Estrogen receptor (ER) expression and function in the pregnant human myometrium: estradiol via ER α activates ERK1/2 signaling in term myometrium

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Abstract

Estrogens are thought to promote labor by increasing the expression of pro-contraction genes in myometrial cells. The specific estrogen receptors ((ERs: ER α and ER β (also known as ESR1 and ESR2)) and G protein-coupled receptor 30 (GPR30; also known as G protein-coupled estrogen receptor 1)) and signaling pathways that mediate these actions are not clearly understood. In this study, we identified the ERs expressed in the pregnant human myometrium and determined a key extranuclear signaling pathway through which estradiol (E₂) modulates expression of the gene encoding the oxytocin receptor (OXTR), a major pro-contraction protein. Using quantitative RT-PCR, we found that *ER α* and *GPR30* mRNAs were expressed in the human pregnant myometrium while *ER β* mRNA was virtually undetectable. While mRNA encoding ER α was the predominant ER transcript in the pregnant myometrium, ER α protein was largely undetectable in myometrial tissue by immunoblotting. Pharmacological

inhibition of 26S proteasome activity increased ER α protein abundance to detectable levels in term myometrial explants, however, indicating rapid turnover of ER α protein by proteasomal processing in the pregnant myometrium. E₂ stimulated rapid extranuclear signaling in myometrial explants, as evidenced by increased extracellularly regulated kinase (ERK1/2) phosphorylation within 10 min. This effect was inhibited by pre-treatment with an ER antagonist, ICI 182 780, indicating the involvement of ER α . Inhibition of ERK signaling abrogated the ability of E₂ to stimulate *OXTR* gene expression in myometrial explants. We conclude that estrogenic actions in the human myometrium during pregnancy, including the stimulation of contraction-associated gene expression, can be mediated by extranuclear signaling through ER α via activation of the ERK/mitogen-activated protein kinase pathway.

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Introduction

Estrogens (mainly estradiol (E₂)) promote labor by stimulating biochemical and physical changes in myometrial cells that augment uterine contractility and excitability (for review, see [Pepe & Albrecht \(1995\)](#)). Studies on various species have shown that E₂ increases the expression of genes in myometrial cells encoding gap junction proteins that form low-resistance connections between myometrial cells to promote synchronized contractions ([Lye *et al.* 1993](#), [Petrocelli & Lye 1993](#), [Kilarski *et al.* 1996, 2000](#)), receptors for uterotonic hormones such as oxytocin and prostaglandin F_{2 α} (PGF_{2 α} ; [Pinto *et al.* 1966, 1967](#), [Nissenson *et al.* 1978](#)), and enzymes such as PG-endoperoxide synthase-2 (PTGS2), the main inducible rate-limiting enzyme for PG synthesis ([Mesiano *et al.* 2002](#)). Although it is generally accepted that these actions are mediated by estrogen receptors (ERs), the specific ERs and

associated signaling pathways are not well characterized in the pregnant human myometrium. Human ERs exist in three principal forms: two classical nuclear ER subtypes, ER α (also called ESR1) and ER β (ESR2; [Warner *et al.* 1999](#), [Dechering *et al.* 2000](#)), and a seven-transmembrane G protein-coupled receptor known as GPR30 (alternatively known as G protein-coupled estrogen receptor 1 (GPER); [Revankar *et al.* 2005](#), [Thomas *et al.* 2005](#)). The purpose of this study was to determine whether these ERs are expressed in the pregnant human myometrium and to investigate the signaling pathways through which they mediate estrogenic actions.

In most species, the pro-labor influence of estrogens is achieved by a marked increase in circulating estrogen concentrations prior to the onset of labor. In human pregnancy, however, the myometrium is exposed to high levels of estrogens in the form of E₂, estrone, and estriol for most of pregnancy and systemic estrogen levels do not change

significantly prior to labor onset (De Hertogh *et al.* 1975, Smith *et al.* 2009). It appears, therefore, that the human myometrium is refractory to the pro-contractile actions of estrogens for most of pregnancy and that the parturition process involves increased myometrial responsiveness to estrogens. We have previously found that myometrial *ERα* mRNA levels rise at parturition and that the levels are strongly correlated with the abundance of mRNAs encoding *PTGS2* and the oxytocin receptor (*OXTR*; Mesiano *et al.* 2002). However, attempts to measure *ERα* protein in pregnant human myometrium by immunohistochemistry (Geimonen *et al.* 1998, Winkler *et al.* 2002, Laudanski *et al.* 2004) or immunoblotting (Geimonen *et al.* 1998) have been largely unsuccessful, and it is well documented that *ERα* expression is substantially reduced during pregnancy compared with the non-pregnant state (Geimonen *et al.* 1998, Benassayag *et al.* 1999). Wu *et al.* (2000) reported that *ERβ* but not *ERα* was detectable by immunoblotting in term human myometrium and suggested that *ERβ* is the principal mediator of estrogenic actions in the pregnant human uterus. However, we found that *ERβ* mRNA was barely detectable by the very sensitive real-time RT-PCR technique in term myometrium (Mesiano *et al.* 2002). Thus, there is a lack of consensus regarding the expression of *ERα* and *ERβ* in the pregnant human myometrium and their role in mediating estrogen actions, which is further complicated by the possibility that GPR30 is also involved in estrogen signaling in myometrial cells (Maiti *et al.* 2011).

Estrogens typically affect target cell function via the genomic mode of steroid hormone action, whereby *ERα* and *ERβ* function as ligand-activated transcription factors to modulate the expression of specific genes. However, many pro-contraction genes that are regulated by E_2 , including *OXTR* and connexin-43 (*GJA1*), lack complete canonical estrogen response elements (EREs) in their promoter regions (Geimonen *et al.* 1996, Gimpl & Fahrenholz 2001). This suggests that E_2 modulates the expression of some genes via alternative mechanisms. One possibility is that E_2 regulates the expression of ERE-containing genes via the classical genomic mode of action and that the products of those genes then regulate the expression of secondary or tertiary gene sets. An alternative possibility is that estrogens activate extranuclear/non-genomic pathways that lead to downstream changes in gene expression (reviewed in Vasudevan & Pfaff (2008), Fox *et al.* (2009), and Prossnitz & Maggiolini (2009)). Unlike the genomic mode of action, the extranuclear mode affects cell function directly by modulating cytoplasmic signaling cascades that impact on the activity of multiple cellular processes and transcription factors. Extranuclear pathways activated by E_2 include the extracellularly regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PKB/AKT) pathways, as well as Ca^{2+} influx and G protein signaling (Vasudevan & Pfaff 2008, Fox *et al.* 2009, Prossnitz & Maggiolini 2009). *ERα*, *ERβ*, and GPR30 have each been found to induce extranuclear signaling, although the

involvement of the different receptors appears to be tissue- and cell-type specific (Vasudevan & Pfaff 2008, Fox *et al.* 2009, Prossnitz & Maggiolini 2009). Rapid activation of the ERK/MAPK signaling pathway by E_2 has been extensively studied in breast cancer cells where this cascade is thought to mediate the proliferative response to estrogens (Migliaccio *et al.* 1996, Razandi *et al.* 2003, Song *et al.* 2002, 2004). Upon ligand binding, *ERα* and *ERβ* can interact with numerous scaffolding proteins and signaling molecules in the cytoplasm, including SRC (Migliaccio *et al.* 1996, Barletta *et al.* 2004), MNAR (Barletta *et al.* 2004), SHC (Song *et al.* 2002, 2004), and G proteins (Kumar *et al.* 2007), and in concert with these proteins stimulate downstream activation of ERK1/2 (also known as p44/42 MAPK). GPR30 activates rapid non-genomic signaling via its coupled G proteins, which activate SRC and the downstream ERK pathway, often by way of extracellular release of heparin-bound epidermal growth factor (HB-EGF) and transactivation of the EGF receptor (reviewed in Maggiolini & Picard (2010)). Madak-Erdogan *et al.* (2008) have found that the extranuclear actions of E_2 mediated by *ERα* controlled ~25% of all E_2 -responsive genes in MCF7 breast cancer cells, highlighting the importance of extranuclear *ERα* signaling in mediating ligand-activated gene expression.

Extranuclear signaling by estrogens is largely unexplored in pregnant human myometrium. In pregnant rats, the abundance of activated ERK in myometrial cells increases with gestation (Oldenhof *et al.* 2002, Serrano-Sanchez *et al.* 2008) and labor onset (Li *et al.* 2003). In animals induced to deliver preterm (PT) by administration of the progesterone receptor antagonist RU486, concurrent inhibition of ERK signaling delayed preterm labor and decreased myometrial contractility (Li *et al.* 2004). These data emphasize the potential importance of this pathway in the regulation of myometrial smooth muscle contractility. In this study, we characterized the ERs expressed in the pregnant human myometrium and determined whether they activate the cytoplasmic ERK signaling cascade in response to E_2 in explant cultures of term human myometrium.

Materials and Methods

Myometrial tissue collection

Lower uterine segment myometrium was obtained following collection of written informed consent from women undergoing cesarean section at MacDonald Women's Hospital, University Hospitals Case Medical Center, Cleveland, OH (IRB approval #11-04-06), and MetroHealth Medical Center, Cleveland, OH (IRB approval #05-00287). A biopsy of myometrium (~0.5 cm³) was excised from the upper margin of the lower segment incision after delivery of the placenta. Tissue was collected from women delivering at PT and not in labor (NIL) (PT-NIL; *n*=6; range 27 w0d–34 w2d), at PT and in labor (IL) (PT-IL; *n*=6; range 24 w1d–36

w6d), at term and NIL (T-NIL; $n=19$; range 37 w3d–41 w6d), and at term and IL (T-IL; $n=11$; range 38 w1d–40 w4d). Cesarean sections were performed in non-laboring women for complications such as pre-eclampsia, breech presentation, or previous cesarean section, while indications for cesarean section during labor included fetal distress, maternal complications, or failure to progress. Samples from pregnancies with induced labor or clinical signs of chorioamnionitis were excluded. Tissues were rinsed in ice-cold PBS and portions of each myometrial specimen were immediately snap-frozen in liquid nitrogen or placed in fixative (10% neutral-buffered formalin). Tissue samples used for explant studies were transported to the laboratory in PBS on ice for further processing. Non-pregnant myometrium was collected from women undergoing surgery for benign gynecological conditions at MacDonald Women's Hospital (IRB approval #06-07-29) and snap-frozen in liquid nitrogen.

Myometrial explant culture

Myometrial tissue samples collected from women at term and NIL were dissected into explants of 2–3 mm³ and placed onto 0.2 µm polycarbonate filters (GE Water & Process Technologies, Trevose, PA, USA) floating on 2 ml of phenol red-free, serum-free DMEM (Sigma) containing 2 mm GlutaMAX (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin in 6-well plates and cultured overnight (16–18 h) at 37 °C in 5% CO₂, 95% air (six to seven explants per well). Media were refreshed and the explants were incubated with media alone or U0126 (10 µM) (Cell Signaling, Beverly, MA, USA), ICI 182 780 (1 µM; Sigma), or vehicle for 30 min. E₂, 1 or 10 nM (Sigma), phorbol myristate acetate (PMA; 100 nM), or vehicle was then added for various times, and the explants were snap-frozen in liquid nitrogen. The viability of myometrial explants in this culture system was confirmed by histological analysis showing few necrotic cells and maintenance of smooth muscle-specific marker expression and RNA integrity (data not shown). For 26S proteasome inhibition experiments, explants were placed into culture and stimulated without delay with vehicle or MG132 (10 µM; Sigma) for 24 h. Nuclear and cytoplasmic protein extracts were then immediately prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell culture and transfection

The hTERT-HM cell line, a telomerase-immortalized myometrial smooth muscle cell line derived from the fundus of a non-pregnant, pre-menopausal woman (Condon *et al.* 2002), was provided by Prof. William Rainey, Medical College of Georgia. Cells were cultured in complete media: phenol red-free DMEM containing 10% charcoal-stripped FCS, 2 mm GlutaMAX, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO₂, 95% air. Cells at 90–95% confluency were

collected by trypsinization, centrifuged, and resuspended in Amaxa Nucleofector Mammalian Smooth Muscle Cell Solution (Lonza, Walkersville, MD, USA) at a concentration of 2×10^6 cells/100 µl solution. siRNA targeted against exon 8 of *ERα* (Silencer Select Pre-Designed siRNA ID s4824; Ambion) or Silencer Select Negative Control #1 siRNA (Ambion) was added to the transfection solution at 375 or 750 nm each. The transfection solution (100 µl) was transferred to an electroporation cuvette and nucleofection was achieved using the Amaxa Nucleofector Device with program A33. The transfection solution was then diluted in 8 ml complete media and plated at 0.5×10^6 cells/35 mm dish. A subset of cells were co-transfected with an expression plasmid encoding full-length *ERα* (1 µg plasmid DNA/ 0.5×10^6 cells) provided by Dr Peter Kushner, University of California at San Francisco. The cells were cultured for 48 h before lysis at 4 °C using CellLytic MT Lysis Buffer (Sigma) supplemented with protease and phosphatase inhibitors (Sigma). T47D and MCF7 human breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were used as positive controls for ER expression.

Immunoblotting

Whole tissue protein extracts were prepared from myometrium by homogenization in CellLytic MT Lysis Buffer supplemented with protease and phosphatase inhibitors on ice. Tissue homogenates were centrifuged at 10 000 *g* at 4 °C for 10 min and the supernatants were collected and assayed for total protein content using the Quick Start Bradford Protein Assay (Bio-Rad). Cell lysates were incubated with reducing sample buffer and subjected to SDS-PAGE (20–200 µg protein/lane) using Pierce 10% Precise Protein Gels (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and non-specific binding was blocked using either 5% BSA (for phospho-antibodies) or 5% skimmed milk (for non-phospho-antibodies) in 20 mM Tris, 150 mM NaCl, pH 7.5, containing 0.1% Tween-20 (TTBS) for 1 h at room temperature. Membranes were incubated with primary antibodies (Table 1) diluted in blocking buffer overnight at 4 °C, then washed, and incubated with secondary antibody (anti-mouse or anti-rabbit IgG-HRP; 1:3000; Cell Signaling) at room temperature for 1 h. The membranes were washed, incubated with Amersham ECL chemiluminescent reagent (GE Healthcare, Piscataway, NJ, USA), and exposed to autoradiography film (Denville Scientific, South Plainfield, NJ, USA). The resultant bands were quantified using digital densitometry (Fujifilm MultiGauge v 3.0 Software; Fuji Photo Film Co., Tokyo, Japan). Where required, the PVDF membranes were dried, re-wet in methanol, and re-probed with antibodies directed against loading control proteins or nuclear markers. The effective stripping of each primary antibody between blots was confirmed by a lack of signal upon incubation with secondary antibody alone with chemiluminescent detection.

Table 1 Primary antibodies used for western blotting

Antibody target	Clone/designation	Species	Supplier	Dilution
ERα	F10	Mouse	Santa Cruz (Santa Cruz, CA, USA)	1:1000
ERα	HC20	Rabbit	Santa Cruz	1:1000
ERα	C-542	Mouse	Calbiochem (Rockland, MA, USA)	1:1000
GPR30	LS-A4272	Rabbit	Lifespan Biosciences (Seattle, WA, USA)	1:500
GAPDH	6C5	Mouse	Santa Cruz	1:50000
LSD1	C69G12	Rabbit	Cell Signaling	1:1000
Phospho-ERK1/2	9101	Rabbit	Cell Signaling	1:1000
Total ERK1/2	9102	Rabbit	Cell Signaling	1:2000

RNA extraction and quantitative RT-PCR

RNA was extracted from myometrial tissues and T47D cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA extracts were treated with DNase (Ambion) to remove residual DNA and quantified by u.v. spectrometry. RNA integrity was determined by agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed to cDNA using the Superscript II or III First-Strand Synthesis Systems for RT-PCR (Invitrogen) with random hexamer primers. Real-time PCR was performed using an ABI PRISM 7500 Sequence Detector with SYBR Green fluorescence detection (Applied Biosystems, Carlsbad, CA, USA). PCR primers (Table 2) were designed using Primer Express Software (Applied Biosystems). The abundance of specific mRNAs in myometrial tissues and explants were determined relative to the abundance of h-caldesmon (*hCaD*) mRNA using the ΔC_t calculation (Livak & Schmittgen 2001). We had determined in previous experiments that the levels of *hCaD* mRNA (which is expressed specifically in smooth muscle) do not change significantly in the myometrium with advancing gestational age (GA) or labor onset (linear regression performed on logarithmically transformed data showed no relationship between *hCaD* mRNA and GA ($r^2=0.0286$, $P=0.464$; $n=21$) or labor status ($r^2=0.0009$, $P=0.897$; $n=21$)). PCR efficiency for each amplicon was calculated using LinRegPCR version 11.1 Software (Ruijter et al. 2009). T47D cell line cDNA served as a positive control for *ERα*, *ERβ*, and *GPR30* expression.

Data analysis

The distribution of data was assessed using the Skewness and Kurtosis test for normality. Comparisons between two groups

of normally distributed data were performed by Student's *t*-test (paired or unpaired as appropriate). Phospho-ERK expression in myometrial explants was assessed by repeated measures ANOVA and differences between treatments were analyzed by paired *t*-test with Bonferroni's correction for multiple comparisons. Linear regression was performed to examine the relationships between a number of variables (*ERα* mRNA, *ERβ* mRNA (logarithmically transformed data), *GPR30* mRNA (logarithmically transformed data), *GPR30* protein, pERK1 expression (square-root transformed data), pERK2 or total ERK expression, or the ratio of pERK1 and pERK2 to total ERK (square-root transformed data)) and GA or labor. Spearman's rank correlation was used to test the relationship between *GPR30* mRNA and protein data. Explant *OXTR* mRNA data were examined using repeated measures ANOVA with Dunnett's *post-hoc* test. The data were analyzed using GraphPad Prism version 5.03 (GraphPad Software, Inc., San Diego, CA, USA) or Stata version 11 (StataCorp LP, College Station, TX, USA) and $P<0.05$ was considered significant for all analyses.

Results

ER expression in the pregnant human myometrium

ERα and *GPR30* mRNAs were readily detectable in pregnant human myometrium (Fig. 1). However, *ERβ* mRNA was detected at extremely low levels by quantitative RT-PCR (qRT-PCR). T47D cell cDNA served as a positive control for *ERβ* expression and confirmed the functionality of our PCR primers. There was no relationship between *ERβ* mRNA abundance and GA or labor status (linear regression performed on log-transformed data; *ERβ* vs GA, $r^2=0.018$,

Table 2 Real-time PCR primer sequences

Target mRNA	Amplicon size (bp)	GenBank accession no.	Forward primer (5'–3')	Reverse primer (5'–3')
ERα	66	NM_000125.3	TGAAAGTGGGATACGAAAAGAC	CATCTCTCTGGCGCTTGTGT
ERβ	101	NM_001437.2	CACGTCAGGCATGCGAGTAA	GCAATCAGCATCTCCAGCAG
GPR30	78	NM_001505.2	TGCACCTTCATGTCGCTCTT	GCGGTCCGAAGCTCATCCA
OXTR	73	NM_000916.3	CTGGACGCTTTTCTTCTCGT	GAAGGCCGAGGCTTCCTT
hCaD	79	NM_033138.2	CAGAGAGGGCAAGGTTGGAA	GCTCGTTCATCTGCTATCTTTT

Figure 1 ER mRNA relative abundance in pregnant human myometrium. The receptor groups include myometrial samples from a range of gestational ages, $n=21$ each (14 non-laboring and seven laboring tissue samples). The box and whiskers plots demonstrate the median values, with the boxes extending to the 25th and 75th percentiles and the whiskers extending to the minimum and maximum values.

$P=0.563$; $ER\beta$ vs labor, $r^2=0.0172$, $P=0.571$; $n=21$). Owing to the very low levels of $ER\beta$ gene expression, it is unlikely that this receptor plays an important role in mediating estrogen signaling in the pregnant human myometrium. $ER\alpha$ mRNA levels did not change significantly with GA (linear regression; $r^2=0.0064$, $P=0.730$; $n=21$) but were significantly higher in myometrial samples from laboring compared with non-laboring women ($r^2=0.1949$, $P=0.045$; $n=21$). The amplification efficiency for all amplicons, as assessed by the LinRegPCR Software, was close to the theoretical efficiency value of 2 ($ER\alpha$, 2.009 ± 0.005 (mean $\pm$ S.E.M.); $ER\beta$, 1.955 ± 0.022 ; GPR30, 1.950 ± 0.007 ; hCaD 2.002 ± 0.006 ; assessed in 17–22 individual PCR reactions each). These comparable amplification efficiencies allow us to assess the relative expression of different ER mRNA species with reasonable confidence, and together with the pronounced differences in ER mRNA levels shown in Fig. 1, lead us to conclude that $ER\alpha$ is the predominantly expressed ER in the pregnant human myometrium at the mRNA level.

$ER\alpha$ protein expression was examined in non-pregnant myometrium and in pregnant myometrium at various gestational stages by immunoblotting (Fig. 2). The representative results shown in Fig. 2A and B were from tissues that were snap-frozen at the time of collection, not from cultured explants. We found robust expression of full-length $ER\alpha$ protein in the non-pregnant myometrium and in MCF7 breast cancer cells. $ER\alpha$ protein levels were substantially lower in pregnant myometrial tissues than in non-pregnant myometrium, which was expected as similar results have been reported previously (Geimonen *et al.* 1998). $ER\alpha$ protein was detected in one of four PT-NIL myometrial samples, two of six PT-IL samples, one of ten T-NIL samples, and two of ten

T-IL samples. Three different $ER\alpha$ antibodies were screened for their ability to detect $ER\alpha$ protein in the pregnant human myometrium; representative results are shown for a monoclonal ($ER\alpha$ F10; Fig. 2A) and a polyclonal ($ER\alpha$ HC20; Fig. 2B) antibody. Both antibodies detected a 66 000 M_r immunoreactive band, which is the expected position of $ER\alpha$, and other bands that did not match in size and therefore likely represented non-specific protein binding. Similar results were achieved with a third $ER\alpha$ antibody (Clone C-542 from Calbiochem, data not shown). The sensitivity of $ER\alpha$ immunodetection was not improved by concentration of the protein extracts by ammonium sulfate precipitation or immunoprecipitation, by protein extraction using different lysis buffers (CellLytic MT vs RIPA buffer), by loading protein amounts of up to 200 μ g/lane, or by decreasing the dilution of the primary antibodies (to 1:250 for $ER\alpha$ F10 and 1:500 for $ER\alpha$ HC-20; data not shown). However, $ER\alpha$ protein was readily detectable in the cytoplasmic and nuclear fractions of myometrial explants cultured with the 26S proteasome

Figure 2 Representative $ER\alpha$ immunoblotting in human myometrium using (A) monoclonal (F10) and (B) polyclonal (HC-20) $ER\alpha$ antibodies. $ER\alpha$ (M_r : 66 000) was readily detectable in non-pregnant (NP) myometrium (50 μ g total protein loaded) and MCF7 cells (5 μ g total protein) but was undetectable in two out of the three representative pregnant myometrial samples shown (50 μ g total protein each). Panels A and B were produced from a single membrane that was probed sequentially with $ER\alpha$ F10, $ER\alpha$ HC-20, and GAPDH antibodies. (C) $ER\alpha$ levels in cytoplasmic (c) and nuclear (n) protein extracts from myometrial explants treated with vehicle or the proteasome inhibitor MG132 (10 μ M) for 24 h. $ER\alpha$ immunoblotting was performed using the F10 antibody. LSD1 is a nuclear protein and served as a marker to validate the separation of cytoplasmic and nuclear proteins. The lanes were from the same gel; non-contiguous lanes are separated by white lines. The experiment was performed twice and the data shown are representative of both experiments. Numbers at the left of panels A and C indicate M_r ($\times 10^{-3}$).

inhibitor MG132 for 24 h (Fig. 2C). This suggests that ERα protein turnover is rapid in human myometrial tissues, leading to low steady-state ERα levels. Additional smaller protein bands were detected by the ERα antibody in the cytoplasm of MG132-treated tissue, which is similar to the data previously reported in HeLa cells (Nawaz *et al.* 1999) and is thought to be the result of non-proteasomal degradation.

We performed further testing of the specificity of ERα immunodetection, since truncated ERα isoforms of various sizes have been reported previously in a number of human cells and tissues following the use of the ERα F10 and HC-20 antibodies (Flouriou *et al.* 2000, Russell *et al.* 2000, Horvath *et al.* 2002, Figtree *et al.* 2003, Penot *et al.* 2005). By RNA interference, we reduced the expression of endogenous or overexpressed ERα protein in hTERT-HM myometrial smooth muscle cells, followed by ERα immunoblotting (Fig. 3). ERα siRNA specifically reduced the expression of full-length 66 000 M_r ERα protein with no effect on other proteins detected by the ERα antibodies. Both endogenous and transfected ERα protein levels were decreased by 87–95% by ERα siRNA. Additionally, the sizes of the extra protein bands detected by the F10 antibody did not correspond to those detected by the HC-20 antibody, and band patterns

produced by these antibodies, respectively, were similar in the tissue extracts and the hTERT-HM cell extracts (Figs 2 and 3). Therefore, we have found no evidence of ERα splice variants in the human myometrium and conclude that immunoreactive bands other than the 66 000 M_r full-length ERα represent non-specific antibody binding.

GPR30 mRNA levels did not vary significantly with GA (linear regression performed on log-transformed data; $r^2=0.1095$, $P=0.143$; $n=21$), but regression analysis indicated that there was a significant relationship between GPR30 mRNA expression and labor ($r^2=0.2286$, $P=0.028$; $n=21$; Fig. 4A). GPR30 mRNA abundance was significantly lower in myometrial samples from laboring compared with non-laboring women ($P=0.0085$, t -test with Welch's correction for unequal variance). GPR30 protein was detected as a 55 000 M_r immunoreactive protein band in the pregnant myometrium (Fig. 4B), as has been reported previously in human endometrium and decidua (Kolkova *et al.* 2010). GPR30 protein levels did not change significantly with GA (linear regression; $r^2=0.0051$, $P=0.770$; $n=19$) or labor onset ($r^2=0.1801$, $P=0.07$; $n=19$; Fig. 4C). The number of myometrial samples included in the PCR analyses is different from the sample number in the protein analyses, as not all tissue samples were of sufficient size to allow for both analyses to be performed. In 13 samples in which both GPR30 mRNA and protein were measured, there was no correlation between mRNA and protein levels (Spearman's rank correlation, $P=0.6401$).

Figure 3 ERα siRNA in hTERT-HM myometrial smooth muscle cells. Cells were transfected with control siRNA or siRNA targeting ERα, and some cells were co-transfected with a plasmid coding for ERα. MCF7 breast cancer cell lysate was used as a positive control for ERα expression. ERα siRNA reduced expression of full-length ERα protein (M_r: 66 000) with no effect on other, apparently non-specific, protein bands detected by the ERα antibodies. The experiment was performed twice and the data shown are representative of both experiments. The results shown were produced from a single membrane that was probed sequentially with ERα F10, ERα HC-20, and GAPDH antibodies; numbers and arrows at the left of the panels indicate M_r ($\times 10^{-3}$).

E₂ stimulates rapid ERK1/2 phosphorylation in pregnant human myometrial explants

E₂ at physiological concentrations (1 or 10 nM) stimulated phosphorylation of ERK1/2 within 30 min in explants of human term myometrium (Fig. 5A). PMA served as a positive control for stimulation of ERK1/2 phosphorylation (fold changes relative to vehicle controls: 1 nM E₂, 1.69 ± 0.27 (mean $\pm$ S.E.M.); 10 nM E₂, 2.02 ± 0.57 ; PMA, 1.68 ± 0.33). The stimulation of ERK phosphorylation by E₂ (10 nM for 10 min) was abrogated by pre-incubation with U0126, a highly specific inhibitor of the upstream MAPK, MAPK kinase 1/2 (MEK1/2), or with the ERα antagonist ICI 182 780 (Fig. 5B; fold changes relative to vehicle controls: E₂, 1.48 ± 0.09 (mean $\pm$ S.E.M.); E₂+U0126, 0.31 ± 0.14 ; E₂+ICI, 0.81 ± 0.11). Contrary to its action on ERα, ICI 182 780 is a GPR30 agonist (Filardo *et al.* 2002, Lucas *et al.* 2010); therefore, its inhibitory effect indicates that the E₂-stimulated activation of ERK/MAPK signaling in the myometrium is mediated by ERα and not by GPR30. Given that ERβ mRNA expression is virtually undetectable in the pregnant myometrium (Fig. 1), the involvement of this ER isoform in the observed rapid ERK signaling can be effectively ruled out. Control experiments have also shown that ERα mRNA continues to be expressed in myometrial explants after 24 h in culture, albeit at $\sim 38\%$ of the levels in tissue snap-frozen at time 0 (data not shown).

Figure 4 GPR30 expression in pregnant human myometrium. (A) *GPR30* mRNA levels in individual patient samples during pregnancy; NIL, not in labor ($n=14$; filled circles); IL, in labor ($n=7$; open circles); * $P=0.0085$; t -test with Welch's correction for unequal variances. (B) Representative GPR30 immunoblot in term myometrium. (C) Myometrial GPR30 protein abundance; NIL, $n=9$; IL, $n=10$.

Effect of MEK inhibition on E_2 -stimulated myometrial *OXTR* gene expression

E_2 (10 nM for 6 h) significantly increased *OXTR* mRNA expression in myometrial explants ($P<0.05$, repeated measures ANOVA with Dunnett's *post-hoc* test; Fig. 5C; 1.4 ± 0.16 -fold increase over vehicle controls (mean $\pm$ s.e.m.)). This effect was blocked by pre-incubation with the MEK inhibitor U0126 (*OXTR* mRNA levels in explants treated with E_2 + U0126 were 1.05 ± 0.15 -fold higher than vehicle controls, no significant difference). U0126 alone had no effect on basal *OXTR* gene expression (1.00 ± 0.18 -fold change compared with controls), and *hCaD* mRNA levels, used as internal reference, were not affected by any of the treatments (repeated measures ANOVA, $P=0.4668$). These results indicate that the MEK/ERK signaling pathway is involved in the stimulation of *OXTR* gene expression by E_2 .

Effect of GA and labor status on ERK1/2 activation in myometrium

ERK2 phosphorylation levels increased significantly in myometrial tissues with advancing GA at collection (Fig. 6A; linear regression performed on data normalized to protein input, $r^2=0.1800$, $P=0.0218$, $n=29$), but there was no relationship between ERK2 phosphorylation and labor status ($r^2=0.0000$, $P=0.9994$). ERK1 phosphorylation levels did not change significantly with either gestation ($r^2=0.0692$, $P=0.1680$) or labor ($r^2=0.0473$, $P=0.2573$) when normalized to protein input (Fig. 6B). Total ERK protein abundance decreased significantly with advancing GA (Fig. 6C; $r^2=0.2990$, $P=0.0021$), leading to a significant increase in the ratio of both pERK2/total ERK (Fig. 6D; $r^2=0.3056$, $P=0.002$) and pERK1/total ERK (Fig. 6E; $r^2=0.1853$, $P=0.02$) with advancing gestation. There was no significant change in total ERK levels or the pERK1 or 2/total ERK ratios with labor onset. Therefore, despite a decrease in total ERK expression, the activity of the ERK pathway increases significantly in the myometrium with advancing gestation as shown by an independent increase in ERK2 phosphorylation. These data suggest that ERK activation occurs during the prelude to active labor, rather than being a consequence of labor. Phosphorylation of ERK1 appears to be increased in order to maintain consistent ERK1 activity with advancing gestation in the face of decreasing total ERK levels.

Discussion

Although estrogens are known to induce myometrial contractions and labor in animal species such as the sheep (Wu *et al.* 2004) and to promote the expression and/or function of contraction-associated proteins in human myometrial smooth muscle cells (Di *et al.* 2001, Knock *et al.* 2001), the genomic targets and signaling pathways controlled by estrogens in the pregnant uterus are poorly understood. Considering the importance of estrogen sensitivity in regulating the contractile capacity of the myometrium, it is surprising that ER expression in the human pregnant myometrium has not been systematically characterized. Our qRT-PCR data indicate that ER α is the most abundantly expressed ER in the myometrium during pregnancy, at least at the level of gene expression. ER α mRNA abundance was significantly higher in the myometrial tissues from women in labor compared with quiescent myometrium, which is similar to data we have reported previously (Mesiano *et al.* 2002, Bisits *et al.* 2005). Myometrial ER β mRNA levels were extremely low. While these data are in conflict with a previous report showing ER β expression in term, non-laboring myometrium (Wu *et al.* 2000), they are in concurrence with our previously published data, which showed extremely low myometrial ER β mRNA levels in a different population of pregnant women and using different PCR primers

Figure 5 (A) Estradiol (E₂; 1 or 10 nM) treatment for 30 min stimulated phosphorylation of ERK1/2 in human myometrial explants, compared to vehicle-treated controls. PMA (100 nM) served as a positive control for stimulation of ERK phosphorylation. The lanes were from the same gel but the PMA lane was non-contiguous. Phospho-ERK levels were quantified relative to total ERK levels and the results are shown as mean ± S.E.M., normalized to vehicle-treated controls. **P* < 0.05, paired *t*-test comparing treatment and control. Fold changes relative to vehicle controls: 1 nM E₂, 1.69 ± 0.27 (mean ± S.E.M.); 10 nM E₂, 2.02 ± 0.57; PMA, 1.68 ± 0.33; (B) E₂ (10 nM for 10 min) stimulated ERK1/2 phosphorylation, which was inhibited by pre-treatment with the MEK inhibitor U0126 (10 μM) or the estrogen receptor antagonist ICI 182 780 (1 μM). The same pattern was seen when explants were stimulated for 30 min (data not shown). Phospho-ERK levels, relative to total ERK levels, are shown as mean ± S.E.M., normalized to vehicle-treated controls. Different letters denote significant differences between groups (*P* < 0.05; repeated measures ANOVA followed by paired *t*-tests with Bonferroni's correction for multiple comparisons; *n* = 4 independent experiments; fold changes relative to vehicle controls: E₂, 1.48 ± 0.09 (mean ± S.E.M.); E₂ + U0126, 0.31 ± 0.14; E₂ + ICI, 0.81 ± 0.11). (C) OXTR mRNA levels in term myometrial explants were increased by stimulation with E₂ (10 nM; for 6 h) and were reduced to basal levels by pre-incubation with the MEK inhibitor U0126 (10 μM). Data are from myometrial tissues obtained from four different women and are presented as mean ± S.E.M. and normalized to vehicle-treated controls. Fold changes relative to vehicle controls: E₂, 1.4 ± 0.16 (mean ± S.E.M.); E₂ + U0126, 1.05 ± 0.15; U0126, 1.00 ± 0.18. **P* < 0.05 (vehicle vs E₂; repeated measures ANOVA with Dunnett's *post-hoc* test).

(Mesiano *et al.* 2002). In both cases, the validity of our ERβ qRT-PCR assays was confirmed by measuring ERβ mRNA in T47D cells. While we observed robust expression of ERα mRNA in the pregnant myometrium, ERα protein expression was virtually undetectable by immunoblotting with three commercially available ERα antibodies. Similar results have been reported previously (Geimonen *et al.* 1998).

Our data using the proteasome inhibitor MG132, however, indicate that ERα mRNA is indeed translated into ERα protein in the pregnant myometrium, but that this protein is targeted for rapid turnover through the ubiquitin–proteasome pathway in the myometrium leading to low steady-state levels. Similar data have been reported for ERα in human breast cancer cell lines (Chu *et al.* 2007) and HeLa cells (Nawaz *et al.* 1999).

The discovery that GPR30 is a membrane-associated ER added a novel dimension to the complexity of estrogen signaling. Controversy exists, however, regarding whether GPR30 is truly an ER (Levin 2009, Langer *et al.* 2010), with several studies showing that GPR30 directly binds E₂ (Revankar *et al.* 2005, Thomas *et al.* 2005, Thomas & Dong 2006) and another study showing that it does not (Otto *et al.* 2008). It also remains unclear whether GPR30 localizes to the plasma membrane (Funakoshi *et al.* 2006, Filardo *et al.* 2007) or to the endoplasmic reticulum (Revankar *et al.* 2005, Otto *et al.* 2008). GPR30 clearly mediates components of estrogen signaling, as evidenced by multiple studies that confer estrogen responsiveness by inducing GPR30 expression or repress estrogen signaling via GPR30 knockdown or inhibition (reviewed in Prossnitz & Maggiolini (2009) and Maggiolini & Picard (2010)). On balance, the current weight of evidence suggests that GPR30 is most likely a bona fide ER, and our data are similar to a recent report showing that GPR30 mRNA and protein are present in the term pregnant human myometrium (Maiti *et al.* 2011). The role of GPR30 in reproduction and especially uterine function during pregnancy is unknown; however, GPR30 knockout mice have unimpaired fertility, retain normal uterine estrogenic responses, and appear to have normal pregnancies and parturition (Wang *et al.* 2008, Isensee *et al.* 2009, Otto *et al.* 2009, Windahl *et al.* 2009). Current data therefore suggest that the role of GPR30 in mediating estrogen actions in the pregnant myometrium is minimal. We have shown that E₂-induced ERK1/2 phosphorylation was inhibited by ICI 182 780 in the myometrium. ICI 182 780 inhibits ERα and ERβ signaling but acts as an agonist on GPR30 (Filardo *et al.* 2002, Lucas *et al.* 2010). Our results therefore indicate that E₂-stimulated ERK1/2 phosphorylation in myometrial explants was mediated by a classical ER and not GPR30. Owing to the lack of ERβ expression in the pregnant human myometrium, we reason that this rapid effect was mediated by ERα. While Maiti *et al.* (2011) showed that a specific activator of GPR30, G1, stimulated ERK1/2 phosphorylation in human myometrial explants, their study did not examine the contribution of GPR30 to E₂ action. Rapid activation of non-genomic signaling via ERα has been reported in other estrogen-responsive tissues and appears to be cell-type specific (Vasudevan & Pfaff 2008); however, this is a novel signaling mechanism in the pregnant myometrium. Studies using ERα inhibition or knockdown techniques, or conversely the induction of ERα expression, have demonstrated the reliance on this receptor for non-genomic estrogen signaling in vascular endothelial cells (Chen *et al.* 1999, Lu *et al.* 2004),

Figure 6 Total and phosphorylated ERK levels in pregnant human myometrium. (A) pERK2 protein levels (expressed as arbitrary densitometry units); (B) pERK1 protein levels; (C) total ERK protein levels; (D) pERK2/total ERK protein ratio; and, (E) pERK1/total ERK protein ratio. For all panels, NIL, not in labor ($n=14$; filled circles); IL, in labor ($n=15$; open circles). Regression analysis showed that myometrial pERK2 abundance increased significantly with advancing gestational age ($P=0.022$) while total ERK abundance decreased significantly ($P=0.002$), and there was no significant change in pERK1 abundance. When normalized to total ERK expression, there was a significant effect of gestational age on both pERK1 ($P=0.02$) and pERK2 ($P=0.002$) levels. There was no effect of labor on total or phosphorylated ERK abundance or on the pERK1 or 2/total ERK protein ratios.

CHO cells (Razandi *et al.* 1999), and breast cancer cells (Pedram *et al.* 2006, Madak-Erdogan *et al.* 2008). Additionally, cells from ER α / β knockout mice have impaired non-genomic E₂ signaling compared with cells from wild-type mice (Abraham *et al.* 2004, Pedram *et al.* 2006). Our data support the concept that, upon ligand activation, ER α interacts directly with cytoplasmic signaling molecules leading to activation of the ERK/MAPK cascade in the pregnant human myometrium.

Our findings support the hypothesis that extranuclear actions of E₂ are important for the induction of pro-contraction gene expression in the myometrium. The MEK inhibitor U0126 completely blocked the ability of E₂ to stimulate increased *OXTR* mRNA expression in myometrial explants (Fig. 5C). These data provide strong evidence that the MEK/ERK cascade is the principal pathway through which E₂ increases *OXTR* mRNA expression in our experimental system. We have not measured changes in the *OXTR* protein levels in this study, although we acknowledge the importance of measuring protein expression to appraise the functional consequences of gene expression changes. *OXTR* protein turnover is expected to be slower than mRNA turnover and is potentially under additional regulation, and we have not validated our explant culture system for the conditions required to evaluate changes in protein levels in response to E₂, which would include longer incubation times. The focus of this study was instead the regulation of the *OXTR* gene by E₂. There is no complete ERE in the human *OXTR* promoter (Gimpl & Fahrenholz 2001), which is in concurrence with the proposal that E₂ stimulates *OXTR* mRNA expression via an indirect mechanism. It has previously been shown that 25% of all E₂-responsive genes in MCF7 cells are altered via activation

of non-genomic signaling mediated by ER α (Madak-Erdogan *et al.* 2008). It will be important in future studies to determine the full spectrum of estrogen-responsive genes and their protein products in the myometrium, in particular those related to contractility, and to discover which of these genes are mediated via direct genomic ER α transcriptional activity vs indirect, non-genomic pathways. This information could influence the design of therapeutics that target the pro-contractile effect of E₂ in the myometrium to treat and/or prevent preterm labor.

Myometrial ERK2 phosphorylation increased significantly with advancing GA in accordance with previously reported results in the pregnant rat uterus (Oldenhof *et al.* 2002, Serrano-Sanchez *et al.* 2008). We observed no change in ERK1/2 phosphorylation in the myometrium with labor, which agrees with a previous report on the rat myometrium (Oldenhof *et al.* 2002) but differs from that of Li *et al.* (2003) who reported increased ERK2 phosphorylation in the rat uterus during labor. While total ERK protein levels do not appear to change in the rat myometrium with pregnancy or labor (Li *et al.* 2003), we saw a decrease in total ERK in human myometrium with advancing gestation. However, the increase in phospho-ERK2 levels, measured independent of total ERK levels, indicates that the activity of this pathway indeed increases with advancing gestation. Paul *et al.* (2011) reported that total ERK2 protein levels in human myometrium increased slightly with labor onset at term and that there was no change in the ratio of pERK2/total ERK2, which largely agrees with our data, although we found no significant change in total ERK protein following labor onset. Our data are the first report of ERK1/2 phosphorylation levels in relation to advancing GA in the human myometrium. Given

the elevated levels of myometrial ERK phosphorylation in late gestation, and the delayed labor caused by inhibition of the ERK pathway in RU486-treated rats (Li *et al.* 2004), we hypothesize that this is an important signaling pathway that is activated as part of the parturition process. Increased ERK phosphorylation may remodel the myometrium into a contractile phenotype by modulating gene expression via activation of transcription factors (Yoon & Seger 2006) and/or by altering the contractile apparatus via phosphorylation of cytoskeletal proteins including hCaD, paxillin, and FAK (Yoon & Seger 2006). While ERK proteins may become phosphorylated in response to many stimuli in addition to estrogens, we hypothesize that ER α -mediated estrogen activation in the uterus may be partially responsible for stimulating the elevated levels of ERK signaling observed at late gestation. Thus, we propose that estrogenic activity in the human pregnant myometrium is predominantly mediated by ER α , which affects gene expression by a combination of non-genomic and genomic signaling. The novel membrane ER, GPR30, is also expressed in the pregnant human myometrium, but its contribution to mediating estrogen action appears minor. Nevertheless, it is important to consider the multiple ERs and pathways of receptor action in determining the molecular mechanisms by which estrogens regulate myometrial contractility in pregnant women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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