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17 β -estradiol inhibits HIV-1 by inducing a complex formation between β -catenin and estrogen receptor on the HIV promoter to suppress HIV transcription

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Abstract

Human Immunodeficiency virus type 1 (HIV-1) disproportionately affects women, accounting for >50% of new HIV infections in adults worldwide. While multiple mechanisms may contribute to a greater degree of HIV infection in women than men, we evaluated the direct effect of 17 β -estradiol, the most bioactive form of estrogen in women, on HIV replication in peripheral blood mononuclear cells (PBMCs). We demonstrate that 17 β -estradiol, in an ER α dependent manner, inhibits HIV replication by activating β -catenin signaling. Specifically, we show for the first time that 17 β -estradiol induces a complex formation between ER α and β -catenin which tether on the HIV LTR at –143 nt site from +1 start site of HIV transcription to repress HIV promoter activity. These studies define a role of 17 β -estradiol in inhibiting HIV replication which may impact HIV pathogenesis in women and add to a growing list of viruses that are inhibited by 17 β -estradiol through ER α engagement.

Keywords

HIV; women; estradiol; female sex hormones; β -catenin; HIV LTR; transcription; retroviruses; pathogenesis

Background

The current HIV pandemic is heavily skewed against women. HIV infection in women accounts for >50% of new HIV infections in adults worldwide. Once infected, emerging data points to differences in HIV pathogenesis and anti-HIV immune responses in women in comparison to men (Gandhi et al., 2002; Meier et al., 2009; Sterling et al., 2005; Weinberg et al., 2011). Female sex hormones, at least in part, may contribute to gender-based differences in HIV acquisition and pathogenesis. Particularly, female sex hormones regulate immune responses both systemically and in the female genital tract by impacting homing of HIV-susceptible cells to mucosal tissue, regulating cytokine production and antimicrobial peptides induction, and influencing lymphocyte responses (Fahey et al., 1999; Fahey and Wira, 2002; Fung et al., 2013; Kaushic et al., 1998; Lu et al., 2002; Quayle et al., 1998; Rodriguez-Garcia, Patel, and Wira, 2013). This intricate balance between host factors under

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female sex hormone regulation and HIV is likely to impact HIV transmission and pathogenesis in women.

Some clinical data suggest that lower estrogen level (e.g progesterone dominant stage) is correlated with higher rate of HIV infection and higher plasma viral load. Specifically, during the periovulatory stage of the menstrual cycle, when estrogen dominates, HIV plasma viral load is decreased (Greenblatt et al., 2000; Hanna, 1999; Money et al., 2003). Further, HIV productive infection of human cervical explant tissue predominately occurs in tissue obtained from women in their secretory phase of the menstrual cycle, a stage where progesterone dominates and counteracts estrogen effects (Saba et al., 2013). Lastly, women on progesterone-based injectable hormonal contraceptives are more likely to become HIV infected and to transmit the virus to their male partners (Heffron et al., 2012). Taken together, these clinical observations suggest that estrogen dominance may be linked to lower susceptibility of cells to HIV infection.

17 β -estradiol, the most potent estrogen found in humans, exerts an anti-viral effect for a number of viruses. It inhibits hepatitis C virus (HCV) infection at multiple steps including attachment, entry, replication, and post-replication (Hayashida et al., 2010; Murakami et al., 2013). It also inhibits hepatitis B virus (HBV) transcription (Wang et al., 2012), limits herpes simplex virus (HSV) primary infection and reactivation (Gillgrass et al., 2010; Vicetti Miguel et al., 2010), and inhibits rubella virus replication (Roehrig, Brawner, and Riggs, 1979). 17 β -estradiol, on the other hand, promotes the replication of adenovirus type 12 (James, Vanderpool, and Roane, 1992). The signal transduction cascade of estrogen is complex and can be engaged by multiple pathways. Canonical estrogen signaling involves the activation of its nuclear receptors, estrogen receptor α (ER α) or estrogen receptor β (ER β), which in turn bind to estrogen responsive elements (ERE) located near or within gene promoters to regulate the transcriptional activity of cognate target genes (Marino, Galluzzo, and Ascenzi, 2006). Estrogen also regulates gene expression via noncanonical signaling whereby (Marino, Galluzzo, and Ascenzi, 2006) estrogen receptors bind to other transcriptional factors such as Sp1 or Ap-1, leading to either their stabilization or recruitment of transcriptional co-regulators, which in turn bind to their cognate promoters to regulate gene expression. Also, estrogen can bind to either its nuclear receptors or other receptors to initiate second messenger signaling leading to physiologic effects. These signal transduction pathways include ERK/MAPK, p38/MAPK, or PI3K/AKT, to name a few (Marino, Galluzzo, and Ascenzi, 2006). Interestingly, many of the reported 17 β -estradiol reported anti-viral effects are mediated by ER α and not ER β (Hayashida et al., 2010; Murakami et al., 2013; Wang et al., 2012; Gillgrass et al., 2010; Vicetti Miguel et al., 2010; Roehrig, Brawner, and Riggs, 1979).

β -catenin signaling represses HIV replication in multiple targets, including PBMCs at the level of transcription (Henderson et al., 2012; Kumar et al., 2008; Li et al., 2011). β -catenin is a transcriptional co-regulator and is the central mediator of the canonical Wnt/ β -catenin signaling pathway (Al-Harhi, 2012). β -catenin binds to members of the TCF/LEF family of transcription factors (TCF1, TCF3, TCF-4, and LEF) to modulate hundreds of genes, including those that regulate cell proliferation and survival. β -catenin expression is regulated at the protein level by a multi-protein destruction complex (GSK-3 β , APC, Axin, and casein kinase 1). This multi-protein complex leads to the phosphorylation, ubiquitination, and proteasomal degradation of β -catenin. When this destruction complex is destabilized, β -catenin is hypophosphorylated and translocates to the nucleus where it binds to TCF/LEF transcription factors, tethering on their cognate DNA binding sites to regulate genes such as CyclinD, C-Myc, Matrix metalloproteinase 7 and 9, and Axin, to name a few (Al-Harhi, 2012).

At least four TCF-4 binding sites have been identified within the HIV promoter at -143 to -136 nt; -336 to -329 nt; +66 to +73 nt; and +186 to +195 nt from the transcription initiation site (Henderson et al., 2012). While all sites have >70% homology to the TCF-4 binding sequence, the -143 site has 100% homology to the TCF-4 core (5'-(A/T)(A/T)CAAAG-3') and is present in approximately one-third of 500 HIV LTR sequences reported in the Los Alamos gene bank (Henderson et al., 2012). TCF-4 binds at a higher affinity at -143 than at any other site (Henderson et al., 2012). Further, β -catenin is tethered on the HIV LTR at the nt-143 site and knockdown of either TCF-4 or β -catenin enhances HIV transcription (Henderson et al., 2012; Narasipura et al., 2012). Dual knock down of β -catenin and TCF4 does not further enhance HIV LTR activity, indicating that these factors work together to repress HIV transcription.

Given that HIV replication is regulated by a complex interplay between virus and host factors and some epidemiologic data linking an estrogen dominant state to lower plasma viral load and infection rate, we directly assessed the role of estrogen (17 β -estradiol) on HIV replication in peripheral blood mononuclear cells (PBMCs). We demonstrate that estrogen through engaging ER α leads to association between ER α and β -catenin that tethers on the HIV LTR at -143 nt from the transcription initiation site to repress HIV transcription. These findings provide the first direct association between ER α and β -catenin on the HIV LTR, impacting HIV transcription. Further, the mechanism revealed here may contribute to some clinical observations linking lower HIV load under an estrogen dominant state (Asin et al., 2008; Greenblatt et al., 2000; Money et al., 2003). Most importantly, these studies add to a growing list of viruses (HCV, HBV, HSV, rubella) that are inhibited by ER through ER α engagement (Gillgrass et al., 2010; Hayashida et al., 2010; Murakami et al., 2013; Roehrig, Brawner, and Riggs, 1979; Vicetti Miguel et al., 2010; Wang et al., 2012).

Materials and Methods

Ethics Statement

This study was approved by Rush Institutional Review Board (09040706-IRB01) and participants' written consent was provided.

Reagents

AZT, 17 β -estradiol, PHTPP (4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol), PPT (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol), anti-total β -catenin antibody, amino acids 768-781 (cat. C2206), and anti-GAPDH antibody (cat. G9545) purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-active β -catenin antibody clone 3C196 purchased from US Biologicals (San Antonio, TX, USA). Anti-ER α antibody clone 62A3 purchased from Cell Signaling (Boston, MA). Anti-ER β antibody clone 14C8 purchased from Abcam (Cambridge, MA, USA). XAV 939 and IWR-1-endo purchased from Tocris Bioscience (Bristol, United Kingdom).

Isolation and treatment of PBMCs

PBMCs were isolated from venous blood of healthy women of reproductive age and not on hormone contraceptives using lymphocyte separation medium (Lonza Biotech, Allendale, NJ, USA) and density centrifugation. The cells were suspended in phenol red free-RPMI complete medium supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine, 1% Penicillin, 1% Streptomycin, and 20 U/ml IL-2. PBMCs were left unstimulated or stimulated with 1 β g/ml each of anti-CD3/CD28 antibodies (BD Biosciences, Franklin Lake, NJ, USA) for 24h at 37°C under 5% CO₂.

Immunofluorescence Staining and Flow Cytometric Analysis

Up to 9-color flow cytometric analyses were performed using a FACSCalibur cytometer with CellQuest (BD) and LSR-II cytometer with Diva software (BD). Aqua Live/Dead staining was used. The following antibodies from BD Biosciences were used for cell surface staining as per instructions: For T cells subsets, CD3- alexafluor 700, CD14-pacific blue, CD8-phycoerythrin-cyanine (PerCP-Cy) 5.5, CD4- allophycocyanin (APC), CD19 APC-H7; for dendritic cell subsets, CD3- PerCP-Cy7, CD123-phycoerythrin (PE), HLA-Dr PerCP, CD14-pacific blue, CD11c-APC, CD56/CD16-alexfluor 700 and CD19/CD20-APC-H7. Proper isotypes were purchased from same vendor for primary stain. Intracellular flow cytometry staining was performed using CytoPerm/fix and CytoWash reagents, according to recommendations of the supplier (BD Biosciences). For ERs staining, 10 β 1 of a FITC-conjugated rabbit monoclonal antibody that recognizes both ER α and ER β (clone SP1, Abcam, Cambridge, MA, USA) was used. For active β -catenin, 200 μ l of 0.05 μ g dilution in PBS of mouse anti-human active β -catenin un-conjugated antibody (clone 3C196, US Biological, Marbelhead, MA) was used followed by 1 μ l of a secondary anti-mouse F(ab')₂ FITC-conjugated antibody at 2mg/ml. The cells were subsequently washed and fixed with 2% paraformaldehyde.

Cell viability, HIV Infection, and p24 assay

Cell viability was assessed by standard MTS assay (Promega, Madison, WI). HIV-1_{Bal} or HIV_{III}B at 2ng HIV p24/10⁶ cells was added to anti-CD3/CD28 stimulated PBMCs and incubated for 4-6 h at 37°C under 5% CO₂. Cells were then washed with PBS three times to remove un-bound virus, suspended in phenol red free-RPMI complete medium with 20 units/ml IL-2, treated as indicated per experimental condition, and plated in 96 well U-bottom plate at 200,000 cells/200 μ l volume of the media in quadruplicates for 7 days at 37°C, 5% CO₂. HIV p24 assay was performed using p24 ELISA kit (SAIC-Frederick, MD) according to the manufacturer's protocol.

Western blot and Immunoprecipitation (IP)

The cells were lysed using RIPA buffer (Sigma) with 10% protease inhibitors and protein content measured by Pierce BCA assay (Thermo Fisher, Barrington, IL). Western blot was performed as described (Narasipura et al., 2012), using anti-active β -catenin antibody at 1:8000, anti-total β -catenin antibody at 1:8000, anti-ER α at 1:1000, anti ER β at 1:1000, and/or anti-GAPDH at 1:30,000. Secondary antibodies included goat anti-mouse HRP (Pierce) at 1:50,000 and goat anti-rabbit HRP (Cell Signaling) at 1:60,000 for GAPDH. Densitometries were measured by ImageJ software. For IP, cell lysate was pre-cleared with 25 μ l of protein A/G magnetic beads (Thermo Scientific), cross-linked with specific antibodies using Protein A/G MagSepharose and SpinTrap Buffer Kit (GE Healthcare, Piscataway, NJ) as outlined by manufacturer. Denatured proteins were then resolved on a 12% SDS gel. IgG controls were run in parallel.

RNA Isolation and real time PCR

Total RNA was isolated from PBMCs using Trizol (Life Technologies, Grand Island, NY). Complimentary DNA synthesis was performed using qScript cDNA Supermix (Quanta BioSciences, Gaithersburg, MD). Real-time PCR was performed employing SsoFast EvaGreen Supermix with low ROX (BioRad, Hercules, CA) in a 7500 real time PCR system (Applied Biosystems, Carlsbad, CA) to quantify target genes. Reaction conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15sec, 60 °C for 1min and 95 °C for 30sec. A melting curve stage was included to make sure that single specific products were amplified devoid of non-specific products or primer-dimers. Fold change in product expression was calculated by relative quantification using the comparative C_T method with

GAPDH as endogenous control. The following primers were used to amplify Wnt/ β -catenin pathway target genes: c-Myc, F- 5'-ACGTCTCCACACATCAGCACAAC and R-5'-TGACACTGTCCAACCTGACCCTCT; CyclinD1, F-5'-TCATGGCTGAAGTCACCTGGT and R-5'-TCCACTGGATGGTTTGTCACTGGA; Axin2, F-5'-ACAACAGCATTGTCTCCAAGCAGC and R-5'-GCGCCTGGTCAAACATGATGGAAT and GAPDH, F-5'-TGACTTCAACAGCGACACCCACT and R-5'-ACCACCCTGTTGCTGTAGCCAAAT.

Analysis of HIV reverse transcription, integration and transcription

Stimulated PBMCs were infected with HIV_{Bal} and treated or untreated as described above. For HIV reverse transcription analysis, infected cells were harvested at 24h, genomic DNA isolated using DNeasy blood and tissue kit (Qiagen, Valencia, CA), quantitative real time PCR was performed to quantify HIV LTR using F-5'- TCAAGTGAGTGCCCGGTT and R-5'-AGCTCCGGTTTCTTTTCGCT primers. For HIV integration analysis, infected cells were harvested at 72h AND Alu-PCR was performed as described (Brussel and Sonigo, 2003). A minus Taq and a minus template controls were included throughout. For HIV transcription analysis, at 72h post infection, cDNA was subjected to real time PCR using the following primers to amplify HIV transcripts: Rev, F-5'-TCCTTGGCACTTATCTGGGACGAT and R-5'-TCCCAGAAGTTCCACAATCCTCGT and Env, F-5'-ACGAGGATTGTGGAACCTTCTGGGA and R-5'-TGGCATTGAGCAAGCTAACAGCAC. GAPDH was used as endogenous control.

Nucleofection and transfection

Stimulated PBMCs were transfected with dominant negative (dn) TCF-4 plasmid (Addgene; Cambridge, MA) and pcDNA backbone (Invitrogen; Carlsbad, CA) using Amaxa T cell Nucleofection kit and Amaxa Program T-23 (Lonza, Walkersville, MA). Jurkat cells were transfected with 0.5 μ g ER α expression plasmid (pEGFP-C1-ER alpha, Addgene, Cambridge, MA), 0.5 μ g of either the WT-LTR or Δ -143-LTR previously described (Henderson et al., 2012) using Lipofectamine 2000 (Invitrogen).

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR

At 48hrs post-transfection, chromatin immunoprecipitation (ChIP) was performed using EMD Millipore EZ Magna ChIPA/G (Temecula, CA) according to manufacturer's protocol with antibodies for total β -catenin (Sigma) and ER α (Thermo). An appropriate IgG (CellSignaling) control antibody was also included. Real time PCR was performed to quantify HIV LTR DNA using above mentioned LTR primers in a 7500 real time PCR system employing SsoFast EvaGreen Supermix with low ROX. Reaction conditions were: 95 °C for 10 min followed by 40 cycles of 95 °C for 15sec, 60 °C for 1min and 95 °C for 30sec. A melting curve stage was included to ensure amplification of a single product. Change in binding was calculated by relative quantification using the comparative threshold cycle (C_T) method. Results were reported as fold change relative to IgG control ($\Delta C_T = C_T \text{ Target} - C_T \text{ IgG control}$; fold change relative to IgG control = $2^{-\Delta C_T}$).

Statistics

When the data was distributed normally, ANOVA and post-hoc tests were used. When the data was not normally distributed, nonparametric analysis was performed. All tests assumed a two-sided significance level of 0.05. GraphPad InStat 3 software (San Diego, CA) was used for data analysis.

Results

17 β -estradiol inhibits HIV replication in PBMCs

PBMCs were stimulated with anti-CD3/CD28 prior to HIV_{Bal} infection then treated with 17 β -estradiol (0-3nM) or AZT (1 μ M). At day seven post infection, HIV replication was monitored by conventional HIV core antigen (p24) ELISA. We demonstrate that 17 β -estradiol at 1.5 and 3nM inhibited HIV replication by approximately 40% (**Fig.1a**). 17 β -estradiol at these two doses also inhibited HIV strain (IIB), a T-tropic/CXCR-4 utilizing strain (**Fig.1b**). None of these 17 β -estradiol doses (0.001-3nM) were cytotoxic to PBMCs, as evaluated by the MTS assay (data not shown).

Robust expression of ER α in PBMCs

To determine which estrogen receptor (ER α or ER β) is involved in 17 β -estradiol-mediated inhibition of HIV replication, we evaluated ER α and ER β expression in PBMCs. Using an antibody that recognizes both ER α and ER β we show that ER expression is >80 % in all lymphocyte subsets and that T cell activation through anti-CD3/CD28 stimulation did not significantly increase ER expression in T cells (**Fig. 2a & b**). Using an antibody that is specific for either ER α or ER β , we demonstrate robust expression of ER α in PBMCs and low level of ER β expression (**Fig.2c**). 17 β -estradiol treatment of PBMCs at doses that inhibited HIV replication (1.5 and 3nM) did not significantly induce ER α or ER β expression (**Fig.2e**)

17 β -estradiol inhibition of HIV replication is dependent on ER α and is at the level of HIV transcription

To determine the role of ER α or ER β in estrogen mediated inhibition of HIV, we used an ER α agonist and an ER β antagonist. We were unable to use an ER α antagonists in PBMCs because ER α antagonists are highly context dependent and in some cases they function as agonist instead of antagonist. For example, 1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1*H*-pyrazole dihydrochloride (known as MPP) depending on the cell type can be an ER α agonist or antagonist (Shang and Brown, 2002). In PBMCs, ER agonists functioned as antagonist (data not shown). For this reason, we used an ER α agonist (4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol, PPT) to assess if engaging ER α leads to HIV inhibition. PPT inhibited HIV replication by approximately 40%, a magnitude that was similar to that mediated by 17 β -estradiol (**Fig.3a**). The ER β antagonist (2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol, PHTPP) did not abrogate the ability of 17 β -estradiol to inhibit HIV replication in PBMCs (**Fig.3b**). Together, these findings indicate that the mechanism by which 17 β -estradiol mediates HIV inhibition is dependent on ER α and is independent of ER β . To determine the step of the HIV life cycle whereby 17 β -estradiol may exert its inhibitory effect, PBMCs were infected with HIV_{Bal} and HIV reverse transcription, integration, and transcription were assessed by conventional real time PCR-based assays. 17 β -estradiol inhibited HIV transcription by approximately 40% and had no effect on HIV reverse transcription or integration (**Fig.4c-e**).

17 β -estradiol induces β -catenin signaling

Given that 17 β -estradiol inhibited HIV transcription and we defined β -catenin through its interaction with TCF-4 as a potent repressor of HIV transcription (Henderson et al., 2012; Kumar et al., 2008; Narasipura et al., 2012), we evaluated the relationship between 17 β -estradiol and β -catenin signaling. 17 β -estradiol induced active β -catenin expression in PBMCs as evaluated by western blot (**Fig. 5a**) and intracellular staining for β -catenin (**Fig. 5b**). 17 β -estradiol also induced classical target genes of β -catenin-mediated transcription such as axin-2, c-Myc, and cyclinD1 (**Fig. 5c**). Inhibition of TCF-4 through transfection

with a dominant negative TCF-4 construct abrogated the ability of 17 β -estradiol to induce axin-2 mRNA, indicating that 17 β -estradiol-mediated induction of axin-2 is β -catenin specific (**Fig. 5d**). Collectively, these data demonstrate that 17 β -estradiol activates β -catenin signaling. This is the first study, to our knowledge, to demonstrate 17 β -estradiol activation of β -catenin signaling in PBMCs.

17 β -estradiol induces the stable association of β -catenin and ER α

We assessed the interaction between ER α and β -catenin in PBMCs by immunoprecipitation (IP) and western blot (WB). While β -catenin was pulled down by ER α IP, the level of β -catenin pulled down by ER α IP was significantly higher when the cells were treated with 17 β -estradiol (**Fig. 6a**). These data indicate that 17 β -estradiol potentiates the interaction between ER α and β -catenin, which maybe driven by higher level of β -catenin in response to 17 β -estradiol or stabilization of β -catenin in presence of 17 β -estradiol to promote its interaction with ER α .

Inhibiting β -catenin abrogates the ability of 17 β -estradiol to inhibit HIV replication

XAV and IWR-1 Endo are two small molecules described to inhibit β -catenin signaling by stabilizing axin-2, a scaffolding protein that is part of the β -catenin destruction complex (Huang et al., 2009). By stabilizing axin-2, β -catenin is phosphorylated, ubiquitinated, and tagged for proteasomal degradation. Treating HIV infected PBMCs in the presence of 17 β -estradiol and XAV or IWR-1endo abrogated 17 β -estradiol inhibition of HIV replication (**Fig. 6b**). Collectively, these data indicate that the mechanism by which 17 β -estradiol mediates HIV inhibition is both ER α dependent and β -catenin dependent.

ER α , β -catenin, and TCF-4 associate with the HIV LTR at -143 nt site from transcription initiation site

We evaluated whether ER α , β -catenin, and TCF-4 are associated with the HIV LTR to repress transcription. β -catenin does not bind DNA directly rather acts as a transcription co-factor by binding to TCF/LEF transcription factors. Recently several TCF-4 binding sites were identified on the HIV LTR (Henderson et al., 2012). One in particular, at -143 from +1 transcription start site (-143 to -136 nt) is where TCF-4 and β -catenin associate with the nuclear matrix protein SMAR1 to pull the HIV DNA towards the nuclear matrix and away from RNA pol II transcriptional machinery (Henderson et al., 2012). We assessed whether ER α tethers at -143 by chromatin immunoprecipitation. Jurkat cells were transfected with ER α plasmid and WT-LTR or an LTR deleted in -143 from the +1 start site (LTR Δ -143). The chromatin was pulled down using IgG or ER α antibody and the DNA amplified for HIV LTR. We show that deletion of -143 dramatically diminished the association of ER α with the HIV LTR at the -143 site (**Fig. 7a**). Further, β -catenin and TCF-4 were confirmed to associate with the HIV LTR at -143 (**Fig. 7b**). Collectively, these data demonstrate that ER α , β -catenin, and TCF-4 all associate at -143 nt site of the HIV LTR.

Discussion

17 β -estradiol levels greatly fluctuate during the menstrual cycle, reaching peak levels in the preovulatory phase (>690 pmol/l to <2,120pmol/l) and declining in the luteal phase (>300 to <710 pmol/l) (Harlow and Ephross, 1995). The level of 17 β -estradiol is also highly variable among women and within the same woman from one menstrual cycle to the next. For this reason, we evaluated a wide dose of 17 β -estradiol effects on HIV replication in PBMCs. We show here that 17 β -estradiol at a physiologic dose reported during the preovulatory phase of women of reproductive age (1.5nM equivalent to 1500pmol/l) (Harlow and Ephross, 1995) inhibits HIV replication in PBMCs at the level of transcription by engaging the association between β -catenin, TCF-4 and ER α at -143 nt site of the HIV LTR. The -143 nt site of the

HIV LTR is of a particular importance because it was recently demonstrated to be associated with β -catenin/TCF-4/and SMAR protein to inhibit POLII docking and repress HIV transcription (Henderson et al., 2012). Based on our data we propose that under low level of 17β -estradiol minimal interaction between ER α and β -catenin occurs, which does not protect β -catenin from degradation. However, under a 17β -estradiol dominant state, the interaction between ER α and β -catenin is induced leading to stabilization of β -catenin, its subsequent translocation into the nucleus, and its association with TCF-4 to repress HIV LTR activity. Although the LTR has estrogen responsive elements (EREs) at 343 nt and -231 nt from transcription initiation site, those sites are not relevant to the association between β -catenin and ER α on the HIV LTR, as deletion of -143nt site alone abrogated the tethering of β -catenin, TCF-4, and ER α on the HIV LTR.

Our findings suggest that fluctuations in 17β -estradiol levels during the menstrual cycle and across the reproductive stage of women may impact HIV load and in turn HIV pathogenesis and transmission. Indeed, some studies reported that HIV genital shedding is highest during menses (low estrogen) and declines during the follicular stage (high estrogen). Other studies found that the menstrual cycle leads to a decline in plasma HIV load in the follicular to mid-luteal stage (high estrogen) without impacting HIV vaginal shedding (Greenblatt et al., 2000; Hanna, 1999; Reichelderfer et al., 2000; Reichelderfer et al., 2002; Villanueva et al., 2002). Mid-secretory levels of estrogen and progesterone, together, were also found to inhibit HIV replication in vitro in PBMCs (Asin et al., 2008). However, depending on the estrogen dose, there was either no effect or negligible effect on HIV replication in this study (Asin et al., 2008). This may be reflection of several experimental factors, the most important of which is the use of charcoal stripping in these studies. In our study, the ability of 17β -estradiol to inhibit HIV replication in PBMCs was conducted without charcoal stripping. Historically, charcoal stripping of FBS was performed to remove endogenous steroid hormones, especially in breast and cervical cancer cell lines that are highly enriched in steroid production. However, emerging evidence indicates that charcoal stripping eliminates other metabolites essential for cellular health (Cao et al., 2009). In our experience, charcoal stripping of FBS compromises the survival of PBMCs (data not shown). For this reason and because untreated cultures serve as background for endogenous 17β -estradiol/steroids levels, we have elected not to use charcoal stripping in our cultures and showed that 17β -estradiol at preovulatory doses inhibits HIV replication and revealed the mechanism by which it does so .

Cross-talk between 17β -estradiol and Wnt/ β -catenin has been described in mouse and human epithelial cells (Hou et al., 2004) (Kouzmenko et al., 2004). We provide the first evidence in human PBMCs that 17β -estradiol induce ER α and β -catenin interaction to potentiate HIV repression. In human and mouse uterine epithelial cells, 17β -estradiol induces β -catenin gene expression and activates a number of Wnt proteins, including Wnt4, Wnt5a, and frizzled receptor 2 (Hou et al., 2004). Further, ER α and β -catenin in human colon and breast cancer cells precipitate within the same immune complexes (Kouzmenko et al., 2004), demonstrating that ER α and β -catenin are physically associated with each other. Exogenous 17β -estradiol further enhances this physical interaction of ER α and β -catenin. The result of ER α and β -catenin physical association is a reciprocal regulation of cognate target genes (Kouzmenko et al., 2004).

ER α represses the replication of a number of viruses, including HCV, HBV, HSV, and rubella (Gillgrass et al., 2010; Hayashida et al., 2010; Murakami et al., 2013; Roehrig, Brawner, and Riggs, 1979; Vicetti Miguel et al., 2010; Wang et al., 2012). We show here that ER α also inhibits HIV replication. These findings indicate that ER α engages direct anti-viral pathways to limit replication of some viruses, which may mediate gender-specific differences in susceptibility to virus infection and/or spread. In the case of HCV, women are

more likely to clear HCV in acute stage than men and female sex hormones have been suggested as a potential factor for better clearance (Bakr et al., 2006). Some studies have also alluded to women having a 2-6 fold lower plasma viral load than men during acute and chronic HIV infection (Delmas et al., 1997; Gandhi et al., 2002; Lemly et al., 2009; Meditz et al., 2011; Sterling et al., 1999; Sterling et al., 2001), although additional studies are needed to assess the direct contribution of female sex hormones on HIV replication and progression in the clinical setting suggest.

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Research Highlights

1. 17β -estradiol, the most bioactive form of estrogen, inhibits HIV replication in PBMCs
2. 17β -estradiol, in an ER α dependent manner, inhibits HIV through engaging β -catenin
3. ER α and β -catenin tether on the HIV LTR to repress HIV promoter activity
4. Our findings add HIV to a growing list of viruses which are inhibited by 17β -estradiol

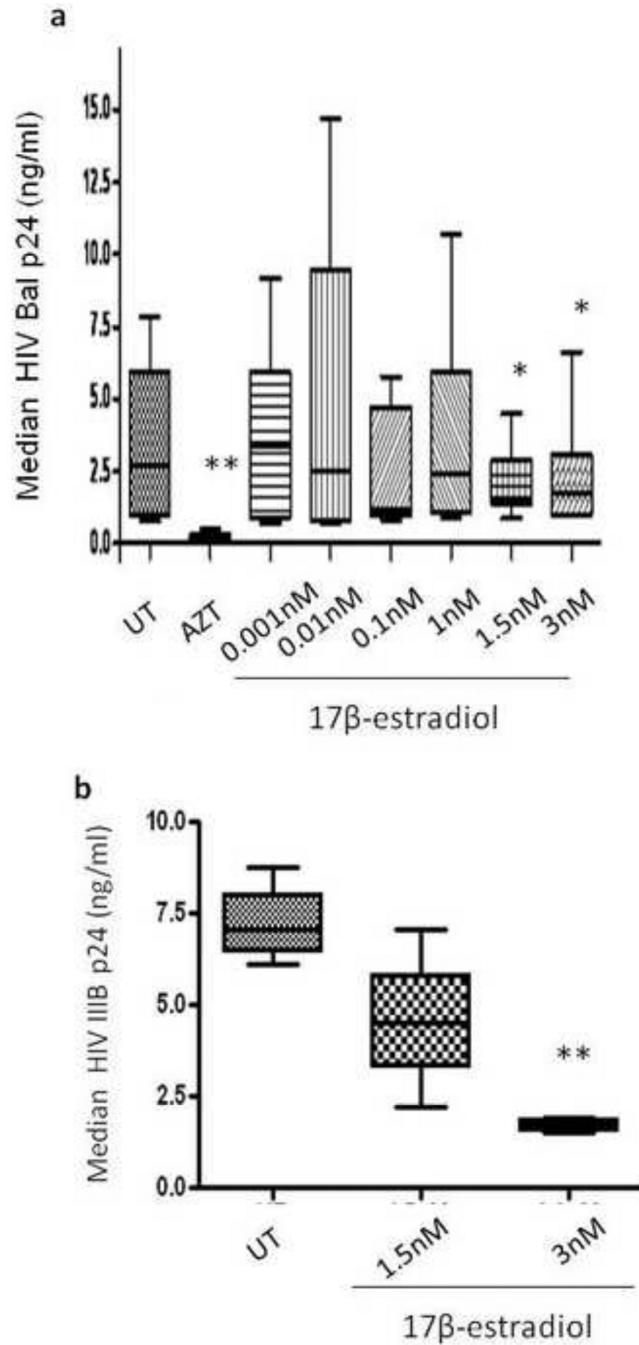


Fig. 1. 17β-estradiol inhibits HIV replication in PBMCs

PBMCs were stimulated with anti-CD3/CD28 antibodies for 48h, infected with HIV_{Bal} (a) or III B (b), re-suspended in phenol red free-RPMI complete medium, and treated with 17β-estradiol (0- 3nM), 1μM AZT, or left untreated. Seven day post-infection, HIV p24 level was measured by conventional ELISA from HIV_{Bal} (a) or HIV III B (b) infected cells. Data is representative of median value from a minimum of four donors, performed in quadruplicates. The bars represent the data range, where the 25th, 50th and 75% median percentile values are shown and analyzed using Mann-Whitney test. *p < 0.05; **p < 0.01

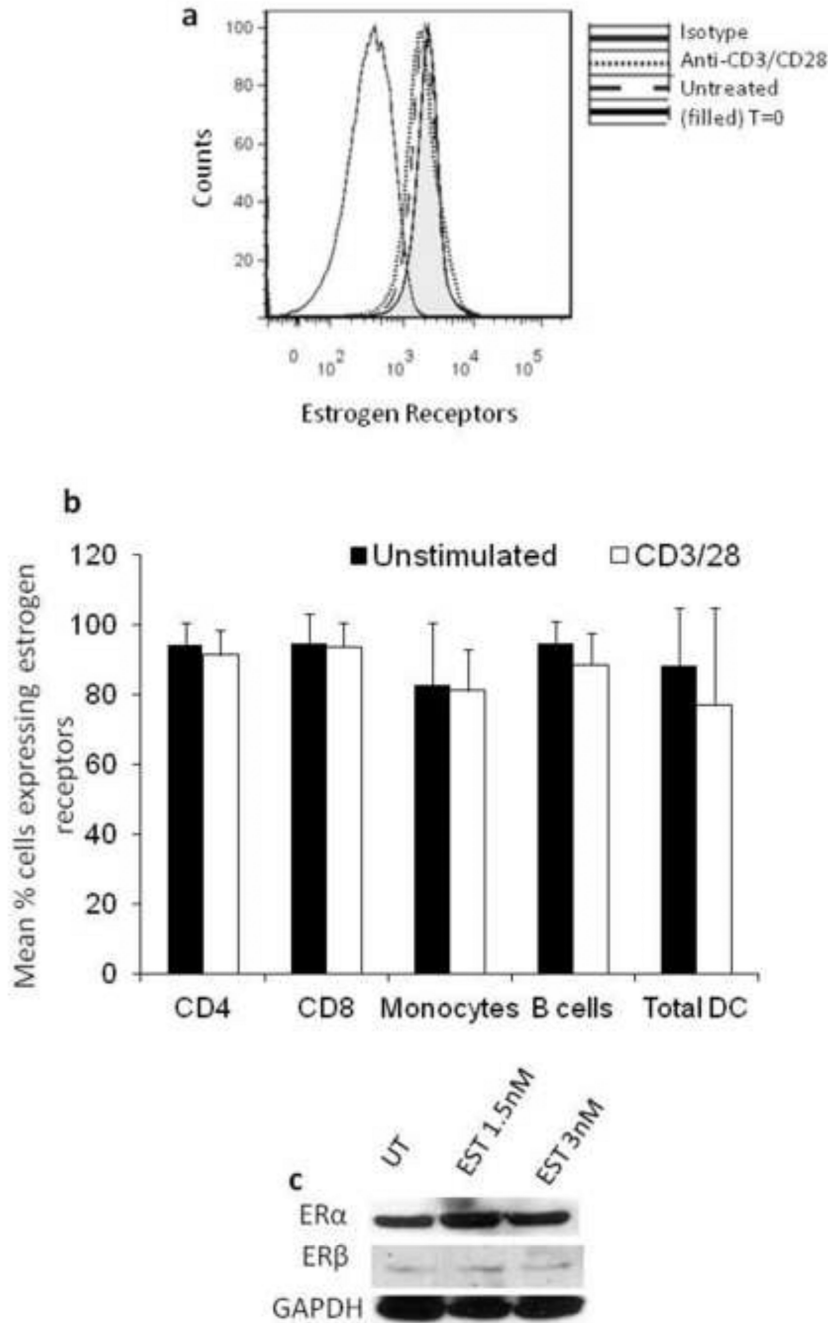


Fig. 2. PBMCs predominately express ERα

PBMCs were stimulated with anti-CD3/CD28 antibodies for 48h, left untreated in (a) and (b) or treated with 1.5nM or 3nM 17β-estradiol in (c). Expression of estrogen receptors (ER) was determined by intracellular flow cytometry (a,b) or by Western blot (c). A representative histogram of expression of 17β-estradiol receptors (ERα and ERβ) in PBMCs is shown in (a) and ER expression in leukocyte subsets is shown in (b). Expression of ERα and ERβ detected by Western blot in PBMCs is shown in (c). Data is representative of a minimum of four donors. Error bars represent standard deviation of the mean.

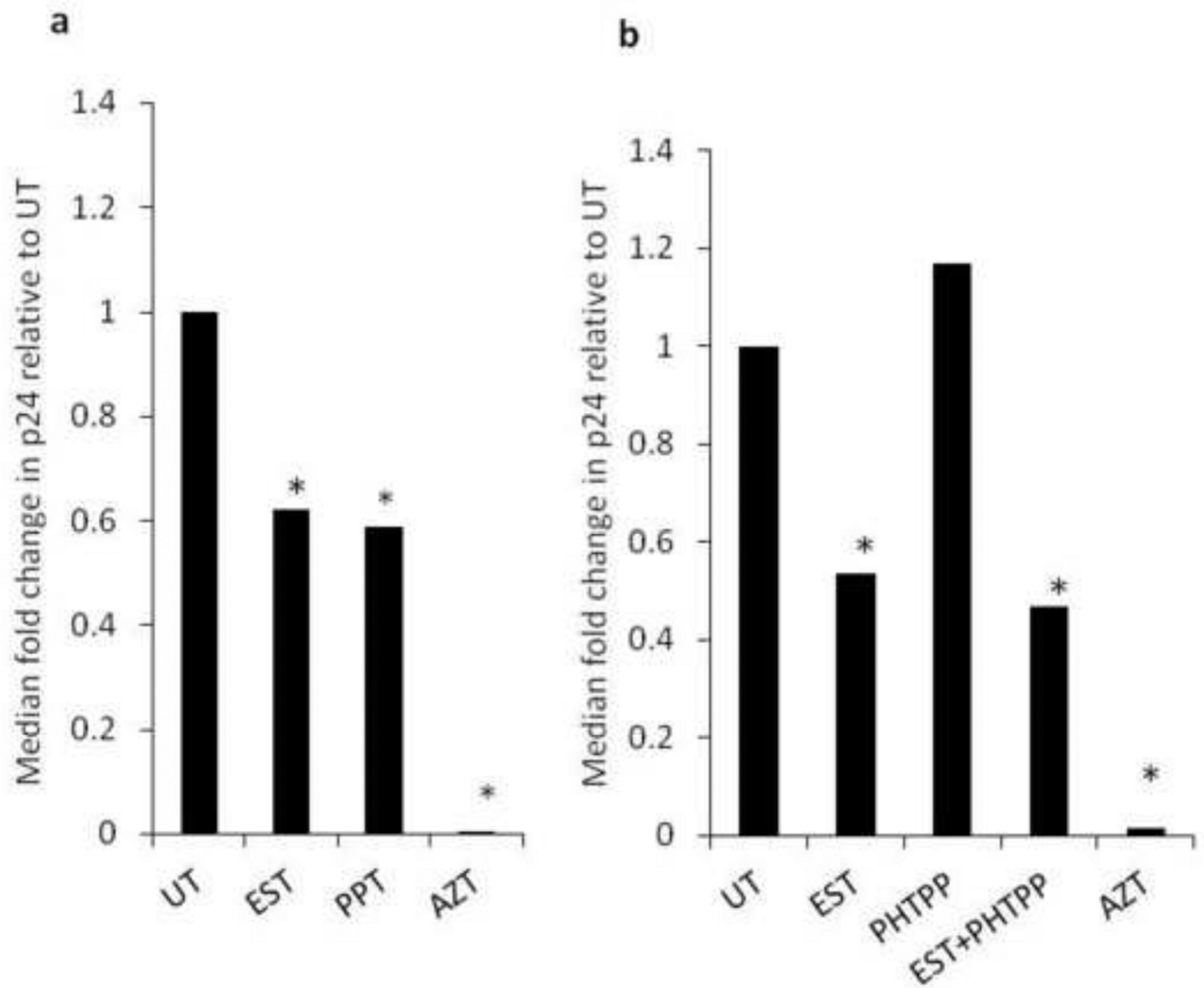


Fig. 3. 17β -estradiol engages $ER\alpha$ and not $ER\beta$ to repress HIV replication

PBMCs were stimulated with anti-CD3/CD28 antibodies, infected with HIV_{Bal}, and treated with 17β -estradiol (1.5nM) with or without an $ER\alpha$ agonist (PPT) at 1mM (**a**) or an $ER\beta$ antagonist (PHTPP) at 100 nM (**b**). HIV p24 ELISA was performed at day seven post infection. Data is representative of a minimum of four donors, performed in quadruplicate. Data was analyzed by Student T-test; * $p < 0.05$.

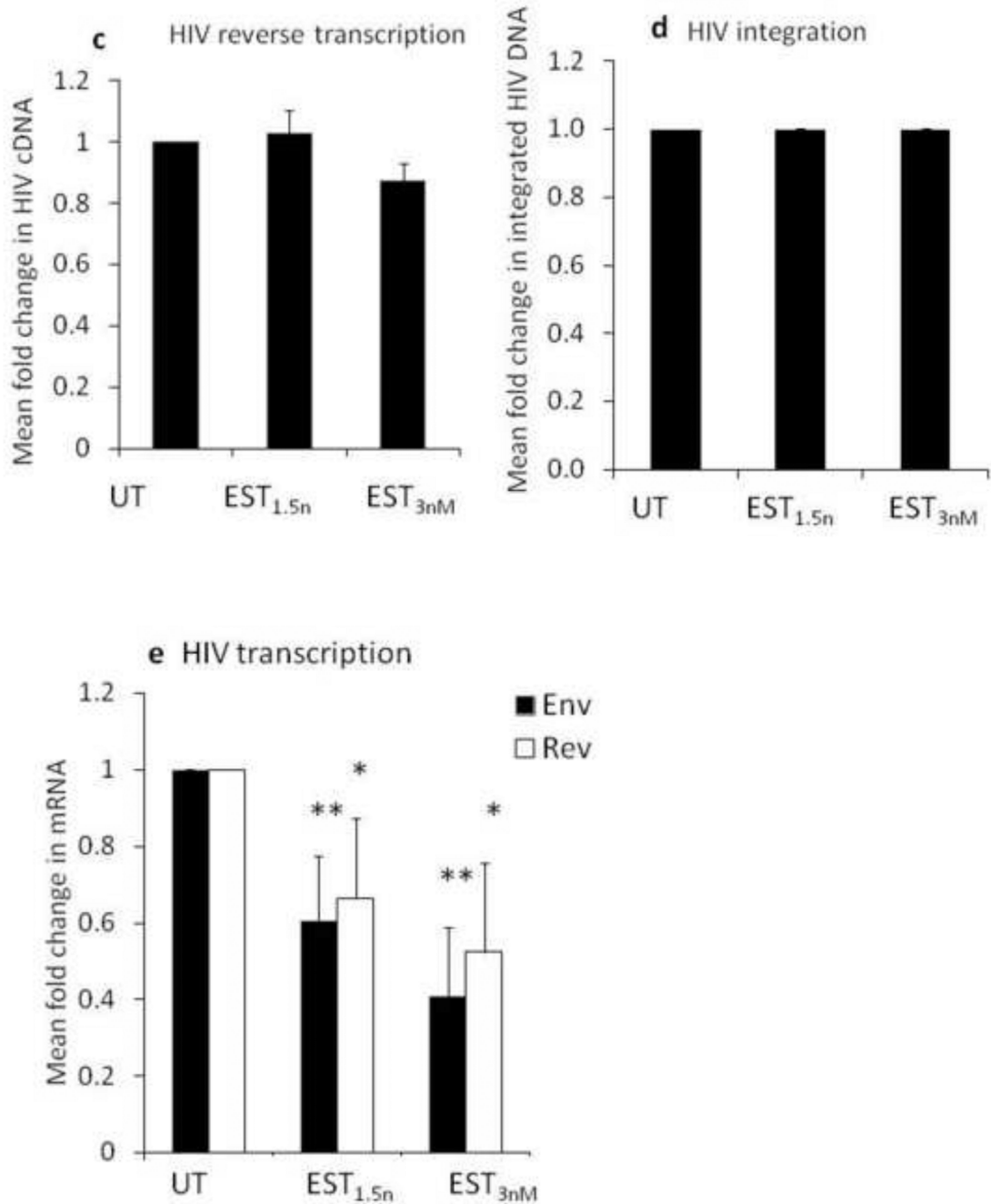


Fig. 4. 17 β -estradiol inhibits HIV transcription

PBMCs were stimulated with anti-CD3/CD28 antibodies, infected with HIV_{Bal} and treated with 17 β -estradiol (1.5nM or 3nM) or left untreated. Genomic DNA was isolated at day one post infection to measure HIV reverse transcription by PCR (a). Genomic DNA was isolated at day three post infection to measure HIV integration by Alu PCR (b). Cellular RNA was isolated at day three post infection to measure HIV transcripts by RT-PCR (c). Data in a-c is representative of a minimum of three experiments. Data was analyzed by Student T-test; *p < 0.05; **p < 0.01.

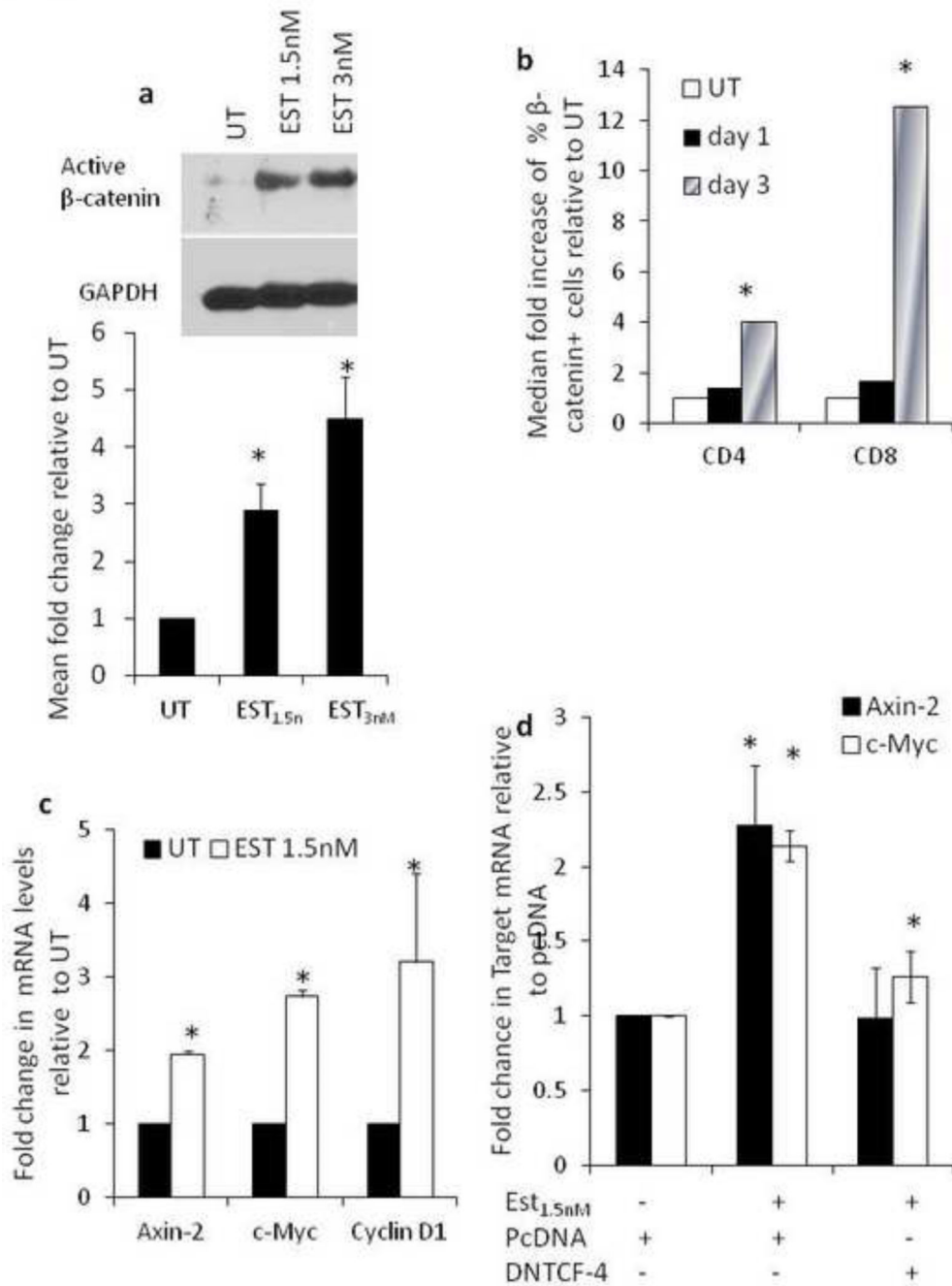


Fig. 5. 17 β -estradiol induces β -catenin signaling

PBMCs were stimulated and treated with 17 β -estradiol (1.5nM) for three days. Western blot and densitometry quantification for active β -catenin measured by Western blot is shown in (a). Flow cytometric analysis of active β -catenin expression in CD4⁺ and CD8⁺ T cells is shown in (b). Level of downstream targets of Wnt/ β -catenin pathway (axin-2, c-Myc, cyclin D-1) post 17 β estradiol treatment performed by real time RT-PCR is shown in (c). GAPDH was used as endogenous control and data shown as fold change relative to untreated cells. In (d) PBMCs were stimulated with antiCD3/CD28 for 48h and nucleofected with a dominant negative plasmid for TCF-4 (dnTCF4) or backbone vector (pcDNA). Post nucleofection the cells were treated with 17 β -estradiol (1.5nM) for three days and axin-2 and c-myc mRNA

level was evaluated by real-time RT-PCR. GAPDH was used as endogenous control. Data is representative of a minimum of three experiments and analyzed by Student T-test; * $p < 0.05$.

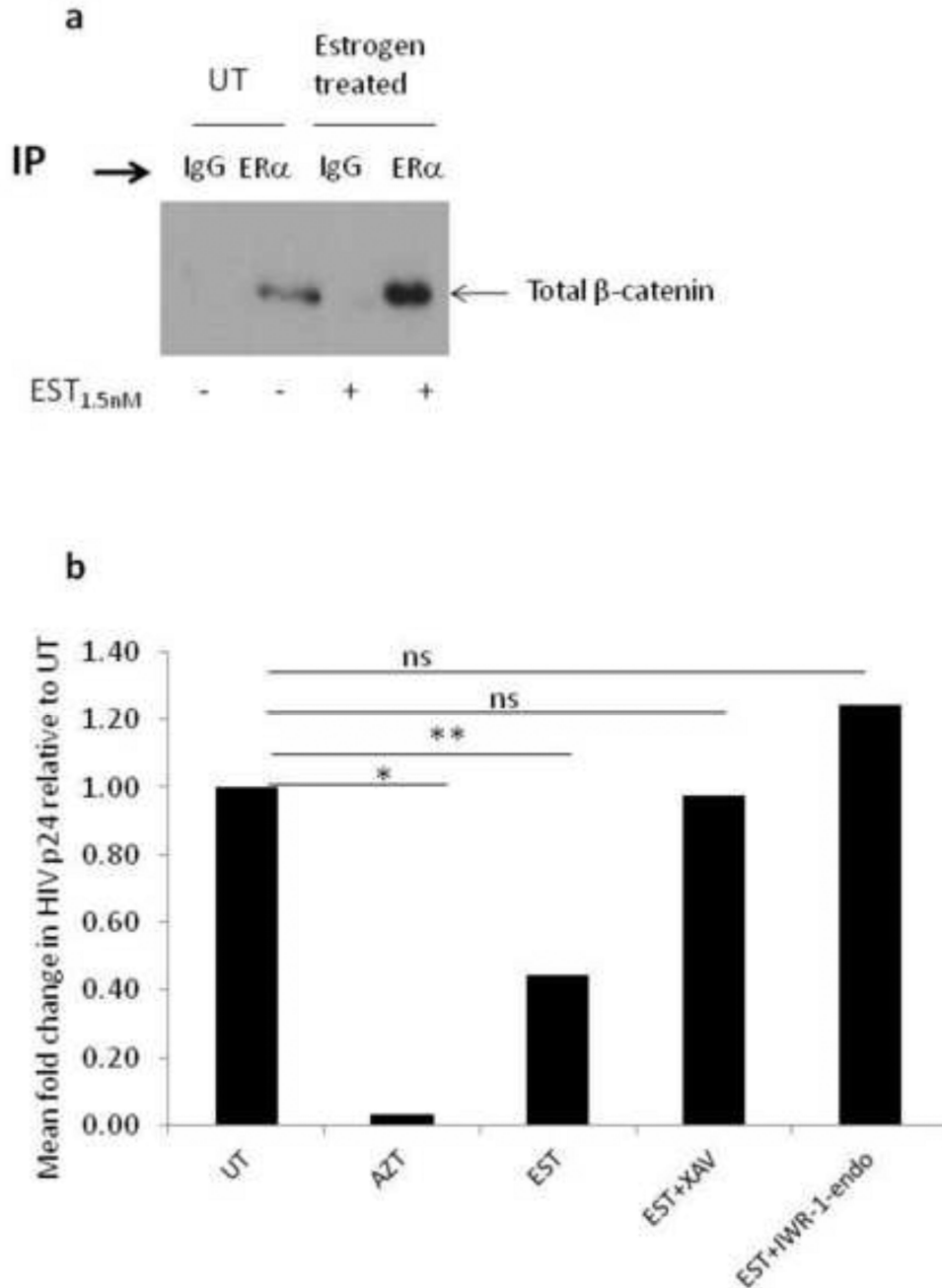


Fig. 6. 17β-estradiol-mediated inhibition of HIV replication is β-catenin-dependent
 PBMCs were stimulated with anti-CD3/CD28 antibodies for 48h then left untreated or treated with 17β-estradiol (1.5nM). At day three, the cells were harvested, lysed, and the protein immunoprecipitated (IP) using ERα antibodies or IgG control then probed with β-catenin (a). In (b) the ability of small molecule inhibitors of β-catenin to abrogate 17β-estradiol-mediated inhibition of HIV replication is shown. Specifically, PBMCs were stimulated with anti-CD3/CD28, infected with HIV_{Bal}, left untreated or treated with 17β-estradiol at 1.5nM with or without small molecule inhibitors of β-catenin (XAV at 1mM and IWR-1-endo at 2.5mM) or treated with 1μM AZT. HIV p24 was measured by ELISA on day seven post-infection. HIV p24 levels from XAV and IWR-1-endo treatments alone,

without 17β -estradiol, was not statistically different than untreated cultures. Data is representative of a minimum of three experiments and analyzed by Student T-test; * $p < 0.05$, NS indicate not significant ($p > 0.05$).

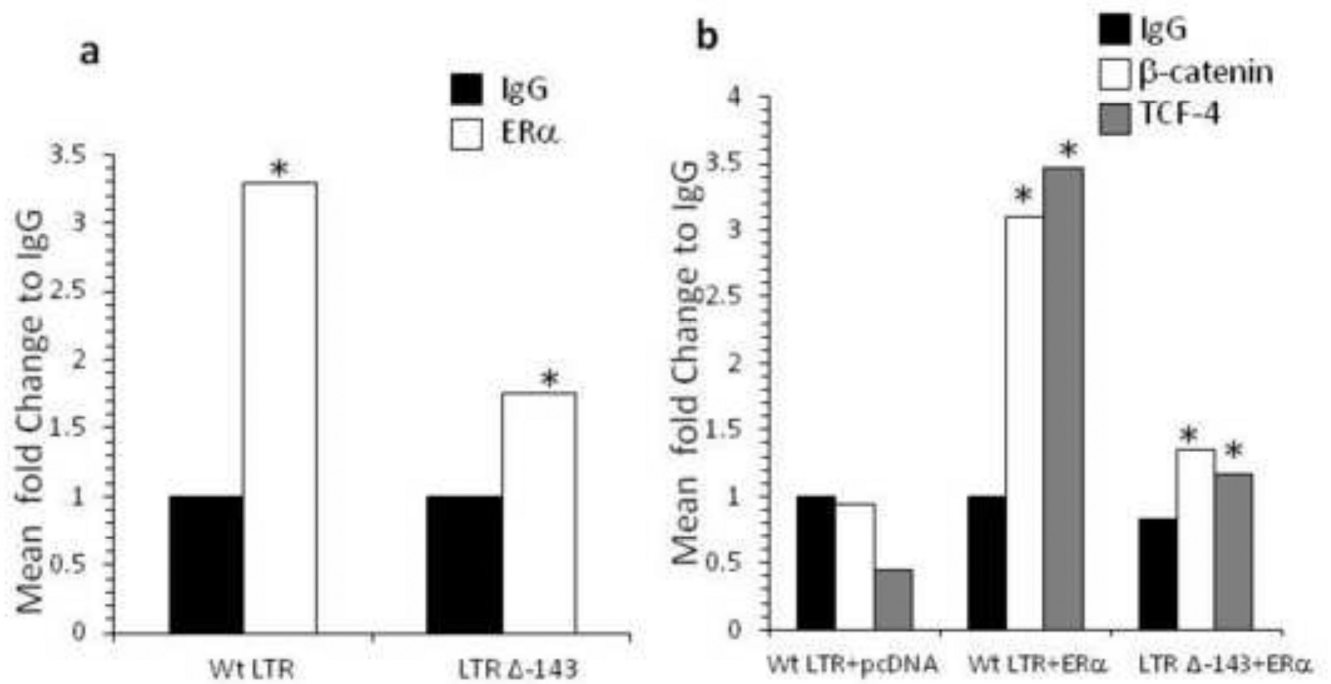


Fig. 7. Tethering of ER α , β -catenin, and TCF-4 at -143 site on the HIV LTR

Jurkat cells were transfected with wild-type (WT) LTR or LTR deleted in -143 (LTR Δ -143, deletion from -143 to -136 nt from +1 site) with or without ER α plasmid. ChIP was performed with either pull down with ER α antibody (**a**) or β -catenin or TCF-4 antibodies (**b**) and DNA amplified for HIV LTR. Data represent fold change in comparison to IgG of at least two independent experiments. Asterisks denote $p < 0.05$ in comparison to IgG determined by Student T-test.