Association of estrogen receptor β with plasma-membrane caveola components: implication in control of vitamin D receptor

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Abstract

This study was designed to provide a direct demonstration of the importance of caveolin-1 in the compartmentalization of estrogen receptor β (ERβ) to the membrane, thus allowing 7β-estradiol (E2) to control vitamin D receptor (VDR) transcription and expression. Our strategy was to obtain cell lines expressing different levels of caveolin-1. To this end, we transfected human embryonic kidney 293 cells with a caveolin-1-expressing vector and obtained three cell-line variants: one expressing high amounts of caveolin-1 (clone A), one expressing low amounts of caveolin-1 (clone B), and one expressing high amounts of the nonfunctional P132L caveolin-1 mutant (clone C), and compared these with parental (wild-type, WT) cells expressing negligible levels of caveolin-1. In clone A, ERβ colocalized to membrane preparations and E2 treatment induced significant ERK 1/2 phosphorylation and enhanced VDR expression. In clones B and C and the WT, ERβ did not localize to membrane preparations and E2 treatment was ineffective at inducing VDR upregulation associated with ERK 1/2 phosphorylation. Luciferase reporter gene expression assays showed that the human VDR promoter is only highly responsive to E2 treatment in clone A, except in the presence of the ER-specific inhibitor ICI182 780. Cotransfection of clone A with the VDR promoter and several mutants of MAPK kinase (MEK) demonstrated that the constitutively active form of MEK significantly increases VDR promoter activation, while the catalytically inactive construct is ineffective in this regard. In clone A cells transfected with an activation protein-1 (AP-1)-luciferase construct, E2 significantly upregulated the promoter activity, while ICI182 780 completely eliminated this E2-mediated effect. Clone A cells transfected with a VDR promoter bearing a targeted mutation towards the AP-1 site showed reduced E2-mediated activation of luciferase activity. Taken together, our data confirm the importance of caveolin-1 in the association of ERβ to the membrane caveolae, allowing ERK 1/2 phosphorylation and upregulation of VDR.

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Introduction

Previous studies have indicated an age-related decrease in the number of vitamin D receptors (VDRs; Horst *et al.* 1990, Takamoto *et al.* 1990). The number of VDRs is a primary determinant of the biological response to $1,25(OH)_2D_3$, as previously shown in osteoblastic cell lines (Dokoh *et al.* 1984, Liel *et al.* 1992) and in human populations in association with *VDR*-gene polymorphism (Gross *et al.* 1996, Arai *et al.* 1997, Harris *et al.* 1997). 17β-Estradiol (E2) has been shown to increase the number of VDRs in the osteoblast-like cell line rat osteosarcoma cells (ROS) 17/2.8, an increase associated with enhanced responsiveness of the cells to $1,25(OH)_2D_3$ (Liel *et al.* 1992). E2 administration has been shown to effectively restore the normal responsiveness of the intestine to $1,25(OH)_2D_3$ in ovariectomized pre- (Gennari *et al.* 1990) and postmenopausal (Heaney *et al.* 1978, Civitelli *et al.* 1988) women. Increased VDR expression following E2 treatment has also been noted in other tissues and cell types, such as the uterus (Walters 1981, Levy *et al.* 1984), liver (Chatterjee *et al.* 2005, Gilad *et al.* 2005), human breast cancer cells (Gilad *et al.* 2005), rat colon, and rat duodenal mucosa (Liel *et al.* 1999, Schwartz *et al.* 2000). However, no evidence has been accrued demonstrating the existence of estrogen-responsive elements within the VDR promoter.

The cellular actions of E2 are thought to be mediated through transcriptional regulation of target genes (Halachmi *et al.* 1994). This process occurs mainly when E2 binds to nuclear estrogen receptor (ER). The resultant complex then binds directly to response elements present on various genes (Nilsson *et al.* 2001) or modifies transcription through protein–protein interactions prior to DNA binding (Pfahl 1993). When E2 binds to ER, the receptor undergoes a conformational change that results in dimerization, binding to specific DNA elements, and transcriptional regulation of target genes (Nahmias & Strosberg 1995, Csikos *et al.* 1998). It is becoming increasingly clear, however, that multiple ligands for the steroid receptor superfamily can modulate cell function through nongenomic actions mediated through the plasma-membrane proteins (Blackmore *et al.* 1991, Nemere *et al.* 1994, Wehling 1995, Nadal *et al.* 1998).

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For example, there is evidence that E2 can trigger a variety of signal-transduction events within seconds to a few minutes. These events include stimulation of adenylate cyclase (Aronica *et al.* 1994), activation of protein kinase C (Setalo *et al.* 2005), and triggering of an intracellular calcium spike (Tesarik & Mendoza 1995). Additionally, a putative cell-membrane ER, the existence of which was first reported more than 20 years ago (Pietras & Szego 1977, 1980), appears capable of activating signal-transduction pathways according to more recent investigations (Marin *et al.* 2003*a*,*b*, 2005, Guerra *et al.* 2004, Gilad *et al.* 2005, Pietras *et al.* 2005, Marquez *et al.* 2006).

The ERK 1/2 cascade has been shown to be involved in cell differentiation, proliferation, and increased cell motility and migration, all responses that can be initiated by estrogens as well. Rapid activation of MAPK by E2 in ROS 17/2.8 cells has provided the first evidence of MAPK activation by E2 through phosphorylation, indicating the involvement of putative plasma-membrane receptors (Castoria *et al.* 2004). Rapid effects exerted by E2 on growth factor-related signaling pathways have also been demonstrated in neuronal cells and E2 activation of the ERK 1/2 signaling pathways by G protein (Filardo *et al.* 2002), suggesting a potential mechanism by which E2 might affect the expression of genes with promoters that do not contain strictly estrogen-responsive elements but are responsive to factors acting through other response elements, such as activation protein-1 (AP-1) and serum response elements (Chaban *et al.* 2004).

Caveolae are flask-shaped structures that serve as platforms for the interaction between a host of signaling proteins in various cell types (Stan 2005). Caveolae actively participate in the regulation of cholesterol trafficking at the plasma membrane, and, in addition, cholesterol has a structural role in the caveolar membrane, contributing to the creation of a specific lipid environment important for protein segregation within rafts (Simons & Ikonen 1997). Caveolin-1, which is a key component of caveola-enriched lipid rafts of the plasma membrane and a structural protein in caveolae, plays a key role in both maintaining the caveolar structure (Rothberg *et al.* 1992) and binding directly to and interacting with different signaling molecules. A link between cholesterol and caveolae has been well documented (Ikonen & Parton 2000). Caveolin-1 binds Src, Grb7, Raf, Ras, MEK, EGF-R, and ER at the plasma membrane, forming a 'signalsome' for rapid activation of intracellular signaling (Couet *et al.* 1997). Caveolae are thought to be formed by the tissue-specific expression of three caveolin isoforms (caveolin-1, -2, and -3), and they can be induced to form in tissues lacking caveolae following transfection with caveolin-1 (Wharton *et al.* 2005).

The human embryonic kidney cell line, human embryonic kidney 293 (HEK-293), expresses low to negligible levels of caveolin-1 (Ravid *et al.* 2005). Following transfection of HEK cell lines with low and high levels of caveolin-1, we obtained cellular models that enabled us to directly dissect the nature of the interactions between E2, caveolar ERβ, the MAPK-signaling pathway, and VDR.

Materials and methods

Materials

Tissue-culture media and antibiotic antimycotic solution supplements were obtained from Biological Industries Ltd (Beit Haemek, Israel). The PhosphoPlus p42/44 MAPK antibody kit was from New England Biolabs, Inc. (Beverly, MA, USA). Monoclonal human anti-VDR antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit anti-ERβ polyclonal antibody was from Chemicon (Temecula, CA, USA), ERα monoclonal antibody was from Cell Signaling Technology Inc. (Beverly, MA, USA), and monoclonal antibodies to caveolin-1 and caveolin-2 were from BD Transduction Laboratories (Franklin Lakes, NJ, USA). Flourescein isothiocyanate (FITC)-conjugated donkey anti-rabbit immunoglobulin G (IgG) $F(ab')_2$ fragments and Texas Red-conjugated donkey anti-mouse IgG $F(ab')_2$ fragments (Jackson Immunoresearch, West Grove, PA, USA) were used for indirect immunofluorescence staining procedures in confocal microscopy.

Polyclonal antibody to β-actin was purchased from Sigma Chemical Co. The enhanced chemiluminescence kit was from Amersham Biosciences. The protein determination kit, based on bicinchoninic acid, was from Pierce (Rockford, IL, USA). ICI182 780 was from Tocris (Bristol, UK). All other biochemicals were from Sigma Chemical Co.

Cell lines, culture conditions, and treatments

HEK-293 cells and HT29 colon cancer cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 1% (v/v) L-glutamine, and 0·2% (v/v) antibiotic antimycotic solution 1 and were maintained under a humidified atmosphere and 5% CO_2 at 37 °C. MCF-7, a human breast cancer cell line, was cultured similarly, but the medium was also supplemented with 0·2% (w/v) insulin solution. Cells were grown to 80–90% confluence and the medium was replaced every other day.

Stable transfections

To generate stable caveolin-1 HEK-293 cells, we utilized a full-length mouse caveolin-1 cDNA subcloned into a

pcDNA3 vector, kindly provided by Dr Mordechai Liscovitch (Weizmann Institute of Science, Rehovot, Israel). DNA (10 μg) was transfected into 6×10^{6} cells in a 100 mm dish using lipofectamine-2000 (GIBCO/BRL) according to the manufacturer's instructions. The selection was carried out in a medium containing 500 μg/ml G418 (Calbiochem, La Jolla, CA, USA) for at least 4 weeks before the experiments. Single colonies were selected using cloning rings (Falcon, Franklin Lakes, NJ, USA), and each line of HEK-293 cells produced was tested for protein levels of caveolin-1.

Two distinctive clones were developed from this procedure, one with high (clone A) and another with low (clone B) expression levels of caveolin-1. The HEK-293 cell lines expressing different levels of caveolin-1 were grown in DMEM containing 500 μg/ml G418.

A point mutation changing proline 132 to leucine was generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The P132L caveolin-1 mutant was constructed by PCR using the full-length caveolin-1 cDNA as template and a sense primer containing the desired mutation. The size of the mutated plasmid was verified by restriction enzyme digestion analysis and the correctly sized plasmid was used for further confirmation of the desired mutation by DNA sequencing. The fidelity of the mutation was confirmed by direct sequencing of the plasmid. The mutated vector was stably transfected into HEK-293 cells. The HEK-293 cell lines expressing high levels of caveolin-1-mutated vector (clone C) were selected in DMEM containing 500 μg/ml G418 as above.

Cells were harvested, washed twice by centrifugation in PBS and then cultured in 50 ml flasks or in six-well plates in DMEM with phenol red (PR) or DMEM without PR supplemented with 10% charcoal-stripped FCS, 1% L-glutamine, and 0·2% antibiotic antimycotic solution.

Expression of ERβ and of caveolin-1 was determined in the membrane (P) and cytosolic (S) preparations of each of the three clones (A, B, and C) and compared with the wild-type control cells (WT).

Cells from the different clones (A, B, and C) and WT cells were treated with E2 (10^{-8} M) or respective control cultures which included ethanol at a final concentration of 0·0067% (v/v) in the medium. Cells were exposed to a medium containing the designated treatments for 4 days, the optimal time period as determined in preliminary experiments (data not shown), and VDR protein expression and extent of P-ERK1/ERK2 phosphorylation were assessed.

To study the involvement of the MAPK signal-transduction pathway in clone A, these cells were treated with different concentrations of E2 in the presence or absence of 10 μM UO126 (Calbiochem). To study the role of ER, clone A cells were treated with 1 μM ICI182 780. Drugs were freshly diluted in culture media for each experiment.

To determine whether E2-induced VDR regulation is mediated by the activity of the hormone at the cell membrane, clone A cells were treated with an E2-BSA conjugate that could not traverse the plasma membrane. Before each experiment, stock solutions of BSA conjugate were incubated with charcoal dextran (0·05 mg/ml) and charcoal (50 mg/ml) for 30 min, centrifuged at 3000 ***g*** for 10 min and filtered through a 0·22 μm filter to obtain E2-BSA free of unbound E2. E2-BSA was dissolved in phenol-free growth medium at 0·2 mg/ml. The concentration of BSA conjugate was adjusted to the values of the free hormone.

Confocal microscopy: For ERβ and caveolin-1 co-staining in the different HEK-293 clones, cells were cultured on chamber slides, fixed with acetone, blocked with 4% fish gelatin, and incubated overnight at 4 °C with rabbit polyclonal anti-ERβ antibody and mouse anti-caveolin-1 antibody. After washing, the slides were incubated for 1 h with the appropriate reporter antibodies (goat anti-rabbit-Texas Red (red, caveolin-1) and FITC-conjugated goat anti-mouse anti-mouse IgG (green, ERβ)). The slides were viewed on a Zeiss LSM 510 (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) laser scanning confocal microscope. Images were obtained using LSM510 software.

Isolation of cell membranes

All clones (A, B, and C) and WT HEK-293 cells were grown to confluence, scraped into cold PBS (137 mM NaCl, 2·7 mM KCl, 10 mM Na_2HPO_4, and 1·8 mM KH_2PO_4) with 5 mM benzamidine, and pelleted by centrifugation at 1000 ***g***. The cells were resuspended in hypotonic buffer (5 mM Tris (pH 7·5), 1 mM $MgCl_2$, 1 mM EGTA, and 0·1 mM EDTA) supplemented with protease inhibitors, 1 μM aprotinin, 10 μM leupeptin, 1 μM pepstatin, and 5 mM benzamidine. The lysates were incubated on ice for 30 min and passed through a 21 gauge needle ten times. Pellets were collected following low-speed (1000 ***g***) centrifugation to remove nuclear debris. The supernatant lysates were then centrifuged at 133 000 ***g*** for 30 min. The membrane pellet was resuspended in PBS containing the protease inhibitors at the concentrations noted earlier.

Cholesterol determinations

Free cholesterol was determined as previously described (Wharton *et al.* 2005). Briefly, cholesterol was extracted from the different HEK-293 cell clones by the Folch method (Lees *et al.* 1964). The lipid phase was hydrolyzed with KOH and ethanol, and a colorimetric assay was used for quantitative analyses.

Transient transfections

The 1·5 kb human VDR promoter fragment, inserted into the basic vector pGL2 containing the luciferase reporter gene, was a generous gift from Prof. H F Deluca (Department of Biochemistry, University of Wisconsin, Madison, WI, USA). We also used the AP-1-Luc vector (Clontech), which contains the luciferase gene driven by the TATA box of the thymidine kinase promoter and an AP-1-dependent enhancer element.

In all transient transfections, a vector expressing β-galactosidase (β-gal) was always cotransfected in order to standardize the transfection assay. Plasmids were transfected using lipofectamine-2000. A nonmodified pGL2 basic vector with no promoter activity was used as a control. Stimulation of the AP-1-Luc vector and the VDR promoter was induced by treatment of the transfected cells for 48 h with 10^{-11} M up to 10^{-7} M E2 in the presence or absence of 10^{-6} M ICI182 780. Luciferase activity was assessed in each sample and standardized in relation to β-gal activity. All experiments were performed in triplicate.

For transient transfection of small interfering (si) RNA against ERβ, HEK-293 clone A cells were plated in a six-well plates with complete medium. When cells reached 50% confluence, old medium was replaced with fresh medium. To knockdown the expression of ERβ, we used commercially available siRNA for ERβ from Invitrogen Corporation. Specific siRNA selected and probed for its efficacy at knocking down ERβ expression was directed against GCAGACCACAAGCCCAA (beginning at codon 956). The control scramble sequence containing the same number of nucleotides was GCAACCAACCCGACGAAAT. Mock control cells were HEK-293 clone A cells that underwent the transfection conditions without incubation with any RNA sequence. To knockdown caveolin-1 in HT-29 cells previously demonstrated to express caveolin-1 and ERβ (Gilad *et al.* 2005), we also used commercially available caveolin-1 siRNA from Invitrogen. Specific siRNA selected and probed for its efficacy to knock down caveolin-1 expression was directed against CCGCATCAACTTGCAGAAA (beginning at codon 583). The control scramble sequence containing the same number of nucleotides was CCGAACTGTTCGACACAAA. Mock control cells were HT-29 cells subjected to the transfection conditions without incubation with any RNA sequence. HT-29 and HEK-293 clone A cells were incubated with lipofectamine-2000 and serum-free medium for 30 min and siRNA was then added and the mixture was incubated for 20 min at room temperature. After 24-h transfection, the expression of ERβ and caveolin-1 was detected to measure the effectiveness of the siRNA treatment. The medium of transfected cells was replaced with fresh medium and treated (or not) with E2 for 6 days (HT-29 cells) or 4 days (HEK-293 clone A cells) in order to detect the extent of VDR protein expression.

MEK constructs

The constitutively active MEK construct (EE-MEK) and its catalytically inactive form (KA-MEK) were a gift from Prof. R Seger (Weizmann Institute of Science). HEK-293 cells were transfected with both the 1·5 kb human VDR promoter and the MEK constructs. Luciferase activity was assessed in each sample and standardized relative to β-gal activity. All experiments were performed in triplicate.

Site-directed mutagenesis of the AP-1-binding site in the VDR promoter

Mutation directed towards the AP-1-binding site of an mVDR-luc plasmid was performed with the QuikChange site-directed mutagenesis kit from Stratagene as described in the user manual. The sequences of the PCR primer were: forward, 5′-GCTTTTCTTCTCGAGAGCGTCAGCTTTCCC-3′; reverse, 5′-GGGGAAAGCTGACGCTCTCGAGAAGAAAAG-3′. The size of the mutated plasmid was verified with restriction enzyme digestion analysis and the correctly sized plasmid was used for further confirmation of the desired mutation by DNA sequencing. The mutated vector was transiently transfected into HEK-293 cells, HT29 colon cancer cells and MCF7 cells, and luciferase activity assessed as already described.

Protein determination

Protein concentration in the different cell lysates was determined by a microbicinchoninic acid-based protein assay using BSA as the standard protein.

Western-blot analysis

Cell lysates or cellular subfractions were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to a nylon-transfer membrane (Amersham Biosciences), blocked in 10^{-3} M Tris-base and 0·1 M sodium chloride, containing 5% (w/v) dry nonfat milk, incubated with monoclonal human anti-VDR antibody, rabbit anti-ERβ polyclonal antibody, ERα monoclonal antibody or antibodies to caveolin-1 and caveolin-2, and subsequently incubated with a secondary antibody coupled to horseradish peroxidase. Proteins were visualized using an ECL kit (Amersham Biosciences).

To determine ERK 1/2 phosphorylation, cells were plated in six-well plates in DMEM-PR and gradually deprived of FCS as follows: cells were exposed for 2 days to 0·5% charcoal-stripped FCS-DMEM-PR, and then to

media devoid of FCS for 24 h including different concentrations of E2. Western blot was performed on cell lysates using a rabbit polyclonal phospho-p42/44 MAPK (Thr202/Tyr204) antibody, or a phospho-Raf antibody, and after stripping the membranes, reactions were performed with their respective antibodies to nonphosphorylated proteins.

RT-PCR analyses

RNA isolation was performed using Tri-Reagent solution (MRC, Cincinnati, OH, USA). RT-PCR assay was performed using the Promega kit assay. The specific selected VDR primers were as follows: 5′-ATGCCATCTG-CATCGTCTC-3′ and 5′-GCACCGCACAGGCTGTCCTA-3′. For siRNA, ERβ transfectants of HEK-293 clone A cells, the specific ERβ primers used were 5′-CAG-CATTCCCAGCAATGTCAC-3′ (ERβ forward) and 5′-GCAGAAGTCAGCATCCCTCTTTG-3′ (ERβ reverse) to give a PCR product of 281 bp. To assess the quality and loading of RNA, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified concomitantly with 5′-GAGCCACATCGCTCAGAC-3′ (GAPDH forward) and 5′-AAATCCCATCACCATCTT-3′ (GAPDH reverse) to give a PCR product of 250 bp. The PCR protocol for all of these primers was 5 min at 94 °C, then 31 cycles (1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C), and finally 10 min at 72 °C.

Statistical analyses

The data presented herein represent means ± standard error (S.E.M). Differences between the control and treatments were evaluated by Student's *t*-test.

Results

Characterization of HEK-293 clones

To demonstrate our cell system's responsiveness to E2 treatment, we determined whether HEK-293 cells express ERs. Western-blot analyses were performed with specific anti-ERα and anti-ERβ antibodies. Cells expressed principally ERβ and very low levels of ERα (Fig. 1A).

Figure 1B shows, by western blotting, the expression levels of the lipid-raft proteins caveolin-1 and caveolin-2 in parental HEK cells and in three cell lines transfected with caveolin-1 cDNA and then selected for high (clone A) and low (clone B) levels of caveolin-1 expression. In clones A and C, caveolin-1 is expressed at significantly higher levels than in clone B or WT HEK-293 cells ($P<0{\cdot}001$). Analysis of the protein lysates revealed that the amount of caveolin-2 in clone A cells expressing high caveolin-1 is also strongly elevated. These data confirm a previous study (Fiucci *et al.* 2002) in which the overexpression of caveolin-1 induced upregulation of caveolin-2. In contrast, in clone C, expressing genetically disrupted nonfunctional caveolin-1, caveolin-2 was destabilized, resulting in reduced caveolin-2 protein levels, in support of previous findings (Drab *et al.* 2001, Razani *et al.* 2001). To determine whether caveolin expression impinges directly upon cholesterol content of transfected HEK-293 cells, we measured free cholesterol in the HEK-293 parental cell line (WT; Table 1) and compared with that in the different HEK-293 clones. High free cholesterol content was measured only in cells from clone A. In contrast to clone A, in cells from clone C expressing the nonfunctional P132L-mutated caveolin-1, the caveolin-1 protein was unable to stabilize caveolin-2 and bind significant amounts of free cholesterol in the cell membrane.

Localization of ERβ and caveolin-1 in the different HEK-293 clones

Subfractionation of cells from clones A, B, and C and the parental WT HEK-293 into pelleted membranous fractions (P) and supernatant cytosolic fractions (S) devoid of nuclei allowed us to detect the expression of the 54 kDa ERβ molecule mainly in fraction P of clone A cells. In clone B cells, a different pattern was observed: expression of the ERβ molecule was less pronounced and equal in fractions P and S (Fig. 2A). Clone C and WT HEK-293 cells expressed very low levels of ERβ in both P and S fractions (Fig. 2A). Caveolin-1 was mainly expressed in the P fractions of clone A and C cells, and to a much lower extent in their S fractions. We concluded that the maximal ERβ expression is observed in plasma-membrane fractions expressing the highest functional caveolin-1 levels.

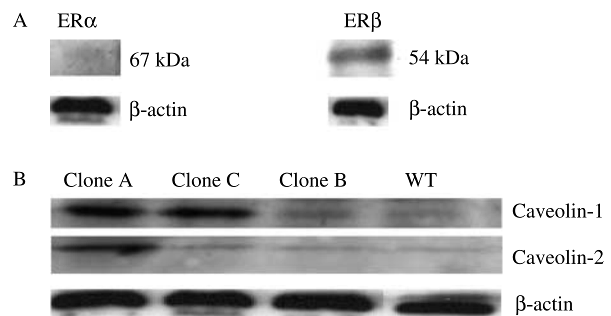

Figure 1 (A) Expression of ERα and ERβ. E2 receptors (ER) were detected by western blot with specific anti-ERα and anti-ERβ antibodies on HEK-293 cells. The blots were stripped and reprobed with anti-β-actin antibody. (B) Caveolin-1 and caveolin-2 protein expression in different HEK-293 clones. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-caveolin-1 antibody. The blots were stripped and reprobed with caveolin-2 antibody. The blots were stripped and reprobed with anti-β-actin antibody.

Table 1 Free cholesterol content of human embryonic kidney 293 (HEK-293) cells

Cell line	**Free cholesterol** (μg/mg protein)
HEK-293 WT	21·3±3·5
HEK-293 clone A	39·3±3·7*
HEK-293 clone B	22·1±2·4
HEK-293 clone C	22·3±2·1

Cellular free cholesterol was determined as described in Materials and methods. Results represent the mean±S.E.M. of three independent determinations. *$P<0{\cdot}01$, Student's *t*-test for the value of clone A when compared with clone B, clone C, and WT HEK-293 cells. Expression levels of caveolin-1 in the different clones and WT cells are as depicted in Fig. 1.

The different clones and WT HEK-293 cells were treated with E2, and ERK 1/2 phosphorylation and VDR protein expression were monitored by western blot. E2 induced ERK 1/2 phosphorylation following 20-min exposure (Fig. 2B) and concomitantly enhanced VDR protein expression only in clone A cells, but not in clone B, clone C or WT HEK-293 cells (Fig. 2C).

To determine the ER localization within plasma membranes in each of the clones, we examined cultured cells for possible colocalization of ERβ with caveolin-1 by confocal immunofluorescence microscopy (Fig. 3A–I). Images of representative cells from clones A, B, and C were obtained after double immunofluorescent staining for ERβ (green) and caveolin-1 (red). Figure 3A, D, and G illustrates ERβ staining, Fig. 3B, E, and H illustrates caveolin-1 staining, and Fig. 3C, F, and I show colocalization. As shown in Fig. 3C, there is significant colocalization of ERβ (green) with caveolin-1 (red). Yellow labeling indicates an extensive colocalization of the two proteins. This technique allows the detection of the presence of ERβ at the cell surface, extensively colocalized with caveolin-1, only in clones expressing caveolin-1. Combined immunostaining (overlapping, indicated in yellow) for ERβ and caveolin-1 occurred on the cell membrane. Clone B cells did not exhibit any measurable caveolin-1 expression and clone C cells, expressing high levels of

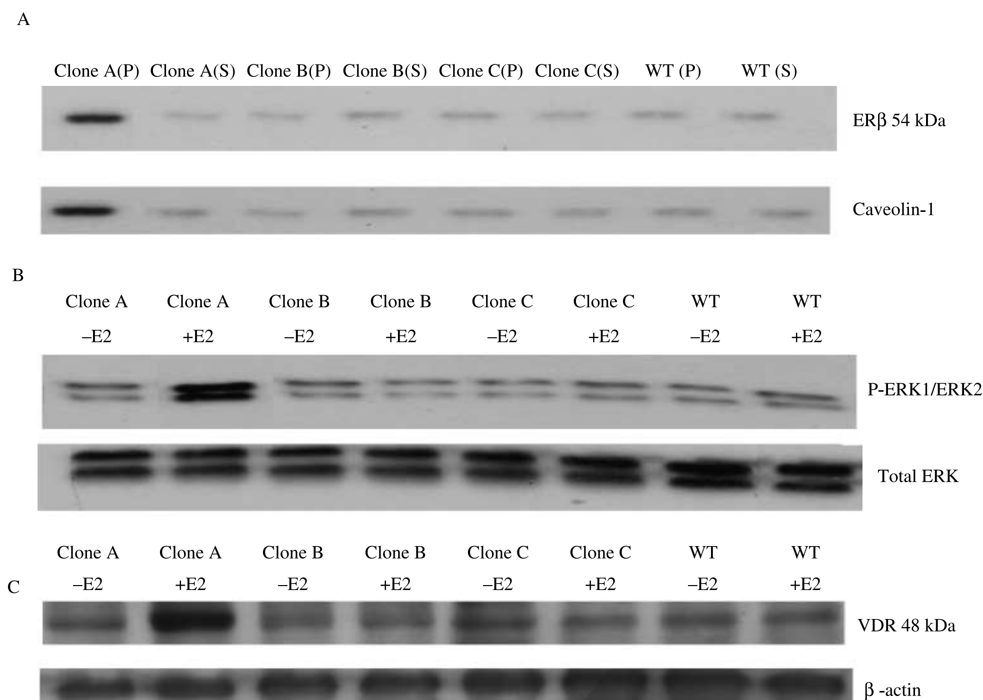

Figure 2 (A) Cellular localization of ERβ in different clones of HEK-293 cells. Immunoblot analyses for ERβ in the cytosol (S) and plasma membrane (P) harvested from the different clones of HEK-293. The blots were stripped and reprobed with caveolin-1 antibody. (B) Effect of E2 on ERK 1/2 phosphorylation in different clones of HEK-293 cells. Cells from the different HEK-293 clones were treated with 10^{-8} M E2 and harvested after 20 min. Total ERK 1/2 was detected with anti-ERK1 and anti-ERK2 antibodies or with anti-phospho-ERK 1/2 antibodies to detect the phosphorylated proteins. Control cells (−E2) were treated with E2 vehicle (ethanol, 0·0067%). The figure is a representative example of four similar, independent experiments. (C) Effect of E2 on VDR expression in different clones of HEK-293 cells. Cells from the different HEK-293 clones were treated with 10^{-8} M E2 and harvested after 4 days of treatment. Medium and treatment were replaced every other day. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. The blots were stripped and re-probed with β-actin, which was used as a loading control. One representative experiment from three identical ones is shown.

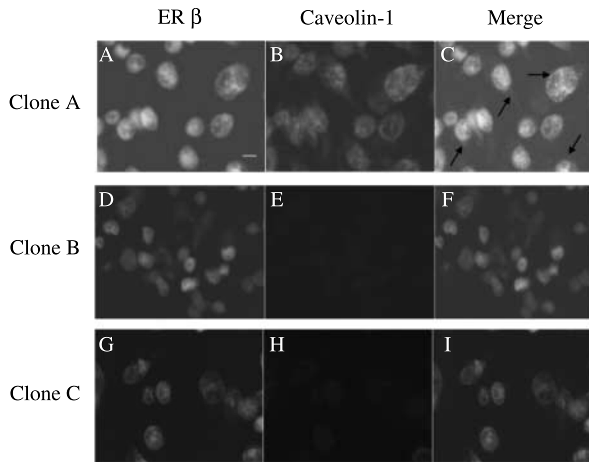

Figure 3 (A–I) Cellular localization of caveolin-1 and ERβ in different clones of HEK-293 cells. Clones A, B, and C were fixed and stained with anti-caveolin-1 and ERβ antibodies. Antibodies to ERβ and caveolin-1 were used to determine colocalization between the two proteins in the different HEK-293 clones, followed by the appropriate reporter antibodies (goat anti-rabbit-Texas Red (red, caveolin-1) and FITC-conjugated goat anti-mouse IgG (green, ERβ)). Merge is an overlay of the caveolin-1 (red) and ERβ (green) signal. Arrowhead indicates ERβ and caveolin-1 colocalization, which takes place only in clone A (see merge, C). In clone B (E), no caveolin 1 was evident, and in clone C, cells expressing high levels of nonfunctional-mutated P132L caveolin-1 protein, caveolin-1 is faintly expressed; however, the distribution is completely different from clone A (H). Bar = 10 μm. The experiments were repeated three times.

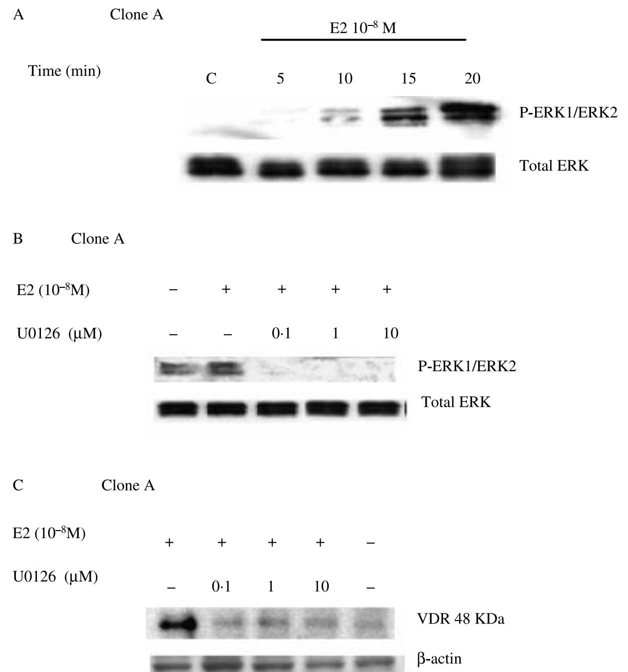

Figure 4 (A) Effect of E2 on ERK 1/2 phosphorylation in clone A of HEK-293 cells. Cells were treated with 10^{-8} M E2 and harvested at the time periods indicated. Total ERK 1/2 was detected with anti-ERK1 and anti-ERK2 antibodies or with anti-phospho-ERK 1/2 antibodies to detect the phosphorylated proteins. Control cells (C) were treated with E2 vehicle (ethanol, 0·0067%). The figure is a representative example of four similar, independent experiments. (B and C) Inhibition of ERK 1/2 phosphorylation and relation to VDR expression. Effect of UO126 on ERK 1/2 phosphorylation (B) and VDR expression (C) in HEK-293 cells. Cells were treated with different UO126 concentrations and with 10^{-8} M E2. Cell extracts were analyzed by western blot using an anti-phospho-ERK 1/2 and anti-ERK 1/2 antibodies, or a VDR antibody. One representative experiment of four identical ones is shown.

nonfunctional mutated P132L caveolin-1 protein, caveolin-1 is faintly expressed; however, the distribution is completely different from clone A (Fig. 3H). Wild-type HEK-293 cells show identical staining distribution of ERβ and caveolin-1 as clone B.

Phosphorylation activation of ERK 1/2 by E2 via the MAPK pathway

We further analyzed clone A cells and assessed whether this clone behaves similar to HT-29 colon cancer cells and MCF7 breast cancer cells (Gilad *et al.* 2005). To assess whether E2 can induce rapid cellular signaling effects in HEK-293 clone A cells, we measured ERK 1/2 phosphorylation. E2 activated ERK 1/2 phosphorylation within 10 min after exposure to a 10^{-8} M concentration (Fig. 4A), with peak activation at 20 min.

To determine whether a direct relationship exists between ERK 1/2 activation and VDR expression in HEK-293 clone A cells, we used the specific MEK 1/2 phosphorylation inhibitor UO126. ERK 1/2 phosphorylation and VDR protein expression were evaluated on western blots. We exposed the cells to different E2 concentrations for 4 days, the time period previously shown to be required for E2 to induce effective upregulation of VDR expression, and to UO126 for the last 2 days of the total 4 days. UO126 completely blocked E2-mediated ERK 1/2 phosphorylation and VDR protein expression at all UO126 concentrations tested (Fig. 4B and C), indicating that a direct relationship exists between these cellular events. Basal phosphorylation of ERK 1/2 in the control cells (without E2 treatment) was observed in these experiments, most likely because cells were exposed to culture media containing FCS.

Effect of E2 on VDR mRNA and protein-expression levels in HEK-293 clone A cells

The effect of different concentrations of E2 on VDR protein expression in HEK-293 clone A cells was

assessed by western-blot analysis of whole-cell lysates. The cells were exposed to a medium containing different concentrations of the hormonal treatments for 4 days, which was found to be the optimal time period in preliminary experiments (data not shown). We found that E2 dose-dependently upregulates VDR expression (Fig. 5A). E2 was also effective at upregulating VDR transcription in HEK-293 clone A cells. We evaluated VDR mRNA expression by reverse transcriptase-PCR analyses following the 4-day hormonal treatments. The effect of E2 on VDR mRNA upregulation was found to be similar to its effect on protein expression (Fig. 5B). The specific ER inhibitor ICI182 780 blocked E2-mediated VDR protein upregulation suggesting that E2 mediates VDR expression via a process involving ERs (Fig. 5C).

Effect of E2-BSA on ERβ localization VDR expression and ERK 1/2 phosphorylation in HEK-293 clone A cells

To determine whether E2-induced VDR regulation is mediated by the activity of the hormone at the cell membrane where caveolin-1 is located, HEK-293 clone A cells were treated with an E2-BSA conjugate, a molecule unable to traverse the plasma membrane. The effect of E2-BSA mimicked that of E2 on VDR protein expression and ERK 1/2 phosphorylation: following exposure to E2-BSA for 10 min, significant ERK 1/2 phosphorylation was detected, similar in pattern and intensity to that with the nonconjugated hormone (Fig. 6A). E2-BSA at all concentrations tested (10^{-10} M up to 10^{-6} M) significantly upregulated VDR expression in HEK-293 cells (Fig. 6B). Clone A cells treated with E2BSA were stained with anti-ERβ antibody and visualized with the reporter secondary antibody FITC-conjugated goat anti-mouse IgG (Fig. 6C).

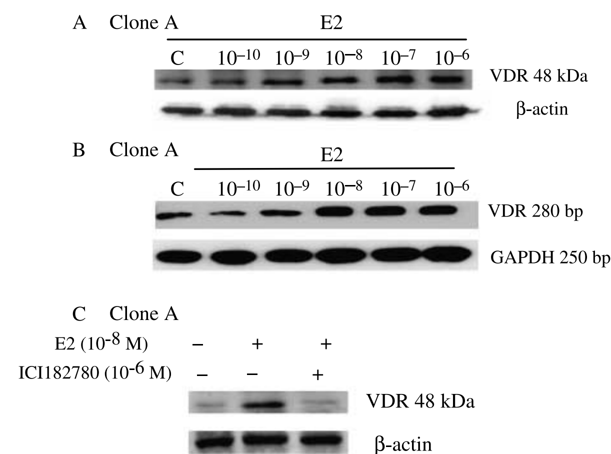

Figure 5 (A) Effect of different E2 concentrations on VDR protein expression in clone A of HEK-293 cells. HEK-293 clone A cells were treated with different E2 concentrations for 4 days. Medium and treatment were replaced every other day. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. Blots were stripped and reprobed with β-actin, which was used as a loading control. One representative experiment from three identical ones is shown. (B) VDR mRNA expression in clone A of HEK-293 cells. HEK-293 cells were treated with different E2 concentrations (10^{-10} to 10^{-6} M), and RNA extracts were analyzed for VDR mRNA expression by RT-PCR and when compared with GAPDH transcription. One representative experiment from three identical ones is shown. (C) VDR protein expression is inhibited by ICI82 780 in clone A of HEK-293 cells. Cells treated with 10^{-8} M E2 in the absence or presence of the ER inhibitor ICI182 780 (10^{-6} M) were analyzed by western blot using anti-VDR antibody. Blots were stripped and reprobed with β-actin, which was used as a loading control. One representative experiment from three identical ones is shown.

The role of ERβ and caveolin-1 on VDR expression

To demonstrate the role of ERβ and caveolin-1 in E2-induced VDR expression, we used RNA interference (RNAi) to block, ERβ (siRNA to ERβ) in HEK-293 clone A cells or caveolin-1 (siRNA to caveolin-1) in HT29 colon cancer cells, previously shown to express caveolin-1 and be amenable to VDR regulation through ERβ (Gilad *et al.* 2005). The specificity and efficiency of each siRNA were tested in transient transfection experiments, by western-blot or RT-PCR analyses.

Figure 7A demonstrates that the selected siRNA completely blocked caveolin-1 expression and concomitant treatment with E2 did not result in enhanced VDR expression (Fig. 7B), as opposed to scramble- or mock-transfected HT-29 cells. The role of ERβ in VDR control by E2 was tested in HEK-293 clone A cells transfected with siRNA ERβ. The transfected clones did not express the ERβ protein (Fig. 7C) or the RNA transcript (Fig. 7D), demonstrating the efficiency of the siRNA treatment. The mock- and scramble-transfected cells did express the protein and transcript. This treatment demonstrated that VDR is directly dependent on functional ERβ expression in HEK-293 clone A cells (Fig. 7E). SiRNA against caveolin-1 and ERβ were effective up to 4 days of E2 treatment (not shown).

Effect of E2 on VDR promoter activation

We performed transient transfection assays with the luciferase reporter vector pGL2 containing the 1·5 kb region of VDR in HEK-293 clones A, B, and C (bearing the P132L mutation) cells. Transfected cells were treated for 48 h with E2 and luciferase activity was recorded for control and E2-treated cells. The most E2-responsive cells were those from HEK-293 clone A, an effect which was not detectable in cells from clones B

and C or from WT HEK-293 cells (Fig. 8A). We again concentrated on clone A cells and treated them with E2 at concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M. These treatments resulted in upregulation of luciferase activity at all E2 concentrations used (Fig. 8B). Similar to E2-mediated VDR expression activity, the specific ER inhibitor ICI182 780 was able to block E2-mediated VDR promoter upregulation in HEK-293 clone A cells, at all E2 concentrations tested, suggesting that E2-induced VDR promoter activity is mediated by ERs. ICI182 780 therefore significantly inhibited activation of VDR promoter (data not shown).

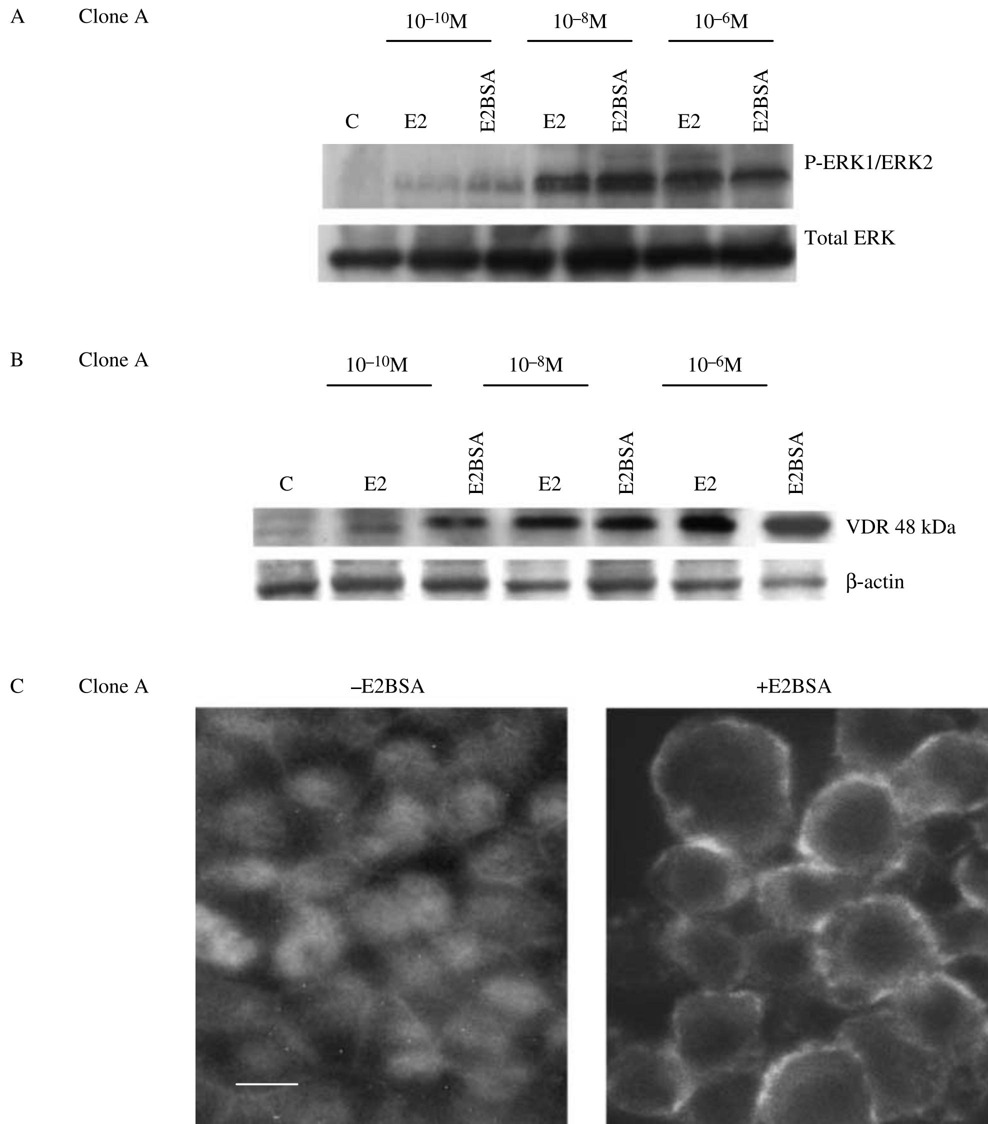

Figure 6 (A) Effect of E2-BSA treatment on ERK 1/2 phosphorylation in clone A of HEK-293 cells. HEK-293 clone A cells were cultured in PR-free DMEM and treated with E2-BSA or E2 for 10 min at concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M. Control cells (C) were exposed to 0·0067% ethanol. Cell extracts were analyzed by western blot using an anti-phospho-ERK 1/2 and total anti-ERK 1/2 antibodies. Clone A cells were treated with E2-BSA 10^{-8} M for 24 h and fixed and immunostained with anti-ERβ antibody. In nontreated clone A cells with E2-BSA, ERβ was expressed at all cell compartments. Following E2-BSA treatment, ERβ was mainly localized in the membrane compartment. One representative experiment of three identical ones is shown. (B) Effect of E2-BSA treatment on VDR expression in clone A of HEK-293 cells. HEK-293 clone A cells were cultured and treated as in A. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-VDR antibody. Blots were stripped and reprobed with β-actin, which was used as a loading control. One representative experiment of three identical ones is shown.

Effect of E2-BSA on VDR promoter activation in HEK-293 clone A cells

HEK-293 clone A cells transiently transfected with the luciferase reporter vector pGL2 containing the 1·5 kb region of VDR were treated for 48 h with E2-BSA at concentrations ranging from 10^{-10} to 10^{-7} M. These treatments resulted in upregulation of luciferase activity at all E2-BSA concentrations tested (Fig. 9), suggesting that E2-induced VDR promoter activity is mediated by membrane-bound ERs.

Effect of ERK 1/2 phosphorylation on VDR promoter activation in HEK-293 clone A cells

Using different MEK constructs, we assessed whether MEK can directly activate the VDR promoter in HEK-293

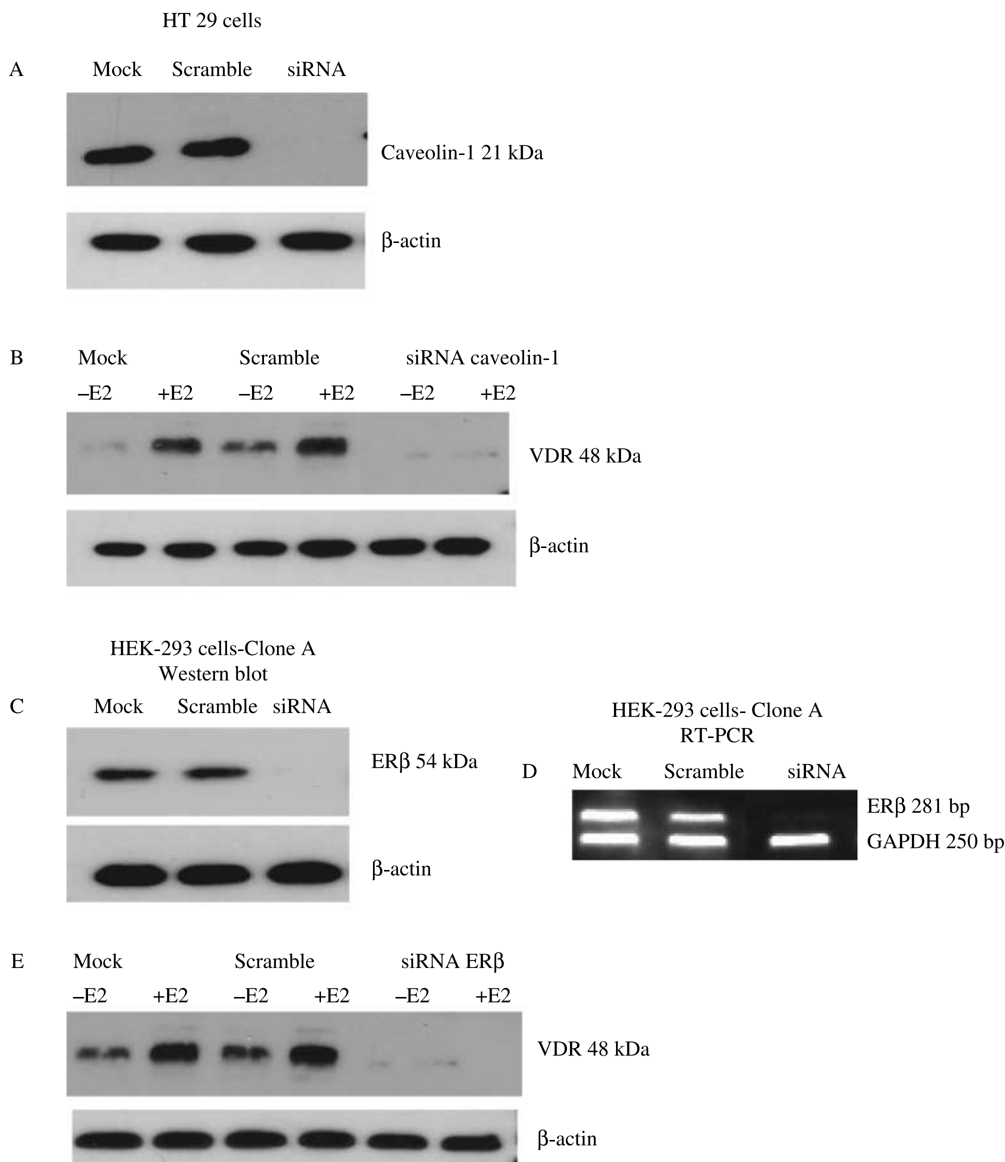

Figure 7 (A) Caveolin-1 siRNA. Caveolin-1 was specifically knocked down in HT-29 cells, demonstrated to express the protein. Mock- and scramble-transfected cells express caveolin-1. (B) Effect of E2 treatment on HT29 cells transfected with caveolin-1 siRNA. Transfection with siRNA to caveolin-1 yields HT-29 cells that are not responsive to E2 treatment in terms of VDR expression. (C and D) ERβ siRNA. ERβ was specifically knocked down in HEK-293 clone A cells. Cells transfected with siRNA to ERβ do not express protein (C) or cDNA transcript (D). (E) Effect of E2 treatment on clone A HEK-293 cells transfected with ERβ siRNA. Transfection with siRNA to ERβ yields clone A HEK-293 cells that are not responsive to E2 treatment in terms of VDR expression.

clone A cells. To this end, the constitutively active MEK construct EE-MEK and its catalytically inactive form KA-MEK were transiently transfected into HEK-293 clone A cells concomitantly with the 1·5 kb human VDR promoter luciferase reporter plasmid. A significant fourfold increase in luciferase activity associated with the VDR promoter was detected in the presence of the constitutively active EE-MEK construct, but not in that of the catalytically inactive KA-MEK construct (Fig. 10). This effect was not seen in clone B cells (data not shown).

Effect of E2 on AP-1 activation

The VDR promoter contains three AP-1-binding sites. AP-1 is a transcriptional activator composed of homo-

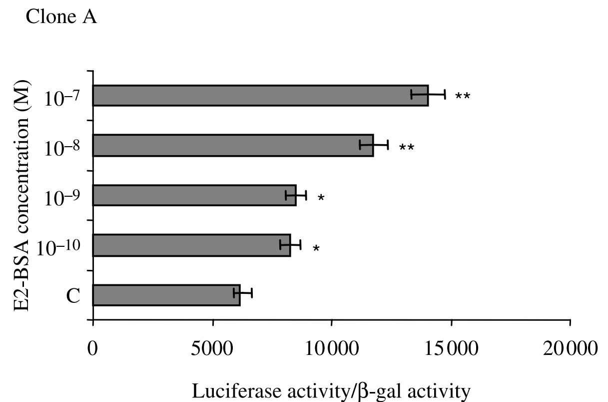

Figure 9 Effect of E2-BSA treatment on VDR promoter activity in clone A of HEK-293 cells. Plasmids containing the 1·5 kb human VDR promoter fragment in a pGL2 basic vector in front of the luciferase reporter gene were transfected into HEK-293 cells. Cells were then treated with different E2-BSA concentrations. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. *$P<0{\cdot}05$, **$P<0{\cdot}001$, when compared with control.

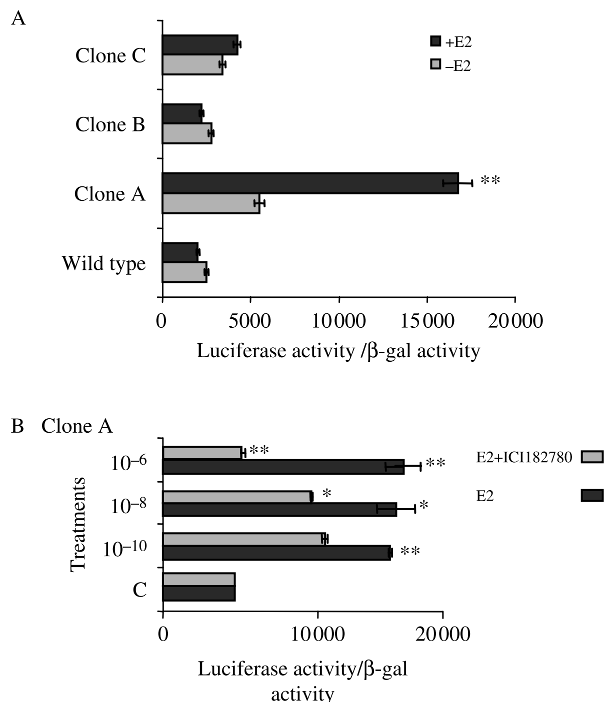

Figure 8 (A) Effect of E2 treatment on VDR promoter activity. Plasmids containing the 1·5 kb human VDR promoter fragment in a pGL2 basic vector in front of the luciferase reporter gene were transfected into the different stable clones (A, B, and C) of HEK-293 cells, and into parental HEK-293 cells. These were then treated with 10^{-8} M E2. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. *$P<0{\cdot}001$, when compared with all untreated control cells (−E2) or to treated (+E2) parental cell line and clones B and C. (B) Effect of E2 treatment on VDR promoter activity in clone A of HEK-293 cells. Plasmids containing the 1·5 kb human VDR promoter fragment in a pGL2 basic vector in front of the luciferase reporter gene were transfected into HEK-293 clone A cells. Cells were then treated with different E2 concentrations in the absence or presence of 10^{-6} M ICI182 780. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. *$P<0{\cdot}05$, when comparing ICI182 780-treated with respective E2-treated; **$P<0{\cdot}01$, when compared with control, without ICI182 780 treatment.

and hetero-dimers of Jun and Fos proteins. It is involved in the activation of many genes. AP-1 activity is subject to complex regulation both transcriptionally and posttranscriptionally. Transcriptional control of *jun* and *fos* gene expression determines the amount and composition of the AP-1 complex. The *jun* and *fos* genes are regulated both positively and negatively and are highly inducible in response to extracellular stimuli and to posttranslational control. AP-1 has been shown to play a key role in the nuclear integration of the Ras-ERK phosphorylation pathway. We therefore assessed whether E2 signaling through MAPK pathways integrates at AP-1 sites within the VDR promoter. To this end, we performed transient transfection assays in HEK-293 clone A cells with the AP-1-Luc vector, which contains the luciferase gene driven by the TATA box of the thymidine kinase promoter and an AP-1-dependent enhancer element, and then assessed the effect of E2. Similar to E2-mediated VDR expression activity, E2 activated AP-1-driven promoter activity, while the specific ER inhibitor ICI182 780 was also able to block E2-mediated AP-1 promoter upregulation, suggesting that E2-induced VDR promoter activity is mediated by ER and integrated at AP-1 sites present within the VDR promoter (Fig. 11A).

To further demonstrate that AP-1 consensus sequences play a key role in regulating E2-mediated VDR activation, we performed site-directed mutagenesis on one of the AP-1 sites of the VDR promoter. The AGAGTCA sequence in the VDR promoter was mutated to AGCGTCA. Site-directed mutagenesis was achieved using a QuikChange mutagenesis kit as instructed in the user's manual and confirmed with enzyme digestion and DNA sequencing. The effect of E2 stimulation was then determined following transient transfections with

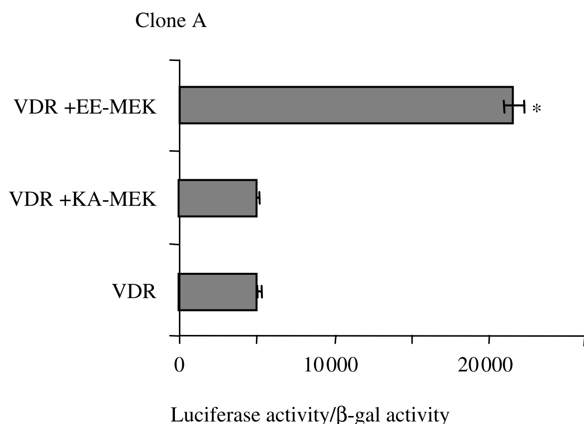

Figure 10 MEK activity and relation to VDR promoter activity in clone A of HEK-293 cells. HEK-293 clone A cells were cotransfected with VDR promoter in the absence or presence of EE-MEK, a constitutively active MEK construct, or KA-MEK, a dominant-negative MEK construct. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. $^{*}P<0{\cdot}001$, $^{**}P<0{\cdot}01$, when compared with control.

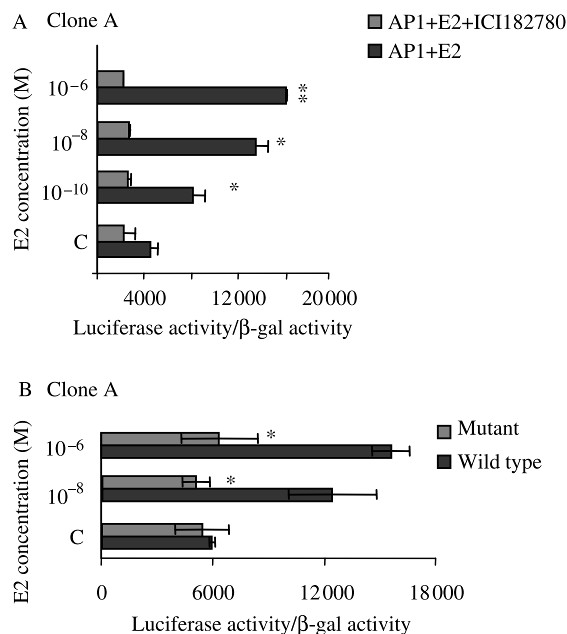

Figure 11 (A) Effect of E2 treatment on AP-1-Luc activity in clone A of HEK-293 cells. Plasmids containing the luciferase gene driven by the TATA box of the thymidine kinase promoter and an AP-1-dependent enhancer element were transfected into HEK-293 cells, which were then treated with different E2 concentrations in the presence or absence of ICI182 780 (10^{-6} M). Cells were analyzed for luciferase activity and standardized relative to β-gal activity. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}001$, when comparing E2 treatment with control and ICI182 780 treatment.

the mutated construct to HEK-293 clone A cells. E2 was unable to activate luciferase activity at any of the concentrations tested, in contrast to the WT promoter (Fig. 11B). Similar studies in HT-29 and MCF-7 cells demonstrated that mutation of the AP-1-binding site in the VDR-promoter similarly results in downregulation of luciferase activity (Fig. 12A and B). These results provide direct evidence that the AP-1 sequence in the VDR promoter plays a key role in the E2-mediated increase in VDR upregulation.

Discussion

This study was undertaken to directly demonstrate that E2–ER interactions leading to enhanced VDR expression take place at the cell surface of epithelial cells, namely in the caveola-enriched domain. We selected HEK-293 cells as our experimental system: these cells express low to barely detectable levels of caveolin-1 protein and concomitantly express ER (in this case, the major ER expressed was ERβ). This cellular system was amenable to manipulation: permanent transfections with caveolin-1 expression vector allowed us to obtain clones of HEK-293 cells expressing 18- to 20-fold higher levels of caveolin-1 (clone A) than the parental WT cells, clones with low caveolin-1 levels (clone B) and clones expressing high levels of nonfunctional mutated P132L caveolin-1 protein (clone C). These clones enabled us to assess E2–ER–caveolin interactions. Caveolin-1 point-mutated at the P132L locus has been previously reported to behave in a dominant-negative manner, causing the mislocalization and intracellular retention of caveolin-1. This P132L mutation leads to formation of misfolded caveolin-1 oligomers that are retained within the Golgi complex and are not targeted to caveolae on the plasma membrane (Lee *et al.* 2002). Evinger and associates (Evinger & Levin 2005) have recently demonstrated that deletion of the caveolin-1 scaffolding domain (amino acid residues 60–100) largely prevents the localization of ERα at the plasma membrane. In the present study, we demonstrate a direct functional consequence of caveolin-1 overexpression in HEK-293 cells, i.e., caveolin-1 overexpression in the transfected HEK-293 cells induced caveolin-2 stabilization and expression and coordinately, significantly increased free cholesterol in the cell. Since caveolin-1 is a cholesterol-binding protein and free cholesterol is principally found in the cell membrane, the present findings lent further support to our previous ones in which a direct correlation was obtained between functional caveolin-1 expression and free cholesterol content in the cell.

Cellular fractionation analyses of the different control and E2-treated parental and selected HEK-293 clones demonstrated specific localization of ERβ to plasma-membrane domains exclusively in clone A, the clone expressing 18- to 20-fold higher levels of functional caveolin-1 than the parental HEK-293 cells. In HEK-293 clone A cells, we showed that a significant fraction of ERβ

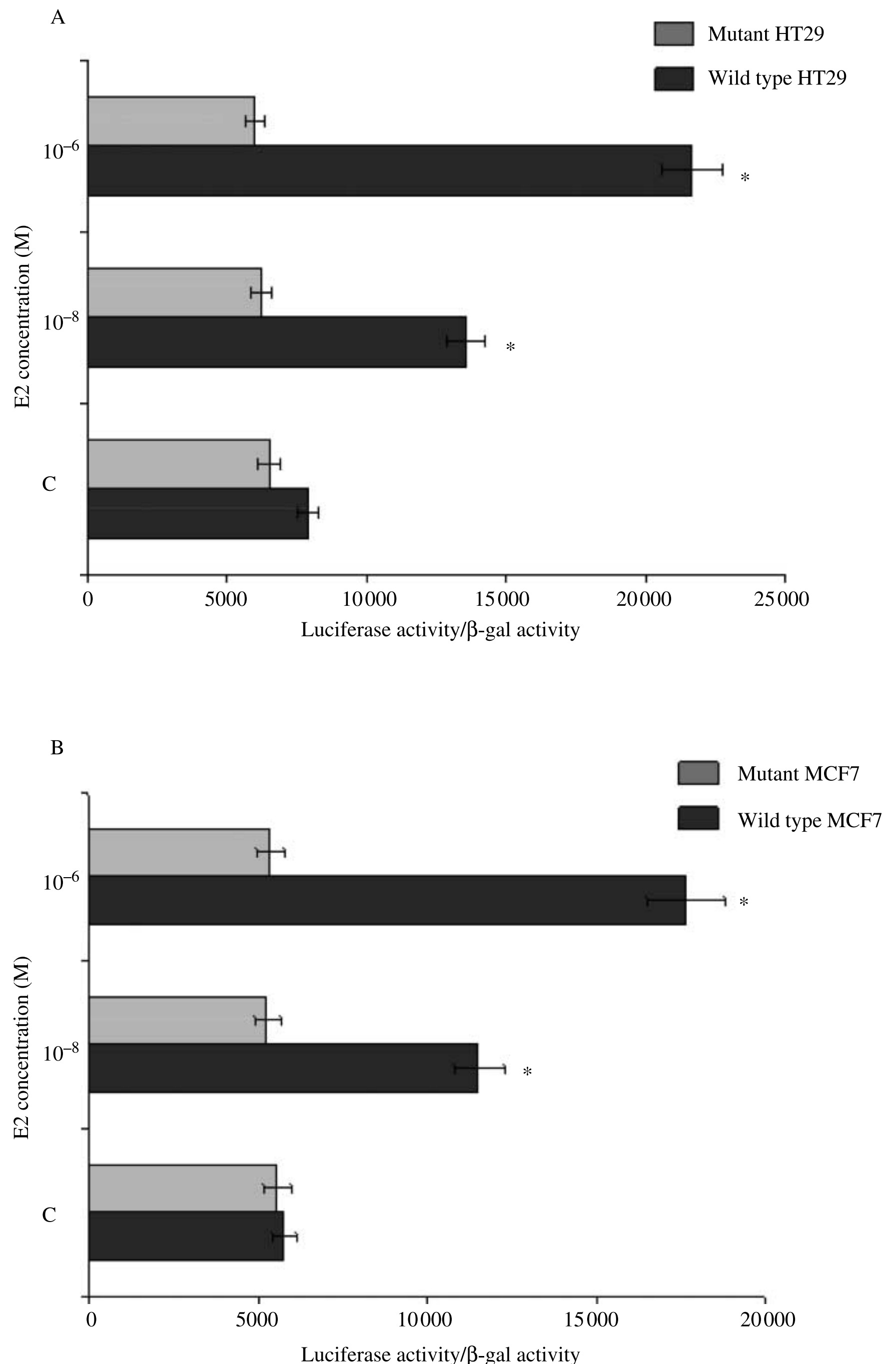

Figure 12 (A) Effect of E2 treatment on VDR promoter activity with mutations in the potential AP-1-binding site of VDR-luc in HT29 colon cancer cells. Plasmids containing the WT 1·5 kb human VDR promoter fragment or following point mutation at one AP-1 site (mutated) integrated front of the luciferase reporter gene (as for Fig. 10B), were transfected into HT29 cells and treated with E2. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. *$P<0{\cdot}001$, when compared with AP-1-mutated vector. (B) Effect of E2 treatment on VDR promoter activity with mutations in the potential AP-1-binding site of VDR-luc in MCF7 breast cancer cells. Plasmids containing the WT 1·5 kb human VDR promoter fragment or following point mutation at one AP-1 site (mutated) integrated front of the luciferase reporter gene (as for Fig. 10B), were transfected into MCF7 cells and treated with E2. Cells were analyzed for luciferase activity and standardized relative to β-gal activity. *$P<0{\cdot}001$, **$P<0{\cdot}01$, when compared with AP-1-mutated vector.

is expressed in the membrane fraction expressing high caveolin-1 levels. Immunocolocalization shown by confocal microscopy indicated extensive overlap of ERβ with caveolin-1 in the whole-cell plasma membrane. This association takes place upon expression of functional caveolin-1 expression. In clone C, expressing the mutated nonfunctional P132L caveolin-1, ERβ was not detected in the plasma membrane. In clone B, expressing low caveolin-1 levels similar to those in the parental WT HEK-293 cells, expression of ERβ in the plasma-membrane domains was negligible. We assume that caveolin anchors ER proteins to the membrane, similar to that which has been suggested for G-protein α-subunit (Couet *et al.* 1997). Additionally, immunostaining analyses allowed us to demonstrate that in E2-BSA clone A treated cells, ERβ is preferentially localized to membrane cellular domains.

The present study directly demonstrates the absolute requirement of functional caveolin-1 expression for E2, following binding to caveolar-membranal ERs, to be able to induce the activation of intracellular signaling that culminates in upregulation of VDR expression.

Several recent studies have indicated that many signaling molecules, such Raf1 and Src family tyrosine kinases, are recruited into caveolae by caveolins, which, through the scaffolding domain, interact with the caveolin-binding motifs in these signal molecules (Kiss *et al.* 2005). These groups of signal molecules can form 'preassembled signaling complexes' on the plasma membrane. Accumulation of receptors together with signal molecules in lipid rafts/caveolae enables them to be in close contact with each other and makes lipid rafts/caveolae the gateways for signals entering into the cells.

E2 was shown to affect VDR transcription and translation in HEK-293 clone A cells. In addition, ER mediated E2's effect on VDR, since the ER-specific inhibitor ICI182 780 was extremely effective at abrogating E2-mediated VDR upregulation.

Similar to our previous findings in HT-29 and MCF-7 cells expressing functional caveolae (Gilad *et al.* 2005), in the present study, E2 induced significant MAPK phosphorylation activities only in clone A, the clone expressing high caveolin-1 levels. These rapid non-genomic effects could take place whether the ER is located within or near the plasma membrane (Watson *et al.* 1999, Norfleet *et al.* 2000, Wade *et al.* 2001, Qi *et al.* 2002). We demonstrate herein that when HEK-293 clone A cells were treated with an E2-BSA conjugate, a compound unable to traverse the plasma membrane, the conjugate was able to upregulate both VDR expression and ERK phosphorylation, in a fashion that very closely mimicked the effect of the free nonconjugated E2.

The inhibition of VDR protein expression with the specific ERK 1/2 phosphorylation inhibitor UO126 supports the notion that E2 activation through ERK 1/2 modulates VDR expression. These data support the concept that MAPK activation plays a central role in the regulation of VDR expression by E2. To further demonstrate whether a direct relationship exists between ERK 1/2 activation and VDR expression, we used the EE-MEK construct which expresses a constitutively activated MEK and the KA-MEK construct which expresses a catalytically inactive MEK. Cotransfection of the EE-MEK construct with the luciferase reporter VDR promoter resulted in enhanced activation of E2-mediated VDR luciferase activation, in contrast to the catalytically inactive KA-MEK construct which was ineffective in this regard, a finding that further supports the notion that estrogen activation through MEK/ERK 1/2 modulates VDR expression.

The absolute requirement of functional caveola-membrane localization of ERs in HEK-293 cells in the framework of regulation of VDR expression by signaling pathways was further clarified using clone C cells transfected with the mutated nonfunctional P132L caveolin-1 expression vector. These experiments demonstrated that appropriate caveola-membrane organization and detection of ERβ in the plasma membrane are directly reflected by MAPK phosphorylation and consequent VDR expression.

Additional convincing results linking caveolin-1 ERβ positioning at the plasma membrane and control of VDR expression by E2 were obtained in experiments in which we knocked down ERβ with siRNA to ERβ in HEK-293 clone A cells and demonstrated that indeed ERβ is directly involved in regulating VDR (Fig. 6). In addition, the role of caveolin-1 in positioning ERβ to the plasma membrane and thereby allowing signaling to take place along with VDR transcription and translation was demonstrated by knocking down caveolin-1 in the caveolin-1- and ERβ-positive colon cancer cell line HT-29.

An additional event downstream of the MAPK phosphorylation reaction is phosphorylation of the nuclear transcription factor c-Jun (Gilad *et al.* 2005), able to induce transcriptional activation at AP-1 sites. These sites are present within the VDR (Qi *et al.* 2002) and are involved in regulation of VDR transcription. Luciferase reporter gene assays with the AP-1-Luc vector revealed that AP-1 is dose-dependently activated by E2 and inhibited by the specific ER inhibitor ICI182 780, just like that which occurred with VDR in clone A cells. The human VDR promoter contains AP-1 sites (Qi *et al.* 2002), and activation of the ERK/MAPK pathway causes induction of fos genes through phosphorylation of ternary complex factors (Kast *et al.* 2003). Fos heterodimerizes with Jun, or Jun homodimerizes to Jun family members, to form the AP-1 complex, which activates gene transcription by binding to the AP-1 element. Furthermore, Jun/Fos heterodimers can lead to increased c-jun transcription through binding to the AP-1 sites in the c-jun promoter (Shaulian & Karin

2001). Our experimental data suggest that a direct signaling connection exists between E2-ERβ, Raf-MAPK pathways, and VDR expression, and that the AP-1 sequence on the VDR promoter plays a key role in mediating this transactivation (Shaulian & Karin 2001), given that point mutations in AP-1 sites resulted in an inactive VDR promoter. These observations were equally demonstrated in HEK-293 clone A cells, HT29, and MCF7 cancer cell lines, indicating that this mechanism is conserved in tumor cells of different origin.

Taken together, our results demonstrate that in VDR regulation by E2, first E2 binds to ERβ when this ER is specifically associated with membranal caveolae. Following E2-ERβ binding, the Ras-Raf-ERK pathway is triggered. These signaling events activate transcription factors, such as c-Jun or c-Fos, to bind to their specific sequences within the VDR promoter (the AP-1-binding site), activity which culminates in upregulation of *VDR* gene transcription and expression.

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References

Arai H, Miyamoto K, Taketani Y, Yamamoto H, Iemori Y, Morita K, Tonai T, Nishisho T, Mori S & Takeda E 1997 A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *Journal of Bone and Mineral Research* **12** 915–921.

Aronica SM, Kraus WL & Katzenellenbogen BS 1994 Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *PNAS* **91** 8517–8521.

Blackmore PF, Neulen J, Lattanzio F & Beebe SJ 1991 Cell surface-binding sites for progesterone mediate calcium uptake in human sperm. *Journal of Biological Chemistry* **266** 18655–18659.

Castoria G, Lombardi M, Barone MV, Bilancio A, Di Domenico M, De Falco A, Varricchio L, Bottero D, Nanayakkara M, Migliaccio A *et al.* 2004 Rapid signalling pathway activation by androgens in epithelial and stromal cells. *Steroids* **69** 517–522.

Chaban VV, Lakhter AJ & Micevych P 2004 A membrane estrogen receptor mediates intracellular calcium release in astrocytes. *Endocrinology* **145** 3788–3795.

Chatterjee B, Echchgadda I & Song CS 2005 Vitamin D receptor regulation of the steroid/bile acid sulfotransferase SULT2A1. *Methods in Enzymology* **400** 165–191.

Civitelli R, Agnusdei D, Nardi P, Zacchei F, Avioli LV & Gennari C 1988 Effects of one-year treatment with estrogens on bone mass, intestinal calcium absorption, and 25-hydroxyvitamin D-1 alpha-hydroxylase reserve in postmenopausal osteoporosis. *Calcified Tissue International* **42** 77–86.

Couet J, Li S, Okamoto T, Ikezu T & Lisanti MP 1997 Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *Journal of Biological Chemistry* **272** 6525–6533.

Csikos T, Chung O & Unger T 1998 Receptors and their classification: focus on angiotensin II and the AT2 receptor. *Journal of Human Hypertension* **12** 311–318.

Dokoh S, Donaldson CA & Haussler MR 1984 Influence of 1,25-dihydroxyvitamin D3 on cultured osteogenic sarcoma cells: correlation with the 1,25-dihydroxyvitamin D3 receptor. *Cancer Research* **44** 2103–2109.

Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC *et al.* 2001 Loss of caveolae, vascular dysfunction, and pulmonary defects in *caveolin-1* gene-disrupted mice. *Science* **293** 2449–2452.

Evinger AJ III. & Levin ER 2005 Requirements for estrogen receptor alpha membrane localization and function. *Steroids* **70** 361–363.

Filardo EJ, Quinn JA, Frackelton AR Jr. & Bland KI 2002 Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Molecular Endocrinology* **16** 70–84.

Fiucci G, Ravid D, Reich R & Liscovitch M 2002 Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene* **21** 2365–2375.

Gennari C, Agnusdei D, Nardi P & Civitelli R 1990 Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D3 in oophorectomized women. *Journal of Clinical Endocrinology and Metabolism* **71** 1288–1293.

Gilad LA, Bresler T, Gnainsky J, Smirnoff P & Schwartz B 2005 Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. *Journal of Endocrinology* **185** 577–592.

Gross C, Eccleshall TR, Malloy PJ, Villa ML, Marcus R & Feldman D 1996 The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican-American women. *Journal of Bone and Mineral Research* **11** 1850–1855.

Guerra B, Diaz M, Alonso R & Marin R 2004 Plasma membrane oestrogen receptor mediates neuroprotection against beta-amyloid toxicity through activation of Raf-1/MEK/ERK cascade in septal-derived cholinergic SN56 cells. *Journal of Neurochemistry* **91** 99–109.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C & Brown M 1994 Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264** 1455–1458.

Harris SS, Eccleshall TR, Gross C, Dawson-Hughes B & Feldman D 1997 The vitamin D receptor start codon polymorphism (FokI) and bone mineral density in premenopausal American black and white women. *Journal of Bone and Mineral Research* **12** 1043–1048.

Heaney RP, Recker RR & Saville PD 1978 Menopausal changes in calcium balance performance. *Journal of Laboratory and Clinical Medicine* **92** 953–963.

Horst RL, Goff JP & Reinhardt TA 1990 Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. *Endocrinology* **126** 1053–1057.

Ikonen E & Parton RG 2000 Caveolins and cellular cholesterol balance. *Traffic* **1** 212–217.

Kast C, Wang M & Whiteway M 2003 The ERK/MAPK pathway regulates the activity of the human tissue factor pathway inhibitor-2 promoter. *Journal of Biological Chemistry* **278** 6787–6794.

Kiss AL, Turi A, Mullner N, Kovacs E, Botos E & Greger A 2005 Oestrogen-mediated tyrosine phosphorylation of caveolin-1 and its effect on the oestrogen receptor localisation: an *in vivo* study. *Molecular and Cellular Endocrinology* **245** 128–137.

Lee H, Park DS, Razani B, Russell RG, Pestell RG & Lisanti MP 2002 Caveolin-1 mutations (P132L and null) and the pathogenesis of breast cancer: caveolin-1 (P132L) behaves in a dominant-negative manner and caveolin-1 (−/−) null mice show mammary epithelial cell hyperplasia. *American Journal of Pathology* **161** 1357–1369.

Lees MB, Carr S & Folch J 1964 Purification of bovine brain white matter proteolipids by dialysis in organic solvents. *Biochimica et biophysica acta* **84** 464–466.

Levy J, Zuili I, Yankowitz N & Shany S 1984 Induction of cytosolic receptors for 1 alpha, 25-dihydroxyvitamin D3 in the immature rat uterus by oestradiol. *Journal of Endocrinology* **100** 265–269.

Liel Y, Kraus S, Levy J & Shany S 1992 Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D receptors in osteoblast-like cells (ROS 17/2.8). *Endocrinology* **130** 2597–2601.

Liel Y, Shany S, Smirnoff P & Schwartz B 1999 Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. *Endocrinology* **140** 280–285.

Marin R, Guerra B, Morales A, Diaz M & Alonso R 2003*a* An ICI 182 780-sensitive, membrane-related estrogen receptor contributes to estrogenic neuroprotective actions against amyloid-beta toxicity. *Annals of the New York Academy of Sciences* **1007** 108–116.

Marin R, Guerra B, Morales A, Diaz M & Alonso R 2003*b* An oestrogen membrane receptor participates in estradiol actions for the prevention of amyloid-beta peptide1-40-induced toxicity in septal-derived cholinergic SN56 cells. *Journal of Neurochemistry* **85** 1180–1189.

Marin R, Guerra B, Alonso R, Ramirez CM & Diaz M 2005 Estrogen activates classical and alternative mechanisms to orchestrate neuroprotection. *Current Neurovascular Research* **2** 287–301.

Marquez DC, Chen HW, Curran EM, Welshons WV & Pietras RJ 2006 Estrogen receptors in membrane lipid rafts and signal transduction in breast cancer. *Molecular and Cellular Endocrinology* **246** 91–100.

Nadal A, Rovira JM, Laribi O, Leon-quinto T, Andreu E, Ripoll C & Soria B 1998 Rapid insulinotropic effect of 17beta-estradiol via a plasma membrane receptor. *FASEB Journal* **12** 1341–1348.

Nahmias C & Strosberg AD 1995 The angiotensin AT2 receptor: searching for signal-transduction pathways and physiological function. *Trends in Pharmacological Sciences* **16** 223–225.

Nemere I, Dormanen MC, Hammond MW, Okamura WH & Norman AW 1994 Identification of a specific binding protein for 1 alpha,25-dihydroxyvitamin D3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *Journal of Biological Chemistry* **269** 23750–23756.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M & Gustafsson JA 2001 Mechanisms of estrogen action. *Physiological Reviews* **81** 1535–1565.

Norfleet AM, Clarke CH, Gametchu B & Watson CS 2000 Antibodies to the estrogen receptor-alpha modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors. *FASEB Journal* **14** 157–165.

Pfahl M 1993 Nuclear receptor/AP-1 interaction. *Endocrine Reviews* **14** 651–658.

Pietras RJ & Szego CM 1977 Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* **265** 69–72.

Pietras RJ & Szego CM 1980 Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. *Biochemical Journal* **191** 743–760.

Pietras RJ, Marquez DC, Chen HW, Tsai E, Weinberg O & Fishbein M 2005 Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. *Steroids* **70** 372–381.

Qi X, Pramanik R, Wang J, Schultz RM, Maitra RK, Han J, DeLuca HF & Chen G 2002 The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D(3)-induced growth inhibition. *Journal of Biological Chemistry* **277** 25884–25892.

Ravid D, Maor S, Werner H & Liscovitch M 2005 Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by upregulation of insulin-like growth factor-I receptors and signaling. *Oncogene* **24** 1338–1347.

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG *et al.* 2001 Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *Journal of Biological Chemistry* **276** 38121–38138.

Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR & Anderson RG 1992 Caveolin, a protein component of caveolae membrane coats. *Cell* **68** 673–682.

Schwartz B, Smirnoff P, Shany S & Liel Y 2000 Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Molecular and Cellular Biochemistry* **203** 87–93.

Setalo G Jr, Singh M, Nethrapalli IS & Toran-Allerand CD 2005 Protein kinase C activity is necessary for estrogen-induced Erk phosphorylation in neocortical explants. *Neurochemical Research* **30** 779–790.

Shaulian E & Karin M 2001 AP-1 in cell proliferation and survival. *Oncogene* **20** 2390–2400.

Simons K & Ikonen E 1997 Functional rafts in cell membranes. *Nature* **387** 569–572.

Stan RV 2005 Structure of caveolae. *Biochimica et Biophysica Acta* **1746** 334–348.

Takamoto S, Seino Y, Sacktor B & Liang CT 1990 Effect of age on duodenal 1,25-dihydroxyvitamin D-3 receptors in Wistar rats. *Biochimica et Biophysica Acta* **1034** 22–28.

Tesarik J & Mendoza C 1995 Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *Journal of Clinical Endocrinology and Metabolism* **80** 1438–1443.

Wade CB, Robinson S, Shapiro RA & Dorsa DM 2001 Estrogen receptor (ER)alpha and ERbeta exhibit unique pharmacologic properties when coupled to activation of the mitogen-activated protein kinase pathway. *Endocrinology* **142** 2336–2342.

Walters MR 1981 An estrogen-stimulated 1,25-dihydroxyvitamin D3 receptor in rat uterus. *Biochemical and Biophysical Research Communications* **103** 721–726.

Watson CS, Norfleet AM, Pappas TC & Gametchu B 1999 Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-alpha. *Steroids* **64** 5–13.

Wehling M 1995 Nongenomic aldosterone effects: the cell membrane as a specific target of mineralocorticoid action. *Steroids* **60** 153–156.

Wharton J, Meshulam T, Vallega G & Pilch P 2005 Dissociation of insulin receptor expression and signaling from caveolin-1 expression. *Journal of Biological Chemistry* **280** 13483–13486.

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