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Estrogen Sulfotransferase/SULT1E1 Promotes Human Adipogenesis

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Estrogen sulfotransferase (EST/SULT1E1) is known to catalyze the sulfoconjugation and deactivation of estrogens. The goal of this study is to determine whether and how EST plays a role in human adipogenesis. By using human primary adipose-derived stem cells (ASCs) and whole-fat tissues from the abdominal subcutaneous fat of obese and nonobese 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 proadipogenic activity of EST in humans was opposite to the antiadipogenic effect of the same enzyme in rodents. Mechanistically, EST promoted adipogenesis by deactivating estrogens. The proadipogenic effect of EST can be recapitulated by using an estrogen receptor (ER) antagonist or ERα knockdown. In contrast, activation of ER in ASCs inhibited adipogenesis by decreasing the recruitment of the adipogenic peroxisome proliferator-activated receptor γ (PPARγ) onto its target gene promoters, whereas ER antagonism increased the recruitment of PPARγ 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 ERα expression and BMI. We conclude that EST is a proadipogenic factor which may serve as a druggable target to inhibit the turnover and accumulation of adipocytes in obese patients.

Obesity is a major health concern with high prevalence. Obesity is defined as a body mass index (BMI) equal to or greater than 30, whereas a BMI equal to or greater than 25 is considered overweight. An estimated 400 million people worldwide are obese. In the United States alone, obesity-related medical complications contribute to an average of 300,000 deaths annually (1).

The obesity epidemic has generated much research attention toward understanding the biochemical regulation of adipose tissue and the development of adipocytes, known as adipogenesis. Adipogenesis is a multifaceted process that is regulated by temporal and spatial expression of a battery of adipogenic genes. When the preadipocytes located in various visceral and subcutaneous fat depots are stimulated by specific mitogenic and adipogenic cues, they begin the differentiation process until maturation is reached (2). This process is accompanied by a dramatic increase in the expression of adipogenic genes, such as the genes for lipoprotein lipase (LPL), fatty acid binding protein 4/adipocyte protein 2 (FABP4/aP2), and the CCAATT enhancer binding proteins α, β, and δ (C/EBPα, -β, and -δ) (3). Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor known as the master regulator of adipogenesis. Activation of PPARγ is required for the induction of several of the aforementioned adipogenic enzymes and transcription factors (3). Differentiation also leads to morphological and biochemical changes in preadipocytes that allow them to store lipids and secrete adipokines (4).

The sex hormones are known to have a marked impact on adipose tissue development, accumulation, and distribution in humans. Men tend to have a more central abdominal accumulation of fat, while women tend to accumulate adipose tissue around the gluteal and femoral area (5). Animal models and human epidemiological studies have shown that, in general, loss of estrogen signaling facilitates increased adipose tissue accumulation. This has been described in ERα and aromatase knockout mice and documented in postmenopausal women (6–9). In contrast, estrogen replacement therapy in older women resulted in reductions in central subcutaneous and visceral adipose tissue (10, 11).

The homeostasis of estrogens is tightly regulated by balanced synthesis and deactivation. Estrogen sulfotransferase (EST, or SULT1E1) is a key enzyme known to catalyze the sulfation of estrogens, leading to their inactivation because of the inability of estrogen sulfates to bind to the estrogen receptor (ER) (12). We recently described a novel role for Est in murine adipogenesis, in which Est functions as a negative regulator of adipogenesis. Est is highly expressed in mouse preadipocytes, and differentiation attenuates the expression of Est (13). Moreover, Est overexpression and ablation inhibited and promoted murine adipogenesis, respectively (13). It is unclear whether the role of EST in adipogenesis is conserved in humans.

In this study, we found that the effect of EST on adipogenesis is highly species specific. EST promoted human adipogenesis by deactivating estrogens. The proadipogenic effect of EST was recapitulated in preadipocytes whose ER was pharmacologically or genetically inhibited. In contrast, pharmacological activation of ER inhibited adipogenesis. We propose EST as a druggable target whose inhibition may be used to inhibit the turnover of adipocytes in obese patients.

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MATERIALS AND METHODS

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. Preadipocytes were isolated from the abdominal subcutaneous fat of 15 obese patients and 3 nonobese (lean) patients who had undergone liposuction or whole-fat removal surgery. 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 demographic information of the 18 patients is available upon request. The whole fats or liposuction aspirates were collected from the abdominal subcutaneous fat of 16 female patients who were nondiabetic nonsmokers and ranged in age from 32 to 59 years. The demographic information of these 16 patients is available upon request. The cells were cultured under standard conditions as reported previously (14). 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.

Lentivirus generation and production. To generate lentivirus expressing EST and mutant EST AAK (EST with a change from GXXGXXK to AXXAXXK in the 3′-phosphoadenosine 5′-phosphosulfate [PAPS]-binding domain), wild-type (WT) EST was cloned by reverse transcription-PCR (RT-PCR) using cDNA from differentiated human adipocytes. WT EST or AAK EST cDNA was cloned into a lentiviral expression plasmid (pWPI) via the Pme1 restriction sites. Lentiviral particles were generated using a second-generation system that contained three plasmids: the transgene expression plasmid (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-μm-pore-size 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. The cDNA for the EST AAK mutant was cloned by overlap extension PCR mutagenesis (15). Preadipocytes were infected at a multiplicity of infection (MOI) of 3 before puromycin selection. Based on our observations, there was no noticeable effect of lentiviral infection and puromycin selection alone on the differentiation.

To generate lentivirus expressing short hairpin RNAs (shRNAs) against EST and ERα (shEST and shERα, respectively), expression plasmids containing shEST and shERα were purchased from Open Biosystems (Pittsburgh, PA). For each knockdown, five sequences were purchased and tested in transient-transfection assays, and the sequence with the most efficient knockdown was chosen for lentiviral production. The targeting sequences for EST and ERα are ATGAGTCTTCACAATTC TAGG (product TRCN0000035880) and TTCCAGAGACTTCAGGG TGCT (product TRCN0000003299), respectively. A scrambled shRNA (shSCR) plasmid was purchased to serve as a control.

Adipocyte differentiation. In all experiments except those in which exogenous estrogen (E_2) was added, differentiation medium 1 (DM1) consisted of DMEM–F-12 medium, 10% standard FBS, 1% Pen-Strep, 33 μM biotin, 100 nM insulin, 17 μM pantothenic acid, 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, and 1 μM rosiglitazone. Differentiation medium 2 (DM2) consisted of DMEM–F-12 medium, 10% FBS, 1% Pen-Strep, 1 μM 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 E_2 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 E_2 at a final concentration of 10 nM.

Quantitative real-time reverse transcription-PCR (RT-PCR). The cDNA was synthesized from 1.0 μg 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).

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 1 min 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 μl of elute was loaded onto 96-well plates. Absorbance was measured at 500 nm using a PerkinElmer plate reader.

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-polyacrylamide 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), ERα (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.

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 150-cm 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 3×10^3 cells per well in 96-well plates for 1, 3, and 5 days. On the appropriate day, 10 μl of MTT reagent was added to each well, and the cells were incubated for 3 h at 37°C. Subsequently, 100 μl 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.

Transient-transfection and luciferase assays. HepG2 cells or 293T cells were plated at a density of 2×10^6 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-ERα, pCMX-PPARγ, and pCMX. The triplicate plasmid amounts for pCMX-CBP, pCMX–β-Gal (where β-Gal is β-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 600 ng, respectively. Cells were transfected and incubated for 24 h. Transfected cells were treated with the appropriate ligand using DMEM without phenol red and DCC FBS, followed by lysis and assays for luciferase and

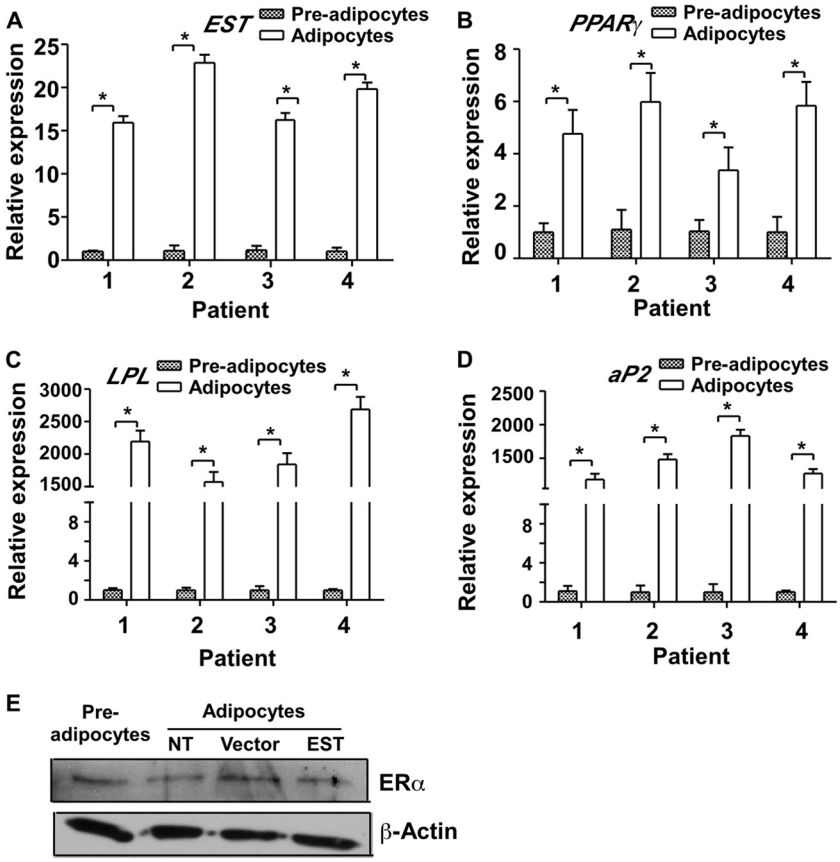

FIG 1 The expression of EST was induced during adipogenesis. (A to D) Preadipocytes were cultured and differentiated in standard medium for 14 days. Total RNA was extracted before and after differentiation, and the expression of EST (A), PPARγ (B), LPL (C), and aP2 (D) was measured by real-time PCR analysis. *, $P < 0.05$. (E) The expression of ERα was measured by Western blotting. NT, nontransfected.

β-Gal activities. The luciferase activities were normalized to β-Gal activities.

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-PPARγ 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 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.

Statistical analysis. When applicable, results are presented as means ± standard deviations (SD). The Student *t* test was used to calculate *P* values. *P* values of less than 0.05 are considered to be significant. Linear regression analysis was performed using the GraphPad Prism software.

RESULTS

The expression of EST was induced during adipogenesis. We have previously reported that the expression of the mouse Est gene was high in preadipocytes, and expression decreased upon differentiation (13). In an effort to determine whether the human EST affects the differentiation of human preadipocytes, we were surprised to find that in four independent cases of human preadipocytes isolated from obese patients, the mRNA expression of EST in preadipocytes was low, and differentiation led to a marked increase in EST expression (Fig. 1A). The induction of EST coincided with the induction of adipocyte differentiation maker genes, such as PPARγ (Fig. 1B), LPL (Fig. 1C), and aP2 (Fig. 1D). Western blotting results showed that the expression of ERα remained constant from preadipocytes to differentiated adipocytes regardless of EST overexpression (Fig. 1E).

Overexpression of EST promoted adipogenesis. To determine the functional relevance of EST induction during adipogenesis, we overexpressed EST in preadipocytes before subjecting them to differentiation. Samples of preadipocytes from four patients were transduced with lentivirus overexpressing EST or the vector control virus and then induced to terminal differentiation for 2 weeks. The overexpression of EST was confirmed by real-time PCR (Fig. 2A) and Western blotting (Fig. 2B). Overexpression of EST promoted adipogenesis, as confirmed by both oil red O staining (Fig. 2C) and gene expression analysis (Fig. 2D). Gene expression changes included the induction of PPARγ, LPL, aP2, and C/EBPα. The C/EBPs are vital for adipogenesis (3, 4, 17). C/EBPα has been shown to facilitate the activation of PPARγ target genes, whereas C/EBPβ and C/EBPδ are important for the transactivation of PPARγ gene expression (18–23). Interestingly, the enhanced adipogenesis in cells infected with EST-expressing

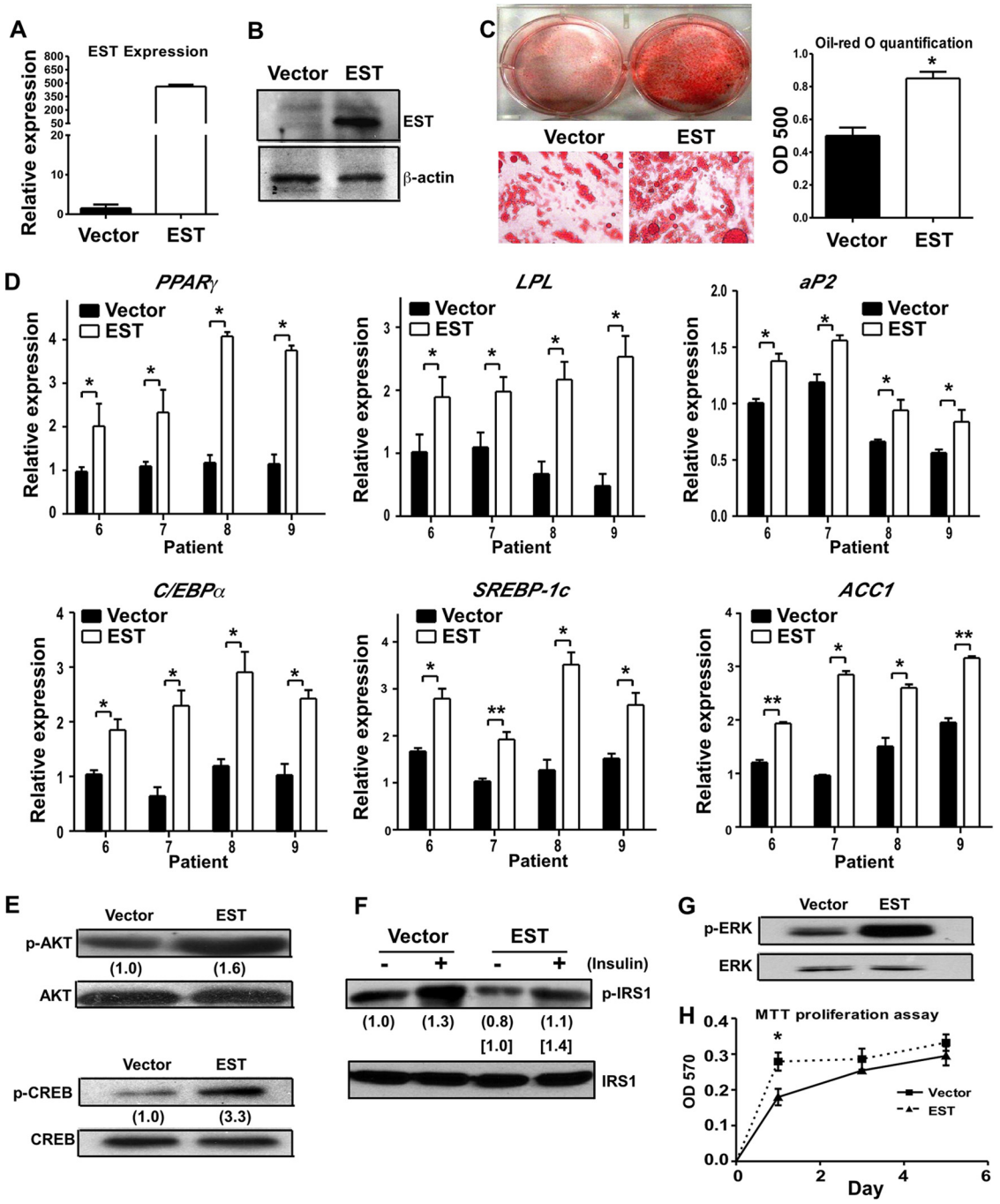

FIG 2 Overexpression of EST promoted adipogenesis. (A and B) Preadipocytes were transduced with vector or EST-expressing lentivirus. The expression of the transduced EST was confirmed by real-time PCR (A) and Western blotting (B). (C) Vector- and EST-expressing cells were induced to differentiate for 14 days in standard differentiation medium before being examined by oil red O staining microscopically (left panel) and quantitatively (right panel). (D) Total RNA was extracted before and after differentiation, and the expression of indicated genes was measured by real-time PCR. (E) Lysates from differentiated cells were subjected to Western blot analysis to detect protein expression of total AKT, phospho-AKT (p-AKT), total CREB, and phospho-CREB (p-CREB). The signals were quantified by using NIH ImageJ software. $n = 3$. (F) Preadipocytes were 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. (G) 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). (H) Preadipocytes were transduced with vector- or EST-expressing lentivirus and grown in triplicate for each time point and examined by an MTT proliferation assay. *, $P < 0.05$; **, $P < 0.01$; ns, statistically not significant. OD500, optical density at 500 nm.

virus was associated with the induction of C/EBPα (Fig. 2D), but the expression of C/EBPβ and C/EBPδ was unchanged (data not shown). The expression of lipogenic genes, including the genes for sterol regulatory element binding protein 1c (SREBP-1c), acetyl coenzyme A (CoA) carboxylase 1 (ACC1) (Fig. 2D), fatty acid synthase (FAS), and stearoyl CoA desaturase 1 (SCD1) (data not shown), was increased in cells transduced with EST-expressing virus. In contrast, the expression of the lipolytic genes for adipose

triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) was not affected by EST overexpression (data not shown).

The enhancement of differentiation in cells infected with EST-expressing virus was associated with increased phosphorylation of AKT and CREB in terminally differentiated cells (Fig. 2E), which was suggestive of increased insulin signaling. However, when the acute insulin response was evaluated in preadipocytes treated with insulin for 10 min, we found that the phosphorylation of the insulin receptor substrate 1 (IRS1) was similarly increased in vector-infected and EST virus-infected cells (Fig. 2F). These results suggested that overexpression of EST alone did not confer increased acute insulin sensitivity. The increased AKT and CREB phosphorylation in terminally differentiated cells might have been secondary to the enhanced differentiation when EST was overexpressed. The phosphorylation of ERK1/2 was also increased in EST virus-infected cells (Fig. 2G). ERK1/2 activation is often linked to cell proliferation. During adipocyte differentiation, ERK1/2 activation was required during the phase of clonal expansion, whereas persistent activation of ERK1/2 may inhibit adipocyte differentiation (24, 25). Consistent with the increased ERK1/2 phosphorylation, the proliferation of cells infected with EST-expressing virus was increased compared to that of vector-infected cells in the early phase of growth, but the difference became insignificant after 3 days of culture when the cells were more confluent (Fig. 2H). Addition of the ERK1/2 inhibitor PD98059 had little effect on the differentiation of either the vector- or EST virus-infected cells (data not shown), suggesting that the difference in proliferation rate in the early phase might not be the key factor responsible for the difference in differentiation.

The enzymatic activity of EST was required for the adipogenic effect of EST. 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 proadipogenic effect, we generated a lentiviral vector expressing a mutant human EST lacking its enzymatic activity. 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 AXX AXXK (AAK), the enzymatic activity of EST was completely abolished (13, 26). 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. 3A). The expression of EST AAK in transduced preadipocytes was confirmed by real-time PCR (data not shown) and Western blotting (Fig. 3B). Oil red O staining showed that overexpression of EST AAK failed to promote differentiation (Fig. 3C). The lack of adipogenic activity of EST AAK was also supported by the lack of induction of adipogenic gene expression (Fig. 3D) and phosphorylation of AKT and ERK (Fig. 3E).

Genetic knockdown or pharmacological inhibition of EST inhibited adipogenesis. Having demonstrated that forced expression of EST promoted adipocyte differentiation, we went on to determine whether downregulation of EST inhibited adipogenesis. In this experiment, samples of human preadipocytes from two patients were infected with lentivirus expressing an shRNA against EST (shEST) or the control scrambled shRNA (shSCR) before they were subjected to differentiation for 2 weeks. The efficiency of EST knockdown was confirmed by real-time PCR (Fig. 4A). Indeed, downregulation of EST inhibited differentiation, as supported by the markedly decreased oil red O staining (Fig. 4B). At the gene expression level, the expression of PPARγ, LPL, aP2, and C/EBPα was decreased in cells infected with shEST-expressing virus (here, shEST virus) (Fig. 4C). The expression of lipogenic genes for SREBP-1c, ACC1, FAS, and SCD-1 was decreased in shEST virus-infected cells (Fig. 4C and data not shown). The expression of lipolytic genes for ATGL and HSL was also decreased in shEST knockdown cells (Fig. 4C). The pattern of lipogenic and lipolytic gene expression was consistent with the suppression of adipogenesis. The phosphorylation of AKT and CREB was also decreased in shEST virus-infected cells (Fig. 4D).

The inhibition of differentiation was also observed when the preadipocytes were differentiated in the presence of the EST inhibitor triclosan. Triclosan, a proposed antimicrobial agent used in many commercial products, inhibits EST from transferring a sulfuryl moiety from PAPS onto E_2 by binding to the E_2-binding site on EST, causing the formation of triclosan-sulfate conjugates instead of the E_2-sulfate conjugates (27, 28). The inhibition by triclosan appeared to be EST specific (27). Our own results showed that triclosan inhibited EST but had little effect on the activity of the hydroxysteroid sulfotransferase SULT2A1 (data not shown). We showed that treatment with triclosan decreased oil red O staining (Fig. 4E) and suppressed the expression of differentiation marker genes for PPARγ, LPL, and aP2 but had little effect on the expression of the endogenous EST (Fig. 4F).

The effects of EST overexpression and knockdown were conserved in preadipocytes isolated from nonobese patients. The above-described experiments were performed on preadipocytes isolated from overweight or obese patients. To determine whether the EST effect on adipogenesis was conserved in nonobese subjects, the EST overexpression and shEST knockdown experiments were repeated in samples of preadipocytes isolated from three nonobese patients (BMI 21.4 to 23.7). As shown in Fig. 5A, lentiviral overexpression of EST increased adipogenic and lipogenic gene expression. In contrast, shEST virus-infected cells showed decreased expression of adipogenic genes, lipogenic genes (Fig. 5B), and lipolytic genes (data not shown). The respective promotion and inhibition of adipogenesis by EST overexpression (Fig. 5C) and knockdown (Fig. 5D) were confirmed by oil red O staining.

Pharmacological antagonism or genetic knockdown of ERα enhanced adipogenesis. A primary function of EST is to sulfonate and deactivate estrogens. The proadipogenic activity of EST and the requirement of the enzymatic activity for the proadipogenic effect of EST led to our hypothesis that EST may have promoted adipogenesis by antagonizing the estrogen activities. Indeed, we showed that treatment of vector-infected human preadipocytes maintained in DCC serum with exogenously added E_2 inhibited differentiation (Fig. 6A), which was in contrast to the previously reported lack of estrogen effect on the differentiation of the mouse preadipocytes (13). The inhibitory effect of E_2 was abolished in EST virus-transduced cells, presumably due to enhanced estrogen deactivation. Interestingly, overexpression of EST alone in the absence of exogenously added E_2 was still sufficient to increase the expression of PPARγ and aP2 although the induction of LPL was not significant (Fig. 6A). The effectiveness of estrogen treatment in vector-infected cells and loss of estrogen effect in EST virus-infected cells was confirmed by the measurement of the expression of the estrogen-responsive genes for insulin-like growth factor binding proteins 2 and 4 (IGFBP2 and IGFBP4) and glutathione

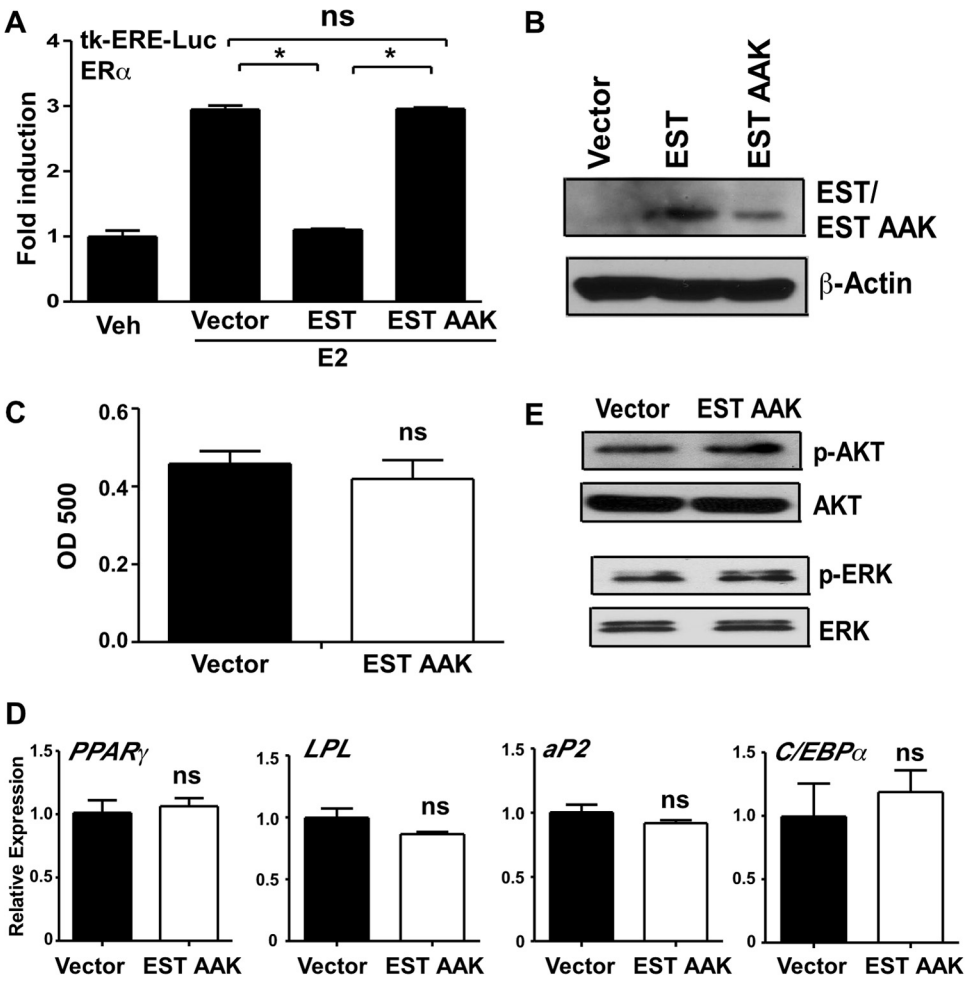

FIG 3 The enzymatic activity of EST was required for the adipogenic effect of EST. (A) The activity of the EST AAK mutant was compared to that of the wild-type EST. 293T cells were transfected with a tk-ERE-Luc reporter gene and ERα, along with EST, EST AAK, or empty vector as indicated. The cells were either treated with 1 nM E_2 or vehicle in phenol red-free DMEM containing 10% DCC FBS. The luciferase activities are normalized against β-Gal activities from the cotransfected CMX-β-Gal vector. Results are shown as fold induction over vehicle, treated in triplicate. (B) Preadipocytes were infected with EST- or EST AAK-expressing lentivirus. The expression of EST and EST AAK was measured by Western blotting. (C to E) Preadipocytes were transduced with vector- or AAK-expressing lentivirus and then induced to differentiate for 14 days before being evaluated for oil red O staining (C), gene expression analysis by real-time PCR (D), and Western blot analysis to detect total AKT, phospho-AKT, total ERK1/2, and phospho-ERK1/2 (E). *, $P < 0.05$; ns, statistically not significant.

peroxidase 3 (GPX3) (Fig. 6B). Estrogens function through the estrogen receptors. Both ERα and ERβ are expressed in adipose tissues, but ERα is the predominant ER isoform in human subcutaneous fat (29). We then used the ER antagonist fulvestrant (ICI 182780) and ERα knockdown to further determine the role of estrogen-ER signaling in human adipocyte differentiation. Fulvestrant is a potent ERα/β antagonist clinically used to treat estrogen-dependent breast cancer (30). The ERα knockdown was achieved by using a lentivirus expressing shRNA against ERα (shERα). The efficiency of ERα knockdown was confirmed by real-time PCR (Fig. 6C, left panel) and Western blotting (Fig. 6C, right panel). Knockdown of ERα did not affect the expression of ERβ (Fig. 6C, left panel).

When subjecting the vector- or EST virus-infected preadipocytes to differentiation in the presence or absence of fulvestrant or in the presence or absence of shERα, we found that treatment of the vector cells with fulvestrant or knockdown of ERα in the vector cells promoted adipogenesis to a level similar to what was observed in vehicle-treated EST cells, as judged by oil red O staining (Fig. 6D), adipogenic and lipogenic gene expression (Fig. 6E), and phosphorylation of AKT and CREB (Fig. 6F). In contrast, treatment of the EST virus-infected cells with fulvestrant or knockdown of ERα in EST virus-infected cells showed little effect in further enhancing adipogenesis (Fig. 6D to F). These results demonstrated that adipogenesis was enhanced when estrogen-ER signaling was downregulated through the use of ER antagonist or ERα knockdown, supporting that the proadipogenic effect of EST may have been mediated through the inhibition of the estrogen-ER signaling pathway.

Molecular mechanism for the inhibitory effect of the estrogen-ERα signaling pathway on adipogenesis. Cross talk between nuclear receptors is an important regulatory mechanism to fine-tune gene expression. The heart of this cross talk could lie in the limited supply of coactivators or corepressors that are shared by two or more nuclear receptors within the same cells. PPARγ is a critical adipogenic nuclear receptor (31, 32). CBP/p300 is an im-

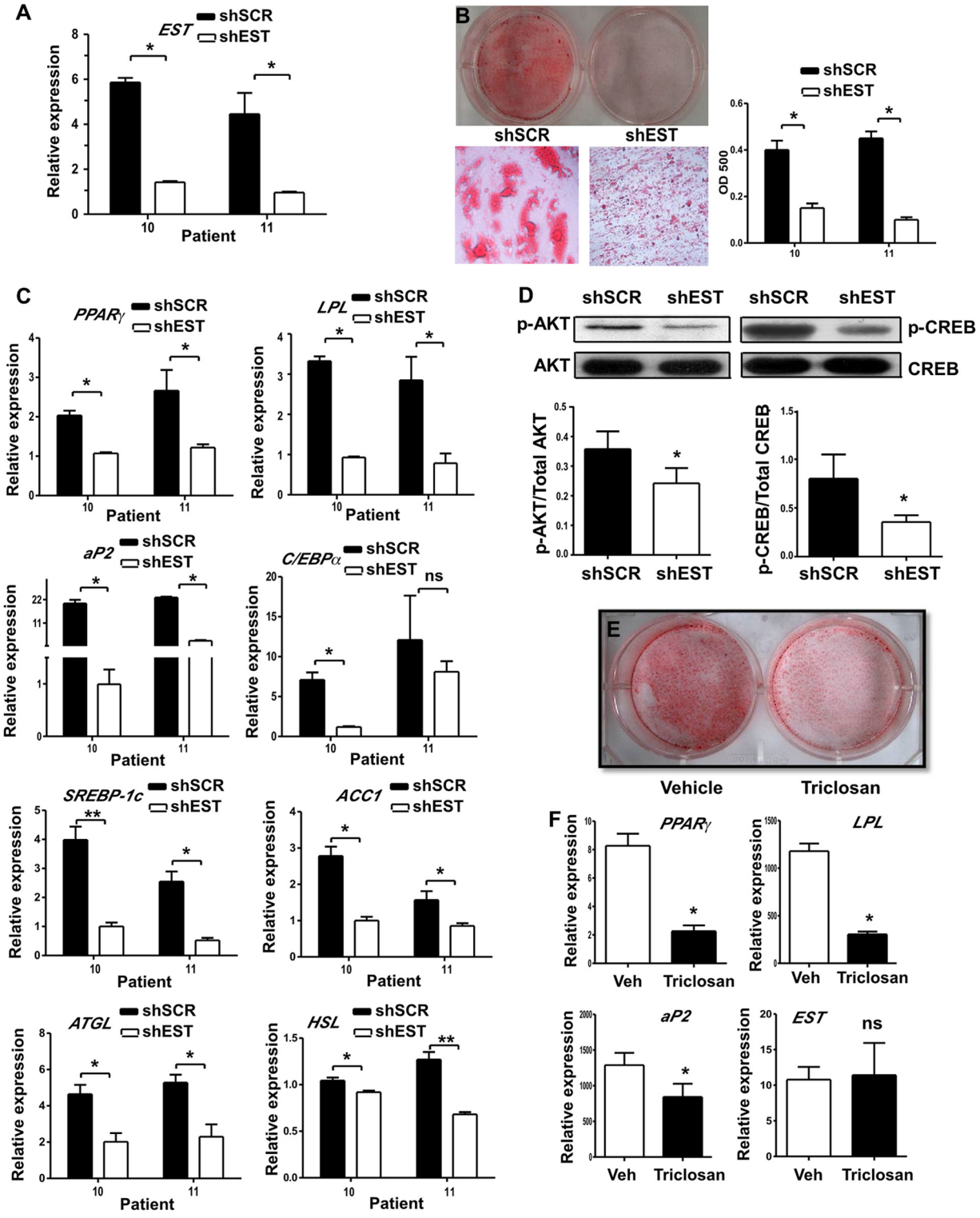

FIG 4 Genetic knockdown or pharmacological inhibition of EST inhibited adipogenesis. (A to D) Preadipocytes were transduced with lentivirus expressing either shEST or a scrambled shRNA (shSCR) and then induced to differentiate for 2 weeks before being evaluated for the efficiency of EST knockdown by real-time PCR analysis (A), oil red O staining and quantification (B), gene expression analysis by real-time PCR (C), and Western blot analysis to detect the expression of total AKT, phospho-AKT, total CREB, and phospho-CREB (D). (E and F) Preadipocytes were induced to differentiate for 2 weeks in the presence of 10 μM triclosan or vehicle (Veh) before being evaluated for oil red O staining (E) and the expression of adipogenic genes and EST (F). *, $P < 0.05$; **, $P < 0.01$; ns, statistically not significant.

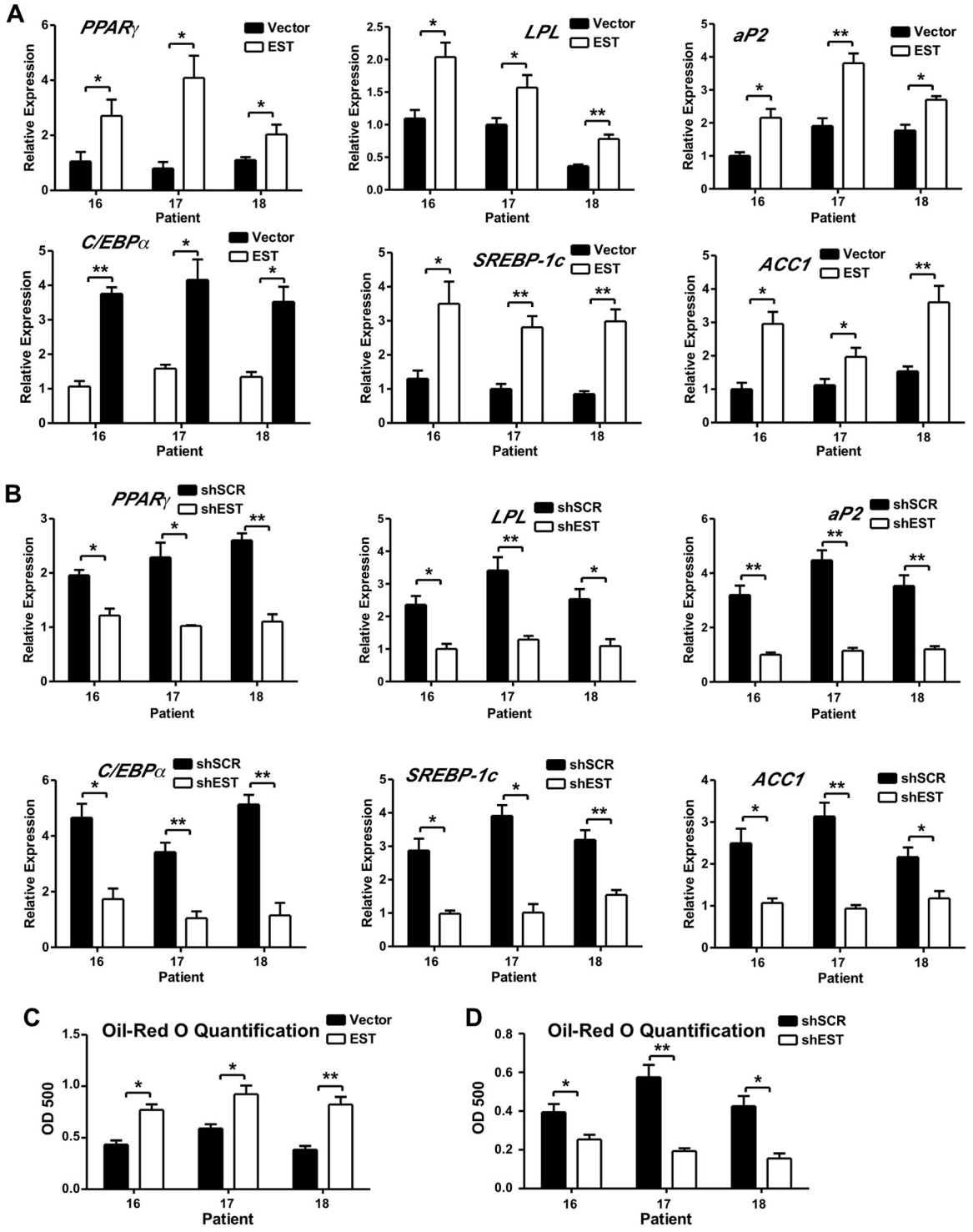

FIG 5 The effects of EST overexpression and knockdown were conserved in preadipocytes isolated from nonobese patients. The BMI range for these three patients is 21.4 to 23.7. (A and B) Cells infected with virus expressing vector and EST (A) or shSCR and shEST (B) were induced to differentiate for 2 weeks before gene expression profiling by real-time PCR. (C and D) The differentiation of cells infected with virus expressing EST (C) and shEST (D) was evaluated by the quantification of oil red O staining. $n = 3$. *, $P < 0.05$; **, $P < 0.01$.

portant coactivator shared by both ERα and PPARγ. We then hypothesized that ERα may antagonize adipogenesis by transsuppressing PPARγ. Indeed, we showed that in transient-transfection and luciferase reporter gene assays, ERα inhibited the PPARγ-mediated activation of a PPAR-responsive reporter gene, tk-PPRE-Luc, in a ligand-dependent manner, whereas this inhibition was attenuated by the cotransfection of CBP (Fig. 7A). Reciprocally, PPARγ inhibited the ERα-mediated activation of an ER-

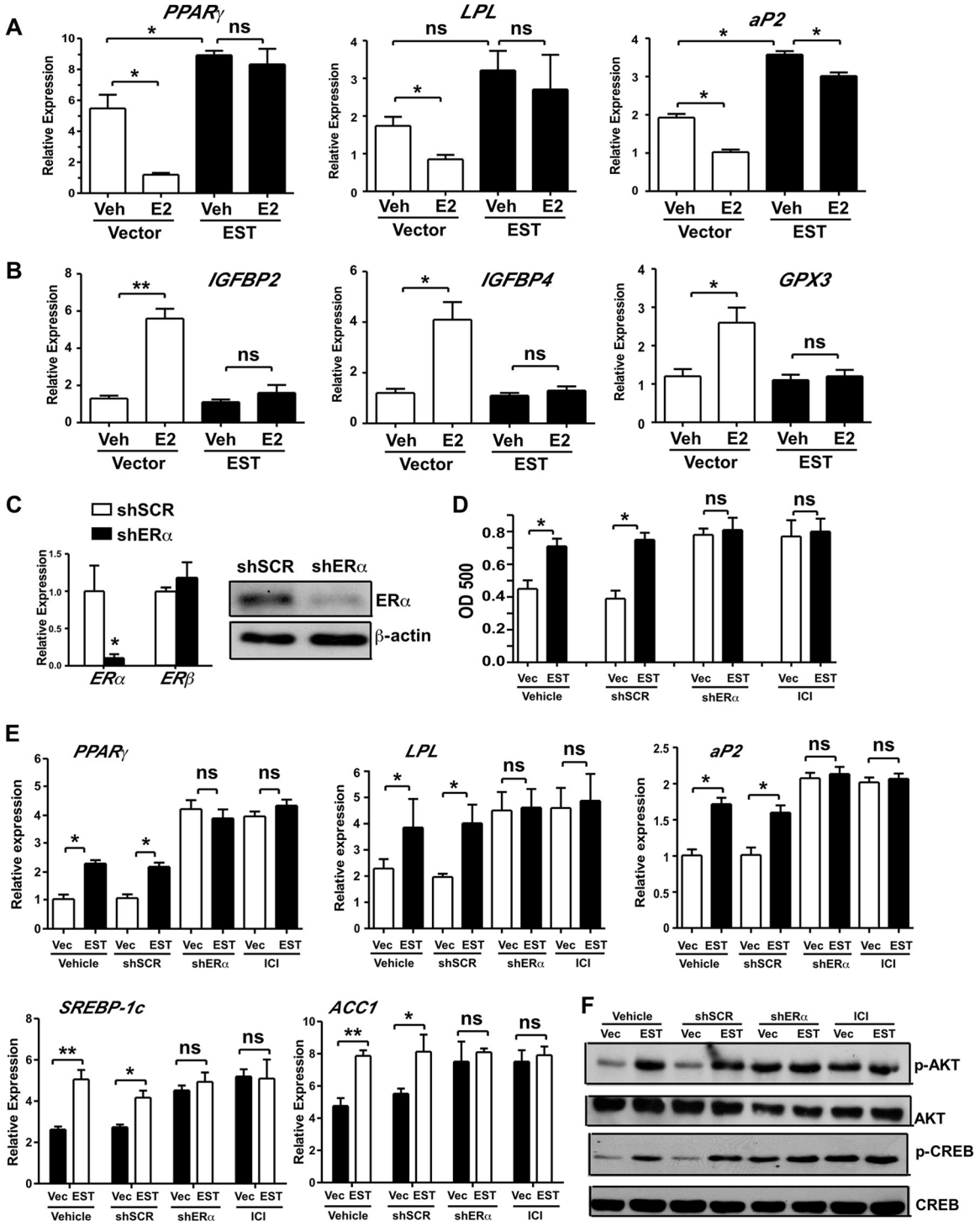

FIG 6 Pharmacological antagonism or genetic knockdown of ERα enhanced adipogenesis. (A and B) Preadipocytes were transduced with either vector- or EST-expressing virus and induced to differentiate in the presence or absence of 10 nM E_2 for 2 weeks before being evaluated for the expression of adipogenic genes (A) and estrogen-responsive genes (B) by real-time PCR. (C) The efficiency of lentiviral shRNA knockdown of ERα was confirmed by real-time PCR (left panel) and Western blotting (right panel). (D to F) Preadipocytes infected with virus expressing vector (Vec) or EST were treated with 100 nM fulvestrant (ICI) or transduced with lentivirus expressing shSCR or shERα and then induced to differentiate for 2 weeks before being evaluated for oil red O staining and quantification (D), expression of adipogenic and lipogenic genes by real-time PCR (E), and Western blot analysis to detect the expression of total AKT, phospho-AKT, total CREB, and phospho-CREB (F). *, $P < 0.05$; **, $P < 0.01$; ns, statistically not significant.

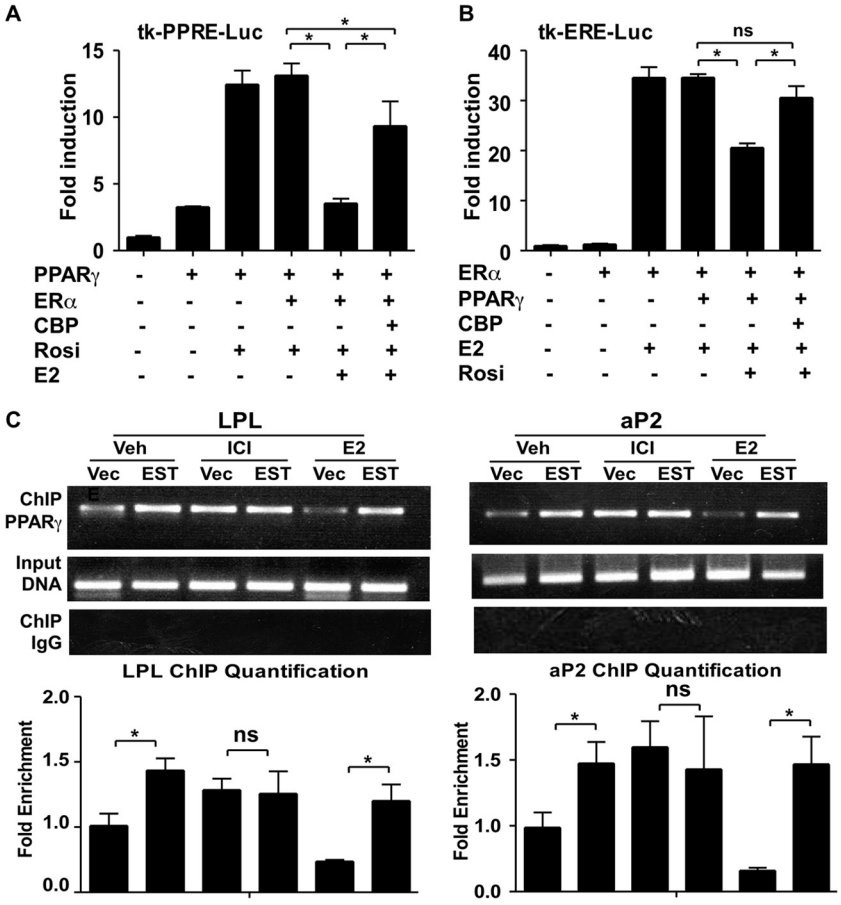

FIG 7 Molecular mechanism for the inhibitory effect of the estrogen-ER signaling pathway on adipogenesis. (A and B) 293T cells were transfected with either the tk-ERE-Luc reporter gene together with ERα and/or CBP (A) or the tk-PPRE-Luc reporter gene together with PPARγ and/or CBP (B). Transfected cells were treated with E_2 (10 nM) and/or rosiglitazone (Rosi; 1 μM) for 24 h before luciferase assay. The luciferase activities were normalized against β-Gal activities from the cotransfected β-Gal vector. Results are shown as fold induction over vehicle-treated triplicates. (C) The vector- or EST-transduced preadipocytes were treated with 100 nM fulvestrant (ICI) or 10 nM E_2 and then induced to differentiate for 2 weeks before being evaluated for the recruitment of PPARγ onto the LPL (left panel) and aP2 (right panel) gene promoters by chromatin immunoprecipitation (ChIP) assay. Quantification was performed by using NIH ImageJ software. *, $P < 0.05$; ns, statistically not significant.

responsive reporter gene, tk-ERE-Luc, in a ligand-dependent manner, and this inhibition was abolished by the cotransfection of CBP (Fig. 7B).

We then used a chromatin immunoprecipitation (ChIP) assay on the vector- and EST virus-infected preadipocytes to determine whether ER activation or antagonism affected the recruitment of PPARγ onto its adipogenic target gene promoters. As shown in Fig. 7C, overexpression of EST led to increased recruitment of PPARγ onto the LPL and aP2 gene promoters, which was consistent with the phenotype of enhanced adipogenesis. Treatment of cells with fulvestrant increased the recruitment of PPARγ in vector-infected cells to a level equal to that seen in the cells infected with EST-expressing virus. In contrast, treatment with E_2 inhibited the recruitment of PPARγ in the vector-infected cells, and this inhibition was normalized in cells infected with EST-expressing virus, likely due to the enhanced estrogen deactivation.

The respective expression levels of EST and ERα were positively and negatively correlated with adiposity in human patients. The proadipogenic activity of EST and the antiadipogenic activity of the estrogen-ER signaling pathway prompted us to determine whether the expression levels of EST and ERα were positively and negatively correlated with adiposity, respectively. In this experiment, total RNAs were isolated from abdomen whole fat or liposuction aspirate from 16 patients, and gene expression was analyzed by real-time PCR. The correlation between gene expression and BMI was analyzed by linear regression. We found a positive and significant correlation between EST expression and BMI (Fig. 8A), as well as an inverse and significant correlation between ERα expression and BMI (Fig. 8B). The correlation between ERβ expression and BMI did not reach statistical significance (Fig. 8C).

DISCUSSION

In this study, we have uncovered a novel role for EST in human adipogenesis. By using preadipocytes isolated from obese and nonobese patients and lentivirus overexpressing or knocking down EST, we showed that EST functioned as a positive regulator of adipogenesis. Moreover, a positive correlation was found between the expression of EST and BMI, whereas a negative correlation was found between the expression of ERα and BMI in human patients. These results were consistent with the clinical observations that loss of estrogen signaling led to increased adi-

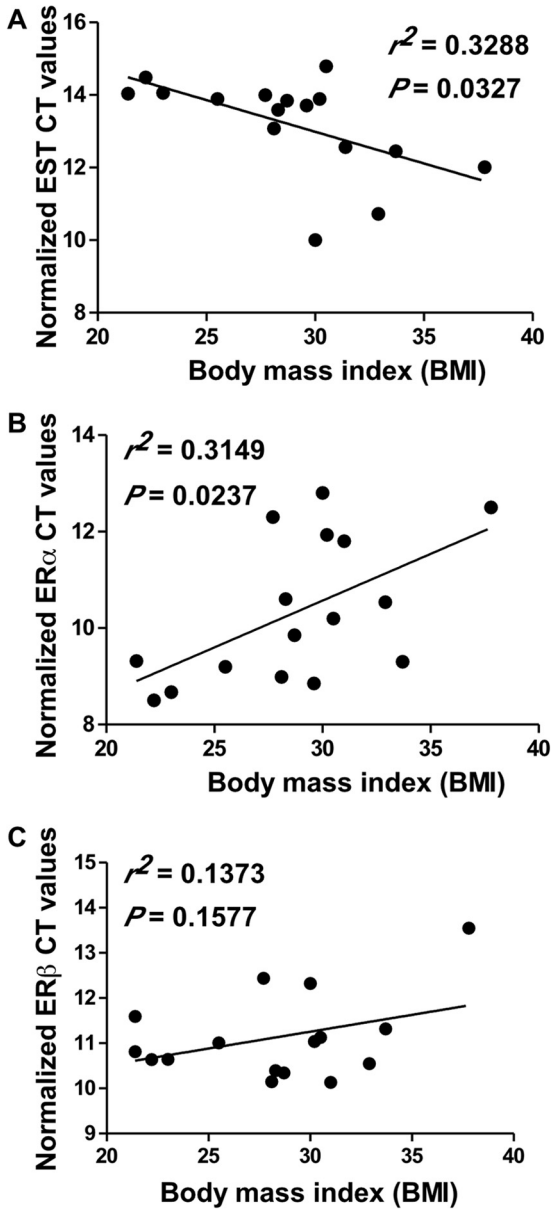

FIG 8 The respective expression of EST and ERα was positively and negatively correlated with adiposity in human patients. (A to C) Total RNAs extracted from whole fat or lipoaspirate of a cohort of 16 patients were subjected to gene expression analysis by real-time PCR. The correlation between body mass index and EST (A), ERα (A), or ERβ (C) 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.

posity (6, 7, 9–11, 33, 34), and there was an inverse correlation between the ERα expression and BMI (35). Mechanistically, EST promoted adipogenesis by deactivating estrogens, and the gain-of-function EST effect can be recapitulated by the use of an ER antagonist or genetic knockdown of ERα. The inhibitory effect of the estrogen-ERα signaling pathway on adipogenesis may have been achieved through the transsuppression of PPARγ by ERα, in which the activation of ERα inhibited the recruitment of PPARγ onto the promoters of PPARγ target genes. In contrast, treatment with an ER antagonist increased the recruitment of PPARγ onto its target gene promoters.

It is interesting that the effect of EST on adipogenesis in humans was opposite to what we reported in mice. Using mouse 3T3-L1 preadipocytes and mouse primary preadipocytes, we found that Est levels were high in preadipocytes and that differentiation led to decreased expression of Est (13). Moreover, forced expression of Est in 3T3-L1 cells inhibited differentiation, whereas the preadipocytes isolated from the Est null mice showed enhanced adipogenesis. In mouse preadipocytes, overexpression of Est prevented the molecular switch from Erk1/2 activation to Akt activation needed to facilitate adipocyte differentiation. Treatment with the Erk1/2 inhibitor PD98059 partially restored the ability of Est-overexpressing cells to differentiate (13). In human preadipocytes, we found that overexpression of EST caused sustained activation of both ERK1/2 and AKT and that the final outcome is promotion of adipogenesis. Moreover, treatment of human preadipocytes with the ERK1/2 inhibitor PD98059 had little effect on the differentiation of either the vector- or EST virus-infected cells.

The species-specific effect of EST may have been due to the differential effect of estrogens on adipogenesis in mice and humans. Although Est enzymatic activity was required for the inhibitory effect of Est on rodent adipogenesis, treatment of vector or Est-overexpressing 3T3-L1 cells with estrogens failed to affect adipogenesis (13), suggesting that an Est substrate(s) other than the estrogens may have been responsible for the effect of Est on mouse adipogenesis. In contrast, in human preadipocytes, treatment with estrogens efficiently suppressed adipogenesis, and the inhibitory effect of estrogens was abolished in EST-overexpressing cells, presumably due to the enhanced estrogen deactivation. The mechanism for the species-specific effect of estrogens and EST on adipogenesis remains to be clearly defined. Nevertheless, our observations underscore the importance of using human cells and human models in understanding human adipogenesis.

The identification of EST as a positive regulator of human adipogenesis may have its clinical and therapeutic implications. In humans, adipocytes from white adipose tissue replace themselves at a rate of approximately 10% each year (36, 37). Additionally, it is believed that the human body establishes a fixed number of adipocytes during early childhood development, adolescence, and puberty. That number is maintained tightly throughout an individual's adult life, despite long-term changes in diet and exercise that may result in a dramatic weight loss. It was reported that even after lipectomy in nonobese female patients, body fat regenerates and redistributes, particularly to the abdominal region (38). This phenomenon was also recapitulated in mouse models (39). It is conceivable that adipocyte differentiation and replenishment play an essential role in maintaining adipose tissue homeostasis. This provides a novel concept of antiobesity therapy, that is, the need to limit adipocyte turnover, either through increased apoptosis of adipocytes or through the inhibition of adipocyte differentiation. One caveat is that increased cell death may cause an inflammatory response that could actually worsen the metabolic syndrome and thus hinder the attempt to increase apoptosis. Our results showed that pharmacological inhibition or genetic knockdown of EST attenuated the adipogenic process, suggesting that inhibition of EST may be an effective weight reduction strategy in the future. It is encouraging that major progress has been made in the identification and characterization of chemical EST inhibitors (27, 40).

Among limitations, it is noted that the age range of our human subjects was quite varied and that all samples were from abdominal subcutaneous fat. Although the effects of EST overexpression and knockdown were observed in preadipocytes isolated from both the obese and nonobese patients, we cannot exclude the possibility that age, menopausal status, steroid hormone level, obesity, and origin of the fat depot affect the phenotypic exhibition. In addition, since overexpression of EST under the estrogen-free DCC cell culture condition can still increase the expression of certain adipogenic marker genes (Fig. 6A), 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.

In summary, we have established that EST is an important positive regulator of adipogenesis in humans. We propose that EST is a druggable target whose inhibition can be used to inhibit the turnover of adipocytes in obese patients.

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