

# **THE ROLE OF ESTROGEN SULFOTRANSFERASE IN HUMAN ADIPOGENESIS**

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# **THE ROLE OF ESTROGEN SULFOTRANSFERASE IN HUMAN ADIPOGENESIS**

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**University of Pittsburgh, 2014**

Estrogen Sulfotransferase (EST/SULT1E1) is a phase II conjugating enzyme which catalyzes the sulfonation of estrogen and estrogen like compounds. EST belongs to a large class of cytosolic sulfotransferases that are widespread in human and animal tissues, and facilitate sulfoconjugation through a donor co-substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS). EST is an estrogen preferring sulfotransferase; among all human sulfotransferases it has the highest affinity for Estradiol (E2).

The goal of this dissertation is to determine whether and how EST plays a role in human adipogenesis. Adipogenesis is the process whereby preadipocytes are stimulated to differentiate into functional mature adipocytes. The role of EST in adipogenesis has been studied in rodents, however in humans no studies have been published that address the role of EST in adipogenesis. In this dissertation we have uncovered a novel role for estrogen sulfotransferase in human adipogenesis. By using human primary adipose-derived stem cells (ASCs) and whole-fat tissues from the abdominal subcutaneous fat of obese and non-obese subjects, we showed that the expression of EST was low in preadipocytes but increased upon differentiation. Overexpression and knockdown of EST in ASCs promoted and inhibited differentiation, respectively. The pro-adipogenic activity of EST in humans was opposite to the anti-adipogenic effect of the same

enzyme in rodents. Mechanistically, EST promoted adipogenesis by deactivating estrogens. The pro-adipogenic effect of EST can be recapitulated by using an estrogen receptor (ER) antagonist or ER $\alpha$  knockdown. In contrast, activation of ER in ASCs inhibited adipogenesis by decreasing the recruitment of the adipogenic peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) onto its target gene promoters, whereas ER antagonism increased the recruitment of PPAR $\gamma$  to its target gene promoters. Linear regression analysis revealed a positive correlation between the expression of EST and body mass index (BMI), as well as a negative correlation between ER $\alpha$  expression and BMI. We conclude that EST is a pro-adipogenic factor which may serve as a druggable target to inhibit the turnover and accumulation of adipocytes in obese patients.

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## **Preface**

This dissertation is dedicated to my family and friends whom have supported me along my journey, especially my mother Dr. Debra Ihunnah and my late father Dr. Anthony Ihunnah.

## ABBREVIATIONS

**ACC-1**, acetyl CoA carboxylase 1; **AF-1**, activation function 1; **AF-2**, activation function 2; **AMPK**, AMP-activated protein kinase; **aP2**, Adipocyte Binding Protein ; **AR**, androgen receptor; **ARE**, androgen receptor responsive element; **ATGL**, Adipocyte Triglyceride Lipase; **BAT**, brown adipose tissue; **BrdU**, bromodeoxyuridine; **CREB**, cAMP-responsive-element-binding protein; **CYP**, Cytochrome P450; **DEX**, Dexamethasone; **DMSO**, Dimethyl sulfoxide; **DR-1**, direct repeat spaced by one nucleotide; **DHT**, dihydrotestosterone; **E2**, 17 $\beta$ -estradiol; **EST**, estrogen sulfotransferase; **ER $\alpha/\beta$** , Estrogen Receptor Alpha and Beta **FABP**, fatty acid binding protein; **FAS**, fatty acid synthase; **FAT**, fatty acid translocase; **FATP**, fatty acid transporter protein; **FBS**, fetal bovine serum; **FFA**, free fatty acid; **HSL**, Hormone Sensitive Lipase, **IGF-1**, insulin-like growth factor 1; **LDLR**, low density lipoprotein receptor; **LPL**, Lipoprotein Lipase; **LXR**, liver X receptor; **NCoR**; nuclear receptor corepressor; **PAPS**, 3'-phosphoadenosine-5'-phosphosulfate; **PPAR $\gamma$** , Peroxisome Proliferator Activated Receptor Gamma; **RXR**, retinoid X receptor; **SCD-1**, stearoyl CoA desaturase-1; **SHP**, short heterodimer partner; **SMRT**, silencing mediator or retinoid and thyroid receptors; **SR**, scavenger receptor; **SRC**, steroid receptor coactivator; **STS**, steroid sulfatase; **SULT**, sulfotransferase; **T**, testosterone; **VLDL**, very low density lipoprotein; **VLDLR**, very low density lipoprotein receptor; **VP**, viral protein 16; **WAT**, white adipose tissue; **WT**, wild type

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## **1.0 CHAPTER ONE: ESTROGEN SULFOTRANSFERASE/ SULT1E1**

### **1.1 INTRODUCTION**

Estrogen Sulfotransferase (EST/SULT1E1) is a phase II metabolic enzyme which catalyzes the sulfonation of estrogen and estrogen like compounds. The human EST gene is located on chromosome 4q13.2, and the EST protein consists of 294 amino acids [1]. EST belongs to a large class of cytosolic sulfotransferases that are widespread in human and animal tissues. facilitate sulfoconjugation through a donor co-substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to a host of endogenous and exogenous compounds such as xenobiotic chemicals, pharmaceuticals, endogenous molecules such as fatty acids, steroids, and neurotransmitters [2, 3]. Sulfonation plays an important role in the metabolism and biotransformation of the aforementioned substances in a number of ways including increasing their water solubility and urinary excretion, bioactivation and bio-inactivation [4].

EST is an estrogen preferring sulfotransferase, among all human sulfotransferases it has the highest affinity for Estradiol (E2) and can also sulfonate estrone (E1). EST also has been shown to sulfonate catecholestrogens and 2-methoxyestradiol [2, 4-6]. The sulfoconjugation of E2 and other estrogens by EST deactivates the hormone, inhibiting its ability to bind to the estrogen receptor alpha or beta (ER $\alpha$ / $\beta$ ). Sulfation of estrogen is reversible by hydrolysis mediated by the cytosolic phosphatase Steroid Sulfatase (STS), creating an EST-STs regulated estrogen reservoir [5].

The regulation of estrogen is the primary function of EST, and the functional effects of this regulation are an integral part of normal mammalian metabolism. EST is expressed in a number of tissue including the mouse and bovine placenta, human mammary and endometrial epithelial cells, guinea pig adrenal gland, mouse uterus; mouse, rat and human testis [7-13]. EST largely regulates local tissue concentrations of estrogen, and it has been shown to be a potent protective enzyme against toxic estrogenic effects in peripheral tissues [14]. Dysregulation of EST has been shown to cause severe physiological consequences in the local tissue which it regulates. In male mice the expression of EST maintains functional integrity of the epididymis by regulating luminal estrogen concentrations [3, 15]. It has been shown that EST is expressed abundantly in the reproductive tract, specifically in the epithelium of the epididymis (corpus and cauda), the vas deferens and small amounts in the prostate and seminal vesicle [15]. This is important because normal functioning of the epididymis is dependent on maintaining a specific ratio of androgen and estrogen activity; moreover it has been shown that sperm motility is greatly reduced in EST knockout mice, compared with wild-type controls [15]. Additionally, estrogen challenge in wild type mice that underwent epididymal ligation also showed marked reductions in sperm quality and motility [15]. These results demonstrate a specific role for EST in the epididymis in reducing local estrogen level and activity.

In human umbilical vein endothelial cells, EST has been shown to regulate the inflammatory response and lipid metabolism [16]. Specifically in the presence of exogenous estrogen, loss of EST using siRNA caused decreased expression of anti-inflammatory cytokines, while pro-inflammatory cytokines were upregulated; moreover PPAR $\gamma$  expression and the expression of its target genes involved in cholesterol metabolism were downregulated, including LDL-R, APO B and FASN [16]. SULT1E1 also plays a role in cancer susceptibility. It has been well established

that estrogens are an integral mediator in hormone dependent cancer. In several cohorts of female patients, mutations in the *SULT1E1* gene caused decreased EST expression in endometrial, ovarian and breast cancer tissue and is associated with poor survival and reoccurrence of malignant tissue [1, 14, 17].

The functional role of Est in the adipogenesis has been studied in animal models, however the overall functional impact is still controversial. Several *in vivo* and *in vitro* studies have shown that in female murine models Est acts a negative regulator of adipogenesis [3, 18]; aP2-Est transgenic mice over-expressing Est in adipocytes have decreased adipocyte size and improved hepatic insulin sensitivity, but reduced adipose tissue glucose uptake [18]. While other *in vivo* murine models have shown that the induction of Est is associated with hepatic insulin resistance, moreover ablation of Est was shown to improve metabolic function in female mice which includes increased energy expenditure, insulin sensitivity, and body composition [19]. Mechanistically, the improved metabolic function was attenuated after ovariectomy which suggests that the metabolic benefit was mediated by increased estrogenic activity in the liver [19].

In humans no studies have been published that address the role of EST in adipogenesis. Recently it was reported that EST is expressed in the subcutaneous adipose tissue of obese patients suffering from metabolic syndrome; additionally that same study reported that a positive correlation was found between the expression of EST and inflammatory cytokine signaling in adipose tissue [20]. Our study sought to examine the functional role of EST in human adipose tissue during adipogenesis.

## 1.2 NUCLEAR RECEPTOR DEPENDENT ROLE IN ADIPOSE TISSUE DEVELOPMENT

### 1.2.1 Peroxisome Proliferator Gamma (PPAR $\gamma$ )

Peroxisome Proliferator Gamma (PPAR $\gamma$ , NR1C3) belongs to the PPAR subfamily, which is part of the nuclear receptor superfamily of ligand activated transcription factors [21]. PPAR $\gamma$  is expressed in a number of tissue types, but its expression is highest in brown and white adipose tissue [21-23]. It is known for its role in lipid metabolism and glucose tolerance, and its dysregulation leads to a number of pathologies including hyperlipidemia, insulin resistance, and coronary artery disease[24]. PPAR gamma is activated by a number of lipid molecules including polyunsaturated fatty acids, nitrated unsaturated fatty acids and eicosanoids [25, 26].

Additionally, PPAR $\gamma$  is the target for the thiazolidinedione (TZD) class of fibrate anti-diabetic drugs [24, 27, 28].

Like many nuclear receptors, activation of PPAR $\gamma$  causes its translocation to the nucleus and heterodimerization with the Retinoid X Receptor (RXR), which increases its affinity for DNA. The PPAR $\gamma$  -RXR heterodimer binds to a short stretch of DNA called the PPAR response Element (PPRE) located in the proximal or distal promoter region of its targets genes. In order for transcription of its target genes to begin, the appropriate coactivators must also form a complex with PPAR $\gamma$ ; these coactivators possess histone acetyltransferase activity and other chromatin remodeling properties [21, 24, 29].

PPAR $\gamma$  has been well established as the most important transcription factor induced during the process of adipogenesis. In some fibroblast models activation of PPAR $\gamma$  alone is sufficient to

induce adipogenesis, while loss of function models *in vivo* and *in vitro* show that adipocytes cannot develop without PPAR $\gamma$  signaling; additionally PPAR $\gamma$  null mice die during embryogenesis. In humans, rare mutations in the DNA and ligand binding domain of PPAR $\gamma$  cause severe lipodystrophy and insulin resistance in these patients [30, 31].

PPAR $\gamma$  functions in adipogenesis as part of a spatial and temporal cascade of transcription factors and enzymes that facilitate the proper development and maintenance of each adipocyte [23]. During differentiation mitogenic and adipogenic cues activate several members of the CAAT/ Enhancer Binding Protein family(C/EBP); (*Covered more extensively in section 1.2.3*) specifically C/EBP $\beta$  and C/EBP $\delta$  work in tandem to promote the activation of PPAR $\gamma$  by directly binding to the promoter region of PPAR $\gamma$  [29, 32]. Once PPAR $\gamma$  has been induced and activated by a ligand it can then induce C/EBP $\alpha$ , which further induces PPAR $\gamma$  in a positive feedback loop; PPAR $\gamma$  can then bind the promoter regions of its target genes including Fatty Acid Binding Protein 4(FABP4)/ Adipocyte factor 2(aP2), Lipoprotein Lipase (LPL), and the Glucose Transporter 4 (GLUT4)[21, 24, 27, 33, 34]. LPL and aP2 are classic markers of adipogenesis, LPL serves as a dual action triglyceride hydrolase as well as bridging factor for membrane receptor mediated lipoprotein uptake; while aP2 serves a potent intracellular transporter that controls metabolism of long and short chain fatty acids as well as other hydrophobic ligands [35, 36]. Throughout this document, these genes (LPL, aP2, C/EBP $\alpha$ ) will be used to measure the extent of PPAR $\gamma$  activation and adipogenesis in various conditions.

### 1.2.2 Estrogen Receptor Alpha/Beta (ER $\alpha$ / $\beta$ )

The Estrogen Receptor alpha and beta (ER $\alpha$ / $\beta$ ) are part of the nuclear receptor superfamily of ligand activated transcription factors (NR3A1 and NR3A2 respectively)[37]. Once activated by the appropriate ligand, these receptors undergo a conformational change in the LBD that promotes dissociation from corepressor proteins, cytosolic heat shock proteins, chaperone proteins and cochaperone proteins [38, 39]. The ER is then shuttled into the nucleus, can homodimerize and bind to short stretches of DNA called estrogen response elements (ERE), which are located in the promoter region of their target genes [40]. In order for the ERs to elicit transcriptional activation, a complex of coregulators must also bind to the receptor at distinct Leucine rich motifs (LeuXxxXxxLeuLeu, LXXLL) [41]. The coregulators that activate transcription are known as coactivators; they possess histone and chromatin remodeling capabilities that allow the circumvention of repressive chromatin features and subsequent activation of gene transcription through general transcription machinery [41, 42].

ER $\alpha$ / $\beta$  is expressed in male and female subcutaneous preadipocytes and adipocytes as well as in visceral adipose tissue, rendering these tissues amenable to changes caused by estrogen signaling [40, 43]. This expression has been found in several other species including mice, rats, and sheep as well [40]. Hormone signaling plays a major role in adipose tissue development, maintenance, and apoptosis. In addition to other critical hormones involved in adipogenesis such growth hormone, thyroid hormone and glucocorticoids; sex hormones also play an integral role in adipose tissue development, and often lead to sexually dimorphic manifestations of adipocyte size, number, depot location, and life cycle [44-47]. Men tend to have a central/abdominal deposition of adipose tissue, whereas females accumulate adipose tissue in the gluteal and

femoral regions [46-50]. Epidemiologic evidence shows that menopause typically leads to a central distribution of adipose tissue, demonstrating the effects sex hormones, and in particular estrogen, have on adipose tissue development and distribution [51-54]. The mechanisms delineating the sexually dimorphic effects of hormone signaling on adipose tissue development are not entirely clear; subcutaneous adipose tissue has a higher concentration of estrogen and progesterone receptors compared to visceral adipose tissue, which has a higher concentration of the androgen receptor [44]. Evidence also suggests that changes in eating and energy expenditure are partly the cause [44, 47]; however a large body of evidence from studies in mice suggests that these differences are due to genetic and transcriptional changes that effect the expression of key proteins involved in adipogenesis [54-56].

In subcutaneous adipose tissue  $ER\alpha$  and  $ER\beta$  are both expressed, however  $ER\alpha$  activation has been shown to effect energy homeostasis and mediate estradiol's genomic effects on body weight and adipogenesis [44]. There are several known anti-adipogenic genomic effects found after estradiol treatment such as reductions in Lipoprotein Lipase concentration (LPL), as well as reduced expression of the Sterol Regulatory Element-Binding Protein (SREBP1c) [40, 54]. LPL is a  $PPAR\gamma$  target gene and an important enzyme expressed during adipogenesis [21]; SREBP1c is an important transcription factor that enhances lipogenesis mainly through the up-regulation of its lipogenic target genes [40]. LPL and SREBP1c both contain an ERE in their promoter region, which suggests that  $ER\alpha$  can directly inhibit the transcription of said genes, culminating in a reduction of adipogenesis and lipogenesis [40, 57, 58].

The genomic effects mediated by estrogen signaling lead to significant functional changes in adipose tissue accumulation and distribution. Several studies have shown that loss of estrogen

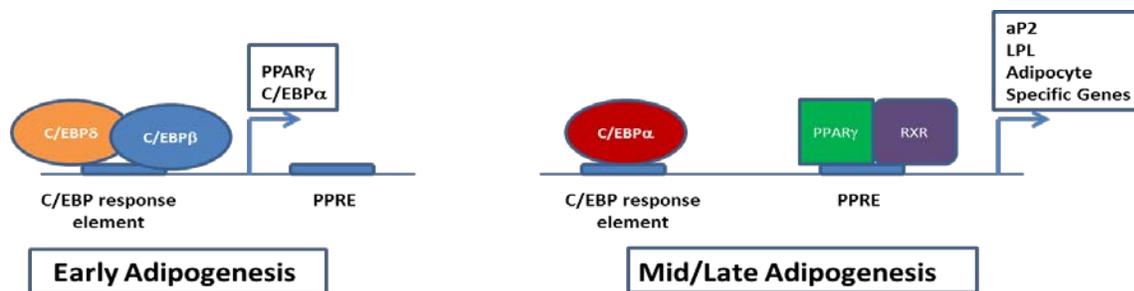
signaling in mice, either through genetic knockout of the ER $\alpha$  receptor, genetic knockout of aromatase, or through ovariectomy lead to a significant increase in adiposity [43, 44, 55]. In humans, it is well documented that post- menopausal women tend to accumulate adipose tissue in the abdominal region, however this can be reversed by estrogen replacement therapy [40, 59]. Men lacking aromatase/CYP19A1, a member of the cytochrome P450 family that catalyzes the aromatization of androgens and converts them to estrogens, have increased body fat, insulin resistance and Type II diabetes, which can also be reversed by estrogen treatment [40, 60, 61].

### **1.2.3 CCAAT Enhancer Binding Protein Family C/EBP family**

The CCAAT binding protein (C/EBP) family of transcription factors is aptly named based on the short stretch of DNA that it binds in the promoter regions of its target genes. This family of proteins is made of several major isoforms, C/EBP alpha, beta, delta, gamma and epsilon [62, 63]. Each of the members of this family contain a conserved basic leucine zipper (bzip) DNA binding domain that promotes the binding of said transcription factors to the CCAAT motif [24, 64]. In contrast, these transcription factors share very little sequence homology in the N-terminal region of the protein which house transrepression and transactivation domains [65]. The C/EBP proteins are expressed in the nucleus of most cell types of the body and are involved in an array of functions including cell cycle regulation, body weight homeostasis, immune and inflammatory responses, macrophage and adipose tissue differentiation [62, 63, 65-72].

In adipose tissue differentiation C/EBP $\beta/\delta/\alpha$  display distinct temporal and spatial expression patterns which regulate adipose tissue development and maintenance [64]. Specifically, these proteins play a critical role in the induction of PPAR $\gamma$  and many PPAR $\gamma$  target genes. It has been demonstrated *in vivo* and *in vitro* that during the initiation of adipogenesis, C/EBP $\beta$  and C/EBP $\delta$

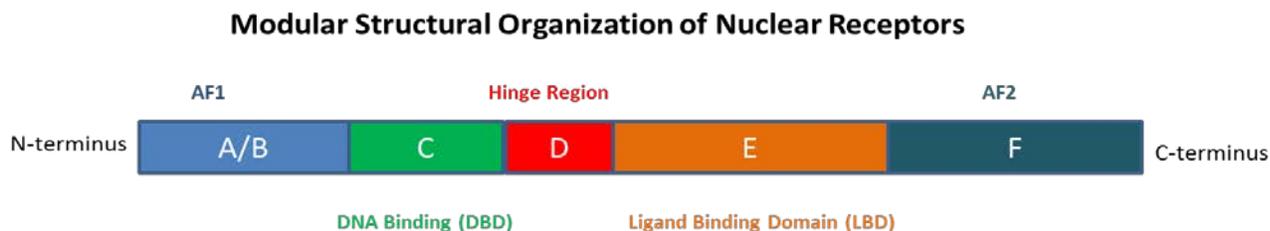
form hetero/homodimers that promote the expression of PPAR $\gamma$  through direct interaction with the distal enhancer region of the PPAR $\gamma$  promoter [32, 73]. Once PPAR $\gamma$  is activated it then promotes the expression of C/EBP $\alpha$ , which further enhances the expression of PPAR $\gamma$  itself by binding to a CCAAT motif in the PPAR $\gamma$  distal promoter [32, 34, 73]. This positive feedback loop is important because many of PPAR $\gamma$ 's target gene promoters also contain CCAAT motifs in their distal promoter regions; C/EBP $\alpha$  binds to these motifs and enhances the expression of adipogenic genes such as LPL, aP2, PEPCCK, and GLUT4 [21, 32, 73]. Additionally, the expression of C/EBP $\alpha$  is critical to maintain insulin sensitivity; it has been shown that the activation of PPAR $\gamma$  in C/EBP $\alpha$ -deficient cells leads to abnormally low insulin sensitivity. Several other papers have confirmed that C/EBP $\alpha$  contributes to the full adipose program beyond simply maintaining PPAR $\gamma$  and PPAR $\gamma$  target gene expression [21, 74].



**Figure 1. C/EBP Family Induction of Adipogenic Genes.** Adopted from : Lefterova, M.I. and M.A. Lazar, *New developments in adipogenesis*. Trends Endocrinol Metab, 2009. **20**(3): p. 107-14. [24]. C/EBP $\beta$  and  $\delta$  bind to their response element early, also C/EBP $\alpha$  and PPAR $\gamma$  binding to its PPRE mid/late during adipogenesis.

### 1.2.4 Nuclear Receptors and Coregulators in Nuclear Receptor Pharmacology

The nuclear receptor (NR) superfamily is made up of 49 ligand activated transcription factors which share considerable structural similarity. These receptors mediate the response of steroids, retinoids, fatty acids, thyroid hormones, and pharmaceuticals to regulate endo- and xenobiotic metabolism, as well as a host of physiological processes [75]. Nuclear receptors are modular proteins that contain the following domains; the N-Terminal (**A-B**) domain which contains the Activation Factor-1 (AF-1) transactivation domain that is activated independent of ligand binding, the (**C**) domain which contains the DNA binding domain which is comprised of two zinc fingers which recognizes short conserved DNA motifs, the (**D**) domain which is a flexible hinge region, the (**E**) domain which contains the ligand binding domain, and the C-Terminal (**F**) domain which contains the Activation Factor-2 domain (AF-2) is a ligand dependent activation domain [41, 75, 76].



**Figure 2. Modular Structure of Nuclear Receptors.**

A general model of nuclear receptor signaling is that in the absence of ligand NRs remain sequestered in the cytosol by corepressors, heat shock proteins, chaperone proteins, and cochaperone proteins [41, 75]. Additionally, these repressor proteins promote an inactive conformation in the LBD that decreases the affinity of the NR for coactivators and its cognate DNA response element [38]. Ligand binding causes a conformational change in the LBD that enhances the binding affinity of the AF2 region and LBD with specific amino acid motifs found in coactivators [77, 78]. Nuclear receptor coactivators contain an “NR box” sequence, a leucine rich stretch of amino acids, LXXLL, or sometimes FXXLF [41, 77]. An array of nuclear receptors contain single or multiple copies of the NR box which dictates the specificity and affinity of each NR for certain coactivators [41]. The recognition of this sequence by various nuclear receptors is based on hydrophobic interactions and hydrogen bonding between specific charged amino acid residues that make up the LBD [79]. A “charge clamp” is formed by a lysine residue in helix 3 and a glutamic acid residue in helix 12 that clamp the NR box  $\alpha$ -helix in place by forming hydrogen bonds with the LXXLL coactivator motif [79]. This motif has been shown to facilitate direct binding of coactivators to nuclear receptors, however only about half of all coactivators contain this motif but they are still able to bind and activate nuclear receptors [41].

The recruitment of coactivators can enhance the ability of nuclear receptors to bind to their specific DNA motif known as a hormone response element (HRE)[41, 80]. Coactivators enhance transcriptional activation in a number of ways; many contain methyltransferase and acetyltransferase activity which is essential for chromatin and histone remodeling [80]. In addition to enzymatic properties, coactivators can act as scaffolding proteins or also convey post translational modifications (PTMs) that act on nuclear receptors and other coactivators [77].

These PTMs can enhance DNA binding and stabilize the transcription complex involved in initiation, elongation, and termination [41, 42, 77, 81]. Common coactivator PTMs include methylation, acetylation, phosphorylation, sumoylation, and ubiquitination[41]. To date over 300 different coactivators have been identified and can affect a host of cellular processes [42]. Some of the most studied coactivators include the steroid receptor coactivator family (SRC) which includes SRC-1, 2, and 3. SRCs are recruited to NRs early during transcription because they serve as scaffolding proteins for other coactivators and transcriptional machinery to bind [42]. Other important coactivators include CREB binding Protein (CBP), which possesses histone acetylase activity and PPAR $\gamma$  co-activator-1a (PGC-1a) which utilizes both phosphorylation and acetylation to regulate NR activity [81].

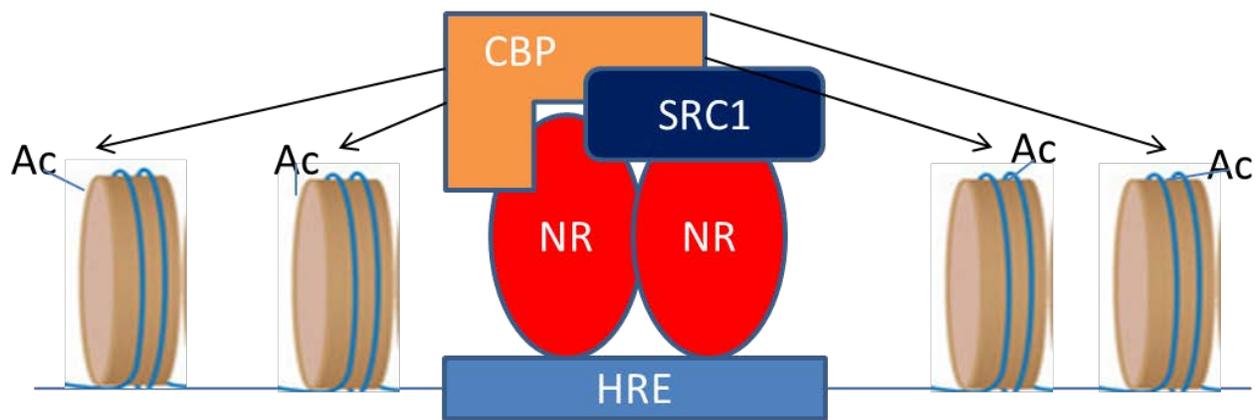
In addition to coactivator binding that enhances NR activation, corepressors are also a class of coregulators that can bind to NRs and alter chromatin structure towards an inactive state. These proteins can recruit histone deacetylases, increase nuclear export, and promote cytoplasmic sequestration of their target NRs [42]. Some of the most well studied corepressors include nuclear corepressor 1/ 2 (NCoR 1 and 2) which use phosphorylation to increase NR nuclear export, as well as SHARP which uses phosphorylation to increase transcriptional repression of its NR targets [81, 82].

The concentration, activity, and affinity of coactivators for nuclear receptors are largely cell dependent and are subject to temporal and spatial changes [83]. Additionally, many nuclear receptors share the same coactivators, therefore competition known as “squelching” or “crosstalk” can occur [41]. This phenomenon can occur naturally when the activation of one nuclear receptor by its ligand inhibits the transcriptional pathway controlled by another nuclear receptor that shares the same coactivator(s) [41, 84-87]. Crosstalk between nuclear receptors can

have significant effects on cell growth, proliferation, and differentiation; sometimes leading to dysregulation of critical metabolic processes [87, 88].

The importance of coregulator function at the molecular level is exemplified by the array of clinical pathologies that manifest from loss or perturbation of coactivator or corepressor signaling. These diseases include but are not limited to Rubinstein-Taybi Syndrome (RTS), Androgen Insensitivity Syndrome (AIS), breast cancer, prostate cancer, Huntington's Disease, and Refetoff Syndrome [89]. RTS is an autosomal dominant inherited disease that is clinically characterized by mental retardation, webbed fingers and toes, pulmonary stenosis, chest abnormalities, vertebral anomalies and sleep apnea [90]. Individuals with RTS possess genomic mutations in the CREB Binding Protein (CBP) gene or complete deletion of said gene. This mutation is so striking because CBP is an extremely important coactivator that can facilitate the activation of several nuclear receptors including the estrogen receptor, thyroid hormone receptor, retinoic acid receptor, glucocorticoid receptor and the vitamin D receptor [90]. Androgen Insensitivity Syndrome is an X-linked recessive disorder that affects males with a karyotype of 46XY but have end-organ androgen insensitivity due to mutations in the androgen receptor (AR) gene and signaling pathway [91]. AIS patients often have a common mutation in the AR that is located in the AF-1 domain of the receptor, impairing its ability to interact with coactivators [89]. Patients with breast cancer tend to have increased expression and activation of the coactivator AIB1 which increases the recruitment of CBP/p300 and Cyclin D to the estrogen receptor [89, 92]. This facilitates increased trans-activation of the ER and also increased signaling to the tumor. Similar mechanisms have been found in prostate cancer patients where increased expression and activation of SRC-1 or TIF2 promotes the transactivation of Cyclin E and the AR [89]. Finally, in patients with Refetoff Syndrome, also known as Resistance to

Thyroid Hormone (RTH), the Thyroid Receptor  $\beta$  disproportionately recruits the corepressor nuclear corepressor 1/2 (NCoR1 or NCoR2) in lieu of coactivator SRC-1[89]. This inhibits TR signaling, and elevates circulating levels of thyroid hormone (T3) and thyroid stimulating hormone (TSH) [92]. Taken together, the trans-activation or repression of a host of nuclear receptors by coregulators is an essential molecular mechanism that can have severe and significant consequences if attenuated or abated.



**Figure 3 Nuclear Receptor Activation is Facilitated by Coactivators**

Coactivators such as SRC1 and CBP contain DNA modifying enzymes such as Histone Acetyltransferase (HAT) that enhance the recruitment of NR's to their DNA response elements and enhance transcriptional activation of NR target genes. *Ac*, Acetyl group; *NR*, Nuclear Receptor; *HRE*, Hormone Response Element; *CBP*, CREB Binding Protein; *SRC1*, Steroid Receptor Coactivator.

## **2.0 CHAPTER II: A PRO-ADIPOGENIC ROLE FOR EST IN HUMAN ADIPOGENESIS AND LIPOGENESIS**

### **2.1 BACKGROUND: MOLECULAR MECHANISM CONTROLLING ADIPOGENESIS AND LIPOGENESIS**

Adipogenesis is the process whereby preadipocytes are stimulated to differentiate into functional mature adipocytes. Adipocytes make up the majority of white adipose tissue (WAT) and brown adipose tissue (BAT); however adipose tissue also contains preadipocytes, macrophages and other immune cells, fibroblasts, and stromal vascular cells [93]. This study focuses on the differentiation of WAT; however BAT is also important for body weight homeostasis, energy metabolism, and thermogenesis. BAT is found in newborn and adult humans, as well as many mammals albeit this type of adipose tissue is not as abundant as WAT [94]. It contains a large amount of mitochondria that utilize several isoforms of Uncoupling Protein (UCP) to generate heat from stored lipids [95]. WAT was once thought of as only a lipid storing compartment, adipose tissue is now regarded as a critical component of the endocrine system with paracrine/autocrine signaling capabilities [64]. Adipose tissue contains connective tissue, nerve tissue, stromal vascular cells and immune cells [96]. Functionally, WAT can regulate food intake and energy expenditure by responding to afferent signals to mobilize fat stores for energy and also by expressing and secreting factors with endocrine function on other organs and the CNS [64, 97]. Interestingly, because adipose tissue is an amalgamation of an array of different tissue and cells types, many of the secreted factors actually are not derived from the adipocyte fraction [98]. Some of the secreted factors include cytokines and cytokine related proteins such as leptin,

TNF $\alpha$ , and Interleukin 6 (IL6). This class of proteins can regulate food intake, satiety, and the release of insulin from the pancreas (Leptin); others such as IL6 and TNF $\alpha$  are secreted by macrophages found in adipose tissue and are positively correlated with obesity, insulin resistance, and impaired glucose tolerance [93]. Adipose tissue also secretes proteins such as adiponectin which can enhance insulin sensitivity and fatty acid oxidation in the liver and muscle, as well as decrease the adhesion of macrophages and subsequent formation of foam cells on the vascular wall [93, 99]. Other proteins secreted by adipose tissue include those involved in fatty acid metabolism and transport such as Lipoprotein Lipase (LPL), Cholesterol Ester Transfer Protein (CETP), and Apolipoprotein E (APO- E); and proteins involved in steroid metabolism such as Aromatase, 17 $\beta$  hydroxysteroid dehydrogenase (17 $\beta$  HSD), as well as 11 $\beta$  hydroxysteroid dehydrogenase 1 ( 11 $\beta$  HSD1) [93]. Additionally, adipose tissue expresses an array of receptors which allow them to respond to afferent signaling and secrete the assortment of the aforementioned proteins. Some of these include traditional hormone receptors such as insulin, glucagon, Growth Hormone (GH), Thyroid Stimulating hormone (TSH), and Angiotensin II Receptors [93]. Adipose tissue also expresses a host of nuclear receptors which include the Glucocorticoid, Thyroid, Estrogen, Androgen, Progesterone, and Vitamin D Receptor [93]. Finally, adipose tissue also expresses cytokine and catecholamine receptors such as Leptin, IL6, TNF $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\beta$ 3, and  $\alpha$ 1/2 adrenergic receptors [93].

Preadipocytes originate from multipotent mesenchymal stem cells (MSC) of the mesodermic embryonic germ layer located in various fat depots in the body (abdominal, back, thigh etc.) [100]; based on their origin, MSC have the ability to differentiate into mature cells of the mesodermic lineage including adipocytes, myocytes, chondrocytes, and osteocytes [97, 100]. WAT development begins before birth and expansion of adipocyte number continues throughout

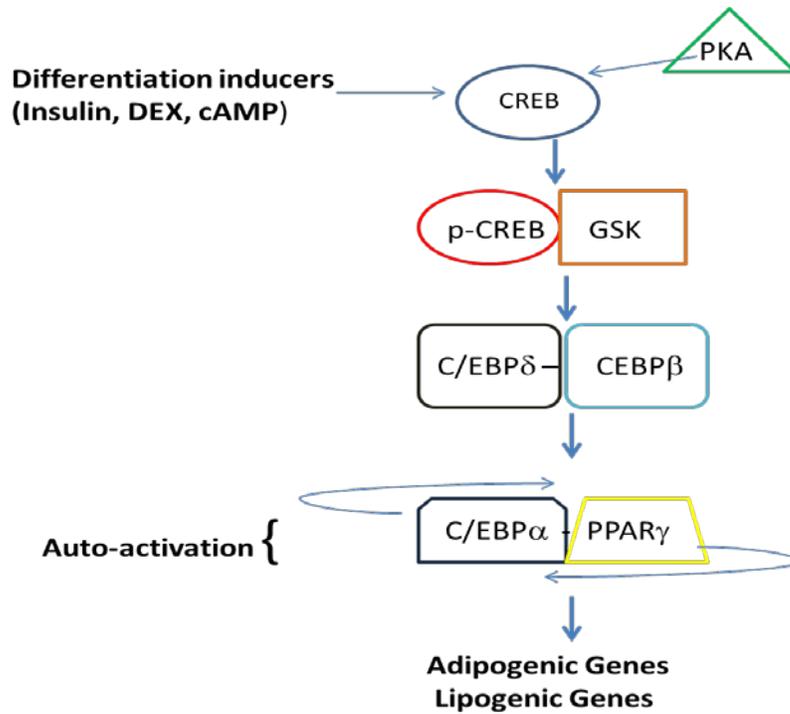
adolescence until a tightly controlled “set point” has been reached by young adulthood [101-103]; recently it has been reported that in adult humans there is approximately a 10% annual turnover of adipocytes to maintain the individual’s “set point” [101, 103].

The process of differentiation /adipogenesis is a highly conserved and controlled event that has been studied extensively, albeit mostly in animal models, but recently in human cell culture and *in vivo* models also. Adipogenesis is characterized by the induction and repression of adipocyte specific genes that morphologically and biochemically transform preadipocytes to functionally mature adipocytes. The induction of adipogenesis begins with the “commitment” of MSC to the adipocyte lineage [100]; the commitment process is not well characterized but it has been suggested that this process may involve promoter hyper- and hypomethylation patterns in genes which facilitate adipogenesis [104, 105]. Commitment is stimulated *in vivo* by increased energy intake and elevated glucose uptake over time [106], as well changes in hormones such as insulin, estrogens, androgens, thyroid hormone, and IGF-1 [44-47]. This can be recapitulated *in vitro* by culturing preadipocytes with media enriched with insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX)[29, 107]. Once cells have been committed, they undergo one final round of clonal expansion followed by growth arrest [100]. Molecularly, the first transcription factors activated are C/EBP $\beta$  and C/EBP $\delta$  [29, 108, 109]; these transcription factors are activated by Krüppel-like factors (KLFs), and phosphorylated by CREB binding protein (CBP), Glycogen Synthase Kinase -3 $\beta$  (GSK3 $\beta$ ), which primes them to bind to their appropriate DNA motifs in the PPAR $\gamma$  distal promoter [24, 100]. PPAR $\gamma$  then binds to its PPRE in the C/EBP $\alpha$  distal promoter and induces its expression, this also leads to activation of a positive feedback loop between PPAR $\gamma$  and C/EBP $\alpha$ , thus potentiating the expression of each transcription factor [21, 110-112]. PPAR $\gamma$  and C/EBP $\alpha$  induce the expression of PPAR $\gamma$  target genes involved in

lipogenesis and adipogenesis such as Lipoprotein Lipase, Fatty Acid Binding Protein (FABP4)/Adipocyte Protein-2(aP2), Glucose Transporter 4 (GLUT4), Phosphoenolpyruvate Carboxykinase 2 (PEPCK), CD36, Perilipin(PLIN1), and others [21, 108]. C/EBP $\alpha$  also is necessary for proper insulin sensitivity; cells lacking C/EBP $\alpha$  are not responsive to insulin stimulation, however this can be reversed once C/EBP $\alpha$  is ectopically expressed [29]. The increased insulin sensitivity mediated by C/EBP $\alpha$  helps to promote the expression of the sterol regulatory element-binding protein 1c (SREBP) which is essential for proper induction of lipogenic genes [100, 113]. SREBP1c (mouse homolog ADD1) is part of a family of helix-loop-helix-leucine zipper proteins that modulate cholesterol metabolism and lipogenesis. The induction of SREBP1c is not directly promoted by PPAR $\gamma$ , however the expression of SREBP1c begins shortly after the expression of PPAR $\gamma$  and CEBP $\alpha$  [114]. SREBP1c is activated by insulin signaling through the PI3K-AKT signaling pathway[115, 116], which facilitates binding to the sterol regulatory element (SRE) or “E-Box” motif found in the promoter region of its target genes which include fatty acid synthase (FASN/FAS), acetyl CoA Carboxylase (ACC), and stearoyl-CoA desaturase (SCD1) [64, 114].

Adipogenesis is a dynamic process, where many environmental cues, transcription factors, enzymes, hormones and signaling molecules influence the induction or inhibition of this process. It has been studied extensively in mice and humans; however, the role that EST plays in adipogenesis is unknown. Our study sought to examine the functional role of EST in human adipose tissue and specifically adipogenesis. The primary function of EST is to modulate estrogen signaling. Estrogen and other sex hormones play a major role in adipose tissue signaling and maintenance, as well as a critical role in adipogenesis. Based on our studies done in animals and our preliminary data, ***we hypothesize that EST can enhance the induction of adipogenesis***

*through its repressive effects on estrogen signaling.* Many factors regulate the overall process of adipogenesis however, the major focus of this study is to analyze the genomic effects that over-expression or knockdown of EST has on the critical transcriptional regulators that mediate the induction of adipogenesis and lipogenesis. In this document we will focus on the affect EST has on PPAR $\gamma$ , and its target genes LPL, aP2 and the C/EBP family including C/EBP  $\alpha$ ,  $\beta$ , and  $\delta$ . Additionally, since adipogenesis and lipogenesis are not mutually exclusive, we will also analyze the key transcription factors involved in the induction of lipogenesis, specifically SREBP1c and its target genes ACC, FASN, and SCD1. Functionally, we can measure the overall outcome of the genomic changes in adipogenic signaling by quantitatively and qualitatively analyzing lipid droplet formation in our adipocytes. We will use these and other techniques to demonstrate the role EST plays in human adipogenesis.



**Figure 4. Adipogenic Transcriptional Cascade.** Figure adopted from: Tang, Q.Q. and M.D. Lane, *Adipogenesis: from stem cell to adipocyte*. *Annu Rev Biochem*, 2012. **81**: p. 715-36 [100] DEX, Dexamethasone; PKA, Protein Kinase A; GSK, glycogen synthase Kinase.

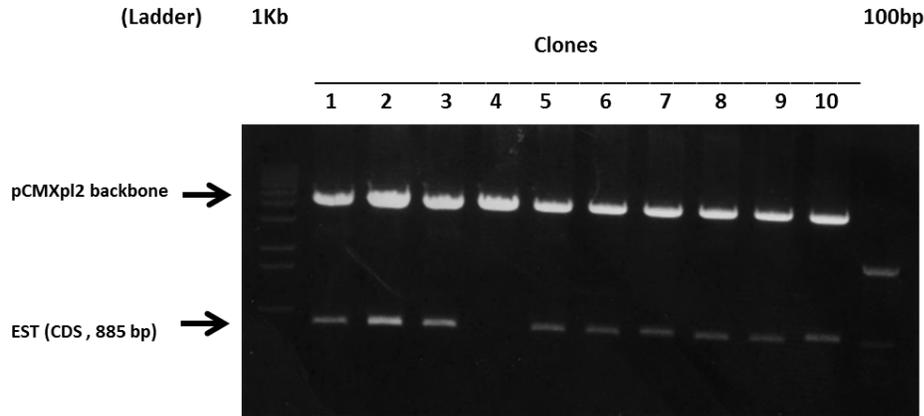
## 2.2 METHODS

### 2.2.1 Patient Population

*ASC and adipose tissue collection:* Human adipose-derived stem cells (ASCs; primary preadipocytes) and whole fat/lipoaspirate were obtained through the Adipose Stem Cell Center, Department of Plastic Surgery, University of Pittsburgh. The whole fat or liposuction aspirates were collected from the abdominal subcutaneous fat of 18 female patients who were non-diabetic nonsmokers and ranged in age from 32 to 59 years. Subsequently, preadipocytes were isolated from the abdominal subcutaneous fat of these 18 patients [15 obese patients and 3 nonobese (lean) patients]. For linear regression analysis, 16 additional obese patients' whole fat or lipoaspirate was analyzed. All experiments were performed on cells from the obese patients except as otherwise specified. The names of the patients were kept anonymous, and all patients used for preadipocyte differentiation experiments were female, non-diabetic nonsmokers and ranged in age from 25 to 56 years. The cells were cultured under standard conditions as reported previously. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium containing 10% standard fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). Each cell line was cultured for no more than four passages. Patient sample and data collection were performed in accordance with the University of Pittsburgh Institutional Review Board Protocol PRO12050016. A complete list of patients used for preadipocytes and whole fat/ liposuction studies can be found in APPENDIX A.

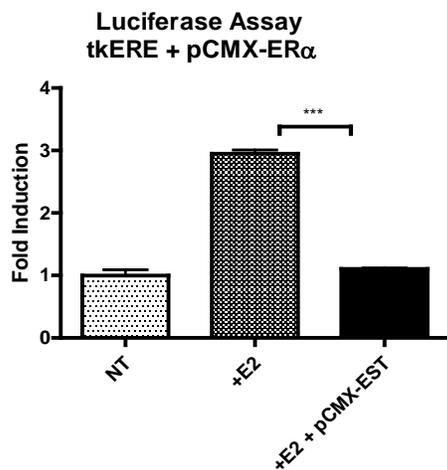
### **2.2.2 Generation of pCMXpl2 EST**

Human EST was cloned using cDNA synthesized from total RNA isolated from differentiated human adipocytes. In short, 1 $\mu$ g of total RNA was aliquoted for cDNA synthesis using the Iscript reverse transcriptase kit. For PCR amplification of the EST coding sequence, primers were designed to amplify the full length EST coding sequence with the HINDIII restriction enzyme sequence flanking the 5' region (forward primer) and the NHEI restriction enzyme sequence flanking the 3' region (reverse primer). This design allowed us to sub-clone the EST coding sequence into the pCMXpl2 expression vector. The pCMXpl2 expression vector and the EST PCR product were digested overnight with HINDIII and NHEI restriction enzymes and gel purified in 1% agarose. Ligation was carried out using T4 DNA ligase at 16° C overnight to produce the pCMXpl2-hEST construct. Ligation products were transformed into JM109 bacterial cells by electroporation and plated overnight on ampicillin-agarose plate. Clones were screened using mini-preps to isolate plasmid DNA. Successful clones were confirmed by digesting the purified pCMXpl2-hEST plasmids with HINDIII and NHEI restriction enzymes to release the SULT1E1 fragment from the vector (Fig 5). For final confirmation of successful cloning, we used primers against the CMV promoter to sequence the purified vector through the University of Pittsburgh Genomics Core. Results can be found in Appendix A. Functional analysis was validated using a transfection and reporter gene assay in which the estrogen deactivating activity of EST was demonstrated (Fig 6).



**Figure 5. pCMXpl2- hEST cloning confirmation**

10 Clones were isolated from ampicillin-agarose plates and plasmid DNA was purified using mini-preps. Clones were digested with HINDIII and NHEI restriction enzymes overnight to release EST coding sequence (CDS) from the plasmid backbone. The results show that 9 out of 10 clones isolated successfully incorporated the EST CDS. One clone was used for functional testing and virus production.

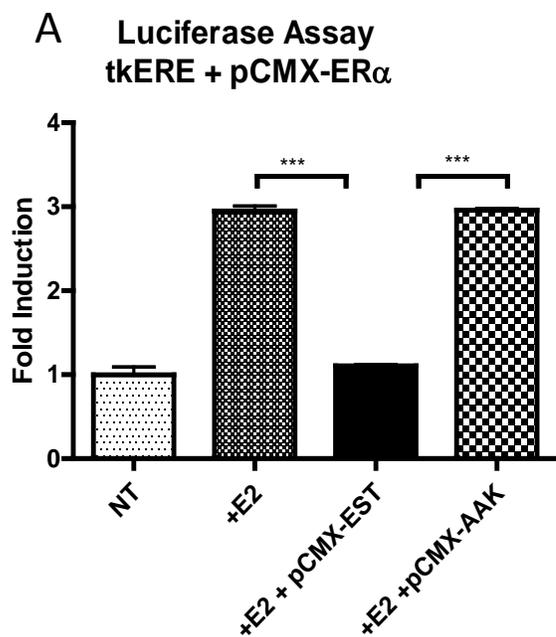


**Figure 6. Functional Analysis to confirm EST Activity.**

tk-ERE-Luc (where ERE is estrogen response element Luc is luciferase) and pCMX-ER $\alpha$  were transfected in triplicate using 293T cells. pCMX-EST was transfected and inhibited the E2 induced ER $\alpha$  activation. pCMX- $\beta$ -Gal (where  $\beta$ -Gal is  $\beta$ -galactosidase), was used to normalize the luciferase signal. Estrogen treatment concentration was 10nM. .  $P < .05^*$ ,  $P < .01^{**}$ ,  $P < .0001^{***}$ .

### **2.2.3 Site Directed Mutagenesis for the Creation of EST-AAK mutant**

3' phosphoadenosine 5' phosphosulfate (PAPS) is the co-substrate and source of the sulfonate group in the sulfonation reaction catalyzed by SULT1E1 [117]. We along with others have reported that by mutating a conserved domain in the P-loop region of the PAPS-binding domain from GxxGxxK (GGK) to AxxAxxK(AAK), the enzymatic activity of EST was completely abolished [3, 118]. To produce an enzymatically dead SULT1E1 virus, we used the PCR based overlap extension method [119]. The PCR product was cloned into a pCMXpl2 expression vector using the restriction enzyme sites HINDIII and NHEI to produce pCMXpl2-AAK-hEST. To confirm the AAK-EST coding sequence and orientation were correct we sequenced the purified plasmid through the University of Pittsburgh Genomics Core. Results can be found in Appendix A. The lack of enzymatic activity of the EST AAK mutant was validated in a transfection and reporter gene assay in which the estrogen-deactivating activity of wild-type EST was abolished in cells transfected with a virus expressing the EST AAK mutant (Fig 7).



**Figure 7 . Functional Analysis to Confirm Loss of Enzymatic Activity in AAK-EST.**

(A) tk-ERE-Luc (where ERE is estrogen response element Luc is luciferase) and pCMX-ER $\alpha$  were transfected in triplicate using 293T cells. pCMX-EST was transfected and inhibited the E2 induced ER $\alpha$  activation, but pCMX-AAK was unable to inhibit E2 induction of luciferase . pCMX- $\beta$ -Gal (where  $\beta$ -Gal is  $\beta$ -galactosidase), was used to normalize the luciferase signal. Estrogen treatment concentration was 10nM.  $P < .05^*$ ,  $P < .01^{**}$ ,  $P < .0001^{***}$ .

#### **2.2.4 Creation and Characterization of Lentivirus to Over-express EST and AAK- EST**

To produce lentivirus expressing EST, the full length coding region was PCR amplified from the pCMXp12- hEST plasmid using primers that incorporated the PME1 restriction enzyme sites flanking the 5' and 3' regions of the SULT1E1 coding sequence. EST was then sub-cloned into the pWPI lentiviral expression vector via the PME1 restriction sites to produce pWPI-EST. To confirm the coding sequence and orientation were correct, we used primers against the EF1 $\alpha$  promoter to sequence the purified plasmid through the University of Pittsburgh Genomics Core. Results can be found in Appendix A.

Lentiviral particles were generated using the second-generation system that contained three plasmids: the transgene expression plasmid (pWPI or pWPI-EST), a packaging plasmid (psPAX2), and an envelope plasmid (pMDG.2). All three plasmids were transfected simultaneously into 293T cells for viral particle packaging, assembly, and amplification using Trans-IT transfection reagent from Mirus (Madison, WI). Viral lysates were collected every 24 h after transfection, filtered with 0.45-um-poresize Millex GV syringe filter units from Millipore (Billerica, MA), pooled, and concentrated with a Lenti-X-Concentrator from Clontech (Mountain View, CA). Titer concentrations were assessed with Lenti-X-Stix from Clontech and by fluorescent examination of the green fluorescence protein that was engineered in the lentiviral vector. Viral lysates were aliquoted and stored at -80°C until use.

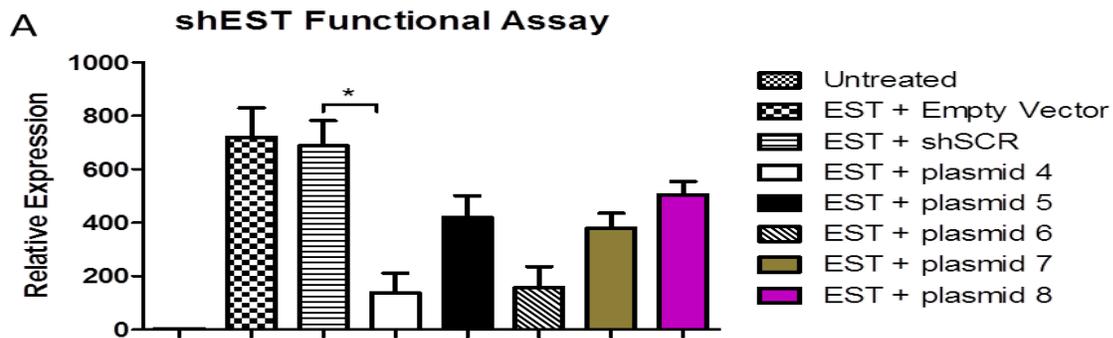
#### *AAK Mutant Virus*

We used PCR to amplify AAK-hEST from the pCMXpl2-AAK-hEST plasmid discussed in section 2.2.3. The amplification incorporated the PmeI restriction enzyme sites flanking the 5' and 3' region of the coding sequence. The PCR product was then sub-cloned into pWPI using the PmeI restriction sites. Lentiviral particles were generated using the second generation lentiviral system as described previously.

#### **2.2.5 Creation and Characterization of Lentivirus to Knockdown EST**

To generate lentivirus expressing short hairpin RNAs (shRNAs) against EST (shEST), expression plasmids containing shEST were purchased from Open Biosystems (Pittsburgh, PA). For each knockdown, five sequences were purchased and tested in transient-transfection assays. In this assay 0.2ug pCMX-EST was transfected into 293T cells, followed by 2ug of one of the 5 plasmids purchased (10:1 knockdown constructs: pCMX-EST); the sequence with the most

efficient knockdown was chosen for lentiviral production (Fig 8). A scrambled shRNA (shSCR) plasmid was purchased to serve as a control. Plasmid sequences for shEST can be found in APPENDIX A. Lentiviral particles were generated using the second generation lentiviral system as described previously.



**Figure 8. EST Knockdown (KD) Functional Assay**

RT-PCR expression. (A) 293T cells were plated in 6 wells plates and grown to 80% confluency. Duplicate co-transfections were performed with 0.2ug pCMX-EST, 2ug pCMX-pl2 (empty vector), 2ug scrambled or knockdown plasmids when applicable. For EST KD, plasmid 4 was chosen for virus production. Sequences can be found in APPENDIX A.

### 2.2.6 Adipocyte Culturing and Differentiation

In all experiments except those in which exogenous estrogen (E2) was added, differentiation medium 1 (DM1) consisted of DMEM/F-12 medium, 10% standard FBS, 1% Pen-Strep, 33uM biotin, 100 nM insulin, 17 uM pantothenic acid, 0.5 mM methylisobutylxanthine, 1 uM dexamethasone, and 1 uM rosiglitazone. Differentiation medium 2 (DM2) consisted of DMEM/F-12 medium, 10% FBS, 1% Pen-Strep, 1 uM dexamethasone, and 100 nM insulin. Confluent preadipocytes were cultured in DM1 for 3 days before being switched to DM2 for 2

weeks to reach terminal differentiation. Culture medium was changed every other day. For differentiation experiments in which E2 was exogenously added, the cells were cultured in phenol red-free DMEM/ F-12 medium and dextran-coated charcoal (DCC)-stripped FBS before the addition of E2 at a final concentration of 10 nM.

### **2.2.7 Gene Expression Analysis**

Total RNA from preadipocytes/adipocytes was isolated using TRIZOL (Invitrogen) reagent.

Total RNA from whole fat tissue or lipoaspirate was collected using the RNEASY Lipid Tissue Mid-Kits (Quiagen). The cDNA was synthesized from 1.0 ug of total RNA by Iscript from Bio-Rad(Hercules, CA). Aliquots of cDNA were amplified on an ABI 7300 Real-Time PCR System from Applied Biosystems (Foster City, CA) using the SYBR green PCR master mix. mRNA expression was normalized against the expression of cyclophilin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A complete list of primers used can be found in APPENDIX A.

Primers were validated using serial dilutions (1fg -10ng) of linearized expression vectors for the target gene or quantified cDNA from differentiated adipocytes to generate efficiency curves. An example of an efficiency curve validating the EST primer can be found in APPENDIX A.

### **2.2.8 Oil Red O Staining and Quantification**

Differentiated six-well culture dishes were washed twice with cold phosphate-buffered saline (PBS) and then prefixed for 1 h with 10% formaldehyde in PBS. After 1 hr, fresh 10% formaldehyde was added, and the cells were incubated overnight at room temperature. The next day, the cells were washed twice with PBS and then incubated in 60% isopropanol for 5 min, followed by drying at room temperature (RT). Cells were incubated with oil red O working solution for 10 minutes and then washed five times with deionized water. Images were acquired microscopically. For quantification of oil red O staining, cells were differentiated in triplicate in

24-well plates, stained with oil red O, and eluted with 100% isopropanol, and 100  $\mu$ L of elute was loaded onto 96-well plates. Absorbance was measured at 500 nm using a PerkinElmer plate reader.

### **2.2.9 Western Blot Analysis**

Cells were lysed with NP-40 lysis buffer containing protease inhibitors and then quantified for protein concentrations by a bicinchoninic acid (BCA) assay kit from Pierce (Rockford, IL). Protein samples were resolved by electrophoresis on 10% SDS-polyacryl-amide gels. For the detection of insulin receptor substrate 1 (IRS1) and its phosphorylation, cell lysates were immunoprecipitated with an IRS1 antibody before being subjected to Western blotting using an IRS1 antibody and phosphotyrosine antibody. After transfer of proteins to polyvinylidene difluoride (PVDF) membranes, the membranes were probed with antibodies against total extracellular signal-regulated kinases 1 and 2 (ERK1/2) (catalog no. sc94; Santa Cruz), phospho-ERK1/2 (catalog no.sc7383; Santa Cruz), total AKT (catalog no. 9272; Cell Signaling), phospho-AKT (catalog no. 9215; Cell Signaling), phospho-CREB (catalog no.87G3; Cell Signaling), total CREB (catalog no. 48H2; Cell Signaling), human EST (catalog no. SAB1400267; Sigma), ER $\alpha$  (catalog no. sc7207;Santa Cruz), IRS1 (catalog no. 2382S; Cell Signaling), and phosphotyrosine (catalog no. ab10321; Abcam). Detection was achieved by using an ECL system from Amersham (Piscataway, NJ). Quantification was performed using the NIH ImageJ software.

### **2.2.10 MTT Cell Proliferation Assay**

An MTT[3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was performed using an assay kit from ATCC (Manassas, VA). Briefly, preadipocytes were grown in 150cm dishes and treated with lentivirus expressing either EST or vector at an MOI of 3. Five

days later, cells were plated in triplicate at a density of  $3 \times 10^3$  cells per well in 96-well plates for 1, 3, and 5 days. On the appropriate day, 10ul of MTT reagent was added to each well, and the cells were incubated for 3hrs at 37°C. Subsequently, 100 ul of detergent was added, and the cells were incubated overnight at RT before a colorimetric assessment was performed using a PerkinElmer plate reader at 570 nm.

### **2.2.11 Transient Transfection Assay**

HepG2 cells or 293T cells were plated at a density of  $2 \times 10^6$  cells per 48-well plate and incubated overnight. Transfection was performed using Trans-IT reagent from Mirus. Plasmids that were used in triplicate at an amount of 300 ng included pCMX-EST, pCMX-EST AAK, pCMX-ER $\alpha$ , pCMX-PPAR $\gamma$ , and pCMX. The triplicate plasmid amounts for pCMX-CBP, pCMX- $\beta$ -Gal (where  $\beta$ -Gal is  $\beta$ -galactosidase), and tk-ERE-Luc (where ERE is estrogen response element Luc is luciferase) or tk-PPRE-Luc (where PPRE is peroxisome proliferator response element) were 50 ng, 200 ng, and 600ng, respectively. Cells were transfected and incubated for 24 h. Transfected cells were treated with the appropriate ligand for 24hrs using DMEM without phenol red and DCC FBS, followed by lysis and assays for luciferase and  $\beta$ -Gal activities. The luciferase activities were normalized to  $\beta$ -Gal activities.

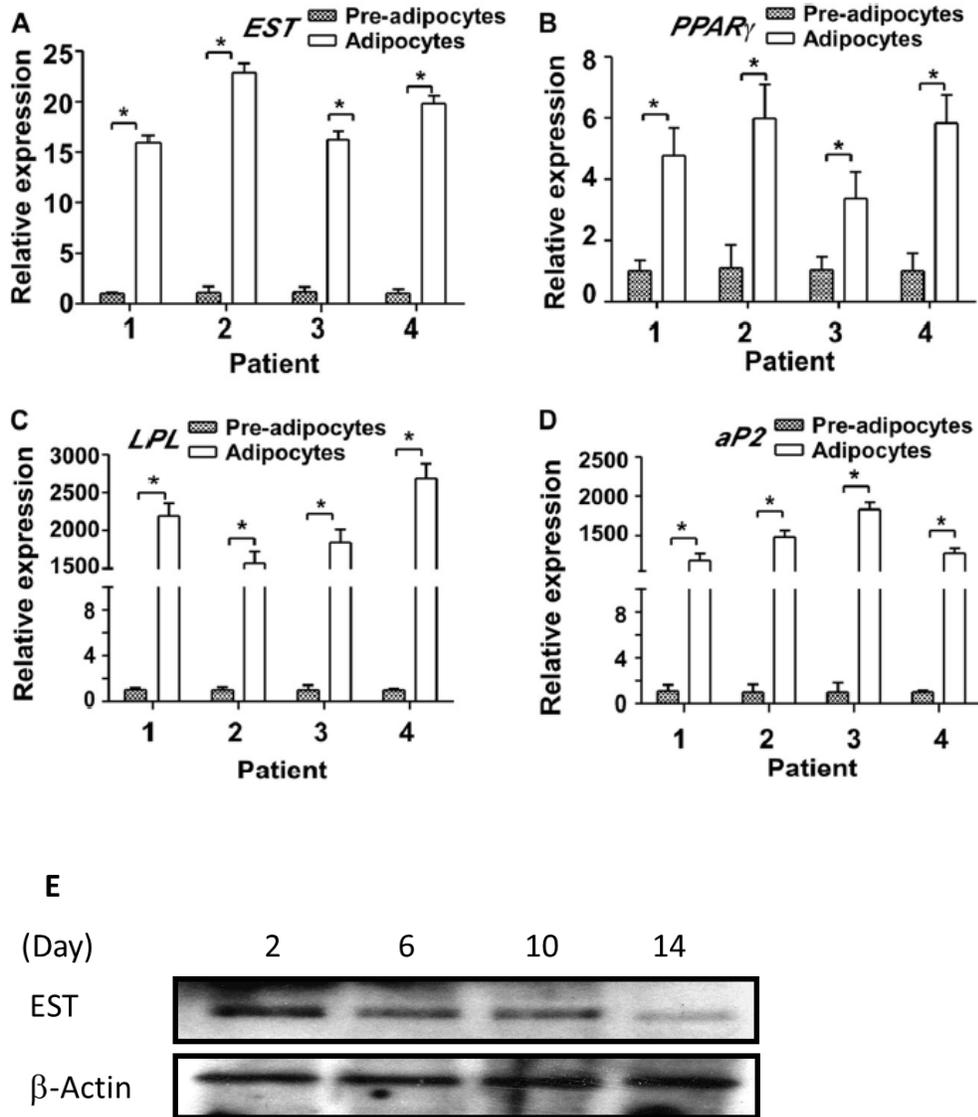
### **2.2.12 Statistical Analysis**

When applicable, results are presented as means  $\pm$  standard deviations (SD) The Student t test was used to compare means of two groups and One-way ANOVA was used to compare the means of three or more groups. P values of less than 0.05 are considered to be significant. Repeated- measures ANOVA was used to compare means of two or more groups across multiple time points (MTT cell proliferation assay). Linear regression analysis was performed using the Graph-Pad Prism software.

## 2.3 RESULTS

### 2.3.1 EST Expression Increases during Adipogenesis

We have previously reported that the expression of the **mouse Est** gene was high in preadipocytes, and expression decreased upon differentiation [3]. In an effort to determine whether the **human EST** affects the differentiation of human preadipocytes we found that in four independent cases of obese human preadipocytes isolated from the abdominal fat depot the mRNA expression of EST in preadipocytes was low, and differentiation led to a marked increase in mRNA expression EST expression (Fig 9A). The protein expression showed that EST is expressed highest at the induction of differentiation, and this expression then begins to diminish by day 14 (Fig 9E). The induction of EST coincided with the induction of adipocyte differentiation maker genes, such as PPAR $\gamma$ , LPL, and aP2/ FABP4 (Fig 9B-D)[88]. These results demonstrate that the regulation pattern of EST in humans is the opposite of what we observed in murine models.



**Figure 9. The Expression of EST was Induced During Adipogenesis.**

The expression of EST was induced during adipogenesis. (A to D) Preadipocytes were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of EST (A), PPAR $\gamma$  (B), LPL (C), and aP2 (D) was measured by real-time PCR analysis. (E) Lysates from differentiated adipocytes were subjected to Western blot analysis to detect protein expression of EST over the course of the 14 day differentiation period \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### **2.3.2 EST is induced by the Glucocorticoid Receptor**

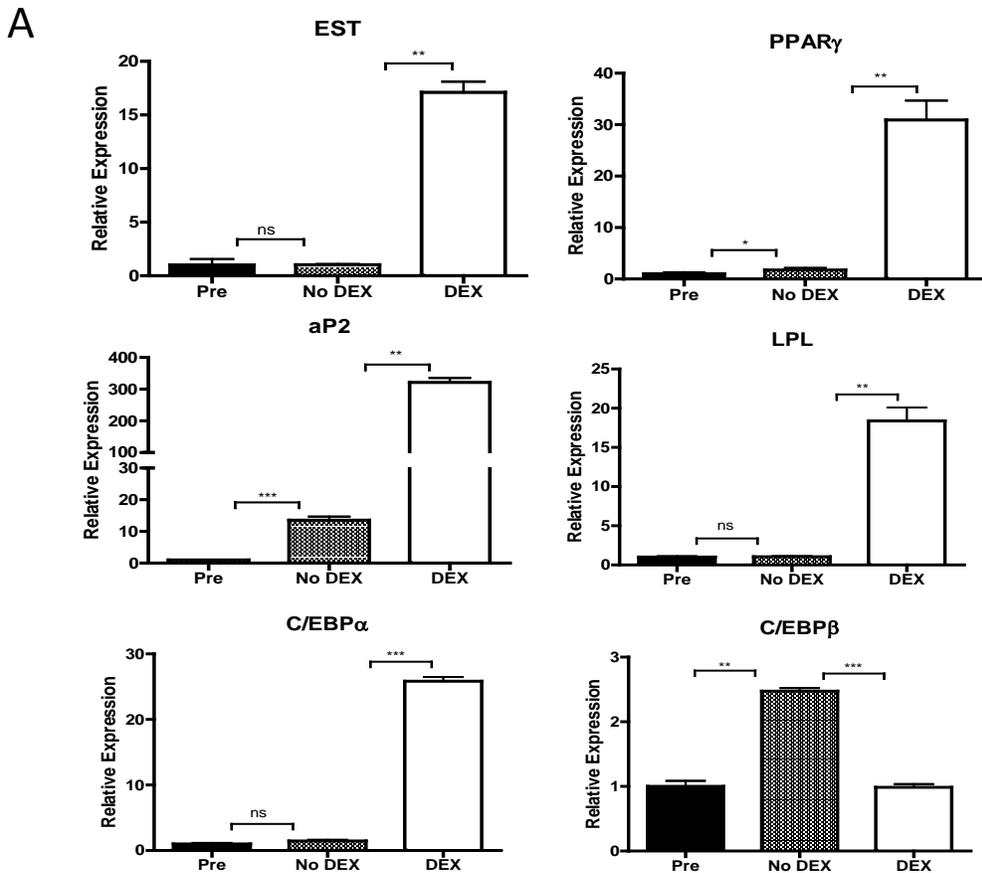
The Glucocorticoid Receptor (GR) is an important nuclear receptor that regulates adipogenesis through its effect on the 5' adenosine monophosphate-activated protein kinase (AMPK) pathway. The activation of the AMPK pathway takes place when the body needs to utilize energy such as during exercise, and therefore leads to a catabolic metabolic state. During AMPK activation, anabolic and energy consuming pathways such as adipogenesis, gluconeogenesis, and protein synthesis are inhibited; while energy producing pathways such as beta oxidation, and glycolysis are stimulated [120].

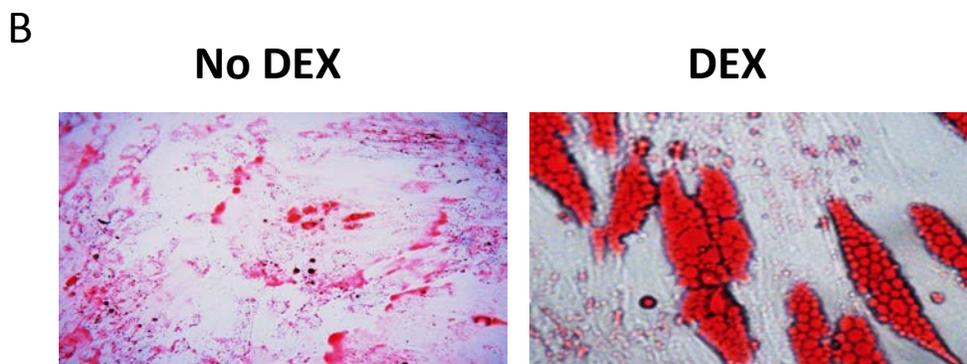
The Glucocorticoid Receptor (GR) has been shown to inhibit AMPK phosphorylation when activated by an agonist such as dexamethasone in rat and human adipocytes [121]. The inhibition of this pathway is essential for the accumulation of lipids in adipose tissue, however glucocorticoid excess such as in Cushing's Syndrome or from exogenous glucocorticoid treatment can lead to an severe accumulation of lipids in visceral and adipose tissue as well as fatty liver [120, 121]. Typically metformin, an AMPK activator, is used to treat endogenous or exogenous glucocorticoid excess which demonstrates the severe impact this pathway exerts on lipid metabolism [122].

Previously, we showed that the human SULT1E1 and mouse Sult1e1 promoters contained functional Glucocorticoid Response Elements (GRE) [123]. Additionally, we showed that pharmacologic activation of GR was sufficient to induce SULT1E1 expression and inhibit MCF-7 cell growth in cell culture and *in vivo* due to estrogen deprivation [123].

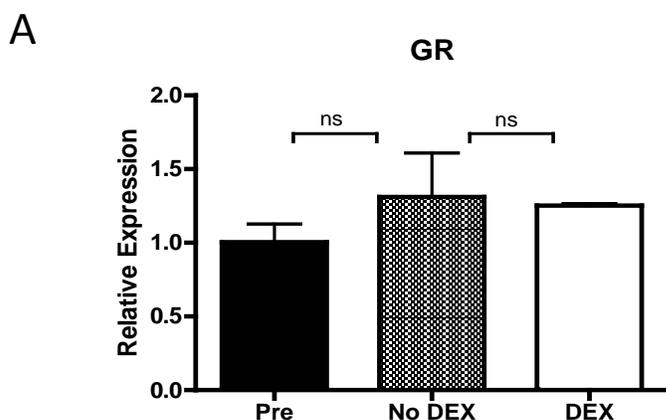
Based on the aforementioned data, we decided to analyze the DEX-GR- SULT1E1 pathway in human adipocytes. One case of preadipocytes was expanded, separated into two groups and

plated for differentiation. One group was treated with standard differentiation media including DEX and the other lacked DEX. Differentiation proceeded for two weeks followed by gene expression analysis and lipid content measurement. We found that in the group differentiated without DEX there was nearly no induction of EST or adipogenic gene expression PPAR $\gamma$ , LPL, aP2, or C/EBP $\alpha$  (Fig 10A). We used oil red O staining to show the paucity of lipid droplets formed in the samples differentiated without DEX (Fig 10B). Additionally, the mRNA expression of the GR remained high throughout the experiment and did not change under either condition (Fig 11). These results strongly suggest that the DEX-GR-SULT1E1 pathway is conserved in human adipocytes and is necessary for efficient induction of EST and adipogenesis.





**Figure 10. DEX deprivation during differentiation fails to induce SULT1E1 expression and adipogenesis.** Preadipocytes were cultured and differentiated in standard medium or medium lacking DEX for 14 days. Total RNA was extracted before and after differentiation, and the expression of (A) EST and adipogenic genes was measured by real-time PCR analysis. (B) Samples were fixed, dried and stained with oil red O. Images were acquired microscopically. \*,  $P < .05$ ; \*\*,  $P < .01$ ; *ns*, *not statistically significant compared to vector*.



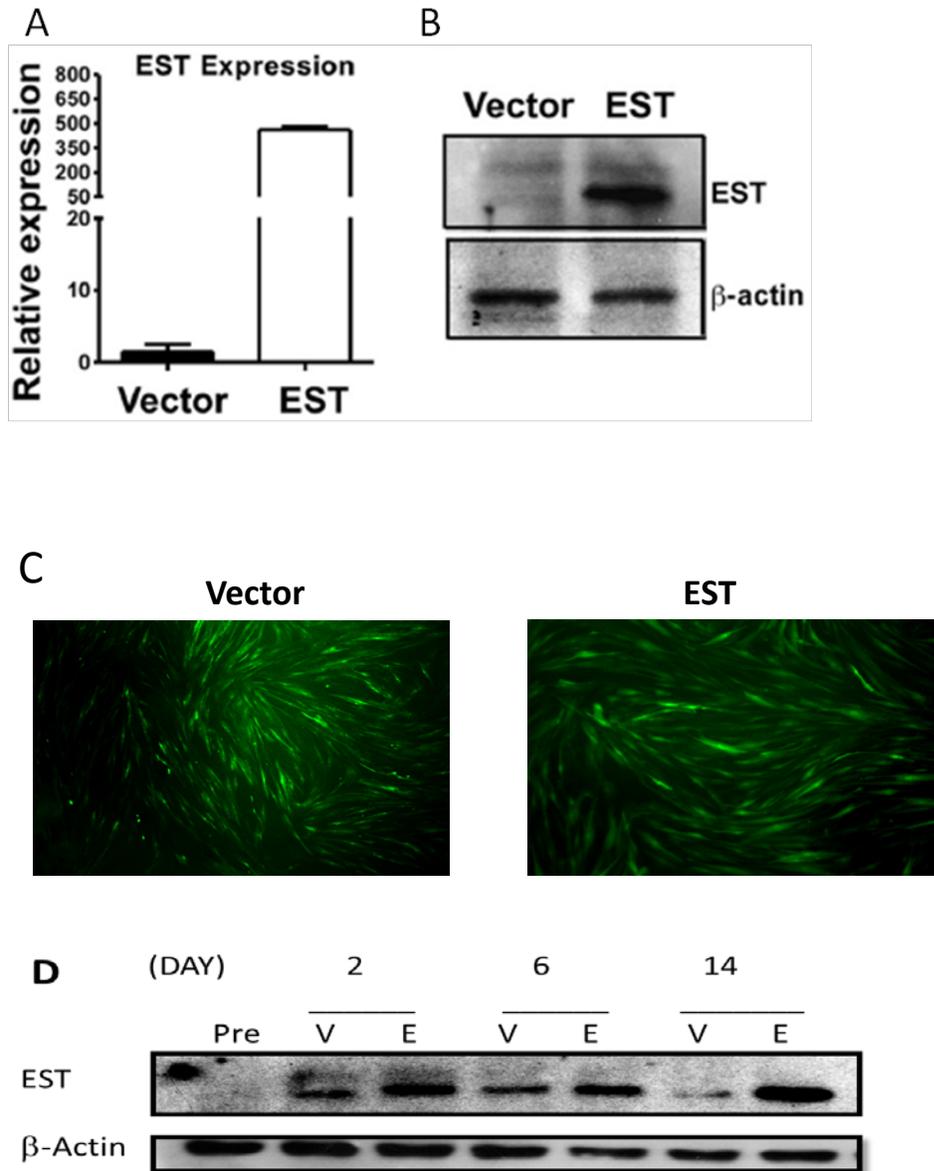
**Figure 11. DEX deprivation during differentiation does not alter GR mRNA expression.** The expression of the GR remained very high (relative to GAPDH) throughout the differentiation process and was unchanged by culturing conditions. CT values for pre, no DEX, and DEX were approximately 21, 20.5, 20.8 respectively; compared to the CT values of the housekeeping gene, GAPDH, approximately 15 for all groups. *ns*, *not statistically significant*.

### **2.3.3 Over-expression of EST Increases Adipogenic and Lipogenic Gene Expression but does not change Lipolytic Gene Expression**

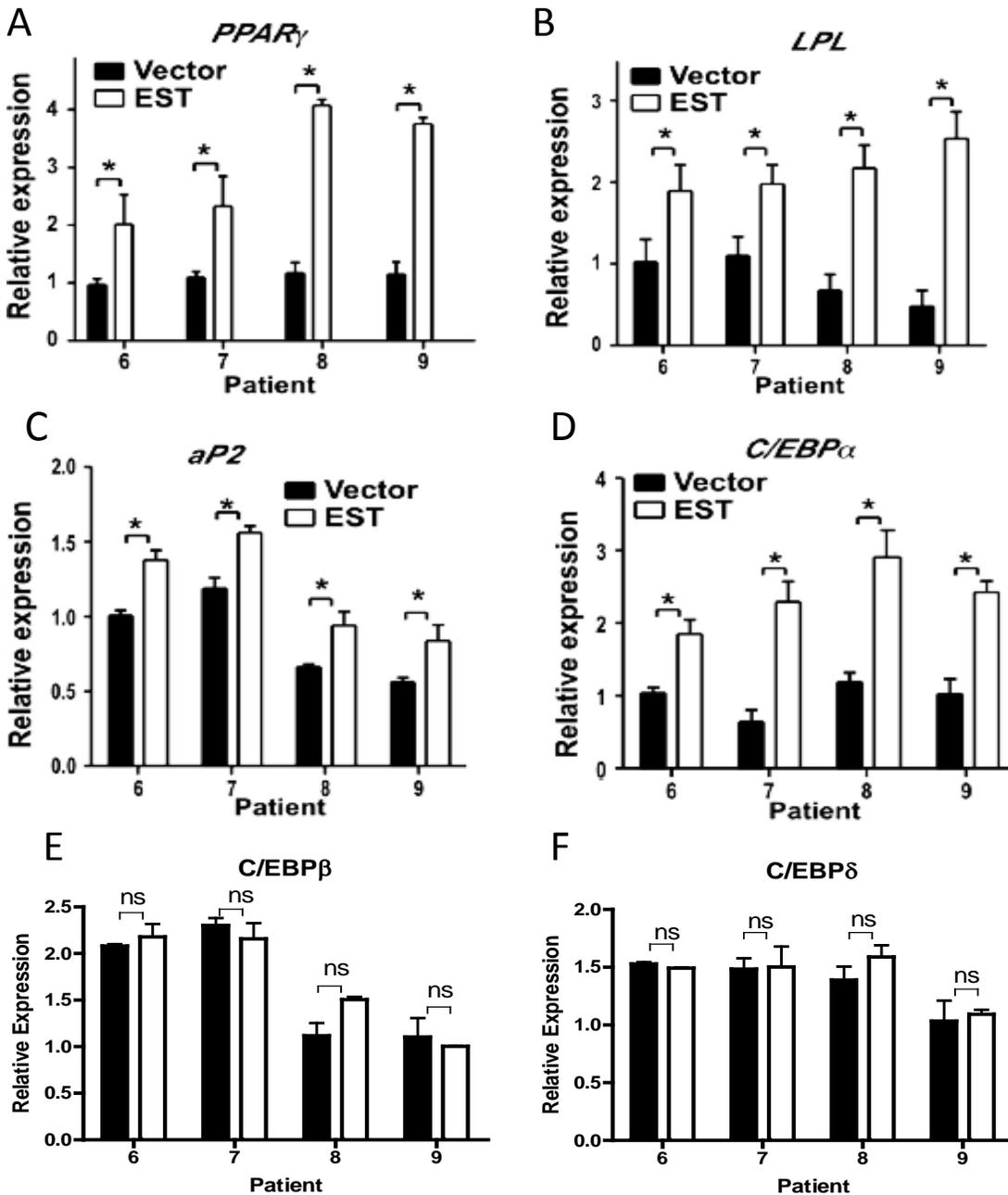
#### **2.3.3.1 Adipogenic mRNA expression in Obese and Lean Patients**

To determine the functional relevance of EST induction during adipogenesis, we overexpressed EST in preadipocytes before subjecting them to differentiation. Four cases of preadipocytes from **obese patients** were transduced with lentivirus expressing EST or the vector control, and then induced to terminal differentiation for 2 weeks. Lentivirus transduction was confirmed via GFP fluorescence microscopy (Fig 12C) and the overexpression of EST was confirmed by real-time PCR (Fig 12A.) and Western blotting (Fig 12B, D.). Overexpression of EST promoted adipogenesis (Fig 13) as confirmed by gene expression analysis. Adipogenic gene expression changes included the induction of PPAR $\gamma$ , LPL, aP2, and C/EBP $\alpha$ ( 13A-D). As stated in the introduction, the C/EBPs are vital for adipogenesis. C/EBP $\alpha$  has been shown to facilitate the activation of PPAR $\gamma$  target genes, whereas C/EBP $\beta$  and C/EBP $\delta$  are important for the transactivation of PPAR $\gamma$  gene expression. We also show that adipogenesis in cells transduced with EST-expressing virus was associated with the induction of C/EBP $\alpha$  (Fig 13D), but the expression of C/EBP $\beta$  and C/EBP $\delta$  was unchanged (Fig 13E-F).

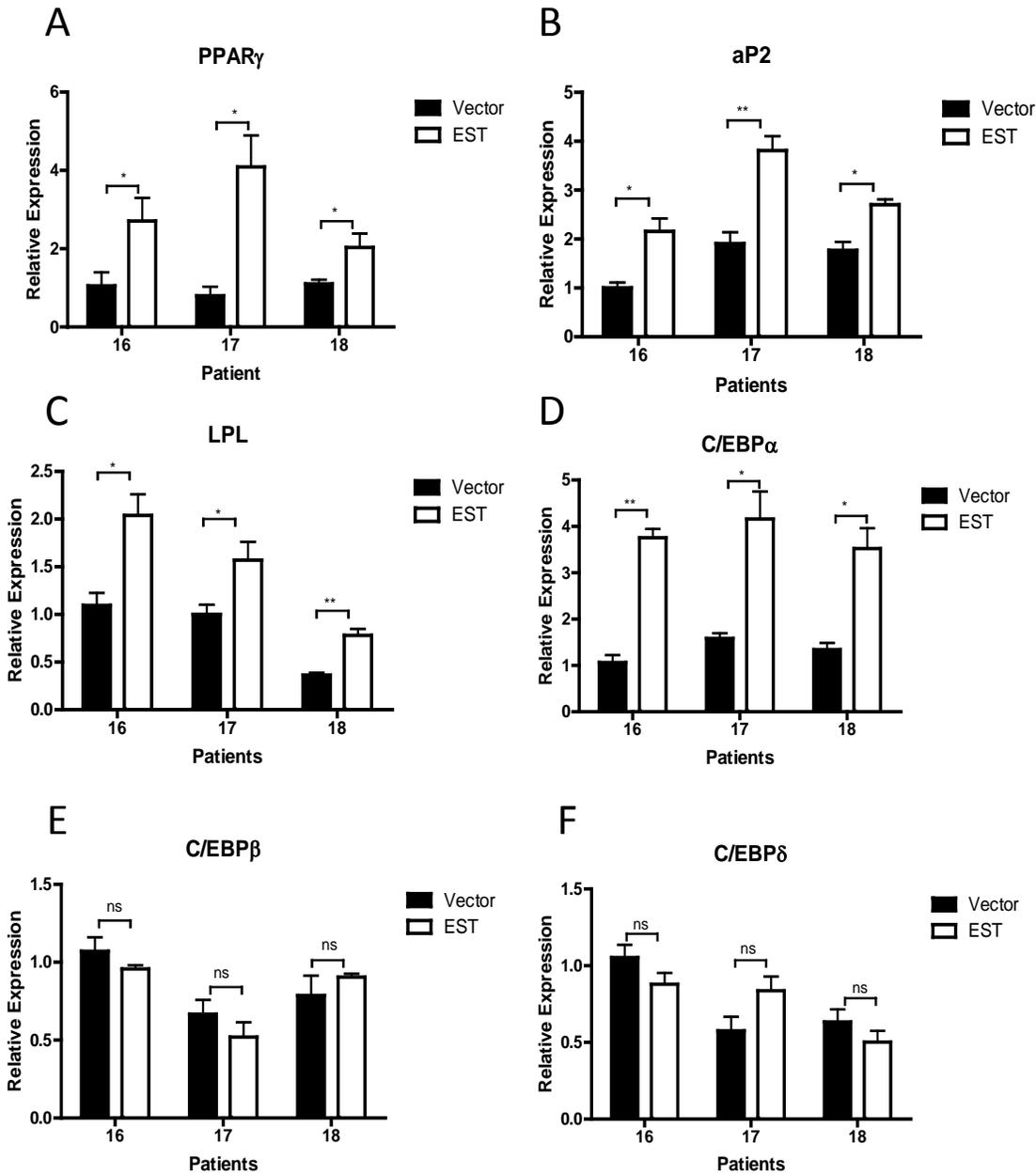
We also transduced three cases of **lean patients** with lentivirus expressing EST and analyzed them to assess if pro-adipogenic metabolic changes were conserved in these samples also. Indeed, over-expression of EST enhanced adipogenesis in these samples as well, (Fig 14A-D) confirmed by genetic analysis of adipogenic genes that showed the induction of PPAR $\gamma$ , LPL, aP2, and C/EBP $\alpha$ , but no change was observed in C/EBP $\beta$  and  $\delta$  (Fig 14E/F). Taken together, these results indicate that EST is a potent enhancer of adipogenic mRNA expression in our cell culture model.



**Figure 12. Lentiviral Transduction and Overexpression of EST.** In preadipocytes EST expression is low, we successfully transduced and overexpressed EST using lentivirus. Total RNA was extracted 3 days after transduction and the expression of EST was measured by (A) Real time PCR (B) Western Blot (C) The lentiviral construct contains a GFP tag which was also used to measure transduction efficiency in vector and EST treated cells (D) Lysates from differentiated vector and EST-virus treated cells were subjected to Western blot analysis to detect protein expression of EST over the 14 day differentiation period. *Pre*, preadipocyte; *V*, vector; *E*, EST.



**Figure 13. Adipogenic Gene Expression in Obese Patients.** Overexpression of EST promoted Adipogenesis in **Obese Patients**. (A-F) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.



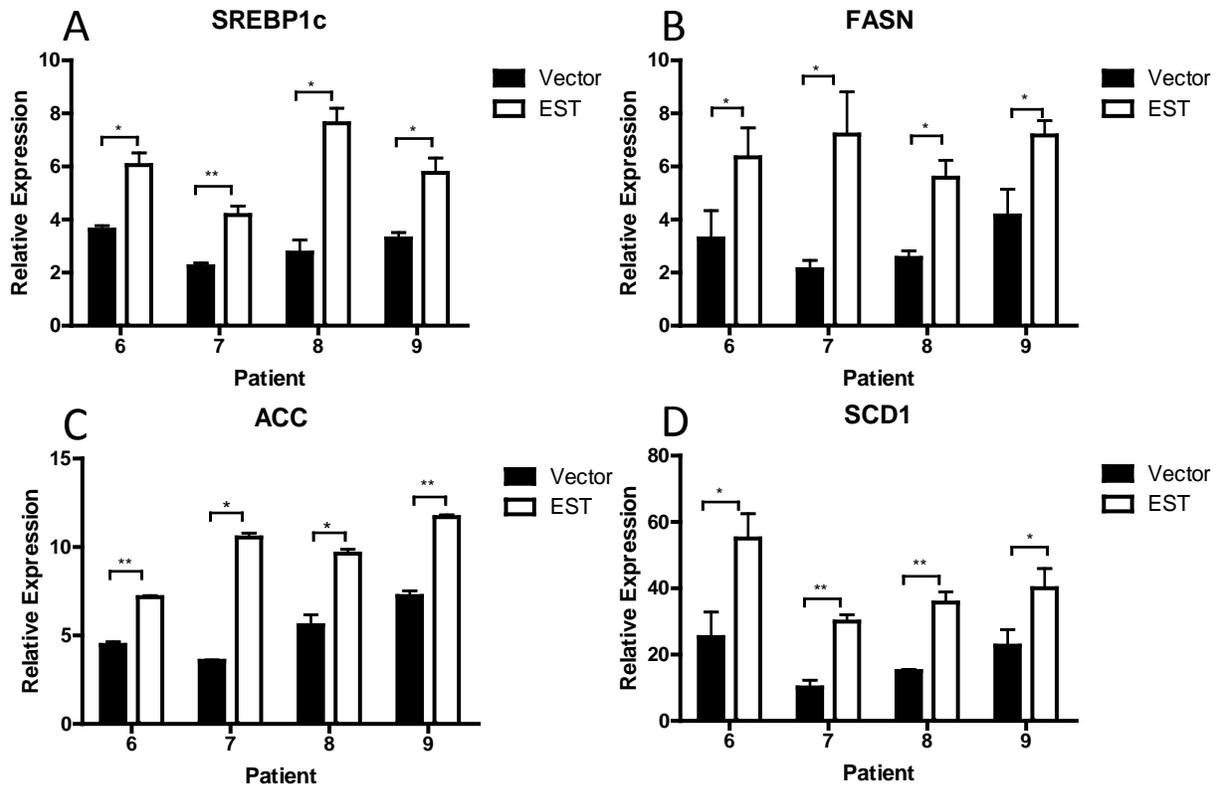
**Figure 14. Lean Patients Adipogenic Gene Expression.**

Overexpression of EST promoted Adipogenesis in **Lean Patients**. (A-F) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### 2.3.3.2 Lipogenic and Lipolytic mRNA expression in Obese and Lean Patients

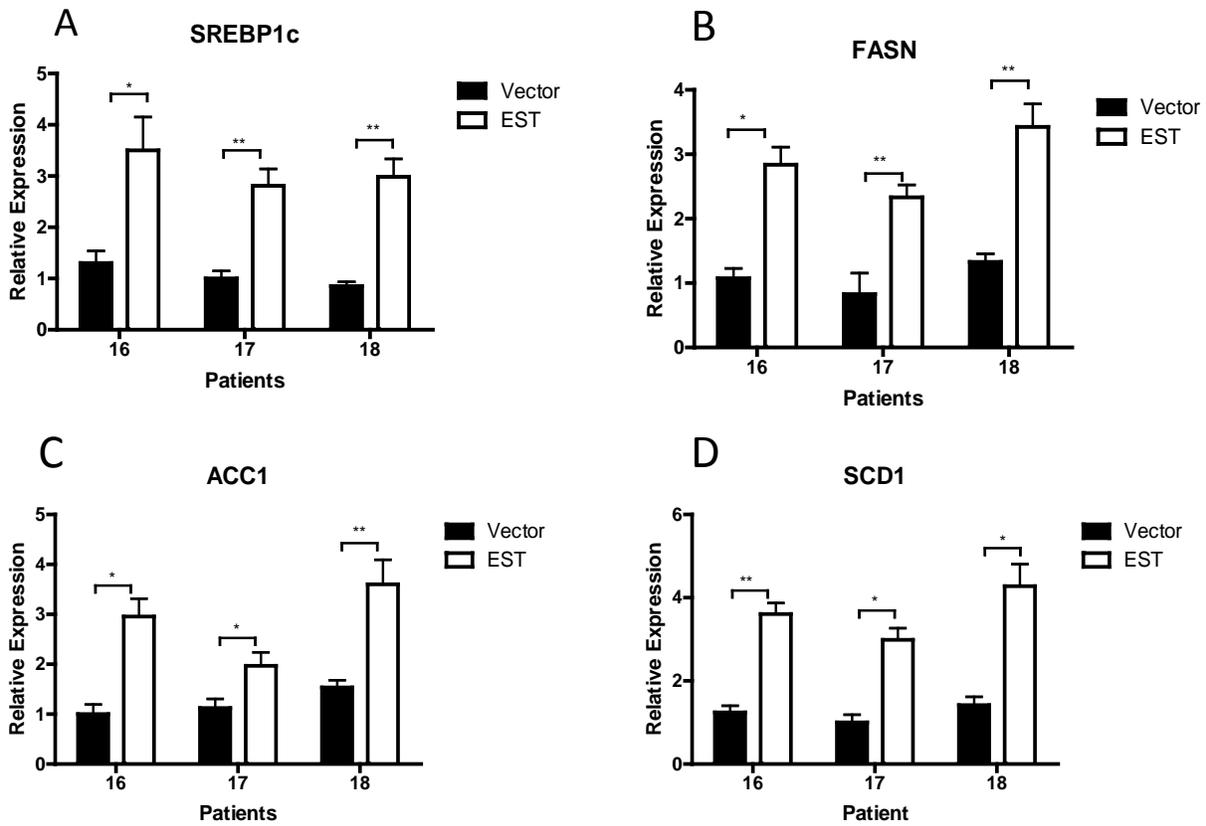
We also analyzed the expression of lipogenic genes, because the adipogenesis and lipogenesis pathways are not mutually exclusive and in fact converge at many points. In the introduction, we discussed the genes which are critical for the induction of lipogenesis, specifically sterol regulatory element binding protein 1c (SREBP1c), acetyl-coenzyme A (CoA) carboxylase1 (ACC1), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1). The activation of these genes is directly and indirectly effected by adipogenic transcription factors and insulin signaling (*section 2.1 background: molecular mechanism controlling adipogenesis and lipogenesis*).

The over-expression of EST in the **overweight/obese cohort** enhanced the expression of several lipogenic genes, including SREBP1c and its target genes ACC, FASN and SCD1 (Fig 15). In contrast, the expression of the lipolytic genes, adipose triglyceride lipase (ATGL) and Hormone sensitive lipase (HSL) were not affected by EST overexpression (Fig 17). The lipogenic and lipolytic results were also recapitulated in 3 independent cases from **lean patients** (Fig 16 and 18). Taken together, these results indicate that EST has the ability to potentiate the mRNA expression of adipogenic and lipogenic genes, while the expression of genes involved in lipolysis is unchanged in obese and lean patients.



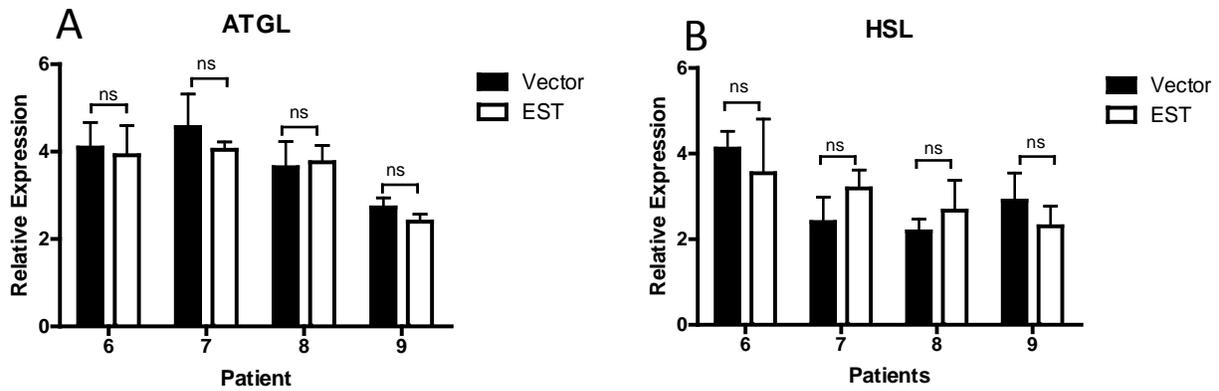
**Figure 15. Obese Patients Lipogenic Gene Expression**

Overexpression of EST promoted Lipogenesis in **Obese Patients**. (A-D) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.



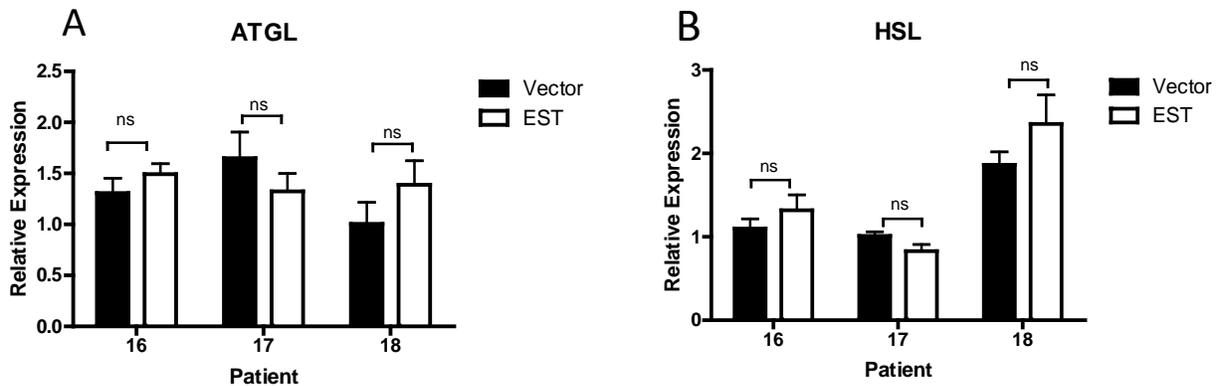
**Figure 16. Lean Patients Lipogenic Gene Expression**

Overexpression of EST promoted Lipogenesis in **Lean Patients**. (A-D) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.



**Figure 17. Obese Patients Lipolytic Gene Expression.**

Overexpression of EST did not change lipolysis in **Obese Patients**. (A-B) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipolytic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; *ns*, not statistically significant compared to vector.



**Figure 18. Lean Patients Lipolytic Genes**

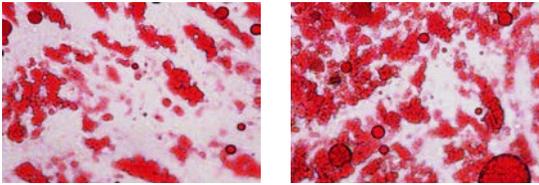
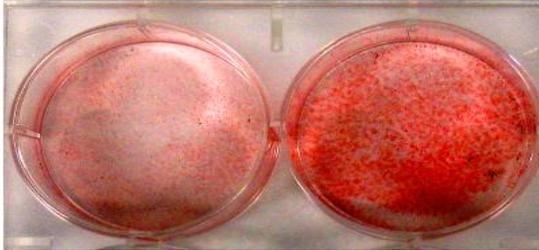
Overexpression of EST did not change lipolysis in **Lean Patients**. (A-B) Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipolytic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; *ns*, not statistically significant compared to vector.

### **2.3.4 Over-expression of EST Increases Lipid Droplet Accumulation in Obese and Lean Patients**

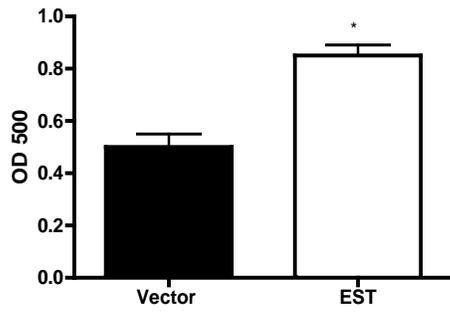
The final outcome of adipogenesis and lipogenesis is the synthesis and storage of fatty acids and triglycerides. We quantitatively and qualitatively measured the accumulation of fatty acids in four **obese patients** using oil red O dye. We found that cells overexpressing EST accumulated significantly more lipid content than cells transduced with vector virus (Fig 19). Additionally, when we analyzed the lipid content in three **lean patients**, we found the same EST promoting effect on lipid accumulation in these cells as well (Fig 20). Taken together, these results indicate that EST has the ability to potentiate adipogenesis and lipogenesis at the molecular genomic level, leading to a significant induction in the number of adipocytes, lipid droplet formation and storage.

A

Patient 6  
Vector EST

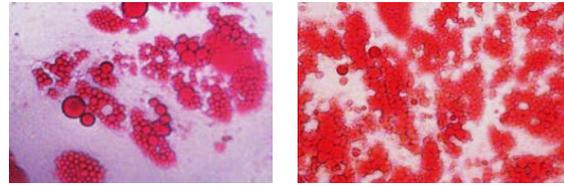


H6 Oil Red O Quantification

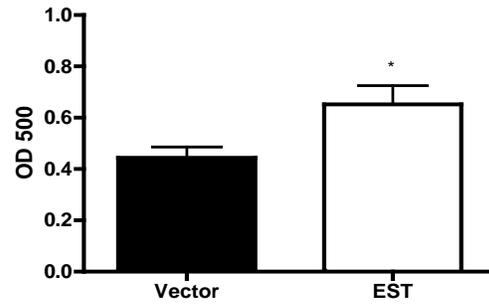


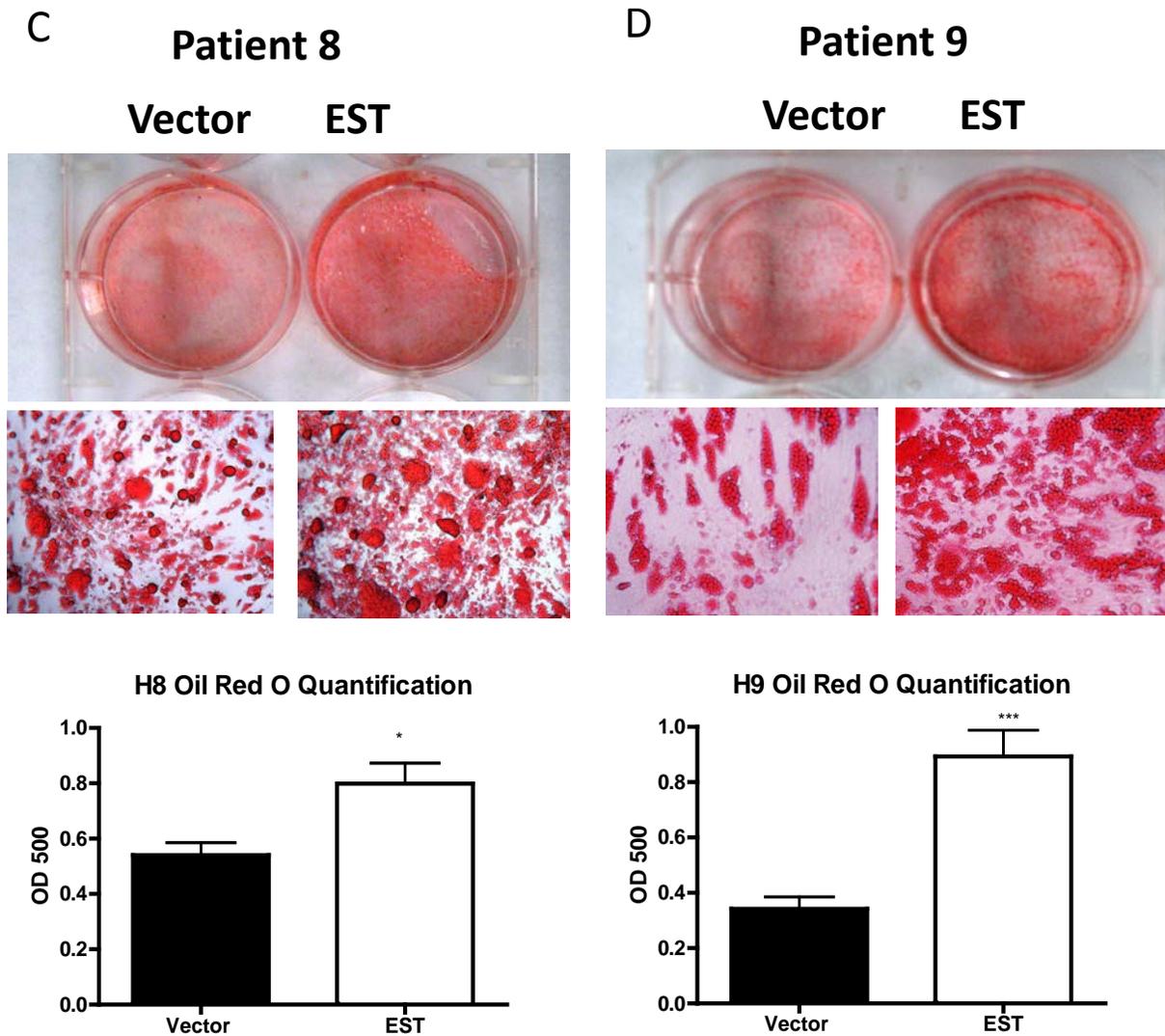
B

Patient 7  
Vector EST



H7 Oil Red O Quantification

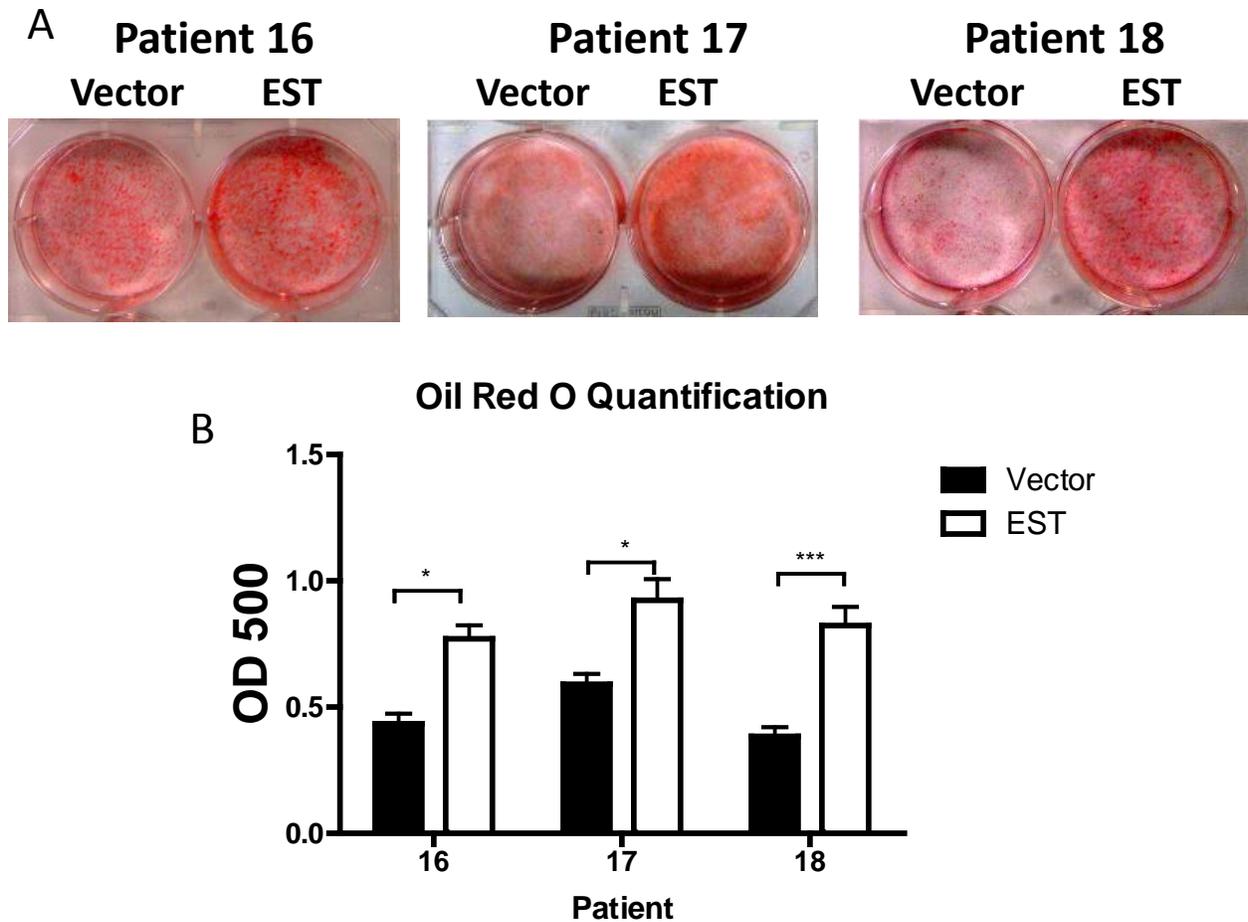




**Figure 19. EST Increases Lipid Droplet Formation in Overweight/Obese Patients**

Preadipocytes from **obese patients** were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. (A-D) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively.

\*,  $P < .05$ ; \*\*,  $P < .01$ ; OD500, optical density at 500nm.



**Figure 20. EST Increase Lipid Droplet Formation in Lean Patients**

Preadipocytes from **lean patients** were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. (A-D) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively. \*,  $P < .05$ ; \*\*,  $P < .01$ ;  $OD_{500}$ , optical density at 500nm.

### 2.3.5 Analysis of EST Over-Expression and Insulin Sensitivity

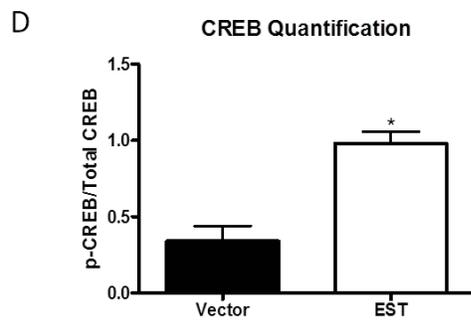
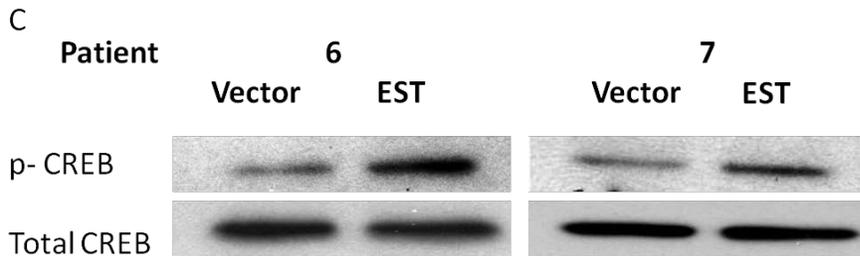
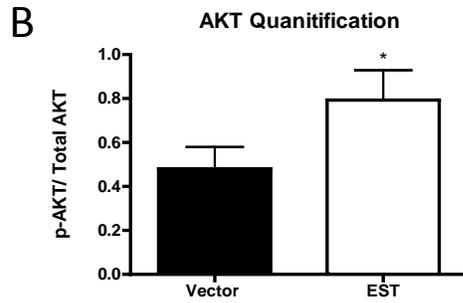
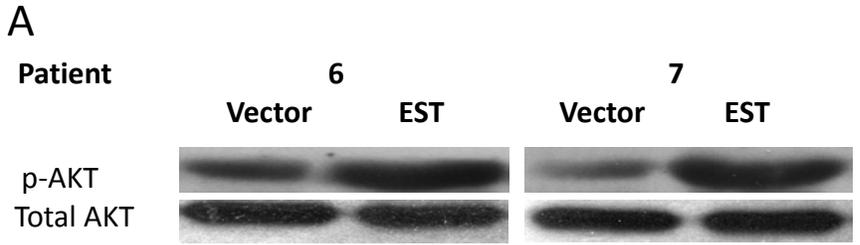
The increase in adipogenesis and lipogenesis, led us to investigate the underlying mechanisms promoting this change. The insulin signaling pathway was analyzed to assess if EST promoted insulin signaling or sensitivity. The Insulin Receptor substrate 1 (IRS-1), the serine/threonine protein kinase B (PKB/AKT) and the cAMP responsive element binding protein (CREB) signaling were examined, because these molecules play an integral role in the insulin signaling pathway. In general the signaling pathway is activated when insulin binds to the insulin receptor, a transmembrane tyrosine kinase receptor, resulting in insulin receptor (IR) autophosphorylation and activation [124]. The activated IR phosphorylates several adaptor/scaffold molecules including the Insulin Receptor Substrate (IRS) family of proteins. The phosphorylation of IRS-1 is a rapid response after insulin treatment and leads to the recruitment of effectors such as phosphoinositide 3-kinase (PI3K), which phosphorylates AKT [124]. Finally, activated AKT can phosphorylate CREB which acts as a transcription factor that can bind to its DNA motif and promote cell survival and differentiation [125, 126].

#### 2.3.5.1 Over-expression of EST Increases AKT and CREB signaling in Obese and Lean Patients

It was found that EST promoted phosphorylation of AKT and CREB in terminally differentiated cells from **obese patients** (Fig 21). Additionally increased AKT signaling in EST transduced cells from **lean patients** was identified (Fig 22).

To analyze if the increased AKT and CREB signaling is a primary effect exerted by EST expression, we transduced preadipocytes with EST and Vector viruses and treated them with insulin for 10 minutes. We found that the phosphorylation of the insulin receptor substrate 1 (IRS1) was similarly increased in vector transduced and EST transduced cells (Fig 23). These

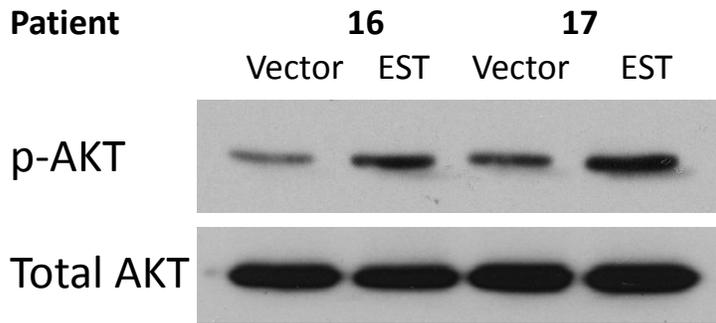
results were surprising because the activation of the inulin signaling pathway typically occurs instantaneously after insulin treatment. This suggests that downstream changes in insulin signaling (AKT and CREB) are secondary effects that may be primarily promoted by changes in adipogenic gene expression. Additionally, as mentioned in the introduction (*C/EBP family*), C/EBP $\alpha$  signaling has been reported to be critical in the regulation of insulin signaling and sensitivity [21, 74]. It is feasible that the increased C/EBP $\alpha$  expression we reported may induce AKT and subsequent CREB signaling, albeit still secondary to the primary genomic changes that are taking place.



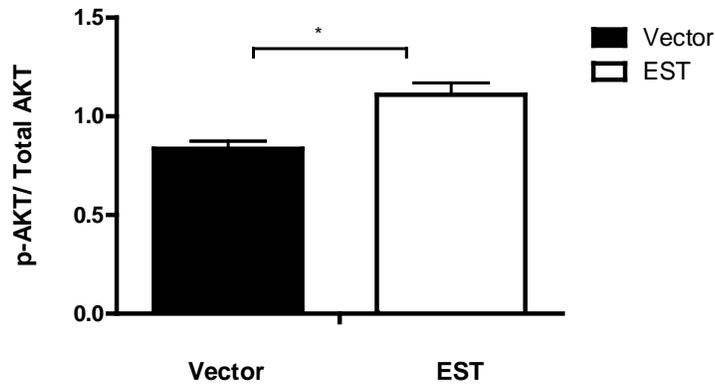
**Figure 21. Obese/Overweight Patients AKT and CREB Signaling**

Lysates from differentiated vector and EST-virus treated cells from obese patients were subjected to Western blot analysis to detect protein expression of (A-B) total AKT, phospho-AKT (p-AKT) and quantification, (C-D) total CREB, and phospho-CREB (p-CREB) and quantification. The signals were quantified by using NIH ImageJ software (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ .

A



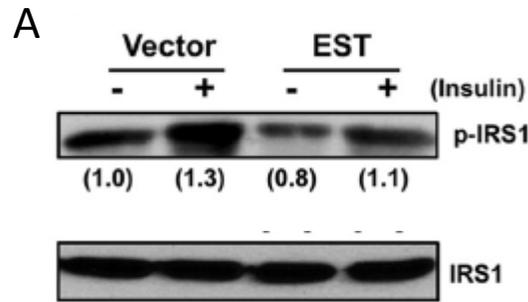
B



**Figure 22. EST Increases AKT signaling in Lean Patients**

Lysates from differentiated cells were subjected to Western blot analysis to detect protein expression of (A-B) total AKT, phospho-AKT (p-AKT). The signals were quantified by using NIH ImageJ software (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ .

### 2.3.5.2 Over-expression of EST does not Enhance IRS-1 Signaling



**Figure 23. EST does not Directly Increase Insulin Signaling**

Preadipocytes from obese patients were transduced with EST or vector viruses and grown to confluency. They were then treated with insulin (100 nM) for 10 min before being evaluated for the protein expression of total IRS1 and phospho-IRS1 by immunoprecipitation and Western blotting. The signals were quantified by using NIH ImageJ software (n=2)

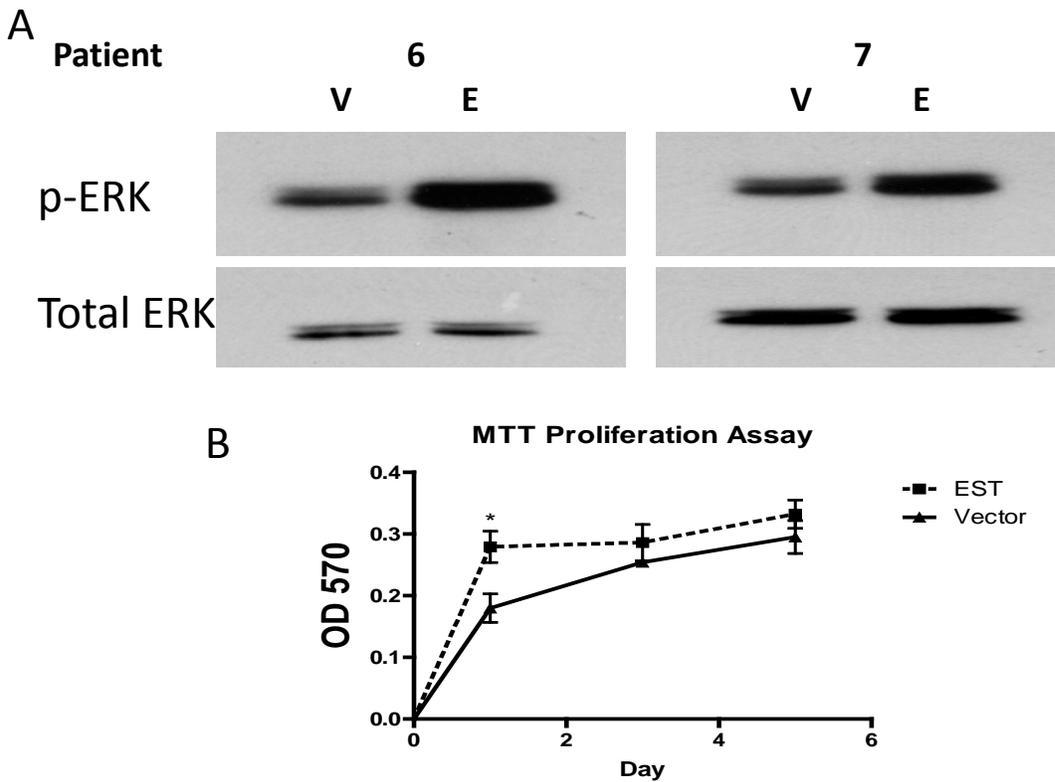
### 2.3.6 Analysis of EST Overexpression and ERK Signaling

#### 2.3.6.1 Over-expression of EST Increases ERK Signaling

The extracellular signal related kinases (ERK1/2) is an essential component of the MAPK signaling pathway that is linked to cell proliferation [127]. Recently it was reported the ERK1/2 signaling may also be important for the recruitment and commitment of undifferentiated preadipocytes to begin the differentiation process [128].

We observed that ERK1/2 signaling was increased in terminally differentiated EST cells compared to vector cells (Fig 24A). Consistent with the increased ERK1/2 phosphorylation, the proliferation of EST cells was indeed increased compared to the vector cells in the early phase of growth, although the difference in proliferation became non-significant after three days of culturing when the cells became more confluent(Fig 24B). To test the significance of ERK

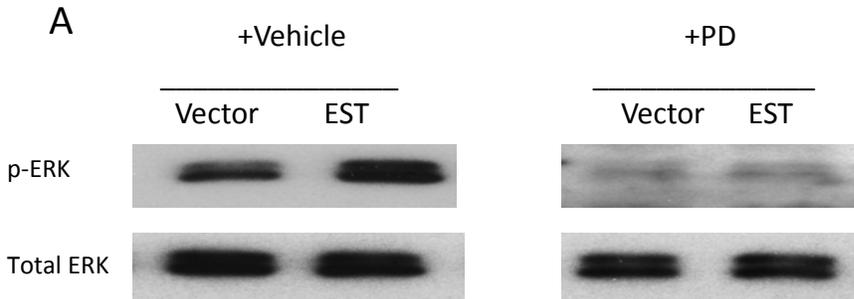
signaling on adipogenesis we grew EST or vector virus transduced cells to confluency and cultured them in differentiation media containing the ERK phosphorylation inhibitor PD98059. Interestingly, the addition of the ERK1/2 inhibitor PD98059 had little effect on the mRNA expression or lipid droplet formation of either the vector or EST cells (Fig 26-27). These results indicate that the increased ERK 1 /2 signaling is most likely a secondary effect since inhibition of said signaling had no effect on adipogenesis in the vector or EST-virus transduced cells.



**Figure 24. ERK Protein Expression and MTT Cell Proliferation Assay**

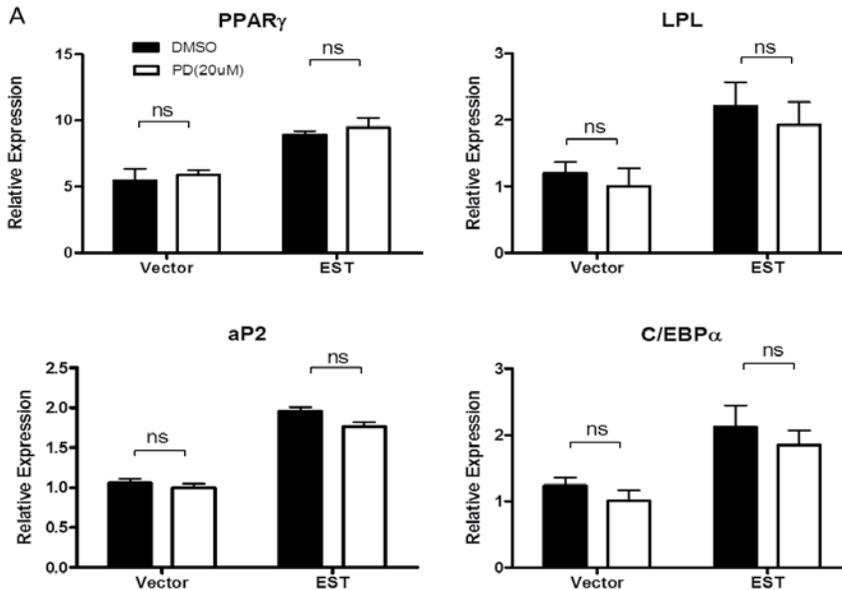
(A) Lysates from differentiated cells were subjected to Western blot analysis to detect the protein expression of total ERK1/2 and phospho-ERK1/2 (p-ERK). (B) Preadipocytes were transduced with vector- or EST-expressing lentivirus and grown in triplicate for each time point and examined by an MTT proliferation assay (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ .

### 2.3.6.2 Pharmacologic Inhibition of ERK Signaling does not ablate EST effect



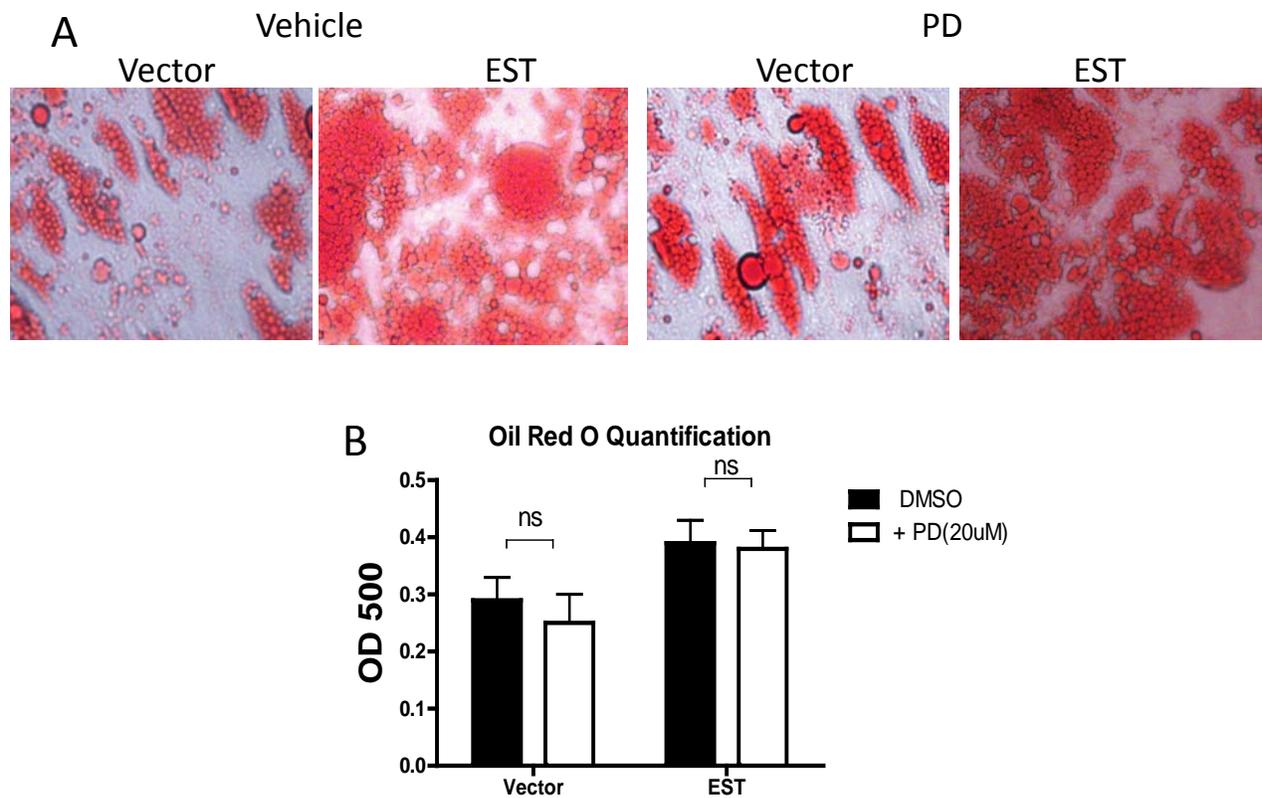
**Figure 25. Pharmacological Inhibition of ERK in obese adipocytes.**

Preadipocytes were grown to confluency then cultured in differentiation media containing the ERK1/2 inhibitor PD98059 for 14 days. Lysates were collected and subjected to Western blot analysis.



**Figure 26. Inhibition of ERK does not affect Adipogenic Gene Expression.**

Preadipocytes from obese patients were grown to confluency then cultured in differentiation media containing the ERK1/2 inhibitor PD98059 for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR (n=1). *ns*, not significant compared to vector.



**Figure 27. Inhibition of ERK does not affect Oil Red O staining**

Preadipocytes from obese patients were transduced with vector or EST-expressing lentivirus, then were cultured in differentiation medium containing the ERK1/2 inhibitor PD98059 for 14 days. (A-B) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively in triplicate groups (n=1). *OD500*, optical density at 500nm; *ns*, not statistically significant compared to vector.

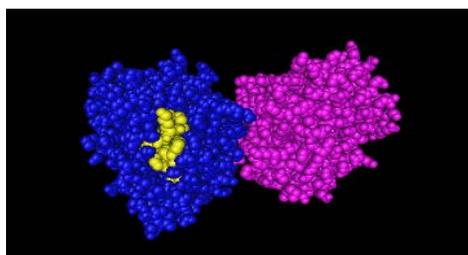
### **2.3.7 Site Directed Mutagenesis of 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) binding domain**

EST catalyzes the transfer of a sulfonate group from the universal sulfonate donor 3-phosphoadenosine 5-phosphosulfate (PAPS) to the estrogens. To assess whether the enzymatic activity of EST is necessary for the pro-adipogenic effect, we generated a lentivirus expressing a mutant human EST lacking enzymatic activity [2]. We along with others have reported that by mutating a conserved domain in the P-loop region of the PAPS-binding domain from GxxGxxK (GGK) to AxxAxxK (AAK), the enzymatic activity of EST was completely abolished[3, 118]. The lack of enzymatic activity of the EST AAK mutant was validated in a transfection and reporter gene assay in which the estrogen-deactivating activity of wild-type EST was abolished in cells transfected with a virus expressing the EST AAK mutant (Materials and Methods 2.2.4). The expression of EST AAK in transduced preadipocytes was confirmed by fluorescence microscopy analysis of GFP and Western blotting (Fig 29). The lack of adipogenic activity of EST AAK was also supported by the lack of induction of adipogenic gene expression (Fig 30) and phosphorylation of AKT and ERK (Fig 30). These results indicate that pro-adipogenic of EST is dependent on its enzymatic activity, and does include passive interaction with other proteins or transcription factors.

**A**

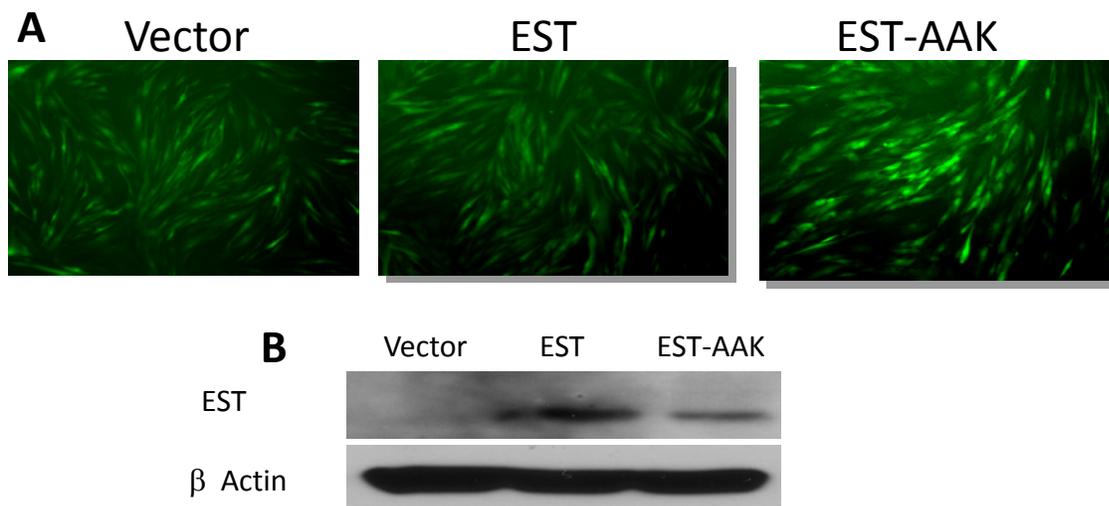
			251		296	
RAT	NP_001166493	(251)	QK	SPFMRKGISGDNRNHF	IVALNESFDKHYQQQMKG	STLQLRTEI
HUMAN	NP_005411	(249)	QK	SPFMRKGITGDNKNHF	IVALNEKFDKHYEQQMK	STLKFRTEI
MOUSE	NP_075624	(250)	QK	SPFMRKGIIGDNKNHF	PEALREAFDEHYKQQMK	CTVKKRMEL
	Consensus	(251)	QKV	SPFMRKGISGDWKNHF	IVALNEKFDKHY	QQMKDSTLKRTEI

The GxxGxxK to AxxAxxK sequence is highlighted in red.



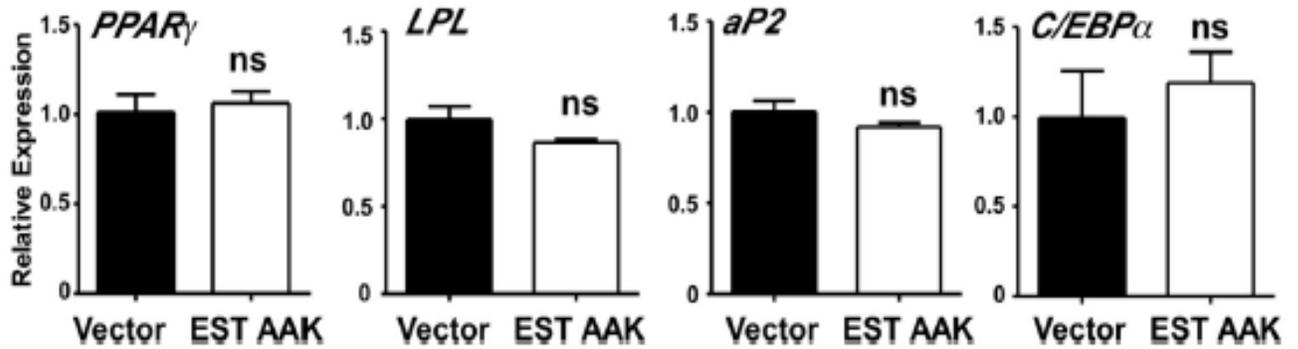
**Figure 28. Multiple Sequence Alignment of PAPS binding domain**

(Upper panel) Multiple sequence alignment of the he P-loop region of the PAPS binding domain in rat, mouse, and human showing conservation across multiple species. (Lower panel) EST dimeric subunit structure with PAPS binding domain highlighted in yellow.

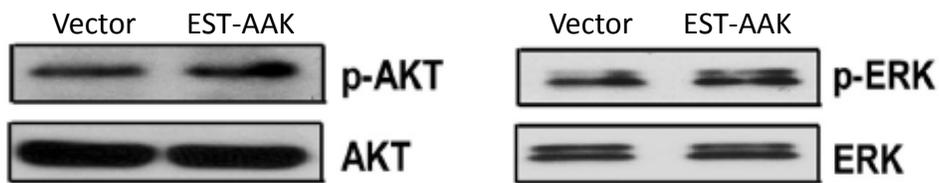


**Figure 29. Transduction of AAK EST, EST, and VECTOR Lentivirus into Preadipocytes**(A) Preadipocytes were transduced with vector, EST, and EST-AAK viruses and analyzed for GFP with fluorescence microscopy and (B) Lysates were collected and subjected to Western blot analysis.

A



B



**Figure 30. Adipogenic Gene Expression and Western Blot Analysis**

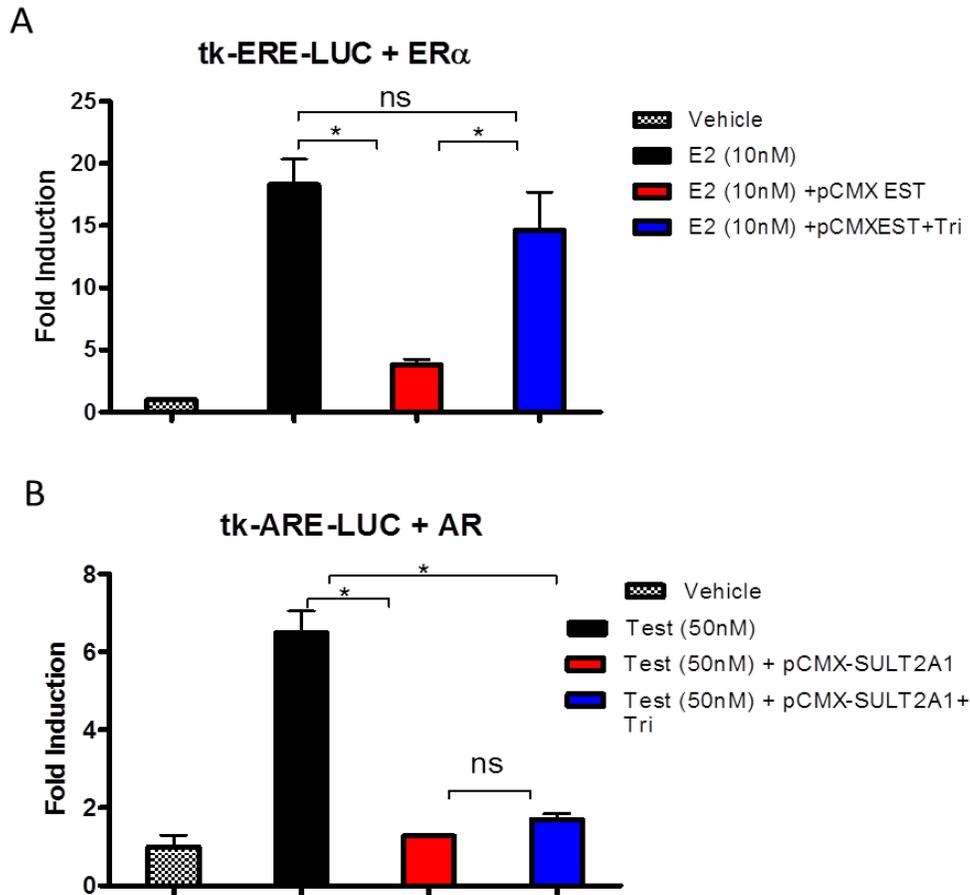
Preadipocytes were transduced with vector- or AAK-expressing lentivirus and then induced to differentiate for 14 days before being evaluated for gene expression analysis by (A) real-time PCR and (B) Western blot analysis to detect total AKT, phospho-AKT, total ERK1/2, and phospho-ERK1/2 (n=1). *ns*, not statistically significant compared to vector.

## **2.4 RESULTS (B) KNOCKDOWN OF EST AND PHARMACOLOGIC INHIBITION IN OBESE AND LEAN PATIENTS INHIBITS ADIPOGENESIS AND LIPOGENESIS**

### **2.4.1 Pharmacologic Inhibition of EST inhibits Adipogenesis**

In the previous section we demonstrated that over-expression of EST promoted adipogenesis and lipogenesis through increased adipogenic/lipogenic gene expression, lipid droplet formation, and increased insulin signaling. We then wanted to determine if the opposite effect would be seen if we inhibited EST.

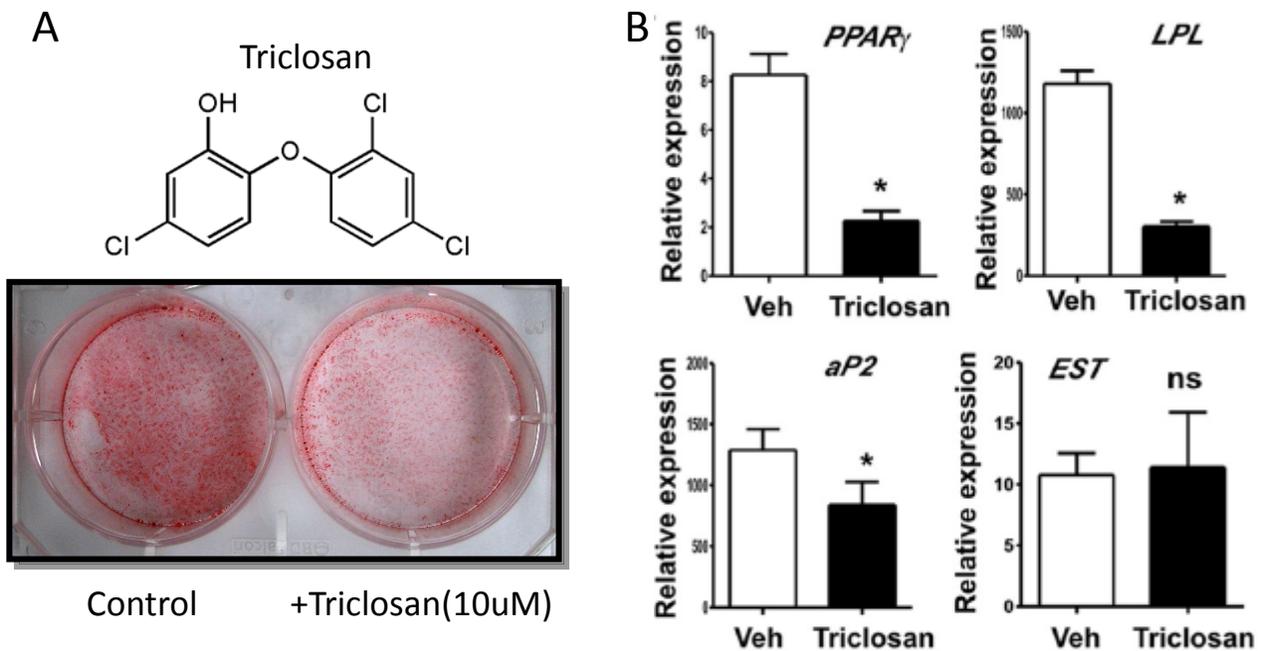
It has been reported that Triclosan, an antimicrobial agent, is an inhibitor of EST [129, 130]. Specifically, Triclosan can bind to the E2 binding pocket in EST causing the formation of Triclosan-sulfate conjugates instead of E2 sulfate conjugates [130]. We tested the specificity of Triclosan inhibition of EST in a luciferase reporter assay where we compared the inhibition of EST to the inhibition of hydroxysteroid sulfotransferase (SULT2A1), a sulfotransferase known to sulfate and deactivate androgens. We transfected tk-ERE-LUC and pCMX-ER $\alpha$  into 293T cells and treated them with 10nM E2. We found that the EST mediated sulfation and repression of E2-ER $\alpha$  activation was abolished when the cells were co-cultured with 10uM Triclosan (Fig 31A). We performed the same assay with tk-AR-LUC and pCMX-AR and found that Triclosan did not inhibit SULT2A1 sulfation and repression on the Androgen Receptor after ligand treatment (Fig 31B).



**Figure 31. Triclosan can Specifically Inhibit EST**

Luciferase Reporter Assay showing Triclosan specificity in 293T cells. (A) tk-ERE-LUC reporter and pCMX-ER $\alpha$  was transfected into 4 triplicate groups. The cells were treated with E2 (10nM), Triclosan (10uM) and transfected with pCMX-EST when applicable. (B) tk-AR-LUC reporter and pCMX-AR was transfected into 4 triplicate groups. The cells were treated with Testosterone (50nM), Triclosan (10uM) and transfected with pCMX- SULT2A1 when applicable. ERE, Estrogen response element; AR, Androgen response element; \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant.

Next we decided to analyze the effects of Triclosan in our differentiation cell culture model. We cultured one patient sample in differentiation media containing 10uM Triclosan for 14 days and found inhibition on the mRNA expression of PPAR $\gamma$  and its target genes LPL and aP2 (Fig 32B). Additionally, we found reduced lipid droplet formation compared to the vehicle treated group (Fig 32A). These results indicate that Triclosan is a specific inhibitor of EST and that pharmacologic inhibition of EST has significant consequences on the adipogenesis in our model.

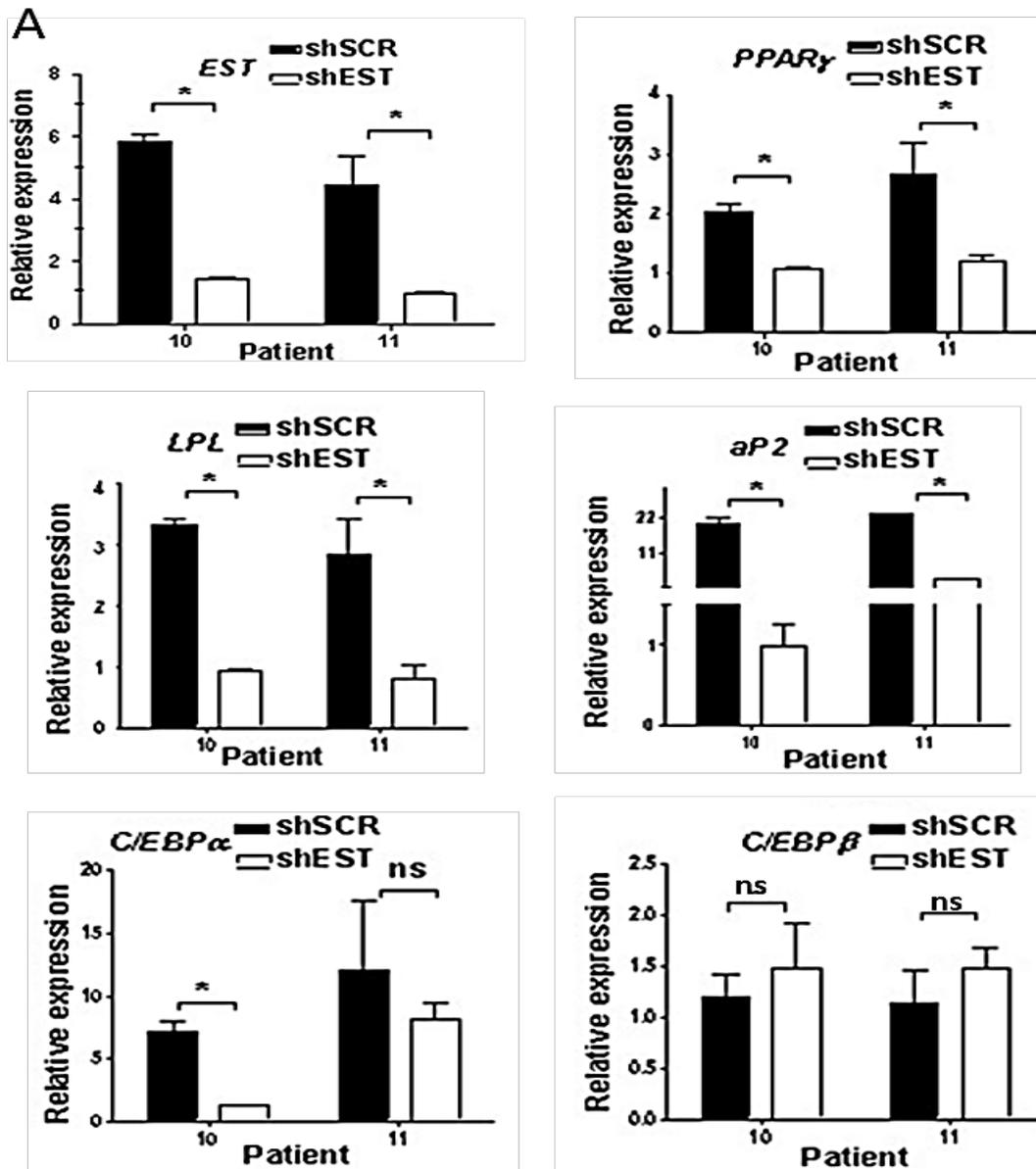


**Figure 32. Triclosan Inhibits Differentiation**

Preadipocytes were induced to differentiate for 2 weeks in the presence of 10 uM Triclosan or vehicle (Veh) before being evaluated for oil red O staining (A) and the expression of adipogenic genes and EST (B). (n=2) \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant

### 2.4.2 Knockdown of EST Inhibits Adipogenic, Lipogenic, and Lipolytic Gene Expression in Obese and Lean Patients

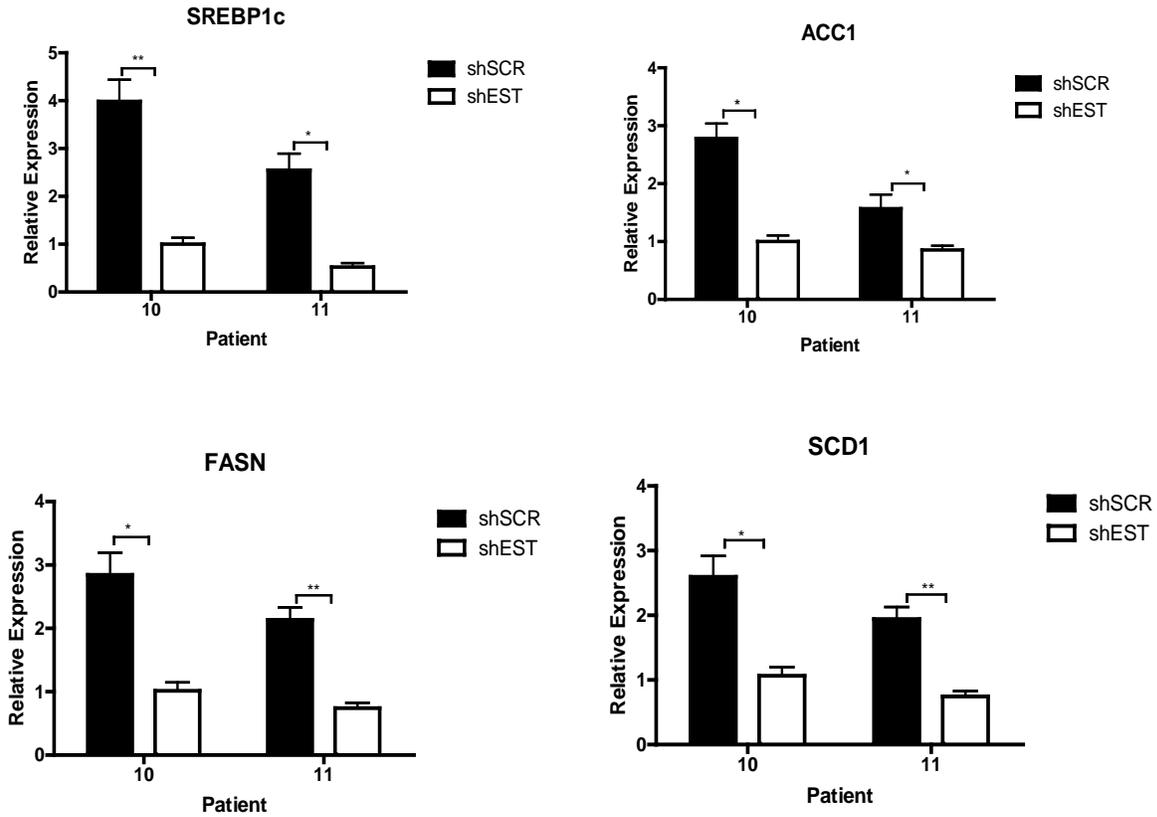
Based on our results from the pharmacologic inhibition of EST using Triclosan, we decided to determine if genetic knockdown could recapitulate the same effects. Additionally, we were concerned that some of the effects that we saw in the Triclosan assay could be based on toxicity. We created two knockdown viruses that express the shRNA against EST, *shEST* and a scrambled control virus, *shSCR* (See virus production in Methods section). Two obese patient samples and 3 lean patients were used to test the adipogenic and lipogenic effect of EST Knockdown (KD). We treated the cells with either *shEST* or *shSCR* virus, and proceeded to terminally differentiate the cells for two weeks. Cells were then analyzed for gene expression, lipid content, and protein expression. The gene expression profile in our **obese patients** confirmed that we had knocked down EST (Fig 33), and additionally that KD of EST caused significant reduction in adipogenic and lipogenic mRNA expression. PPAR $\gamma$  and its target genes LPL and a P2 were both significantly inhibited compared to *shSCR* (Fig 33). C/EBP $\alpha$  was only inhibited in one obese patient sample, but similar to the over-expression model there was no change in C/EBP $\beta$  expression (Fig 33). There was also an inhibition of the lipogenic and lipolytic genetic profile in the **obese patients**; specifically an attenuation of SREBP1c, FASN, SCD1, and ACC (Fig 34) expression as well as ATGL, and HSL (Fig 35) respectively. Further, the results from the adipogenic, lipogenic and lipolytic RT-PCR analysis in obese patients, were recapitulated in the 3 lean patients tested (Fig 36-38). Taken together, these findings indicate that EST KD has a significant impact on adipogenesis and lipogenesis at the genomic level. The ability to reduce the mRNA expression of the aforementioned adipogenic and lipogenic enzymes is an important and novel function of EST in adipocytes.



**Figure 33. Knock down of EST Inhibits Adipogenic Gene Expression in Obese Patients**

Knockdown of EST attenuated adipogenesis in **Obese Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

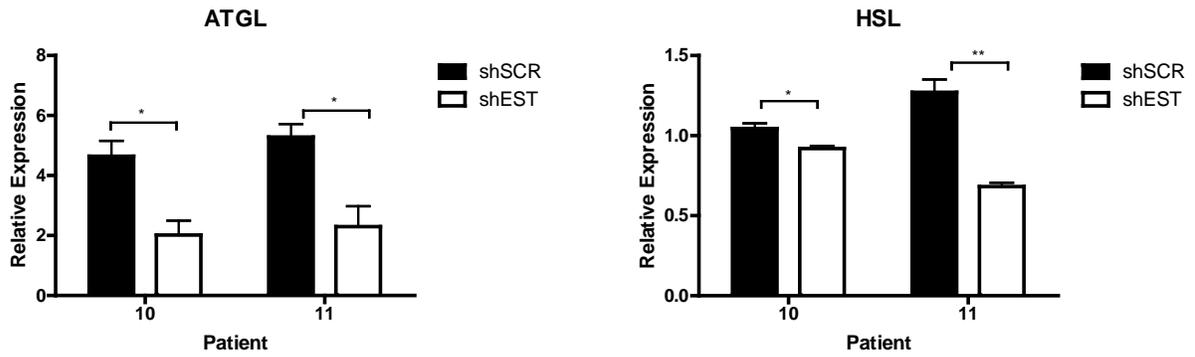
A



**Figure 34. Knockdown of EST Inhibits Lipogenic Gene Expression in Obese Patients**

Knockdown of EST attenuated lipogenesis in **Obese Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; *ns*, not statistically significant compared to vector.

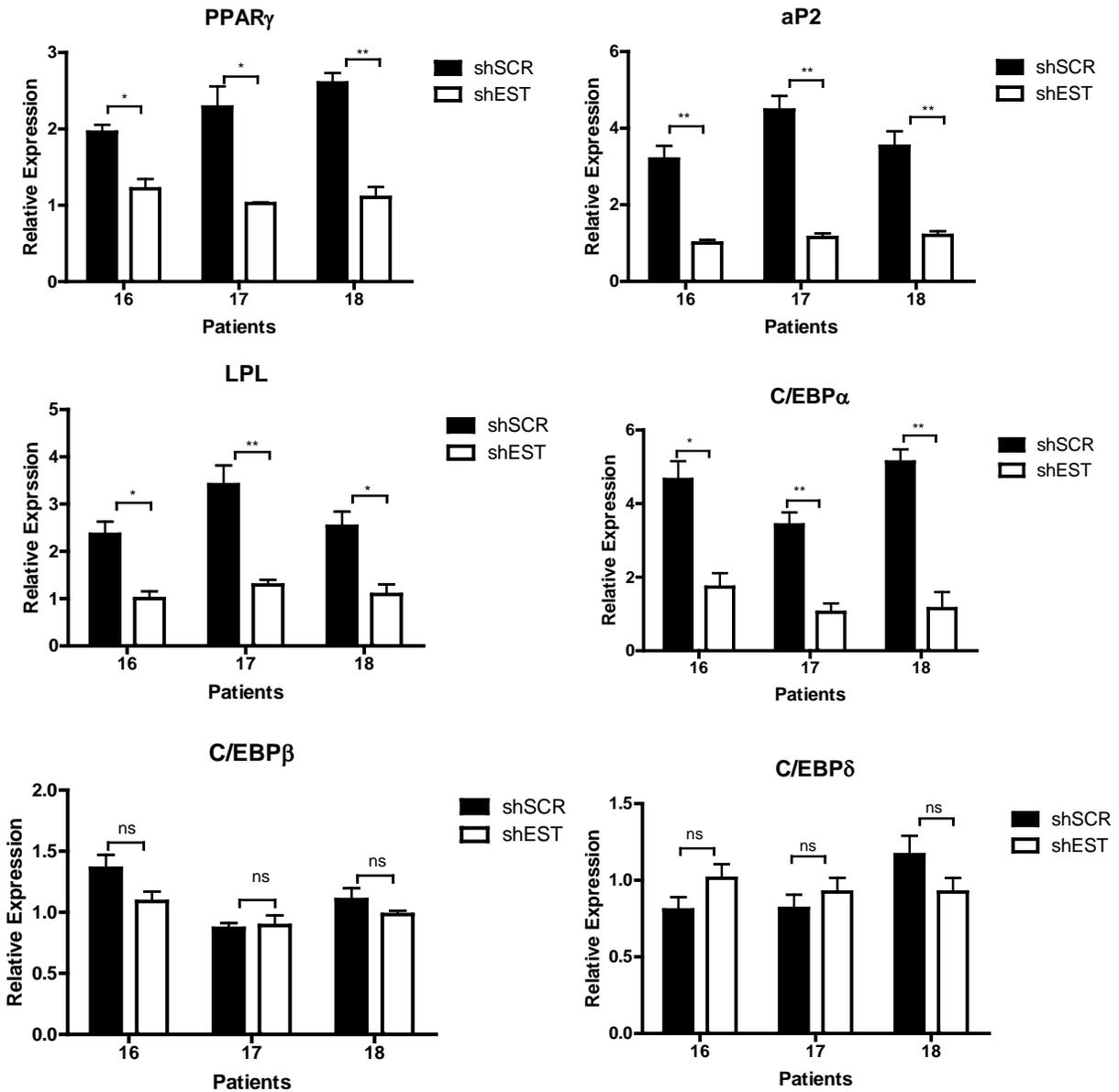
A



**Figure 35. Knockdown of EST Inhibits Lipolytic Gene Expression in Obese Patients**

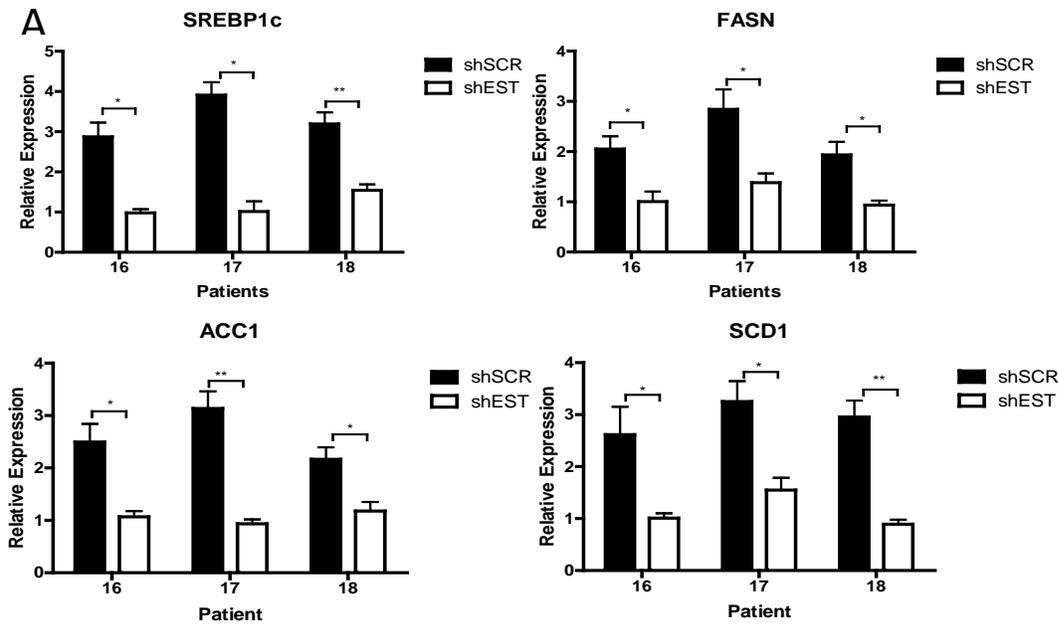
Knockdown of EST attenuated lipolysis in **Obese Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipolytic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ .

A

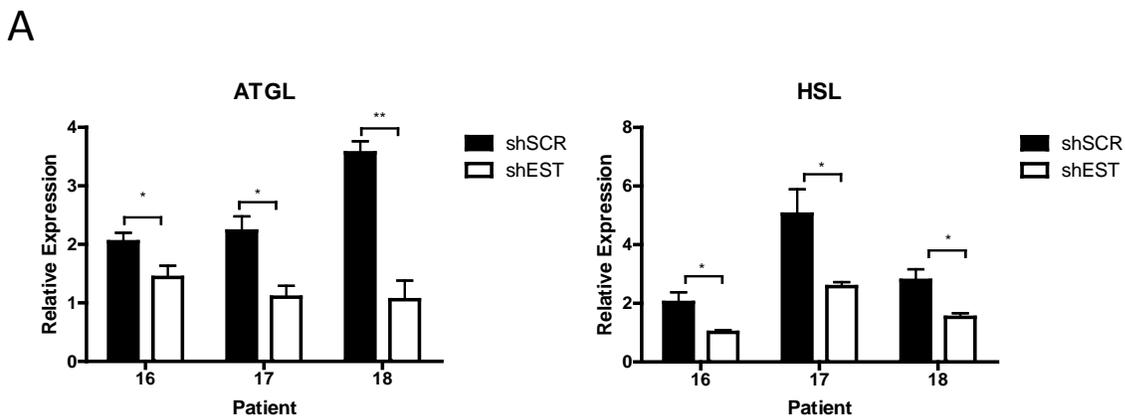


**Figure 36. EST Knockdown Inhibits Adipogenic Gene Expression in Lean Patients**

Knockdown of EST attenuated adipogenesis in **Lean Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.



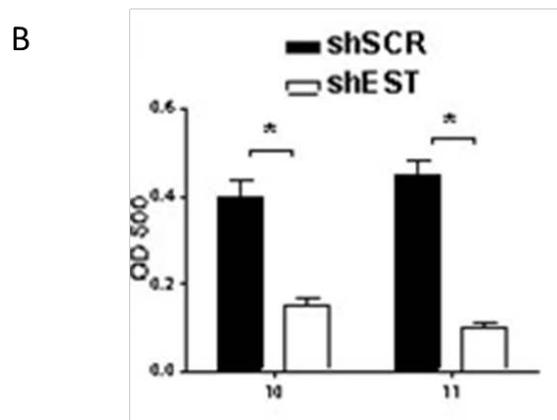
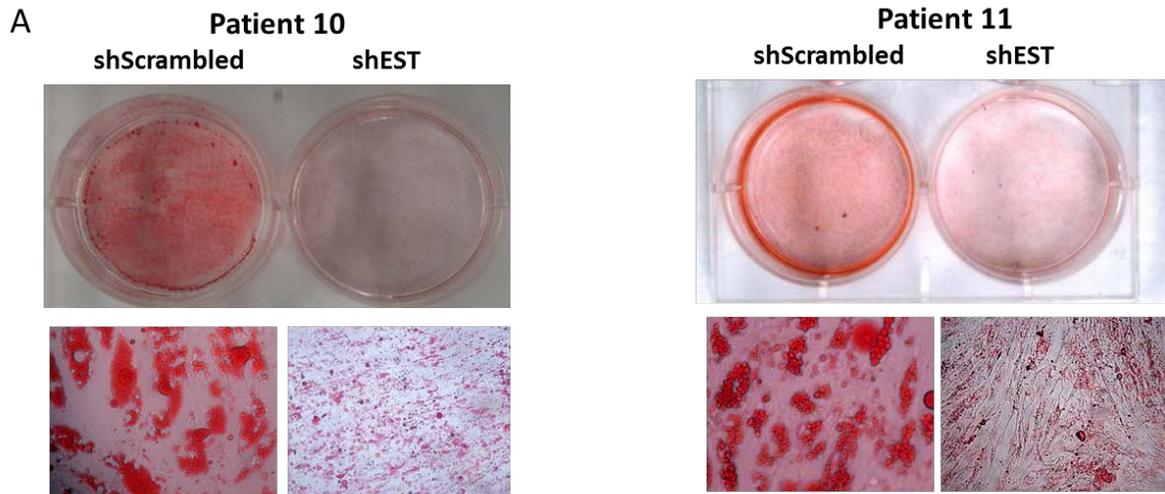
**Figure 37. Knockdown of EST Inhibits Lipogenic Gene Expression in Lean Patients**  
 Knockdown of EST attenuated lipogenesis in **Lean Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ .



**Figure 38. Knockdown of EST Inhibits Lipolytic Gene Expression in Lean Patients**  
 Knockdown of EST attenuated lipolysis in **Lean Patients**. (A) Preadipocytes were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of indicated lipolytic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$

### **2.4.3 EST Knockdown Inhibits Lipid Droplet Accumulation in Obese and Lean Patients**

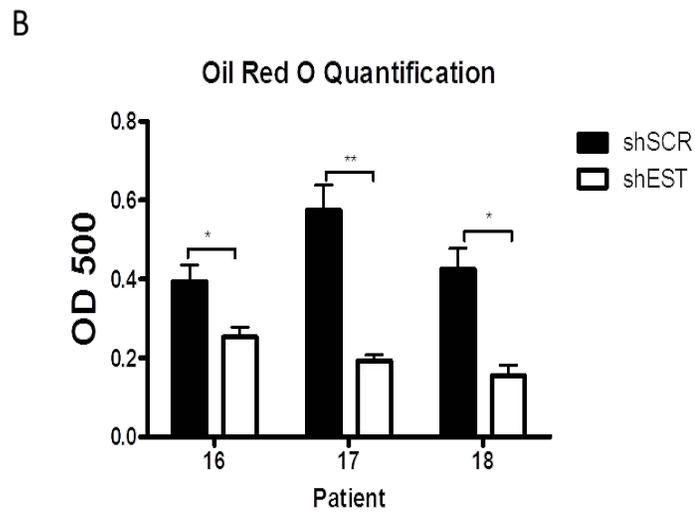
As stated in the previous section detailing the EST over-expression experiments, the functional endpoint of adipogenesis and lipogenesis is the synthesis and storage of fatty acids and triglycerides. We quantitatively and qualitatively measured the accumulation of fatty acids in two **obese patients** using oil red O dye and NIH ImageJ software. The knockdown of EST had a marked inhibitory impact on the synthesis and storage of fatty acids compared to the shSCR control treated cells (Fig 39). Microscopically, the shEST cells remained morphologically similar to fibroblasts, with small lipid droplets forming in a scattered pattern; compared to the shSCR cells which had many lipid clusters throughout the dish (Fig 39). Additionally, we recapitulated these results in 3 **lean patients** as well (Fig 40). These results suggest that the loss of EST mRNA expression in obese and lean patients leads to significant attenuation of lipid storage and synthesis.



**Figure 39. Knockdown of EST Inhibits Lipid Droplet Formation in Obese Patients**

Preadipocytes from **obese patients** were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. (A-B) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively.

*\**,  $P < .05$ ; *\*\**,  $P < .01$ ; OD500, optical density at 500nm.



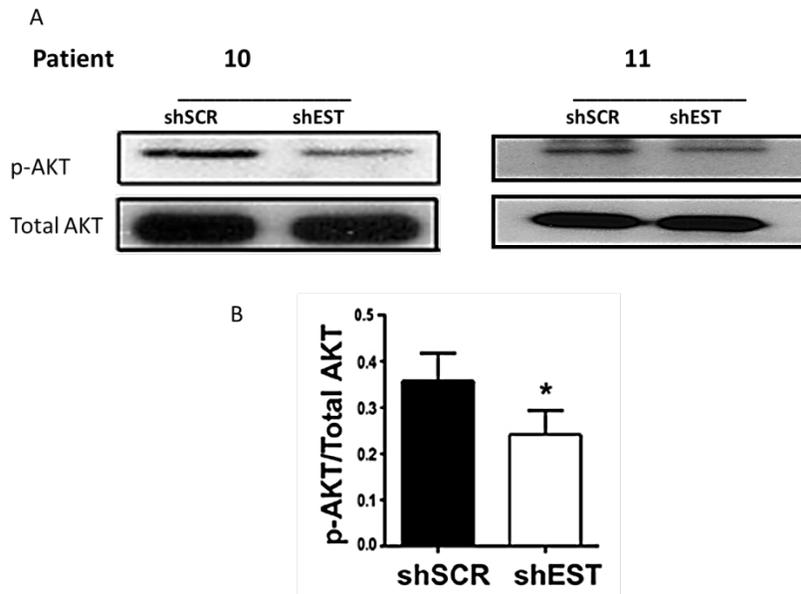
**Figure 40. Knockdown of EST Inhibits Lipid Droplet Formation in Lean Patients**

Preadipocytes from **Lean patients** were transduced with shSCR or shEST-expressing lentivirus, then were cultured and differentiated in standard medium for 14 days. (A-B) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively.

*\**,  $P < .05$ ; *\*\**,  $P < .01$ ; *OD500*, optical density at 500nm.

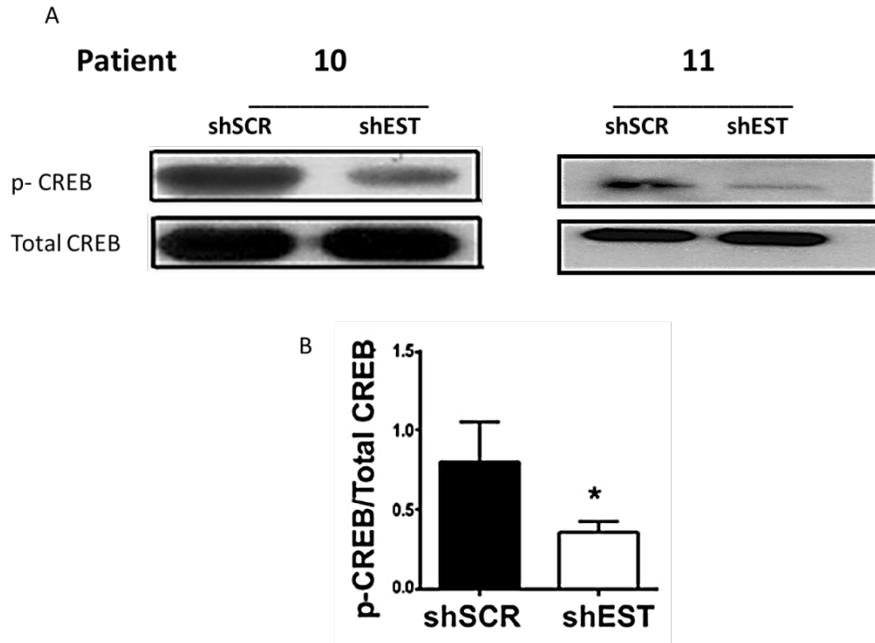
#### 2.4.4 EST Knockdown Inhibits AKT and CREB signaling

Similar to the over-expression experiments, we decided to analyze the insulin signaling pathway to assess if EST KD attenuated insulin signaling or sensitivity. Again we analyzed AKT and CREB activation, two integral signaling molecules in the insulin secondary messenger pathway. Here we found that EST KD did inhibit AKT and CREB signaling compared to shSCR in obese patients (Fig 41-42). Due to limitations in available cells, we were unable to analyze this pathway in lean patients, however based on the gene expression and oil red O results, we are confident that the attenuation of AKT and CREB is also reflected in lean patients.



**Figure 41. Knockdown of EST Inhibits AKT Signaling in Obese Patients**

Lysates from differentiated shSCR and shEST-virus treated cells from obese patients were subjected to Western blot analysis to detect protein expression of (A-B) total AKT, phospho-AKT (p-AKT), The signals were quantified by using NIH ImageJ software (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ .



**Figure 42. Knockdown of EST Inhibits CREB signaling in Obese patients**

Lysates from differentiated shSCR and shEST virus treated cells from obese patients were subjected to Western blot analysis to detect protein expression of (A-B) total CREB, and phospho-CREB (p-CREB). The signals were quantified by using NIH ImageJ software. \*,  $P < .05$ ; \*\*,  $P < .01$ .

## 2.5 DISCUSSION

These studies were driven by observations that EST had a repressive role on adipogenesis in mice, however we have uncovered the opposite effect in human ASCs; here we show a novel functional role for EST in human adipogenesis. To summarize the major findings of this section:

- (1) In preadipocytes the mRNA expression of EST is low and differentiation causes increased expression of EST mRNA. Moreover we observed the protein expression of EST is highest at the beginning of differentiation and the expression begins to diminish during the 14 day culture period (Fig 9).
- (2) Dexamethasone treatment during differentiation induces EST mRNA expression, and differentiation without DEX treatment causes no induction of EST, moreover, previously we published data which shows that there is a glucocorticoid response element in the promoter region of EST [123](Fig 10).
- (3) Over-expression of EST using lentivirus leads to significant genomic changes in obese and lean patients which include an enhanced induction of PPAR $\gamma$  and PPAR $\gamma$  target genes (LPL and aP2) and C/EBP compared to vector (Fig 13-14) . Additionally, we observed a promotion of lipogenic signaling mRNA that included SREBP1c, ACC, FASN, and SCD1(Fig 15-16). We did not see a difference in lipolytic mRNA signaling between the vector and EST groups (Fig 17-18).
- (4) Over-expression of EST in obese and lean patients caused an increase in lipid droplet formation compared to vector treated cells as noted using Oil Red-O staining and quantification (Fig 19-20).
- (5) Over-expression of EST markedly increased the terminal protein expression of AKT, CREB, and ERK 1/2 in obese patients (Fig 21, 24) and enhanced AKT signaling in lean

patients (Fig 22). However we did not see a primary difference in rapid (10 minute treatment) IRS1 signaling between the vector and EST treated cells (Fig 23). Moreover, the pharmacologic inhibition of ERK1/ 2 signaling during adipogenesis had no effect on enhanced adipogenic mRNA expression or lipid droplet formation caused by EST over-expression (Fig 25-27).

- (6) Genetic knock-down and pharmacologic inhibition of EST in obese and lean patients caused the opposite effects as those in the over-expression model. Specifically, we observed a marked decrease in adipogenic, lipogenic, and lipolytic mRNA signaling; lipid droplet formation, and AKT/ CREB signaling (Fig 33-42).
- (7) The changes observed from over-expressing or knocking down EST are dependent on the enzymatic activity of EST as shown in section 2.3.7, where we created an enzymatically dead mutant that had no effect on adipogenic gene signaling, or AKT/CREB activity.

Our results demonstrate that EST acts as a positive regulator of adipogenesis in human abdominal preadipocytes and adipocytes taken from both lean and obese female patients. We observed that at the mRNA level, EST expression is low in preadipocytes and differentiation leads to a marked induction of this expression (Fig 9A-F). Moreover, our time course of EST protein expression indicates that EST is expressed highest at the beginning of differentiation and this expression begins to diminish over the course of the 14 day differentiation period (Fig 9E). Additionally, we demonstrated that without Dexamethasone treatment during differentiation, EST is not induced at the mRNA level along with most other adipogenic enzymes and markers. These results strongly suggest that the DEX-GR-SULT1E1 pathway elucidated in MCF-7 cells and *in vivo* mouse models [123] is conserved in human adipocytes and is necessary for efficient induction of EST and adipogenesis. These results also show that along with the array of other

adipogenic transcription factors and enzymes, EST displays temporal and spatial expression during adipogenesis. Moreover, since EST is highly expressed at the beginning of differentiation, this suggests that EST is important for the commitment of preadipocytes to mature into functional adipocytes.

The preadipocytes taken from our patient population of lean and obese females undergo both genomic and functional changes that promote or attenuate adipogenesis and lipogenesis as a result of EST over-expression or knock down, respectively. The promotion of adipogenesis and lipogenesis at the genomic and lipid level was coupled with increased AKT, CREB, and ERK protein signaling; conversely we observed the opposite when EST was knocked- down. The fact that EST is highly expressed at the beginning of differentiation and diminishes over the course of the 14 day differentiation period indicates that this may be a critical regulatory mechanism to mediate a healthy level of adipocyte formation and lipid synthesis. When we over-expressed EST we ectopically caused sustained expression of EST throughout the entire course of differentiation, which facilitated increased adipogenesis (Fig 10). Likewise, when we knocked down the mRNA expression of EST, the lack of induction at beginning of differentiation attenuated the expression of adipogenic and lipogenic genes. Taken together our data suggests that EST has the ability to mediate the response of the preadipocyte to stimulation by adipogenic cues. The mechanisms through which EST can mediate the adipogenic response are unknown and became the next focus of our experiments.

We observed after terminal differentiation that the overexpression of EST increased AKT/CREB signaling, while the opposite was seen after knockdown of EST. Initially we analyzed whether EST is primarily increasing insulin signaling through AKT/CREB, subsequently leading to increased adipogenic and lipogenic mRNA signaling and lipid accumulation. To do this we used

Co-Immunoprecipitation assays (Co-IP) to detect initial changes in IRS-1 phosphorylation between EST and vector transduced cells (Fig 23). Our assay showed that after 10 minutes of insulin treatment there was no difference in IRS-1 phosphorylation between vector and EST-virus transduced cells (Fig 23).

Additionally, we observed that EST-virus transduced cells had increased ERK 1/2 protein signaling (Fig 24). Therefore we analyzed if modulating ERK signaling was a primary signaling effect facilitated by EST-overexpression that lead to increased adipogenic/lipogenic mRNA signaling. To test this we cultured vector and EST cells in standard differentiation media containing an ERK inhibitor for two weeks (Fig 25-27). Interestingly, we found that this did not attenuate the adipogenesis promoting effects of EST. Collectively, these data suggest that the promotion of AKT, CREB, and ERK 1/2 signaling is not the primary driving force behind EST stimulated adipogenesis. These are most likely secondary effects that become apparent in terminally differentiated cells which are transduced with EST virus.

The primary role of EST is to sulfate and deactivate estrogen, and based on the vast array of data detailing the effects of estrogen in abdominal subcutaneous adipose tissue (covered in section 1.2.2 ER $\alpha$ / $\beta$ ), the observations shown here strongly suggest that EST is promoting adipogenesis by diminishing the repressive effects imposed by estrogen signaling. Adipogenesis is activated at the genomic level primarily through PPAR $\gamma$  activation. Mechanistically, it is feasible that by inhibiting estrogen signaling coactivator recruitment is shifted toward PPAR $\gamma$ . Once PPAR $\gamma$  is robustly activated it subsequently promotes a commensurate adipogenic and lipogenic response. Increased PPAR $\gamma$  signaling can directly increase its own target genes (LPL and aP2) as well as C/EBP $\alpha$  (Fig 4). Additionally, as reported in section 1.2.3, C/EBP $\alpha$  activation is paramount for

proper insulin signaling [21, 74]. The observed increase in C/EBP $\alpha$  signaling after EST-virus transduction may facilitate increased downstream promotion of AKT and CREB signaling. Moreover, as discussed in *section 2.1*, the induction of SREBP1c and its target genes is promoted by C/EBP $\alpha$  induced insulin signaling, which would explain the increased lipogenic mRNA signaling [115, 116]. Collectively, these changes can synergistically enhance lipid droplet formation and lipid accumulation, and therefore can also cause the reverse effect shown when we knock down EST. In the next chapter we will mechanistically evaluate the link between EST, estrogen signaling and adipogenesis in our cell culture model. We will evaluate changes in coactivator recruitment, or squelching (1.2.4 Nuclear Receptors and Coregulators in Nuclear Receptor Pharmacology), that may determine the extent of PPAR $\gamma$  and PPAR $\gamma$  target gene activation in the presence of estrogen or lack thereof. Additionally, we will discuss the role that estrogen signaling traditionally exerts over AKT, CREB, and ERK signaling and evaluate why we have observed changes in these signaling molecules which are inconsistent with literature data.

### **3.0 CHAPTER III: PHARMACOLOGIC INHIBITION AND KNOCKDOWN OF ER $\alpha$ IN OBESE PATIENTS ENHANCES ADIPOGENESIS AND LIPOGENESIS**

#### **3.1 BACKGROUND**

The primary role of EST is to inhibit estrogen signaling; our results from the previous chapter strongly suggest that the changes in estrogen signaling mediated by EST over-expression or knockdown cause the promotion or attenuation of adipogenesis and lipogenesis, respectively. For more information on estrogen signaling in adipocytes see *section 1.2.2 ER $\alpha$ / $\beta$* . It is known that the ER $\alpha$ / $\beta$  are expressed in preadipocytes and adipocytes, and that ER $\alpha$  typically mediates signaling involved energy metabolism and adipose tissue accumulation [44], therefore we decided to verify the presence and function of ER $\alpha$  in our cell culture model. Additionally, we will mechanistically evaluate the link between EST, estrogen signaling and adipogenesis in our cell culture model.

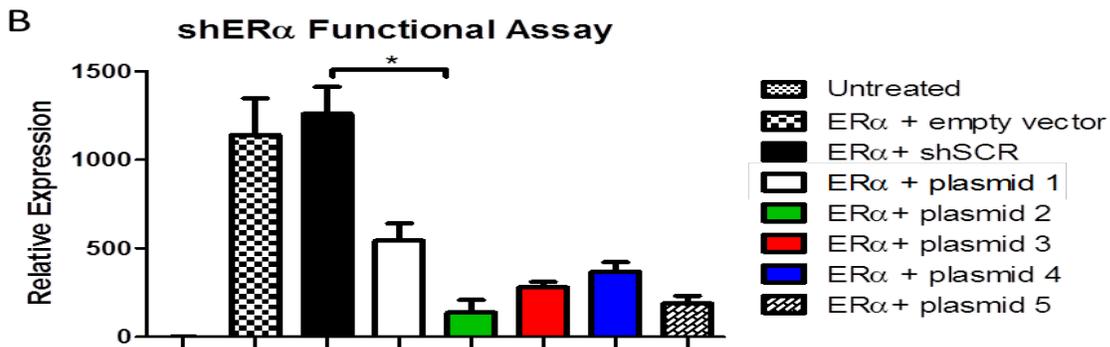
## 3.2 METHODS

### 3.2.1 Patient Population

*ASC and adipose tissue collection:* Human adipose-derived stem cells (ASCs; primary preadipocytes) and whole fat/lipoaspirate were obtained through the Adipose Stem Cell Center, Department of Plastic Surgery, University of Pittsburgh. The whole fat or liposuction aspirates were collected from the abdominal subcutaneous fat of 18 female patients who were nondiabetic nonsmokers and ranged in age from 32 to 59 years. Subsequently, preadipocytes were isolated from the abdominal subcutaneous fat of these 18 patients [15 obese patients and 3 nonobese (lean) patients]. For linear regression analysis, 16 additional obese patients' whole fat or lipoaspirate was analyzed. All experiments were performed on cells from the obese patients except as otherwise specified. The names of the patients were kept anonymous, and all patients used for preadipocyte differentiation experiments were female, nondiabetic nonsmokers and ranged in age from 25 to 56 years. The cells were cultured under standard conditions as reported previously. Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 medium containing 10% standard fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep). Each cell line was cultured for no more than four passages. Patient sample and data collection were performed in accordance with the University of Pittsburgh Institutional Review Board Protocol PRO12050016.

### 3.2.2 Creation and Characterization of Lentivirus to Knockdown ER $\alpha$

To generate lentivirus expressing short hairpin RNAs (shRNAs) against ER $\alpha$  (shER $\alpha$ ), expression plasmids containing shER $\alpha$  were purchased from Open Biosystems (Pittsburgh, PA). For each knockdown, five sequences were purchased and tested in transient-transfection assays. In this assay 0.2ug pCMX-ER $\alpha$  was transfected into 293T cells, followed by 2ug of one of the 5 plasmids purchased (10:1 knockdown constructs: pCMX-ER $\alpha$ ); the sequence with the most efficient knockdown was chosen for lentiviral production (Fig 43). A scrambled shRNA (shSCR) plasmid was purchased to serve as a control. Plasmid sequences for shER $\alpha$  can be found in APPENDIX A. Lentiviral particles were generated using the second generation lentiviral system as described previously.



**Figure 43 ER $\alpha$  Knockdown (KD) Functional Assay**

293T cells were plated in 6 wells plates and grown to 80% confluency. Duplicate co-transfections were performed with 0.2ug pCMX-ER $\alpha$ , 2ug pCMX-pl2 (empty vector), 2ug scrambled or knockdown plasmids when applicable. For ER $\alpha$  KD, plasmid 2 was chosen for virus production. Sequences can be found in APPENDIX A.

### **3.2.3 Adipocyte Culturing and Differentiation**

In all experiments except those in which exogenous estrogen (E2) was added, differentiation medium 1 (DM1) consisted of DMEM/F-12 medium, 10% standard FBS, 1% Pen-Strep, 33uM biotin, 100 nM insulin, 17 uM pantothenic acid, 0.5 mM methylisobutylxanthine, 1 uM dexamethasone, and 1 uM rosiglitazone. Differentiation medium 2 (DM2) consisted of DMEM/F-12 medium, 10% FBS, 1% Pen-Strep, 1 uM dexamethasone, and 100 nM insulin. Confluent preadipocytes were cultured in DM1 for 3 days before being switched to DM2 for 2 weeks to reach terminal differentiation. Culture medium was changed every other day. For differentiation experiments in which E2 was exogenously added, the cells were cultured in phenol red-free DMEM/ F-12 medium and dextran-coated charcoal (DCC)-stripped FBS before the addition of E2 at a final concentration of 10 nM.

### **3.2.4 Gene Expression Analysis**

Total RNA from preadipocytes/adipocytes was isolated using TRIZOL (Invitrogen) reagent. Total RNA from whole fat tissue or lipoaspirate was collected using the RNEASY Lipid Tissue Mid-Kits (Quiagen). The cDNA was synthesized from 1.0 ug of total RNA by Iscript from Bio-Rad(Hercules, CA). Aliquots of cDNA were amplified on an ABI 7300 Real-Time PCR System from Applied Biosystems (Foster City, CA) using the SYBR green PCR master mix. mRNA expression was normalized against the expression of cyclophilin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A complete list of primers used can be found in APPENDIX A. Primers were validated using serial dilutions (1fg -10ng) of linearized expression vectors for the target gene or quantified cDNA from differentiated adipocytes to generate efficiency curves. An example of an efficiency curve validating the EST primer can be found in APPENDIX A.

### **3.2.5 Oil Red O Staining and Quantification**

Differentiated six-well culture dishes were washed twice with cold phosphate-buffered saline (PBS) and then prefixed for 1 h with 10% formaldehyde in PBS. After 1 h, fresh 10% formaldehyde was added, and the cells were incubated overnight at room temperature. The next day, the cells were washed twice with PBS and then incubated in 60% isopropanol for 5 min, followed by drying at room temperature (RT). Cells were incubated with oil red O working solution for 10 minutes and then washed five times with deionized water. Images were acquired microscopically. For quantification of oil red O staining, cells were differentiated in triplicate in 24-well plates, stained with oil red O, and eluted with 100% isopropanol, and 100  $\mu$ L of elute was loaded onto 96-well plates. Absorbance was measured at 500 nm using a PerkinElmer plate reader.

### **3.2.6 Western Blot Analysis**

Cells were lysed with NP-40 lysis buffer containing protease inhibitors and then quantified for protein concentrations by a bicinchoninic acid (BCA) assay kit from Pierce (Rockford, IL). Protein samples were resolved by electrophoresis on 10% SDS-polyacryl-amide gels. For the detection of insulin receptor substrate 1 (IRS1) and its phosphorylation, cell lysates were immunoprecipitated with an IRS1 antibody before being subjected to Western blotting using an IRS1 antibody and phosphotyrosine antibody. After transfer of proteins to polyvinylidene difluoride (PVDF) membranes, the membranes were probed with antibodies against total extracellular signal-regulated kinases 1 and 2 (ERK1/2) (catalog no. sc94; Santa Cruz), phospho-ERK1/2 (catalog no.sc7383; Santa Cruz), total AKT (catalog no. 9272; Cell Signaling), phospho-AKT (catalog no. 9215; Cell Signaling), phospho-CREB (catalog no.87G3; Cell Signaling), total CREB (catalog no. 48H2; Cell Signaling), human EST (catalog no. SAB1400267; Sigma),

ER $\alpha$  (catalog no. sc7207; Santa Cruz), IRS1 (catalog no. 2382S; Cell Signaling), and phosphotyrosine (catalog no. ab10321; Abcam). Detection was achieved by using an ECL system from Amersham (Piscataway, NJ). Quantification was performed using the NIH ImageJ software.

### **3.2.7 Transient Transfection Assay**

HepG2 cells or 293T cells were plated at a density of  $2 \times 10^6$  cells per 48-well plate and incubated overnight. Transfection was performed using Trans-IT reagent from Mirus. Plasmids that were used in triplicate at an amount of 300 ng included pCMX-EST, pCMX-EST AAK, pCMX-ER $\alpha$ , pCMX-PPAR $\gamma$ , and pCMX. The triplicate plasmid amounts for pCMX-CBP, pCMX- $\beta$ -Gal (where  $\beta$ -Gal is b-galactosidase), and tk-ERE-Luc (where ERE is estrogen response element Luc is luciferase) or tk-PPRE-Luc (where PPRE is peroxisome proliferator response element) were 50 ng, 200 ng, and 600ng, respectively. Cells were transfected and incubated for 24 h. Transfected cells were treated with the appropriate ligand for 24hrs using DMEM without phenol red and DCC FBS, followed by lysis and assays for luciferase and  $\beta$ -Gal activities. The luciferase activities were normalized to  $\beta$ -Gal activities.

### **3.2.8 Chromatin Immunoprecipitation (ChIP) Assay**

Chromatin immunoprecipitation (ChIP) was performed according to a standard protocol (16). In brief, preadipocytes were plated in duplicate six-well plates, infected with either the vector or EST-expressing virus (here, EST virus), grown to confluence, and differentiated. Cross-linking was performed by the addition of formaldehyde, followed by sonication to shear the DNA. Immunoprecipitation was performed using an anti-PPAR $\gamma$  antibody (catalog no. ab45036) from Abcam, followed by elution using protein A magnetic beads (catalog no. S1425S) from NEB

(Ipswich, MA). Duplicate eluates and 2% of the input DNA were amplified by RT-PCR, and the PCR products were resolved on a 1% agarose gel. Quantification was performed by using the NIH ImageJ software. Fold enrichment was calculated as precipitated DNA versus input DNA.

### **3.2.9 Statistical Analysis**

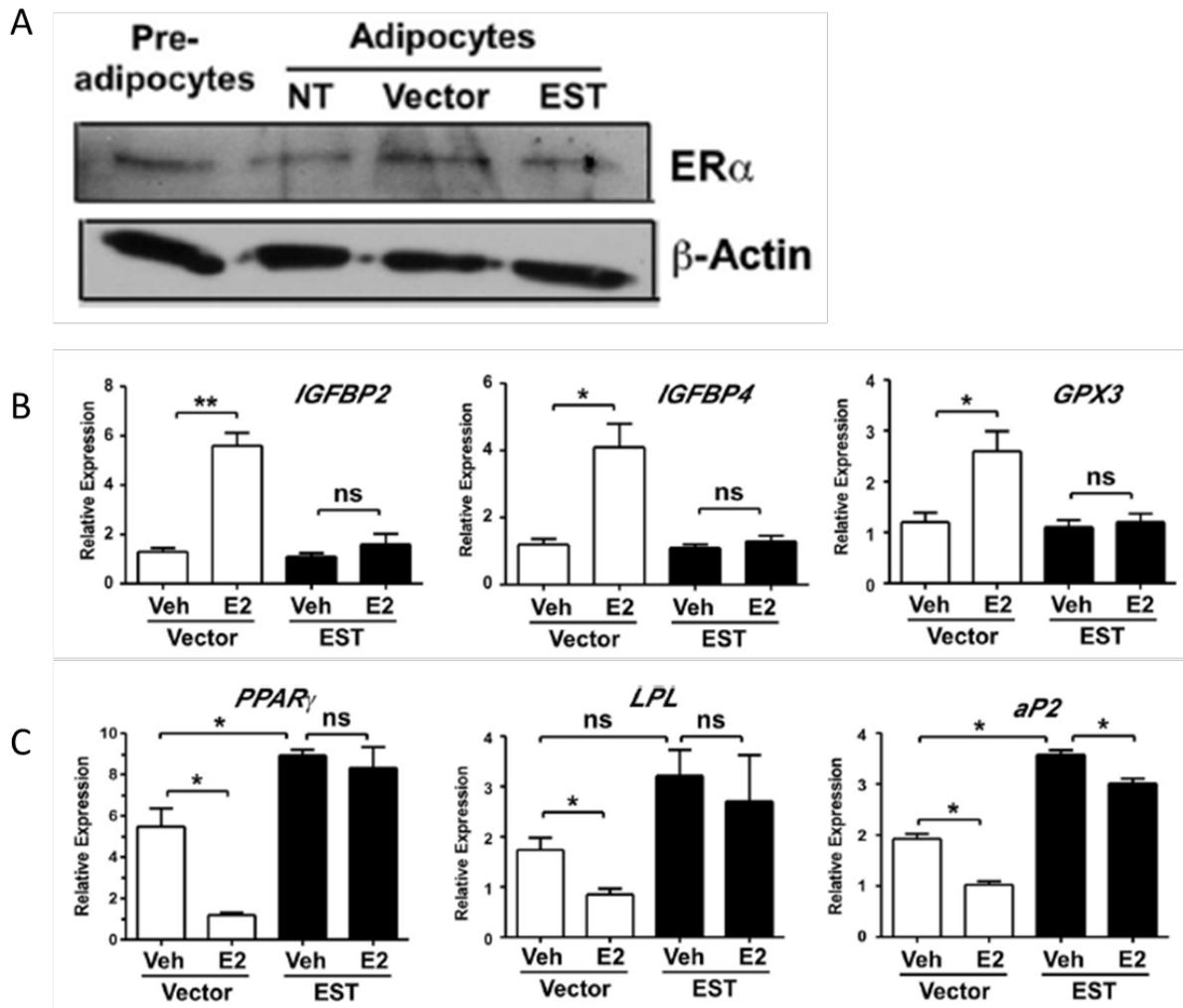
When applicable, results are presented as means  $\pm$  standard deviations (SD). The Student t test was used to compare means of two groups and One-way ANOVA was used to compare the means of three or more groups. P values of less than 0.05 are considered to be significant.

Repeated-measures ANOVA was used to compare means of two or more groups across multiple time points (MTT cell proliferation assay). Linear regression analysis was performed using the Graph-Pad Prism software.

### 3.3 RESULTS

#### 3.3.1 Analysis of Estrogen Receptor Expression, Estrogen Response and Estrogen Treatment in Obese Preadipocytes

We performed Western blots to probe for the presence of ER $\alpha$  in untreated preadipocytes and adipocytes, as well as vector and EST-virus treated adipocytes (Fig 44A). We found that ER $\alpha$  was expressed equally in all four conditions. Additionally, we verified that ER $\alpha$  was functionally active in our cell model by culturing vector and EST virus transduced cells in media containing dextran coated charcoal stripped (DCC) FBS with or without 10nM E2, and analyzed the response of ER $\alpha$  target genes; Insulin like growth factor binding protein 2 and 4 (IGFBP2 and 4), as well as Glutathione Peroxidase 3 (GPX3) [131-133]. We found that in vector virus transduced cells, E2 treatment caused a marked induction of said E2 response genes; however EST virus transduced cells showed no activation after E2 treatment (Fig 44B), confirming the functional response of both ER $\alpha$  and EST (Fig 44B). Interestingly, we also observed that EST marginally retained its adipogenesis promoting characteristics in cell culture media formulated with DCC-FBS (Fig 44C).



**Figure 44. ER $\alpha$  is expressed in preadipocytes/adipocytes**

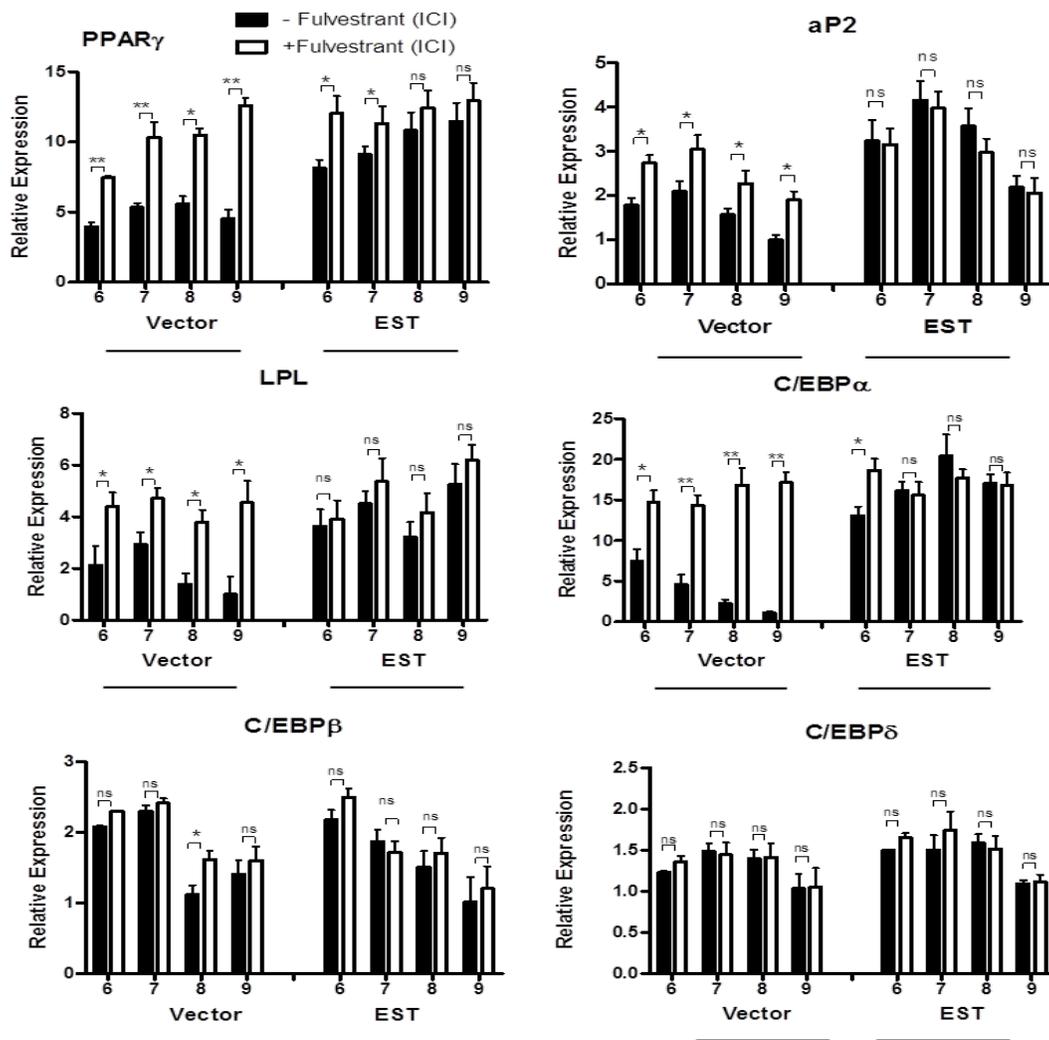
(A) Total lysate was collected from untreated preadipocytes and adipocytes, as well as vector and EST-virus treated adipocytes. NT, no transduction (virus) (B) Vector and EST virus transduced cells were cultured in DMEM without phenol red containing DCC- FBS and grown to confluency. Cells were treated for 24 hours in with 10nM E2 or vehicle. Total RNA was collected for RT-PCR analysis of ER $\alpha$  target genes. (C) Vector and EST virus transduced cells were cultured in DMEM without phenol red containing DCC- FBS and grown to confluency and differentiated for 14 days. Total RNA was collected for RT-PCR analysis of adipogenic genes. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### 3.3.2 Pharmacologic Inhibition of ER $\alpha$ Increases Adipogenic Gene Expression

In the previous section we established the expression and function of ER $\alpha$  and estrogen signaling in our preadipocytes/adipocytes. As described in the introduction (Section 1.2.2 ER $\alpha$ / $\beta$ ) the attenuation of estrogen signaling shown in knock-out animal models [55], post-menopausal women [59], and rare mutations in the aromatase gene have led to increased adipose tissue accumulation and obesity [61]. In chapter 2, the over-expression of EST strongly inhibited estrogen signaling and led to increased expression of adipogenic and lipogenic mRNA, and promoted the formation of lipid droplets; whereby the opposite effect is seen when we knockdown the expression of EST, most likely because of increased bioactive estrogen and increased estrogen signaling. We decided to test if this could be recapitulated when we cultured and differentiated our preadipocytes in the presence of a pharmacologic inhibitor of ER $\alpha$ . We decided to use the ER $\alpha$  antagonist Fulvestrant/Falsodex (ICI 182, 780 Tocris Bioscience). Fulvestrant is an estrogen receptor antagonist that competitively binds to the estrogen receptor and down-regulates the ER protein in human breast cancer cells; it is used clinically to treat post-menopausal women with estrogen receptor dependent metastatic breast cancer [134, 135]. We used the preadipocytes from the initial EST over-expression experiment to conduct the ER $\alpha$  inhibitor studies. Once again, four patients' preadipocytes were treated with vector or EST-virus and differentiated in media with Fulvestrant (ICI) or vehicle. We used RT-PCR to analyze changes in gene expression resultant of the ICI treatment. We did indeed find that Fulvestrant (ICI) increased the adipogenic mRNA expression of vector- virus transduced cells nearly to levels identical with vehicle treated EST-virus transduced cells (Fig 45). Additionally, we did not find significant additional promotion of adipogenic mRNA in Fulvestrant treated EST-virus transduced cells (Fig 45).

Taken together these results indicate that pharmacologic antagonism of ER $\alpha$  is sufficient to promote adipogenesis at the genetic level; additionally these results suggest that the modulation of estrogen signaling by EST causes the promotion of adipogenesis.

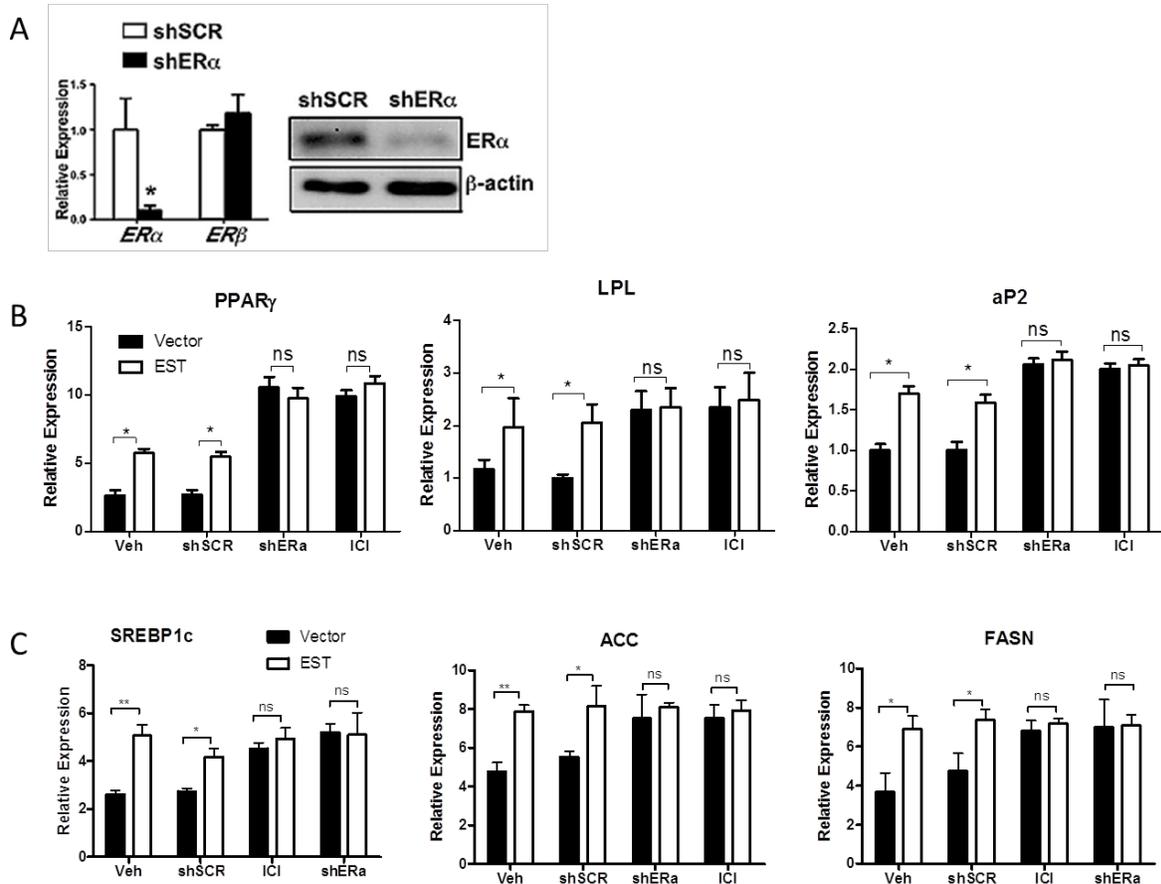
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**Figure 45. Pharmacologic antagonism of ER $\alpha$  can promote adipogenic mRNA expression**  
 Preadipocytes were transduced with vector or EST-expressing lentivirus, then were cultured and differentiated in standard medium with Fulvestrant (100nM) or vehicle for 14 days. (A) Total RNA was extracted before and after differentiation, and the expression of indicated adipogenic genes was measured by real-time PCR. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### **3.3.3 Pharmacologic Inhibition and Knockdown of ER $\alpha$ Increases Adipogenic and Lipogenic Gene Expression**

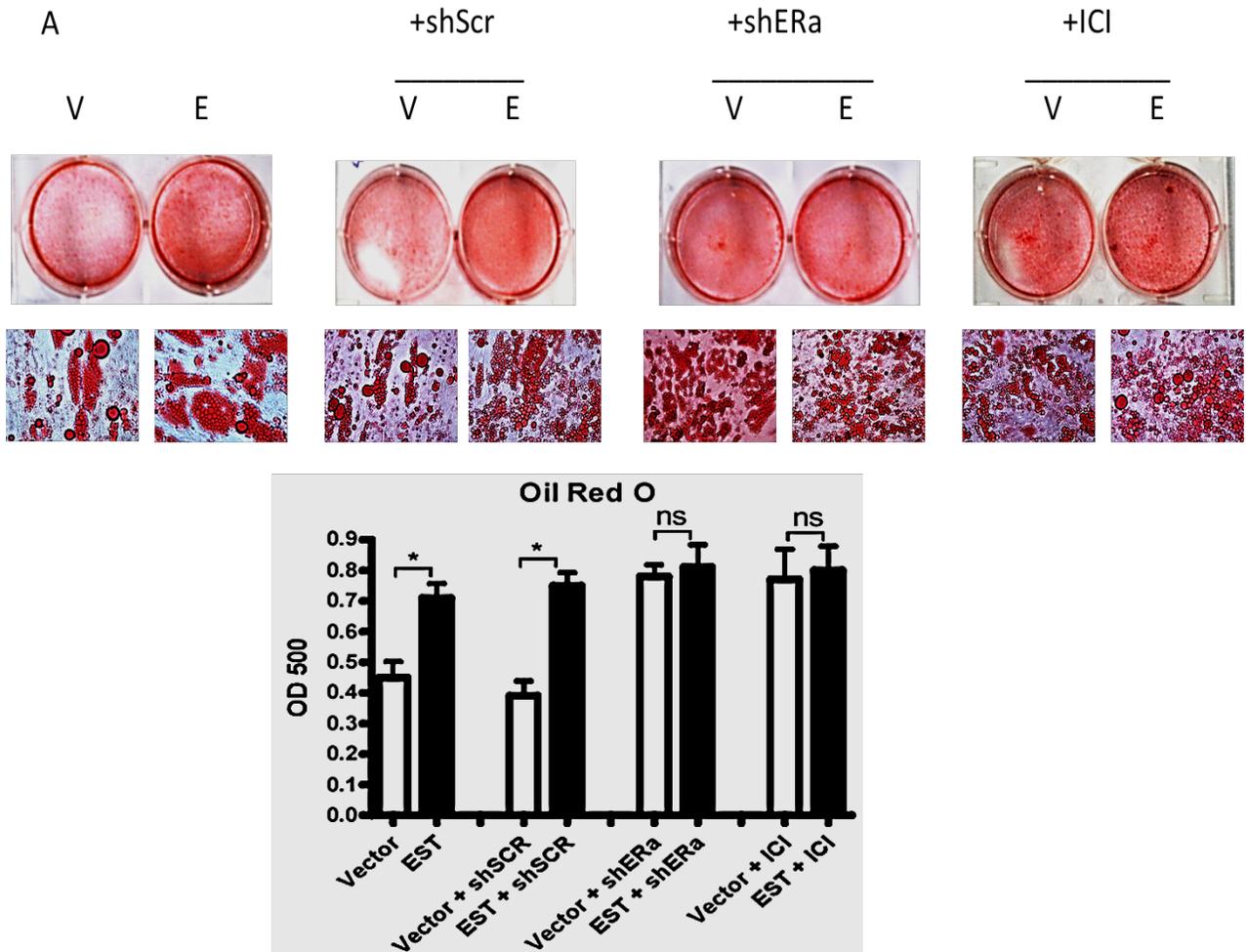
Our results from the previous section provided the impetus to compare the pharmacologic inhibition of ER $\alpha$  to genetic knockdown of ER $\alpha$ . For this experiment we used two patients' preadipocytes and treated them with vector or EST virus and additionally with shSCR or shER $\alpha$  virus or Fulvestrant (ICI). We confirmed that vector virus treated cells that were either transduced with shER $\alpha$  or ICI achieved the same promotion of adipogenic and lipogenic mRNA expression as vehicle treated EST-virus transduced adipocytes (Fig 46B-C). Also, we did not see significant additional promotion of adipogenesis or lipogenesis in EST- virus transduced cells treated with Fulvestrant (ICI) or transduced with shER $\alpha$  (Fig 46B-C). Taken together, these results confirm that EST can promote or inhibit adipogenesis and lipogenesis by directly mediating the ER $\alpha$  signaling pathway.



**Figure 46. Antagonism and Genetic Knockdown of ERα promotes adipogenic mRNA expression.** Preadipocytes were transduced with vector or EST-expressing lentivirus and additionally with shSCR or shERα-virus. Cells were then cultured and differentiated in standard medium with Fulvestrant (100nM) or vehicle for 14 days. (A) Cells lysates were analyzed for the expression of ERα after knockdown. Total RNA was extracted before and after differentiation, and the expression of indicated (B) adipogenic genes or (C) lipogenic genes was measured by real-time PCR, (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### **3.3.4 Pharmacologic Inhibition and Knockdown of ER $\alpha$ Increases Lipid Droplet Accumulation**

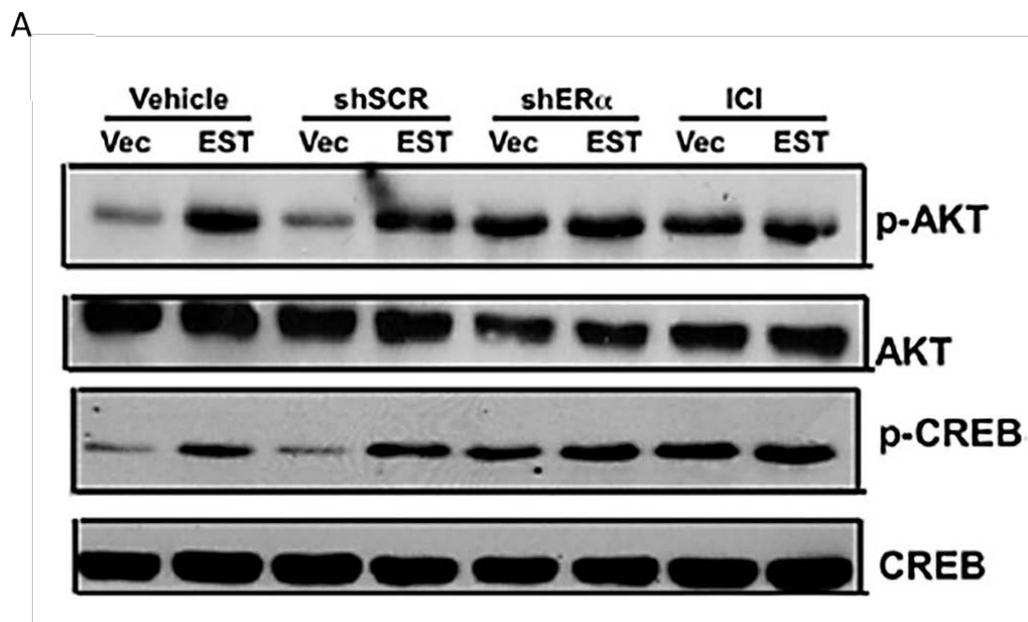
We also quantified lipid droplet formation from the two aforementioned patients' adipocytes. In addition to the promotion of adipogenic and lipogenic mRNA expression, the genetic knockdown or antagonism of ER $\alpha$  also enhanced lipid droplet formation and lipid accumulation as measured by oil red O staining and quantification (Fig 47). In shER $\alpha$  - vector cells or vector cells treated with ICI, the quantified oil red O concentration (OD) was equal to EST transduced cells. We did not find any additional promotion of lipid droplet formation in EST transduced cells treated with the ICI compound or transduced with shER $\alpha$ .



**Figure 47. Knockdown or Antagonism of ER $\alpha$  Increases Lipid Droplet Formation and Lipid Accumulation.** Preadipocytes were transduced with vector or EST-expressing lentivirus and additionally with shSCR or shER $\alpha$ -virus. Cells were then cultured and differentiated in standard medium with Fulvestrant (100nM) or vehicle for 14 days. (A) Cells were then stained with oil red o dye, and examined microscopically as well as quantitatively, (n=2). \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector; OD500, optical density at 500nm.

### 3.3.5 Pharmacologic Inhibition and Knockdown of ER $\alpha$ Increases AKT and CREB signaling

Additionally, we tested if the antagonism or genetic knockdown of ER $\alpha$  could also affect insulin signaling in a manner similar to EST over-expression. We found that in vector treated cells, knockdown of ER $\alpha$  or antagonism with the ICI compound increased AKT and CREB signaling commensurate to that seen in the EST over-expression cells treated with vehicle or transduced with shSCR. Additionally, there was no increase in AKT or CREB signaling in the EST-shER $\alpha$  transduced cells or EST transduced ICI treated cells.



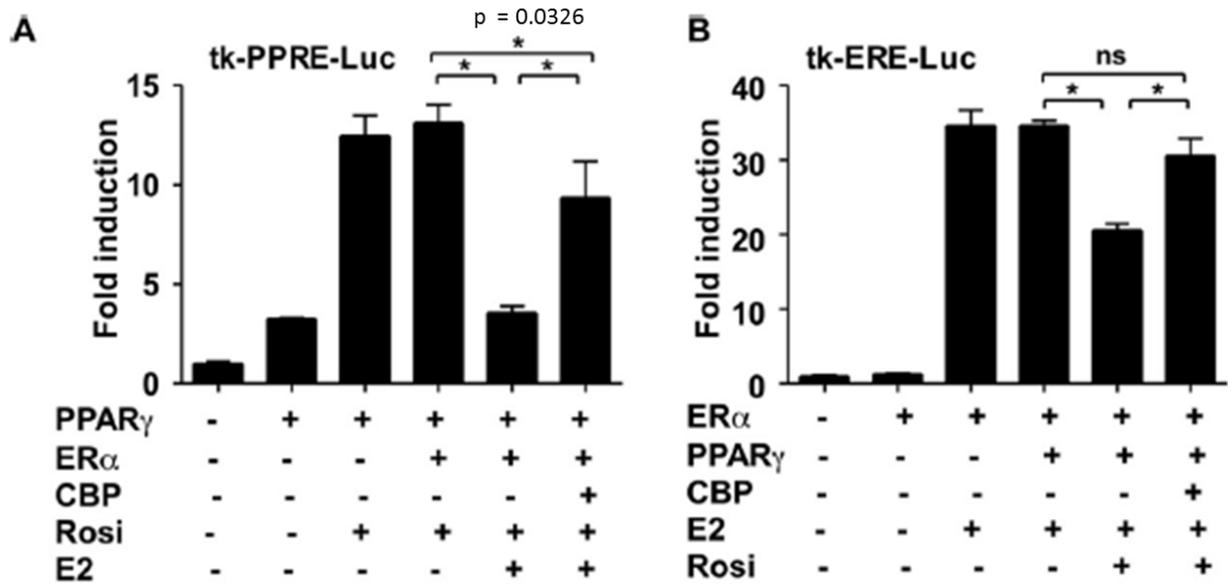
**Figure 48. Knockdown or Antagonism of ER $\alpha$  Increases AKT and CREB Signaling.**

Lysates from Preadipocytes transduced with vector or EST-expressing lentivirus and additionally with shSCR or shER $\alpha$ -virus and cultured and differentiated in standard medium with Fulvestrant (100nM) or vehicle for 14 days were subjected to Western blot analysis to detect protein expression of (A) total AKT, phospho-AKT (p-AKT), total CREB, and phospho-CREB (p-CREB). The signals were quantified by using NIH ImageJ software. (n=2) \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### **3.3.6 Molecular mechanism for the inhibitory effect of the estrogen-ER signaling pathway on adipogenesis: Luciferase Assay and Chromatin Immunoprecipitation**

As stated in the introduction (1.2.3 Nuclear receptor coactivators), the regulation of nuclear receptor transcriptional activation after ligand binding largely depends on the recruitment of coactivators to the ligand binding domain or AF2 domain [81]. The recruitment of coactivators enhances the ability of nuclear receptors to bind to their specific DNA motif known as a hormone response element [82]. Many nuclear receptors share and compete for coactivators subsequent to ligand binding. This competition is known as “crosstalk”, whereby the activation of one nuclear receptor and its signaling pathway can inhibit the activation of another nuclear receptor and its pathway [41]. It has been established that crosstalk between nuclear receptors is an important regulatory mechanism that can fine-tune transcriptional activation or repression of specific nuclear receptor target genes [87].

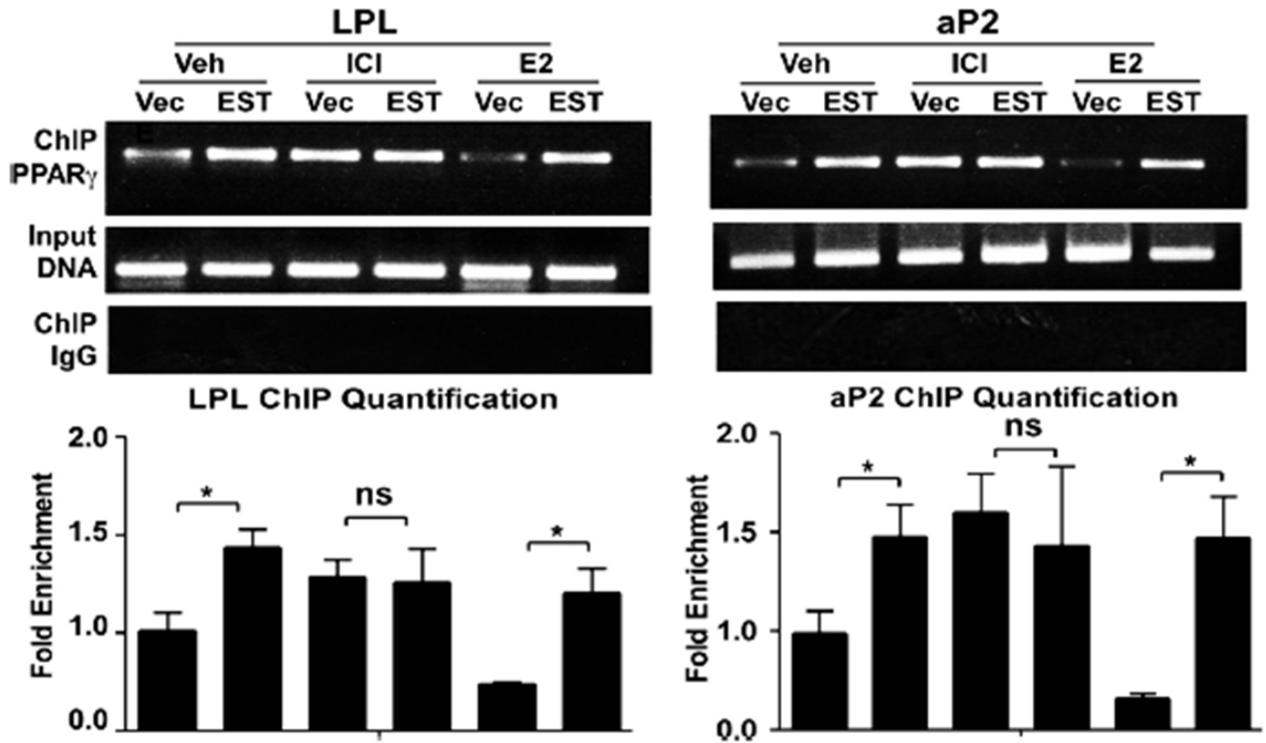
ER $\alpha$  and PPAR $\gamma$  both share the coactivator CREB binding protein (CBP) [136, 137]. CBP contains histone acetylase activity that allows for potent transcriptional activation of each NR's target genes. We used a transient transfection and luciferase reporter gene assay to show that ER $\alpha$  inhibited the PPAR $\gamma$  mediated activation of a PPAR-responsive reporter gene, tk-PPRE-Luc, in a ligand-dependent manner; however this inhibition was attenuated by the co-transfection of CBP (Fig 49). Reciprocally, PPAR $\gamma$  inhibited the ER $\alpha$  mediated activation of an ER responsive reporter gene, tk-ERE-Luc, in a ligand-dependent manner, and this inhibition was abolished by the co-transfection of CBP. Taken together, these results demonstrate that the coactivator CBP plays an integral role in the transcriptional activation of both PPAR $\gamma$  and ER $\alpha$ . Finally, crosstalk between ER $\alpha$  and PPAR $\gamma$  is directly influenced by CBP's abundance or lack thereof.



**Figure 49. Nuclear Receptor Co-Activator CBP can Rescue ER $\alpha$ /PPAR $\gamma$  Crosstalk Effect.** (A and B) 293T cells were transfected with either the tk-ERE-Luc reporter gene together with ER $\alpha$  and/or CBP (A) or the tk-PPRE-Luc reporter gene together with PPAR $\gamma$  and/or CBP (B). Transfected cells were treated with E2 (10 nM) and/or rosiglitazone (Rosi; 1  $\mu$ M) for 24 h before luciferase assay. The luciferase activities were normalized against  $\beta$ -Gal activities from the co-transfected  $\beta$ -Gal vector. Results are shown as fold induction over vehicle-treated triplicates. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

Based on the results of the luciferase assay, we decided to use a chromatin immunoprecipitation assay (ChIP) to biochemically analyze the effect that E2 or ICI treatment had on the binding of PPAR $\gamma$  to the promoter region of its target genes LPL and aP2. Preadipocytes were transduced with vector or EST-virus and differentiated for two weeks in standard media containing ICI or E2. Here we showed that in vector-virus transduced cells, ICI treatment enhanced PPAR $\gamma$  binding to levels commensurate with EST transduced vehicle treated cells; while E2 treatment attenuated PPAR $\gamma$  binding in vector virus transduced cells (Fig 50). The binding of PPAR $\gamma$  was enhanced in EST virus transduced cells and they remained unaffected by E2 treatment which is consistent with the increased adipogenic effect (Fig 50). Taken together, these results show that EST can promote adipogenesis through direct inhibition of estrogen signaling and indirect transcriptional activation of PPAR $\gamma$ . The over-expression of EST inhibited E2-ER $\alpha$  activation, which promoted CBP enhanced recruitment of PPAR $\gamma$  to the promoter region of its targets genes and subsequent activation of these genes.

A

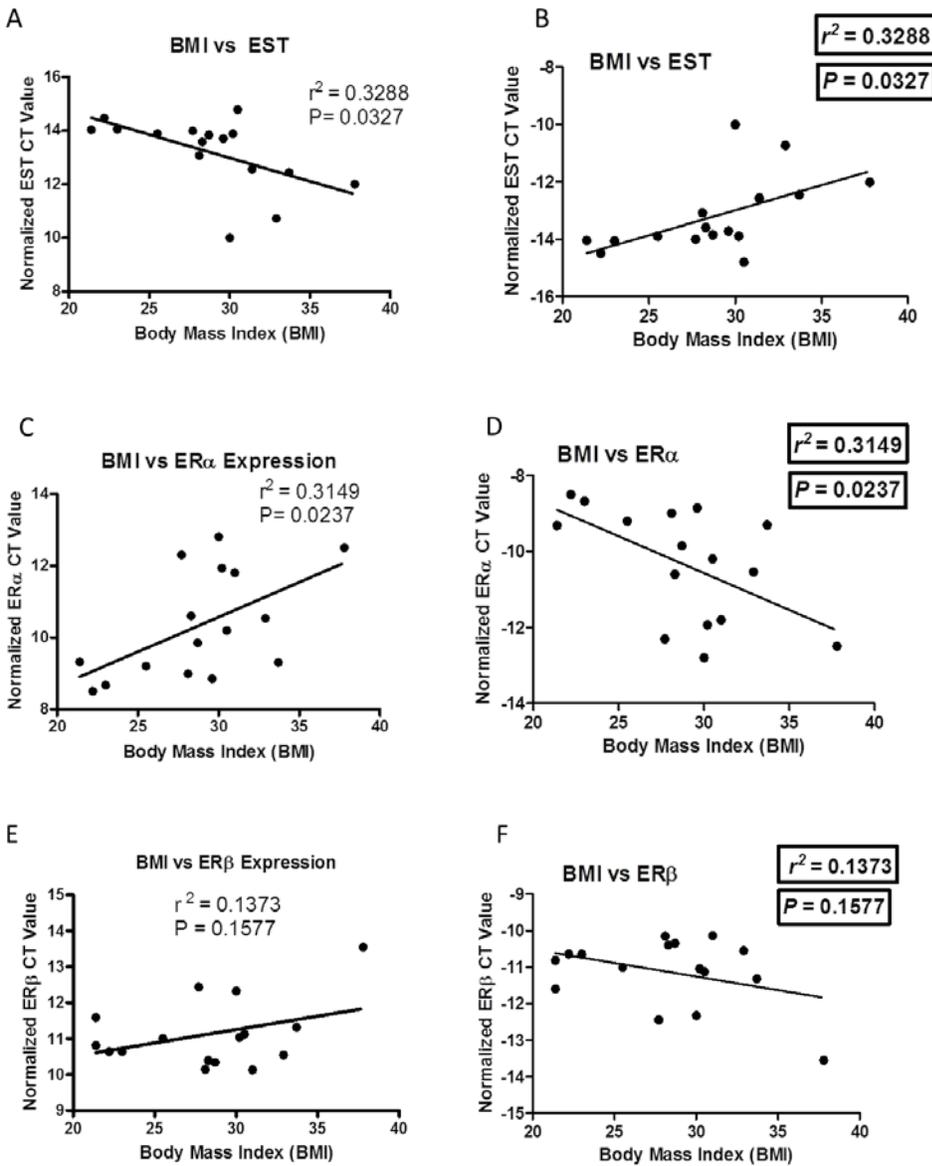


**Figure 50. CHIP Assay showing the effect of E2/ICI treatment on the recruitment of PPAR $\gamma$  to the promoters of two of its adipogenic target genes LPL and aP2**

The vector- or EST-transduced preadipocytes were treated with 100nM fulvestrant (ICI) or 10 nM E2 and then induced to differentiate for 2 weeks before being evaluated for the recruitment of PPAR $\gamma$  onto the LPL (left panel) and aP2 (right panel) gene promoters by chromatin immunoprecipitation (ChIP) assay.(n=2) Quantification was performed by using NIH ImageJ software. \*,  $P < .05$ ; \*\*,  $P < .01$ ; ns, not statistically significant compared to vector.

### **3.3.7 Linear Regression Analysis of ER $\alpha$ , ER $\beta$ , EST Gene Expression versus Body Mass Index from whole fat or lipoaspirate of Obese Patients**

The pro-adipogenic effect of EST over-expression combined with the anti-adipogenic effect of estrogen signaling provided the impetus for us to examine if a correlation existed between the expression of EST or ER $\alpha$  and BMI. We isolated total RNA from whole fat or lipoaspirate from 16 obese patients and used RT-PCR and linear regression to analyze the expression of EST and ER $\alpha$  compared to BMI. We found a positive and significant correlation between the expression of EST and BMI; additionally we found that there was an inverse and significant correlation between the expression of ER $\alpha$  and BMI (Fig 51). Taken together, these results provide physiological evidence that the mRNA expression of EST and ER $\alpha$  has a significant and functional effect on adiposity in the cohort studied in our experiments. It should be noted that the initial depiction of these results were confusing on the surface due to the fact that a higher CT value indicated lower mRNA expression and vice versa. Therefore, we multiplied the final CT values by (-1) in order to present the results in a way that the slope of the linear regression line matched the correlation (Fig 51).



**Figure 51. Linear Regression of Whole Fat/ Lipoaspirate EST, ER $\alpha$ , ER $\beta$ , mRNA Expression**

Total RNAs extracted from whole fat or lipoaspirate of a cohort of 16 obese patients were subjected to gene expression analysis by real-time PCR. The correlation between body mass index (BMI) and EST (A-B), ER $\alpha$  (C-D), or ER $\beta$  (E-F) gene expression was analyzed by linear regression analysis. The expression levels of EST and ERs were presented as normalized threshold cycle values, in which a higher threshold cycle value indicates low gene expression. The expression is normalized to GAPDH. To avoid confusion, the normalized CT values were multiplied by (-1) so that the slope of the linear regression line matched the correlation.

### 3.4 DISCUSSION

We have identified a mechanistic paradigm which describes the novel role for EST in human adipogenesis. In the previous chapter we demonstrated that EST over-expression leads to increased adipogenesis and lipogenesis, while EST knock down causes the opposite effect. In this chapter, our goal was to gain a mechanistic understanding of the observations shown in the previous chapter. Here we summarize the major findings in this chapter:

- (1) Pharmacologic inhibition or genetic knockdown of ER $\alpha$  in preadipocytes/adipocytes can recapitulate the same adipogenic and lipogenic promoting effects seen when preadipocytes are transduced with EST over-expression virus (Fig 48).
- (2) Estrogen signaling antagonizes PPAR $\gamma$  signaling and vice versa through squelching or coactivator competition for CBP ( Fig 49). Moreover, EST directly inhibits estrogen signaling which indirectly increases the binding of PPAR $\gamma$  to the promoters of its target genes (LPL, aP2) (Fig 50)
- (3) Pharmacologic inhibition or genetic knockdown of ER $\alpha$  in preadipocytes/adipocytes can increase terminal AKT and CREB protein expression commensurate to that seen in EST overexpression cells (Fig 48).
- (4) In whole fat or lipoaspirate, we observed a positive correlation between EST mRNA expression and BMI in 16 obese patients. Additionally, we found an inverse correlation between ER $\alpha$  mRNA expression and BMI (Fig 51).

In this chapter our main focus was to understand the connection between EST, estrogen signaling, and the activation of PPAR $\gamma$  and its target genes leading to the promotion of adipogenesis and lipogenesis. We showed that the ER $\alpha$  was present and functional in our

adipocytes and preadipocytes, and that virus treatment did not adversely affect the protein expression of the ER $\alpha$  (Fig 44).

To gain a better understanding of the global nature of estrogen antagonism in adipogenesis, we added a pharmacologic inhibitor of ER $\alpha$ / $\beta$  (Fulvestrant, ICI), to our standard differentiation media. After 14 days in cell culture, we found that vector cells treated with the inhibitor also displayed a significant augmentation of adipogenesis that was commensurate to EST-virus transduced cells (Fig 47). This was a clear indication that targeting the estrogen signaling pathway, either the substrate or the receptor, caused significant changes in adipose tissue development. We used lentivirus expressing shRNA to knock down ER $\alpha$  (shER $\alpha$ ) to confirm that changes in ER $\alpha$  signaling, as opposed to ER $\beta$ , caused the observed adipogenesis promoting effects. Again, we found increased mRNA expression of adipogenic and lipogenic genes, as well increased AKT and CREB signaling (Fig 48); all of which were comparable to that seen in EST-virus transduced cells.

We know that because both ER $\alpha$  and PPAR $\gamma$  are nuclear receptors, the possibility that they shared coactivators is very high. In fact it was reported in several papers that both ER $\alpha$  and PPAR $\gamma$  do share the coactivator CREB Binding Protein (CBP) [136, 137]. Based on this fact, we used transient luciferase and reporter gene assays to determine if PPAR $\gamma$  and ER $\alpha$  could antagonize each other in a ligand dependent manner and if this could be attenuated by CBP transfection. The luciferase assay confirmed that these two nuclear receptors can in fact antagonize each other in a ligand depend manner and this antagonism can be diminished by increased expression of CBP (Fig 49). Moreover, we used a biochemical assay (ChIP) to show that EST can promote the recruitment of PPAR $\gamma$  to the promoters of its target genes by

antagonizing estrogen (Fig 50). It is important to note that the increased induction of PPAR $\gamma$  target genes may also have a positive effect on the expression of PPAR $\gamma$  itself. PPAR $\gamma$  is known as a “lipid sensor” because it can be activated by dietary fatty acids, and their metabolites [138]. LPL, the PPAR $\gamma$  target gene measured throughout this study, maintains a dual functional role as a triglyceride hydrolase and also as a bridging factor for heparan sulfate proteoglycan mediated lipoprotein uptake [35]. The increased induction of LPL subsequently can increase the intracellular concentration of fatty acids that are taken up from the media serum, thus providing increased stimuli for PPAR $\gamma$  gene expression.

The mechanisms that caused increased AKT, CREB, and MAPK/ERK protein expression after EST overexpression/ER $\alpha$  antagonism/ER $\alpha$  knockdown, and the opposite after EST knockdown are still unclear. Estrogen signaling has been reported to have an immediate effect on AKT and ERK/MAPK signaling that is mediated through non-nuclear mechanisms [45, 139]. In human endothelial cells, estrogen treatment caused a biphasic response. First, estrogen treatment facilitated the rapid release of intracellular calcium stores and marked increase in cytoplasmic calcium concentration leading to the activation of MAPK/ERK [140, 141]. The activation of MAPK was shown to be dependent on the ability of the ER $\alpha$  to mobilize and increase intracellular calcium signaling [140]. This was quickly followed by the activation of PI3 kinase. Mechanistically, the ER $\alpha$  has been shown to directly interact with the catalytic subunit (p85) of the PI3 kinase causing increased phosphorylation of IRS1, recruitment and phosphorylation of the PI3 kinase and subsequent recruitment and phosphorylation of AKT [139, 142]. We would expect that in the EST and vector cells treated with insulin, the differential effect that loss of estrogen signaling (EST cells) would have on IRS1 and subsequent AKT and CREB signaling would be apparent immediately, however this was not the case (Fig 23). This result was actually

supported by findings from Simoncini et. al., that showed that E2 treatment did not have a potentiating effect on the IRS1, the PI3 kinase or AKT signaling in endothelial cells that were already stimulated by insulin [139]. Since we did not see a difference in rapid IRS1 signaling (proxy for PI3K signaling) but we did see a significant difference in AKT and CREB after terminal differentiation, this suggests that the effect of estrogen signaling or lack thereof in our cell culture model is actually a nuclear or genomic effect. We can only speculate based on the observations made that it is most likely a result of increased PPAR $\gamma$  activation because PPAR $\gamma$  has the ability to induce C/EBP $\alpha$  and vice versa [34, 73]. This is important because the expression of C/EBP $\alpha$  is critical for proper insulin signaling during adipogenesis[21, 74] (covered in the discussion of chapter 2); therefore the downstream effects of enhanced C/EBP $\alpha$  induction most likely caused increased AKT/CREB signaling that were not apparent early in the adipogenic cascade (Fig 23 IRS-1) but became apparent in the later stages of adipogenesis (terminally differentiated AKT/CREB westerns). It is plausible to extend the conclusion that the changes in PI3K/AKT and CREB signaling takes place through a genomic effect exerted by ER $\alpha$  and not the classical rapid signaling mechanism can also be extended to the changes that were observed in ERK signaling after terminal differentiation. We did not analyze the rapid activation of the MAPK pathway in our cell culture model but we did use an inhibitor of MAPK to test if the activation of this pathway was essential for the augmented adipogenesis seen in EST cells (Fig 25-27). The inhibitor did not have any effect on terminal differentiation which suggests that this also may be a secondary genomic effect that is attributed to the overall change in ER $\alpha$  and PPAR $\gamma$  activation at the genomic level. It is also possible that the changes in AKT and MAPK/ERK signaling are mediated by the G- protein estrogen receptor (GPR30/GPER). The GPR30 is a membrane bound GPCR localized to the endoplasmic reticulum that can bind

estrogen and mediate an estrogenic effect[143]. GPR30's estrogenic effects can also be inhibited by the classical estrogen receptor antagonists Tamoxifen and Fulvestrant (ICI 182,780) [144, 145]. GPR30 is expressed in a number of different mouse and human tissue types including brain, ovary, prostate, lung, endothelial and adipose tissue [145, 146]. Functionally, it has been shown that GPR30 is an integral mediator of many signal transduction pathways involved in the maintenance of the cardiovascular system, neuroendocrine and endocrine system, and is involved in the regulation of energy homeostasis and body weight maintenance [143, 145-147]. Whilst still controversial, GPR30 KO causes sexual dimorphic changes in obesity, energy expenditure, and body weight in male and female mice [145]. Additionally, many of the secondary messenger signaling pathways that were mentioned in the previous paragraph and were once attributed exclusively to ER $\alpha$ , have now been shown to additionally or exclusively actually involve GPR30 [143]. More specifically, the rapid non genomic signaling pathways that activate MAPK/ERK and subsequently PI3K and AKT are emerging as GPR30 mediated pathways. It was shown in ER negative SKBR3 breast cancer cells that E2 treatment led to the rapid activation of MAPK/ERK pathway through GPR30, and culminated with *c-fos* expression [148]. In COS7 and SKBR3 cells it was shown that estrogen treatment could activate GPR30 leading to the phosphorylation of the PI3K and subsequent nuclear membrane accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) molecules and finally accumulation and activation of Akt [149]. GPR30 is expressed in mouse adipose tissue, however it is not known if it is also expressed in human adipose tissue [145]. It is possible that the non-genomic effects that we have observed in AKT and MAPK signaling in our cell culture models could be attributed to GPR30 mediated cell signaling. While the rapid effects mediated by GPR30 have been established in several cell culture models, the slower signaling that we evaluated in terminally

differentiated adipocytes remains unclear albeit a provocative explanation to the observations seen in our experiments.

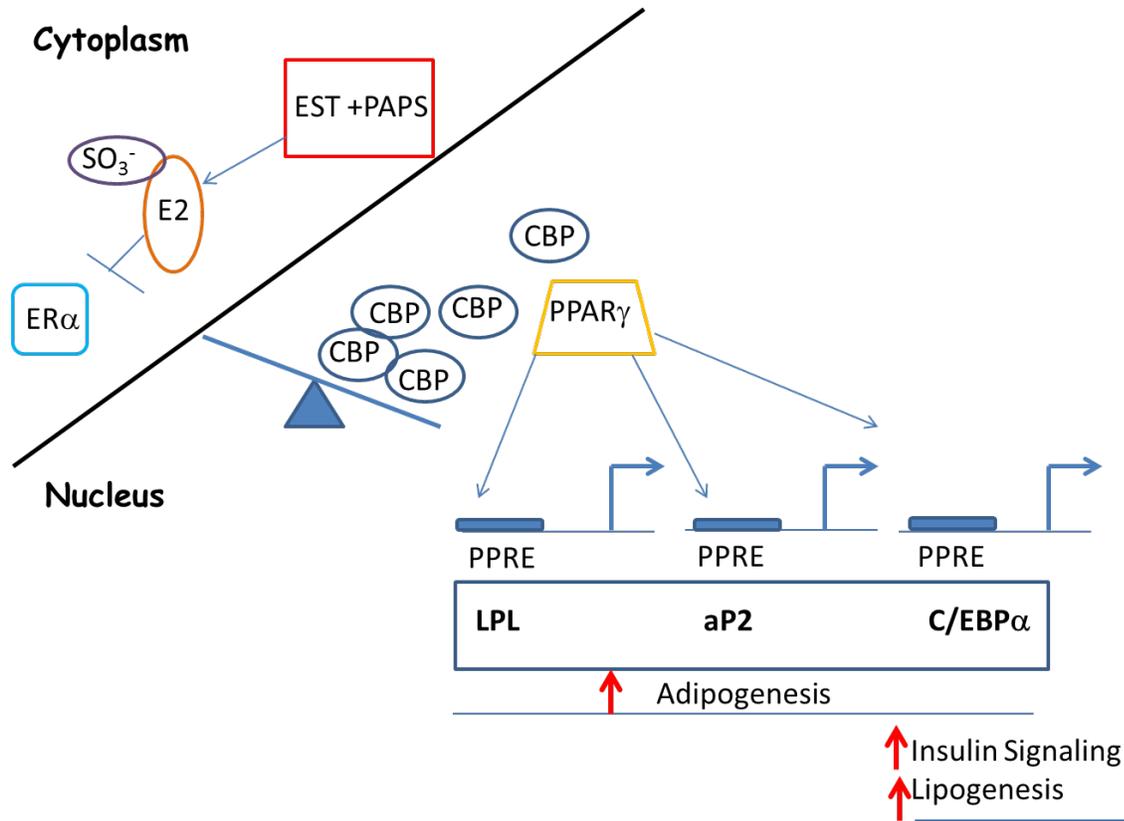
Lipogenic gene expression was also evaluated in this document. Increased expression of lipogenic genes was observed in EST overexpression cells as well as ER $\alpha$  knockdown/pharmacologic inhibition studies. As aforementioned in the chapter 2 discussion, SREBP1c promotes lipogenic mRNA signaling (ACC, FASN, and SCD1); moreover it is activated by increased insulin sensitivity and signaling [115, 116]. Our results suggest that the increased AKT/CREB signaling, which is mediated by increased C/EBP $\alpha$  signaling, led to the enhanced lipogenic signaling facilitated by SREBP1c.

These experiments have provided a clear picture as to the role of EST in human adipogenesis. In general our data suggests that EST serves as mediator of estrogenic effects in subcutaneous adipose tissue. Specifically, in subcutaneous adipocytes estrogen has an overall inhibitory effect on adipogenesis and lipogenesis; EST has the ability to mitigate that effect. During the early stages of adipogenesis EST is highly expressed and therefore able to deactivate estrogen and inhibit the repressive effects facilitated by estrogen signaling. This promotes increased activation of PPAR $\gamma$  and PPAR $\gamma$  target genes thus facilitating adipogenesis. As the adipogenic process begins to wane, the expression of EST decreases to allow the repressive effects of estrogen signaling to return and moderate the level of mature adipocyte development. The EST-Estrogen signaling pathway thus serves to facilitating proper induction of adipogenic genes to develop functional adipocytes, while simultaneously protecting adipocytes from an over stimulation of adipogenic genetic signaling. A general mechanism of EST action in adipogenesis is depicted in (Fig 52).

Finally, since we did not have a suitable animal model, we isolated RNA from whole fat or lipoaspirate from a cohort of obese patients and observed that in mature adipocytes the mRNA expression of EST positively correlates with BMI. Additionally, the inverse correlation of ER $\alpha$  mRNA expression, which is complementary to other data found in the literature, supported our experiments in cell culture which showed the antagonism of estrogen signaling led to increased adiposity. We cannot conclude from this data that EST is sole cause for increased BMI, however this does provide a provocative indication that EST does have a major role in adipogenesis beyond what we found in cell culture.

The overall limitations to this study include a small sample size and the fact that this was done entirely in cell culture with no *in vivo* model. Additionally, we found that cells taken from the abdominal region were most responsive and therefore we only included abdominal preadipocytes. Although the effects of EST overexpression and knockdown were observed in preadipocytes isolated from both the obese and non-obese patients, we cannot exclude the possibility that age, menopausal status and steroid hormone level may confound our results. Finally, since overexpression of EST under the estrogen-free DCC cell culture condition can still marginally increase the expression of certain adipogenic marker genes (Fig. 44), we cannot exclude the possibility of an off-target effect due to the overexpression of EST, as well as the existence of additional EST substrates that may also have an effect on adipogenesis. It should be noted that there are several limitations of this model to evaluate if EST mediated inhibition of estrogen signaling is the primary reason for increased adipogenesis in EST overexpression cells. For example, the stripped serum effectively removes all lipid molecules involved in cell signaling as well as many growth factors necessary for adequate differentiation. We did in fact, observe that these cells did not differentiate as robustly as the preadipocytes in STD-FBS (not

shown). The stripped serum also removes other hormones involved in adipogenesis such as androgens, progesterone, and thyroid hormones. While these are not EST substrates they do play a role in adipogenesis and therefore could confound our results.



**Figure 52. EST regulation of Adipogenesis and Lipogenesis**

The sulfation of E2 and inhibition of estrogen signaling, “tips the CBP scale” toward PPAR $\gamma$  facilitating increased activation of PPAR $\gamma$  and target genes.

## **4.0 CHAPTER IV: CONCLUSIONS AND PERSPECTIVES**

### **4.1 PHARMACEUTICAL POTENTIAL OF EST AS A DRUGGABLE TARGET**

Our findings are interesting when viewed in the context of weight loss, and speculation can be made as to whether EST is a viable druggable target for the reduction of body fat. It has been shown that in adult humans there is approximately a 10% annual turnover of adipocytes [103] and similar findings have been demonstrated in animal models [150]. Moreover the adipocyte number that each person contains is fixed in childhood and surprisingly even weight loss, which decreases the lipid volume per cell, does not decrease the set number of fat cells [101-103]. Additionally, a randomized study of 36 non-obese pre-menopausal female patients whom had undergone liposuction showed that one year after surgery, body fat in both visceral and subcutaneous depots had returned preferentially in the abdominal region [151].

Traditional weight loss strategies have proven to be effective at reducing adipocyte size; however an increasing amount of data shows that this does not reduce adipocyte number. This may be the reason why in many cases weight loss attempts fail and any reductions are quickly regained or even surpassed. A new paradigm could revolve around the idea that an effective weight loss strategy should include a targeted approach to reduce adipocyte turnover by attenuating adipogenesis or increasing adipocyte death. Adipocyte death has been shown to primarily closely resemble necrosis as opposed to apoptosis; it tends to include macrophage infiltration and inflammation that is worsened in obese individuals with high intracellular fat

content [152]. Several *in vitro* studies have analyzed the ability of naturally occurring compounds, such as resveratrol to induce cell death, however an *in vivo* model has not been analyzed[153]. Increased adipocyte death may be an attractive strategy, however rigorous *in vivo* testing is necessary to ensure that the increased inflammatory response does not worsen individual health outcomes.

An inhibition of adipogenesis may be a safer strategy to increase to stave off the re-accumulation of adipose tissue after weight loss. We showed in *Figure 9* that EST protein is expressed highest at the beginning of adipogenesis and begins to diminish towards the end of differentiation. It is plausible to speculate that an EST inhibitor given locally after weight loss surgery or during and intense weight loss period could essentially “re-calibrate” the adipocyte number set point. Some caveats to this approach are the apparent reduction of insulin signaling we observed after EST knockdown. Whilst we were not able to pinpoint a mechanism for the changes observed in AKT/CREB signaling, it has been shown in conditional PPAR $\gamma$  knockout murine models that loss of PPAR $\gamma$  signaling in adipose tissue leads to hepatic insulin resistance[154]. Since loss of PPAR $\gamma$  signaling is the suspected mechanism that causes an attenuation of adipogenesis in the EST knockdown cells, this strategy should also be met with caution.

## **4.2 HUMAN AND MOUSE SPECIES- SPECIFIC DIFFERENCES IN THE REGULATION OF ADIPOGENESIS BY EST**

These studies were primarily driven by the results from previous publications conducted in our lab and others using murine animal models as well as primary and stable mouse cells lines.

Several key differences in the regulation of EST were found between mice and humans which we will highlight here. Overall, the results from mouse studies demonstrated that in female mice Est is a negative regulator of adipogenesis; in primary adipocyte cell cultures the over-expression of Est inhibited adipogenesis and knockdown enhanced it [3, 18]. Moreover this was associated with decreased expression of PPAR $\gamma$ , LPL and FAS [18]. This of course is the opposite of the regulation identified here in humans. Moreover, in mice, it was found that the repressive effect from the over-expression of Est coincided with a loss of Akt signaling and sustained Erk 1/2 expression during terminal differentiation [3]. Based on the results from the mouse study we concluded that the repressive effect found when we over-expressed EST was mediated by the inability of these cells to activate Akt signaling and instead maintained sustained activation of Erk 1/2 [3]. Additionally, when we treated murine EST and vector cells with physiological concentrations of E2, we found no reduction in adipogenesis in the vector cells, nor did we observe any change in adipogenesis in the Est cells [3]. Finally, in the mouse study we were not able to elucidate a direct mechanism, such as a substrate other than E2 that linked Est to the changes in Erk, Akt, or the adipogenic gene expression [3].

In humans the over-expression of EST increased both AKT and ERK 1/2 while knockdown of EST inhibited both of these signaling molecules. Additionally, we found that in humans when vector cells were treated with E2 this caused marked reductions in adipogenic gene expression and lipid droplet formation but EST cells were protected. Mechanistically we found that

estrogen-induced inhibition of adipogenesis was caused by coactivator competition for CBP between ER $\alpha$  and PPAR $\gamma$  [88].

The driving force behind the phenotype that we observed in the human study was the opposing effects estrogen signaling had on PPAR $\gamma$  [88]. In the mouse study we did not observe any effect attributed to estrogen signaling that altered adipogenesis. This does contradict many published articles which cite that E2 treatment in murine cell lines such as 3T3-L1 cells caused marked reduction in adipogenesis. The cause of this discrepancy is unclear, however we did cite that we only used physiological concentrations of E2 in our experiments [155], while many other published articles used supraphysiological concentrations of E2 or overexpressed ER $\alpha$  [57]. Since several studies relied on supraphysiological concentrations of estrogen, this suggests that in these cell lines the expression of ER $\alpha$  may be diminished compared to primary cells, such as those used in our model. Differences in the way mice and humans metabolize estrogen could also be the cause of the discrepancy seen between mice and humans. Mice and human adipocytes express EST, Steroid sulfotransferase (STS) and Aromatase (CYP19A1) [3, 18, 20, 40, 43, 88, 156, 157]. It is known that in addition to ER $\alpha$  and Er $\beta$ , GPR30 is expressed in mouse adipocytes [145], however it is not known whether GPR30 is expressed in human adipocytes. If estrogen can be metabolized by both ER $\alpha$  and GPR30 in mice but exclusively by ER $\alpha$  in humans this could be the reason why varying estrogen concentration had such a significant genomic impact (PPAR $\gamma$  and target genes) in human adipocytes but not in rodents. The opposing role of EST in adipogenesis between humans and mice is also not well understood. Literature data consistently shows that in mice and humans, loss of estrogen signaling leads to increased adiposity [44, 54, 56, 158-160], therefore it is interesting that several *in vivo* and *in vitro* studies have shown that in female murine models Est acts a negative regulator of adipogenesis [3, 18]; aP2-Est transgenic

mice over-expressing Est in adipocytes have decreased adipocyte size [18]. The mechanism through which Est exerts its negative effects on rodent adipogenesis has not been clearly defined in the publications detailing said observations. It is clear that local factors present in specific WAT depots must modulate the response to estrogen inactivation by EST. There is still the possibility that an unknown substrate for EST may exist. This is supported by our findings in *Fig 48* which show that in media lacking estrogen EST was still able to marginally enhance the expression of PPAR $\gamma$  and one of its target genes.

In conclusion the true cause of species-species difference between human and mouse EST remains unknown; however additional experiments to elucidate an unknown substrate could be quite beneficial to our understanding of this enzyme.

## APPENDIX A.

### Adipose Derived Stem Cell patients and Experiments

Patient number	Sex	Age	BMI	Fat depot	Diabetic	Experiments performed
1	F	30	28.1	Abdominal	N	Initial real-time PCR
2	F	31	50	Abdominal	N	Initial real-time PCR
3	F	56	31.3	Abdominal	N	Initial real-time PCR
4	F	33	29.9	Abdominal	N	Initial real-time PCR
5	F	40	24.7	Abdominal	N	Triclosan treatment, Dexamethasone Depletion
6	F	43	32.5	Abdominal	N	Initial EST overexpression and 2nd EST overexpression with ICI treatment
7	F	44	27.7	Abdominal	N	Initial EST overexpression and 2nd EST overexpression with ICI treatment
8	F	34	32.9	Abdominal	N	Initial EST overexpression and 2nd EST overexpression with ICI treatment
9	F	25	29.5	Abdominal	N	Initial EST overexpression and 2nd EST overexpression with ICI treatment
10	F	50	28.3	Abdominal	N	shEST knockdown
11	F	37	32.3	Abdominal	N	shEST knockdown
12	F	52	26.6	Abdominal	N	PD98059 treatment
13	F	46	37.8	Abdominal	N	EST AAK mutant
14	F	45	25.5	Abdominal	N	shERa knockdown, ICI, EST Overexpression
15	F	34	28.1	Abdominal	N	shERa knockdown, ICI, EST Overexpression, CHIP Assay
16 (Lean)	F	47	21.8	Abdominal	N	EST Overexpression, shEST knockdown
17 (Lean)	F	44	23.7	Abdominal	N	EST Overexpression, shEST knockdown
18 (Lean)	F	32	21.4	Abdominal	N	EST Overexpression, shEST knockdown

### Whole Fat Removal/Liposuction Patients

BMI	Age	Sex	Fat Depot
28.7	53	F	Abdominal
23	35	F	Abdominal
22.2	43	F	Abdominal
27.7	44	F	Abdominal
32.9	34	F	Abdominal
30.5	43	F	Abdominal
30.2	53	F	Abdominal
29.6	59	F	Abdominal
28.1	34	F	Abdominal
28.3	50	F	Abdominal
33.7	39	F	Abdominal
31.4	41	F	Abdominal
37.8	46	F	Abdominal
21.4	32	F	Abdominal
30	36	F	Abdominal
25.5	45	F	Abdominal

***pCMXpl2-hEST* SEQUENCING RESULTS FROM THE UNIVERSITY OF PITTSBURGH GENOMICS CORE.**

Sequencing was done using the CMV promoter. The HINDIII sequence is highlighted in red followed by the EST sequence and flanked by NHE1 highlighted in orange. The EST sequence was validated with NCBI and is highlighted blue.

NNNNNNNNNTCNNNNNCGGTNCCGGGCCCCCTCGAG**AAGCTT**ATGAATTCTGAACTTGAC  
TATTATGAAAAGTTTGAAGAAGTCCATGGGATTCTAATGTATAAAGATTTTGTCAAATATTG  
GGATAATGTGGAAGCGTTCAGGCAAGACCAGATGATCTTGTTCATTGCCACCTACCCTAAAT  
CTGGTACAACCTGGGTTAGTGAAATTGTGTATATGATCTATAAAGAGGGTGTGTGGAAAAG  
TGCAAAGAAGATGTAATTTTAAATCGAATACCTTTCCTGGAATGCAGAAAAGAAAACCTCAT  
GAATGGAGTAAAACAATTAGATGAGATGAATTCTCCTAGAATTGTGAAGACTCATTTGCCAC  
CTGAACTTCTTCTGCCTCATTTTGGGAAAAGGATTGTAAGATAATCTATCTTTGCCGGAATG  
CAAAGGATGTGGCTGTTTCCTTTTATTATTTCTTTCTAATGGTGGCTGGTCATCCAAATCCTG  
GATCCTTTCCAGAGTTTGTGGAGAAATTCATGCAAGGACAGGTTCCCTTATGGTTCCCTGGTAT  
AAACATGTAAAATCTTGGTGGGAAAAGGAAAGAGTCCACGTGTACTATTTCTTTTCTACGA  
AGACCTGAAAGAGGATATCAGAAAAGAGGTGATAAAATTGATACATTTCCCTGGAAAGGAAG  
CCATCAGAGGAGCTTGTGGACAGGATTATACATCATACTTCGTTCCAAGAGATGAAGAACA  
ATCCATCCACAAATTACACAACACTGCCAGACGAAATTATGAACCAGAAATTGTCGCCCTTC  
ATGAGAAAGGGAATTACAGGAGACTGGAAAAATCACTTTACAGTAGCCCTGAATGAAAAAT  
TTGATAAACATTATGAGCAGCAAATGAAGGAATCTACACTGAAGTTTCGAACTGAGATCTAA  
**GCTAGC**TAGGTAGCTAGAGGATCTTTGTGAANNAANCNTTACTTCTGNGGTGTGACATNANT  
NGGACAAACTACCTACAGAGATTTNAAGCTNTNNGNAAATNNNAANNTTTNNGNNGNNNN  
NGNNNNNACTACNGANNCTNNNNNTNNNNNNNNNTNNNNANNNNNNNNTNGNANCTNANNN  
NNNNGNNGNCCNNNNNNNNNGNNNNNNNNNNNNNNNAAANNNNNTTNGCNNNCN

***pWPI-EST* SEQUENCING RESULTS FROM THE UNIVERSITY OF PITTSBURGH GENOMICS CORE**

Sequencing was done using the EF1 alpha promoter primer, however it did not sequence the full length EST. The 5' Restriction enzyme **PME1** followed by the EST sequence was confirmed. **PMEI** restriction enzyme sequence highlighted in yellow, followed by EST coding sequence starting with ATG.

NNNNNNNNNNNNNNNNNNNTCGTGANNNNNNCGACNTTTAAATTTAATTAATCTCGACGGT  
ATCGGTAACTTTTAAAAGAAAAGGGGGGATTGGGGGGTACAGTGCAGGGGAAAGAATAGT  
AGACATAATAGCAACAGACATACAACTAAAGAATTACAAAACAAATTACAAAATTCAA  
AATTTTATCGATCACGAGACTAGCCTCGAG**GTTTAAAC**ATGAATTCTGAACTTACTATTAT  
GAAAAGTTTGAAGAAGTCCATGGGATTCTAATGTATAAAGATTTTGTCAAATATTGGGATAA  
TGTGGAAGCGTTCAGGCAAGACCAGATGATCTTGTTCATTGCCACCTACCCTAAATCTGGTA  
CAACCTGGGTAGTGAAATTGTGTATATGATCTATAAAGAGGGTGTGTGGAAAAGTGCAA

AGAAGATGTAATTTTTAATCGAATACCTTTCCTGGAATGCAGAAAAGAAAACCTCATGAATG  
GAGTAAAACAATTAGATGAGATGAATTCTCCTAGAATTGTGAAGACTCATTGGCCACCTGAA  
CTTCTTCCTGCCTCATTGTTGGGAAAAGGATTGTAAGATAATCTATCTTTGCCGGAATGCAAAG  
GATGTGGCTGTTTCTTTTATTATTTCTTTCTAATGGTGGCTGGTCATCCAAATCCTGGATCCT  
TTCCAGAGTTTGTGGAGAAATTCATGCAAGGACAGGTTCCCTTATGGTTCCTGGTATAAACAT  
GTAAAATCTTGGTGGGAAAAGGGAAAAGAGTCCACGTGTACTATTTCTTTTCTACGAAGACCT  
GAAAGANGATATCAGAAAAGANGTGATAAAATTGATACATTTCTGGAAAGGAAGCCATCA  
GAGGAGCTTGTGGACAGGNTTATACATCATACTTCGTTCCAAGAGATGAAGAACAATCCATC  
CACAAATTACACAACACTGCCAGACGANNTNATGAACCANANNTGTCGCCCTTCATGAGAN  
NGNNNTACNGNAGACTGGNAAATCACTTNCNGTANCCNNAATGAAAAANTNGNNNAAN  
ATNATNAGCNNCNANTNNGNNTATCTACNNNNNANNNN

### ***pCMX-AAK-EST* SEQUENCING RESULTS FROM THE UNIVERSITY OF PITTSBURGH GENOMICS CORE**

Confirmation of mutant sequence (**Red highlight portion** is the 3' region of SULT1E1, confirming the orientation is correct).

NNNNNNNNNNNNNGNNNNNTGGTNNCCAATCCTGGATCCTTTCNGAGTTTGTGGAGAAA  
TTCATGCAAGGACAGGTTCTTATGGTTCCTGGTATAAACATGTAAAATCTTGGTGGGAAA  
GGGAAAGAGTCCACGTGTACTATTTCTTTTCTACGAAGACCTGAAAGAGGATATCAGAAA  
GAGGTGATAAAATTGATACATTTCTGGAAAGGAAGCCATCAGAGGAGCTTGTGGACAGGA  
TTATACATCATACTTCGTTCCAAGAGATGAAGAACAATCCATCCACAAATTACACAACACTG  
CCAGACGAAATTATGAACCGAAAATTGTCGCCCTTCATGAGAAAAG

**GCCATTACAGCCGACTGGAAA**AATCACTTTACA

A x x A x x K

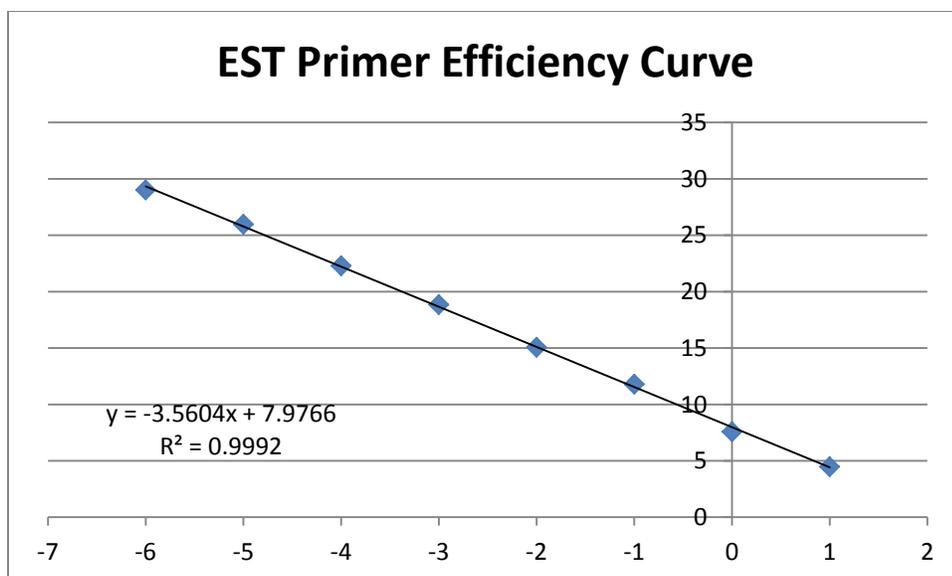
GTAGCCCTGAATGAAAAATTTGATAAACATTATGAGCAGCAAATGAAGGAATCTACTACTGA  
AGTTTCGAACT**GAGATCTA**AGCTAGCTAGGTAGCTAGAGGATCTTTGTGAAGGAACCTTACT  
TCTGTGGTGTGACATAATTGGACAAACTACCTACAGAGATTTAAAGCTCTAAGGTAAATATA  
AAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTGTGTATTTTAGATTCCA  
ACCTATGGAAGTATGAAATGGGAGCAGTGGTGGAAATGCCTTTAATGAGGAAAACCTGTTTTG  
CTCAGAAGAAATGCCATCTAGTGATGATGAGGCTACTGCTGACTCTCAACATTCTACTCCTC  
CAAAAAGAAGAGAAAGGTAGAAGACCCCAAGGACTTTCCTTCAGAATTGCTAAGTTTTTT  
GAGTCATGCTGTGTTTAGTAATAGAACTCTTGCTTGCTTTGCTATTTACACCACAAAGGAAA  
AAGCTGCACTGCTATACAAGAAAATTATGGAAAAATATTTGATGTATAGTGCCTTGACTAGA  
GATCATAATCAGCCATACCACATTTGTAGAGGTTTTACNTGCTTTAAAAAACCTCCCACACC  
TCCCCNGNANCTGAAACATAAAATGNATGN

## shRNA SEQUENCES FROM OPEN BIOSYSTEMS

The targeting sequences for EST and ER $\alpha$  are ATGAGTCTTCACAATTCTAGG (product TRCN0000035880) and TTCCAGAGACTTCAGGGTGCT (product TRCN0000003299), respectively. Both purchased from Open Biosystems.

## RT- PCR PRIMER SEQUENCES

Gene	Forward	Reverse
EST	AGAGGAGCTTGTGGACAGGA	GGCGACAATTTCTGGTTCAT
LPL	TCCAAGGAAGCCTTTGAGAA	TTGCTGCTTCTTTTGGCTCT
ATGL	TACAGAGAGGCACTGCCCCGGCT	AAGGCCACGTTGGTGCAGAAGA
SREBBP1c	CTTGAGCTGCGTGGCTTCCAAC	CTTGAGCTGCGTGGCTTCCAAC
GAPDH	CTCAAGGGCATCCTGGGCTACA	TGGTCGTTGAGGGCAATGCC
CYCLOPHILLIN	TGGTCGTTGAGGGCAATGCC	CACATGCTTGCCATCCAACCACT
FASN	GCCGTGGACCTGATCATCAAGA	ATGGTACTTGGCCTTGGGTGTGTA
SCD1	TGGGGTGAACCTTTTCAAACAGTG	CCGGGGGCTAATGTTCCTGTCATA
ACC	CCGGATGCAGGAGAAGGGTGTTAT	GGGTTGGCATTGTGGATTTCTTC
aP2	CCTTTAAAAATACTGAGATTTCTTCA	GGACACCCCCATCTAAGGTT
C/EBP $\alpha$	AGCAGGAGAAGGCCAAGG	CCCGGGTAGTCAAAGTCG
C/EBP $\beta$	GGGCCCTGAGTAATCGCTTAA	ATAAACAGCAACAAGCCCG
C/EBP $\delta$	AGATGCAGCAGAAGTTGGTGGA	CTGGGCAGCTGCTTGAAGAACT
HSL	GAGATGTCGCTGTCTAGCTGAGACA	GTCCATGGGGCTCAGTCATTT
GR	TCTCTTCAGTTCTTAAGGACGG	TGGAGTTTCTTCCCTCTTGAC
ER $\alpha$	AGGGATGAGGGGAAATGCGTAGAA	GCTGTACAGATGCTCCATGCCTTT
Er $\beta$	AGGGATGAGGGGAAATGCGTAGAA	GGATCATGGCCTTGACACAGAGAT
PPAR $\gamma$	GGGGTACCATGGGTGAA ACTCT	CTAGCTAGC CTAGTACAAGTCCT
IGFBP4	GAGATCGAGGCCATCCAGGAAA	TTTTGGCGAAGTGCTTCTGCAG
IGFBP5	GGGGTTTGCTCAACGAAAAGA	TGTGTTTGGGCCGGAAGATCTT
GPX3	ATTTCCAGCTCTTTGAGAAAGGGG	GCGGATGTCGTGAACCTTCATG



(Above) Here we show the graph of the efficiency curve readout where the x-axis is  $\log_{10}$  of the concentration of linearized plasmid (concentration shown below) and the y-axis is the corresponding Ct value (average triplicate). (Below) Linearized plasmid (p CMXpl2 hEST) concentration,  $\log_{10}$  and corresponding average Ct readout. *E*, efficiency; % *E*, percent efficiency of primer pair.

ng	Log <sub>10</sub>	Avg CT
10	1	4.47
1	0	7.575
0.1	-1	11.795
0.01	-2	15.06
0.001	-3	18.845
0.0001	-4	22.29
0.00001	-5	25.965
0.000001	-6	29.02
	<b>E</b>	<b>% E</b>
	$10^{(-1/\text{slope})}$	$(E-1)^* 100$
	1.909269867	90.92699

1. Suzuki, T., et al., *Steroid sulfatase and estrogen sulfotransferase in normal human tissue and breast carcinoma*. J Steroid Biochem Mol Biol, 2003. **86**(3-5): p. 449-54.
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