tissue sections shown (entire section, not just what was shown). Images were captured using 10x objective for 100x total magnification.
Figure 5. Summary results of steroid sulfatase immunoreactivity for single tissue slides. Slides were scored blindly for immunoreactivity on a scale of 0-3 (see Materials and Methods). N=3 for all groups. Bars represent the mean ± 1 S.E.M. Different letters represent significantly different means (Dunn’s post hoc test, P<0.05). ER+=ER+/PR+ breast tumor, ER-=ER-/PR- breast tumor.
**Figure 6. Immunohistochemical analysis of steroid sulfatase for tissue microarray of various human tissues.** See Materials and Methods for the exact procedure. Duplicate slides were used. One slide was treated with purified steroid sulfatase antiserum (left side of each pair) and the other slide was treated with purified preimmune serum (right side of each pair). Slides were counterstained with hematoxylin. Slides were scored blindly for immunoreactivity on a scale of 0-3 (see Materials and Methods). Numbers after tissue name represent the score assigned to the individual tissue sections shown (entire section, not just what was shown). Images were captured using 10x objective for 100x total magnification.
Steroid sulfatase immunoreactivity for various human tissues.

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Figure 7. Steroid sulfatase immunoreactivity score for each of the tissues represented on the normal human organ tissue microarray.
2.2 Project II. Distribution of Steroid Sulfatase in Various C57/BL6 Mouse Tissues

2.21 Hypothesis And Objectives

Model: Mouse tissues obtained from female wild-type mice of the C57/BL6 strain

Rationale:

Our ultimate goal is to study steroid sulfatase in a physiological system. A mouse model appears to be the most feasible for our purposes. However, steroid sulfatase has never been described in mouse tissues. In order for us to utilize the mouse as a model, certain fundamental questions concerning steroid sulfatase needed to be addressed, such as documenting the presence and activity, as well as mRNA presence of steroid sulfatase.

Hypothesis:

Steroid sulfatase, a fairly ubiquitous enzyme, will be present in all of the mouse tissues to be evaluated, albeit at varying levels. Additionally, steroid sulfatase mRNA will follow a similar pattern to that of tissue.

Objectives:

1. To examine steroid sulfatase activity in various mouse tissues using enzyme activity assays

Experiments to address Objective 1:

   a. Steroid sulfatase conversion assays of tissue microsomes in the presence and absence of known steroid sulfatase inhibitors, DU-14 and EMATE
   b. Steroid sulfatase conversion assays of tissue cytosol

2. To examine the presence and distribution of steroid sulfatase protein in various mouse tissues

Experiments to address Objective 2:
a. Western blotting with the steroid sulfatase antibody, STS-275

3. To examine the presence of steroid sulfatase mRNA in various tissues

Experiments to address Objective 3:

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

2.22 Project II Abstract

Little information is available on the role of steroid sulfatase in various tissues under physiological conditions. The mouse is an ideal model organism for such studies, due to the amount of collateral information on endocrinology of this species, and due to the availability of many strains and transgenic constructs. Unfortunately, almost nothing is known about steroid sulfatase tissue distribution in the mouse. Thus, we sought to determine the activity and presence of the steroid sulfatase enzyme and the presence 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). We used three independent lines of evidence to assess the presence of steroid sulfatase protein and mRNA; enzyme activity, immunoreactivity, and RT-PCR.

All three techniques indicated that each of the six mouse tissues had detectable sulfatase protein and steroid sulfatase mRNA expression. However, there were considerable differences among tissues in activity, the sizes of the immunoreactive bands, and in amount of mRNA detected. 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 these mouse tissues (liver, lung, muscle, kidney, uterus
and ovary) all have the ability to convert sulfated steroids, such as estrone sulfate, into biologically potent steroids, such as 17b-estradiol. Such conversions may be important in the in situ synthesis of active steroids in peripheral tissues.

2.23 Introduction

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 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, 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 (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). This enzyme is also thought to be important in the local production of steroids in bone (Reed et al., 2005).

Steroid sulfatase is widely distributed among human tissues. Its presence has been well established in both placenta and breast, and this enzyme has also been documented to occur 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 (Dibbelt et al., 1994; Salido et al., 1990).

Although steroid sulfatase has been reasonably well studied in humans, there is only anecdotal information about the distribution and abundance of this enzyme in rodents. Steroid sulfatase has been purified from mouse liver and partially characterized (Mortaud et al., 1995) and the gene encoding murine steroid sulfatase has been identified (Salido et al., 1996). van der Eerden et al., (2004) demonstrated that steroid sulfatase mRNA was present during bone maturation in male and female rats; thereby, suggesting that this enzyme is important for development and maintenance of bone.

Given the known and potential physiological and pathological roles of steroid sulfatase, it is important to have more information about the distribution of this enzyme 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 steroid sulfatase in various mouse tissues using enzyme assays, immunoassays and RT-PCR.

2.24 Materials and Methods

Animals

Mice used in this study were wild type adult female C57/BL6, ages 8.5 months and 10.5 months. Five mice were euthanized, after which tissues (liver, lung, skeletal muscle from the thigh, kidney, uterus and ovaries) were collected and immediately frozen in liquid nitrogen.
Chemicals

$^{3}$H-estrone sulfate (ammonium salt, [6,7-$^{3}$H(N)]; 57 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Radioinert steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT). The steroid sulfatase inhibitors DU-14 and EMATE were gifts from Dr. Tom Li (Ohio State University).

Tissue preparation

For preparation of crude homogenates, mouse tissues were minced with scissors and added to 1:5 w:v ice-cold Tris-HCl buffer (50 mM Tris-HCl, pH 7.5), then homogenized using three 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). Tissues were either used immediately or were frozen at –80°C.

For preparation of microsomes, mouse tissues were minced with scissors and added to 1:5 w:v ice-cold Tris-sucrose buffer (50 mM Tris-HCl, 25 mM sucrose pH 7.5), then homogenized using three 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). The nuclear fraction was pelleted by centrifugation at 2500xg for 10 min at 4°C. The resulting supernatant was removed to Beckman ultracentrifuge tubes (11x60mm) (Beckman Coulter Inc., Fullerton, CA) and centrifuged at 107,000xg for 1h 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. The supernatant was used as the cytosol fraction. Microsomes and cytosol were frozen at –80°C until use. Protein concentrations of microsomes and cytosol were determined by BCA assay (Pierce Chemical Co., Rockford, IL).
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 water as the dilutent. Duplicate concentrations (in µg/tube) were 100, 50, 25, 12.5, 6.25, and 3.125. Duplicate tubes of MC3T3-E1 microsomal samples were prepared using 20µl of microsomal suspension or 50µl of cytosol. Absorbances were read at 562 nm using a spectrophotometer (Thermospectronic, Genesys 20; Waltham, MA, USA).

Denaturing polyacrylamide electrophoresis

Mouse tissue microsomes and were dissolved in 100ml Tris-HCl Buffer (pH 7.5). Proteins from mouse tissue microsomes 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 2x Laemmli sample buffer (BioRad) containing 10% β-mercaptoethanol and boiled for 4 min. Samples (10µl) were loaded into wells, and proteins were electrophoretically separated at 30 mA per gel until the dye front reached the bottom of the gel. Gels were either stained or used in immunoblotting.

Immunoblotting

After electrophoresis, proteins were transferred electrophoretically for two h at 70 volts to a PVDF membrane (BioRad) in transfer buffer (25mM Tris, 192mM glycine, 20% methanol). Western blotting was performed on mouse tissue microsomal proteins using an antibody (STS-275) generated against a peptide representing a conserved region of mammalian steroid sulfatase (Selcer et al., 2007). This peptide was identified using a
multiple alignment of mouse, rat and human steroid sulfatase amino acid sequences.

After transfer, the PVDF membranes were washed three times in Tris-saline buffer (50mM Tris-HCl, 154mM NaCl, pH 7.5) for 10 min at room temperature, and then blocked for 30 min with blocking reagent (5% nonfat dry milk in Tris-saline buffer). Membranes were then incubated with steroid sulfatase antibody (1:500 dilution in blocking reagent) overnight in a sealed plastic bag at room temperature. The membranes were washed again three times for 10 min in Tris-Tween buffer (Tris saline buffer + 0.05% Tween 20), then incubated with secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase, BioRad) at a 1:1000 dilution in blocking buffer, for two h. The membranes were washed in Tris-Tween 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, the reaction was stopped by washing the membrane in water for 10 min.

**Steroid sulfatase assay**

$^3$H-estrone sulfate was diluted in Tris-HCl buffer (50mM, pH 7.5) and 100µl (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 50µl would yield a concentration of 10 mM in the final assay volume. The inhibitors DU-14 and estrone-3-O-sulfamate (EMATE) were dissolved in Tris HCl buffer. These solutions (100µl) were added to the assay tubes to achieve the appropriate final concentration of each inhibitor (1µM). Mouse tissue homogenates or microsomes were diluted with Tris-HCl buffer to achieve the final desired concentration of membranes (25 to 200 mg) in 200µl of buffer (500µl final volume). The assay tubes were pre-incubated for 5 min at 37°C in a water
bath. The assay was initiated by addition of the homogenates or microsomes (200µl) to the tubes containing the compounds. Control tubes with no inhibitor, tubes without sample (to control for spontaneous hydrolysis), and tubes with boiled sample (100°C, 10 min) 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 2,500xg for 10 min at 24°C. Duplicate one-ml aliquots were removed from the organic phase of the samples and added to 5ml of scintillation cocktail. The aliquots were counted in a liquid scintillation counter (Packard Instrument Co.) for determination of product formation. The experiments were run 4 times, with duplicate tubes for each experiment.

RNA extraction

Total RNA from mouse tissues were isolated using Absolutely RNA® Miniprep Kit (Stratagene, La Jolla, CA) according to manufacturer’s instructions. Frozen tissues were fractured, the sample quickly weighed (25-40 mg), and returned to liquid nitrogen. Mouse tissues were then minced with scissors and homogenized in 600µl of lysis buffer using two 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). RNA homogenate was transferred to a Prefilter Spin Cup seated in a 2ml receptacle tube and spun at 12,000xg for 5m. The spin cup was removed and the filtrate was retained. An equal volume of 70% ethanol was added to each sample and vortexed for 5 sec. Mixture was then transferred to an RNA Binding Spin Cup seated in a fresh receptacle tube and spun at 12,000xg for 1 min. The filtrated was discarded, and 600µl of 1X Low Salt Wash Buffer was added and spun at 12,000xg for 1min. The filtrate was discarded and the sample was again spun at 12,000xg for 2 min. 55µl DNase
solution was added directly to the spin cup and incubated at 37°C for 25 min. 600µl of 1X High Salt Wash Buffer was added to spin cup and spun at 12,000xg for 1 min. The filtrate was discarded, and 600µl 1X Low Salt Wash Buffer was added and spun at 12,000xg for 1 min. The filtrate was once again discarded and 300µl 1X Low Salt Wash Buffer was added and spun at 12,000xg for 2 min to dry the fiber matrix. The spin cup was then transferred to a 1.5ml microcentrifuge tube and 100µl Elution Buffer was added directly to the spin cup. The sample was incubated for 2 min at room temperature and then spun at 12,000xg for 1 min. The elution step was then repeated for maximum RNA yield. RNA was quantified using a spectrophotometer (ThermoSpectronic, Genesys 8). Purity of nucleic acid preparation was assessed by 260:280 ratio. The amount of RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at -80°C until use.

Reverse transcriptase polymerase chain reaction

PCR primers for RT-PCR were selected from the complete coding sequence for Mus musculus steroid sulfatase (GenBank accession no. U37545). One set of steroid sulfatase primers was devised. The forward primer has the sequence (5’ to 3’) AGC ACG AGT TCC TGT TCC ACT ACT (FB). The reverse primer has the sequence (5’ to 3’) AAG TTG GGC GTG TAG AAG GC (RB). The expected RT-PCR product from this primer pair is 100 base pairs. PCR primers for RT-PCR were selected from the complete coding sequence for Mus musculus peptidylprolyl isomerase A (cyclophilin) (GenBank accession no. NM008907). One set of cyclophilin primers was devised. The forward primer has the sequence (5’ to 3’) TAT CTG CAC TGC CAA GAC TG (mCPH-F1). The reverse primer has the sequence (5’ to 3’) ACA GTC GGA AAT GGT GAT CT
(mCPH-R1) (Davis et al., unpublished). The expected RT-PCR product from this primer pair is 143 base pairs. Cyclophilin was used as an internal standard.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed using the SuperScript® One-Step RT-PCR with Platinum™ Taq Kit (Invitrogen Life Technologies) according to manufacturer’s instructions. One µg of template RNA, one µl of RT / Platinum™, twenty-five µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM MgSO₄), one µl of forward primer (150pmol), one µl of reverse primer (150pmol), DEPC-treated water added to make total volume 50 µl. Ingredients were placed into a sterile 0.5 ml tube, vortexed gently, heated to 55°C for ten minutes, and then spun in a microcentrifuge for 30 seconds at 4,000xg. PCR reactions were then placed into a thermal cycler (MJ Research, Inc., Watertown, MA). The program for amplification included the reverse transcription step, which consisted of a 30 min hold at 55°C and a 2 min hold at 94°C. Next, the cDNA was amplified over 30 cycles of: 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing of primers), and 1 min 60°C (extension). The PCR reaction was terminated with a final extension of 10 min at 72°C, and then held at 4°C. The PCR products were then separated in a 2% agarose gel containing 5ml of 1 mg/ml ethidium bromide solution. The gel was run in 1x TAE running buffer (40 mM Tris-acetate, 2 mM Na2-EDTA-2H2O) for 1 h at 70 volts. Ten µl of PCR product were mixed with 2µl 10x loading dye (41% w/v Bromophenol blue, 25% w/w Ficoll). A 1000-bp DNA ladder (Invitrogen Life Technologies) was used as the standard.
Statistics

Statistics were run using Prism 4.0 for Macintosh (GraphPad Software, San Diego, CA). Probabilities of \( P < 0.05 \) were considered significant.

2.25 Results

Steroid sulfatase enzyme activity in mouse tissues

Steroid sulfatase activity in crude homogenates (Fig. 8) varied considerably among tissue types (One-way ANOVA, \( F=63.78; \ 5, 23 \ \text{df}; \ P < 0.001 \)). Liver and uterus had the highest levels of steroid sulfatase, with lung and muscle having intermediate levels and kidney and ovary showing the lowest levels. Steroid sulfatase activity of liver and uterine homogenates were not statistically different from each other, but were significantly higher than all other tissues (Newman-Keuls post hoc test, \( P < 0.05 \)). Ovarian homogenates had significantly lower steroid sulfatase activity than all other tissues.

We used the known steroid sulfatase inhibitors EMATE and DU-14 to test whether the observed enzyme activity could be blocked. EMATE virtually eliminated all steroid sulfatase activity in all tissue homogenates (data not shown). DU-14 (Fig. 9) substantially reduced (liver) or virtually eliminated (lung, muscle, kidney, uterus, ovary) steroid sulfatase activity of the tissue homogenates.

Steroid sulfatase activity was compared in microsomes from pooled tissues from liver, lung, kidney and muscle (Fig. 10). Uterus and ovary were excluded due to lack of tissue. Liver, lung, and muscle all had similar levels of microsomal steroid sulfatase
activity, at approximately 1.0 fmol/µg protein. Kidney had significantly lower steroid sulfatase at 0.27 fmol/µg protein. Steroid sulfatase activity of the corresponding cytosol fractions from the same tissues were also measured (Fig. 11). In general, all cytosols showed substantially lower (about 50-fold) steroid sulfatase activity than the corresponding microsomes. Liver cytosol was the highest of all tissues, with lung, kidney and muscle significantly lower.

*Steroid sulfatase protein in tissues, by Western blot*

Western blotting, using our newly developed steroid sulfatase antibody, STS-275 (Selcer et al., 2007; see Project 1), indicated the presence of immunoreactive bands in microsomes from all four tissues (Fig. 12). However, there were differences among tissues. For liver, four strongly immunoreactive bands were apparent, at approximately 128 kDa, 40 kDa, 30 kDa and 17 kDa. For kidney, one strongly immunoreactive band was present at 100 kDa. For muscle, two strongly immunoreactive bands were present at 195 kDa and 40 kDa. Lung tissue showed a single weakly immunoreactive band at approximately 65 kDa.

*Steroid sulfatase mRNA analysis by RT-PCR*

Reverse transcriptase polymerase chain reaction, using primers specific for the mouse steroid sulfatase encoding gene, resulted in a single product for all tissues, when resolved using agarose gel electrophoresis (Fig. 13). The size of this band was identical to the expected size of the product (100 bp) for the primer pair used. Thus, in all of the tissues represented, steroid sulfatase mRNA was present.
2.26 Discussion

In this study, we have demonstrated the activity and presence of the steroid sulfatase enzyme and the presence of steroid sulfatase mRNA, in a variety of mouse tissues, using three independent lines of evidence; enzyme activity, immunoreactivity, and RT-PCR. Our results indicate that these mouse tissues (liver, lung, muscle, kidney, uterus and ovary) 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 \textit{in situ} synthesis of active steroids in peripheral tissues.

Steroid sulfatase activity was measured in crude tissue homogenates and in microsomal preparations of various mouse tissues, using conversion assays with $^3$H-estrone sulfate (E$_1$S) as substrate. Measurable steroid sulfatase activity was observed in all tissues for both homogenates and microsomes. For the tissue homogenates, which represent the average of five different mice, liver and uterus had substantially higher steroid sulfatase activity than did the other four tissues. Liver has been previously documented to have high levels of steroid sulfatase activity (Munroe and Chang, 1987; Daniel and Chang, 1990; Shankaran et al., 1991; Iwamori, 2005). Similarly, our data on human tissues (Selcer et al., 2007; see Chapter 3) found high levels of hepatic steroid sulfatase using immunohistochemistry. Our 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).
The specific steroid sulfatase inhibitor estrone-3-O-sulfamate (EMATE) completely eliminated steroid sulfatase activity of all tissue homogenates, while the inhibitor DU-14 significantly reduced activity (both at 1µM). DU-14 has previously been shown to be a weaker inhibitor of steroid sulfatase than EMATE (Kolli et al., 1999), but it has the advantage of not being estrogenic. The finding that EMATE and DU-14 inhibit steroid sulfatase activity under the conditions of our assay provides further evidence that the activity measured in this study is legitimate steroid sulfatase, because these compounds are known to be specific inhibitors of this enzyme (Howarth et al., 1994; Kolli et al., 1999).

Steroid sulfatase activity was also measured in microsomes derived from pooled tissues from liver, lung, kidney and muscle. Microsomes from all four tissues showed detectable steroid sulfatase activity, with liver, lung and muscle having higher levels than kidney. This is not surprising, because steroid sulfatase is known to be concentrated in the microsomal fraction after differential centrifugation (Dibbelt et al., 1989). Cytosols from each of the four tissues also showed some steroid sulfatase activity, albeit 50 times lower than the microsomes, when compared on a per microgram protein basis. Interestingly, the pattern of steroid sulfatase activity for tissue cytosols was different from that of the microsomes, with liver having significantly more cytosolic steroid sulfatase than the other three tissues. It is unclear, what, if any, significance there is to this observation. Cytosolic steroid sulfatase could reflect enzyme that is shaken loose from the membranes during the preparation process, or it could reflect a soluble form of the enzyme. There are reports of a soluble form of steroid sulfatase (Keinanen et al., 1983).
In Western blotting experiments using microsomal preparations from the various mouse tissues, the new steroid sulfatase antibody, STS-275, cross-reacted with one or more protein bands in each tissue. However, there were differences among tissues. For liver, four strongly immunoreactive bands were apparent, at approximately 128 kDa, 40 kDa, 30 kDa and 17 kDa. For kidney, one strongly immunoreactive band was present at 100 kDa. For muscle, two strongly immunoreactive bands were present at 195 kDa and 40 kDa. Lung tissue showed a single weakly immunoreactive band at approximately 65 kDa. The monomeric molecular weight calculated from the primary sequence is 63 kDa (Stein et al., 1989). SDS-PAGE analysis of purified placental steroid sulfatase has produced values of 65 kDa (Hernandez –Guzman et al., 2001) and 73 kDa (Suzuki et al., 1992). Thus, only mouse lung microsomes have immunoreactivity against a protein band of similar size to that of human placental microsomes. Interestingly, the largest band in liver (128 kDa) corresponds exactly to the size of the largest band in the mouse MC3T3-E1 cells (see chapter 5). In a study of mouse liver, Mortaud et al. (1995) suggested that the two larger bands they observed (68 kDa and 60 kDa) in Western blotting were subunits of a 128 kDa steroid sulfatase (based on gel filtration) and that the differences between the bands might be due to differences in carbohydrate content of the subunits. Thus, the 128 kDa band we observed in mouse liver and mouse MC3T3 cells might reflect this proposed dimer. Regarding the 195 kDa band present in muscle, it is possible that this is a nonspecific reaction to the protein myosin, which is prevalent in muscle. Frequently, we observe a band appearing at 195 kDa in the SDS-PAGE standard lane, which corresponds to myosin. This occurs regardless of the antibody used in the Western blot. If the 195 kDa band is ignored, then muscle tissue reveals a single cross-reactive
band at about 40 kDa, identical in size to a band present in liver.

Western blotting, using MC3T3-E1 cell microsomes, revealed three major bands of immunoreactivity at approximately 128 kDa, 90 kDa, and 84 kDa (DiFrancesca and Selcer, submitted; see Project 3). It should be noted that we also found extensive size variation (from 65 to 85 kDa) among microsomes produced from human placenta, human breast cancer cell lines MDA-MB-231 and MCF-7, and rat liver (Selcer et al., 2007; see Project 1). The source of this size variation among tissues is unclear. It may represent different levels of glycosylation of the protein in the different tissues, as steroid sulfatase is known to be a glycoprotein (Stein et al., 1989; Reed et al., 2005). Alternatively, it may represent different sized proteins, or protein products resulting from differential cleavage in different tissues. There were no protein inhibitors used in our tissue preparation, so differences in tissue-specific proteases might explain the results. Overall, the Western blotting data suggest that the new steroid sulfatase antiserum recognizes steroid sulfatase in various tissues from the mouse. STS-275 may therefore be useful in studies of steroid sulfatase distribution or regulation for the mouse.

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 sequence (GenBank accession no. U37545). On an agarose gel, a single prominent cDNA band resulted from the RT-PCR in each of the six tissues, and this band had the expected size for the primer pair used (100 bp). While steroid sulfatase mRNA was present in all six of the tissues examined, no attempt was made to quantify these differences, as this type of RT-PCR is not quantifiable. Using the cyclophilin bands for comparison, muscle reflects low levels
of mRNA in the reaction. Thus, we conclude that these mouse tissues did contain mRNA for steroid sulfatase. The presence of steroid sulfatase mRNA in these tissues is consistent with the presence of steroid sulfatase activity. The cDNA generated from these primers were sequenced and found to be identical to mouse steroid sulfatase cDNA (DiFrancesca and Selcer, submitted; see Project 3).

In conclusion, enzyme assays, Western blotting and RT-PCR all indicate that the six mouse tissues we analyzed all have steroid sulfatase protein and steroid sulfatase mRNA expression. This is consistent with reports from other species that steroid sulfatase is widely distributed among tissues. The role of steroid sulfatase remains to be determined for most tissues, but it is likely that it serves as a local mechanism for providing active steroids to tissues from inactive precursors in circulation.
Figure 8. Single point conversion assay in mouse tissue homogenates. Steroid sulfatase activity in various mouse tissue homogenates, as determined by $^3$H-estrone sulfate conversion assay. Tissue homogenates were prepared as indicated in Materials and Methods. n=5 for all tissue types. Bars represent the mean ± 1 S.E.M. Different letters represent differences among means (One way analysis of variance with Newman-Keuls post hoc, P<0.05). Abbreviations are: Li = Liver, Lu = Lung; M = Muscle; K = Kidney; Ov = Ovary; Ut = Uterus.
Figure 9. Inhibition of steroid sulfatase conversion in mouse tissue homogenates.

Steroid sulfatase conversion assay for mouse tissue homogenates incubated in the presence of \(^3\)H-estrone sulfate using radioinert estrone sulfate as substrate with and without the steroid sulfatase inhibitor with and without DU-14. Each group represents tissue homogenates pooled collectively from 5 animals. Abbreviations are: Li = Liver, Lu = Lung; M = Muscle; K = Kidney; Ov = Ovary; Ut = Uterus.
Figure 10. Mouse tissue microsomal single point conversion assay. Steroid sulfatase conversion assay for mouse tissue microsomes incubated in the presence of $^3$H-estrone sulfate, using estrone sulfate as substrate. Mouse tissue microsomes were prepared as indicated in Materials and Methods. Data represent three independent experiments, run in duplicate. Bars represent the mean ± 1 S.E.M. Different letters represent differences among means (One way analysis of variance with Newman-Keuls post hoc, P<0.05).
Figure 11. Mouse tissue cytosolic single point conversion assay. Steroid sulfatase conversion assay for mouse tissue cytosolic fractions incubated in the presence of $^3$H-estrone sulfate, using estrone sulfate as substrate. Mouse tissue cytosolic fractions were prepared as indicated in Materials and Methods. Data represent three independent experiments, run in duplicate. Bars represent the mean ± 1 S.E.M. Different letters represent differences among means (One way analysis of variance with Newman-Keuls post hoc, P<0.05).
Figure 12. Western blot showing immunoreactivity of steroid sulfatase antibody, STS-275, with microsomes prepared from various mouse tissues. Microsomal proteins were separated by SDS-PAGE (B) and transferred to a PVDF membrane (A) for immunostaining with a specific steroid sulfatase antibody. Western blotting was performed as indicated in Materials and Methods. Antibody dilution was 1:500. Abbreviations are: S: molecular weight markers; Li = liver; K = kidney; M = muscle; Lu = lung.
Figure 13. Reverse transcriptase polymerase chain reaction of various mouse tissues using mouse-specific steroid sulfatase and cyclophilin primers on a 2% agarose gel, stained with ethidium bromide. Primer sequences and reverse transcriptase polymerase chain reaction methods were performed as indicated in Materials and Methods. Lane assignments: Li = Liver, Lu = Lung; M = Muscle; K = Kidney; Ov = Ovary; Ut = Uterus, -RNA = No RNA added; -Taq = No –RT added.
2.3 PROJECT III. Characterization of Steroid Sulfatase in MC3T3-E1, a Mouse Pre-Osteoblastic Cell Line

2.31 Hypothesis and Objectives

Model: MC3T3-E1 mouse pre-osteoblastic cell line derived from newborn mouse calvaria from the C57/BL6 strain

Rationale:

Local estrogen production commonly occurs in many peripheral tissues including bone. Osteoblasts, in particular, are known to be a site of in situ estrogen formation. It appears that estrogen formed locally is important for normal bone physiology. Steroid sulfatase provides a mechanism for the production of estrogen at the local tissue level. MC3T3-E1 cells is a mouse pre-osteoblast cell line that is frequently used to study both normal and abnormal bone physiologies. However, steroid sulfatase has never been characterized in this mouse bone cell line.

Hypothesis:

Steroid sulfatase provides a pathway for conversion of inactive steroid precursors to the active estrogens that are required for maintenance of bone. Therefore, this enzyme will be present and active in bone tissue, in particular in mouse MC3T3-E1 pre-osteoblast cells.

Objectives:

1. To demonstrate the presence of the steroid sulfatase protein in the MC3T3-E1 cell line

   Experiments to address Objective 1:
   
   a. Western blot for steroid sulfatase using MC3T3-E1 microsomes

2. To demonstrate the activity of steroid sulfatase in MC3T3-E1 cells
Experiments to address Objective 2:

a. Whole-cell (intact) steroid sulfatase conversion assay

b. Steroid sulfatase conversion assay using MC3T3-E1 microsomes in the presence and absence of a known steroid sulfatase inhibitor, EMATE

c. Determination of the $K_m$ value for both E₁S and DHEAS using MC3T3-E1 microsomes

d. E₁S and DHEAS competition assay using MC3T3-E1 microsomes

3. To demonstrate the presence of steroid sulfatase mRNA in MC3T3-E1 cells

Experiments to address Objective 3:

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

2.32 Project III Abstract

Regulation of bone density is partly dependent upon steroid hormones, with estrogens playing a particularly important role. Inactive conjugated estrogens may serve as precursors to active estrogens because of their high levels in blood, especially in post-menopausal women. The enzyme steroid sulfatase is required for the conversion of sulfo-conjugated estrogens into unconjugated estrogens. Steroid sulfatase has not been well characterized in mouse bone. The purpose of this study was to characterize steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line. Substantial steroid sulfatase activity was found to be present in MC3T3-E1 whole cells in culture. This activity was significantly reduced in the presence of the steroid sulfatase inhibitor estrone-3-O-sulfatmate (EMATE). Steroid sulfatase activity was also detected in microsomes
prepared from MC3T3-E1 cells, using both $^3$H-E$_1$S and $^3$H-DHEAS as tracers; however, $^3$H-E$_1$S was converted at a faster rate than $^3$H-DHEAS. EMATE significantly reduced steroid sulfatase activity of the microsomes. The $K_m$ of steroid sulfatase activity for microsomal preparations averaged 86 $\mu$M when using estrone sulfate as the substrate and 64 $\mu$M when using DHEAS as the substrate. Western blotting of MC3T3-E1 microsomes for steroid sulfatase was performed using a specific polyclonal antibody (STS-275) generated against a peptide based on a conserved region of the steroid sulfatase protein in human, rat, and mouse. Three bands of cross-reactivity were evident in the Western blots, ranging from 84 to 128 kDa, which is similar to the size range previously reported for steroid sulfatase. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate the presence of steroid sulfatase mRNA in MC3T3-E1 cells, using specific primers. A single cDNA band of the expected size (100bp) was present in the RT-PCR reactions and the sequence of this cDNA was identical to the known sequence for mouse steroid sulfatase. Thus, three lines of evidence (enzyme activity, immunoassay, and RT-PCR) indicate the presence of steroid sulfatase in the MC3T3-E1 mouse bone cell line. The existence of steroid sulfatase in these cells suggests that this enzyme may play a role in regulation of bone density in mice.

2.33 Introduction

The structural integrity of bone is dependent on a balance between the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Steroid hormones play important roles in the regulation of bone architecture (Compston, 2001; Riggs et al., 2002; Vanderschueren et al., 2004). Estrogens, in particular, appear to conserve bone
density. They can act directly on bone, decreasing bone turnover and maintaining a balance between bone formation and bone resorption (Rochira et al., 2001; Heshmati et al., 2002; Riggs et al., 2002, Raisz, 2005; Lerner, 2006; Zallone, 2006).

Conjugated steroids in blood may serve as precursors for active steroid hormones in a variety of tissues, including bone. Sulfated steroids, particularly estrone sulfate and dehydroepiandrosterone sulfate, are present at substantial 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).

Bone cells have been shown to contain all of the enzymes necessary (Fig. 14) for estrogen metabolism (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; Reed et al., 2005). Dehydroepiandrosterone sulfate and estrone sulfate, both abundantly circulating steroid precursors, are converted to dehydroepiandrosterone and estrone, respectively, via steroid sulfatase. Dehydroepiandrosterone can then be converted to androstenedione by 3β-hydroxysteroid dehydrogenase-D5/D4 isomerase (3β-HSD). Androstenedione can be converted to estrone by aromatase or to testosterone by 17β-hydroxysteroid dehydrogenase. Dehydroepiandrosterone can also be converted to androstenediol by 17β-keto-reductase. Androstenediol is an androgen with estrogenic properties (Lardy et al., 2005). Estrone can be converted to the more potent 17β-estradiol by the enzyme 17β-hydroxysteroid dehydrogenase and testosterone can be converted to 17β-estradiol by aromatase. Thus, using pathways beginning with steroid sulfatase, bone cells have the ability to metabolize abundant steroid precursors, particularly dehydroepiandrosterone
Sulfate and estrone sulfate, to biologically potent estrogens such as 17β-estradiol.

Steroid sulfatase is a microsomal enzyme that is classified as an aryl sulfatase C (EC 3.1.6.2.). An aryl sulfatase C enzyme cleaves the sulfate group from sulfate esters of phenol- or 3β-hydroxysteroids (Reed et al., 2005). This enzyme has been shown to be present in both human and rat bone (Fujikawa et al., 1997; Muir et al., 2004; van der Eerden et al., 2004; van der Eerden et al., 2002; Raobaikady et al., 2005). Steroid sulfatase has been purified from mouse liver and partially characterized (Mortaud et al., 1995) and the gene encoding murine steroid sulfatase has been identified (Salido et al., 1996); however, steroid sulfatase activity has not yet been demonstrated in mouse bone cells.

Herein we document for the first time the presence of steroid sulfatase in a mouse bone cell line, MC3T3-E1, using enzyme activity, immunoassays, and RT-PCR.

2.34 Materials and Methods

Chemicals and Reagents

$^3$H-estrone sulfate (ammonium salt, [6,7-3H(N)]; 49 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Radioinert steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT). Estrone 3-O-sulfamate (EMATE) was synthesized according to the procedure described by Howarth et al. (1994).

Cell Culture Methods

Steroid sulfatase activity was assessed in the mouse pre-osteoblastic bone cell line MC3T3-E1. Cells were obtained from the American Type Culture Collection (Rockville,
MD). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). Growth medium, used for routine growth of MC3T3-E1 cells, was 90% (v/v) Dulbecco’s Modified Eagle Media containing 10% (v/v) heat-inactivated fetal calf serum (HyClone, Logan, UT), 10mg/ml penicillin/streptomycin (HyClone). Cells were routinely grown in Falcon 100mm tissue culture dishes (Beckton Dickinson & Co., Franklin Lakes, NJ) in 12ml growth medium.

**Intact Cell Steroid Sulfatase Assay**

MC3T3-E1 cells were seeded into Falcon 6-well tissue culture plates (Beckton Dickinson and Co.) at a density of approximately 200,000 cells/well and incubated in growth medium overnight to allow them to adhere. Two mls of medium were added to individual 35mm tissue culture dishes, which was used as a control for spontaneous hydrolysis. After incubation, the medium in all wells, including controls, was replaced with 2ml of growth media containing $^3$H-estrone sulfate (100,000 dpm/ml) and radioinert estrone sulfate (1µM) in the presence or absence of a specific inhibitor, estrone 3-O-sulfamate (EMATE). Three wells were incubated in the absence of EMATE (control) and three wells were incubated in the presence of EMATE. After 18 h of incubation, 0.5ml of medium was aliquoted into each of two 13x100 borosilicate glass tubes. Three ml 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 medium and $^3$H-estrone sulfate with no cells (18 h incubation). The experiments were repeated 6 times, with seven wells per experiment (three control wells (no EMATE), three treatment wells (EMATE), and one 35 mm dish with medium only).

Preparation of MC3T3-E1 Microsomes

Pre-confluent cultures of MC3T3-E1 cells were scraped off the surface of the dishes and the cells were pelleted by centrifugation (1000xg for 10 min). Pellets were resuspended in 1:5 w:v in ice-cold Tris-sucrose buffer (50mM Tris- HCl, 25mM sucrose, pH 7.5) and homogenized using three 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). The nuclear fraction was pelleted by centrifugation at 2500xg for 10 min at 4°C. The resulting supernatant was removed to Beckman ultracentrifuge tubes (11x60mm) (Beckman Coulter Inc., Fullerton, CA) and centrifuged at 107,000xg for 1h at 4°C to yield the microsomal fraction. The resulting pellet was resuspended in 50mM 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).

Steroid Sulfatase Activity of MC3T3-E1 Microsomes

$^3$H-estrone sulfate was diluted in Tris-HCl buffer (50mM, pH 7.5) and 100µl (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 100µl would yield a concentration of 10mM in the final assay volume. Estrone 3-O-sulfamate (EMATE) was dissolved in Tris-HCl buffer. This solution (100µl) was added to the assay tubes to
achieve the appropriate final concentration of inhibitor (1mM). MC3T3-E1 microsomes were diluted with Tris-HCl buffer to achieve the final desired concentration of membranes (25 to 200 mg) in 200µl of buffer (500µl final volume). The assay tubes were pre-incubated for 5 min at 37°C in a water bath. The assay was initiated by addition of the microsomes (200µl) to the tubes containing the compounds. Control tubes with no inhibitor, tubes without microsomes (to control for spontaneous hydrolysis), and tubes with boiled microsomes (100°C, 10 min) were incubated simultaneously. After 30 min of incubation at 37°C, 3ml of toluene was added for extraction of unconjugated steroids. The samples were vortexed for 1 min. and centrifuged at 2500xg for 10 min at 24°C. Duplicate one-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 4 times, with duplicate tubes for each experiment.

Double reciprocal plot for estimation of $K_m$ values

$^3$H-estrone sulfate, or $^3$H-dehydroepiandrosterone, was diluted in Tris-HCl buffer (50mM, pH 7.5) and 100µl (100,000 dpm) was added to the assay tubes. Radioinert estrone sulfate, or dehydroepiandrosterone sulfate, was dissolved in ethanol such that addition of 50µl would yield the following concentration: 100µM, 50µM, 25µM, 12.5µM, 6.25µM, 3.125µM. MC3T3-E1 microsomes were diluted with Tris-HCl buffer to achieve the final desired concentration of membranes (25 to 200 mg) in 200µl of buffer. The assay tubes were pre-incubated for 5 min at 37°C in a water bath prior to addition of the microsomes. The assay was initiated by addition of the microsomes (200µl) to the tubes. Tubes with varying concentrations of radioinert sulfated steroids,
control tubes without radioinert sulfated steroids, and tubes without microsomes (to control for spontaneous hydrolysis) were incubated for 30 min at 37°C simultaneously. Then 3 ml of toluene was added for extraction of unconjugated steroids. The samples were vortexed for 1 min and centrifuged at 2500xg for 10 min at 24°C. Duplicate one-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.

K_m was determined by linear regression of the reciprocal of the velocity versus the reciprocal of the substrate concentration (µM) using Lineweaver-Burke calculations within the Prism analysis software (GraphPad Software, San Diego, CA). The assays for both 3H-E_1S and 3H-DHEAS were independently performed five times. Protein concentrations of the microsomal suspensions were determined by BCA assay (Pierce Chemical Co.).

**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 water as the diluent. Duplicate concentrations (in µg/tube) were 100, 50, 25, 12.5, 6.25, and 3.125. Duplicate tubes of MC3T3-E1 microsomes were prepared using 100µl of homogenate suspension. Absorbances were read at 562 nm using a spectrophotometer (Thermospectronic, Genesys 20; Waltham, MA, USA).

**Western Blot**

Western blotting was performed on MC3T3-E1 microsomes using an antibody generated 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 sequences (Selcer et al., 2007).

MC3T3-E1 microsomes were dissolved in 100µl Tris-HCl Buffer (pH 7.5). Proteins from MC3T3-E1 microsomes 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 2x Laemmli sample buffer (BioRad) containing 10% β-mercaptoethanol and boiled for 4 min.

Samples (10µl) 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, the gels were either stained with Coomassie blue dye, or the proteins were transferred electrophoretically for two hours at 70 volts to a PVDF membrane (BioRad) in transfer buffer (25mM Tris, 192mM glycine, 20% methanol).

After transfer, the PVDF membranes were washed three times in Tris-saline buffer (50mM Tris-HCl, 154mM NaCl, pH 7.5) for 10 min at room temperature, and then blocked for 30 min with blocking reagent (5% nonfat dry milk in Tris-saline buffer). Membranes were then incubated with steroid sulfatase antibody (1:500 dilution in blocking reagent) overnight in a sealed plastic bag at room temperature. The membranes were washed again three times for 10 min in Tris-Tween buffer (Tris saline buffer + 0.05% Tween 20), then incubated with secondary antibody (goat anti-rabbit IgG coupled to horseradish peroxidase, BioRad) at a 1:1000 dilution in blocking buffer, for two h. The membranes were washed in Tris-Tween 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, the reaction was
stopped by washing the membrane in water for 10 min.

**RNA Isolation**

Total RNA from MC3T3-E1 cells was isolated using TRIzol™ Reagent (Invitrogen Life Technologies) according to manufacturer’s instructions. Cells were lysed directly in the 100 mm culture dishes by incubating 1ml per 10 cm$^2$ of TRIzol™ Reagent for 3 min. Cells were then scraped using a cell scraper and then incubated for another 2 min in the TRIzol™ Reagent. One ml of homogenate was mixed with 200µl of chloroform and centrifuged at 12,000xg for 15 min at 4°C. The supernatant was removed and 500µl of isopropyl alcohol was added and centrifuged at 12,000xg for 10 min at 4°C. The resulting RNA pellet was washed with 1000µl of 75% ethanol, centrifuged at 7000xg for 5 min at 4°C, air-dried, and resuspended in 100µl of diethylpyrocarbonate-treated water. Following a 10 min incubation at 55-60°C, RNA was quantified using a spectrophotometer (ThermoSpectronic, Genesys 8). Purity of nucleic acid preparation was assessed by 260:280 ratio. The amount of RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at 80°C until use.

**Selection of primers for polymerase chain reaction (PCR)**

PCR primers for RT-PCR were selected from the complete coding sequence for *Mus musculus* steroid sulfatase (GenBank accession no. **U37545**). One set of primers was devised. The forward primer start position is 1592 and has the sequence (5’ to 3’) AGC ACG AGT TCC TGT TCC ACT ACT (FB). The reverse primer start position is 1691 and has the sequence (5’ to 3’) AAG TTG GGC GTG TAG AAG GC (RB). The expected RT-PCR product from this primer pair is 100 base pairs. Primers were devised and synthesized by Integrated DNA Technologies, Inc. (IDT) (Coralville, IA).
**Reverse transcriptase polymerase chain reaction (RT-PCR)**

RT-PCR was performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit (Invitrogen Life Technologies) according to manufacturer’s instructions. One mg of template RNA, one µl of RT / Platinum®, 25µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM MgSO₄), one µl of forward primer (150pmol), one µl of reverse (150pmol) primer, DEPC-treated water added to make total volume 50 µl. Ingredients were placed into a sterile 0.5ml tube, vortexed gently, heated to 55°C for 10 min, and then spun in a microcentrifuge for 30 sec at 4000xg.

PCR reactions were then placed into a thermal cycler (MJ Research, Inc., Watertown, MA). The program for amplification included the reverse transcription step, which consisted of a 30 min hold at 55°C and a 2 min hold at 94°C. Next, the cDNA was amplified over 30 cycles of: 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing of primers), and 1 min 60°C (extension). The PCR reaction was terminated with a final extension of 10 min at 72°C, and then held at 4°C. The PCR products were then separated in a 2% agarose gel containing 5ml of 1 mg/ml ethidium bromide solution. The gel was run in 1X TAE running buffer (40 mM Tris-acetate, 2 mM Na₂-EDTA-2H₂O) for 1 h at 70 volts. Ten ml of PCR product was mixed with 2ml 10X loading dye (41% w/v Bromophenol blue, 25% w/w Ficoll). A 1000-bp DNA ladder (Invitrogen Life Technologies) was used as the standard.

**Sequencing of PCR product**

DNA was prepared for sequencing using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Template DNA was sequenced
with the use of Big Dye Terminators (Applied Biosystems, Foster City, CA) and primers (IDT) specific for *Mus musculus* steroid sulfatase cDNA sequence. The forward primer start position is 1592 and has the sequence (5’ to 3’) AGC ACG AGT TCC TGT TCC ACT ACT (FB). The reverse primer start position is 1691 and has the sequence (5’ to 3’) AAG TTG GGC GTG TAG AAG GC (RB). The expected RT-PCR product is 100 bp.

Reactions were purified with the use of Sephadex columns and analyzed on an ABI 310 (Applied Biosystems, Foster City, CA) under standard conditions. DNA sequences were analyzed using BLASTN algorithm for steroid sulfatase cDNA sequence based on mRNA.

Statistical Analysis

Statistics were run using Prism 4.0 for Macintosh (GraphPad Software, San Diego, CA). Probabilities of P < 0.05 were considered significant.

2.35 Results

Whole-Cell Conversion Assay

MC3T3-E1 cells were incubated overnight (18 h) in the presence of $^3$H-estrone sulfate and in the presence or absence of estrone 3-O-sulfamate (EMATE), a potent, irreversible inhibitor of steroid sulfatase (Fig. 15). Steroid sulfatase activity of MC3T3-E1 homogenates was found to be approximately 0.018 pmol/million cells/18h. This activity was virtually eliminated in the presence of EMATE.

Microsomal Conversion Assay

In a conversion assay using microsomal preparations from MC3T3-E1 cells (Fig. 16), steroid sulfatase activity was compared using either $^3$H-estrone sulfate ($E_1S$) or $^3$H-
dehydroepiandrosterone sulfate (DHEAS) as tracers, with either radioinert E₁S or radioinert DHEAS as substrate (10μM). Steroid sulfatase activity was approximately three-fold greater in the presence of ³H-E₁S compared to ³H-DHEAS (Panel A), and the difference was statistically significant (One way analysis of variance; F=5.79; 3, 11 df; P< 0.05). EMATE significantly reduced activity in the presence of ³H-E₁S (Panel B; t=4.75; 2 df, P<0.05) and ³H-DHEAS (not shown).

Double-Reciprocal Plot

Enzyme kinetics experiments using microsomal preparations from MC3T3-E1 cells were performed to determine the $K_m$ value of steroid sulfatase in this cell line (Fig. 17). The $K_m$ was determined to be 83.48 μM ± 8.25 (SEM) using E₁S as substrate and 64.25 μM ± 3.08 (SEM) using DHEAS as substrate (not shown).

Western blotting

In Western blotting experiments (Fig. 18) using microsomal preparations from MC3T3-E1 cells, the steroid sulfatase antibody, STS-275 (Selcer et al., 2007; see Project 1), cross-reacted with three protein bands with approximate molecular weights of 128 kDa, 90 kDa and 84 kDa.

Reverse transcriptase PCR for steroid sulfatase

Reverse transcriptase polymerase chain reaction (Fig. 19), using primers based on the known sequence of the mouse steroid sulfatase encoding gene, resulted in a single cDNA band when resolved on a 2% agarose gel stained with ethidium bromide. This band had the expected size (100 base pairs) for this primer pair. Furthermore, sequencing of the cDNA product revealed that it had the identical sequence to that expected for amplification of the region of the mouse steroid sulfatase mRNA corresponding to the
primers specific for the mouse steroid sulfatase encoding gene used.

2.36 Discussion

In this study, we have demonstrated the activity and presence of the steroid sulfatase enzyme and the presence of steroid sulfatase mRNA, in the MC3T3-E1 mouse pre-osteoblastic cell line, using three independent lines of evidence; enzyme activity, immunoreactivity, and RT-PCR. Our results indicate that this cell line has the ability to convert sulfated steroids, such as dehydroepiandrosterone sulfate and estrone sulfate, into biologically potent steroids, such as 17β-estradiol. Such conversions may be important in the *in situ* synthesis of active steroids in bone cells (see Fig. 14).

Steroid sulfatase activity was demonstrated in whole MC3T3-E1 cells in culture, using $^3$H-estrone sulfate as substrate. The rate of conversion of estrone sulfate to estrone was determined to be 0.018 pmol/million cells/18 h. In contrast, the human osteoblast-like cell line MG-63 was found to hydrolyze estrone sulfate at a rate of 482 fmol/million cells/20 hours (Purohit et al., 1992). The presence of steroid sulfatase activity in bone cells provides them with the ability to convert circulating sulfo-conjugated precursors into active steroids (Reed et al., 2005).

Steroid sulfatase activity also was measured in microsomal preparations of MC3T3-E1 cells, using conversion assays with $^3$H-estrone sulfate (E$_1$S) or $^3$H-dehydroepiandrosterone sulfate (DHEAS) as tracer. Substantial steroid sulfatase activity was observed in microsomes using either ligand; however, the conversion was greater for $^3$H-E$_1$S. The observation that conversion of E$_1$S is greater than that for DHEAS is consistent with previous reports for the human bone cell lines MG-63 and HOS.
(Fujikawa et al., 1997) and for mouse liver (Keinanen et al., 1983). The importance of E₁S being converted at a faster rate than DHEAS is unclear. However, Keinanen et al. (1983) indicated that DHEAS does not reach appreciable levels in mouse blood; suggesting that it may not be an important substrate for conversion in mice. The specific steroid sulfatase inhibitor estrone-3-O-sulfamate (EMATE) completely eliminated steroid sulfatase activity in the presence of either E₁S or DHEAS (not shown) as substrate. This observation is in agreement with results reported by deGooyer et al. (2001), who found that EMATE inhibited steroid sulfatase activity of the human osteoblast-like cells lines MG-63 and HOS TE-85. The finding that EMATE inhibits steroid sulfatase activity under the conditions of our assay provides further evidence that the activity measured in this study is legitimate steroid sulfatase, because EMATE is known to be a specific inhibitor of this enzyme (Howarth et al., 1994).

Western blotting, using microsomal preparations of MC3T3-E1 cells, revealed three major bands of immunoreactivity at approximately 128 kDa, 90 kDa and 84 kDa. Salido et al. (1996) cloned the mouse steroid sulfatase gene and predicted that the mature enzyme consists of 602 amino acids and has a molecular weight of 64 kDa. Our results should be considered in light of those of Mortaud et al. (1995), who showed three immunoreactive bands of steroid sulfatase purified from mouse liver, using an antibody (STS-275) generated against purified steroid sulfatase protein. They discussed two bands, which were estimated to be approximately 68 kDa and 60 kDa; however, it is evident from their gel figure that a third smaller band (43 kDa) is also present. Mortaud et al. (1995) suggested that the two larger bands were subunits of a 128 kDa steroid sulfatase homodimer (based on gel filtration) and that the differences between the bands might be
due to differences in carbohydrate content of the subunits. It is unclear what the third, smaller band, represents; however, it may be a breakdown product of one of the larger polypeptides. Our largest band was 128 kDa, the size that Mortaud et al. (1995), indicated for the steroid sulfatase dimer.

We used reverse transcriptase polymerase chain reaction (RT-PCR) to assess whether or not steroid sulfatase mRNA was present in MC3T3-E1 cells, using primers we designed based on the known mouse steroid sulfatase sequence (GenBank accession no. U37545). On an agarose gel, a single prominent cDNA band resulted from the RT-PCR, and this band had the expected size for the primer pair used (100 bp). Also, the sequence of this band exactly matched with the expected sequence for the primer pair used. Thus, we conclude that the MC3T3-E1 cells did contain mRNA for steroid sulfatase. Similarly, Fujikawa et al. (1997) reported expression of steroid sulfatase mRNA in the human osteoblast cell lines HOS and MG-63, and van der Eerden et al. (2004) found steroid sulfatase mRNA present in osteoblasts and osteoclasts of rat tibial metaphyseal bone. Interestingly, they reported that steroid sulfatase mRNA was present in both immature and mature rat bone cells. Janssen et al. (1999) also found steroid sulfatase expression throughout differentiation of the cell line SV-HFO. The presence of steroid sulfatase mRNA at all stages of bone cell differentiation and development suggest that this enzyme may have an important role throughout bone life.

Bone density is maintained by the formative action of osteoblasts and the degradative action of osteoclasts (Compston, 2001; Riggs et al., 002; Vanderschueren et al., 2004). Estrogens play a central role in regulating this process by acting to maintain bone density (Rochira et al., 2001; Heshmati et al., 2002; Raisz, 2005; Lerner, 2006,
The estrogens acting on bone may be of ovarian origin, or they may be from \textit{in situ} synthesis from circulating inactive steroid precursors (Reed et al., 2005). The most abundant circulating steroid precursors in humans are estrone sulfate and dehydroepiandrosterone sulfate. The \textit{in situ} pathways by which these are converted to active estrogens begin with steroid sulfatase. Thus, steroid sulfatase may be important in providing estrogens needed for proper maintenance of bone density.

Steroid sulfatase is present in various mammalian tissues including placenta, liver, skin, gonads, breast, breast cancer, and endometrial cancer (Reed et al., 2005; Fujikawa et al., 1997; Selcer et al., 2007). Steroid sulfatase activity or steroid sulfatase gene expression has been previously shown in bone cells of both humans (Purohit et al., 1992; Fujikawa et al., 1997; Muir et al., 2000; Raobaikady et al., 2005) and rats (Fujikawa et al., 1997; Muir et al., 2004; van der Eerden et al., 2004; van der Eerden et al., 2002). Steroid sulfatase activity and gene expression has also been reported in mouse liver and other tissues (Mortaud et al., 1995; Salido et al., 1996; Keinanen et al., 1983). Our results demonstrate for the first time the activity, presence and gene expression of steroid sulfatase in mouse bone cells. Knowledge of the presence of steroid sulfatase in mouse bone should allow for improved physiological studies of the role of this enzyme in bone growth and maintenance because of the relative ease of manipulation of the steroid environment in mice and due to the extensive number of mouse models available for study, such as the estrogen-receptor knockout mice (Schomberg et al., 1999).
Figure 14. Role of steroid sulfatase in metabolism of estrogen in bone.

Abbreviations are: ER = estrogen receptor; X = blockade by inhibitors.
Figure 15. Whole-cell single point conversion assay. Steroid sulfatase activity in MC3T3-E1 cells, as determined by $^3$H-estrone sulfate conversion assay. Cells were incubated overnight in the presence or absence of the steroid sulfatase inhibitor, estrone-3-O-sulfamate (EMATE). Whole-cell conversion assay was preformed as indicated Materials and Methods. Bars represent the mean ± 1 S.E.M. Data represent six independent experiments, run in triplicate.
Figure 16A.

Figure 16B.

Figure legend on next page.
Figure 16. Microsomal single point conversion assay in MC3T3-E1 cells.

**Panel A.** A steroid sulfatase conversion assay for MC3T3-E1 microsomes incubated in the presence of \(^3\)H-E\(_1\)S or \(^3\)H-DHEAS using radioinert E\(_1\)S or DHEAS as substrates. Bars represent the mean ± 1 S.E.M. Different letters represent differences among means (One way analysis of variance with Newman-Keuls post hoc, P<0.05). Data represent 4 independent assays, run in duplicate.

**Panel B.** Steroid sulfatase conversion assay for MC3T3-E1 microsomes incubated in the presence of \(^3\)H-E\(_1\)S and using radioinert E\(_1\)S as substrate with and without estrone-3-O-sulfamate (EMATE). Asterisk indicates significant difference between means (Students \(t\)-test, P<0.05). Data represent 4 independent assays, run in duplicate.
Figure 17. Enzyme kinetics of steroid sulfatase in MC3T3-E1 cells. Double reciprocal plot used for calculating the $K_m$ value of steroid sulfatase in microsomes prepared from MC3T3-E1 cells using $^3$H-estrone sulfate as tracer and various concentrations of radioinert estrone sulfate as substrate. Microsomes were prepared and enzyme kinetics experiments were preformed as indicated in Materials and Methods. Data represent 5 independent assays, run in duplicate.
Figure 18. Western blot showing immunoreactivity of a steroid sulfatase antibody, STS-275 with microsomal proteins from MC3T3-E1 cells. Microsomal proteins were separated by SDS-PAGE gel (A) and transferred to a PVDF membrane (B) for immunostaining. Western blotting was performed as indicated in Materials and Methods. Antibody dilution was 1:400. Microsomal protein concentration used in the SDS-PAGE was 60µg/lane. Abbreviations are S = molecular weight markers, 1 and 2 are duplicate lanes of microsomal proteins.
Figure 19. Reverse transcriptase polymerase chain reaction of MC3T3-E1 cells using mouse-specific steroid sulfatase primers on a 2% agarose gel, stained with ethidium bromide. Primer sequences and reverse transcriptase polymerase chain reaction methods were preformed as indicated in Materials and Methods. Lane assignments: L = size ladder, B = blank, and MC3T3 = MC3T3-E1 cell RNA extract.
2.4 Project IV. Activity and Expression of Steroid Sulfatase During the Differentiation Process in the Mouse MC3T3-E1 Pre-osteoblast Cell Line

2.41 Hypothesis and Objectives

Rationale:
MC3T3-E1 cells progress through various stages of bone cell differentiation under the influence of estrogen and osteogenic supplement. It has been shown in rats and humans that steroid sulfatase is present throughout differentiation. However, there is no information on steroid sulfatase during differentiation of mouse bone cells, including MC3T3-E1.

Hypothesis:
Differentiation of the 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.

Objectives:
1. Develop an assay to monitor osteoblast differentiation in MC3T3-E1 cells

Experiments to address Objective 1:

a. Measure growth in the presence and absence of osteogenic supplement as well as in the presence or absence of ICI 182,780

b. Measure alkaline phosphatase expression during the 21 day process in the presence and absence of osteogenic supplement as well as in the presence or absence of ICI 182,780
c. Follow osteocalcin mRNA expression during the 21 day process in the
   presence and absence of osteogenic supplement

2. Measure steroid sulfatase activity throughout differentiation in MC3T3-E1 cells

Experiments to address Objective 2:

a. Whole-cell steroid sulfatase conversion assay in the presence and absence
   of osteogenic supplement as well as in the presence or absence of ICI
   182,780

3. To examine steroid sulfatase mRNA levels throughout differentiation in MC3T3-
   E1 cells

Experiments to address Objective 3:

a. RT-PCR using primers specific for the mouse steroid sulfatase specific
   encoding gene in the presence and absence of osteogenic supplement as
   well as in the presence or absence of ICI 182,780

2.42 Project IV Abstract

Osteoblasts proceed through a well-defined process of proliferation, matrix
maturation, and extracellular mineralization. Because estrogens have been shown to be
important in normal osteoblast function, steroid sulfatase may be necessary during the
differentiation of these cells. Our previous results indicated that the mouse pre-osteoblast
cell line MC3T3-E1 contained significant levels of steroid sulfatase. However, steroid
sulfatase expression and activity during osteoblast differentiation has not yet been
demonstrated in this mouse bone cell line. Therefore, our next objective was to
determine if steroid sulfatase is present throughout differentiation and to examine its
possible role in the differentiation process. Our laboratory developed a 21-day protocol suitable to assess the expression and activity of steroid sulfatase in MC3T3-E1 cells during the differentiation process. Steroid sulfatase expression was assessed by RT-PCR, and steroid sulfatase activity was determined using a whole-cell $^3$H-E$_1$S conversion assay. Cells were grown in whole medium alone (OS-), whole medium containing a commonly used osteogenic supplement to enhance differentiation (OS+) or whole medium containing an estrogen receptor blocker (ICI). Differentiation was assessed by alkaline phosphatase enzyme activity and by osteocalcin mRNA expression. Osteogenic supplement significantly retarded growth, a result consistent with enhanced differentiation. Indeed, alkaline phosphatase activity peaked much earlier in the OS+ cells than in OS- cells. Interestingly, alkaline phosphatase peaked even earlier, and reached higher levels, in the ICI cells. Steroid sulfatase activity showed a peak on Days 7 and 14 in OS- cells, a smaller peak on Day 14 in OS+ cells and no peak in the ICI cells. Steroid sulfatase mRNA levels was present throughout the differentiation process under all test conditions. These data indicate that steroid sulfatase activity varies during differentiation, being higher in the early stages of differentiation. Furthermore, our data indicate that estrogen receptor mediated processes are not required for differentiation. The exact role of increased steroid sulfatase activity during differentiation remain to be elucidated.

2.43 Introduction

The skeletal system has a variety of functions including providing structural support for the body and protection for internal organs (Compston, 2001, Lerner, 2005;
Walsh et al., 2006). Bone microarchitecture is partly regulated by steroid hormones (Compston, 2001; Riggs et al., 2002; Vanderschueren et al., 2004; Walsh et al., 2006). Estrogens, in particular, play a primary role in promoting bone health (Janssen et al., 1999; Compston, 2001; Riggs et al., 2002; Raisz et al., 2005). Estrogens can act directly on bone, decreasing bone turnover and maintaining a balance between bone formation and bone resorption (Riggs et al., 2002; Rochira et al., 2001; Heshmati et al., 2001; Raisz, 2005; Lerner, 2006; Zallone, 2006). Bone tissue serves as an extragonadal site for conversion of sulfated steroids into active steroid hormones (Simpson et al., 2000; Simpson and Davis, 2001; Simpson, 2003; Reed et al., 2005). Bone cells have been shown to contain all of the enzymes necessary (Fig. 14) for estrogen metabolism (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; Reed et al., 2005). Moreover, estrone sulfate and dehydroepiandrosterone sulfate are present at substantial levels in peripheral circulation (Reed et al., 2005), and the estrogen needed for bone density maintenance may be provided by the conversion of these precursors to active estrogens (Purohit et al., 1992; Fujikawa et al., 1997; Muir et al., 2004). Steroid sulfatase converts conjugated steroids into biologically active steroids. Therefore, steroid sulfatase may be an important enzyme in the local estrogen metabolic pathway required for normal bone physiology.

Steroid sulfatase is a microsomal enzyme that is classified as an aryl sulfatase C (EC 3.1.6.2.). An aryl sulfatase C enzyme cleaves the sulfate group from sulfate esters of phenol- or 3β-hydroxysteroids (Reed et al., 2005). This enzyme has been shown to be present in both human and rat bone (Fujikawa et al., 1997; Muir et al., 2004; van der
Eerden et al., 2004; van der Eerden et al., 2002; Raobaikady et al., 2005). Steroid sulfatase has been purified from mouse liver and partially characterized (Mortaud et al., 1995) and the gene encoding murine steroid sulfatase has been identified (Salido et al., 1996). In addition, we have described steroid sulfatase activity, immunoactivity, and mRNA expression in the mouse MC3T3-E1 preosteoblastic cell line (DiFrancesca and Selcer, submitted; see Chapter 5). van der Eerden et al. (2004), demonstrated that steroid sulfatase mRNA was present during bone maturation in male and female rats, thereby suggesting that this enzyme is important for development and maintenance of bone. Because it was the only enzyme in the local estrogen metabolic pathway found to be expressed at all time points examined during rat bone development by van der Eerden et al., (2004), it is reasonable to assume that steroid sulfatase is required throughout both proliferation and differentiation in osteoblasts.

Bone cells, in particular osteoblasts, proceed through a well-defined process of proliferation, matrix maturation, and extracellular mineralization (Janssen et al., 1999; Beck et al., 2001). Matrix formation is accompanied by the induction of specific genes associated with the osteoblast phenotype. Alkaline phosphatase, osteopontin, and osteocalcin are among the genes associated with the formation of the matrix. Induction of the differentiation process in MC3T3-E1 cells can be accomplished by exposing the cells to ascorbic acid, which stimulates deposition of a collagenous extracellular matrix and, and a source of organic phosphate, which triggers the formation of discrete zones of hydroxyapatite containing mineral deposits within the matrix. The sequence of events from induction to the formation of mineralized foci proceeds in a tightly regulated order over a period of 2-3 weeks (Beck et al., 2001). Estrogen has been shown to be instrumental in
normal osteoblast function (Janssen et al., 1999; Compston, 2001; Riggs et al., 2002; Raisz et al., 2005). Because steroid sulfatase provides a local pathway for estrogen metabolism in bone tissue, it is reasonable to believe that steroid sulfatase is necessary during both the proliferative and differentiation phases of bone cells. Recently, Janssen et al., (1999) reported that both activity levels and mRNA expression levels of steroid sulfatase remain the same throughout the differentiation process in human osteoblasts. However, steroid sulfatase activity and mRNA expression during osteoblast differentiation have not yet been demonstrated in mouse bone cells. One obstacle to the broad-range study of the presence and activity of steroid sulfatase in differentiation of osteoblasts is the time span of the differentiation process.

To get a profile of steroid sulfatase activity and mRNA expression during all phases of differentiation (proliferation, matrix accumulation, and mineralization), we chose to measure both conversion of tritiated estrone sulfate to tritiated estrone and steroid sulfatase mRNA at Days 3, 7,14, and 21 following the addition of the osteogenic supplement medium, which induces differentiation. Day 0 represents cells at roughly 95% confluency. In addition to covering the various differentiation phases, these particular time points correspond to time points at which expression patterns of osteoblast differentiation markers have been previously studied (Beck et al., 2001).

Osteoblasts undergoing differentiation progress through three major phases. These phases include an early proliferative phase that continues for several days following confluency, a period of collagen deposition and maturation of the matrix, and terminal differentiation in which the mature matrix is mineralized. Days 0 to 7 correlate to the early proliferation period, during which time the cells undergo active replication.
Between Days 3 and 4, cells begin to exit the cell cycle and decrease in their replication activity, after which they move into the collagen matrix deposition phase. Collagen type I is induced early; its expression declines by Day 14 as matrix deposition nears completion. Osteocalcin levels peak around Day 14, which marks the transition into the terminal differentiation period (Beck et al., 2001).

Because estrogen is a critical component in the maintenance of normal bone physiology, we hypothesize that steroid sulfatase is required during both the proliferative phase and the differentiation phase of osteoblasts. We further hypothesize that, in the presence of ICI 182, 780, alkaline phosphatase activity will be similar to the activity levels in cells incubated in growth medium alone. Moreover, steroid sulfatase activity will not be affected by the presence of ICI 182, 780.

Using three independent lines of evidence, we have previously demonstrated the presence, activity, and expression of steroid sulfatase in MC3T3-E1 pre-osteoblasts (DiFrancesca and Selcer, submitted; see Chapter 5). However, before we can determine the importance of steroid sulfatase during the progression of differentiation, we must first determine if it is present and active throughout the process. To address the expression and activity of steroid sulfatase during osteoblast differentiation, we again chose to use the MC3T3-E1 newborn mouse calvarial-derived cell line (Sudo et al., 1983), because of its ability to differentiate along the osteoblast lineage (Quarles et al., 1992; Franceschi et al., 1994), and because it is an established clonal cell line that maintains tightly linked controls between proliferation and differentiation (Beck et al., 2001). Thus, these cells provide an excellent model for studying steroid sulfatase throughout the differentiation pathway.
Herein, we document for the first time the presence of steroid sulfatase throughout the MC3T3-E1 pre-osteoblast proliferation and differentiation phases, using both enzyme activity and RT-PCR. Using the known estrogen receptor inhibitor, ICI 182,780 (Wakeling et al., 1991), we also demonstrate that estrogen receptor mediated processes are not required during the MC3T3-E1 osteoblast differentiation process.

2.44 Methods and Materials

Chemicals and Reagents

$^3$H-estrone sulfate (ammonium salt, $[6,7-^3$H(N)]-; 49 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Radioinert steroids were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was Ultima Gold (Packard Instrument Co., Meriden, CT). Estrone 3-O-sulfamate (EMATE) was synthesized according to the procedure described by Howarth et al. (1994).

Cell Culture Methods

Steroid sulfatase activity was assessed in the mouse pre-osteoblastic bone cell line MC3T3-E1. Cells were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media and reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). Growth medium, used for routine growth of MC3T3-E1 cells, was 90% (v/v) Dulbecco’s Modified Eagle Media containing 10% (v/v) heat-inactivated fetal calf serum (HyClone, Logan, UT), 10 mg/ml penicillin/streptomycin (HyClone). Cells were routinely grown in Falcon 100mm tissue culture dishes (Beckton Dickinson & Co., Franklin Lakes, NJ) in 10 ml growth medium. To initiate pre-osteoblast cell differentiation down the osteoblast lineage, osteogenic supplement media was used.
Osteogenic supplement media was 90% (v/v) Dulbecco’s Modified Eagle Media containing 10% (v/v) heat-inactivated fetal calf serum (HyClone), 10mg/ml penicillin/streptomycin (HyClone) supplemented with 50µg/ml ascorbic acid (Sigma Chemical Company, St. Louis, MO), 10µM β-glycerophosphate (Sigma), and 10nM dexamethasone (Sigma). Cells used for differentiation assays were routinely plated in Falcon 100 mm tissue culture dishes in 10ml osteogenic supplement medium. Media was changed every 3 to 4 days. Cells used in the inhibition assays were routinely plated in Falcon 100mm tissue culture dishes in 10 ml osteogenic supplement medium supplemented with the estrogen receptor blocker, 100nM ICI 182,780.

Steroid sulfatase activity, alkaline phosphatase activity, and mRNA expression were measured on Days 0, 3, 7, 14, and 21 after the addition of either differentiation media or inhibitor media. On Day 0, cells were approximately 95% confluent. Cells were washed 1x with phosphate buffered saline (Sigma-Aldrich Co., St. Louis, MO) and incubated in respective treatment medium. With the exception of Day 0, each day was represented with three plates, one for each treatment. A minimum of 3 independent assays were performed.

Growth Assay

MC3T3-E1 cells were seeded into Falcon 6-well plates (Becton Dickenson and Co.) at a density of 50,000 cells/well and incubated in growth media for 18 h to allow them to adhere. Cell density was measured on Days 2, 4, 6, 8, 10, and 12. One 6-well plate was used for each day of measurement. After incubation, the media was removed, the cells were washed once with phosphate buffered saline (Sigma-Aldrich Co., St. Louis, MO), and 2 mls of either growth media or growth media containing osteogenic
supplement was added to respective wells. Cells were grown over a 12-day period. Media was then removed and 200µl MTT (Dimethylthiazol tetrazolium bromide) (Sigma Chemical Co., St. Louis, MO) was incubated at 37°C for 3 h. Following incubation, the MTT was completely removed and replaced with 1000µL of acidic isopropanol. The plate was placed onto a plate shaker for 10 min to solubilize the membrane and then four 200µL samples from each well were removed and placed into a 96-well microtiter plate and read at an absorbance of 595nm using the BioRad Microplate reader (Model 3550).

**Intact Cell Steroid Sulfatase Assay**

MC3T3-E1 cells were seeded in 100 mm tissue culture plates (Beckton Dickinson and Co.) at a density of approximately 500,000 cells/well and allowed to grow to 95% confluence, which was considered Day 0. Twenty-four hours prior to assays, media was removed and replaced with 10ml of growth media, differentiation media, or inhibitor media containing ³H-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. Three ml of toluene was added to each tube for extraction of unconjugated steroids. The mixture was vortexed for 1 min and then centrifuged at 2500xg for 10 min at 24°C 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 5ml of scintillation cocktail was added. Radioactivity was counted in a Packard Tri-carb scintillation counter at 50% efficiency for ³H. The conversion values obtained for all treatments were adjusted for spontaneous product formation by subtracting the value obtained for wells containing growth medium and ³H-estrone sulfate with no cells from wells containing treatment medium and ³H-estrone sulfate with cells. The
experiments were repeated 6 times, with one plate for each treatment per day. Each treatment for each day had one 100 mm dish with media only.

*Preparation of MC3T3-E1 Crude Homogenates*

Medium was removed and cells were washed 2 times with phosphate buffered saline (Sigma-Aldrich Co.). Plate was covered in trypsin EDTA (Sigma-Aldrich Co.) for approximately 5 min. Trypsin was then removed and plate was allowed to sit for approximately 30 sec. Four mls of growth media was then added to neutralize the trypsin. Two mls of media with cell mixture was added to one conical tube designated for RNA isolation and 2 mls of media with cell mixture was added to another conical tube designated for crude homogenates. Three mls of fresh growth media was added to each conical tube. Samples were then centrifuged at 1000xg for 5 min to obtain a loose pellet. Samples were immediately placed on ice. The supernatant was removed and the pellets were resuspended in 2mls of 2mM 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 water as the dilutent. Duplicate concentrations (in µg/tube) were 100, 50, 25, 12.5, 6.25, and 3.125. Duplicate tubes of MC3T3-E1 crude homogenate samples were prepared using 25µl 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 were incubated in the presence of ABC buffer consisting of equal parts 1.55M 2-amino-2-methyl propanol (Sigma), 20mM p-nitrophenol phosphate (Sigma) and 10mM magnesium chloride (Sigma) for 1 h, after which absorbance readings were taken at 400nm on a microplate reader (BioRad Model 3550).

**RNA Isolation**

Total RNA from MC3T3-E1 cells was isolated using Absolutely RNA® Miniprep Kit (Stratagene, La Jolla, CA) according to manufacturer’s instructions. Media was removed. Cells were trypsinized, collected into a 15ml conical tube and centrifuged at 1000xg for 5 min. Most of the supernatant was then removed. The residual supernatant was used to resuspend the cells. Resuspension was transferred to a microcentrifuge tube and spun at 3000xg for 5 min at 4°C. The supernatant was removed and the resulting RNA pellet was resuspended in 600µl of lysis buffer. The sample was then homogenized using two 30 sec bursts of a BioSpec Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK). RNA homogenates were then immediately stored at -80°C.

Once all samples were collected, RNA isolation procedure was carried out. RNA homogenate was transferred to a Prefilter Spin Cup seated in a 2 ml receptacle tube and spun at 12,000xg for 5m. The spin cup was removed and the filtrate was retained. An equal volume of 70% ethanol was added to each sample and vortexed for 5 sec. Mixture was then transferred to an RNA Binding Spin Cup seated in a fresh receptacle tube and spun at 12,000xg for 1 min. The filtrated was discarded, and 600µl of 1X Low Salt Wash Buffer was added and spun at 12,000xg for 1min. The filtrate was discarded and the
sample was again spun at 12,000xg for 2 min. 55 µl DNase solution was added directly to the spin cup and incubated at 37 °C for 25 min. 600 µl of 1X High Salt Wash Buffer was added to spin cup and spun at 12,000xg for 1 min. The filtrate was discarded, and 600 µl 1X Low Salt Wash Buffer was added and spun at 12,000xg for 1 min. The filtrate was once again discarded and 300 µl 1X Low Salt Wash Buffer was added and spun at 12,000xg for 2 min to dry the fiber matrix. The spin cup was then transferred to a 1.5 ml microcentrifuge tube and 100 µl Elution Buffer was added directly to the spin cup. The sample was incubated for 2 min at room temperature and then spun at 12,000xg for 1 min. The elution step was then repeated for maximum RNA yield. RNA was quantified using a spectrophotometer (ThermoSpectronic, Genesys 8). Purity of nucleic acid preparation was assessed by 260:280 ratio. The amount of RNA extracted was determined by ultraviolet light absorption at 260 nm. RNA samples were stored at -80 °C until use.

Selection of primers for polymerase chain reaction (PCR)

PCR primers for RT-PCR were selected from the complete coding sequence for Mus musculus steroid sulfatase (GenBank accession no. U37545). One set of steroid sulfatase primers was devised. The forward primer has the sequence (5’ to 3’) AGC ACG AGT TCC TGT TCC ACT ACT (FB). The reverse primer has the sequence (5’ to 3’) AAG TTG GGC GTG TAG AAG GC (RB). The expected RT-PCR product from this primer pair is 100 base pairs. PCR primers for RT-PCR were selected from the complete coding sequence for Mus musculus peptidylprolyl isomerase A (cyclophilin) (GenBank accession no. NM008907). One set of cyclophilin primers was devised. The forward primer has the sequence (5’ to 3’) TAT CTG CAC TGC CAA GAC TG (mCPH-
The reverse primer has the sequence (5’ to 3’) ACA GTC GGA AAT GGT GAT CT (mCPH-R1) (Davis et al., unpublished). The expected RT-PCR product from this primer pair is 143 base pairs. Cyclophilin was used as an internal standard. PCR primers for RT-PCR were selected from the complete coding sequence for Mus musculus osteocalcin (GenBank accession no. NM007541). One set of osteocalcin primers was devised based on a previous study using this primer set (Lee et al., 2006). The forward primer has the sequence GTG AGC TTA ACC CTG CTT GTG A (5’ to 3’) (mOCN-F). The reverse primer has the sequence ACT GAA CTT GAC CGT ACA TGC GTT TGT AGG CGG TCT TC (5’ to 3’) (mOCN-R). The expected RT-PCR product from this primer pair is 98 base pairs. Primers were devised and synthesized by Integrated DNA Technologies, Inc. (IDT) (Coralville, IA).

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed using the SuperScript™ One-Step RT-PCR with Platinum® Taq Kit (Invitrogen Life Technologies) according to manufacturer’s instructions. One µg of template RNA, one µl of RT / Platinum®, twenty-five µl of 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP and 2.4 mM MgSO₄), one µl of forward primer (150pmol), one µl of reverse primer (150pmol), DEPC-treated water added to make total volume 50 µl. Ingredients were placed into a sterile 0.5 ml tube, vortexed gently, heated to 55°C for ten minutes, and then spun in a microcentrifuge for 30 seconds at 4,000xg.

PCR reactions were then placed into a thermal cycler (MJ Research, Inc., Watertown, MA). The program for amplification included the reverse transcription step, which consisted of a 30 min hold at 55°C and a 2 min hold at 94°C. Next, the cDNA was
amplified over 30 cycles of: 30 sec at 94°C (denaturation), 30 sec at 50°C (annealing of primers), and 1 min 60°C (extension). The PCR reaction was terminated with a final extension of 10 min at 72°C, and then held at 4°C. The PCR products were then separated in a 2% agarose gel containing 5µl of 1 mg/ml ethidium bromide solution. The gel was run in 1X TAE running buffer (40 mM Tris-acetate, 2 mM Na₂-EDTA-2H₂O) for 1 h at 70 volts. Ten µl of PCR product was mixed with 2µl 10X loading dye (41% w/v Bromophenol blue, 25% w/w Ficoll). A 1000-bp DNA ladder (Invitrogen Life Technologies) was used as the standard.

**Statistical Analysis**

Statistics were run using Prism 4.0 for Macintosh (GraphPad Software, San Diego, CA). Probabilities of P < 0.05 were considered significant.

### 2.45 Results

**Growth Analysis**

A cellular proliferation assay (MTT Assay) was carried out in the presence of growth medium and in the presence or absence of osteogenic supplement (Fig. 20). The assay was carried out over the course of 12 days. Cells that were incubated in growth medium (OS-) grew steadily over the 12 days, while cells that were treated with osteogenic supplement medium (OS+) grew more slowly compared to the untreated cells. The difference between OS+ and OS- media became apparent on Day 6 and continued through Day 12. Two-way analysis of variance revealed significant differences between day (F=134.06; 5, 35 df; P<0.05) and treatment (F=317.77; 1, 35 df; P<0.05) with significant interaction (F=48.70; 5, 35 df; P<0.05) between day and treatment.
**Alkaline Phosphatase Assay**

Alkaline phosphatase activity levels were measured in MC3T3-E1 crude homogenate preparations of cells on Days 0, 3, 7, 14, and 21 for all treatments (Fig. 21). Alkaline phosphatase levels were adjusted according to the total protein concentration in each sample. Alkaline phosphatase levels increased steadily in the cells incubated in growth medium alone. Alkaline phosphatase increased to a higher level in cells treated with osteogenic supplement medium, and even higher in cells treated with osteogenic supplement media containing 100nM ICI 182,780. Two-way analysis of variance revealed significant differences among day (F=110.72; 3, 50 df; P<0.05) and treatment (F=131.86; 2, 50 df; P<0.05) with significant interaction (F=28.59; 6, 50 df; P<0.05) between day and treatment.

**Whole-Cell Conversion Assay**

MC3T3-E1 cells were incubated overnight (18 h) in the presence of $^3$H-estrone sulfate for each time point and treatment represented in the differentiation experiment (Fig. 23). In cells incubated in growth medium (OS-), steroid sulfatase activity incrementally increased through Day 14, after which steroid sulfatase activity decreased to approximately the same level as that of Day 3. In cells treated with osteogenic supplement medium (OS+), steroid sulfatase activity significantly increased between Days 7 and 14, and then decreased by Day 21. In contrast, for the cells treated with osteogenic supplement media containing 100nM ICI 182,780, there was neither a statistical difference nor an obvious trend in steroid sulfatase activity over the 21-day period. Two-way analysis of variance revealed significant differences among day
(F=3.07; 3, 63 df; P<0.05) and treatment (F=9.43; 2, 63 df; P<0.05) with significant interaction (F=2.43; 6, 63 df; P<0.05) between day and treatment.

**Reverse transcriptase PCR for steroid sulfatase and osteocalcin**

Reverse transcriptase polymerase chain reaction using, primers based on the known sequence of mouse steroid sulfatase, resulted in a single cDNA band when resolved on a 2% agarose gel stained with ethidium bromide. These bands had the expected size (100 base pairs) for this primer set. In addition, reverse transcriptase PCR using primers based on the known mouse cyclophilin sequence and known sequence of mouse osteocalcin, resulted in a single cDNA band for each primer pair when resolved on a 2% agarose gel stained with ethidium bromide. The bands for each resulted in the expected band sizes of 143 base pairs for cyclophilin and 98 base pairs for osteocalcin.

Bands corresponding to the expected cDNA size were evident on all days in all treatments. Additionally, steroid sulfatase mRNA levels did not show any obvious changes during MC3T3-E1 osteoblast differentiation (Fig. 24 and 25). Moreover, steroid sulfatase mRNA expression appeared to remain constant during differentiation in those cells treated with the estrogen receptor blocker, ICI 182,780. In contrast, transcripts for osteocalcin appeared as the cells progress through differentiation (Fig. 22). There was no apparent osteocalcin mRNA on Days 0 or 3, a faint band for Day 7, and strong bands on Days 14 and 21 for OS+. There were no apparent bands for OS-. Bands representing cyclophilin transcripts were present in all days examined.
2.46 Discussion

We developed a protocol to study steroid sulfatase throughout the differentiation process of the mouse MC3T3-E1 pre-osteoblast cells. Using this protocol, we were able to demonstrate both steroid sulfatase activity and presence of mRNA during osteoblast differentiation. To first demonstrate the feasibility of this assay, we showed that we are able to induce differentiation by comparing the proliferation of cells incubated in growth medium compared to cells treated with the 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 are in the differentiation phase. This is consistent with other studies that have shown reduced growth in the presence of osteogenic supplement during the induction of MC3T3-E1 cellular differentiation (Beck et al., 1998; Beck et al., 2001). We confirmed differentiation induction using alkaline phosphatase protein expression and osteocalcin mRNA expression. Alkaline phosphatase and osteocalcin can be used to validate differentiation, as both are phenotypic markers of osteoblast differentiation (Malaval et al., 1999; Beck et al., 2001). The activity of alkaline phosphatase increased over the course of the MC3T3-E1 differentiation process in those cells treated with osteogenic supplemental media. These results indicate that the MC3T3-E1 cells, under this treatment, are able to differentiate to mature osteoblasts. Although alkaline phosphate activity did increase over time in the cells incubated in growth media, this was expected, as MC3T3-E1 cells are capable of undergoing differentiation without the addition of a supplemental media. However, the differentiation process in cells not treated with the osteogenic supplement media occurred at a much slower rate, consistent with previous
studies (Sudo et al., 1983; Quarles et al., 1992). Likewise, mRNA expression of osteocalcin is indicative of MC3T3-E1 osteoblastic differentiation. Osteocalcin was present on Days 14 and 21 with OS+; however, there were no apparent bands for Days 14 and 21 with OS-. This was not due to lack of mRNA in assay as indicated by the presence of cyclophilin from same samples. Osteocalcin is only expressed as the matrix begins the mineralization process (Malaval et al., 1999; Beck et al., 2001), and is therefore a late marker of differentiation. Thus, presence of osteocalcin mRNA is another line of evidence demonstrating progression of these cells towards the mature osteoblast phenotype. As demonstrated by both alkaline phosphatase expression and osteocalcin mRNA expression, cells are moving down the osteoblast lineage upon treatment with osteogenic supplement media.

Steroid sulfatase mRNA was found to be present throughout the entire differentiation process in the MC3T3-E1 cells. There were no apparent differences in steroid sulfatase mRNA levels throughout this process. However, this technique was not designed for quantitation; steroid sulfatase transcript levels would need to be measured by a quantitative method in order to determine if message levels indeed remain constant throughout differentiation. Nonetheless, our findings are consistent with the results obtained by Janssen et al. (1999), who demonstrated that steroid sulfatase mRNA levels remained the same throughout the differentiation process in human fetal osteoblast cells. Interestingly, no apparent differences in steroid sulfatase mRNA levels were detected in the cells treated with the estrogen blocker, ICI 182, 780 throughout differentiation, nor were any obvious differences detected when compared with the mRNA levels of the other treatment groups. Overall, these results were not surprising. According to Reed et
al., 2005, the steroid sulfatase gene does not resemble either a housekeeping gene or a tightly regulated gene. It does, however, have a basal promoter, which suggests constitutive expression. These results also suggest that steroid sulfatase is not transcriptionally regulated under the confines of our conditions. Reed et al. (2005) has speculated that steroid sulfatase is most probably regulated via a post-transcriptional mechanism. Yet, it is unclear what this mechanism is.

Janssen et al. (1999) is the only published study to date that examined steroid sulfatase mRNA expression and activity in pre-osteoblast progression toward mature osteoblasts. Using a human fetal osteoblast cell line, SV-HFO, Janssen et al., (1999) examined steroid sulfatase activity by measuring the conversion of E$_1$S to E$_1$ in cell extracts obtained over a 21-day differentiation period. No changes in sulfatase activity in the cell extracts during differentiation were reported. Additionally, semiquantitative analysis of PCR products revealed no changes in steroid sulfatase transcript levels during differentiation in the SV-HFO cells (Janssen et al., 1999). Our results are similar to the findings of Janssen et al., (1999), who used a human fetal osteoblast cell line to semi-quantitate steroid sulfatase message levels. Nonetheless, Janssen et al. (1999) did not compare steroid sulfatase mRNA or activity levels between cells undergoing differentiation and cells in active proliferation; nor did they compare steroid sulfatase mRNA levels between cells treated with ICI.

We uncovered some unexpected results concerning steroid sulfatase activity during differentiation. We expected the activity levels to be similar among the groups, but we found that activity increased on Days 7 and 14 in the OS- group. It also increased on Day 7 in the OS+ group. There was no such increase in the ICI group. From our
activity data, it appears that steroid sulfatase activity is upregulated during MC3T3-E1 osteoblast proliferation, particularly during the earlier phases of proliferation (up to Day 7). These data also suggest that steroid sulfatase activity is at least partly mediated by estrogen, because, in contrast to significantly increased steroid sulfatase activity in the cells incubated in OS+ (Days 3 and 7), there were no differences in steroid sulfatase activity in cells treated with the estrogen blocker, ICI 182, 780. ICI 182, 780 blocks the ability of 17β-estradiol to bind to the estrogen receptor (Wakeling et al., 1991). Although we are assuming that 17β-estradiol is present in our system (from 10% fetal calf serum), it is important to note that no attempt was made to measure the levels of 17β-estradiol throughout the duration of differentiation.

The role of estrogen in differentiation of bone is not clear. To date, there have been no studies on the direct effect of 17β-estradiol on the differentiation of MC3T3-E1 cells. Moreover, there has only been one other study that has addressed steroid sulfatase expression during the differentiation process (Janssen et al., 1999). From our results, it does not appear that 17β-estradiol is necessary for the differentiation of MC3T3-E1 cells under our test conditions. However, it may be important in proliferation.

Treatment with ICI 182, 780 does not affect the ability of this cell line to differentiate towards the mature osteoblast phenotype. Quite the opposite, it appears that blocking the action of the estrogen receptor allows the cells to differentiate more rapidly. Moreover, it appears that 17β-estradiol may affect the expression of alkaline phosphatase, as significantly higher levels of alkaline phosphate activity are present in the cells under the blockade of estrogen. Our results also indicate that ICI 182, 780 does not affect steroid sulfatase mRNA expression during MC3T3-E1 differentiation.
From our findings, it appears that estrogen acting through an estrogen receptor mechanism is not required during the differentiation process. Our results indicate that differentiation of the MC3T3-E1 cells progress even when the estrogen receptor is blocked, as the ICI 182,780 data suggest. Janssen et al. (1999) demonstrated that the human osteoblast cells are capable of both oxidative (e.g. estradiol to estrone) and reductive (e.g. estrone to estradiol) reactions. They report that the oxidative reaction was stronger than the reductive reaction at any phase of differentiation of the SV-HFO cells. Furthermore, they reported a decrease in aromatase mRNA expression and activity, which is an enzyme that is necessary for conversion of androgens to estrogens. These observations suggest that estrogen does not play a direct role in osteoblast differentiation. Our results confirm their findings.

In summary, we were able to devise an assay to study the expression and activity of steroid sulfatase during the differentiation of mouse MC3T3-E1 pre-osteoblast cells. Our hypothesis regarding the consistent expression of steroid sulfatase mRNA was confirmed. However, unexpectedly the activity of steroid sulfatase did change throughout differentiation and demonstrated very different results between cells treated with osteogenic supplement and osteogenic supplement plus an estrogen blocker. The role that estrogen and steroid sulfatase play in active proliferation of MC3T3-E1 remains to be elucidated. Additionally, the role that steroid sulfatase may play between Days 7 and 14 in cells undergoing differentiation needs to be further examined.
Figure 20. Preliminary growth analysis of MC3T3-E1 cells treated with osteogenic supplement medium versus untreated cells. Cells were incubated in either growth medium or growth medium containing osteogenic supplement for 12 days. Following each specific time point, cell density was determined by an MTT Assay. Data represent three independent experiments, run in duplicate. Bars represent the mean ± 1 S.E.M. Asterisks indicate significant difference between means (Bonferroni’s post hoc, * = P<0.05. *** = P<0.001). Abbreviations are: OS+ = growth medium with osteogenic supplement; OS- = growth medium alone
Figure 21. Alkaline phosphatase activity in MC3T3-E1 cells treated with growth medium (OS-), growth medium containing osteogenic supplement (OS+), and growth medium containing osteogenic supplement plus the estrogen receptor blocker, ICI 182, 780 (ICI). Alkaline phosphatase activity, as determined by p-nitrophenol phosphate hydrolysis, were measured in cell homogenates over a 21-day period. Cell homogenates were prepared as indicated in Materials and Methods. A BCA assay was performed (as indicated in Materials and Methods) to determine protein concentrations for each cell homogenate sample. Data represent three independent experiments. Bars represent the mean ± 1 S.E.M. Different letters indicate significant difference between means for days within a treatment group (Bonferroni’s post hoc, P<0.05). Abbreviations are: OS+ = growth medium with osteogenic supplement; OS- = growth medium alone; ICI = growth media with both osteogenic supplement and ICI 182,780.
Figure 22. Reverse transcriptase polymerase chain reaction of MC3T3-E1 cells using mouse-specific osteocalcin primers on a 2% agarose gel, stained with ethidium bromide. Primer sequences and reverse transcriptase polymerase chain reaction methods were performed as indicated in Materials and Methods. Lane assignments: numbers = days on which RNA was collected; - = growth medium; + = medium containing osteogenic supplement.
Figure 23. Whole-cell conversion assay on MC3T3-E1 cells treated with growth medium (OS-), growth medium containing osteogenic supplement (OS+), and growth medium containing osteogenic supplement plus the estrogen receptor blocker, ICI 182,780 (ICI). Steroid sulfatase activity in MC3T3-E1 cells, as determined by $^3$H-estrone sulfate conversion assay. Data represent three independent experiments, run in duplicate. Bars represent the mean ± 1 S.E.M. Different letters indicate significant difference between means for days within a treatment group (Bonferroni’s post hoc, P<0.05). Abbreviations are: OS+ = growth medium with osteogenic supplement; OS- = growth medium alone; ICI = growth media with both osteogenic supplement and ICI 182,780.
Figure 24. Reverse transcriptase polymerase chain reaction of MC3T3-E1 cells using mouse-specific steroid sulfatase or cyclophilin primers on a 2% agarose gel, stained with ethidium bromide. Primer sequences and reverse transcriptase polymerase chain reaction methods were performed as indicated in Materials and Methods. The samples were run for 30 cycles in the thermocycler. Lane assignments: numbers = days on which RNA was collected; - = growth media alone; + = media containing osteogenic supplement; I = media containing osteogenic supplement and ICI 182,780.
Figure 25. Reverse transcriptase polymerase chain reaction of MC3T3-E1 cells using mouse-specific steroid sulfatase or cyclophilin primers on a 2% agarose gel, stained with ethidium bromide. Primer sequences and reverse transcriptase polymerase chain reaction methods were performed as indicated in Materials and Methods. These samples were run for 25 cycles in the thermocycler. Lane assignments: numbers = days on which RNA was collected; - = growth media alone; + = media containing osteogenic supplement; I = media containing osteogenic supplement and ICI 182,780.
Chapter 3

Summary And Future Directions

Project I. Distribution of Steroid Sulfatase in Normal Human Tissues and Breast Cancers

Our laboratory generated a steroid sulfatase antibody, STS-275 (Selcer et al., 2007; see Project 1), to utilize in the evaluation of steroid sulfatase in normal human tissues and malignant breast tissues. Our data confirm the utility of this antibody in the assessment of the presence and distribution of this enzyme. Preliminary data regarding the protein levels of steroid sulfatase in breast cancers compared to normal breast tissue demonstrated that the levels are indeed higher in hormone dependent breast cancer, thereby suggesting that steroid sulfatase could be used as a prognosis indicator. These data are based on 3 tissue samples from each tissue type. The findings from our study are consistent with previous reports, which have indicated that steroid sulfatase protein and expression are higher in hormone dependent breast cancer compared with hormone independent and normal breast tissue.

We have developed a protocol that could be used to assess steroid sulfatase in a variety of tissues, either as fresh tissues or as paraffin-embedded tissues on slides as single samples or a microarray. This protocol is ideal to perform a robust study using American samples on tissues from a tissue bank. The tissues would require clinical and
outcome data, including stage of disease, recurrence information, diagnosis, race, tumor grade, nodal status, and receptor status. These parameters could then be used to assess the prognostic value of steroid sulfatase in the United States demographic.

**Project II. Steroid Sulfatase Distribution and Activity in Mouse Tissues**

Our results demonstrate that steroid sulfatase is present and active in the mouse tissues that we examined. These findings are consistent with reports on steroid sulfatase distribution and activity in other mammalian species.

We now have addressed the fundamental question about the presence and distribution of steroid sulfatase in mouse tissues, which provides the impetus for using the mouse as a physiological model in which to examine steroid sulfatase regulation and function. Because the steroid environment in mice is easily manipulated, our results should help to enhance the utility of mouse physiological models for the study of steroid sulfatase.

**Project III. Characterization of Steroid Sulfatase in Mouse Bone Cells**

We have demonstrated for the first time, three lines of evidence for the activity and presence of steroid sulfatase enzyme and the presence of steroid sulfatase mRNA in the MC3T3-E1 mouse pre-osteoblast cells. The data generated from enzyme activity assays, Western blotting, and RT-PCR indicate that this cell line is capable of converting inactive steroids into biologically potent steroids. These findings are consistent with previous studies that have demonstrated the presence and activity of steroid sulfatase in osteoblasts derived from several other mammalian species, including humans and rats.

**Project IV. Steroid Sulfatase Activity and Expression During Differentiation of MC3T3-E1 Mouse Bone Cells**
We developed a protocol for the study of proliferation and differentiation in the MC3T3-E1 cell line. We found that OS+ induces differentiation at the expense of proliferation. We further found that ICI induced differentiation at the expense of proliferation. Furthermore, steroid sulfatase appears to be induced during the proliferative / differentiation interface. When this interface is prolonged (OS-), steroid sulfatase increases. When differentiation is induced rapidly, steroid sulfatase remains constant. Thus, there appears to be a time during late proliferation when steroid sulfatase is regulated, at least, its activity.

We have preliminary data that suggest that this cell line will proliferate when estrone sulfate is the only source of estrogen. However, our findings were inconclusive. Because we have a better understanding of the proliferative / differentiation phases of the MC3T3-E1 cells, we can now develop an assay to adequately address the role that estrogens, in particular, sulfated estrogens play in growth. Steroid sulfatase appears to be upregulated during both the early proliferative period and the transition of proliferation into differentiation; therefore, it seems logical to design a growth assay to around the time points when steroid sulfatase activity appears to be most critical. By designing a growth assay with this time period in mind, we can address if steroid sulfatase plays an important role in proliferation when estrone sulfate is the sole source of estrogen by providing a mechanism for in situ estrogen biosynthesis.
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Figure A1. Estrogen cellular transport. 17 β-estradiol can enter the cell and bind to the ER, and thereby activate gene transcription. 17 β-estradiol can also act through a non-genomic mechanism; it can bind to the membrane ER and elicit a signal transduction pathway that leads to gene transcription. Estrone can bind directly to the ER, although its affinity is lower than that of 17 β-estradiol. Once estrone enters the cell it can either bind to the ER and activate gene transcription, or it can be converted to 17 β-estradiol. Estrone sulfate can enter the cell and bind to steroid sulfatase, which can convert it to estrone. Estrone can either bind to the ER and activate gene transcription, or it can be converted to 17 β-estradiol, which can then induce gene transcription (Matthews and Gustaffson, 2006). Abbreviations are: ER= estrogen receptor.
Figure A2. MC3T3-E1 cells captured at both low density and high density. Cells were grown in growth medium only as indicated in Methods and Materials. Images were captured using a 10x objective with 100x total magnification.
**Figure 3A. Morphological features of MC3T3-E1 cells captured during the differentiation process.** Images are representative of cellular morphology throughout differentiation. Images were captured on each day represented and for each treatment group. Cells were grown and treated as indicated in Methods and Materials. Cells did not survive for capturing on Day 21 in OS+ treatment and Day 14 in OS- Treatment.

Abbreviations are: OS- = growth medium alone; OS+ = growth medium with osteogenic supplement; ICI = growth medium with both osteogenic supplement and ICI-182,780; Nodule = nodule formation. Images were captured using a 10x objective with 100x total magnification.
Appendix II

Expanded Abstract

The enzyme steroid sulfatase cleaves the sulfate group from 3\(\beta\)-hydroxysteroid sulfates, including two abundantly circulating steroids, dehydroepiandrosterone and estrone sulfate. Steroid sulfatase is widely distributed among mammalian tissues, and it has been suggested to play a role in the in situ conversion of inactive steroids to their biologically active forms in a variety of tissues. Two tissues in which steroid sulfatase may be particularly important are breast cancer and bone. Our laboratory is interested in the role of steroid sulfatase; consequently, for my dissertation, I worked on four projects characterizing steroid sulfatase in human and mouse tissues and investigating the role of this enzyme in mouse bone cells.

Project I. Distribution of Steroid Sulfatase in Normal Human Tissues and Breast Cancers

Recent evidence indicates that steroid sulfatase could be a prognostic indicator in breast cancer. Our laboratory generated a steroid sulfatase antibody (STS-275) to examine the presence and distribution of steroid sulfatase in a variety of tissues, including breast carcinomas. STS-275 was used in an immunohistochemical analysis of individual human tissue slides as well as a human tissue microarray. For single tissues, human placenta and liver showed strong positive staining against the steroid sulfatase antibody (STS-275). Estrogen receptor positive (ER+) / progesterone receptor positive (PR+) breast cancers also showed relatively strong levels of steroid sulfatase immunoreactivity. Normal human breast showed moderate levels of steroid sulfatase immunoreactivity, while ER-/PR- breast cancer showed weak immunoreactivity. This confirms previous reports that steroid sulfatase is higher in hormone-dependent breast cancers. For the
tissue microarray, most tissues showed some detectable level of steroid sulfatase immunoreactivity, but there were considerable differences among tissues, with skin and lymph nodes having the highest immunoreactivity and brain tissues having the lowest. These data reveal the utility of immunohistochemistry in evaluation of steroid sulfatase activity among tissues. The newly developed antibody, STS-275 should be useful in studies of both humans, and rats.

**Project II. Steroid Sulfatase Distribution and Activity in Mouse Tissue**

Another objective of our laboratory is to investigate the role of steroid sulfatase in a physiological model system. The mouse is an ideal model organism due the to amount of collateral information on endocrinology of this species, and due to the availability of many strains and transgenic constructs. However, little is known about steroid sulfatase in the mouse. Thus, we sought to determine the activity and presence of the steroid sulfatase enzyme and the presence 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). We used three independent lines of evidence to assess the presence of steroid sulfatase protein and mRNA; enzyme activity, immunoreactivity, and RT-PCR.

All three techniques indicated that each of the six mouse tissues had detectable sulfatase protein and steroid sulfatase mRNA levels. However, there were considerable differences among tissues in activity, the sizes of the immunoreactive bands, and in amount of mRNA detected. 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 these mouse tissues (liver, lung, muscle, kidney, uterus and ovary)
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.

Project III. Characterization of Steroid Sulfatase in Mouse Bone Cells

Steroid sulfatase may provide a local mechanism for 17β-estradiol needed to maintain bone health, as estrogens play a central role in the regulation of bone density. However, this enzyme has not been characterized in mouse bone. Therefore, we characterized steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line. Substantial steroid sulfatase activity was found to be present in MC3T3-E1 whole cells in culture. This activity was significantly reduced in the presence of the steroid sulfatase inhibitor estrone-3-O-sulfatmate (EMATE). Steroid sulfatase activity was also detected in microsomes prepared from MC3T3-E1 cells, using both 3H-estrone sulfate (E1S) and 3H-dehydroepiandrosterone (DHEAS) as tracers; however, 3H-E1S was converted at a faster rate than 3H-DHEAS. EMATE significantly reduced steroid sulfatase activity of the microsomes. The K_m of steroid sulfatase activity for microsomal preparations averaged 86 µM when using estrone sulfate as the substrate and 64 µM when using DHEAS as the substrate. Western blotting of MC3T3-E1 microsomes for steroid sulfatase was performed using a specific polyclonal antibody, STS-275 generated against a peptide based on a conserved region of the steroid sulfatase protein in human, rat, and mouse. Three bands of cross-reactivity were evident in the Western blots, ranging from 79 to 128 kDa, which is similar to the size range previously reported for steroid sulfatase. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate the presence of steroid sulfatase mRNA in MC3T3-E1 cells, using specific primers. A single
cDNA band of the expected size (100bp) was present in the RT-PCR reactions and the sequence of this cDNA was identical to the known sequence for mouse steroid sulfatase. Thus, three lines of evidence (enzyme activity, immunoassay, and RT-PCR) indicate the presence of steroid sulfatase in the MC3T3-E1 mouse bone cell line. The existence of steroid sulfatase in these cells suggests that this enzyme may play a role in regulation of bone density in mice.

**Project IV. Steroid Sulfatase Activity and Expression During Differentiation of MC3T3-E1 Mouse Bone Cells**

Osteoblasts proceed through a well-defined process of proliferation, matrix maturation, and extracellular mineralization. Because estrogens have been shown to be important in normal osteoblast function, steroid sulfatase may be necessary during the differentiation of these cells. Our previous results indicated that the mouse pre-osteoblast cell line MC3T3-E1 contained significant levels of steroid sulfatase. However, steroid sulfatase mRNA levels, expression and activity during osteoblast differentiation have not yet been demonstrated in this mouse bone cell line. Therefore, our next objective was to determine if steroid sulfatase is present throughout differentiation and to examine its possible role in the differentiation process. I developed a 21-day protocol suitable to assess the expression and activity of steroid sulfatase in MC3T3-E1 cells during the differentiation process. Steroid sulfatase mRNA levels was detectable by RT-PCR, and steroid sulfatase activity was determined using a whole-cell $^3$H-E$_1$S conversion assay. Cells were grown in whole medium alone (OS-), whole medium containing a commonly used osteogenic supplement (OS+) to enhance differentiation or whole medium containing an estrogen receptor blocker (ICI). Differentiation was assessed by alkaline phosphatase enzyme activity and by osteocalcin mRNA expression. Osteogenic
supplement significantly retarded growth, a result consistent with enhanced 
differentiation. Indeed, alkaline phosphatase activity peaked much earlier in the OS+ 
cells than in OS- cells. Interestingly, alkaline phosphatase peaked even earlier, and 
reached higher levels, in the ICI cells. Steroid sulfatase activity showed a peak on Days 7 
and 14 in OS- cells, a smaller peak on Day 14 in OS+ cells and no peak in the ICI cells. 
In contrast, steroid sulfatase mRNA was present on all days and under all test conditions. 
These data indicate that steroid sulfatase activity varies during differentiation, being 
higher in the early stages of differentiation. Furthermore, our data indicate that estrogen 
is not required for differentiation.

In summary, my dissertation project has provided new data on the distribution of 
steroid sulfatase in human and mouse tissues. It has provided a characterization of steroid 
sulfatase in various mouse tissues, and in a mouse bone cell line. Furthermore, it has 
given us insight into the changes in steroid sulfatase during different phases of mouse 
bone cell differentiation.