THE ROLE OF STEROID SULFATASE IN ENERGY HOMEOSTASIS AND INFLAMMATION

by

Mengxi Jiang

Bachelor of Science, Zhengzhou University, 2009

Submitted to the Graduate Faculty of

School of Pharmacy in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

School of Pharmacy

This dissertation was presented

by

Mengxi Jiang

It was defended on

July 16th, 2014

and approved by

Regis R. Vollmer, Professor, Pharmaceutical Sciences

Song Li, Professor, Pharmaceutical Sciences

Kyle W. Selcer, Associate Professor, Biological Sciences

Dissertation Advisor: Wen Xie, Professor, Pharmaceutical Sciences

THE ROLE OF STEROID SULFATASE IN ENERGY HOMEOSTASIS AND INFLAMMATION

Mengxi Jiang

University of Pittsburgh, 2014

The steroid sulfatase (STS)-mediated desulfation is a critical metabolic mechanism that regulates the chemical and functional homeostasis of endogenous and exogenous molecules. In this study, I first showed that the hepatic expression of STS was induced in the high-fat diet (HFD) and ob/ob models of obesity and type 2 diabetes. Over-expression of STS in the liver of transgenic mice alleviated HFD and ob/ob models of obesity and type 2 diabetes, including reduced body weight, improved insulin sensitivity, and decreased hepatic steatosis and inflammation. Interestingly, STS exerted its metabolic benefit through sex-specific mechanisms. In female mice, STS may have increased hepatic estrogen activity and consequently improved the metabolic functions; whereas ovariectomy abolished this protective effect. In contrast, the metabolic benefit of STS in males may have been accounted for by the male-specific decrease of inflammation in white adipose tissue (WAT) and skeletal muscle, as well as a pattern of skeletal muscle gene expression that favors energy expenditure.

Chronic inflammatory liver diseases are associated with estrogen excess and feminization in men. The estrogen excess was reasoned to be due to liver damage that weakens the liver's ability to breakdown estrogen. In this study, I showed that STS may have mediated the inflammation induced estrogen increase in chronic liver disease. Bioinformatic and IHC analysis

demonstrated that STS was induced in liver samples from patients with chronic inflammatory liver diseases. In contrast, several enzymes involved in estrogen inactivation were down-regulated in diseased livers, including the phase I enzymes CYP1A2 and CYP3A4 and the phase II enzyme estrogen sulfotransferase (EST). Mechanistically, I showed that STS is an NF-κB target gene, whose induction facilitates the conversion of inactive estrogen sulfates to estrogens. On the other hand, loss of STS or blocking estrogen signaling increased NF-κB transcriptional activity and inflammation. These elucidated the activation of negative feedback loop, that is, STS induced by NF-κB in hepatocytes enhanced estrogen activity, which in turn suppresses NF-κB-mediated inflammation.

In summary, I have uncovered novel roles and mechanisms of STS in energy homeostasis and inflammation, which may facilitate the development of novel interventions for metabolic syndrome and chronic inflammatory liver disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

This thesis is dedicated to my father, who inspired my curiosity about the secret of nature. And to my mother, who has always been a great source of motivation and unconditional love for me.

ABBREVIATION

ACC, acetyl-CoA carboxylase; **ACTH**, adrenocorticotropic hormone; **ADHD**, attention deficit hyperactivity disorder; **AMPK**, AMP-activated protein kinase; **bFGF**, basic fibroblast growth factor; **BPA**, bisphenol A; **ChIP**, chromatin immunoprecipitation; **DCC**, dextran coated charcoal; **DHEA**, dehydroepiandrosterone; **DOX**, doxycycline; **EMSA**, Electrophoretic mobility shift assay; **ER**, estrogen receptor; **EST**, estrogen sulfotransferases; **FABP**, fatty acid binding protein; **FAS**, fatty acid synthase; **FAT/CD36**, fatty acid translocase; **FGly**, formylglycine; **G6pase**, glucose-6-phosphatase; **GABAA**, gamma-aminobutyric acid A; **GEO**, Gene Expression Omnibus; **GLUT4**, glucose transporter 4; **HCC**, hepatocellular carcinoma; **HFD**, high-fat diet; **IGF-I**, insulin-like growth factor-1; **IHC**, immunohistochemistry; **IκBs**, inhibitors of NF-κB; **IKK**, IκB kinase; **IL-1**, interleukin-1; **IL-6**, interleukin-6; **IL-8**, interleukin-8; **IPGTT**, intraperitoneal glucose tolerance test; **IRS**, insulin receptor substrate; **ITT**, insulin tolerance test; **LPL**, lipoprotein lipase; **LXR**, liver X receptor; **MAPK**, mitogen-activated protein kinases; **MCP-1**, monocyte chemoattractant protein-1; **MSD**, multiple sulfatase deficiency; **NF-κB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **NMDA**, N-methyl-D-aspartic acid; **OATPs**, organic anion transporting polypeptides; **PDTC**, pyrrolidine dithiocarbamate; **PEPCK**, phosphoenolpyruvate carboxykinase; **PMA**, Phorbol 12-myristate 13-acetate; **POMC**, pro-opiomelanocortin; **PPAR α**, peroxisome proliferator-activated receptor α; **SCD-1**, stearoyl CoA desaturase-1; **SOAT**, organic anion transporter; **SREBP-1c**, sterol

regulatory element-binding protein 1c; **StAR**, steroidogenic acute regulatory protein; **STS**, steroid sulfatase; **SULT2B1b**, cholesterol sulfotransferases; **SULTs**, sulfotransferases (SULTs); **SUMF1**, sulfatase modifying factor 1; **TBE**, Tris borate-EDTA; **TG**, transgenic; **TLRs**, toll-like receptors; **TMA**, tissue microarray; **TNFα**, tumor necrosis factor α; **TRE**, tetracycline response element; **tTA**, tetracycline transactivator; **VMN**, ventromedial hypothalamic nucleus; **WAT**, white adipose tissue; **WT**, wild type

1.0 CHAPTER I: AN OVERVIEW OF STS AS A STEROID-ACTIVATING ENZYME

Aryl sulfatase C, also commonly known as STS, is a member of the sulfatase family which is responsible for the cleavage of sulfate esters (CO-S) and sulfamates (CN-S). STS-mediated desulfation and sulfotransferases (SULTs)-catalyzed sulfation are reversible reactions that tightly regulate steroid homeostasis. The sulfation reaction usually increases the water solubility and decreases the bioactivity of steroids. However, rather than being readily excreted, steroid sulfates act as a reservoir for regenerating active steroids through the STS-mediated desulfation(*1*). STS is the only sulfatase which catalyzes the hydrolysis of steroid sulfates and produces unconjugated steroids in biological systems. Inactivation of STS from point mutation or gene deletions results in X-linked ichthyosis - due to excess deposition of cholesterol sulfate in the skin (*2*). The STS-mediated desulfation of estrogen sulfate is a major route for local estrogen synthesis in malignant breast tissues, which makes STS as an attractive target for treatment of hormone-dependent cancers(*3*). STS regulates the homeostasis of dehydroepiandrosterone (DHEA)/DHEA sulfate, which are neurosteroids regulating brain functions. Dysfunction of STS has been associated with ADHD(*4*). STS also regulates the steroidogenesis through increasing the translation and half-life of the steroidogenic acute

regulatory protein (StAR) (*5*). In the introduction section, I will briefly review the molecular biology and biological function of STS under different pathological conditions.

1.1 THE STS GENE, FROM DNA TO PROTEIN

The human STS gene is located on the short arm of the X-chromosome. It escapes X-inactivation, a process that inactivates one X-chromosome in females to correct for the gene dosage differences between the genders (*6*). There is a pseudogene for STS on the Y-chromosome, which is transcriptionally inactive. The STS gene contains 10 exons spanning over 146 kb. Alternatively spliced transcripts with different first exons were identified in different tissues and cell types. However, the divergence is restricted to the N-terminal signal peptide, which is post-transcriptionally cleaved and thus does not change the enzymatic activity of STS(*7-9*). The 2.4 kb cDNA encodes a protein of 583 amino acids with an N-terminal endoplasmic reticulum-targeting signal peptide of 21-23 amino acids and potential N-glycosylation sites which are important for protein folding and stability(*10*). Mutation studies showed that both the N-terminal and C-terminal regions are critical for the enzymatic activity of STS. C-terminal mutants had dominant negative effects on the activity of wild-type STS (*11*). Human STS has been purified and crystallized (*12*). The “mushroom-like” tertiary structure of STS consists of a polar spherical domain with the catalytic site facing toward the ER lumen, and a transmembrane domain with two antiparallel alpha helices anchoring the enzyme into the ER membrane. Similar to other members of the sulfatase family, STS undergoes a unique yet conserved post-translational modification of a cysteine residue into formylglycine (FGly) at the

catalytic site for the activation of enzyme activity. This process is encoded by the sulfatase modifying factor 1 (SUMF1) gene (*13*). Mutations in the SUMF1 gene severely diminish catalytic activity of all sulfatases, resulting in multiple sulfatase deficiency (MSD), an autosomal recessive lysosomal storage disorder (*14*). The mouse Sts gene is pseudoautosomal, which is inherited in an autosomal pattern and escapes X-inactivation. However, there is a substantial divergence between mouse and human STS gene. The mouse steroid sulfatase gene spans over 9 kb, which is much smaller than that of the human gene, and it is only around 60% identical to human gene at the nucleotide level and amino acid level.

1.2 REGULATION OF STS AT TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL LEVEL

Little is known about the regulation of STS at transcriptional level. Characterization of STS promoter revealed that it resembled neither a tightly regulated gene-which contains a TATA box to position the RNA polymerase nor a housekeeping gene-which is usually GC rich and contains binding sites for SP1. On the other hand, the STS promoter is GC poor and lacks a TATA box and SP1 binding sites(*15*). Four transcription start sites were mapped over a 50bp region. The upstream regulatory elements contain three enhancers and one repressor region. The promoter activity could be detected by luciferase assay in the human placental choriocarcinoma JEG-3 cells, which have high STS activity. Transfection of the promoter into other cell lines, such as the COS-1 monkey kidney epithelial cells and cervical cancer HeLa cells did not produce detectable luciferase activity (*15*). Later, six promoters were reported to drive STS expression

with different first exons in various tissue and cell types. These results suggested tissue-specific regulation of STS transcription (*16*).

STS expression was markedly induced in breast tumor tissues compared with normal tissues, which was associated with higher local estrogen levels and poor prognosis (*17-19*). This regulation may be mediated by cytokines and growth factors. The proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) increased the activity of STS in MCF-7, a hormone-dependent human breast cancer cell line, without affecting its mRNA expression. Transfection of the promoter-less cDNA of STS into the MCF-7 cells increased its activity, which can be further enhanced by adding IL-6 and TNFα. These data suggests that the induction of STS by IL-6 and TNFα in MCF-7 cells was mediated by mechanisms other than transcriptional regulation, such as post-translational modifications or changes in substrate availability (*20*). Insulin-like growth factor-1 (IGF-I) and basic fibroblast growth factor (bFGF) dose-dependently stimulated STS activity in breast cancer cell lines. The induction of STS activity by cytokines and growth factors may explain the increased estrogen levels in breast tumor tissues even in postmenopausal women who have low circulating estrogen concentrations(*17*). Interleukin-1 (IL-1) increased STS activity in MCF-7 cells. However, it decreased the expression and activity of STS in an IL-1 receptor-dependent manner in human endometrial stromal cells-suggesting cell type-specific regulation (*21-22*). The phosphatidylinositol 3-kinase/Akt pathway transduces growth factor and cytokine signals through phosphorylation of its substrates and promotes cell survival and tumor progression(*23*). Recently, this pathway has been reported to mediate the induction of STS expression and activity

by growth factors and cytokines in the PC-3 human prostate cancer cells and human keratinocytes (*24-26*).

Besides cytokines and growth factors, steroid hormones have also been reported to induce STS at enzymatic level (*27-28*). Castration significantly reduced STS activity in adrenal, heart and liver in rats (*29*). On the other hand, estrogen sulfate administration enhanced the enzymatic activity of STS in rats-suggesting that steroid hormones induced STS through substrate supply (*30*). Stress-stimulated adrenocorticotropic hormone (ACTH) secretion induced STS activity in rat adrenals (*31*). The ACTH-mediated secretion of adrenal steroid hormones (*32-33*) may contribute to the induction of STS in response to stress.

1.3 BIOLOGICAL ROLES OF STS

The biological roles of STS are dependent on its tissue distribution and expression, as well as its substrate affinity and availability. Multiple organs and tissues express STS in small quantities. Tissue levels of STS vary under different physiological and pathological conditions. STS is mainly expressed in target tissues of the reproductive tract, with the highest expression identified in the placenta (*34-35*). Placental STS plays a critical role in the formation of estriol from the fetoplacental unit. Low or absent estriol levels in pregnancy were associated with STS deficiency and miscarriages (*14*). The subcellular localization of STS is mainly on the ER membrane, but STS has also been found in the Golgi, endosomes and plasma membranes in smaller fractions (*10*).

The substrates of STS are primarily steroid sulfates, such as cholesterol sulfate, pregnenolone sulfate, estrogen sulfates, DHEA sulfate and androsterone sulfate. For the above substrates, the Km resides in the low micromolar to low nanomolar range. Sulfate metabolite of xenobiotics that are mimics of steroid sulfate can also enter the active site of STS and be efficiently hydrolyzed, such as resveratrol sulfate (*36*) and BPA (Bisphenol A) sulfate (*37*). Sulfation of steroids or steroid mimics changes their polarity and bioavailability, making them either circulating as a reservoir for regeneration or being rapidly excreted. In addition, sulfation changes the structures of the molecules and affects their binding affinity or preference of the corresponding receptors, which further influence downstream signaling pathways in many physiological and pathological processes.

Transport of steroid sulfates contributes to the tissue availability of STS substrate. The hydrophilic and charged steroid sulfates are unable to cross the lipophilic cell membranes. However, cell membrane transporters have been reported to uptake steroid sulfates before they are hydrolyzed, including the organic anion transporter (SOAT) and the organic anion transporting polypeptides (OATPs). SOAT specifically transports steroid sulfates in a sodium-dependent and bi-directional manner in the adrenal gland, testes, placenta and other peripheral tissues; while OATP transport steroid sulfates, as well as other substrates in a sodium-independent manner. Uptake of estrogen sulfate and DHEA sulfate by OATP was stimulated by prostaglandins. (*38-41*).

The STS-mediated desulfation pathway is a major route for local estrogen synthesis. There are three forms of estrogen, E1 (Estrone), E2 (Estradiol) and E3 (Estriol), among which E2

is the most active and major circulating estrogen in premenopausal women. E2 is mainly produced in the ovaries by aromatization of testosterone. In men and postmenopausal women, however, E2 is synthesized locally in extragonadal tissues such as breast, brain, bone and adipose tissue rather than being uptaken from the circulation (*42-43*). Estrone sulfate has a higher concentration and longer half-life than estrone in the circulation. STS switches the estrogen metabolism in target cells by desulfating estrogen sulfates to active estrogens and promoting estrogen activity. The expression of STS was markedly induced in malignant breast tissues compared with normal breast tissues and was positively associated with local estrogen levels, tumor size, metastatic potential and poor prognosis (*17-18, 44-45*). This makes STS an attractive target for the treatment of hormone-dependent cancers, such as breast, endometrial, ovarian and prostate cancer. Currently, Irosustat (STX64) is under clinical trial for treating endocrine-resistant breast cancer with some encouraging results (*46*). In addition, traditional Chinese herbs and food additive have been shown to inhibit the expression and activity of STS *in vitro* (*47-49*). Combination of STS inhibitor and other antiproliferative agents may be effective against hormone-dependent cancers with less drug resistance (*50*).

Another important substrate of STS-cholesterol sulfate-is one of the most abundant steroid sulfates present in human circulation and also plays a critical regulatory role in the skin during barrier formation. Cholesterol sulfate is generated by cholesterol sulfotransferase (SULT2B1b) in the epidermis and is cleaved to produce free cholesterol in the outer epidermis. Cholesterol sulfate regulates epidermal differentiation, desquamation and barrier function(*51*). Inactivation of STS from point mutation or gene deletions results in X-linked ichthyosis, one of the most common human inborn errors (approximately 1 in 6000 males) of metabolism detected

in different ethnic groups (*52-55*). A 10-fold increased deposition of cholesterol sulfate due to loss of function of STS delays the desquamation process and results in hyperkeratosis, which is characterized by scaling and thickening of the skin (*56-59*). Accompanied with X-linked ichthyosis are extracutaneous manifestations including cryptorchidism, seizures, corneal opacity, pyloric hypertrophy, acute lymphoblastic lymphoma and abdominal wall defect (*60*). However, it is doubtful whether there is a causal relationship between the loss of function of STS and these clinical conditions since flanking regions of the STS gene may have also been deleted in patients with X-linked ichthyosis.

DHEA and DHEA sulfate are primarily secreted by the adrenal cortex, but they are also synthesized in the central nervous system and regulate brain functions (*61*). DHEA sulfate act as gamma-aminobutyric acid A (GABAA) receptor antagonist; whereas DHEA act as GABAA receptor agonist. These neurosteroids also modulate the activity of N-methyl-D-aspartic acid (NMDA) and sigma-1 receptors (*62-63*). It has been reported that circulating DHEA levels are inversely correlated with the symptomatology of attention deficit hyperactivity disorder (ADHD), a mental disorder with attention deficits, hyperactivity and impulsivity (*64*). STS-which is relatively highly expressed in both human and mouse brains-may regulate DHEA/DHEA sulfate levels and affect the vulnerability for developing ADHD and other endophenotypes. Indeed, STS dysfunction has been associated with ADHD. Human subjects with deletion or mutation of the STS gene are at higher risk of developing ADHD and other neurologic manifestations (*65-66*). Polymorphism within STS has been associated with ADHD (*67*). Reduced activity of STS has been suggested to cause neural disturbances such as postpartum psychosis in the mothers (*68*). Besides human studies, genetic

loss or pharmacological blockade of STS gene in mice lead to attention deficits, altered impulsivity and increased aggressive behavior, which mimicked human ADHD (*69*).

BPA is a not only a widely used plasticizer, but also an endocrine disruptor through binding to estrogen receptor (ER). On the other hand, BPA sulfate binds poorly to the ER. However, BPA sulfate treatment enhanced the growth of ER-positive MCF-7 cells and free BPA levels inside the cells, which was due to conversion of BPA sulfate to the active BPA by the action of STS. Therefore, STS-mediated desulfation may increase the estrogenic potential of environmental estrogens (*37*). STS substrates also include a natural compound resveratrol, a naturally occurring chemopreventive agent present in grapes and wine (*36*). Compared with resveratrol, resveratrol sulfate showed poor cytotoxicity in human breast cancer cells (*70*). Differential expression of STS in malignant and non-malignant human breast tissues affected the sulfation metabolism of resveratrol, which dramatically lowered the bioavailability and increased the clearance of resveratrol. Therefore, STS may impact the therapeutic efficacy of resveratrol by modulating its local metabolism inside the tumors.

2.0 CHAPTER II: HEPATIC OVER-EXPRESSION OF STS AMELIORATES MOUSE MODELS OF OBESITY AND TYPE 2 DIABETES THROUGH SEX-SPECIFIC MECHANISMS

2.1 ESTROGEN SIGNALING IN ENERGY HOMEOSTASIS

Estrogen is not only a key reproductive steroid hormone, but it also has great implications in glucose and energy metabolism. Estrogen exhibits an overall protective role in preventing obesity and type 2 diabetes (*71*). Postmenopausal women face increased risk of developing obesity and insulin resistance. On the other hand, estrogen replacement therapies ameliorate metabolic disorders in both women and animal models (*72-74*). Estrone sulfate is the major active component in conjugated equine estrogens that are commonly used in hormone replacement therapy. Treatment with conjugated equine estrogens improved glycemic control and blood lipid profiles in postmenopausal, type 2 diabetic women (*75*). ERα knockout mice developed impaired insulin sensitivity and obesity (*76*). Mice deficient of aromatase, the enzyme that converts androgens to estrogens, exhibited increased adiposity and fatty liver, which were normalized by estrogen treatment (*77-78*). Human subjects lacking ERα or aromatase also developed insulin resistance (*79-80*). Despite its known metabolic benefit, a systemic treatment

with estrogens is limited by its potential side effects, such as increased risk for cardiovascular disease and tumor promotion (*81*).

Estrogen signaling regulates energy homeostasis in both the central nervous system and the peripheral tissues. In the hypothalamic neurons, estrogen signaling controls food intake and energy expenditure, which appear to be mediated mainly by ERα. Loss of ERα in ventromedial hypothalamic nucleus (VMN) neurons in mice resulted in hypometabolism and obesity without affecting food intake. On the other hand, deletion of ERα in hypothalamic pro-opiomelanocortin (POMC) neurons caused hyperphagia, while the energy expenditure was not changed. These results indicate that estrogen signaling in distinct hypothalamic neurons regulates different aspects of energy homeostasis (*82*). In addition to the direct effects on the central nervous system, estrogen also regulates body weight homeostasis through the hormone leptin. Leptin is a catabolic hormone secreted from the WAT that reduces food intake and increases energy expenditure. Leptin receptors are co-localized with ERα in the brain-suggesting their close interactions (*83*). Estrogen regulates the expression of leptin receptor and enhances the sensitivity of leptin centrally (*84-85*).

The liver plays an important role in lipid homeostasis by regulating the synthesis, catabolism and transport of lipids. The high insulin level in insulin-resistance state stimulates the expression of the sterol regulatory element-binding protein 1c (SREBP-1c), a master regulator of hepatic lipogenesis and a mediator through which many other transcription factors orchestrate lipid homeostasis (*86*). Activation of SREBP-1c consequently regulates several rate-limiting enzymes involved in lipogenesis, including fatty acid synthase (FAS), acetyl-CoA

carboxylase (ACC) and stearoyl CoA desaturase-1 (SCD-1), which contribute to increased hepatic lipogenesis and steatosis, and fuel the progression of insulin resistance (*87*). The lipid-reducing effect of estrogen has been reported in rodents and humans. Anti-estrogen therapy in women resulted in abnormal lipid profile and liver steatosis (*88*). Aromatase knockout mice developed severe liver steatosis and hepatic insulin resistance (*77*). ERα knockout mice showed increased expression of lipogenic genes and decreased expression of genes for lipid transport in the livers (*89*). On the other hand, estrogen treatment reduced the expression of lipogenic genes and liver steatosis of HFD-fed mice in an ERα-dependent manner (*90*). Mechanistically, estrogen compromises the liver X receptor (LXR)-induced expression of SREBP-1c and triglyceride accumulation in an ER-dependent manner. Activated ERα directly binds to LXR and inhibits its coactivator recruitment on the SREBP-1c promoter-thus reducing the expression of SREBP-1c and lipid accumulation in the liver (*91*).

Estrogen regulates WAT distribution and lipid metabolism. Men and women display different patterns of fat distribution: Men have more intra-abdominal fat, which is a risk factor of metabolic syndrome (*92*); whereas women have more subcutaneous WAT than men in general. Postmenopausal women and ovariectomized mice tend to have the male-patterned fat distribution, which can be prevented by estrogen replacement therapy (*93-97*). STS may also be participated in fat distribution through regulating estrogen homeostasis. Compared with men, E2 levels were higher in subcutaneous WAT in women, along with higher expression of STS (*98*). ERα knockout mice and aromatase knockout mice showed adipocyte hypertrophy (*76-77*), while estrogen treatment prevented lipid accumulation in aromatase knockout mice by reducing lipid synthesis and uptake. Administration of estrogen to postmenopausal women and ovariectomized

mice reduced the expression of genes involved in lipogenesis and fatty acid uptake in adipocytes (*99-100*). In cultured adipocytes and WAT, estrogen also suppresses the expression and activity of lipoprotein lipase (LPL) and inhibits the breakdown of triglycerides into free fatty acids (*101-102*), key mediators of insulin resistance in liver and muscle (*103*). In addition, estrogen signaling also regulates insulin sensitivity in the WAT. Estrogen activated Akt in 3T3-L1 adipocytes in an ER-dependent manner (*104*). Macrophage infiltration into the WAT greatly contributes to obesity-induced insulin resistance (*105*). Ovariectomized mice displayed increased immune cell infiltration and expression of inflammation markers in WAT (*106*). Conditioned media from macrophages lacking ERα impaired insulin-stimulated Akt phosphorylation and glucose uptake in cultured adipocytes (*107*). These data suggest that the estrogen signaling in immune cells plays an important role in adipose tissue inflammation and whole-body insulin sensitivity.

Insulin-stimulated glucose uptake mainly occurs in skeletal muscle, which is mediated by the glucose transporter 4 (GLUT4). GLUT4 can be activated by several different-but intertwined-signaling pathways, including the insulin signaling pathway, the AMP-activated protein kinase (AMPK) pathway and mitogen-activated protein kinases (MAPK) pathway (*108-109*). Estrogen acts on key proteins involved in these pathways, which results in phosphorylation and activation of MAPK and AMPK (*110-111*) and consequent activation of GLUT4 and glucose disposal. Estrogen treatment also restored HFD-induced insulin resistance in mice by increasing the abundance of insulin receptor substrate (IRS) and phosphorylation of Akt in the skeleton muscle (*112*). It should be noted that while ERα promotes glucose disposal and insulin sensitivity, ERβ may suppress GLUT4 activity and cause insulin resistance (*113*). More studies

are needed to identify the metabolic function of ERα and ERβ in the muscle. Estrogen also promotes insulin sensitivity in the muscle by enhancing lipid oxidation and suppressing inflammation. Estrogen treatment enhanced lipid oxidation *in vitro* and *in vivo* by increasing the expression of peroxisome proliferator-activated receptor α (PPARα) and its target genes involved in oxidative metabolism (*114-115*). On the other hand, reduction in markers of fatty acid oxidation, enhanced lipid content and impaired insulin sensitivity in the muscle was observed in ERα knockout mice (*116*). Accumulation of bioactive lipid intermediates can induce inflammation and impair insulin actions. ERα knockout mice displayed accumulation of the bioactive ceramides and diacylglycerol and induction of pro-inflammatory markers in the muscle (*116*). By contrast, Estrogen administration reduced neutrophil infiltration and expression of the inflammation marker in the skeletal muscle of ovariectomized rodents and post-menopausal women (*117-118*).

β-cell failure plays a key role in the pathogenesis of type 2 diabetes. Estrogen treatment prevents β-cell failure in several rodent models of type 2 diabetes (*119*). β-cell expresses multiple ERs including ERα, ERβ and the G protein-coupled ER. Studies using genetic loss-of-function approaches revealed that ERα raises glucose-stimulated insulin biosynthesis, decreases lipotoxicity and promotes β-cell survival; ERβ enhances glucose-stimulated insulin secretion, and GPER promotes insulin secretion, improves lipid homeostasis and reduces β-cell apoptosis. These effects may be mediated by extranuclear ERs in the β-cells (*119*).

The estrogen homeostasis is tightly regulated by sulfation and desulfation. Estrogens can be sulfated and deactivated by the EST (SULT1E1) (*120*). Unlike estrogen, estrogen sulfate

cannot bind to ER and thus are biologically inactive, but they have higher concentrations and prolonged half-life in the circulation, acting as a reservoir for regenerating active estrogen by the STS-mediated desulfation (*1*). It is believed that STS is the only enzyme responsible for desulfating estrogen sulfates. STS gene deletion or mutation caused X-linked ichthyosis, which is often associated with reproductive abnormalities due to disrupted steroid hormone homeostasis (*121*).

It has been previously reported that EST affected mouse models of type 2 diabetes in a sex-specific manner (*122*). Loss of *Est* improved metabolic function in ob/ob and HFD-fed female mice as a result of decreased estrogen deprivation and increased estrogenic activity in the liver. In contrast, *Est* ablation in ob/ob males exacerbated the diabetic phenotype due to the loss of pancreatic β cells caused by increased WAT inflammation. Although STS is known to counteract with EST to enhance estrogen activity, whether STS can affect the pathogenesis of obesity and type 2 diabetes through its regulation of the estrogen homeostasis, and whether the effect of STS has sex specificity has not been reported.

In this report, I showed that hepatic over-expression of STS elicited metabolic benefits in both sexes but via distinct mechanisms. The metabolic benefit in female STS mice was likely mediated by increased hepatic estrogen activity, whereas the protective effect of STS in males may have been accounted for by decreased inflammation in WAT and skeletal muscle. My results have uncovered a novel function of STS in regulating energy metabolism and improving insulin sensitivity.

2.2 METHODS

2.2.1 Generation of STS transgenic mice, diet and drug treatment, body composition analysis, and indirect calorimetry

The human STS cDNA was cloned into the TRE-SV40 transgene cassette (*123*) to construct the TRE-STS transgene. Transgenic production was performed at University of Pittsburgh Transgenic Core Facility. The TRE-STS/FABP-tTA double transgenic or "STS" mice were generated by cross-breeding the TRE-STS mice with the FABP-tTA mice (*124*) and maintained in the C57BL/6J background.

The majority of the subsequent mating was set up between the TRE-STS/FABP-tTA double transgenic mice and FABP-tTA single transgenic mice of the opposite sex, in which I observed 55.3% of female pups and 52.7% of male pups were double transgenic and expressed the STS transgene. These mating results suggested a Mendelian segregation of the transgene. The obS mice (ob/ob mice expressing the STS transgene) were generated by crossing the transgene into the ob/ob background. When necessary, doxycycline (DOX, 2 mg/ml) was given in drinking water one week before the HFD treatment and until the completion of the experiment. For estrone sulfate treatment, mice were treated with 75 mg/kg/day estrone-3-sulfate from Sigma for 5 days by oral gavage (*125*). HFD (Cat # S3282) with 60% of total calories coming from animal fat (mainly lard) was purchased from Bio-serv (Frenchtown, NJ). The HFD contains 37% saturated fatty acid, 47% monounsaturated fatty acid and 16% polyunsaturated fatty acid. Body composition by EchoMRI and indirect calorimetry by Oxymax Indirect

Calorimetry System were performed as previously described (*126*). The use of mice in this study has complied with relevant federal guidelines and institutional policies.

2.2.2 Intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT), and euglycemic-hyperinsulinemic clamp

IPGTT was performed in 16-h (5:00 pm to 9:00 am) fasted mice with an intraperitoneal injection of D-glucose at 1g/kg body weight for ob/ob mice or at 2 g/kg body weight for other genotypes. ITT was performed in 6-h (9:00 am to 3:00 pm) fasted mice with an intraperitoneal injection of insulin at 1.5 units/kg body weight for ob/ob mice, or at 0.5 unit/kg body weight for other genotypes. For euglycemic-hy-perinsulinemic clamp study, 16-h fasted mice were constantly infused with [3-^{3}H] glucose at 0.05 μCi/min through a right jugular vein catheter at the basal state. During the clamp state, the mice were infused with a primed dose of human insulin from Novo Nordisk (Princeton, NJ) at 300 mU/kg body weight, followed by a constant insulin infusion at 2.5milliunits/kg/min. [3-^{3}H] glucose was infused at 0.1 μCi/min. A variable rate of 20% glucose was infused at the same time to maintain blood glucose range between 120 and 140mg/dl. Blood glucose levels were monitored every 10 min. 20 μl of blood were sampled at the end of the basal state and clamp state for plasma [3-^{3}H] glucose measurement. Liver, skeletal muscle and adipose tissue were harvested at the end of the clamp experiment to assess the insulin signaling (*127*).

2.2.3 Measurement of hepatic estrogen activities by liver luciferase reporter gene transfection

Mouse livers were transfected with the estrogen receptor responsive tk-ERE-Luc reporter gene by hydrodynamic injection. Hepatic luciferase activity was assessed 16 h after transfection and normalized to the protein concentrations (*128*). When necessary, ovariectomized and tk-ERE-Luc transfected mice were subcutaneously injected with 2.5 mg/kg estrone sulfate 6 h after transfection.

2.2.4 UPLC-MS/MS analysis of hepatic estrogens and estrogen sulfates

UPLC/MS-MS were carried out with a Waters Acquity UPLC system connected with the Xevo TQ triple quadrupole mass spectrometer (*129*). The analyses were performed using Electro Spray Ionization (ESI) in positive ion mode, capillary voltage of 3.0 kV, extractor cone voltage of 3 V and detector voltage of 500 V. Desolvation gas flow was maintained at 600 L/h. Source temperature and desolvation temperatures were set at 150 and 350 °C, respectively. The collision energy was varied to optimize daughter ions. Analytical separations on the UPLC system were conducted using an Acquity UPLC BEH C18 1.7 µm column (1 X 50 mm) at a flow rate of 0.2 ml/min. The gradient started with 100% A (0.1% formic acid in H_2O) and 0% B (0.1% formic acid in CH_3CN) and changed to 20% A over 5 min, then 0 % A over 2 min. Finally over 1 min it was changed back to original 100% A and column was equilibrated at 100% A for 2min,

resulting in a total separation time of 10 min. The elutions from the UPLC column were introduced to the mass spectrometer and resulting data were analyzed and processed using MassLynx 4.1 software. Reference standards were used to optimize the UPLC/MS conditions prior to analysis.

2.2.5 Measurement of serum biochemistry

Serum levels of estradiol (Diagnostic Systems Laboratories, Webster, TX), total triglycerides and cholesterol (Stanbio Laboratory, Boerne, TX), and insulin and leptin (Crystal Chem, Downers Grove, IL) were measured by using commercial assay kits.

2.2.6 Hepatocyte glucose production assay

Glucose production was measured from mouse primary hepatocytes as described before (*130-131*). Briefly, primary hepatocytes were incubated in glucose-free Dulbecco's Modified Essential Medium from Gibco (Grand Island, NY) containing 20 mM sodium lactate and 2 mM sodium pyruvate for 2 h in the presence or absence of 10 μM forskolin. Glucose production was measured from medium using a glucose assay kit from Sigma (St Louis, MO) and was normalized to protein concentrations.

2.2.7 Northern blotting, real-time PCR analysis, western blotting, and immunohistochemistry

Total RNA was isolated using TRIZOL reagent from Invitrogen. Northern blot analysis was performed as previously described (*132*). ^{32}P-labeled full-length STS cDNA was used to probe the transgene expression. For real-time PCR analysis, cDNA was synthesized by reverse transcription with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real-time PCR was performed with ABI 7300 real-time PCR system. Data were normalized against cyclophilin. Primary antibodies used for Western blot analysis include phospho-Akt (serine 473) (cat # 9271) and total Akt (cat # 9272) from Cell Signaling (Danvers, MA), and STS (cat# ab62219) from Abcam (Cambridge, MA). The anti-insulin antibody (cat # 3014) used for immunohistochemistry was purchased from Cell Signaling (Danvers, MA). The islet area was measured using ImageJ from the National Institutes of Health (Bethesda, MD).

2.2.8 Statistical analysis

Results are expressed as means ± SD. Statistical significance was determined by unpaired two-tailed student's t test or analysis of variance (ANOVA). The Bonferroni test was used for pot hoc comparisons. See Appendix A for details on statistical analyses used for each figure in the document. A P value less than 0.05 is considered as statistically significant.

2.3 RESULTS

2.3.1 The hepatic expression of STS was induced in obese mice and by fasting, and the creation of transgenic mice expressing STS in the liver

HFD-fed mice and chow diet-fed ob/ob mice are commonly used rodent models of obesity and type 2 diabetes. Both models are associated with marked hepatic steatosis. In profiling the gene expression in HFD-fed WT mice and ob/ob mice, I found that the hepatic expression of endogenous mouse Sts was increased in both models (Fig. 1A). Sts was also induced in chow-fed mice by fasting (Fig. 1B), during which the mouse liver accumulates large amounts of triglycerides (*133*).

To understand the functional relevance of this STS induction and to investigate the metabolic role of STS *in vivo*, I created the tetracycline-responsive STS transgenic mice by crossing two transgenic mouse lines: the FABP-tTA line expresses the tetracycline transactivator (tTA) in the liver and small intestine under the fatty acid binding protein (FABP) gene promoter (17); and the TRE-STS line expresses STS under the control of the tetracycline response element (TRE). In the double transgenic mice, tTA proteins bind to TRE and initiate the expression of the transgenic STS; whereas administration of DOX can silence the transgene expression (Fig. 1C). The expression of the transgenic STS and its silencing by DOX in the liver and small intestine was confirmed by Northern blotting (Fig. 1D). The transgene was absent in a panel of none-targeting tissues, including brain, kidney, skeletal muscle, brown adipose tissue, and WAT. The protein expression of the transgene in the liver was confirmed by Western blotting (Fig. 1E). The transgenic expression in the liver was also confirmed at the enzymatic level by using the tritium-

labeled estrone sulfate as substrate (Fig. 1F). It is noted that the hepatic expression of the transgene was comparable between the male and female TG mice (Fig. 1G).

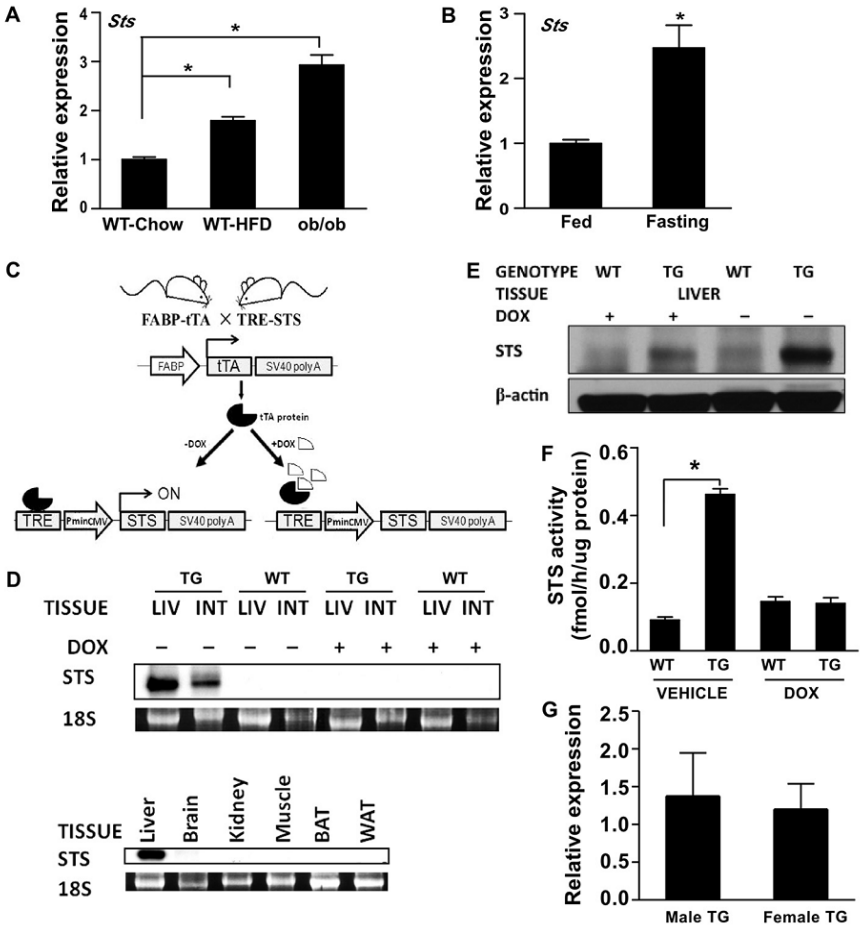

Figure 1. The hepatic expression of STS was induced in obese mice and by fasting and creation of transgenic mice expressing STS in the liver.

A, hepatic mRNA expression of mouse Sts in chow-diet fed WT mice, HFD-fed WT mice, and ob/ob mice. B, effect of 16-h fasting on hepatic Sts mRNA expression in chow diet-fed WT mice. C, schematic representation of the Tet-off STS transgenic system. FABP, fatty acid binding protein; tTA, tetracycline-controlled transactivator;SV40 poly(A), simian virus 40 polyadenylation signal; TRE, tetracycline-response element;Pmin CMV, minimal human cytomegalovirus promoter. D, mRNA expression of the transgene was determined by Northern blot analysis using the STS cDNA as the probe. The 18 S rRNA was used as a loading control. WT, WT mice; TG, STS transgenic mice; LIV, liver; INT, small intestine; BAT, brown adipose tissue. E, the expression of STS protein was measured by Western blotting. F, the hepatic STS enzymatic activity was determined by an estrone sulfate conversion assay and was normalized against protein concentrations. G, the hepatic expression of STS in female and male transgenic mice was measured by real-time PCR. $n \geq 4$ for each group. *, $p < 0.05$. *Error bars*, S.D.

2.3.2 Over-expression of STS ameliorated HFD-induced obesity, insulin resistance, and hepatic steatosis in female mice

When analyzing the metabolic phenotype, I found no significant changes in body weight, body composition (Fig. 2A), IPGTT and ITT (Fig. 2B). However, when challenged with HFD, the female STS mice gained less body weight compared to their WT counterparts (Fig. 2C). Body composition analysis showed that the reduced body weight gain in STS mice was mainly due to decreased fat mass, because the lean mass was comparable between the two genotypes (Fig. 2C). The serum level of leptin was decreased in STS mice (Table 1), consistent with their decreased adiposity. The body weight is maintained by balanced energy intake and energy expenditure. While the food intake was comparable between the two genotypes, the oxygen consumption and energy expenditure were significantly higher in STS mice (Fig. 2D).

Obesity is closely associated with insulin resistance. I went on to measure the insulin sensitivity in HFD-fed mice by IPGTT. The STS mice had lower glucose level after a 16-h fast, but their serum insulin level was comparable with that of the WT counterparts (Table 1), and they also showed improved IPGTT performance (Fig. 2E, left two panels). Interestingly, the STS mice and WT mice performed similarly in ITT (Fig. 2E, right two panels). The inhibition of hepatic glucose production by STS was recapitulated in isolated mouse primary hepatocytes, as the hepatocytes isolated from the STS mice showed blunted forskolin-induced glucose production (Fig. 2F). Hyperinsulinemic euglycemic clamp was then performed to further assess the effect of STS on hepatic and peripheral insulin sensitivity. The STS mice showed a 50% higher glucose infusion rate compared to WT mice during the clamp period (Fig. 2G, left panel), indicating better insulin sensitivity. The STS mice also displayed lower glucose production at

basal state, which may have accounted for the reduced fasting hyperglycemia. More dramatic suppression of hepatic glucose production in STS mice was observed during the insulin-stimulated clamp state (Fig. 2G, right panel). To gain insight into the improved insulin sensitivity in STS mice, the insulin-stimulated Akt phosphorylation was measured in three major metabolic tissues: liver, skeletal muscle and WAT. Increased Akt phosphorylation was observed in the liver, but not in the skeletal muscle and WAT (Fig. 2H), suggesting a liver-specific improvement of insulin sensitivity. The hepatic expression of the gluconeogenic enzymes glucose-6-phosphatase (*G6pase*) and phosphoenolpyruvate carboxykinase (*Pepck*) was suppressed in STS mice (Fig. 2I).

The HFD-fed STS mice also showed relief of hepatic steatosis, as evidenced by a reduced number and size of lipid droplets within the hepatocytes (Fig. 3A), as well as decreased liver triglyceride level (Fig. 3B). Interestingly, the expression of major genes responsible for lipogenesis (Fig. 3C) and fatty acid oxidation (Fig. 3D) was not different between WT and STS mice. The circulating levels of triglycerides and cholesterol were also not affected (Table 1). The hepatic fatty acid translocase (FAT/CD36), a fatty acid transporter responsible for the uptake of fatty acids from the circulation (17), was down-regulated in STS mice (Fig. 3E). Sustained liver steatosis is known to be associated with inflammation. Consistent with their relief of steatosis, the STS mice showed decreased hepatic expression of the inflammatory marker genes, such as the *Tnfα* and monocyte chemoattractant protein-1 (*Mcp-1*) (Fig. 3F). The suppression of inflammatory gene expression was liver-specific, because the expression of *Tnfα* and *Mcp-1* was not affected in the WAT and skeletal muscle of the STS mice (Fig. 3F). The metabolic benefit in

HDF-fed female STS mice was achieved without affecting the pancreatic islet morphology and area (Fig. 3G).

The metabolic benefit was transgene-dependent, because silencing the transgene by treating the STS mice with DOX normalized the body weight and body composition (Fig. 4A), energy expenditure (Fig. 4B), IPGTT performance (Fig. 4C), gluconeogenic gene expression (Fig. 4D), and inflammation marker gene expression (Fig. 4E).

TABLE 1. Metabolic profile of HFD-fed WT and STS transgenic female mice

	WT	TG
Serum leptin (ng/mL)	29.56±0.36	22.76 ±2.96*
Fasting glucose (mg/dL)	153.3±14.67	112.4±7.05*
Fasting insulin (ng/mL)	2.1±0.52	1.69±0.36
Serum triglyceride (mg/dL)	104.5±7.65	99.99±4.88
Serum total cholesterol (mg/dL)	125.1±8.21	116.8±5.06

*$P < 0.05$.

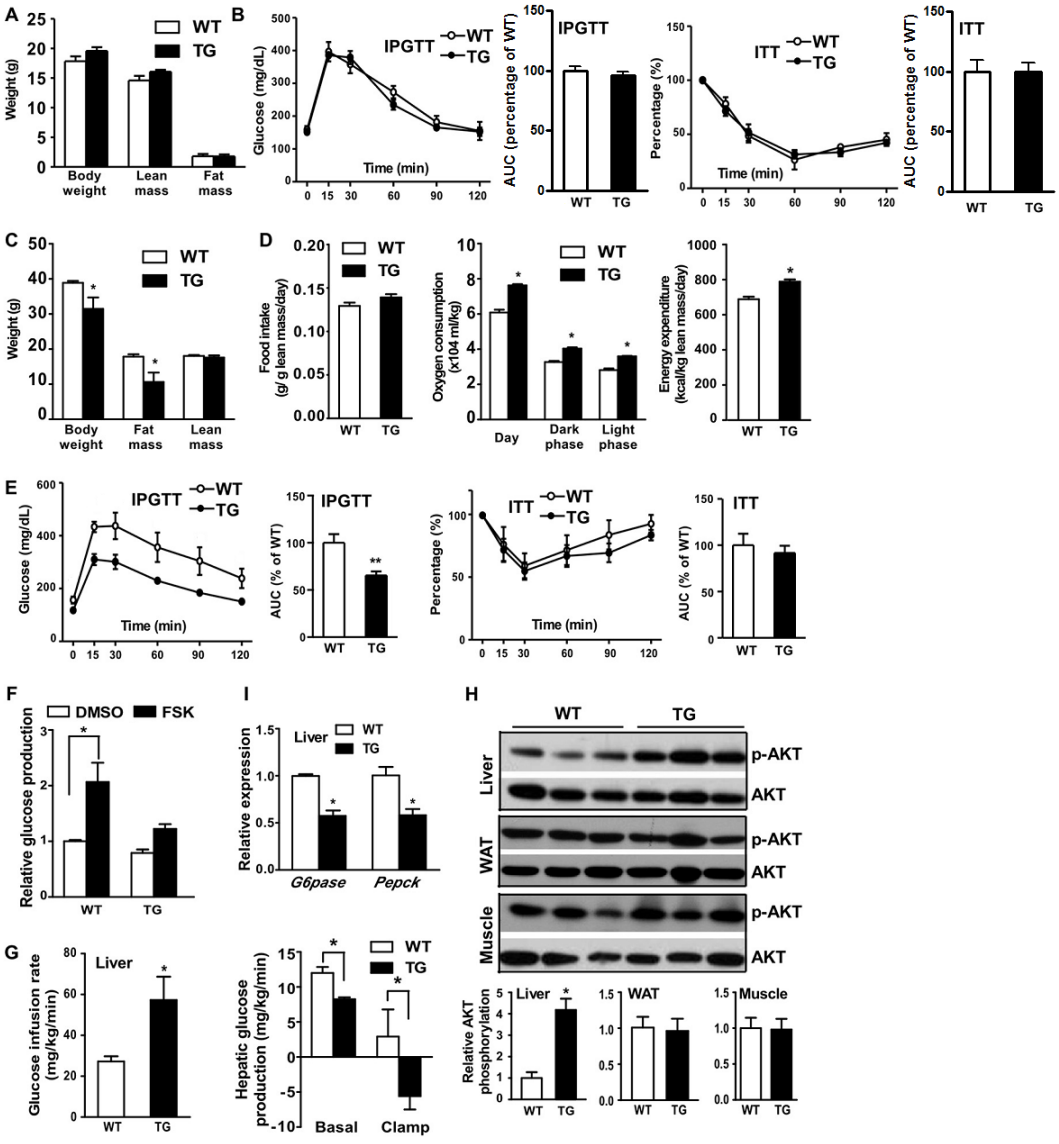

Figure 2. Over-expression of STS alleviated HFD-induced obesity and improved insulin sensitivity.

All mice are female. A and B, mice maintained on a chow diet were analyzed for body weight and body composition (A) and IPGTT and ITT (B).C–H, mice were fed with HFD for 20 weeks before analysis. C–E, body weight and body composition (C); food intake, oxygen consumption, and energy expenditure (D); and IPGTT and ITT (E) of HFD-fed mice. The quantifications of the IPGTT and ITT results are shown as the area under the curve. F, primary hepatocytes were measured for forskolin (FSK)-stimulated glucose production. G, glucose infusion rate (left) and hepatic glucose production (right).H, Western blot analysis of insulin-stimulated Akt phosphorylation in the liver, WAT, and skeletal muscle at the completion of euglycemic-hyperinsulinemic clamp. Arbitrary units in the bottom panels represent the ratio of phosphorylated Akt (p-AKT) to total Akt. I, hepatic expression of gluconeogenic enzyme genes was measured by real-time PCR. $n \geq 4$ for each group. *, $p < 0.05$.; **, $p < 0.01$, TG versus WT, or the comparisons are labeled. *Error bars*, S.D.

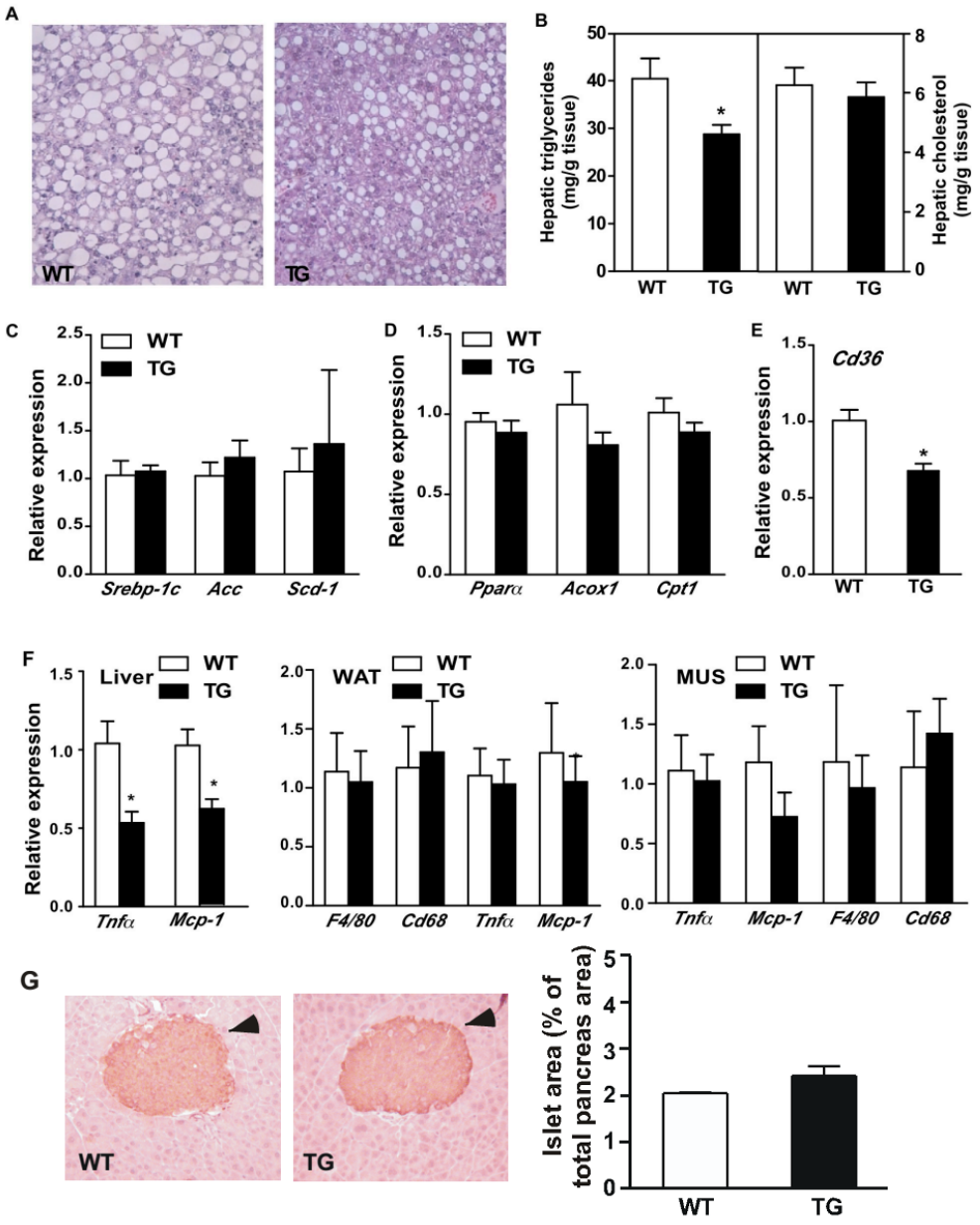

Figure 3. Over-expression of STS alleviated HFD-induced hepatic steatosis and inflammation.

Mice are the same as those shown in Fig. 2, C–H. A, histological analysis of liver sections by H&E staining. B, hepatic lipid levels. C–E, the hepatic expression of genes responsible for lipogenesis (C), fatty acid oxidation (D), and fatty acid uptake (E) in the liver was measured by real-time PCR. F, the expression of pro-inflammatory genes and/or macrophage marker genes in the liver (left), WAT (middle), and skeletal muscle (right) was measured by real-time PCR.G, immunostaining of insulin and quantification of total islet area. Arrowheads indicate islets. $n \geq 4$ for each group. *, $p < 0.05$, TG versus WT. *Error bars*, S.D.

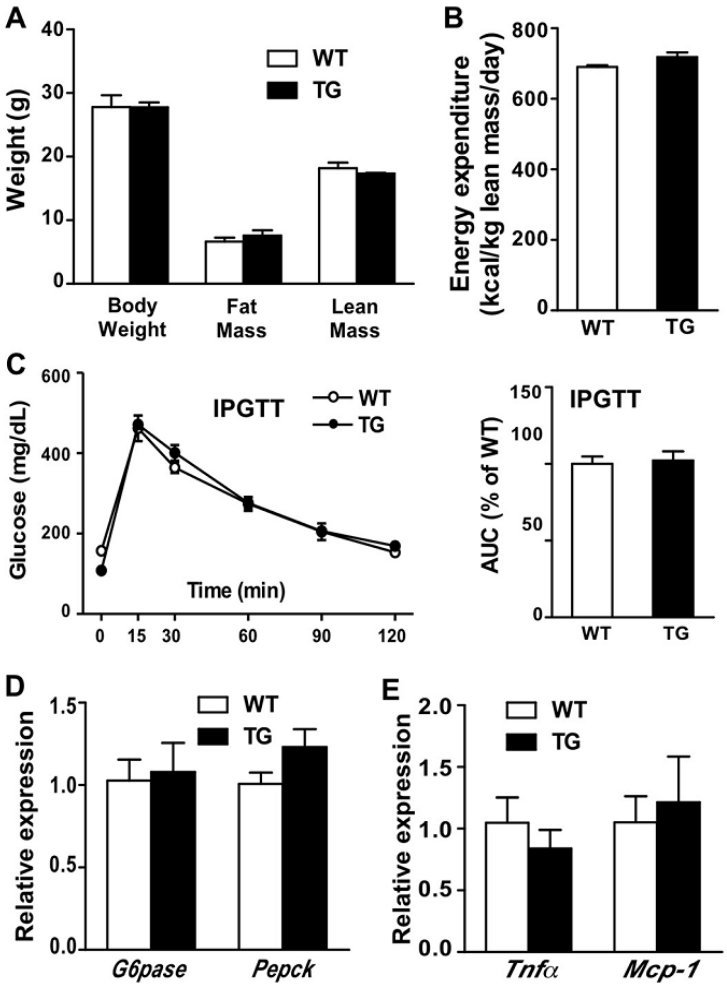

Figure 4. Silencing the transgene expression abolished the metabolic benefit.

A–E, body weight and body composition (A), energy expenditure (B), IPGTT (C), and the expression of hepatic gluconeogenic genes (D) and inflammation marker genes (E) in doxycycline-treated and HFD-fed female WT and STS mice. $n \geq 4$ for each group. *Error bars*, S.D.

2.3.3 The metabolic benefit in female STS mice was estrogen-dependent

Estrogen sulfates are the preferred STS substrates existing in high concentrations *in vivo* (*1, 134*). Because estrogens are known for their activity in improving metabolic functions (*71*), I hypothesized that the STS transgene may have exerted its metabolic benefit by converting estrogen sulfates to active estrogens and enhancing estrogen signaling in the liver. Indeed, the

STS mice showed increased and decreased liver concentrations of estrone and estrone sulfate, respectively (Fig. 5A). The enhanced hepatic estrogen activity in STS mice was also supported by the observation that in the presence or absence of estrone sulfate treatment, the activity of an estrogen-responsive luciferase reporter gene tk-ERE-Luc was increased when transfected into the mouse liver (Fig. 5B). Despite the increased hepatic estrogen activity, neither the serum estradiol concentration nor the length of the estrous cycle was affected in STS mice (Fig. 5C). These results suggested the liver-specific increase of estrogen activity in STS mice.

To determine whether the metabolic benefit was mediated by estrogens, I eliminated the primary source of estrogens from 5-week-old pre-pubertal female mice by ovariectomy before challenging them with HFD. Ovariectomy completely abolished the metabolic benefit of the STS transgene in body weight and body composition (Fig. 5D), IPGTT performance (Fig. 5E) and hepatic expression of gluconeogenic genes, inflammatory marker genes, and *Cd36* (Fig. 5F). These results suggested that the metabolic benefit in female STS mice was estrogen-dependent.

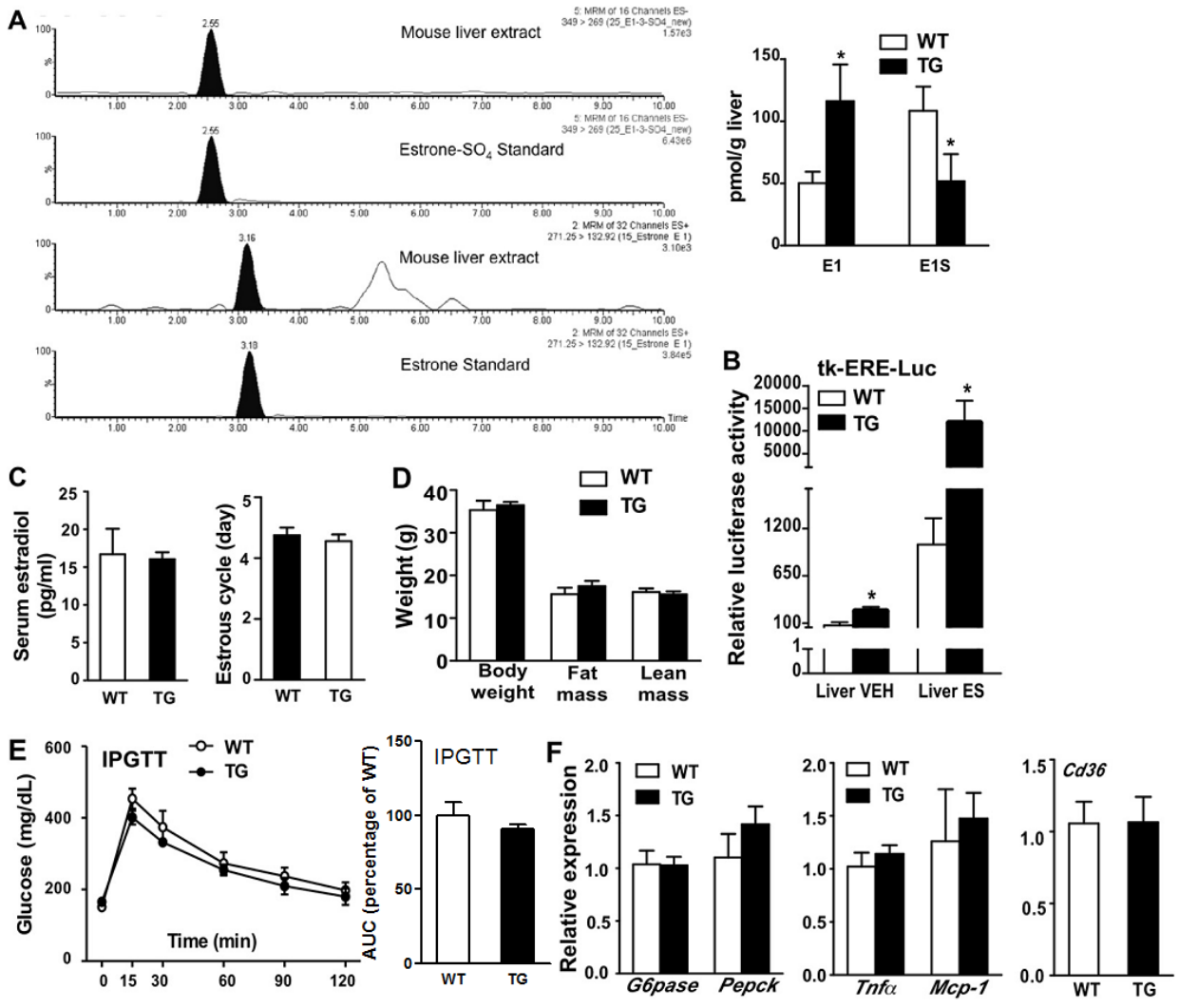

Figure 5. The metabolic benefit in female STS mice was estrogen-dependent.

All mice are female. A, increased hepatic estrogen levels in HFD-fed female STS transgenic mice. The estrogen and estrogen sulfate were extracted from mouse liver and analyzed by the LC-MS method. Shown are the chromatographs of the estrone and estrone sulfate standards and the mouse liver extracts (left) and quantification of the results (right). The data were normalized against liver weight. E1, estrone; E1S, estrone sulfate. B, enhanced hepatic estrogen activity in female STS mice treated with vehicle (VEH) or estrogen sulfate (ES), as shown by a liver-specific transfection of the estrogen-responsive luciferase reporter gene tk-ERE-Luc. The data were expressed as the percentage of VEH-treated WT mice. C, serum estradiol levels (left) and estrus cycle length (right) were measured in intact mice. The length of vaginal estrus cycle was determined by vaginal smears. D–F, body weight and body composition (D), IPGTT (E), and the expression of hepatic gluconeogenic genes (F, left), inflammation markers (F, middle) and fatty acid transporter (F, right) in ovariectomized mice fed with HFD for 20 weeks. $n \geq 4$ for each group. *, $p < 0.05$, TG versus WT. *Error bars*, S.D.

2.3.4 Over-expression of STS improved metabolic function in ob/ob mice

The leptin-deficient ob/ob mice represent a genetic model of obesity and type 2 diabetes. To determine the metabolic effect of STS on this model, I bred the STS transgene into the ob/ob background. The resulting ob/ob-STS transgenic mice were termed obS mice. Both ob/ob and obS mice were maintained on chow diet. Although they were not less obese, the female obS mice showed an improved body composition by having increased lean mass (Fig. 6A), improved IPGTT performance (Fig. 6B), and suppression of gluconeogenic (Fig. 6C) and pro-inflammatory (Fig. 6D) gene expression in the liver. The obS mice also exhibited ameliorated liver steatosis (Fig. 6E) without affecting the expression of genes involved in lipogenesis and fatty acid oxidation (Fig. 6F). The serum levels of triglyceride and cholesterol were not affected (data not shown). The morphology and area of pancreatic islets were indistinguishable between the ob/ob and obS mice (Fig. 6G). These phenotypes were consistent with those observed in the HFD model.

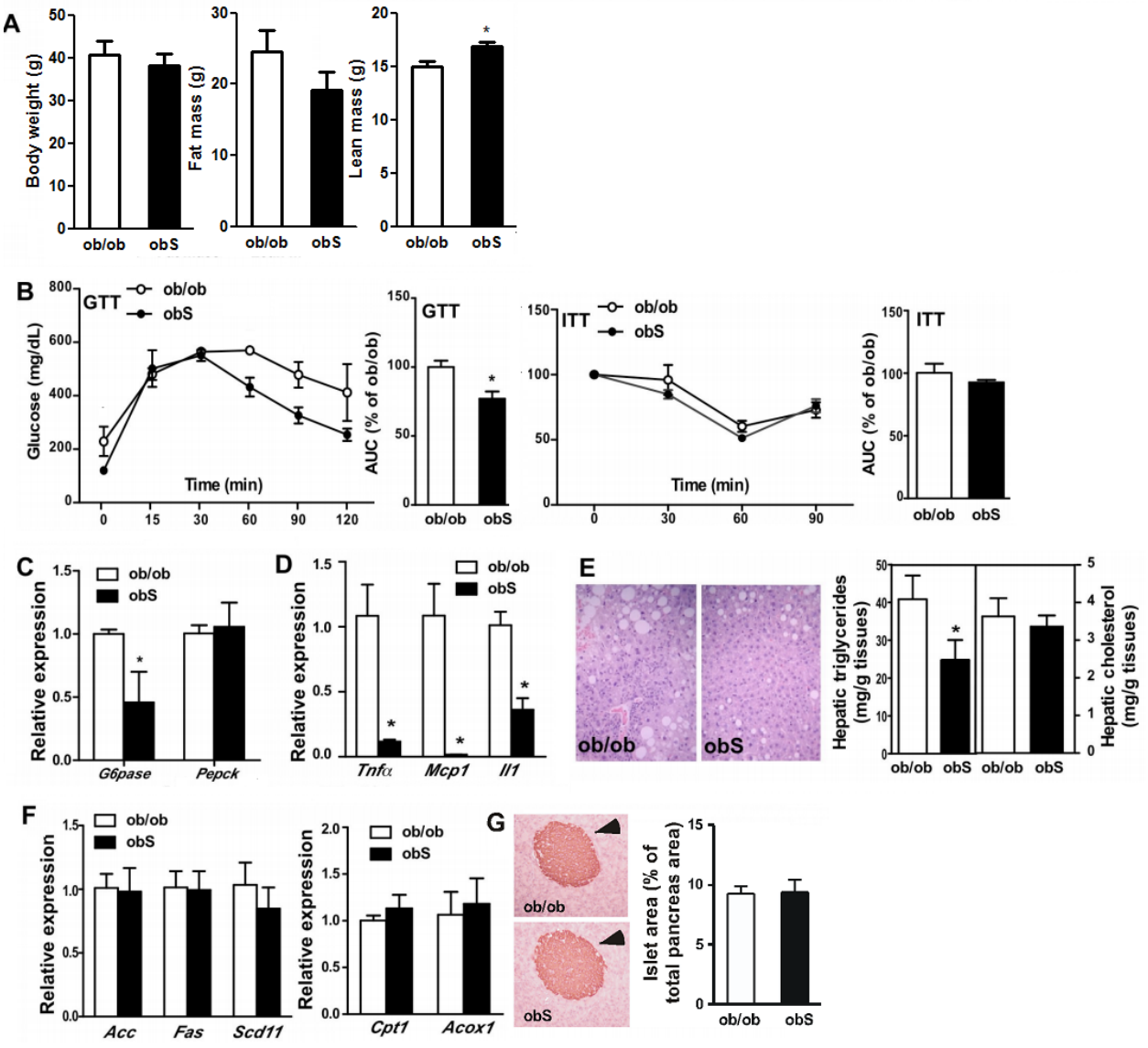

Figure 6. Over-expression of STS improved metabolic function in ob/ob mice.

The female ob/ob and obS mice were maintained on a chow diet. A–D, body weight and body composition (A), IPGTT and ITT (B), and the expression of hepatic gluconeogenic genes (C) and proinflammatory marker genes (D). The quantifications of the IPGTT and ITT results are shown as the area under the curve. E, histological analysis of liver sections by H&E staining (left) and measurement of hepatic triglyceride and cholesterol levels (right).F, expression of genes responsible for hepatic lipogenesis (left) and fatty acid oxidation (right), as determined by real-time PCR.G, immunostaining of insulin and quantification of total islet area. Arrowheads, islets. $n \geq 4$ for each group. *, $p < 0.05$, obS versus ob/ob. *Error bars*, S.D.

2.3.5 Treatment with estrogen sulfate improved insulin sensitivity in ob/ob and HFD models

Estrogen administration has been reported to improve insulin sensitivity in both ob/ob mice and HFD-fed mice (*90, 135*). The induction of STS in the ob/ob and HFD models (Fig. 1A) led to the hypothesis that administration of estrone sulfate in these models may result in the same estrogenic benefit-due to the increased STS-mediated conversion of estrone sulfates to estrogens in the liver. As shown in Fig. 7A, administration of estrone sulfate gradually reduced the fed glucose levels in ob/ob mice and maximized its glucose-lowering effect by day 5. The estrone sulfate-treated mice also showed improved IPGTT performance in both the ob/ob (Fig. 7B, top two panels) and HFD (Fig. 7C, left two panels) models. Interesting, the estrone sulfate treatment did not improve ITT performance in ob/ob mice (Fig. 7B, bottom two panels), but did so in the HFD model (Fig. 7C, right two panels).

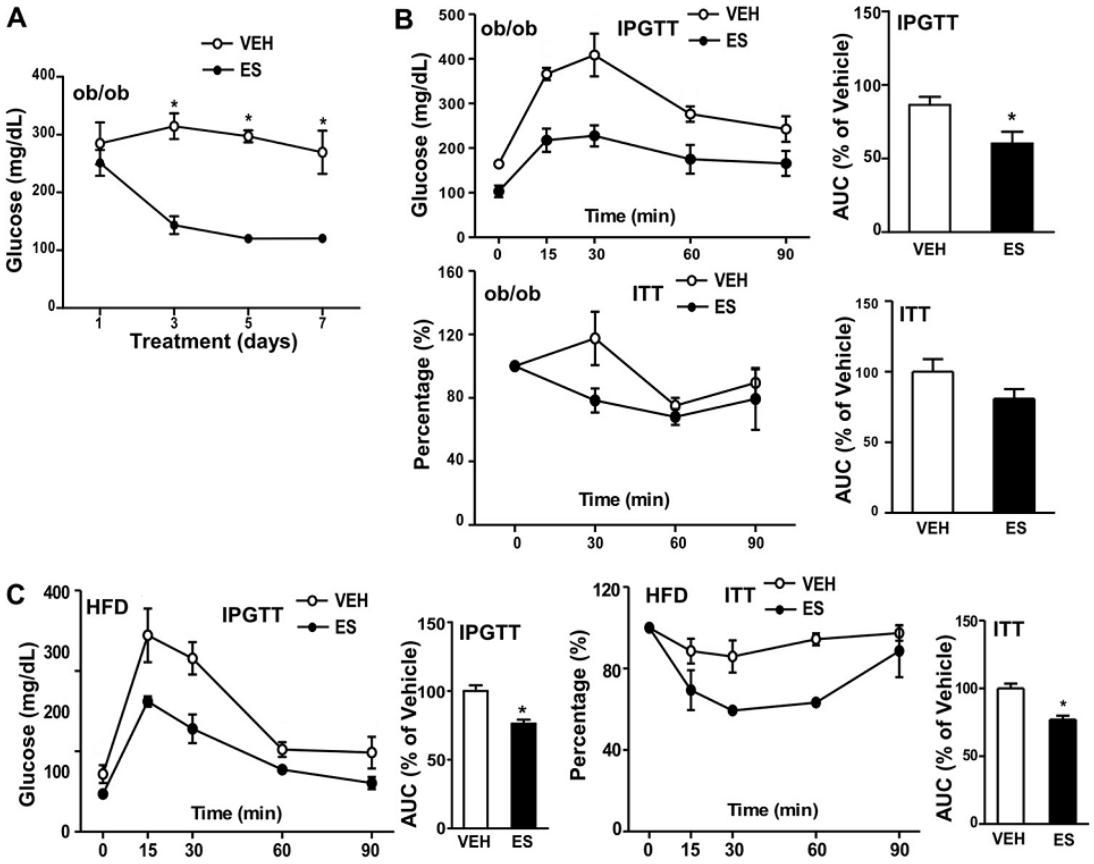

Figure 7. Estrogen sulfate improved insulin sensitivity in ob/ob mice and HFD-fed mice.

A and B, female ob/ob mice were maintained on a chow diet. Shown are fed glucose levels (A) and IPGTT and ITT (B) of vehicle (VEH) or estrone sulfate (ES)-treated ob/ob mice. C, WT female mice were fed with HFD for 16 weeks. Shown are IPGTT and ITT of mice treated with VEH or ES. The quantifications of the IPGTT and ITT results are shown as the area under the curve. $n \geq 4$ for each group. *, $p < 0.05$, ES versus VEH. *Error bars*, S.D.

2.3.6 Over-expression of STS improved metabolic function in HFD-fed male mice with distinct mechanisms

Unlike their female counterparts, the male STS mice showed no increased activation of the tk-ERE-Luc reporter gene when transfected into their livers (Fig. 8A). However, the STS males also showed protection from the HFD challenge. Although their body weight was not affected, the HFD-fed STS males exhibited reduced fat mass and increased lean mass (Fig. 8B).

In addition, the HFD-fed STS males showed increased oxygen consumption and energy expenditure (Fig. 8C), improved IPGTT performance but unchanged ITT performance (Fig. 8D), and suppression of the gluconeogenic genes and pro-inflammatory genes (Fig. 8E) in their livers. The HFD-fed STS males also showed inhibition of hepatic steatosis, as revealed by liver histology and hepatic triglyceride levels (data not shown). These results were in general agreement with those observed in the HFD-fed STS females. The improvement of IPGTT performance and inhibition of gluconeogenic gene expression (data not shown) were retained in HFD-fed STS males following castration, suggesting the sex hormones are dispensable for the metabolic benefit of STS in males.

In understanding the mechanism of metabolic benefit in STS males, I found that the WAT of STS males showed fewer signs of inflammation, including reduced crown-like structures, and decreased expression of macrophage and inflammation marker genes (Fig. 8F). The inhibition of WAT inflammation was not seen in STS females (Fig. 3F). In addition, I observed decreased expression of macrophage and inflammation marker genes, increased expression of the glucose transporter *Glut4,* and increased expression of genes responsible for mitochondrial biogenesis and fatty acid oxidation in the skeletal muscle of STS males (Fig. 8G), which was also absent in STS females (Fig. 3F) (data not shown). The transgene had little effect on the area of pancreatic islets either in the HFD model (Fig. 8H, left panel) or the obS model (Fig. 8H, right panel).

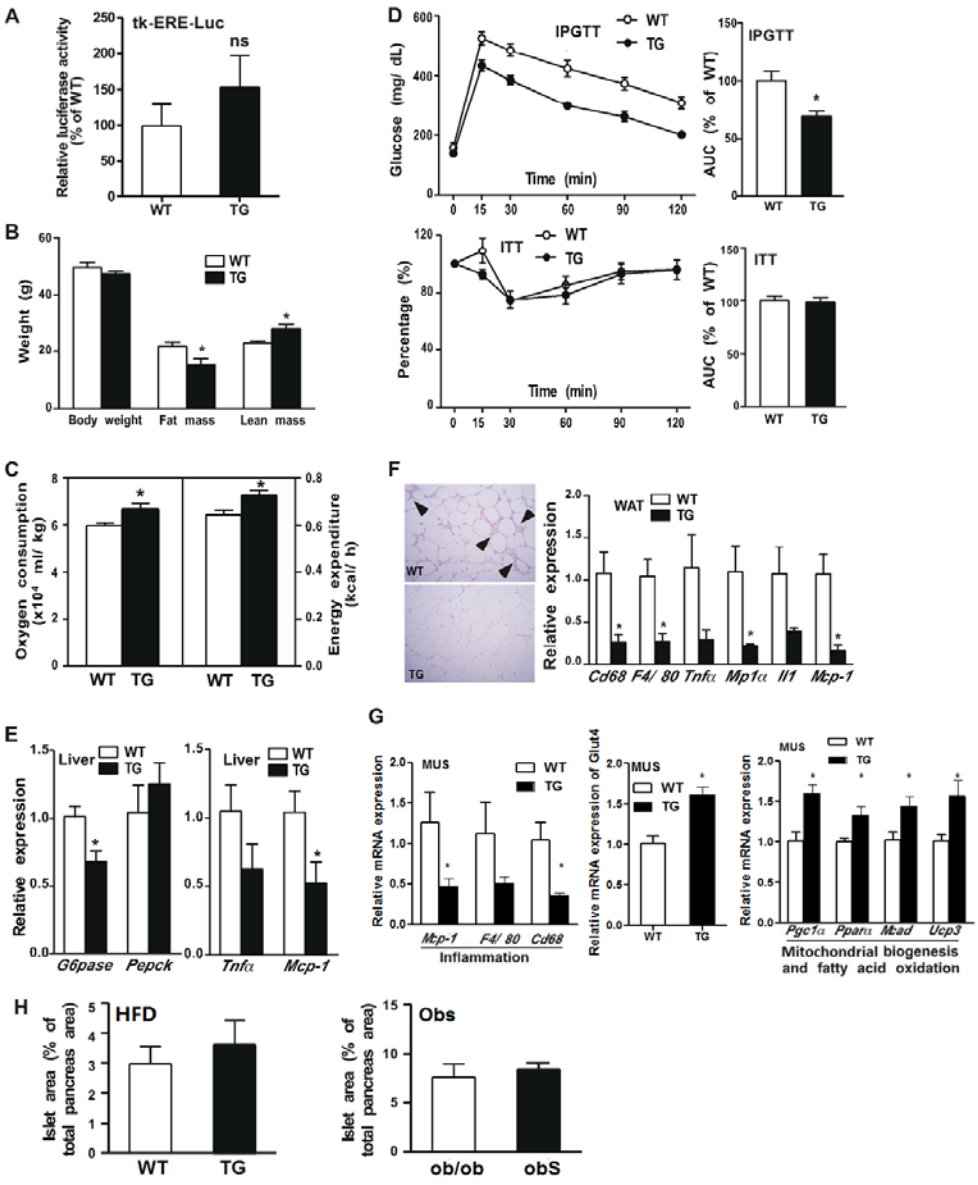

Figure 8. Over-expression of STS improved metabolic functions in male mice via distinct mechanisms.

All mice are male. A, lack of change in hepatic estrogen activity in male STS mice as shown by a liver-specific transfection of the estrogen-responsive luciferase reporter gene tk-ERE-Luc. B–E, mice were fed with HFD for 20 weeks before analysis. Body weight and body composition (B), oxygen consumption and energy expenditure (C), IPGTT and ITT (D), and expression of hepatic gluconeogenic genes (E, left) and inflammatory marker genes (E, right). F, H&E staining (left) and expression of macrophage and inflammatory marker genes (right) in abdomen adipose tissue. Arrowheads(left), crown-like structures. G, gene expression in the skeletal muscle as measured by real-time PCR. $n \geq 4$ for each group. H, quantification of total islet area based on insulin immunostaining. *, $p < 0.05$. *Error bars*, S.D.

2.4 DISCUSSION

This study reported the induction of hepatic STS in mouse models of obesity and insulin resistance, as well as during the transition from fed to fasting. The induction of STS may represent a protective response against metabolic stress because over-expression of STS in the liver improved metabolic function in both the HFD and ob/ob models of obesity and type 2 diabetes.

The metabolic benefits of the STS transgene in female mice may have resulted from enhanced hepatic estrogen production as evidenced by the increased estrogen level and activity in the livers of STS mice, whereas ovariectomy abolished the protective effect of STS. The sulfation-desulfation pathways are important to maintain the local estrogen homeostasis. Under normal conditions, the mouse liver has a low basal expression of EST, but a substantial constitutive expression of STS, suggesting a dominant estrogenic activity is required to maintain hepatic estrogen levels and normal physiology. However, in type 2 diabetes, EST is dramatically and specifically induced in the liver (*136*), which may have increased estrogen deprivation and contributed to the development of insulin resistance (Fig.15). In the transgenic mice, over-expression of STS may have regenerated active estrogens, compensated for reduced estrogen signaling, and thus protected mice from insulin resistance.

Although estrogens are known for their activities in preventing metabolic syndrome, their effector tissues or cell types remain to be clearly defined. It has been suggested that estrogen signaling in the central nervous system controls food intake, energy metabolism, and

body weight homeostasis (*82*). In the pancreas, estrogen actions protect β cell from death and promote insulin biosynthesis and secretion (*119*). Estrogen decreases lipogenesis and adipogenesis in adipose tissue, and enhances fatty acid oxidation in skeletal muscle. Estrogen also reduces inflammation in the muscle and adipose tissue, which altogether improves insulin sensitivity in these peripheral tissues (*71*). My results provided direct evidence that a liver-specific enhancement of estrogen activity was sufficient to confer the metabolic benefit. The enhanced estrogen activity in STS mice was likely localized to the liver, because the circulating levels of estradiol were similar between WT and STS mice, and the increased insulin-stimulated Akt phosphorylation in STS mice was also liver-specific. The confinement of insulin sensitization to the liver may also explain why improved IPGTT performance, but not ITT performance, was observed in HFD-fed STS mice and chow-fed obS mice.

A systemic treatment with estrogens has been limited by their potential side effects (*81*). My results raised the promise of using liver-specific activation of estrogen signaling to harness the metabolic benefit of estrogens and avoid unwanted side effects. It is encouraging that targeted estrogen delivery has been successfully explored. In one such example, targeted estrogen delivery was achieved by fusing the glucagon-like peptide-1 with estrogen, which improved glucose and lipid metabolism without inducing endocrine toxicity and oncogenicity (*137*). In addition to direct use of estrogens, my results suggested that the delivery of the hormonally inactive estrogen sulfate may be a practical approach to enhance estrogen activity in tissues bearing a high basal or inducible expression of STS, such as the liver.

Having demonstrated the sex-specific effect of *Est* ablation on glucose homeostasis (*122*), it was surprising to note that over-expression of STS improved glucose homeostasis in both male and female mice. EST is expressed in both the liver and adipose tissue. The anti-diabetic and pro-diabetic effect of *Est* ablation in females and males may be due to the loss of *Est* in the liver and adipose tissue, respectively (*122*). In the current study, the STS transgene was expressed in the liver but not in the adipose tissue, which may explain the consistent benefit in both sexes. Interestingly, the mechanism for the protective effect of STS appeared to be sex-specific. The protection in female mice was estrogen-dependent, but the protection in males may be independent of sex hormones, because the hepatic estrogen activity did not increase, and castration failed to abolish the protective effect in male STS mice. Instead, the metabolic benefit in males might have been accounted for by the male-specific inhibition of inflammation in the adipose tissue and skeletal muscle, as well as a pattern of skeletal muscle gene expression that favors energy expenditure. Another notable difference between the STS transgenic and *Est* null model is their impact on the pancreatic islets and β-cell mass. *Est* ablation caused a male-specific loss of β-cell mass in the ob/ob background, which was reasoned to be due to the WAT inflammation (15). In contrast, the STS transgene had little effect on the pancreatic islets either in the HFD model or ob/ob model regardless of the sex of the mice.

The STS transgene was not targeted to the adipose tissue and skeletal muscle, so I cannot exclude the possibility that the metabolic benefit of STS in these two tissues was secondary. Indeed, I found that the serum concentration of DHEA, which can be converted from DHEA sulfate by STS, was increased in male STS mice (data not shown). DHEA is known to

have multiple beneficial effects on the liver and extra-hepatic tissues in preventing the progression of metabolic syndrome (*138*).

The X-linked ichthyosis is resulted from inactivation of STS by complete deletion or point mutations (*2*). STS polymorphisms have been associated with vulnerability to attention-deficit/hyperactivity disorder (*65, 67*). It would be interesting to know whether the polymorphisms of STS are associated with altered estrogen signaling and susceptibility to obesity and insulin resistance in human populations. In addition, several STS inhibitors are being developed for the treatment of breast cancer in postmenopausal women (*46, 139*). It is imperative to evaluate the effect of STS inhibitors on glucose and energy metabolism in order to avoid their potential side effects.

The 60 kcal% HFD I used is a widely used research diet to induce obesity and type 2 diabetes in rodents (*140*). However, I understand that the 60 kcal% HFD can be toxic to non-adipose tissues including the pancreatic beta cells (*141*). A future use of HFD with lower fat content is necessary to exclude the possibility that the toxicity of the 60 kcal% HFD may have impacted the phenotypic exhibition or interpretation.

In summary, the current study has uncovered a novel function of STS in regulating energy metabolism and improving insulin sensitivity through sex-specific mechanisms. The liver-specific activation of the estrogen signaling pathway was sufficient to confer metabolic benefit. I proposed that liver-specific STS induction or estrogen/estrogen sulfate delivery may represent novel approaches to manage metabolic syndrome.

3.0 CHAPTER III: ACTIVATION OF A NEGATIVE FEEDBACK LOOP INVOLVING INFLAMMATION AND STS-MEDIATED ESTROGEN HOMEOSTASIS

3.1 ESTROGEN SIGNALING IN INFLAMMATION

Abnormal estrogen metabolism in liver disease has been identified for decades (*142*). Concomitants of liver diseases are clinical signs and symptoms like palmar erythema, spider nevus(*143*), gynecomastia and infertility due to disturbed homeostasis of endocrine steroid hormones, especially estrogen. Ample studies have reported increased estrogen levels, reduced estrogen metabolites levels and signs of endocrine disturbance in patients with chronic liver disease, such as liver cirrhosis, hepatocellular carcinoma (HCC), or alcoholic liver disease. (*144-149*). The hormone levels are associated with the severity of liver disease (*150*) while treating patients towards improved liver function resulted in regression of the signs of endocrine disturbance (*151*). Liver is the primary site of estrogen metabolism through the phase I oxidation reaction, which is mainly catalyzed by CYP1A2 and CYP3A4, and the phase II conjugation reaction mainly mediated by EST. It is believed that damage to the liver impairs its capacity to metabolize estrogen, resulting in increased estrogen levels in the circulation (*152*). However,

there are reports that changes in steroid hormone levels may occur before severe liver dysfunction has appeared (*153*). Whether the estrogen levels are increased through other mechanisms than pure liver damage is not known.

EST and STS catalyze the sulfation-desulfation pathway of estrogen, respectively, which is a reversible process of metabolic conjugation and dissociation, rather than destruction of estrogen. The sulfation pathway, rather than the glucuronidation pathway, seemed to be specifically affected in chronic inflammatory liver diseases, since the level of major estrogen glucuronides was not changed (*154*). STS gene deletion or mutation is often associated with reproductive manifestations, such as cryptorchidism in males and failed labor progression in females, which may be due to disrupted steroid hormone homeostasis (*121*). STS is induced in malignant breast tissues compared with nonmalignant tissues(*17-18, 44-45*). Cytokines have been suggested to regulate the expression and activity of STS, although with very different results. IL-1 decreased the expression and activity of STS in endometrial stromal cells(*21*). In contrast, IL-6 and TNFα increased STS activity in breast cancer cells, but probably due to post-translational mechanisms (*20, 155*). Therefore, little is known about the transcriptional regulation of STS, especially in the major estrogen-metabolizing organ: liver.

The development of many chronic inflammatory liver diseases are more common in men than in women (*156*). The prognosis of female HCC patients is also much better than male patients (*157*). Although the liver is not a classic target organ of sex steroid hormones, it has been shown to express functional ER and regulate liver functions in response to estrogen. Therefore, estrogen may influence the progression from hepatitis to cirrhosis and HCC (*158*).

In this study, I provide evidence that STS is a novel target gene of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which is induced in clinical liver specimens from patients with chronic inflammatory diseases. Using human hepatocytes, I tested the hypothesis that a negative feedback loop exists in which induction of STS upon NF-κB activation increases active estrogen levels and in turn inhibits NF-κB-mediated inflammation.

3.2 METHODS

3.2.1 Bioinformatic analysis

The Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) database (*159*) was mined for STS expression in chronic inflammatory liver disease. The dataset (GSE28619) containing 15 samples from alcoholic hepatitis patients and 7 control liver samples were analyzed on Affymetrix Human Genome U133 Plus 2.0 microarrays.

3.2.2 Immunohistochemical analysis, western blotting and RT-PCR analysis

For immunohistochemistry (IHC) analysis, commercially available tissue microarray (TMA) slides of human liver diseases (LV1201, US Biomax, Inc., Rockville, MD) were used. They were provided in single core per case with clinical information of sex, age, and pathological diagnosis. 1 out of 30 cases of cirrhosis was excluded because of lack of

hepatocytes. IHC staining of the TMA blocks was performed using monoclonal anti-STS antibody (Abcam, dilution 1:50) and heat-induced antigen-retrieval procedures. The stained slides were evaluated by a surgical pathologist (Y.G.) in a blinded fashion. Liver samples were scored according to staining intensity as weakly positive when a faint brown staining was detectable and as strongly positive when a dark brown staining was detectable using the l00x magnification. For western blot analysis, the anti-STS antibody was used with 1:200 dilution. For RT-PCR analysis, total RNA was isolated using TRIZOL reagent from Invitrogen. cDNA was synthesized by reverse transcription with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real-time PCR was performed with ABI 7300 real-time PCR system. Data were normalized against GAPDH.

3.2.3 Cell culture and drug treatment

Human livers were obtained through the Liver Tissue Procurement and Distribution System, and human primary hepatocytes were isolated by three-step collagenase perfusion as described before (*160*). Primary hepatocytes were maintained in hepatocyte maintenance medium from Cambrex. The hepatoma cell line Huh7 and HEK 293 cell line were originally obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Invitrogen Corp.), 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Life Technologies, Invitrogen) at 37 ℃. Chemicals used in this study include: Estrone sulfate (Cat #E0251; Sigma), estradiol sulfate (cat # E9505; Sigma), STX64 (cat # S1950; Sigma), Fulvestrant (ICI 182,780) (cat# I4409; Sigma), LPS 0111:B4 (cat # L4391; Sigma), Recombinant human TNF-alpha (cat # 210-TA; R&D

Systems), Phorbol 12-myristate 13-acetate (PMA) (cat# P8139, Sigma) and pyrrolidine dithiocarbamate (PDTC) (cat# P8765, Sigma). For PDTC treatment, cells were pretreated with PDTC for 30 min prior to the treatment of NF-κB activators.

3.2.4 STS activity assay

The hepatic STS enzymatic activity was determined from cell microsomes by an estrone sulfate conversion assay as described before(*161*). The enzyme activity was normalized against protein concentrations.

3.2.5 Measurement of estrogen levels in cell culture medium

For estrogen measurement, cells were cultured in phenol red free DMEM and dextran-coated charcoal (DCC)-stripped FBS. Cell culture medium was removed and centrifuged at 15,000g for 10 min. The estradiol content of the supernatant was measured by an ELISA assay kit (Cat # 582251; Cayman Chemicals). Estrogen concentration was normalized by cellular protein concentration.

3.2.6 Plasmid construction, transient transfections, and luciferase assay

The 5′-regulatory sequences of human STS promoter was analyzed for potential NF-κB binding sites by using JASPAR matrices(*162*). The wild-type luciferase reporter (nt −447 to +150) was generated by PCR amplification using a template of human genomic DNA kindly provided by Dr. Jiang Li. The following primers were used for PCR: forward,

5’- GGGGTACCCTCTGCAGTTAGCAAACC-3’ and reverse, 5’-GAAGATCTCTCTGTTCATGGTTTCTGCA-3’. The PCR product was digested with Kpn1 and BglII and inserted into the same enzyme-digested pGL3-basic luciferase reporter plasmid (Promega, Madison, WI). The shortened luciferase reporter (nt −56 to +150) was generated using the following primers: forward, 5’-GGGGTACCAAAAGAATGCAGCAACTA-3’ and reverse, 5’-GAAGATCTCTCTGTTCATGGTTTCTGCA-3’. Deletion mutants of NF-κB sites within the STS reporter plasmids were generated by PCR-mediated mutagenesis using the following primers: NF-κB-1 mutation forward, 5’-TCACCTGAAACTGAGAAAAGAATGCAGCAA-3’ and reverse, 5’-CTTTTCTCAGTTTCAGGTGATCCTGGTAAA-3’; NF-κB-2 mutation forward, 5’-AGGGTTGAAAATAGATTAAACTCCCGTTCA-3’ and reverse, 5’-TTTAATCTATTTTCAACCCTCTCTTATACCT-3’; NF-κB-3 mutation forward, 5’-TCACCTGAAACTGAGAAAAGAATGCAGCAA-3’ and reverse, 5’-CTTTTCTCAGTTTCAGGTGATCCTGGTAAA-3’. All mutated plasmids were confirmed by sequencing (model 373XL sequencer, ABI). HEK 293 cells were transfected with a luciferase reporter constructs and expression vectors in 48-well plates using polyethyleneimine polymer (kindly provided by Dr. Xiang Gao). When necessary, cells were stimulated with drugs for 24 hours before the luciferase assay. For experiments in which estrogen and estrogen sulfate were added or estrogen signaling was evaluated, the cells were cultured in phenol red free DMEM and DCC stripped FBS. Relative reporter activity was calculated by comparing with empty vector-transfected or vehicle-treated cells. All transfections were performed in triplicate. The human STS siRNA (J-009602-09) and control scrambled siRNA (D-001810-10) were purchased from Dharmacon Research. Transfection of siRNA was performed using DharmaFECT transfection

reagent 4 (Dharmacon) according to the manufacturer's protocols. After siRNA transfection, the cells were collected at 24 hours for mRNA analysis, and at 48 hours for protein analysis.

3.2.7 Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis

EMSA was performed using an *in vitro* translated p65 protein prepared from T7 Quick Coupled Transcription/Translation System (Promega) and [^{32}P]-labeled DNA probes. The EMSA oligonucleotide sequences are as follows: WT NF-κB-1 forward, 5'-CGAAACCTGGGCTTTCCAAAA-3' and reverse, 5'-CGATTTTGGAAAGCCCAGGTT-3'; NF-κB-1 mutant forward, 5'- CGAAACCTTCGCTTTCC AAAA-3' and reverse, 5'-CGATTTTGGAAAGCGAAGGTT-3'; WT NF-κB-2 forward, 5'- CGAGAGGGGGGAAAATCCCTTAA-3' and reverse, 5'-CGATTAAGGGATTTTCCCCCCTC-3'; NF-κB-2 mutant forward, 5'-CGAGAGGGTTGAAAATAGATTAA-3' and reverse, 5'-CGATTAATCTATTTTCAACCCTC-3'; WT NF-κB-3 forward, 5'-CGACACCTGAAACTCCCAAAA-3' and reverse, 5'-CGATTTTGGGAGTTTCAGGTG-3'; NF-κB-3 mutant forward, 5'-CGACACCTGAAACTGAGAAAA-3' and reverse, 5'-CGATTTTCTCAGTTTCAGGTG-3'. The oligonucleotide was annealed and end-labeled with ^{32}P. The binding reaction mixture consists of 2 μL of radioactive probe, 4 μL of p65 protein, 1 μL of poly(dI-dC)(Cat # P4929; Sigma), 4 μL of 5*binding buffer and sterile water to bring the total volume up to 20 μL. For competition assay, a 200-fold excess of unlabeled oligonucleotide was added to the reactions. For supershift assays, the binding reaction mixture

was pre-incubated with 1 μL of anti-p65 antibody (D14E12; Cell Signaling Technology) for 10 min at room temperature before adding the probe. The binding mixtures were incubated at room temperature for 20 min before running on 5% polyacrylamide gel in 0.5 x Tris borate-EDTA (TBE) at 4 °C for 1–3 h. The gel was dried and autoradiographed. Result of a representative experiment was shown.

ChIP assay was performed in Huh7 cells treated with vehicle, 50ng/mL PMA or 40ng/mL TNFα for 6 hours. Anti-p65 antibody (D14E12; Cell Signaling Technology) or normal serum (IgG) was used for ChIP analysis following a two-step cross-linking method as described by Nowak et al.(*163*). The PCR primers for NF-κB binding sites within STS promoter are: NF-κB-1 forward, 5′-CACTTGTCACTTAGCAGCA-3′ and reverse, 5′-GACCAGGCATTTCTTTCC-3′; NF-κB-2 forward, 5′-GCCTGGTCATTGACCCAGAC-3′ and reverse, 5′-AGGAACAGGCCAGTGACATG-3′; NF-κB-3 forward, 5′-GACACAAATCATACCGAAGG-3′ and reverse, 5′-GGCAGATAATGTGAGGCGGA-3′; IL-8 forward, 5'-CAGAGACAGCAGAGCACAC-3' and reverse, 5'-ACGGCCAGCTTGGAAGTC-3'.

3.2.8 Statistical analysis

Results are presented as means ± SD. The Student's *t*-test was used to calculate significance between two group means. Analysis of variance (ANOVA) was used to calculate significance between multiple group means (see Appendix A for details). Fisher's exact test was used to calculate statistical significance for categorical data. For correlation analysis, mRNA expression data were log-transformed and analyzed by Spearman rank correlation. $P < 0.05$ was considered as statistically significant.

3.3 RESULTS

3.3.1 Induction of STS expression is frequent in chronic liver disease

A search of the GEO database revealed that STS was up-regulated in patients with alcoholic hepatitis (GSE28619)(*164*), (Fig. 9A). Concomitant of STS induction was the reduction of phase I and phase II enzymes involved in estrogen metabolism: CYP1A2, CYP3A4 and EST (Fig. 9B), which supports the clinical observation of impaired estrogen inactivation in liver disease(*152*). Further analysis revealed significant inverse correlations between the mRNA expression of STS and CYP1A2 (Spearman R = -0.6894; P = 0.0004), CYP3A4 (Spearman R = -0.6940; P = 0.0003) and EST (Spearman R = -0.7741; P < 0.0001). The activated estrogen formation pathway and suppressed estrogen elimination pathway may together increase estrogen level and bioactivity during the progression of alcoholic hepatitis. On the other hand, the expression of Interleukin-8 (IL-8) was up-regulated in hepatitis livers (Fig. 9C). IL-8, an NF-κB target gene, has been shown to be strongly activated in patients with chronic liver diseases and correlated with liver function and hepatic macrophages accumulation(*165*). The positive correlation between the mRNA expression of STS and IL-8 (Fig. 9D) suggests a possible association between inflammation and STS.

To validate the microarray data, I performed an IHC staining of STS expression in a TMA of control and cirrhosis liver specimens (Fig. 9E). IHC analysis showed a homogeneous cytoplasmic staining pattern in STS stained hepatocytes. The negative control in which the primary antibody was omitted did not reveal any detectable staining-suggesting the staining was STS-specific. I categorized patient samples into weak or strong staining groups according to the

immunoreactivity of STS. Fig. 9E depicted representative examples of weak and strong staining intensity. Of the 29 cirrhosis samples examined, 22 (75.86%) showed strong staining for STS and 7 (24.14%) showed weak staining. Analysis of the association between STS expression and clinicopathological factors among the 43 patients revealed that the presence of cirrhosis was strongly associated with STS expression (Table 2) (P =0.018), while the staining intensity of STS did not correlate with gender or age (Table 2). In summary, the induction of the STS gene expression is a very common event in chronic inflammatory liver disease.

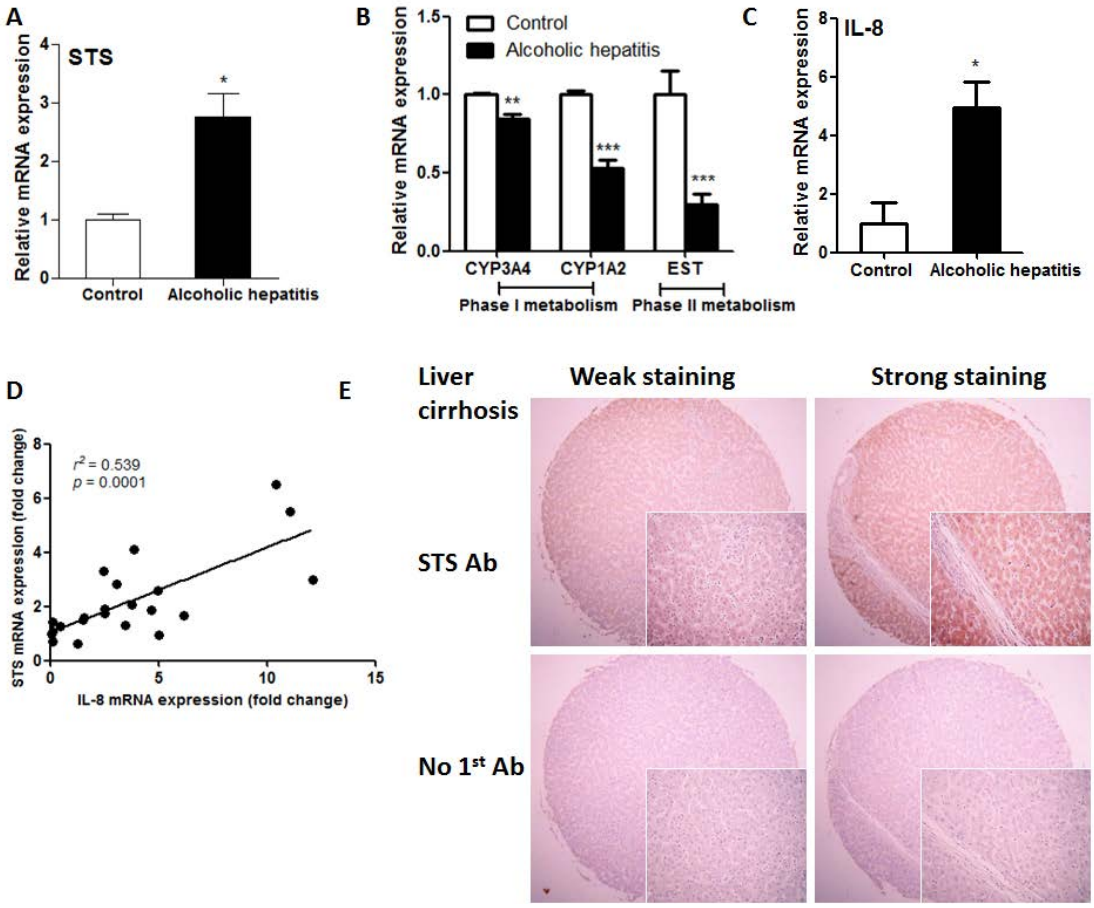

Figure 9. STS was induced in patients with chronic inflammatory liver diseases.
A to C, mRNA expression of human STS (A), CYP3A4, CYP1A2 and EST (B) and IL-8 (C) in patients with alcoholic hepatitis (GSE28619). D, correlation between the expression of IL-8 and STS in control and alcoholic hepatitis patients. E, IHC analysis of TMA of liver cirrhosis representing different STS expression. The top two panels are representative cases showing weak and strong expression of STS in cirrhosis patients. The bottom two panels are their corresponding controls without primary anti-STS antibody. *, p≤0.05; **, p≤0.01; ***, p≤0.001, control versus alcoholic hepatitis group.

TABLE 2. Immunohistochemical expression of STS in cirrhosis patients and controls in relation to clinicopathologic features

				STS		
Variable	Categorization	*n*	%	Weak	Strong	P-value
Age (years)						0.215
	≤50	21	48.84	10	11	
	>50	22	51.16	6	16	
Gender						0.304
	Female	11	25.58	6	5	
	Male	32	74.42	10	22	
Cirrhosis						0.018*
	Absence	14	32.56	9	5	
	Presence	29	67.44	7	22	

A statistical analysis was conducted with two-sided Fisher's exact test for all the parameters. *P <0.05.

3.3.2 Activation of NF-κB induced STS expression and activity in human hepatocytes

The NF-κB family of transcription factors modulates various aspects of the inflammatory responses, and chronic NF-κB activity has been implicated in several liver diseases including hepatitis, liver fibrosis, cirrhosis and hepatocellular carcinoma(*166*). To test the effect of NF-κB activation on the expression of STS, I treated human primary hepatocytes with LPS, which

signals through toll-like receptors (TLRs) to stimulate NF-κB. LPS increased STS mRNA expression as determined by real-time PCR analysis (Fig. 10A). The induction of STS seemed to be human-specific because LPS had little effect on hepatic expression of mouse *Sts* (Fig. 10B). Despite the induction of STS, the expression of estrogen-deactivating enzymes was down-regulated upon LPS treatment, including CYP3A4, CYP1A2 and EST (Fig. 10C), which was consistent with the *in vivo* data. Consistent with increased mRNA level of STS gene, the protein expression (Fig. 10D) and enzymatic activity (Fig. 10E) of STS were also induced by LPS in human primary hepatocytes. To investigate whether activation of NF-κB is necessary for STS induction, I analyzed the induction of STS in the presence of a selective NF-κB inhibitor PDTC. As shown in Fig. 10D, pretreatment with PDTC abolished the induction of STS by LPS. These results support that STS expression is dependent on NF-κB signaling pathway. Besides the human primary hepatocytes, I also used the hepatic cell line Huh7 as another *in vitro* model to study the effect of NF-κB activation on the expression of STS. NF-κB activators TNF-α and PMA act through TNF receptor (*167*) and protein kinase C (*168*) to stimulate NF-κB activity, respectively. The same as in human primary hepatocytes, I also observed the induction of STS gene at mRNA and protein levels in an NF-κB-dependent manner (Fig. 10 F and G).

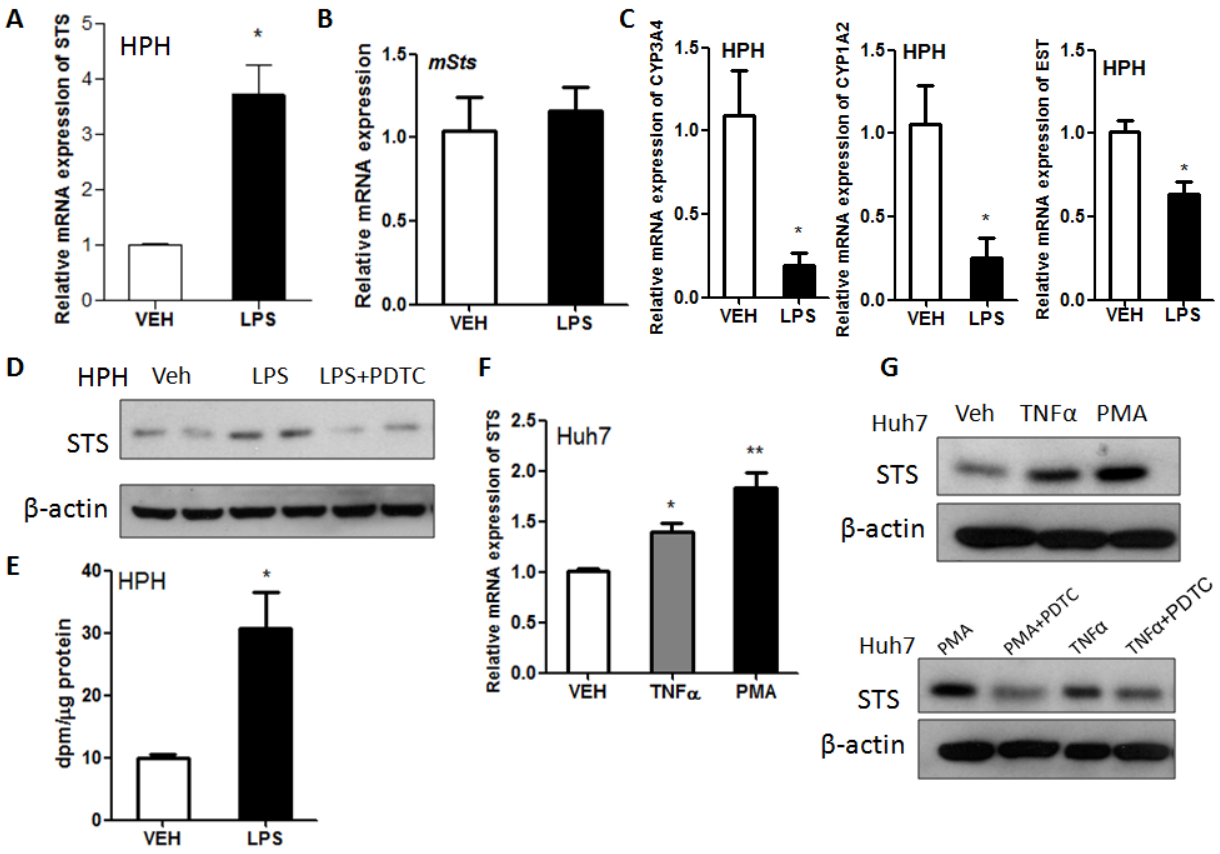

Figure 10. Induction of STS in human hepatocytes through the NF-κB signaling pathway.

A, mRNA expression of STS in human primary hepatocytes (HPH) treated with vehicle (VEH) or LPS (1μg/ml) for 24 hours. B, hepatic mouse *Sts* expression in CD-1 mice treated with either vehicle (VEH) or LPS (5 mg/kg) for 16h. N=3 for each group. C, mRNA expression of CYP3A4, CYP1A2 and EST in VEH- or LPS (1μg/ml) -treated HPH. D, protein expression of STS in HPH treated with VEH, LPS, or LPS plus PDTC (50 μM) for 24 hours. E, enzymatic activity of STS in HPH treated with VEH or LPS. F and G, mRNA expression (F) and protein expression (G, top panel) of STS in huh7 cells treated with vehicle, PMA (50ng/mL) or TNFα (40ng/mL) for 24 hours. Effect of PDTC on PMA- or TNFα-induced STS expression in huh7 cells (G, bottom panel). *, p≤0.05; **, p≤0.01 compared with the vehicle group.

3.3.3 STS is an NF-κB target gene

Sequence inspection of the 5'-flanking region of the STS gene revealed three potential NF-κB binding motifs located between nucleotides -50 and -500 (Fig. 11A). EMSA was used to assess the ability of these binding sites to form complexes with p65 protein, a major subunit of

NF-κB transcription complex (Fig. 11B). I made STS probes containing individual NF-κB binding site as well as their corresponding mutants. The WT probes formed protein-DNA complexes with p65 and showed mobility similar to that of the complex formed with the κB site. The positive control κB site is derived from the MHC class II-associated invariant chain gene(*169*). Mutations of the putative NF-κB binding site attenuated or eliminated the p65 binding, suggesting site-specific binding. The binding specificity was further confirmed by competition experiments, in which excess amounts of unlabeled WT probes, but not mutant probes, out-competed the p65 binding with the labeled WT probes. The p65 antibody was included to the binding reaction to identify the protein bound to STS probes, which decreased the mobility of the protein-DNA complex with a supershift. All three NF-κB binding sites within STS promoter can specifically bind to p65 protein, with nucleotides -279/-270 showing the strongest binding affinity and nucleotides -66/-57 showing the weakest binding affinity. To examine whether the NF-κB-binding motifs within the STS promoter are transcriptional active, I linked the WT, mutant and shortened STS promoters to the luciferase gene. These resulting luciferase reporters were transfected into human HEK293 cells (Fig. 11C). Co-transfection of p65 induced the luciferase gene expression driven by WT STS promoters. On the other hand, elimination or mutation the NF-κB binding sites markedly attenuated or abolished the p65-induced luciferase activity. Consistent with the EMSA result, the most proximal site at -66/-57 showed weakest basal and p65-induced luciferase activity. I assessed the *in situ* binding of p65 by using ChIP assay performed on chromatin isolated from TNFα-stimulated Huh7 cells. The p65 protein showed association with all three putative binding sites within the STS promoter after stimulation, as with the classical NF-κB target gene IL-8 (Fig. 11D).

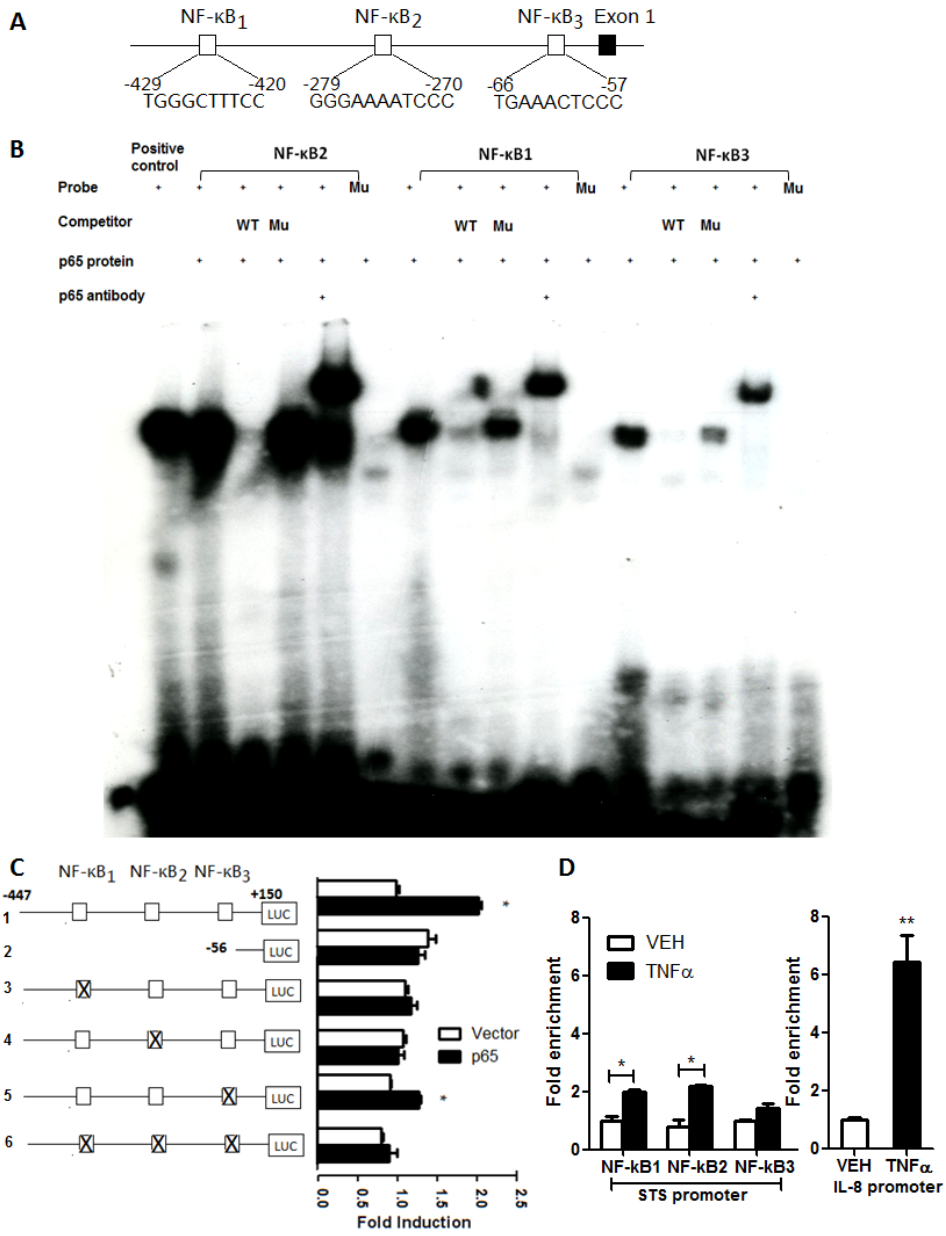

Figure 11. STS is a transcriptional target of NF-κB.

A, Schematic diagram of the 5'-flanking region of STS gene. Note the presence of three NF-κB binding sites. B, EMSA of NF-κB binding sites derived from the human STS promoter. C, Analysis of STS promoter activity by luciferase reporter assay performed in HEK293 cells. Results are fold induction over the luciferase activity of the empty vector pGL-3 and represent means ± SD from triplicate assays. D, ChIP assay performed with Huh7 cells using anti-p65 antibody or normal serum (IgG), with the primers amplifying different regions of the STS promoter. IL-8 promoter region was included as a positive control. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle or vector group.

3.3.4 Activation of a negative feedback loop involving STS and NF-κB

Estrogen sulfates are the preferred STS substrates existing in high concentrations *in vivo* (*1*, *134*). I hypothesized that the induction of STS by NF-κB activators may convert estrogen sulfates to active estrogens and enhance estrogen signaling in the hepatic cells. Indeed, PMA treatment increased the estrogen levels in cell culture supernatants from Huh7 cells, which were abolished by knocking down STS (Fig. 12A). The knockdown efficiency of STS was shown at both mRNA and protein levels (Fig. 12B). The estrogen may be converted from serum estrogen sulfate since the "estrogen free" DCC stripped FBS used for cell culture contains relatively high level of estrogen sulfate (data not shown) despite very low level of estrogen. Consistent with increased estrogen levels, the estrogen activity, as demonstrated by the estrogen-responsive luciferase reporter gene, was also enhanced by PMA treatment. Adding estrogen sulfate, the STS substrate, further enhanced the PMA-induced luciferase activity (Fig. 12C). The induction of estrogen activity was dependent on NF-κB, because pretreatment with PDTC blocked the PMA induced luciferase activity (Fig. 12D). In addition, the STS inhibitor STX64 or the ER antagonist ICI significantly attenuated or abolished the PMA-induced ERE-luciferase activity, suggesting that the NF-κB induced estrogen activity was mediated by STS (Fig. 12D). Consistently, the expression of estrogen-responsive genes was induced by NF-κB activation in an STS-dependent manner (Fig. 12E and F).

Given the well-known anti-inflammatory activities of estrogen (*170*), I hypothesized that the induction of STS in response to NF-κB activation may serve to terminate the NF-κB response through the estrogen signaling pathways. The PMA-induced transcriptional activation of NF-κB was blocked by estrogen as determined by the NF-κB-responsive luciferase activity (Fig. 13A).

Estrogen sulfate also moderately reduced the NF-κB-luciferase activity-probably due to its conversion into estrogen by endogenous STS (Fig. 13A). Indeed, after co-transfection of the STS expression vector, the NF-κB activity was comparable between the estrogen sulfate group and the estrogen treatment group (Fig. 13A). On the other hand, the STS inhibitor STX64 or the estrogen receptor antagonist ICI further enhanced the PMA-induced NF-κB luciferase activity (Fig. 13B), as well as the expression the NF-κB target genes (Fig. 13C and E). Besides the pharmacological inhibitor, I also used the siRNA to knockdown STS gene. Consistently, knocking down of STS also enhanced the expression of NF-κB target genes (Fig. 13. D). These results suggest that the STS-mediated estrogen signaling suppressed NF-κB activity and inflammation in hepatocytes.

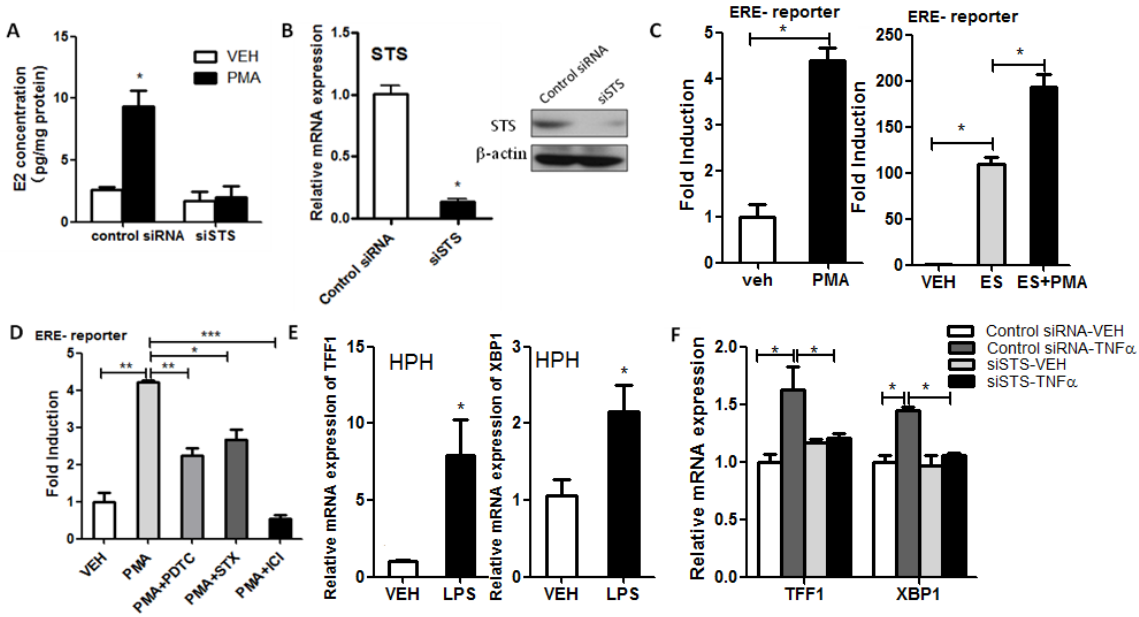

Figure 12. Activation of NF-κB enhanced estrogen activity in human hepatocytes.
A, E2 levels in cell culture supernatant from control siRNA or siSTS-transfected Huh7 cells treated with either vehicle(VEH)- or PMA (50ng/mL) for 24 hours. B, knock-down efficiency of siSTS as determined at mRNA level (left panel) by RT-PCR analysis and protein level (right panel) by western blotting. C, ERE-luciferase activity in Huh7 cells treated with VEH, PMA, 1μM estrone sulfate (ES) or both PMA and ES. D, Effect of 25 μM PDTC, 10 μM STX64 or 100 nM ICI on PMA-induced ERE-luciferase activity. E, Effect of LPS (1μg/mL) on the expression of estrogen-responsive genes in human primary hepatocytes. F, Effect of TNFα (40ng/mL) on the expression of estrogen-responsive genes in Huh7 cells transfected with control siRNA or siSTS. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ compared with the vehicle group, or the comparisons are labeled.

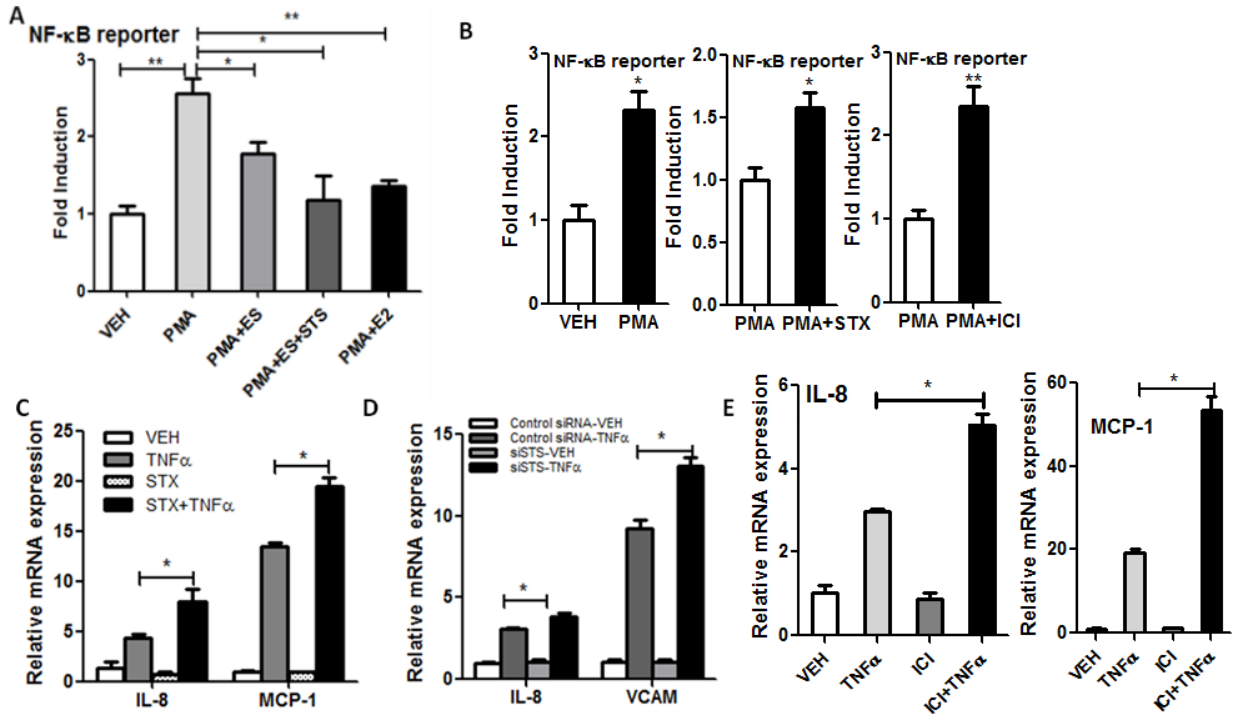

Figure 13. STS-mediated estrogen signaling suppressed NF-κB activity.

A, treatment of 100nM estrone sulfate (ES) or 10nM estradiol (E2) attenuated the PMA (50ng/mL)-induced NF-κB transcriptional activity in Huh7 cells transfected with empty vector or STS expression vector. B, effect of 10 μM STX64 or 100 nM ICI on PMA-induced NF-κB luciferase activity. Effect of blocking STS by pharmacological inhibitor STX64 (C) or genetic knockdown (D) on TNFα (40ng/mL)-induced expression of NF-κB target genes. E, effect of ICI on the expression of NF-κB target genes induced by TNFα. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group, or the comparisons are labeled.

3.4 DISCUSSION

Chronic inflammatory liver diseases are commonly associated with estrogen excess. The conventional hypothesis is that liver damage reduces the liver's ability to inactivate the estrogen. My study first suggested that the increased expression and activity of STS during inflammation may have contributed to the altered estrogen metabolism during chronic liver diseases. In contrast to the induction of STS, several of the estrogen-metabolizing enzymes was down-regulated in diseased livers, including CYP1A2, CYP3A4 and EST. CYP1A2 and CYP3A4 catalyze the phase I metabolism of estrogen and generate 2-hydroxy metabolites in human livers. The reduction of CYP1A2 and CYP3A4 is consistent with the previous report of reduced expression of estrogen-metabolizing enzymes in liver disease (*171*), and a significant impairment of 2-hydroxy estrogen metabolites in cirrhosis patients(*172*). Mechanistically, the expression of these estrogen metabolizing genes has been subjected to transcriptional and post-transcriptional regulations mediated by several transcription factors and kinases (*173*). Therefore, the decreased elimination and increased formation of active estrogen may together increase estrogen level and bioactivity during chronic inflammatory liver diseases (Fig. 15).

The STS expression was also significantly correlated with the expression of IL-8, a major pro-inflammatory cytokine and NF-κB target gene, suggesting a possible association between STS and NF-κB signaling. I, therefore, examined the effect of NF-κB activation on hepatocytes and found an NF-κB-dependent upregulation of the STS gene at mRNA, protein and enzyme levels. Furthermore, NF-κB-induced STS is functional in catalyzing the conversion of estrogen sulfate to estrogen as evidenced by the NF-κB- and STS-dependent increase in estrogen level and

activity. Since STS is expressed in many tissues and cell types, I cannot exclude the possibility that STS in extra-hepatic tissues and cells may also contribute to estrogen increase in inflammatory liver diseases. The induction of STS seemed to be human-specific because LPS treatment did not affect the mouse Sts expression in the liver. This result may be due to the substantial divergence between mouse Sts and human STS gene: The mouse Sts gene spans over 9 kb, which is much smaller than human STS, and it is only around 60% identical to human STS at the nucleotide level and amino acid level. This result may suggest the gap between mouse models and human disease. However, further studies using mouse models of chronic liver diseases are needed to validate this result.

Previous characterization of human STS promoter revealed that it resembled neither a tightly regulated gene-which contains a TATA box to position the RNA polymerase; nor a housekeeping gene-which is usually GC rich and contains binding sites for Sp1. On the other hand, the STS promoter is GC poor and lacks the TATA box and Sp1 binding sites(*15*). Here I identified novel regulatory element in the human STS promoter which is critical for NF-κB induced gene expression in hepatocytes. The DNA element is composed of three closely positioned putative NF-κB binding sites: site 1 (-429 to -420), site 2(-279 to -270) and site 3(-66 to -57). EMSA, luciferase and ChIP data revealed that site 1 and site 2 better resembled the canonical NF-κB binding site and contributed to the NF-κB induced expression of the STS gene than did site 3. However, all three sites may be required for maximal promoter activity since mutation of all three binding sites most profoundly compromised both the basal and inducible promoter activity. These results suggest that the activation of the STS gene may require more than one NF-κB binding site.

STS is likely to play a protective role against NF-κB-mediated inflammation, because loss of STS by pharmacological inhibition or genetic knockdown increased NF-κB transcriptional activation and its target gene expression. The beneficial role of STS may be due to increased estrogen level and activity, since blocking estrogen receptor achieved the same effects in enhancing NF-κB transcriptional activity as did inhibition of STS. The NF-κB family of transcription factors modulates various aspects of the inflammatory responses, and chronic NF-κB activity has been implicated in several liver diseases including hepatitis, liver fibrosis, liver cirrhosis and HCC(*166*). In the canonical NF-κB pathway, the extracellular stimulators activate the IκB kinase (IKK) complex. The IKK complex phosphorylates and degrades the inhibitors of NF-κB (IkBs), thus frees the NF-κB complex from IκBs in the cytoplasm. Activated NF-κB is translocated to the nucleus, binds to promoters of target gene and recruits coactivators to promote gene transcription. ER has been shown to inhibit NF-κB activity at almost each, and every step mentioned above, including (a) Blocking IKK activity; (b) Inhibiting IκB degradation; (c) Hindering DNA binding by NF-κB; (d) Competing with NF-κB for coactivator binding, and (e) Inhibiting NF-κB-mediated transcriptional activation by binding directly to DNA-bound NF-κB (*174*). It should be noted that the NF-κB activation-mediated inflammation in hepatocytes plays a dual role in the pathogenic progression of liver diseases. In early stages of liver diseases, NF-κB prevents hepatocytes cell death by inducing antiapoptotic genes. In late stages, however, NF-κB also promotes the survival of hepatocytes harboring oncogenic mutations-a major source of HCC (*166*). Future studies are required to clarify the function of STS-mediated estrogen increase in acute versus chronic inflammatory liver diseases. Despite its known anti-inflammation effect, a systemic treatment with estrogen may cause feminization in men. Given

the inducibility of STS expression during inflammation, targeted delivery of the hormonally inactive estrogen sulfate may avoid the unwanted side effects while maintaining the benefits of estrogen.

In conclusion, my results provided a new endocrine basis for the estrogen increase in chronic liver diseases, and confirmed the presence of negative feedback loop that is triggered by initial activation of NF-κB, which induces STS expression in hepatocytes and converts inactive estrogen sulfate metabolites into active estrogens. The resulting estrogens may inhibit NF-κB-mediated inflammation via the ER signaling, thereby completing the negative feedback loop (Fig. 14). These findings indicate the critical role of the hepatic microenvironment in regulating estrogen activity, and identify STS-mediated bidirectional interaction between the estrogen signaling and inflammatory response.

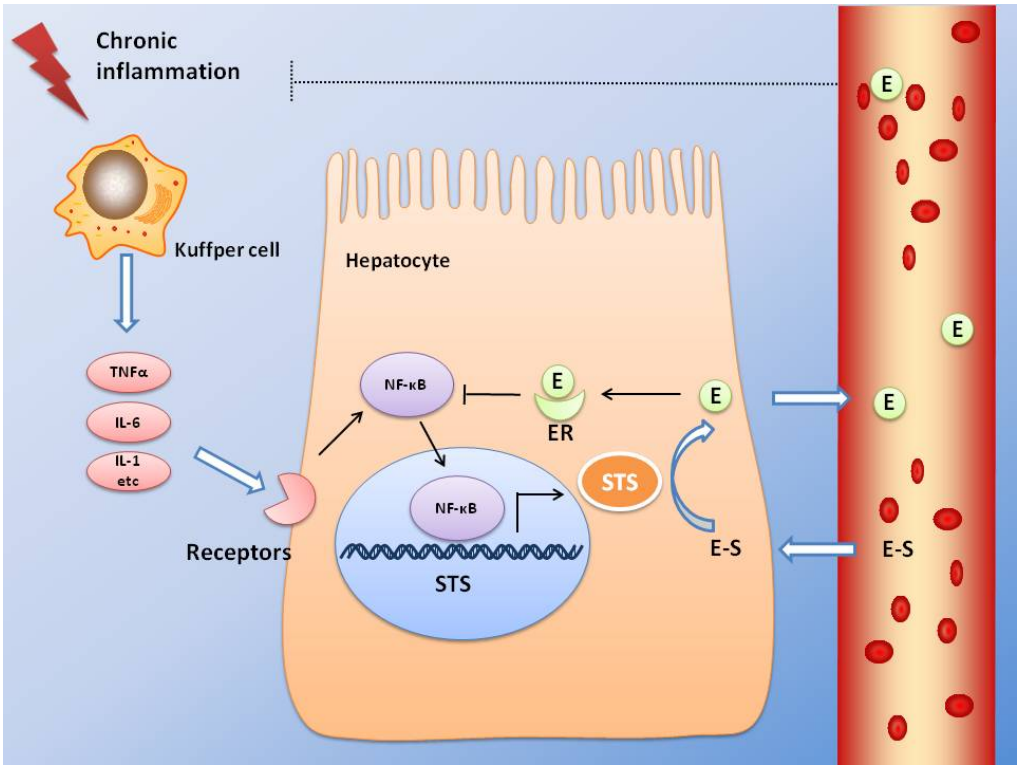

Figure 14. Reciprocal regulation between inflammation and STS-mediated estrogen signaling.

During chronic inflammation, activated macrophages release proinflammatory cytokines such as TNFα, IL-6 and IL-1, which act on neighboring hepatocytes to elicit NF-κB activation. NF-κB induces the expression of STS, which converts inactive estrogen sulfate to active estrogen and increases estrogen level in the circulation. On the other hand, activation of estrogen signaling by STS may suppress the NF-κB response and inhibit inflammation.

4.0 CHAPTER IV: SUMMARY AND PERSPECTIVES

The research goal of this dissertation is to elucidate the role of the STS-mediated estrogen homeostasis in energy metabolism and inflammation. Estrogen is not only a key hormone in reproduction, but it also has great implications in glucose and energy metabolism. Estrogens exhibit an overall protective role in preventing obesity and type 2 diabetes. Estrogen homeostasis is tightly regulated by sulfation and desulfation. The STS-mediated desulfation is a critical metabolic mechanism that regulates the chemical and functional homeostasis of estrogen. Here I showed that over-expression of STS in the liver of transgenic mice alleviated HFD and ob/ob models of obesity and type 2 diabetes, including reduced body weight, improved insulin sensitivity, and decreased hepatic steatosis and inflammation. Interestingly, STS exerted its metabolic benefit through sex-specific mechanisms. In female mice, hepatic over-expression of STS elicited liver-specific increase of estrogen activity and improvement of the metabolic functions; whereas ovariectomy abolished this protective effect (Fig. 15). In contrast, the STS males displayed extra-hepatic metabolic benefits, including the male-specific decrease of inflammation in WAT and skeletal muscle, as well as a pattern of skeletal muscle gene expression that favors energy expenditure. My results have uncovered a novel function of STS in energy and glucose metabolism. Liver-specific STS induction or estrogen/estrogen sulfate delivery may represent a novel approach to manage metabolic syndrome.

The decreased body weight and adiposity in STS females may have resulted from their enhanced energy expenditure. The STS transgene was not expressed in the brain, which modulates energy expenditure. Therefore, certain metabolic information from the transgenic livers may affect the brain via humoral or the neuronal pathway. In STS females, the increased estrogen activity was likely localized to the liver because the circulating estradiol levels were similar between WT and STS mice. Thus, it may be the neuronal pathway that mediates the liver-to-brain communication. Neuronal pathway from the liver has been reported to regulate energy expenditure and insulin sensitivity (*175*). However, how hepatic STS affects autonomic afferents in transmitting metabolic information to the brain, and whether estrogen or other mediators are involved in this process are remained to be studied.

The phenotype of STS males is rather intriguing. Since the STS transgene was not targeted to the adipose tissue and skeletal muscle, it is likely that the transgenic liver releases protective mediators to benefit extra-hepatic tissues. Serum profile analysis showed that serum DHEA, which can be converted from DHEA sulfate by STS, was increased in STS males. *STS* mutant mice were reported to have decreased serum DHEA levels(*176*). Polymorphism of STS (SNP accession no. rs_13648) was associated with plasma DHEA and DHEA sulfate concentrations and body composition in response to exercise. Individuals with the more DHEA-responsive G allele had greater fat mass loss than individuals with a common A allele only(*177*). DHEA has multiple beneficial effects during the progression of metabolic syndrome, including increasing lean body mass, reducing fat mass, lowering inflammation and improving insulin sensitivity (*178-182*), which phenocopied the metabolic benefits observed in STS males. It might be possible that the enhanced circulating DHEA in male STS mice contributed to the systemic

protection. However, whether and why the elevation of circulating DHEA followed a male-specific pattern require further studies. In the future, transgenic mice with adipose tissue- or skeletal muscle-specific over-expression of STS, which have been developing in our lab, can be used directly to study the local metabolic function of STS. These genetic models will serve to dissect the metabolic function of STS-mediated steroid homeostasis in different tissue or cell types.

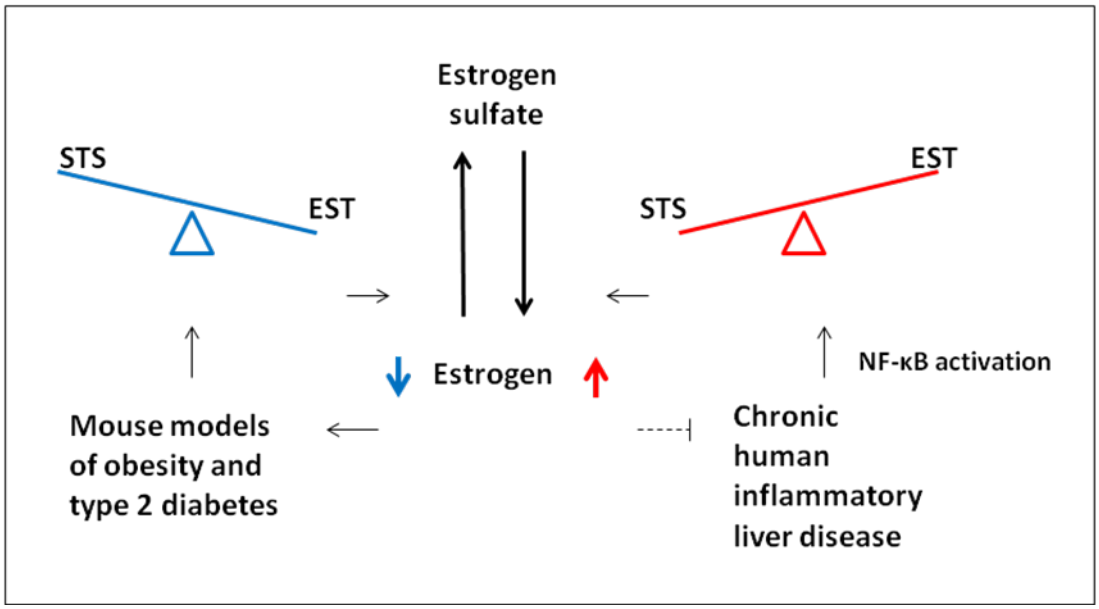

Figure 15. Schematic illustration of the role of the STS-mediated estrogen homeostasis in energy homeostasis and inflammation.

Under physiological conditions, estrogen homeostasis is maintained by the balanced sulfation and desulfation reactions catalyzed by EST and STS. However, in mouse models of type 2 diabetes, *EST* is dramatically induced in the liver, which overrides the small increase of *STS*, resulting in increased estrogen deprivation and contributing to the development of insulin resistance. During chronic inflammation, activation of NF-κB induces the expression of STS. On the other hand, EST was down-regulated, which together increase estrogen level and bioactivity in the circulation. STS-mediated activation of estrogen signaling may suppress the NF-κB response and inhibit inflammation.

Abnormal estrogen metabolism in liver disease has been identified for decades. Concomitants of liver diseases are clinical signs and symptoms indicating estrogen excess. Liver is the primary site of estrogen metabolism through phase I oxidation and phase II conjugation reactions. The estrogen excess was reasoned to be due to liver damage that weakens the liver's capacity to breakdown estrogen. Estrogen may participate in the progression of chronic liver diseases. The developments of many chronic inflammatory liver diseases are more common in men than in women. The prognosis of female HCC patients is also much better than male patients. In this dissertation study, I showed that STS may mediate the inflammation induced estrogen increase in chronic liver disease. Bioinformatic and IHC analysis demonstrated that STS was induced in liver samples from patients with chronic liver diseases, such as hepatitis and cirrhosis, despite of down-regulation of several enzymes involved in estrogen inactivation. EMSA, luciferase assay and ChIP analysis revealed that STS is an NF-κB target gene, whose induction facilitates the conversion of inactive estrogen sulfates to estrogens. On the other hand, loss of STS or blocking estrogen signaling increased NF-κB transcriptional activity and inflammation (Fig. 15). These studies define a novel mechanism of estrogen increase during inflammatory liver disease and identify STS as a mechanistic linker between estrogen action and inflammatory response.

Although blocking STS-mediated estrogen signaling increased NF-κB transcriptional activity and inflammation at cellular level, the *in vivo* role of estrogen increase in liver disease is unclear. Liver disease induced estrogen excess is usually considered harmless and often reversible as liver function improves. However, whether the estrogen increase merely serves as a marker of liver dysfunction or it may affect liver function, and the progression of chronic liver

disease is not known. Moreover, NF-κB activation represents a double-edged sword in the pathogenic progression of liver diseases. Although chronic activation of NF-κB-mediated inflammatory response promotes the development HCC, activation of NF-κB in early stages of liver disease also fights against infection and prevents hepatocyte cell death. Therefore, future studies are needed to identify the point when NF-κB activation becomes oncogenic, so that more precise treatment can be launched. Systemic estrogen treatment is limited by potentially adverse effects such as tumor promotion and risk of cardiovascular disease. Estrogen may also cause feminization and other adverse effects in males, the majority of HCC cases. In addition, estrogen administration to patients with liver disease may enhance the workload of the already embarrassed liver. Therefore, it requires rigorous validation before using estrogen-related therapy in the treatment of chronic liver disease.

APPENDIX A

STATISTICS

Figure 1, panel A. One-way analysis of variance. $P<0.05$. Bonferroni's multiple comparison test. WT-chow vs WT-HFD, $P<0.05$. WT-chow vs ob/ob, $P<0.05$.

Figure 1, panel B. Student's t-test. Two tailed. Fed vs Fasting, $P<0.05$.

Figure 1, panel F. One-way analysis of variance. $P<0.05$. Bonferroni's multiple comparison test. WT-VEHICLE vs TG-VEHICLE, $P<0.05$. WT-DOX vs TG-DOX, $P>0.05$.

Figure 1, panel G. Student's t-test. Two tailed. Male TG vs Female TG, $P>0.05$.

Table 1. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Serum leptin, $P<0.05$. Fasting glucose, $P<0.05$. Fasting insulin, $P>0.05$. Serum triglyceride, $P>0.05$. Serum total cholesterol, $P>0.05$.

Figure 2, panel A. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 2, panel B, IPGTT. Two-way repeated measures analysis of variance, WT vs TG. $P>0.05$. Student's t-test, two tailed, WT-AUC vs TG-AUC, $P>0.05$. ITT. Two-way repeated measures analysis of variance, WT vs TG. $P>0.05$. Student's t-test, two tailed, WT-AUC vs TG-AUC, $P>0.05$.

Figure 2, panel C. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Body weight, $P<0.05$. Lean mass, $P>0.05$. Fat mass, $P<0.05$.

Figure 2, panel D. Food intake. Student's t-test. Two tailed. WT vs TG. P>0.05. Oxygen consumption. Two-way analysis of variance. WT vs TG. P<0.05. Bonferroni posttests. Day, P<0.05; dark phase, P<0.05; light phase, P<0.05. Energy expenditure. Student's t-test. Two tailed. WT vs TG. P<0.05.

Figure 2, panel E. IPGTT. Two-way repeated measures analysis of variance, WT vs TG. P<0.05. Student's t-test. Two tailed. WT-AUC vs TG-AUC, P<0.05. ITT. Two-way repeated measures analysis of variance, WT vs TG. P>0.05. Student's t-test. Two tailed. WT-AUC vs TG-AUC, P>0.05.

Figure 2, panel F. Two-way analysis of variance. DMSO vs FSK. P<0.05. Bonferroni posttests. WT, P<0.05. TG, P>0.05.

Figure 2, panel G. Student's t-test. Two tailed. WT vs TG. Glucose infusion rate, P<0.05. Hepatic glucose production. Two-way analysis of variance. WT vs TG. P<0.05. Bonferroni posttests. Basal, P<0.05. Clamp, P<0.05.

Figure 2, panel H. Student's t-test. Two tailed. WT vs TG. Relative AKT phosphorylation, liver, P<0.05; WAT, P>0.05; muscle, P>0.05.

Figure 2, panel I. Two-way analysis of variance. WT vs TG. P<0.05. Bonferroni posttests. G6pase, P<0.05. Pepck, P<0.05.

Figure 3, panel B. Student's t-test. Two tailed. WT vs TG. Hepatic triglycerides, P<0.05. Hepatic cholesterol, P>0.05.

Figure 3, panel C. Two-way analysis of variance. WT vs TG. P>0.05.

Figure 3, panel D. Two-way analysis of variance. WT vs TG. P>0.05.

Figure 3, panel E. Student's t-test. Two tailed. WT vs TG. P<0.05.

Figure 3, panel F. Liver. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Tnfα, $P<0.05$; Mcp-1, $P<0.05$. WAT. Two-way analysis of variance. WT vs TG. $P>0.05$. MUS. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 3, panel G right. Student's t-test. Two tailed. WT vs TG. $P>0.05$.

Figure 4, panel A. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 4, panel B. Student's t-test. Two tailed. WT vs TG. $P>0.05$.

Figure 4, panel C. Two-way repeated measures analysis of variance, WT vs TG. $P>0.05$. Student's t-test. Two tailed. WT-AUC vs TG-AUC. $P>0.05$.

Figure 4, panel D. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 4, panel E. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 5, panel A right. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. E1, $P<0.05$. E1S, $P<0.05$.

Figure 5, panel B. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. VEH, $P<0.05$. ES, $P<0.05$.

Figure 5, panel C. Student's t-test. Two tailed. WT vs TG. Serum estradiol, $P>0.05$. Estrous cycle, $P>0.05$.

Figure 5, panel D. Two-way analysis of variance. WT vs TG. $P>0.05$.

Figure 5, panel E. Two-way repeated measures analysis of variance, WT vs TG. $P>0.05$. Student's t-test. Two tailed. WT vs TG.$P>0.05$.

Figure 5, panel F. Two-way analysis of variance. WT vs TG, Left, $P>0.05$. Middle, $P>0.05$. Right. Student's t-test. Two tailed. WT vs TG.$P>0.05$.

Figure 6, panel A. Student's t-test. Two tailed. ob/ob vs obS. Body weight, $P>0.05$. Fat mass, $P>0.05$. Lean mass, $P<0.05$.

Figure 6, panel B. IPGTT. Two-way repeated measures analysis of variance, ob/ob vs obS. $P<0.05$. Student's t-test. Two tailed. ob/ob-AUC vs obS-AUC. $P<0.05$. ITT. Two-way repeated measures analysis of variance, ob/ob vs obS. $P>0.05$. Student's t-test. Two tailed. ob/ob-AUC vs obS-AUC. $P>0.05$.

Figure 6, panel C. Two-way analysis of variance. ob/ob vs obS. $P>0.05$. Bonferroni posttests. G6pase, $P<0.05$. Pepck, $P>0.05$.

Figure 6, panel D. Two-way analysis of variance. ob/ob vs obS. $P<0.05$. Bonferroni posttests. Tnfα, $P<0.05$. Mcp1, $P<0.05$. Il1, $P<0.05$.

Figure 6, panel E. Student's t-test. Two tailed. ob/ob vs obS. Triglycerides, $P<0.05$. Cholesterol, $P>0.05$.

Figure 6, panel F. Two-way analysis of variance. ob/ob vs obS. Left. $P>0.05$. Right. $P>0.05$.

Figure 6, panel G. Student's t-test. Two tailed. ob/ob vs obS. $P>0.05$.

Figure 7, panel A. Two-way repeated measures analysis of variance. VEH vs ES. $P<0.05$. Bonferroni posttests. Day 1, $P>0.05$. Day 3, $P<0.05$. Day 5, $P<0.05$. Day 7, $P<0.05$.

Figure 7, panel B. IPGTT. Two-way repeated measures analysis of variance. VEH vs ES. $P<0.05$. Student's t-test. Two tailed. VEH-AUC vs ES-AUC, $P<0.05$. ITT, Two-way repeated measures analysis of variance. VEH vs ES. $P>0.05$. Student's t-test. Two tailed. VEH-AUC vs ES-AUC, $P>0.05$.

Figure 7, panel C. IPGTT. Two-way repeated measures analysis of variance. VEH vs ES. $P<0.05$. Student's t-test. Two tailed. VEH-AUC vs ES-AUC, $P<0.05$. ITT, Two-way repeated measures analysis of variance. VEH vs ES. $P<0.05$. Student's t-test. Two tailed. VEH-AUC vs ES-AUC, $P<0.05$.

Figure 8, panel A. Student's t-test. Two tailed. WT vs TG, $P>0.05$.

Figure 8, panel B. Two-way analysis of variance. WT vs TG. $P>0.05$. Bonferroni posttests. Body weight, $P>0.05$. Lean mass, $P<0.05$. Fat mass, $P<0.05$.

Figure 8, panel C. Student's t-test. Two tailed. WT vs TG. Energy expenditure, $P<0.05$. Oxygen consumption, $P<0.05$.

Figure 8, panel D. Two-way repeated measures analysis of variance. WT vs TG. $P<0.05$. Student's t-test. Two tailed. WT-AUC vs TG-AUC, $P<0.05$. ITT, Two-way repeated measures analysis of variance. VEH vs ES. $P>0.05$. Student's t-test. Two tailed. WT-AUC vs TG-AUC, $P>0.05$.

Figure 8, panel E. Two-way analysis of variance. WT vs TG. Left. $P>0.05$. Bonferroni posttests. G6pase, $P<0.05$. Pepck, $P>0.05$. Right. $P<0.05$. Bonferroni posttests. Tnfα, $P>0.05$. Mcp-1, $P<0.05$.

Figure 8, panel F. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Cd68, $P<0.05$. F4/80, $P<0.05$. Tnfα, $P<0.05$. Mip1α, $P<0.05$. Il1, $P<0.05$. Mcp-1, $P<0.05$.

Figure 8, panel G. Left. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Mcp-1, $P<0.05$. F4/80, $P>0.05$. Cd68, $P<0.05$. Middle. Student's t-test. Two tailed. WT vs TG, $P<0.05$. Right. Two-way analysis of variance. WT vs TG. $P<0.05$. Bonferroni posttests. Pgc1α, $P<0.05$. Pparα, $P<0.05$. Mcad, $P<0.05$. Ucp3, $P<0.05$.

Figure 8, panel H. Student's t-test. Two tailed. Left, WT vs TG. $P>0.05$. Right, ob/ob vs obS. $P>0.05$.

Figure 9, panel A. Student's t-test. Two tailed. Control vs Alcoholic hepatitis. $P<0.05$.

Figure 9, panel B. Two-way analysis of variance. Control vs Alcoholic hepatitis. $P<0.05$. Bonferroni posttests. CYP3A4, $P<0.05$. CYP1A2, $P<0.05$. EST, $P<0.05$.

Figure 9, panel C. Student's t-test. Two tailed. Control vs Alcoholic hepatitis. IL-8, $P<0.05$.

Figure 9, panel D. Regression between the mRNA expression of STS and that of IL-8. r^2=0.5390, P<0.01. y=0.3151x+1.045

Table 2. Fisher's exact test. Age vs STS expression. P>0.05. Gender vs STS expression. P>0.05. Cirrhosis vs STS expression. P<0.05.

Figure 10, panel A. Student's t-test. Two tailed. VEH vs LPS. P<0.05.

Figure 10, panel B. Student's t-test. Two tailed. VEH vs LPS. P>0.05.

Figure 10, panel C. Student's t-test. Two tailed. VEH vs LPS. CYP3A4, P<0.05. CYP2A1, P<0.05. EST, P<0.05.

Figure 10, panel E. Student's t-test. Two tailed. VEH vs LPS.P<0.05.

Figure 10, panel F. One-way analysis of variance. P<0.05. Bonferroni's multiple comparison test. VEH vs TNFα, P<0.05. VEH vs PMA. P<0.05.

Figure 11, panel C. Two-way analysis of variance. Vector vs p65. P<0.05. Bonferroni posttests. Student's t-test. Two tailed. Vector vs p65. Promoter 1, P<0.05. Promoter 2, P>0.05. Promoter 3, P>0.05. Promoter 4, P>0.05. Promoter 5, P<0.05. Promoter 6, P>0.05.

Figure 11, panel D. Two-way analysis of variance. VEH vs TNFα. P<0.05. Bonferroni posttests. STS promoter, NF-κB1, P<0.05; NF-κB2, P<0.05; NF-κB3, P>0.05. IL-8 promoter. Student's t-test. Two tailed. VEH vs TNFα. P<0.05.

Figure 12, panel A. Two-way analysis of variance. VEH vs PMA. P<0.05. Bonferroni posttests. Control siRNA, P<0.05. siSTS, P>0.05.

Figure 12, panel B. Student's t-test. Two tailed. Control siRNA vs siSTS. P<0.05.

Figure 12, panel C. Student's t-test. Two tailed. VEH vs PMA. P<0.05. One-way analysis of variance. P<0.05. Bonferroni's multiple comparison test. VEH vs ES. P<0.05. ES vs ES+PMA. P<0.05.

Figure 12, panel D. One-way analysis of variance. $P<0.05$. Bonferroni's multiple comparison test. VEH vs PMA. $P<0.05$. PMA vs PMA+PDTC. $P<0.05$. PMA vs PMA+STX. $P<0.05$. PMA vs PMA+ICI. $P<0.05$.

Figure 12, panel E. Student's t-test. Two tailed. VEH vs LPS. TFF1, $P<0.05$. XBP1, $P<0.05$.

Figure 12, panel F. Two-way analysis of variance. Treatment. $P<0.05$. Bonferroni posttests. Control siRNA-VEH vs Control siRNA-TNFα. TFF1, $P<0.05$. XBP1, $P<0.05$. Control siRNA-TNFα vs siSTS-TNFα. TFF1, $P<0.05$. XBP1, $P<0.05$.

Figure 13, panel A. One-way analysis of variance. $P<0.05$. Bonferroni's multiple comparison test. VEH vs PMA. $P<0.05$. PMA vs PMA+ES. $P<0.05$. PMA vs PMA+ES+STS. $P<0.05$. PMA vs PMA+E2. $P<0.05$.

Figure 13, panel B. Student's t-test. Two tailed. VEH vs PMA, $P<0.05$. PMA vs PMA+STX, $P<0.05$. PMA vs PMA+ICI, $P<0.05$.

Figure 13, panel C. Two-way analysis of variance. Treatment. $P<0.05$. Bonferroni posttests. TNFα vs TNFα+STX. IL-8, $P<0.05$. MCP-1, $P<0.05$.

Figure 13, panel D. Two-way analysis of variance. Treatment. $P<0.05$. Bonferroni posttests. Control siRNA-TNFα vs siSTS-TNFα. IL-8, $P<0.05$. VCAM, $P<0.05$.

Figure 13, panel E. IL-8. One-way analysis of variance. $P<0.05$. Bonferroni's multiple comparison test. TNFα vs TNFα+ICI, $P<0.05$. MCP-1. One-way analysis of variance. Bonferroni's multiple comparison test. TNFα vs TNFα+ICI, $P<0.05$.

BIBLIOGRAPHY

1. Reed, M. J., Purohit, A., Woo, L. W., Newman, S. P., and Potter, B. V. (2005) Steroid sulfatase: molecular biology, regulation, and inhibition, *Endocr Rev 26*, 171-202.
2. Webster, D., France, J. T., Shapiro, L. J., and Weiss, R. (1978) X-linked ichthyosis due to steroid-sulphatase deficiency, *Lancet 1*, 70-72.
3. Purohit, A., Woo, L. W., and Potter, B. V. (2011) Steroid sulfatase: a pivotal player in estrogen synthesis and metabolism, *Mol Cell Endocrinol 340*, 154-160.
4. Stergiakouli, E., Langley, K., Williams, H., Walters, J., Williams, N. M., Suren, S., Giegling, I., Wilkinson, L. S., Owen, M. J., O'Donovan, M. C., Rujescu, D., Thapar, A., and Davies, W. (2011) Steroid sulfatase is a potential modifier of cognition in attention deficit hyperactivity disorder, *Genes Brain Behav 10*, 334-344.
5. Sugawara, T., and Fujimoto, S. (2004) The potential function of steroid sulphatase activity in steroid production and steroidogenic acute regulatory protein expression, *Biochem J 380*, 153-160.
6. Berletch, J. B., Yang, F., and Disteche, C. M. (2010) Escape from X inactivation in mice and humans, *Genome Biol 11*, 213.
7. Zaichuk, T., Ivancic, D., Scholtens, D., Schiller, C., and Khan, S. A. (2007) Tissue-specific transcripts of human steroid sulfatase are under control of estrogen signaling pathways in breast carcinoma, *J Steroid Biochem Mol Biol 105*, 76-84.
8. Nardi, A., Pomari, E., Zambon, D., Belvedere, P., Colombo, L., and Dalla Valle, L. (2009) Transcriptional control of human steroid sulfatase, *J Steroid Biochem Mol Biol 115*, 68-74.
9. Valle, L. D., Toffolo, V., Nardi, A., Fiore, C., Bernante, P., Di Liddo, R., Parnigotto, P. P., and Colombo, L. (2006) Tissue-specific transcriptional initiation and activity of steroid sulfatase complementing dehydroepiandrosterone sulfate uptake and intracrine steroid activations in human adipose tissue, *J Endocrinol 190*, 129-139.
10. Stein, C., Hille, A., Seidel, J., Rijnbout, S., Waheed, A., Schmidt, B., Geuze, H., and von Figura, K. (1989) Cloning and expression of human steroid-sulfatase. Membrane topology, glycosylation, and subcellular distribution in BHK-21 cells, *J Biol Chem 264*, 13865-13872.
11. Sugawara, T., Nomura, E., and Hoshi, N. (2006) Both N-terminal and C-terminal regions of steroid sulfatase are important for enzyme activity, *J Endocrinol 188*, 365-374.
12. Hernandez-Guzman, F. G., Higashiyama, T., Osawa, Y., and Ghosh, D. (2001) Purification, characterization and crystallization of human placental estrone/dehydroepiandrosterone sulfatase, a membrane-bound enzyme of the endoplasmic reticulum, *J Steroid Biochem Mol Biol 78*, 441-450.
13. Cosma, M. P., Pepe, S., Annunziata, I., Newbold, R. F., Grompe, M., Parenti, G., and Ballabio, A. (2003) The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases, *Cell 113*, 445-456.
14. Rommerskirch, W., and von Figura, K. (1992) Multiple sulfatase deficiency: catalytically inactive sulfatases are expressed from retrovirally introduced sulfatase cDNAs, *Proc Natl Acad Sci U S A 89*, 2561-2565.
15. Li, X. M., Alperin, E. S., Salido, E., Gong, Y., Yen, P., and Shapiro, L. J. (1996) Characterization of the promoter region of human steroid sulfatase: a gene which escapes X inactivation, *Somat Cell Mol Genet 22*, 105-117.

16. Dalla Valle, L., Toffolo, V., Nardi, A., Fiore, C., Armanini, D., Belvedere, P., and Colombo, L. (2007) The expression of the human steroid sulfatase-encoding gene is driven by alternative first exons, *J Steroid Biochem Mol Biol 107*, 22-29.
17. van Landeghem, A. A., Poortman, J., Nabuurs, M., and Thijssen, J. H. (1985) Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue, *Cancer Res 45*, 2900-2906.
18. Utsumi, T., Yoshimura, N., Takeuchi, S., Maruta, M., Maeda, K., and Harada, N. (2000) Elevated steroid sulfatase expression in breast cancers, *J Steroid Biochem Mol Biol 73*, 141-145.
19. Utsumi, T., Yoshimura, N., Takeuchi, S., Ando, J., Maruta, M., Maeda, K., and Harada, N. (1999) Steroid sulfatase expression is an independent predictor of recurrence in human breast cancer, *Cancer Res 59*, 377-381.
20. Newman, S. P., Purohit, A., Ghilchik, M. W., Potter, B. V., and Reed, M. J. (2000) Regulation of steroid sulphatase expression and activity in breast cancer, *J Steroid Biochem Mol Biol 75*, 259-264.
21. Matsuoka, R., Yanaihara, A., Saito, H., Furusawa, Y., Toma, Y., Shimizu, Y., Yanaihara, T., and Okai, T. (2002) Regulation of estrogen activity in human endometrium: effect of IL-1beta on steroid sulfatase activity in human endometrial stromal cells, *Steroids 67*, 655-659.
22. Purohit, A., Reed, M. J., Morris, N. C., Williams, G. J., and Potter, B. V. (1996) Regulation and inhibition of steroid sulfatase activity in breast cancer, *Ann N Y Acad Sci 784*, 40-49.
23. Courtney, K. D., Corcoran, R. B., and Engelman, J. A. (2010) The PI3K pathway as drug target in human cancer, *J Clin Oncol 28*, 1075-1083.
24. Sung, C. H., Im, H. J., Park, N., Kwon, Y., Shin, S., Ye, D. J., Cho, N. H., Park, Y. S., Choi, H. K., Kim, D., and Chun, Y. J. (2013) Induction of steroid sulfatase expression in PC-3 human prostate cancer cells by insulin-like growth factor II, *Toxicol Lett 223*, 109-115.
25. Hattori, K., Yamaguchi, N., Umezawa, K., and Tamura, H. (2012) Interferon gamma induces steroid sulfatase expression in human keratinocytes, *Biol Pharm Bull 35*, 1588-1593.
26. Suh, B. Y., Jung, J. J., Park, N., Seong, C. H., Im, H. J., Kwon, Y., Kim, D., and Chun, Y. J. (2011) Induction of steroid sulfatase expression by tumor necrosis factor-alpha through phosphatidylinositol 3-kinase/Akt signaling pathway in PC-3 human prostate cancer cells, *Exp Mol Med 43*, 646-652.
27. Schneider, G., French, A., Bullock, L. P., and Bardin, C. W. (1971) Absence of androgen stimulation of hepatic steroid sulfatase in the female rat, *Endocrinology 89*, 308-310.
28. Lam, S. T., and Polani, P. E. (1985) Hormonal induction of steroid sulphatase in the mouse, *Experientia 41*, 276-278.
29. Snyder, V. L., Turner, M., Li, P. K., El-Sharkawy, A., Dunphy, G., and Ely, D. L. (2000) Tissue steroid sulfatase levels, testosterone and blood pressure, *J Steroid Biochem Mol Biol 73*, 251-256.
30. Barth, A., Romer, W., and Oettel, M. (2000) Influence of subchronic administration of oestrone-3-O-sulphamate on oestrone sulphatase activity in liver, spleen and white blood cells of ovariectomized rats, *Arch Toxicol 74*, 366-371.
31. Dominguez, O. V., Valencia, S. A., and Loza, A. C. (1975) On the role of steroid sulfates in hormone biosynthesis, *J Steroid Biochem 6*, 301-309.
32. Higashi, S., and Aizawa, Y. (1980) Effect of ACTH on adrenal estrogens, *Jpn J Pharmacol 30*, 273-278.
33. Odell, W. D., and Parker, L. N. (1984) Control of adrenal androgen production, *Endocr Res 10*, 617-630.
34. Miki, Y., Nakata, T., Suzuki, T., Darnel, A. D., Moriya, T., Kaneko, C., Hidaka, K., Shiotsu, Y., Kusaka, H., and Sasano, H. (2002) Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues, *J Clin Endocrinol Metab 87*, 5760-5768.
35. Suzuki, T., Miki, Y., Nakata, T., Shiotsu, Y., Akinaga, S., Inoue, K., Ishida, T., Kimura, M., Moriya, T., and Sasano, H. (2003) Steroid sulfatase and estrogen sulfotransferase in normal human tissue and breast carcinoma, *J Steroid Biochem Mol Biol 86*, 449-454.
36. Miksits, M., Wlcek, K., Svoboda, M., Thalhammer, T., Ellinger, I., Stefanzl, G., Falany, C. N., Szekeres, T., and Jaeger, W. (2010) Expression of sulfotransferases and sulfatases in human breast cancer: impact on resveratrol metabolism, *Cancer Lett 289*, 237-245.
37. Stowell, C. L., Barvian, K. K., Young, P. C., Bigsby, R. M., Verdugo, D. E., Bertozzi, C. R., and Widlanski, T. S. (2006) A role for sulfation-desulfation in the uptake of bisphenol a into breast tumor cells, *Chem Biol 13*, 891-897.

38. Geyer, J., Wilke, T., and Petzinger, E. (2006) The solute carrier family SLC10: more than a family of bile acid transporters regarding function and phylogenetic relationships, *Naunyn Schmiedebergs Arch Pharmacol 372*, 413-431.
39. Roth, M., Obaidat, A., and Hagenbuch, B. (2012) OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies, *Br J Pharmacol 165*, 1260-1287.
40. Moitra, K., Silverton, L., Limpert, K., Im, K., and Dean, M. (2011) Moving out: from sterol transport to drug resistance - the ABCG subfamily of efflux pumps, *Drug Metabol Drug Interact 26*, 105-111.
41. Pizzagalli, F., Varga, Z., Huber, R. D., Folkers, G., Meier, P. J., and St-Pierre, M. V. (2003) Identification of steroid sulfate transport processes in the human mammary gland, *J Clin Endocrinol Metab 88*, 3902-3912.
42. Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Davis, S., and Jones, M. (1999) Local estrogen biosynthesis in males and females, *Endocr Relat Cancer 6*, 131-137.
43. Fishman, J., Nisselbaum, J. S., Menendez-Botet, C. J., and Schwartz, M. K. (1977) Estrone and estradiol content in human breast tumors: relationship to estradiol receptors, *J Steroid Biochem 8*, 893-896.
44. Miyoshi, Y., Ando, A., Hasegawa, S., Ishitobi, M., Taguchi, T., Tamaki, Y., and Noguchi, S. (2003) High expression of steroid sulfatase mRNA predicts poor prognosis in patients with estrogen receptor-positive breast cancer, *Clin Cancer Res 9*, 2288-2293.
45. Suzuki, T., Nakata, T., Miki, Y., Kaneko, C., Moriya, T., Ishida, T., Akinaga, S., Hirakawa, H., Kimura, M., and Sasano, H. (2003) Estrogen sulfotransferase and steroid sulfatase in human breast carcinoma, *Cancer Res 63*, 2762-2770.
46. Stanway, S. J., Purohit, A., Woo, L. W., Sufi, S., Vigushin, D., Ward, R., Wilson, R. H., Stanczyk, F. Z., Dobbs, N., Kulinskaya, E., Elliott, M., Potter, B. V., Reed, M. J., and Coombes, R. C. (2006) Phase I study of STX 64 (667 Coumate) in breast cancer patients: the first study of a steroid sulfatase inhibitor, *Clin Cancer Res 12*, 1585-1592.
47. Zhang, Y., Qian, R. Q., and Li, P. P. (2009) Shikonin, an ingredient of Lithospermum erythrorhizon, down-regulates the expression of steroid sulfatase genes in breast cancer cells, *Cancer Lett 284*, 47-54.
48. Zhou, N., Han, S. Y., Zhou, F., and Li, P. P. (2014) Anti-tumor effect of Shu-gan-Liang-Xue decoction in breast cancer is related to the inhibition of aromatase and steroid sulfatase expression, *J Ethnopharmacol*.
49. Yang, B., Bhattacharyya, S., Linhardt, R., and Tobacman, J. (2012) Exposure to common food additive carrageenan leads to reduced sulfatase activity and increase in sulfated glycosaminoglycans in human epithelial cells, *Biochimie 94*, 1309-1316.
50. Purohit, A., and Foster, P. A. (2012) Steroid sulfatase inhibitors for estrogen- and androgen-dependent cancers, *J Endocrinol 212*, 99-110.
51. Strott, C. A., and Higashi, Y. (2003) Cholesterol sulfate in human physiology: what's it all about?, *J Lipid Res 44*, 1268-1278.
52. Canueto, J., Ciria, S., Hernandez-Martin, A., Unamuno, P., and Gonzalez-Sarmiento, R. (2010) Analysis of the STS gene in 40 patients with recessive X-linked ichthyosis: a high frequency of partial deletions in a Spanish population, *J Eur Acad Dermatol Venereol 24*, 1226-1229.
53. Matsumoto, J., Ariyoshi, N., Ishii, I., and Kitada, M. (2013) Functional characterization of seven single-nucleotide polymorphisms of the steroid sulfatase gene found in a Japanese population, *J Hum Genet 58*, 267-272.
54. Abdel-Hamed, M. F., Hussein, H. A., Helmy, N. A., and Elsaie, M. L. (2010) The detection of steroid sulfatase gene deletion (STS) in Egyptian males with X-linked ichthyosis, *J Drugs Dermatol 9*, 1192-1196.
55. Craig, W. Y., Roberson, M., Palomaki, G. E., Shackleton, C. H., Marcos, J., and Haddow, J. E. (2010) Prevalence of steroid sulfatase deficiency in California according to race and ethnicity, *Prenat Diagn 30*, 893-898.
56. Shapiro, L. J., Weiss, R., Buxman, M. M., Vidgoff, J., Dimond, R. L., Roller, J. A., and Wells, R. S. (1978) Enzymatic basis of typical X-linked icthyosis, *Lancet 2*, 756-757.
57. Shapiro, L. J., Yen, P., Pomerantz, D., Martin, E., Rolewic, L., and Mohandas, T. (1989) Molecular studies of deletions at the human steroid sulfatase locus, *Proc Natl Acad Sci U S A 86*, 8477-8481.
58. Alperin, E. S., and Shapiro, L. J. (1997) Characterization of point mutations in patients with X-linked ichthyosis. Effects on the structure and function of the steroid sulfatase protein, *J Biol Chem 272*, 20756-20763.

59. Williams, M. L., and Elias, P. M. (1981) Stratum corneum lipids in disorders of cornification: increased cholesterol sulfate content of stratum corneum in recessive x-linked ichthyosis, *J Clin Invest 68*, 1404-1410.
60. Hernandez-Martin, A., Gonzalez-Sarmiento, R., and De Unamuno, P. (1999) X-linked ichthyosis: an update, *Br J Dermatol 141*, 617-627.
61. Mathur, C., Prasad, V. V., Raju, V. S., Welch, M., and Lieberman, S. (1993) Steroids and their conjugates in the mammalian brain, *Proc Natl Acad Sci U S A 90*, 85-88.
62. Park-Chung, M., Malayev, A., Purdy, R. H., Gibbs, T. T., and Farb, D. H. (1999) Sulfated and unsulfated steroids modulate gamma-aminobutyric acidA receptor function through distinct sites, *Brain Res 830*, 72-87.
63. Bergeron, R., de Montigny, C., and Debonnel, G. (1996) Potentiation of neuronal NMDA response induced by dehydroepiandrosterone and its suppression by progesterone: effects mediated via sigma receptors, *J Neurosci 16*, 1193-1202.
64. Strous, R. D., Spivak, B., Yoran-Hegesh, R., Maayan, R., Averbuch, E., Kotler, M., Mester, R., and Weizman, A. (2001) Analysis of neurosteroid levels in attention deficit hyperactivity disorder, *Int J Neuropsychopharmacol 4*, 259-264.
65. Kent, L., Emerton, J., Bhadravathi, V., Weisblatt, E., Pasco, G., Willatt, L. R., McMahon, R., and Yates, J. R. (2008) X-linked ichthyosis (steroid sulfatase deficiency) is associated with increased risk of attention deficit hyperactivity disorder, autism and social communication deficits, *J Med Genet 45*, 519-524.
66. Puri, P. K., Reddi, D. M., Spencer-Manzon, M., Deak, K., Steele, S. U., and Mikati, M. A. (2012) Banding pattern on polarized hair microscopic examination and unilateral polymicrogyria in a patient with steroid sulfatase deficiency, *Arch Dermatol 148*, 73-78.
67. Brookes, K. J., Hawi, Z., Kirley, A., Barry, E., Gill, M., and Kent, L. (2008) Association of the steroid sulfatase (STS) gene with attention deficit hyperactivity disorder, *Am J Med Genet B Neuropsychiatr Genet 147B*, 1531-1535.
68. Davies, W. (2012) Does steroid sulfatase deficiency influence postpartum psychosis risk?, *Trends Mol Med 18*, 256-262.
69. Davies, W., Humby, T., Kong, W., Otter, T., Burgoyne, P. S., and Wilkinson, L. S. (2009) Converging pharmacological and genetic evidence indicates a role for steroid sulfatase in attention, *Biol Psychiatry 66*, 360-367.
70. Miksits, M., Wlcek, K., Svoboda, M., Kunert, O., Haslinger, E., Thalhammer, T., Szekeres, T., and Jager, W. (2009) Antitumor activity of resveratrol and its sulfated metabolites against human breast cancer cells, *Planta Med 75*, 1227-1230.
71. Mauvais-Jarvis, F., Clegg, D. J., and Hevener, A. L. (2013) The role of estrogens in control of energy balance and glucose homeostasis, *Endocr Rev 34*, 309-338.
72. Carr, M. C. (2003) The emergence of the metabolic syndrome with menopause, *J Clin Endocrinol Metab 88*, 2404-2411.
73. Margolis, K. L., Bonds, D. E., Rodabough, R. J., Tinker, L., Phillips, L. S., Allen, C., Bassford, T., Burke, G., Torrens, J., and Howard, B. V. (2004) Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial, *Diabetologia 47*, 1175-1187.
74. Zhu, L., Brown, W. C., Cai, Q., Krust, A., Chambon, P., McGuinness, O. P., and Stafford, J. M. (2013) Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance, *Diabetes 62*, 424-434.
75. Friday, K. E., Dong, C., and Fontenot, R. U. (2001) Conjugated equine estrogen improves glycemic control and blood lipoproteins in postmenopausal women with type 2 diabetes, *J Clin Endocrinol Metab 86*, 48-52.
76. Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B., and Cooke, P. S. (2000) Increased adipose tissue in male and female estrogen receptor-alpha knockout mice, *Proc Natl Acad Sci U S A 97*, 12729-12734.
77. Jones, M. E., Thorburn, A. W., Britt, K. L., Hewitt, K. N., Wreford, N. G., Proietto, J., Oz, O. K., Leury, B. J., Robertson, K. M., Yao, S., and Simpson, E. R. (2000) Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity, *Proc Natl Acad Sci U S A 97*, 12735-12740.

78. Hewitt, K. N., Pratis, K., Jones, M. E., and Simpson, E. R. (2004) Estrogen replacement reverses the hepatic steatosis phenotype in the male aromatase knockout mouse, *Endocrinology 145*, 1842-1848.
79. Smith, E. P., Boyd, J., Frank, G. R., Takahashi, H., Cohen, R. M., Specker, B., Williams, T. C., Lubahn, D. B., and Korach, K. S. (1994) Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man, *N Engl J Med 331*, 1056-1061.
80. Morishima, A., Grumbach, M. M., Simpson, E. R., Fisher, C., and Qin, K. (1995) Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens, *J Clin Endocrinol Metab 80*, 3689-3698.
81. Billeci, A. M., Paciaroni, M., Caso, V., and Agnelli, G. (2008) Hormone replacement therapy and stroke, *Curr Vasc Pharmacol 6*, 112-123.
82. Xu, Y., Nedungadi, T. P., Zhu, L., Sobhani, N., Irani, B. G., Davis, K. E., Zhang, X., Zou, F., Gent, L. M., Hahner, L. D., Khan, S. A., Elias, C. F., Elmquist, J. K., and Clegg, D. J. (2011) Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction, *Cell Metab 14*, 453-465.
83. Diano, S., Kalra, S. P., Sakamoto, H., and Horvath, T. L. (1998) Leptin receptors in estrogen receptor-containing neurons of the female rat hypothalamus, *Brain Res 812*, 256-259.
84. Rocha, M., Bing, C., Williams, G., and Puerta, M. (2004) Physiologic estradiol levels enhance hypothalamic expression of the long form of the leptin receptor in intact rats, *J Nutr Biochem 15*, 328-334.
85. Ainslie, D. A., Morris, M. J., Wittert, G., Turnbull, H., Proietto, J., and Thorburn, A. W. (2001) Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y, *Int J Obes Relat Metab Disord 25*, 1680-1688.
86. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver, *J Clin Invest 109*, 1125-1131.
87. Hughes, P. J., Lee, J. S., Reiner, N. E., and Brown, G. (2008) The vitamin D receptor-mediated activation of phosphatidylinositol 3-kinase (PI3Kalpha) plays a role in the 1alpha,25-dihydroxyvitamin D3-stimulated increase in steroid sulphatase activity in myeloid leukaemic cell lines, *J Cell Biochem 103*, 1551-1572.
88. Redig, A. J., and Munshi, H. G. (2010) Care of the cancer survivor: metabolic syndrome after hormone-modifying therapy, *Am J Med 123*, 87 e81-86.
89. Bryzgalova, G., Gao, H., Ahren, B., Zierath, J. R., Galuska, D., Steiler, T. L., Dahlman-Wright, K., Nilsson, S., Gustafsson, J. A., Efendic, S., and Khan, A. (2006) Evidence that oestrogen receptor-alpha plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver, *Diabetologia 49*, 588-597.
90. Bryzgalova, G., Lundholm, L., Portwood, N., Gustafsson, J. A., Khan, A., Efendic, S., and Dahlman-Wright, K. (2008) Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice, *Am J Physiol Endocrinol Metab 295*, E904-912.
91. Han, S. I., Komatsu, Y., Murayama, A., Steffensen, K. R., Nakagawa, Y., Nakajima, Y., Suzuki, M., Oie, S., Parini, P., Vedin, L. L., Kishimoto, H., Shimano, H., Gustafsson, J. A., and Yanagisawa, J. (2014) Estrogen receptor ligands ameliorate fatty liver through a nonclassical estrogen receptor/Liver X receptor pathway in mice, *Hepatology 59*, 1791-1802.
92. Wajchenberg, B. L. (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome, *Endocr Rev 21*, 697-738.
93. Bouchard, C., Despres, J. P., and Mauriege, P. (1993) Genetic and nongenetic determinants of regional fat distribution, *Endocr Rev 14*, 72-93.
94. Lovejoy, J. C., Champagne, C. M., de Jonge, L., Xie, H., and Smith, S. R. (2008) Increased visceral fat and decreased energy expenditure during the menopausal transition, *Int J Obes (Lond) 32*, 949-958.
95. Genazzani, A. R., and Gambacciani, M. (2006) Effect of climacteric transition and hormone replacement therapy on body weight and body fat distribution, *Gynecol Endocrinol 22*, 145-150.
96. Haarbo, J., Marslew, U., Gotfredsen, A., and Christiansen, C. (1991) Postmenopausal hormone replacement therapy prevents central distribution of body fat after menopause, *Metabolism 40*, 1323-1326.
97. Stubbins, R. E., Holcomb, V. B., Hong, J., and Nunez, N. P. (2012) Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance, *Eur J Nutr 51*, 861-870.
98. Wang, F., Vihma, V., Soronen, J., Turpeinen, U., Hamalainen, E., Savolainen-Peltonen, H., Mikkola, T. S., Naukkarinen, J., Pietilainen, K. H., Jauhiainen, M., Yki-Jarvinen, H., and Tikkanen, M. J. (2013) 17beta-

Estradiol and estradiol fatty acyl esters and estrogen-converting enzyme expression in adipose tissue in obese men and women, *J Clin Endocrinol Metab 98*, 4923-4931.

99. D'Eon, T. M., Souza, S. C., Aronovitz, M., Obin, M. S., Fried, S. K., and Greenberg, A. S. (2005) Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways, *J Biol Chem 280*, 35983-35991.
100. Lundholm, L., Zang, H., Hirschberg, A. L., Gustafsson, J. A., Arner, P., and Dahlman-Wright, K. (2008) Key lipogenic gene expression can be decreased by estrogen in human adipose tissue, *Fertil Steril 90*, 44-48.
101. Homma, H., Kurachi, H., Nishio, Y., Takeda, T., Yamamoto, T., Adachi, K., Morishige, K., Ohmichi, M., Matsuzawa, Y., and Murata, Y. (2000) Estrogen suppresses transcription of lipoprotein lipase gene. Existence of a unique estrogen response element on the lipoprotein lipase promoter, *J Biol Chem 275*, 11404-11411.
102. Hamosh, M., and Hamosh, P. (1975) The effect of estrogen on the lipoprotein lipase activity of rat adipose tissue, *J Clin Invest 55*, 1132-1135.
103. Delarue, J., and Magnan, C. (2007) Free fatty acids and insulin resistance, *Curr Opin Clin Nutr Metab Care 10*, 142-148.
104. Kim, J. Y., Jo, K. J., Kim, B. J., Baik, H. W., and Lee, S. K. (2012) 17beta-estradiol induces an interaction between adenosine monophosphate-activated protein kinase and the insulin signaling pathway in 3T3-L1 adipocytes, *Int J Mol Med 30*, 979-985.
105. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A., and Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance, *J Clin Invest 112*, 1821-1830.
106. Rogers, N. H., Perfield, J. W., 2nd, Strissel, K. J., Obin, M. S., and Greenberg, A. S. (2009) Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity, *Endocrinology 150*, 2161-2168.
107. Ribas, V., Drew, B. G., Le, J. A., Soleymani, T., Daraei, P., Sitz, D., Mohammad, L., Henstridge, D. C., Febbraio, M. A., Hewitt, S. C., Korach, K. S., Bensinger, S. J., and Hevener, A. L. (2011) Myeloid-specific estrogen receptor alpha deficiency impairs metabolic homeostasis and accelerates atherosclerotic lesion development, *Proc Natl Acad Sci U S A 108*, 16457-16462.
108. Hardie, D. G., Ross, F. A., and Hawley, S. A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis, *Nat Rev Mol Cell Biol 13*, 251-262.
109. He, A., Liu, X., Liu, L., Chang, Y., and Fang, F. (2007) How many signals impinge on GLUT4 activation by insulin?, *Cell Signal 19*, 1-7.
110. Ronda, A. C., Vasconsuelo, A., and Boland, R. (2010) Extracellular-regulated kinase and p38 mitogen-activated protein kinases are involved in the antiapoptotic action of 17beta-estradiol in skeletal muscle cells, *The Journal of endocrinology 206*, 235-246.
111. Rogers, N. H., Witczak, C. A., Hirshman, M. F., Goodyear, L. J., and Greenberg, A. S. (2009) Estradiol stimulates Akt, AMP-activated protein kinase (AMPK) and TBC1D1/4, but not glucose uptake in rat soleus, *Biochem Biophys Res Commun 382*, 646-650.
112. Riant, E., Waget, A., Cogo, H., Arnal, J. F., Burcelin, R., and Gourdy, P. (2009) Estrogens protect against high-fat diet-induced insulin resistance and glucose intolerance in mice, *Endocrinology 150*, 2109-2117.
113. Barros, R. P., and Gustafsson, J. A. (2011) Estrogen receptors and the metabolic network, *Cell Metab 14*, 289-299.
114. Maher, A. C., Akhtar, M., and Tarnopolsky, M. A. (2010) Men supplemented with 17beta-estradiol have increased beta-oxidation capacity in skeletal muscle, *Physiol Genomics 42*, 342-347.
115. Fu, M. H., Maher, A. C., Hamadeh, M. J., Ye, C., and Tarnopolsky, M. A. (2009) Exercise, sex, menstrual cycle phase, and 17beta-estradiol influence metabolism-related genes in human skeletal muscle, *Physiol Genomics 40*, 34-47.
116. Ribas, V., Nguyen, M. T., Henstridge, D. C., Nguyen, A. K., Beaven, S. W., Watt, M. J., and Hevener, A. L. (2010) Impaired oxidative metabolism and inflammation are associated with insulin resistance in ERalpha-deficient mice, *Am J Physiol Endocrinol Metab 298*, E304-319.
117. Tiidus, P. M., Holden, D., Bombardier, E., Zajchowski, S., Enns, D., and Belcastro, A. (2001) Estrogen effect on post-exercise skeletal muscle neutrophil infiltration and calpain activity, *Can J Physiol Pharmacol 79*, 400-406.

118. Dieli-Conwright, C. M., Spektor, T. M., Rice, J. C., Sattler, F. R., and Schroeder, E. T. (2009) Hormone therapy attenuates exercise-induced skeletal muscle damage in postmenopausal women, *J Appl Physiol (1985) 107*, 853-858.
119. Tiano, J. P., and Mauvais-Jarvis, F. (2012) Importance of oestrogen receptors to preserve functional beta-cell mass in diabetes, *Nat Rev Endocrinol 8*, 342-351.
120. Falany, C. N. (1997) Enzymology of human cytosolic sulfotransferases, *FASEB J 11*, 206-216.
121. Hazan, C., Orlow, S. J., and Schaffer, J. V. (2005) X-linked recessive ichthyosis, *Dermatol Online J 11*, 12.
122. Gao, J., He, J., Shi, X., Stefanovic-Racic, M., Xu, M., O'Doherty, R. M., Garcia-Ocana, A., and Xie, W. (2012) Sex-specific effect of estrogen sulfotransferase on mouse models of type 2 diabetes, *Diabetes 61*, 1543-1551.
123. Saini, S. P., Sonoda, J., Xu, L., Toma, D., Uppal, H., Mu, Y., Ren, S., Moore, D. D., Evans, R. M., and Xie, W. (2004) A novel constitutive androstane receptor-mediated and CYP3A-independent pathway of bile acid detoxification, *Mol Pharmacol 65*, 292-300.
124. Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R. M., and Xie, W. (2006) A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway, *J Biol Chem 281*, 15013-15020.
125. Borthwick, E. B., Houston, M. P., Coughtrie, M. W., and Burchell, A. (2001) The antihyperglycemic effect of estrone sulfate in genetically obese-diabetic (ob/ob) mice is associated with reduced hepatic glucose-6-phosphatase, *Horm Metab Res 33*, 721-726.
126. Gao, J., He, J., Zhai, Y., Wada, T., and Xie, W. (2009) The constitutive androstane receptor is an anti-obesity nuclear receptor that improves insulin sensitivity, *J Biol Chem 284*, 25984-25992.
127. Cani, P. D., Knauf, C., Iglesias, M. A., Drucker, D. J., Delzenne, N. M., and Burcelin, R. (2006) Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor, *Diabetes 55*, 1484-1490.
128. Al-Dosari, M. S., Knapp, J. E., and Liu, D. (2006) Activation of human CYP2C9 promoter and regulation by CAR and PXR in mouse liver, *Mol Pharm 3*, 322-328.
129. Gaikwad, N. W. (2013) Ultra performance liquid chromatography-tandem mass spectrometry method for profiling of steroid metabolome in human tissue, *Anal Chem 85*, 4951-4960.
130. He, J., Gao, J., Xu, M., Ren, S., Stefanovic-Racic, M., O'Doherty, R. M., and Xie, W. (2013) PXR ablation alleviates diet-induced and genetic obesity and insulin resistance in mice, *Diabetes*.
131. Gong, H., Jarzynka, M. J., Cole, T. J., Lee, J. H., Wada, T., Zhang, B., Gao, J., Song, W. C., DeFranco, D. B., Cheng, S. Y., and Xie, W. (2008) Glucocorticoids antagonize estrogens by glucocorticoid receptor-mediated activation of estrogen sulfotransferase, *Cancer Res 68*, 7386-7393.
132. Xie, W., Barwick, J. L., Downes, M., Blumberg, B., Simon, C. M., Nelson, M. C., Neuschwander-Tetri, B. A., Brunt, E. M., Guzelian, P. S., and Evans, R. M. (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR, *Nature 406*, 435-439.
133. Lin, X., Yue, P., Chen, Z., and Schonfeld, G. (2005) Hepatic triglyceride contents are genetically determined in mice: results of a strain survey, *Am J Physiol Gastrointest Liver Physiol 288*, G1179-1189.
134. Hawkins, R. A., and Oakey, R. E. (1974) Estimation of oestrone sulphate, oestradiol-17beta and oestrone in peripheral plasma: concentrations during the menstrual cycle and in men, *The Journal of endocrinology 60*, 3-17.
135. Gao, H., Bryzgalova, G., Hedman, E., Khan, A., Efendic, S., Gustafsson, J. A., and Dahlman-Wright, K. (2006) Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3, *Mol Endocrinol 20*, 1287-1299.
136. Leiter, E. H., and Chapman, H. D. (1994) Obesity-induced diabetes (diabesity) in C57BL/KsJ mice produces aberrant trans-regulation of sex steroid sulfotransferase genes, *J Clin Invest 93*, 2007-2013.
137. Finan, B., Yang, B., Ottaway, N., Stemmer, K., Muller, T. D., Yi, C. X., Habegger, K., Schriever, S. C., Garcia-Caceres, C., Kabra, D. G., Hembree, J., Holland, J., Raver, C., Seeley, R. J., Hans, W., Irmler, M., Beckers, J., de Angelis, M. H., Tiano, J. P., Mauvais-Jarvis, F., Perez-Tilve, D., Pfluger, P., Zhang, L., Gelfanov, V., DiMarchi, R. D., and Tschop, M. H. (2012) Targeted estrogen delivery reverses the metabolic syndrome, *Nat Med 18*, 1847-1856.

138. Aoki, K., Nakajima, A., Mukasa, K., Osawa, E., Mori, Y., and Sekihara, H. (2003) Prevention of diabetes, hepatic injury, and colon cancer with dehydroepiandrosterone, *J Steroid Biochem Mol Biol 85*, 469-472.
139. Maltais, R., and Poirier, D. (2011) Steroid sulfatase inhibitors: a review covering the promising 2000-2010 decade, *Steroids 76*, 929-948.
140. Petro, A. E., Cotter, J., Cooper, D. A., Peters, J. C., Surwit, S. J., and Surwit, R. S. (2004) Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse, *Metabolism 53*, 454-457.
141. Schaffer, J. E. (2003) Lipotoxicity: when tissues overeat, *Current opinion in lipidology 14*, 281-287.
142. Adlercreutz, H. (1970) Oestrogen metabolism in liver disease, *The Journal of endocrinology 46*, 129-163.
143. Li, C. P., Lee, F. Y., Hwang, S. J., Chang, F. Y., Lin, H. C., Lu, R. H., Hou, M. C., Chu, C. J., Chan, C. C., Luo, J. C., and Lee, S. D. (1999) Spider angiomas in patients with liver cirrhosis: role of alcoholism and impaired liver function, *Scand J Gastroenterol 34*, 520-523.
144. Green, J. R., Mowat, N. A., Fisher, R. A., and Anderson, D. C. (1976) Plasma oestrogens in men with chronic liver disease, *Gut 17*, 426-430.
145. Farinati, F., De Maria, N., Marafin, C., Fagiuoli, S., Della Libera, G., and Naccarato, R. (1995) Hepatocellular carcinoma in alcoholic cirrhosis: is sex hormone imbalance a pathogenetic factor?, *Eur J Gastroenterol Hepatol 7*, 145-150.
146. Nagasue, N., Ogawa, Y., Yukaya, H., Ohta, N., and Ito, A. (1985) Serum levels of estrogens and testosterone in cirrhotic men with and without hepatocellular carcinoma, *Gastroenterology 88*, 768-772.
147. Pignata, S., Daniele, B., Galati, M. G., Esposito, G., Vallone, P., Fiore, F., Ricchi, P., and Pergola, M. (1997) Oestradiol and testosterone blood levels in patients with viral cirrhosis and hepatocellular carcinoma, *Eur J Gastroenterol Hepatol 9*, 283-286.
148. Kley, H. K., Nieschlag, E., Wiegelmann, W., Solbach, H. G., and Kruskemper, H. L. (1975) Steroid hormones and their binding in plasma of male patients with fatty liver, chronic hepatitis and liver cirrhosis, *Acta Endocrinol (Copenh) 79*, 275-285.
149. Myking, O., Aakvaag, A., and Digranes, O. (1987) Androgen-oestrogen imbalance in men with chronic alcoholism and fatty liver, *Alcohol Alcohol 22*, 7-15.
150. Gavaler, J. S. (1995) Alcohol effects on hormone levels in normal postmenopausal women and in postmenopausal women with alcohol-induced cirrhosis, *Recent Dev Alcohol 12*, 199-208.
151. Long, R. S., and Simmons, E. E. (1951) The liver and estrogen metabolism; report of cases, *A.M.A. archives of internal medicine 88*, 762-769.
152. GLASS, S. J., EDMONDSON, H. A., and SOLL, S. N. (1940) SEX HORMONE CHANGES ASSOCIATED WITH LIVER DISEASE, *Endocrinology 27*, 749-752.
153. Becker, U. (1993) The influence of ethanol and liver disease on sex hormones and hepatic oestrogen receptors in women, *Dan Med Bull 40*, 447-459.
154. Myking, O. L., and Digranes, O. (1984) Conjugated and unconjugated plasma oestrogens in men with chronic alcoholism and in normal men, *J Steroid Biochem 20*, 799-801.
155. A Purohit, L. J. D., D Y Wang, N G Coldham, M W Ghilchik and M J Reed. (1997) Paracrine control of oestrogen production in breast cancer, *Endocr Rel Cancer*, 323–330.
156. Shimizu, I. (2012) Preventive Female Sex Factors Against the Development of Chronic Liver Disease
126.
157. El-Serag, H. B., Mason, A. C., and Key, C. (2001) Trends in survival of patients with hepatocellular carcinoma between 1977 and 1996 in the United States, *Hepatology 33*, 62-65.
158. Shi, L., Feng, Y., Lin, H., Ma, R., and Cai, X. (2014) Role of estrogen in hepatocellular carcinoma: is inflammation the key?, *J Transl Med 12*, 93.
159. Barrett, T., Troup, D. B., Wilhite, S. E., Ledoux, P., Evangelista, C., Kim, I. F., Tomashevsky, M., Marshall, K. A., Phillippy, K. H., Sherman, P. M., Muertter, R. N., Holko, M., Ayanbule, O., Yefanov, A., and Soboleva, A. (2011) NCBI GEO: archive for functional genomics data sets--10 years on, *Nucleic Acids Res 39*, D1005-1010.
160. Strom, S. C., Pisarov, L. A., Dorko, K., Thompson, M. T., Schuetz, J. D., and Schuetz, E. G. (1996) Use of human hepatocytes to study P450 gene induction, *Methods in enzymology 272*, 388-401.
161. Selcer, K. W., and Difrancesca, H. M. (2012) Characterization of steroid sulfatase in the MC3T3-E1 mouse pre-osteoblastic cell line, *Steroids 77*, 696-702.

162. Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W. W., and Lenhard, B. (2004) JASPAR: an open-access database for eukaryotic transcription factor binding profiles, *Nucleic Acids Res 32*, D91-94.
163. Nowak, D. E., Tian, B., and Brasier, A. R. (2005) Two-step cross-linking method for identification of NF-kappaB gene network by chromatin immunoprecipitation, *BioTechniques 39*, 715-725.
164. Affo, S., Dominguez, M., Lozano, J. J., Sancho-Bru, P., Rodrigo-Torres, D., Morales-Ibanez, O., Moreno, M., Millan, C., Loaeza-del-Castillo, A., Altamirano, J., Garcia-Pagan, J. C., Arroyo, V., Gines, P., Caballeria, J., Schwabe, R. F., and Bataller, R. (2013) Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis, *Gut 62*, 452-460.
165. Zimmermann, H. W., Seidler, S., Gassler, N., Nattermann, J., Luedde, T., Trautwein, C., and Tacke, F. (2011) Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis, *PLoS One 6*, e21381.
166. Sun, B., and Karin, M. (2008) NF-kappaB signaling, liver disease and hepatoprotective agents, *Oncogene 27*, 6228-6244.
167. Ding, W. X., and Yin, X. M. (2004) Dissection of the multiple mechanisms of TNF-alpha-induced apoptosis in liver injury, *Journal of cellular and molecular medicine 8*, 445-454.
168. Holden, N. S., Squires, P. E., Kaur, M., Bland, R., Jones, C. E., and Newton, R. (2008) Phorbol ester-stimulated NF-kappaB-dependent transcription: roles for isoforms of novel protein kinase C, *Cellular signalling 20*, 1338-1348.
169. Pessara, U., and Koch, N. (1990) Tumor necrosis factor alpha regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-kappa B-like factor to a promoter element, *Mol Cell Biol 10*, 4146-4154.
170. Straub, R. H. (2007) The complex role of estrogens in inflammation, *Endocrine reviews 28*, 521-574.
171. Villeneuve, J. P., and Pichette, V. (2004) Cytochrome P450 and liver diseases, *Current drug metabolism 5*, 273-282.
172. Zumoff, B., Fishman, J., Gallagher, T. F., and Hellman, L. (1968) Estradiol metabolism in cirrhosis, *J Clin Invest 47*, 20-25.
173. Aitken, A. E., Richardson, T. A., and Morgan, E. T. (2006) Regulation of drug-metabolizing enzymes and transporters in inflammation, *Annu Rev Pharmacol Toxicol 46*, 123-149.
174. Kalaitzidis, D., and Gilmore, T. D. (2005) Transcription factor cross-talk: the estrogen receptor and NF-kappaB, *Trends Endocrinol Metab 16*, 46-52.
175. Uno, K., Katagiri, H., Yamada, T., Ishigaki, Y., Ogihara, T., Imai, J., Hasegawa, Y., Gao, J., Kaneko, K., Iwasaki, H., Ishihara, H., Sasano, H., Inukai, K., Mizuguchi, H., Asano, T., Shiota, M., Nakazato, M., and Oka, Y. (2006) Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity, *Science 312*, 1656-1659.
176. Trent, S., Dennehy, A., Richardson, H., Ojarikre, O. A., Burgoyne, P. S., Humby, T., and Davies, W. (2012) Steroid sulfatase-deficient mice exhibit endophenotypes relevant to attention deficit hyperactivity disorder, *Psychoneuroendocrinology 37*, 221-229.
177. Riechman, S. E., Fabian, T. J., Kroboth, P. D., and Ferrell, R. E. (2004) Steroid sulfatase gene variation and DHEA responsiveness to resistance exercise in MERET, *Physiol Genomics 17*, 300-306.
178. Kawano, H., Yasue, H., Kitagawa, A., Hirai, N., Yoshida, T., Soejima, H., Miyamoto, S., Nakano, M., and Ogawa, H. (2003) Dehydroepiandrosterone supplementation improves endothelial function and insulin sensitivity in men, *J Clin Endocrinol Metab 88*, 3190-3195.
179. Weiss, E. P., Villareal, D. T., Fontana, L., Han, D. H., and Holloszy, J. O. (2011) Dehydroepiandrosterone (DHEA) replacement decreases insulin resistance and lowers inflammatory cytokines in aging humans, *Aging (Albany NY) 3*, 533-542.
180. Aoki, K., Kikuchi, T., Mukasa, K., Ito, S., Nakajima, A., Satoh, S., Okamura, A., and Sekihara, H. (2000) Dehydroepiandrosterone suppresses elevated hepatic glucose-6-phosphatase mRNA level in C57BL/KsJ-db/db mice: comparison with troglitazone, *Endocr J 47*, 799-804.
181. Coleman, D. L., Leiter, E. H., and Schwizer, R. W. (1982) Therapeutic effects of dehydroepiandrosterone (DHEA) in diabetic mice, *Diabetes 31*, 830-833.
182. Sato, K., Iemitsu, M., Aizawa, K., Mesaki, N., Ajisaka, R., and Fujita, S. (2012) DHEA administration and exercise training improves insulin resistance in obese rats, *Nutr Metab (Lond) 9*, 47.