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Effects of combined progesterone and 17β-estradiol treatment on the transcriptome of cultured human myometrial smooth muscle cells

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Abstract

A transcriptomic analysis of cultured human uterine smooth muscle cells (hUtSMCs) was performed in order to examine gene expression profiles in smooth muscle in an environment containing the two major steroid hormones that regulate the human myometrium in physiological states associated with estrous, pregnancy, labor, and pathophysiological states such as leiomyoma and endometrial cancer. hUtSMCs were treated with progesterone (P4) and 17β-estradiol (E2) individually and in combination, in the presence and absence of RU486 (mifepristone). Transcription of many genes was modulated in the presence of P4 or E2 alone but almost six times more genes were transcriptionally modulated in the presence of the P4/E2 hormone combination. In total 796 annotated genes were significantly differentially expressed in the presence of both P4 and E2 relative to their expression in untreated cells. Functional withdrawal of progesterone by addition of RU486 effectively reversed almost all transcriptional changes caused by P4/E2 treatment. Gene ontology analysis of differentially expressed genes revealed a strong association between P4/E2 treatment and down-regulated expression of genes involved in cell communication, signal transduction, channel activity, inflammatory response and differentiation. Up-regulated processes included cell survival, gene transcription, steroid hormone biosynthesis, muscle development, insulin receptor signalling and cell growth.

Introduction

The two steroid hormones, estrogen and progesterone, play vital roles in maintaining pregnancy and initiating labor (51) and more generally in myometrial physiology and contractility. Progesterone maintains uterine quiescence during pregnancy, while estrogen induces uterine contraction at labor (49-51). These hormones fluctuate relative to each other throughout life from puberty, through the fertile years, to the post-fertile years and also within these life stages during specific physiological states such as estrous, pregnancy, labor and menopause. Furthermore, both hormones have profound effects on pathophysiological conditions such as leiomyoma, endometriosis and endometrial cancer.

In maternal physiology these hormones induce changes that prepare the mother for pregnancy and aid in conceptus development (11). In most species, a sharp decline in circulating progesterone and an increase in estrogen are observed towards parturition (11, 38). However, in humans, no marked difference in the levels of circulating progesterone or estrogen is noted before or at term (51). Hence, in humans, withdrawal of progesterone and responsiveness of the myometrium to circulating estrogen during labor is believed to occur at a functional level, with changes in hormone receptors playing a more important role than the circulating levels of the hormone themselves (50). Understanding the molecular mechanisms underlying the transition of the uterus from a relatively quiescent organ to an actively contracting organ is of utmost importance in predicting and treating disorders of pregnancy, including preterm labor.

Several functional genomics studies have been carried out across a range of species, including humans, in order to understand the molecular changes underlying the transition of the quiescent uterus to a rhythmic and actively contracting organ during parturition (1, 5, 8, 12, 13, 16, 17, 26, 31, 53, 56, 60). In their analysis of all published microarray-based transcriptomic analyses of labouring and non-laboring myometrium, Breuiller-Fouche and colleagues identified little overlap between results from different studies (10). However, each study has identified key molecular regulators and pathways that have broadened our understanding of the complex mechanisms underlying myometrial quiescence and activation. Differences in the cascade of events that precede human labor, compared to well-studied

animal models, makes extrapolation of conclusions from model organisms to humans difficult. The stringency in ethical regulations and difficulty in obtaining patient samples understandably makes human studies challenging. Therefore, developing a model cell culture system to study biological events associated with progesterone and estrogen is both relevant and important.

The human myometrium is composed of two distinct smooth muscle layers with a surface epithelium and a supporting capillary network and stromal cells. Clearly a smooth muscle cell culture system cannot model all the intricate interactions within the native myometrium *in vivo*, nor can it replicate the complex cell signalling and hormonal interactions, as well as other physiological and pharmacological changes that occur in the human myometrium in pregnancy and at the onset of labor. Isolated *in vitro* cell culture systems, while not mimicking the pregnant laboring human myometrium, can, however yield important clues as to the roles played by the smooth muscle cells – roles that can be obscured in whole tissue studies. As a result, studies of this nature can also provide important clues as to the overall myometrial system, which can inform the complex physiological events occurring *in vivo* in pregnant myometrium at the onset of labor.

No transcriptomic study has yet been carried out to investigate the combined effects of 17β-estradiol (E2) and progesterone (P4), and the impact of the progesterone inhibitor (RU486) on cultured uterine smooth muscle cells in relation to myometrial contractility, though an *in vitro* study has recently been published on the gene expression profile of immortalised human myometrial cells primarily in response to progesterone (69). Global transcriptomic profiling of cultured human uterine smooth muscle cells (hUtSMCs) following combined P4 and E2 steroid hormone treatments, including combined hormone treatment in the presence of the progesterone antagonist RU486 to functionally withdraw progesterone as occurs at human labor, is presented here.

Materials and methods

Cell culture and treatment:

The primary human uterine smooth muscle cells (hUtSMCs) used were obtained from Lonza (CLONETICS™ Uterine Smooth Muscle Cell Systems, CC-2562) and are identical to the Cambrex product described in other published studies (20, 70). It has previously been shown that E2 treatment of these hUtSMC cells produces effects expected of steroid hormones on myometrial cells, for example, both mRNA and protein expression of previously reported E2-responsive genes, CD38 (23) and GJA1 (also known as connexin CX43) (22) responded as expected to E2 treatment (14). As a control for the Lonza CLONETICS™ cells, normal primary smooth muscle myometrial cells were isolated from a pregnant non-labouring patient, with ethical approval having been obtained through the University College Hospital Galway ethics committee, and informed consent obtained from donors.

hUtSMCs were cultured in complete Dulbecco's modified essential medium (DMEM), supplemented with 10 % (v/v) fetal bovine serum (FBS) until 50 % confluent. Cells were preconditioned overnight with Phenol Red-free DMEM (PRF-DMEM), supplemented with 10 % (v/v) double charcoal stripped fetal bovine serum (CS-FBS) (Biosera). Cells were subsequently starved of endogenous steroids by incubation in PRF-DMEM supplemented with 2 % (v/v) CS-FBS, henceforth referred to as control media, until the cells were 80 % confluent. The time schedule for additions to the hUtSMCs is outlined in Figure 1.

In brief, at 100 % confluence the following additions were made to flasks 1-6: (1) no addition, (2) 10 nM 17β-estradiol (E2) (Sigma Aldrich), (3) & (6) 100 nM progesterone (P4) (Sigma Aldrich), and (4) & (5) P4 + E2 (P4/E2 samples). 48 h after these additions, flasks (5) and (6) were supplemented with 1 μM RU486 (Sigma Aldrich). Flasks (4) and (5) had been primed at 80 % confluence by supplementation with 100 nM progesterone (P4). All samples were harvested 72 h after hormonal treatments at 100% confluence. Each single experiment consisted of all six treatments and each experiment was repeated three times on different occasions (18 samples).

Final concentrations of E2 (14), P4 and RU486 were determined empirically. For this study, the optimal level of P4 addition was determined by real time RT-PCR quantification of the BCL2 gene, a

known P4-responsive gene (48, 72), over the range 10-500 nM P4 (data not shown). Induced expression did not increase substantially above 100 nM P4. Again following the gene expression level of BCL2, it was shown that 1μM RU486 significantly inhibited the effects of 100 nM P4. These concentrations are in line with similar studies (41, 47, 72).

RNA isolation and quantitative RT-PCR:

RNA was isolated using the RNeasy Mini Kit following the manufacturer's protocol (Qiagen). RNA was quantified using the NanoDrop 1000 (Thermo Scientific) and the quality checked using a Bioanalyzer 2100 (Agilent Technologies). Polymerase chain reaction (PCR) was performed using the GoTaq® DNA Polymerase Kit (Promega) according to manufacturer's protocol. The list of genes and corresponding primers sequences obtained from PrimerDepot (19) (http://primerdepot.nci.nih.gov/) are provided in Table 1. Reverse transcription (RT) was carried out using 20 U Superscript II Reverse Transcriptase (Invitrogen) with 2 μg RNA and 50 pmol Oligo dT. Real-time RT-PCR was carried out using the QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect primers for BCL2 (QT00025011), TTPA (QT00012040) and EMILIN2 (QT00092246) and subjected to 40 cycles of amplification in an ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems). All gene quantities were normalised to the housekeeping gene (GAPDH). Relative expression was evaluated by the ΔΔCt method.

Microarray hybridization and data analysis:

Isolated RNA was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion) following the manufacturer's protocol. cDNA was hybridised against the Operon Human Genome Array-Ready Oligo Set Microarrays™ ((AROS) Version 4.0). The slides contained 35,035 oligonucleotide probes representing approximately 25,100 unique genes and 39,600 transcripts, excluding control oligos. All hybridizations were carried out using the OpArray (Operon) hybridization and wash solutions as recommended by the manufacturer. Slides were scanned at 543 nm (Cy3) with a ScanArray Express HT scanner (Perkin Elmer) and quantified using GenePix 6.0 software (Axon Instruments, Inc.). For this particular dataset all slides were scanned three times

successively and the median raw values only were inputted into GeneSpring. All data normalization and analyses were carried out in GeneSpring GX12.0 (Agilent Technologies). Data was log transformed and normalized by the Percentile Shift Method using the 75th percentile. Subsequently all normalized data was baseline transformed to the median of all samples. Intensity values for all entities (35,357) were initially filtered to exclude values which were less than 50 in at least 2 of 6 conditions (CON, E2, P4, P4/E2, P4/E2 + RU486 or P4 + RU486). Significantly differentially expressed genes were identified based on log values using a moderated *t*-test (68), with a *P*-value cut-off of 0.05 and a fold change minimum of 1.5 fold. No multiple testing correction was applied. All data has been submitted to the Gene Expression Omnibus (GSE59231).

Gene ontology and transciptional analysis of genes:

A web-based system for the detection of over-represented conserved transcription factor binding sites, oPOSSUM, was used to analyse sequences of specific, apparent co-regulated, gene sets (33). Initially the genes differentially expressed on addition of individual hormones, P4 or E2, were analysed. Transcription factor binding sites were considered significant if either the Z-score was greater than 10 or the Fisher score was greater than 5. Gene ontology (GO) analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery, v6.7) (21). Some gene annotation was not recognized by DAVID and some genes had no GO annotation. Individual analyses were carried out on upregulated, downregulated and pooled regulated genes. The biological process GO category was given most weight though cell component and molecular function categories were noted where relevant, and other pathway and term analysis approaches using Panther, KEGG and PIR keywords (all within DAVID) were also included.

Statistical analysis:

All qPCR experiments were carried out in triplicate. Results are expressed as mean +/- standard deviation. *P*-values were used as a measure of the magnitude of the change and inter-subject variability among the treated and control samples. *P*-values were calculated by Student's *t*-test using

the GraphPad program (http://www.graphpad.com/quickcalcs/ttest2.cfm) and http://www.physics.csbsju.edu/stats/t-test_bulk_form.html.

Results

Transcriptomic analysis of hUtSMCs after hormone treatment:

Cells were (1) untreated, or treated with (2) E2 alone, (3) P4 alone, (4) P4 and E2 in combination (P4/E2), (5) RU486 after P4/E2 incubation (P4/E2 + RU486) and (6) RU486 after P4 incubation (P4 + RU486). Cells in samples 4 & 5 were primed with P4 (see Figure 1). Unlike other model organisms (both primate and non-primate), the human myometrium is exposed to high concentrations of both estrogen and progesterone during pregnancy (3). To mimic this steroid hormone environment, cultured hUtSMCs were exposed to both 10 nM E2 and 100 nM P4 (concentrations correlating to circulating levels in mid to late pregnancy). P4 additions were made 24 h prior to E2 additions to mimic the earlier elevation of circulating P4 levels in pregnancy. A total of 18 slides were analysed representing all conditions (CON, E2, P4, P4/E2, P4/E2 + RU486 and P4 + RU486), with each condition analysed in three independent experiments.

A total of 796 genes ($P<0.05$, fold change>1.5-fold) showed significant differential expression following P4/E2 co-treatment of which 364 genes were up-regulated and 432 down-regulated (Supplemental data Table 1). Fifty three genes showed fold changes exceeding 5-fold following P4/E2 treatment, of which the top 20 are shown (Table 2). Although 796 genes were significantly modulated transcriptionally by the combination of P4/E2 only 28 and 23 of these genes also showed significantly altered transcriptional regulation by the individual hormones E2 and P4 respectively (Figure 2). This suggests that the combined effect of the hormones was much greater than the sum of their individual effects. There were also genes whose expression suggested transcriptional modulation by the individual hormones (106 by P4, 110 by E2) and an absence of modulation by the combination of hormones (Figure 2). This may indicate antagonistic effects of the individual hormones.

To identify genes that were differentially expressed in response to addition of the P4 antagonist RU486, the expression profile of cells co-treated with P4/E2 was compared to that of cells co-treated with P4/E2 to which RU486 was subsequently added (see Figure 1). The expression of 666 fully annotated genes (>1.5-fold) was altered in cultured hUtSMCs treated with P4/E2 + RU486 compared

to cells treated with P4/E2. The expression of 304 genes was up-regulated and of 362 genes was down-regulated in this experimental group (Supplemental data Table 2). Seventy two genes showed fold changes exceeding 5-fold following P4/E2 + RU486 treatment, of which the top 20 are shown (Table 3).

The expression profiles of the significant P4/E2 differentially regulated genes (796 genes) showed modulated expression in some of the other conditions (E2, P4, P4 + RU486) but the vast majority showed reversal of transcriptional modulation in P4/E2 + RU486 conditions. Similarly the expression profiles of the significant P4/E2 ± RU486 differentially regulated genes (666 genes) showed modulated expression in some of the other conditions but the vast majority showed reversal of transcriptional modulation on addition of RU486. This suggested that virtually all P4/E2 regulated genes were also modulated by RU486 even though only approximately 25 % of genes were common to both sets. To show the similarity of the two sets all P4/E2 modulated genes and all P4/E2 ± RU486 modulated genes were separately clustered across all conditions investigated; both populations showed significant up- or down-regulation upon P4/E2 treatment and a return to near untreated levels following RU486 treatment. All regulated genes (here, for clarity, we only show the P4/E2 ± RU486 group, not the two groups) were clustered into five expression profile sets across all conditions (Figure 3).

The genes of the major cluster (cluster 1) showed no altered transcriptional regulation by P4, but considerable up-regulation by the P4/E2 combination. RU486 restored expression levels to approximately those of the control. This cluster was mirrored by a second large cluster (cluster 2) that showed down-regulated transcription by the P4/E2 combination. RU486 again reversed P4/E2 effects. A small cluster (cluster 3) was similar to cluster 2 but in addition these genes showed down-regulated transcription following RU486 treatment of P4 treated cells. Cluster 4 genes were down-regulated by E2 alone but up-regulated by the P4/E2 combination. Cluster 5 showed the strongest down-regulation of expression by the P4/E2 combination. Very few genes showed an expression profile different from the patterns shown in Figure 3. Comparison of the expression profiles of P4/E2 + RU486 treated cells

with cells treated with E2 alone, indicated that there were very few genes whose expression was differentially modulated between these two conditions, and insufficient to identify any novel GO term (data not shown). This and additional comparisons (e.g. CON vs P4+RU486 and CON vs P4/E2+RU486) generated gene lists for which GO analysis extracted very few terms of significance. Therefore, there was no novel functional category outside that of its role as a progesterone antagonist that could be ascribed to the effect of RU486.

Since gene expression is a measure of the combined actions of different transcription factors, it was of interest to investigate how the genes of each of the five expression profile clusters differed in their transcription factor binding site makeup. Using the 796 genes differentially regulated by P4/E2 as representative of the five clusters, oPOSSUM, a web-based system for the detection of over-represented conserved transcription factor binding sites, was used to analyse sequences of the gene set (33). Initial interrogation of genes differentially expressed on addition of individual hormones, P4 or E2, identified a number of significant transcription factor binding sites. Two transcription factors from the hormone-nuclear receptor family (NR1H2::RXRA and NR3C1) were overrepresented on E2 treatment and four from the Rel family (NFATC2, NF-kappaB, RELA, NFKB1) of transcription factors were overrepresented on P4 treatment (Table 4). Three of the five clusters of P4/E2 regulated genes (clusters 1, 2 and 3) showed significantly overrepresented binding sites for transcription factors. Of note, the putative transcription factors associated with clusters 1 and 3 genes showed a high degree of overlap, including forkhead (FOX) factors, NKx factors, ARID3A, PDX1, HOXA5, SRY, and PRRX2, yet the cluster 1 and cluster 3 gene expression profiles were almost opposites. Furthermore, forkhead factors, NKx factors and ARID3A were also significantly overrepresented in the E2 treated samples.

Quantitative and semi-quantitative RT PCR:

Microarray analysis was validated by quantitative or semi-quantitative RT-PCR on selected genes (Figure 4). In samples co-treated with P4 and E2, the down-regulation of TTPA (>19-fold in microarray and >5-fold in qPCR, $P<0.005$) was confirmed by qRT-PCR (Figure 4A). The signals for

both BCL2 probes on the array were too weak to escape data filtering but qPCR revealed >5-fold up-regulation (P<0.005). In the experimental group where P4 was functionally withdrawn from the P4/E2 co-treated system by the addition of RU486, real time qRT-PCR and semi-quantitative RT-PCR analyses confirmed differential gene expression of EMILIN2 (down-regulated, 10-fold) and TTPA (highest up-regulated gene identified, 31-fold) (Figure 4B). In addition, semi-quantitative RT-PCR was carried out on eight genes (four down-regulated - NDUFA10, MYL6, SRD5A2 and PLCD4, and four up-regulated - DHRS7C, LEPROT, C10orf99 and SCN4A) identified as differentially expressed in P4/E2 + RU486-treated samples compared to (P4/E2)-treated hUtSMCs (Figure 4C). Differential expression of all selected genes identified by microarray analysis was confirmed by RT-PCR (Figure 4C, D).

Gene Ontology analysis of differentially expressed genes:

To understand the biological significance of the differentially expressed genes identified following addition of RU486 to P4/E2-treated hUtSMCs, gene ontology (GO) analysis was performed using DAVID (21). Of the original significant 666 genes identified, GO analysis identified significant terms for 397 genes. The most enriched biological process cluster, which included mostly up-regulated genes, related to signal transduction (Figure 5). In addition, using the other two GO categories (cell component and molecular function), it was clear that channel activity, transcription and translation were very significant but that most activity was associated with the plasma membrane of the cell. Other gene ontology pathway and term analysis approaches using Panther, KEGG and PIR keywords (all within DAVID) reflected the initial GO analysis, though additional informative terms such as androgen and estrogen metabolism and cell surface receptor mediated signal transduction were also significant. The direction of regulation was clear for genes assigned to some GO terms: transcription, translation and metabolism terms included genes that were mainly down-regulated whereas cell communication, response to external stimulus and immune response genes were generally up-regulated. Transport (solutes and ions) processes were associated with both up- and down-regulated genes.

Similarly, GO analysis of the 796 genes differentially expressed in hUtSMCs treated with P4/E2, identified GO terms for 535 genes (data not shown). The most significant biological process terms were cell communication, signal transduction, differentiation, apoptosis, transcription, sensory perception and inflammatory response. Because P4/E2 + RU486 treatment returned genes up- or down-regulated in P4/E2 conditions to near control levels the gene ontology terms for both analyses (Figure 5) were similar.

Discussion

This study describes gene expression changes in response to estradiol and progesterone either alone or in combination, in an *in vitro* culture system of myometrial smooth muscle cells. In addition, it identifies transcriptional alterations elicited by the progesterone antagonist RU486 on P4/E2 treated cells, when added to simulate functional withdrawal of progesterone in the cell culture system, as occurs at the onset of human labour. A large number of genes were significantly transcriptionally modulated by estradiol (110) or progesterone (106) individually, but the most striking altered expression profiles were obtained under the combined P4/E2 treatment conditions (796 genes). The addition of RU486 to P4/E2 treated cells led to altered expression transcriptional profiles for 666 genes. Our study revealed genes and pathways related to those identified in previous studies that analysed the global transcriptomic profiles in pregnant and laboring myometrium, in humans and other species (32, 53, 62, 69). The *in vitro* analysis of transcriptional alterations in hUtSMCs in response to two of the main steroid regulators provides important insights into the role played by a key individual cell type, the smooth muscle cell, in all physiological and pathophysiological states where these two hormones fluctuate. The main biological processes reported to be altered in the human myometrium during pregnancy and parturition (for which most data are available) are muscle contraction, ion channels and transporters, cell proliferation, immune and inflammatory responses and coagulation (53).

The human uterine smooth muscle cell culture system used in these studies, clearly does not replicate the complex interplay between different cell types in the uterus *in vivo*, during pregnancy and at labor. This *in vitro* model provides information on the specific and direct response of myometrial smooth muscle cells to different treatments. As with similar *in vitro* models, this cell culture model system enables analysis of the effect of different treatments on specific cell types, in isolation; each type of study, in its own way, provides evidence of potential biological events occurring *in vivo*. These findings, therefore, provide the basis for future investigations in human myometrial tissue *ex vivo* and *in vivo*. The use of hUtSMCs to assess the effects of a broad range of natural biological compounds, including steroid hormones, and potential tocolytic agents on uterine smooth muscle cells has been

reported in previous publications by ourselves and by others (4, 14, 27, 39) . Confirmed expression of the oxytocin receptor (OXTR), calponin (CNN1) and smoothelin (SMTN) genes (71) (data not shown) and immunofluorescence staining of smooth muscle α-actin (55) attested to the smooth muscle origin of the cells.

Expression of both endogenous estrogen and progesterone receptors in myometrial smooth muscle cells is reported to decrease rapidly in culture (74). However, expression of estrogen receptor-alpha (ER-α, now named ESR1) preferentially promotes the expression of progesterone receptors (29). Microarray analysis confirmed the expression of ESR1 (ER-α), ESR2 (ER-β), PGR and other related receptors (OXTR, ESRRA, ESRRB, mPRA, mPRB, mPRG and PGRMC1) in hUtSMCs. qRT-PCR showed levels of the OXTR and ESR1 receptors at the approximate levels in pregnant non-labouring myometrial cells (data not shown). Estrogen-responsiveness of these cells had been shown previously (14) and progesterone responsiveness was confirmed by the >5-fold up-regulation of BCL2 gene, a known P4 responsive gene (48, 72) (data not shown).

Progesterone is generally referred to as the hormone that promotes myometrial relaxation (2, 11). Its withdrawal at any stage of pregnancy initiates muscle contraction leading to labor (52). G protein-coupled receptor (GPCR) activation and reversible Ca^{2+}-dependent phosphorylation of MLC, catalysed by myosin light chain kinase (MLCK), is a major determinant of smooth muscle contraction (7, 34). The regulation of contractility is described by gene ontology terms such as G protein-coupled signalling, cell communication, channel activity (voltage-gated cation channels), all of which were very significant in the analysis of the significantly modulated genes in P4/E2 and RU486 treated cells in this study. Most of the genes associated with the G protein-coupled signalling GO term were transcriptionally up-regulated following the addition of RU486 to P4/E2 treated cells, and down-regulated in the presence of P4/E2 compared to untreated cells. This is in agreement with the association of muscle relaxation (as modelled by P4/E2 conditions) with a general desensitization of G protein-coupled signalling. The neuropeptide FF receptor 1 (GPR147) gene was up-regulated 6.2-fold following the addition of RU486 and down-regulated 8.0-fold by P4/E2 alone. GPR73L1, also known as prokineticin receptor 2 (PROKR2), which is known to influence contraction-related effects

in smooth muscle (63), was up-regulated 5.3-fold in P4/E2 treated cells following the addition of RU486.

Another G protein-coupled receptor, the β2 adrenergic receptor (Q13715_HUMAN) mRNA was down-regulated (3.8-fold) following the addition of RU486 to P4/E2 treated cells. β-AR stimulates protein kinase A via cAMP, which opposes the contractile actions of PLC, phosphorylates myosin light chain kinase (MLCK) and inhibits phosphorylation of the myosin light chain (MLC). Other studies have found that β-adrenergic receptor (β-AR) induction by progesterone plays an important role in uterine relaxation during pregnancy, and our *in vitro* transcriptomic results support this role (67). A previous study demonstrated GPCR transcriptional up-regulation of the α1 adrenergic receptor and a number of other GPCRs in laboring rat myometrium (32). Other transcriptionally modulated players in the machinery of MLC phosphorylation were significantly down-regulated following addition of RU486 to P4/E2 treated cells, including: NUAK1 (NUAK family of SNF1-like kinase 1) (3.8-fold) (73); ADRBK2 (β-adrenergic receptor kinase 2) (58) (4.2-fold); and MYL6 (myosin light chain, smooth muscle-specific) (30) (5.2-fold).

The PLC family of proteins, plays an important role in the activation of myometrial contractility and induction of labor (24, 64). Activation of any of the several forms of PLC stimulates Ca^{2+} release from internal stores and the activation of contraction-associated proteins (6, 45). The expression of phospholipase C, beta 1 (PLCB1), delta 4 (PLCD4) and gamma 2 (PLCG2) were all significantly down-regulated in cultured hUtSMCs following addition of RU486 to P4/E2 treated cells.

Contraction of myometrial smooth muscle cells is synchronized across the tissue by the concerted action of gap junctions and channels that contribute to efficient cell communication. In the presence of P4/E2, cell communication was the most significant gene ontology term relating to transcriptionally down-regulated genes. Gap junction genes GJA8 (Connexin 50, Cx50) and GJA3 (Connexin 46, Cx46) were significantly down-regulated, 3.5-fold and 1.7-fold respectively, in hUtSMCs treated with P4/E2 and up-regulated following the addition of RU486. In the lens tissue of the eye, Cx50 has been associated with calmodulin/Ca^{2+} activity, phosphorylation of myosin light

chain, and interaction with aquaporin 0 (46). The results reported in this study, correlate with previous studies in pregnant non-laboring and laboring rat and mouse myometrium, reporting down-regulation of gap junctions during pregnancy and their transcriptional activation at labor (32, 62). The expression of aquaporin 5 (AQP5), one of several related aquaporin channels that control water transport across cells, has been shown to be regulated by both estrogen and progesterone (35, 61). Aquaporin 5 was down-regulated transcriptionally (2.9-fold) on addition of RU486 to P4/E2 treated hUtSMCs, in agreement with the results of a study by Helguera and colleagues in pregnant labouring rat myometrium, where AQP5 mRNA was found to be up-regulated during pregnancy and down-regulated at labor (32).

Ca^{2+} ion movement and transport are essential components of the muscle contraction process. Equally essential is the range of ion transporters (K^+, Na^+, Cl^-) that maintain an electrochemical gradient across the cell membrane and thereby regulate Ca^{2+} ion movement. Almost without exception, expression of genes associated with ion transport and cation channel activity were up-regulated following the addition of RU486 to P4/E2 treated hUtSMCs. The potassium voltage-gated channel (KCNQ2) was up-regulated 8.5-fold following RU486 addition and was down-regulated 7.4-fold in P4/E2 treated cells, relative to untreated cells. The voltage-gated, type 4 sodium channel, (SCN4A) showed similar large fold transcriptional changes, which were confirmed by qPCR. Other up-regulated channels following addition of RU486 included KCNJ10, KCNC2, SCN2B and the voltage dependent calcium channels CACNA1F and CACNA1C. Our results on ion channel expression are in agreement with previous studies comparing transcriptomes in pregnant and labouring mouse and rat myometrium, which also reported the up-regulation of calcium ion channel transcription at labor (32, 62).

Activation of immune and inflammatory responses was observed following the addition of RU486 to P4/E2 treated cells, however, as expected in our *in vitro* model there was little evidence of the major *in vivo* reported effectors IL8, IL6 and CXCL6. One significantly up-regulated (1.8-fold) gene was the regulator of calcineurin 2 (RCAN2), also known as calcipressin-2 (DSCR1L1). Expression of the RCAN family of proteins is induced by the NFAT transcription factors. Binding motifs for NFAT

were most notably overrepresented in the P4/E2 regulated genes (Table 4) suggesting that NFAT factors are important regulators of gene expression in these hormonal conditions (59). IL1RN was down-regulated 6.0-fold following addition of RU486, and since it antagonizes the pro-inflammatory interleukin 1, its down-regulation is essentially pro-inflammatory.

During the early stages of pregnancy, myometrial cells proliferate rapidly to accommodate the growing fetus (66) and reach a hypertrophic phase by mid-gestation (65). Late pregnancy in the rat myometrium is marked by an increase in apoptosis. In our system with P4/E2 treatment there was a balance of up- and down-regulated apoptosis-related genes but on addition of RU486, pro-apoptotic genes were in general down-regulated. Elastin microfibril interface-located protein 2 (EMILIN2) expression was significantly down-regulated in P4/E2 + RU486-treated hUtSMCs compared to P4/E2-treated cells. EMILIN2 binds to TRAIL receptor DR4 to induce apoptosis (54). The role of ribosomal proteins in regulating cell proliferation and as inducers of cell death is well studied. In the present study, ribosomal protein RPL23 was down-regulated (5.7-fold) in hUtSMCs treated with P4/E2+RU486. RPL23 blocks the interaction between tumor suppressor gene p53 and MDM2, activating p53 and thereby inhibiting cell proliferation (75). These results correlate with those of a previous study in mouse myometrium, which also found that pro-apoptotic genes were down-regulated at labor (62). BCL2 expression, an important determinant of cell proliferation, differentiation and tumorigenesis, was up-regulated 4.4-fold as determined by real time qRT-PCR under the P4/E2 treatment conditions, as were two other BCL2 family members, BCL2A1 and BCL2L10, on the microarray (18). CUL1 (cullin 1), another key regulator of proliferation and apoptosis (43, 77), was strongly up-regulated (9.6-fold) in P4/E2 treated cells. A core component of an E3 ubiquitin-protein ligase complex that targets specific proteins for degradation, CUL1 may also target cell cycle arrest proteins, therefore up-regulation of CUL1 can clearly have a key regulatory role in determining proliferation or apoptosis. These results concur with transcriptional activation of genes associated with cell division, proliferation and growth in rat myometrium, which were demonstrated to be up-regulated during pregnancy and down-regulated at labor (32).

A number of genes, known to be involved in the hormonal regulation of uterine contractility during pregnancy and labor, were identified as dysregulated as a result of the hormonal treatments of hUtSMCs. Steroid 5-alpha-reductase (SRD5A2) gene expression was down-regulated (>5-fold) in human myometrial smooth muscle cells treated with P4/E2 + RU486, compared to P4/E2 treated cells. Under the same conditions, aldo-keto reductase family 1, member D1 (AKR1D1), otherwise known as steroid 5-beta-reductase (SRD5B1), was up-regulated (>5-fold). Both genes are important regulators of progesterone metabolism and hence progesterone levels (15, 42).

TTPA, or alpha-tocopherol transfer protein, was up-regulated (>30-fold) following the addition of RU486 to P4/E2-treated cells. The importance of TTPA for the maintenance of pregnancy has been shown by the failure of TTPA knockout mice to reach term after implantation due to severe defects in placental development, which can be rescued with α-tocopherol (vitamin E) supplementation (36). Gap junctions in myometrial strips can be protected from chemical damage by administration of α-tocopherol (40).

In oPOSSUM analysis (Table 4) of P4/E2 regulated genes one common motif is that for NFAT. It was also overrepresented in the E2-regulated genes but not evident in the P4-regulated genes. Five isoforms of NFAT are known; for four isoforms, signalling is mediated by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin and all are expressed in human myometrial cells (59). It has been shown that NFAT can respond to oxytocin pulses and regulate transcription by controlled translocation between the nucleus (on) and cytoplasm (off). NFAT could therefore be playing an important role in parturition by linking oxytocin and muscle contraction to NFAT transcriptional activation via Ca^{2+} levels and calcineurin (59). There is also an overrepresentation of forkhead transcription factor-binding sites in genes regulated by the P4/E2 combination treatment. Forkhead transcription factors have been associated with TA-rich ESR1 sites as opposed to GC-rich ESR2 sites (44). oPOSSUM analysis (Table 4) also identified genes regulated by the P4/E2 combination treatment with binding motifs for transcription factors NK-2/NK-3 (NKx2-5, NKx3-1 or NKx3-2), HOXA5 and ARID 3A. Nkx2-5 belongs to the NK-2 homeobox family and has been implicated in myocardial development, in both cardiomyocytes and smooth muscle (76). In an *in vitro* model of

smooth muscle differentiation, the promoter of the smooth muscle gamma isoactin gene has been shown to bind NK-2 and serum response factor (SRF) in intestinal smooth muscle cells (57). In uterine smooth muscle cells myocyte enhancer factor 2 (MEF2) is also bound in this complex (57). Previous studies have also reported the up-regulation of several transcription factors associated with the transition from pregnant not-in-labor to laboring myometrium in rat, mouse and human (8, 16, 25, 28, 32, 53, 62, 69).

Conclusions

In the past decade, several functional genomic studies have been conducted to decipher the complex molecular mechanisms involved in pregnancy and parturition across species. The key events that regulate pregnancy and parturition are diverse and often species-specific making extrapolation from model species to the human condition difficult. Understanding the underlying mechanisms in humans is of utmost importance for the diagnosis and treatment of preterm and post-term labor. Establishing an *in vitro* myometrial cell culture model system to study human pregnancy and labor, therefore, has many associated benefits. In this study we have modelled the effects of two major steroid hormones to identify differentially expressed genes associated with the physiology of pregnancy, labor, estrous and menopause, a model which also has possible implications for the pathophysiology of leiomyoma and endometrial cancer. Exposure of hUtSMCs to steroid hormonal conditions similar to those of the pregnant non-laboring myometrium (P4/E2 treatment) altered the expression of genes favouring muscle cell relaxation, increased cell proliferation and growth, and reduced immune and inflammatory responses. These findings were similar to previously published results on *in vivo* myometrial samples (32). When progesterone was functionally withdrawn from the P4/E2 co-treated cell culture system, by using the progesterone antagonist RU486, the altered expression of genes favoured myometrial contraction, as seen by enrichment of biological processes such as muscle contraction, cell growth arrest and apoptosis, and immune and inflammatory response. While *in vitro* studies on isolated myometrial smooth muscle cells do not represent the full range of biological events occurring *in vivo*, they do allow the analysis of the specific effects of steroid hormones, individually and in combination, as well as the effects of functional progesterone withdrawal on myometrial cells.

The findings in this study and other *in vitro* studies, provide important insights into hormonal regulation of myometrial quiescence and contractility, and form a basis for further research studies using freshly isolated myometrium *ex vivo* as well as *in vivo* studies using intact myometrium. This model could add to the debate about whether labor is an inflammatory response per se or an inflammatory response triggered by the fetus (9, 37)). In the present cell culture model system the foreign body/fetus is absent, yet treatment of hUtSMCs with hormonal conditions of laboring myometrium triggered the alteration of gene expression that favoured inflammatory responses. This would suggest that the inflammatory response elicited during parturition may be an effect of functional progesterone withdrawal, though it should be noted that the major cytokine response was absent. Overall, it may be concluded that the human uterine smooth muscle cell culture system developed here behaved in accordance with reported *in vivo* myometrial responses. Thus this model cell culture system may be used for the functional study of various aspects of human myometrial physiology.

Figure 1:

Experimental strategy for hormone additions to hUtSMCs.

Cells starved of endogenous steroids (see Materials and Methods) were grown to 100 % confluency (T = 0 h) and treated with 10nM E2 and/or 100nM P4. After 48 h RU486 was added to appropriate cells. All cells were grown for 72 h beyond the point of reaching 100 % confluency. Samples that were P4 primed had P4 added when the cells were approximately 80 % confluent, i.e. ~T = minus 24 h.

Figure 2:

Relationship of significant genes upon individual or combination additions of hormones.

The number of genes (with annotation) significantly regulated ($P<0.05$, fold change >1.5-fold) under P4/E2 conditions in relation to the number of genes significantly regulated by the individual P4 and E2 hormones.

Figure 3:

Expression profile clustering of genes significantly differentially expressed on treatment of hUtSMCs with P4/E2.

Genes significantly regulated ($P<0.05$, fold change >1.5) on addition of RU486 to hUtSMCs in P4/E2 conditions were clustered into five groups across all conditions (k-means clustering by Euclidean distance). The clusters are best displayed as box plots where the boxes (shaded) represent the 95% limits of the log2 normalized (and baseline centred) signal intensities and spots indicate outliers. (The total number here exceeds 666 genes because partially annotated genes were included).

Figure 4:

PCR validation of differentially expressed genes following treatments.

(A) hUtSMCs were treated with 100 nM P4 + 10 nM E2 and harvested after 72 h. Total RNA was extracted from the samples and untreated control, and analysed by qRT-PCR. Fold changes are relative to untreated control cells. (B) qRT-PCR validation of EMILIN2 and TTPA after treatment with 100 nM P4, 10 nM E2 and co-treated with 1μM RU486 24 h prior to harvest at 72 h. Fold changes are expression levels in the presence of RU486 relative to its absence. (C) Gel image from semi-quantitative PCR validation of selected genes after P4/E2 + RU486 treatment. (D) Densitometry analysis of intensity values of image C. The fold differences in each group were calculated by normalizing the values to the housekeeping gene (GAPDH) and comparing with corresponding untreated sample. Each value corresponds to the average from three independent experiments plotted (+/-SD). Statistically significant differences between the treated and control samples were determined using the Student *t*-test (* $P<0.05$; ** $P<0.005$). For semi-quantitative PCR amplification cycles were controlled to avoid the plateau effect.

Figure 5:

Gene Ontology enrichment analysis.

Enriched gene ontology terms for differentially expressed genes identified following RU486 addition to P4/E2 treated hUtSMCs. All analyses were implemented within DAVID (21). The analysis focused on gene ontology (GO) biological process (BP) terms but included molecular function (MF), cellular component (CC), Panther (PAN) (http://www.pantherdb.org/) and KEGG (http://www.genome.jp/kegg/) terms where these gave additional information. The prefix is the number of genes included in that term.

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Figure 1:

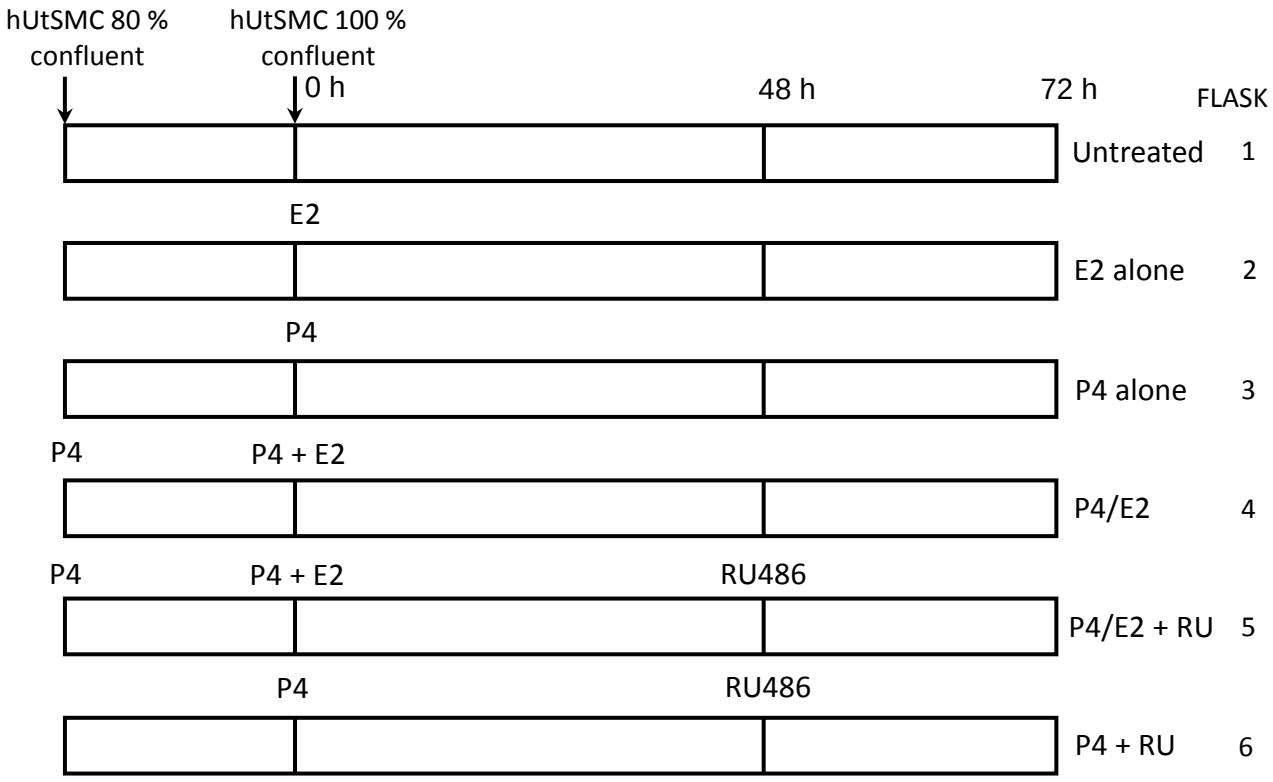
hUtSMC 80 % confluent
hUtSMC 100 % confluent
0 h
48 h
72 h
FLASK
Untreated
1
E2
E2 alone
2
P4
P4 alone
3
P4
P4 + E2
P4/E2
4
P4
P4 + E2
RU486
P4/E2 + RU
5
P4
RU486
P4 + RU
6

Figure 2:

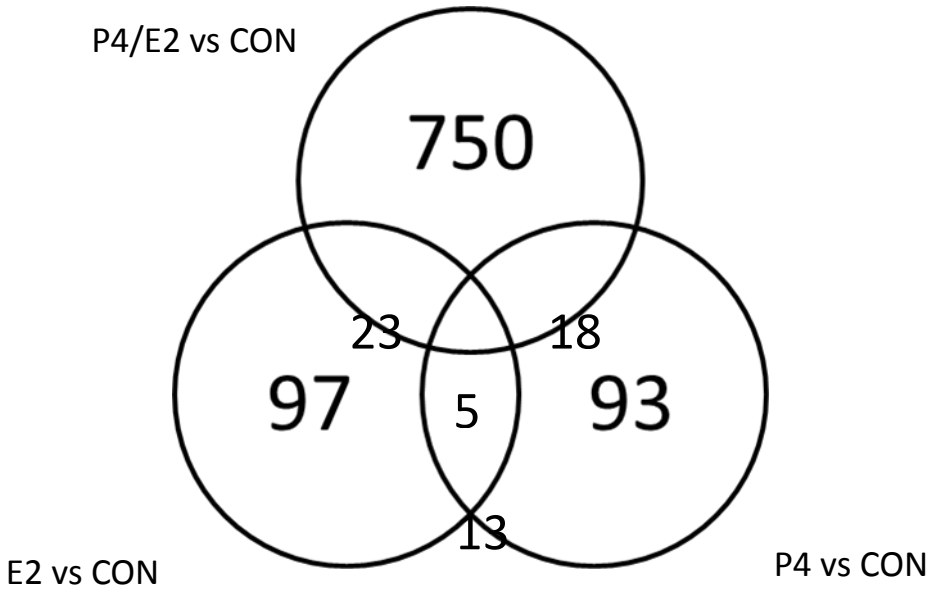

Figure 3:

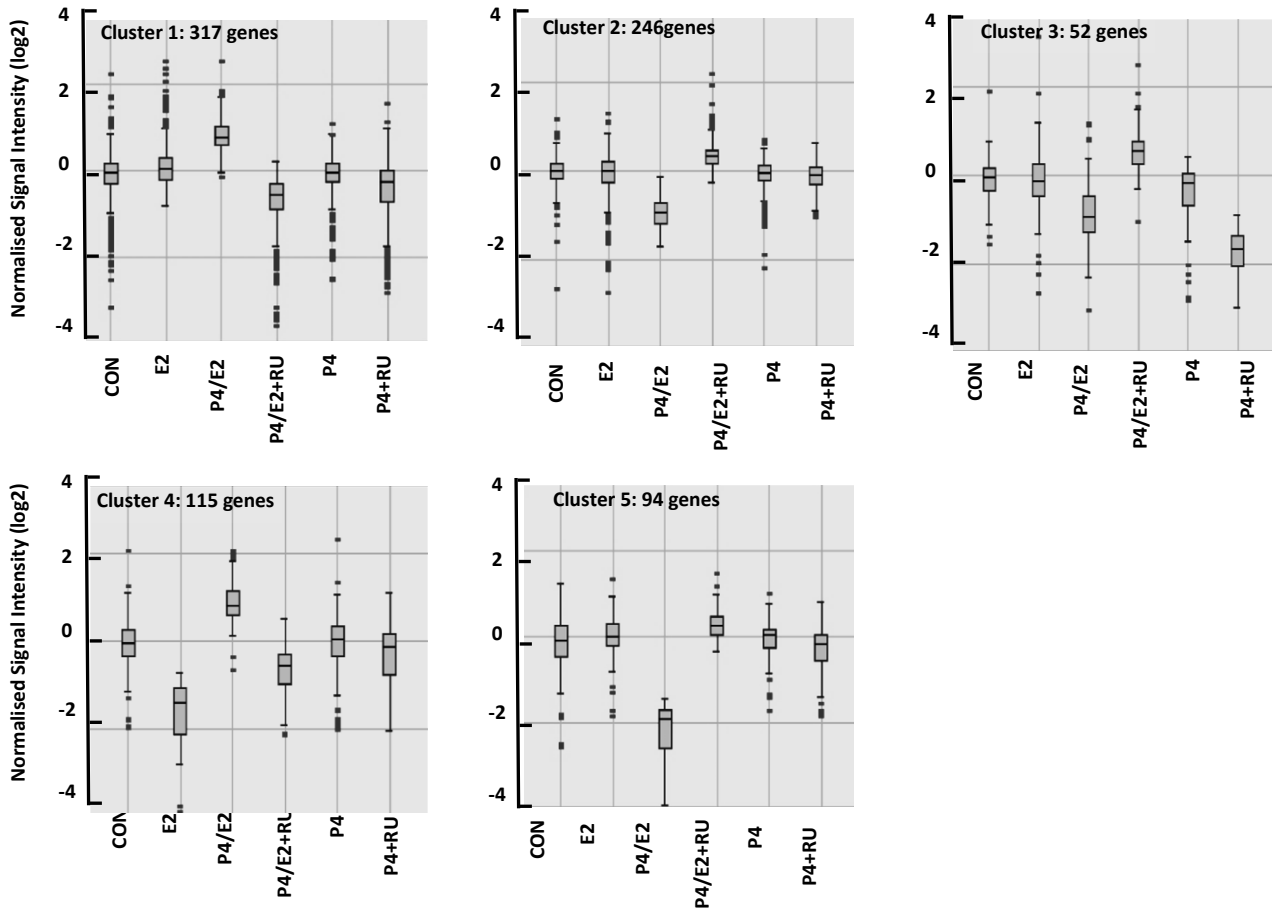
Cluster 1: 317 genes
Cluster 2: 246genes
Cluster 3: 52 genes
Cluster 4: 115 genes
Cluster 5: 94 genes
Normalised Signal Intensity (log2)
4
2
0
-2
-4
CON
E2
P4/E2
P4/E2+RU
P4
P4+RU

Figure 4:

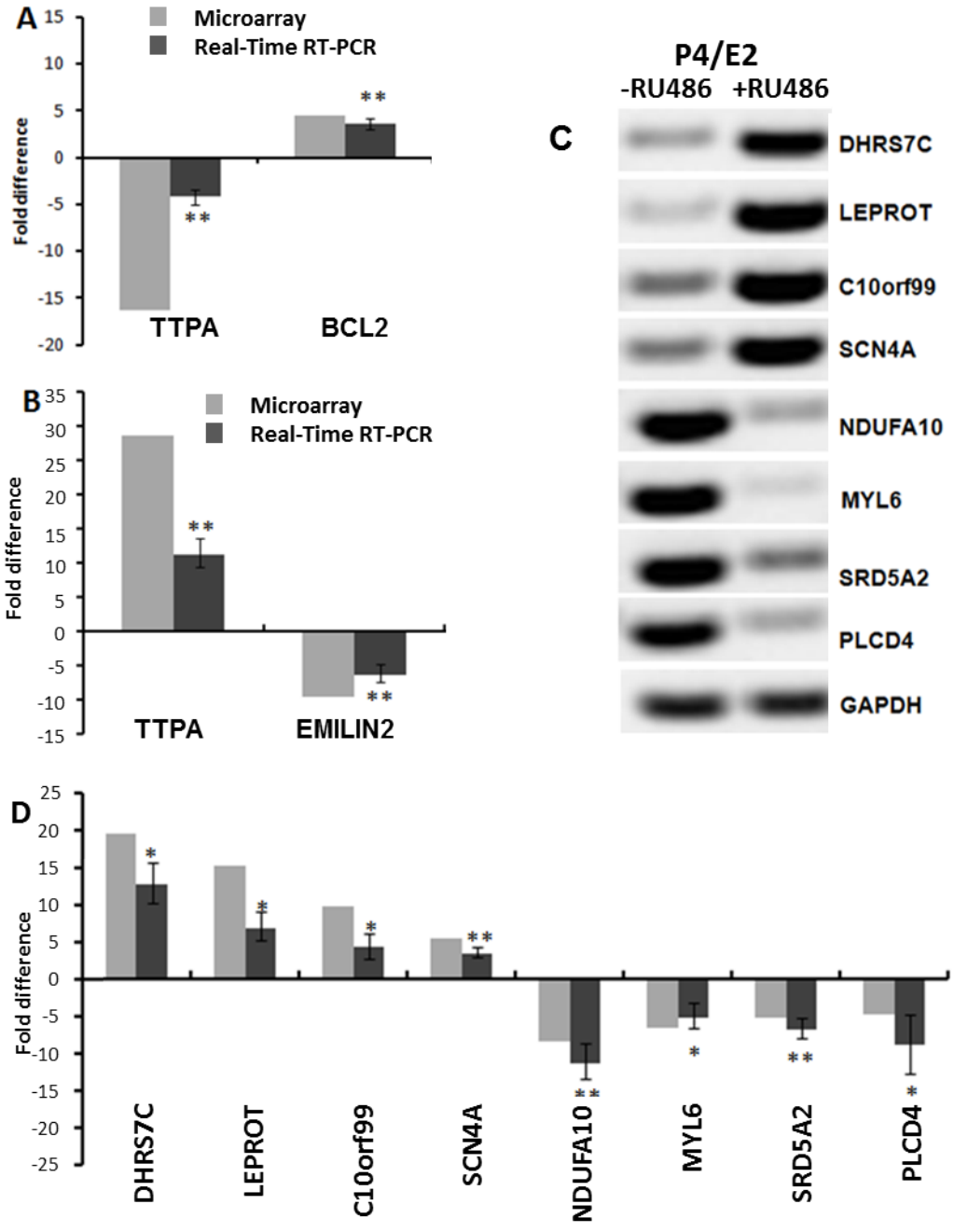
A
Microarray
Real-Time RT-PCR
Fold difference
TTPA
BCL2
B
Microarray
Real-Time RT-PCR
Fold difference
TTPA
EMILIN2
C
P4/E2
-RU486
+RU486
DHRS7C
LEPROT
C10orf99
SCN4A
NDUFA10
MYL6
SRD5A2
PLCD4
GAPDH
D
Fold difference
DHRS7C
LEPROT
C10orf99
SCN4A
NDUFA10
MYL6
SRD5A2
PLCD4

Figure 5:

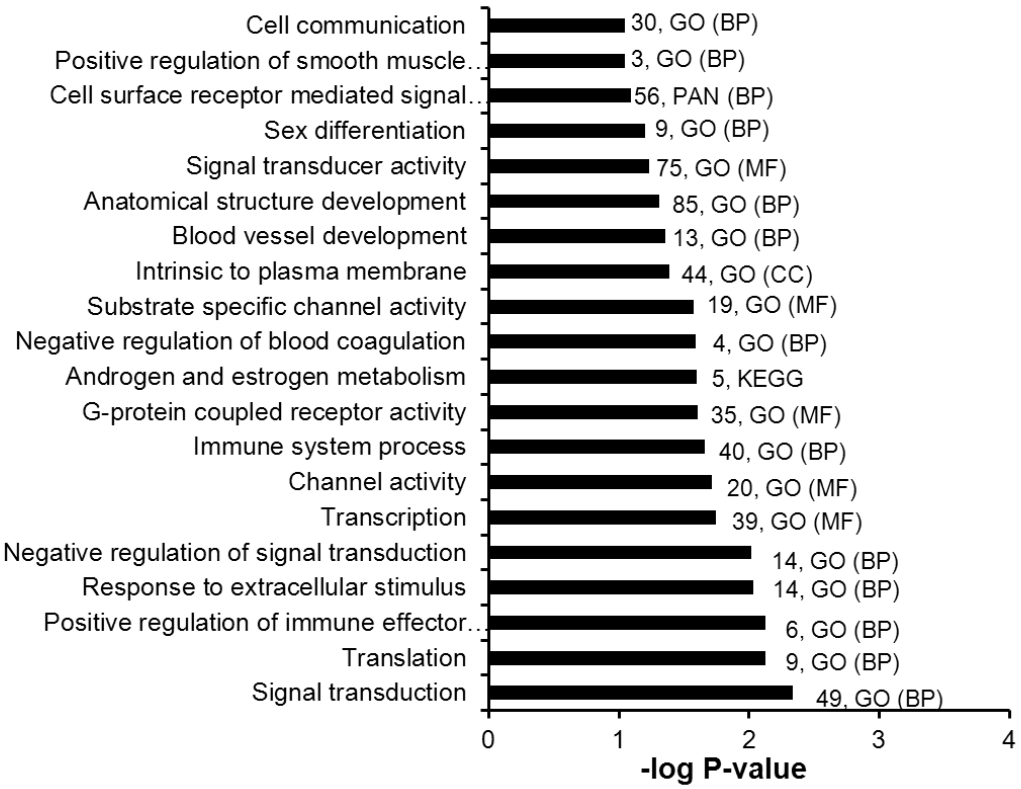
Cell communication
30, GO (BP)
Positive regulation of smooth muscle…
3, GO (BP)
Cell surface receptor mediated signal…
56, PAN (BP)
Sex differentiation
9, GO (BP)
Signal transducer activity
75, GO (MF)
Anatomical structure development
85, GO (BP)
Blood vessel development
13, GO (BP)
Intrinsic to plasma membrane
44, GO (CC)
Substrate specific channel activity
19, GO (MF)
Negative regulation of blood coagulation
4, GO (BP)
Androgen and estrogen metabolism
5, KEGG
G-protein coupled receptor activity
35, GO (MF)
Immune system process
40, GO (BP)
Channel activity
20, GO (MF)
Transcription
39, GO (MF)
Negative regulation of signal transduction
14, GO (BP)
Response to extracellular stimulus
14, GO (BP)
Positive regulation of immune effector…
6, GO (BP)
Translation
9, GO (BP)
Signal transduction
49, GO (BP)
0
1
2
3
4
-log P-value

Table 1. Genes selected for PCR reaction with primer sequence

Gene Symbol	RefSeq ID	Forward primer	Reverse primer
ESR1	NM_001122742	AGGTGCCCTACTACCTGGAG	CTTCGTAGCATTTGCGGAGC
SMTN	NM_134270	CTGTCTGGCCCCAAAGAGAC	TTGTTCACTCGGGTCTGCTC
CNN1	NM_001299	CAGAGAAGCAGGAGCGGAAA	CTGGGTACTCGGGAGTCAGA
OXTR	NM_000916	CAAGCTCATCTCCAAGGCCA	GGCTCAGGACAAAGGAGGAC
DHRS7C	NM_001105571	GCTGATGCTCCCCCTGCTGC	CACCAGCCTTGCCCCACCTG
LEPROT	NM_017526	GAGATGGCGTGGAAAATCAG	CCCCAGTTCGGGAGACAT
C10orf99	NM_207373	GTTCTCCGCTGTCTGGAGTC	TAGCCCCAACTCAACAAACC
SCN4A	NM_000334	AGGTTGTTCATCTCGCCATC	CTCACCGTCTTCCTCATGGT
NDUFA10	AF453834	GTACCCAGGGCTGTACTTGC	CTGCGATTACTGGTTCAGGA
MYL6	NM_079425	ATTCACACAGGGAAAGGCAC	TATGAAGCGTTTGTGAGGCA
SRD5A2	NM_000348	ATTTCCAGTGCAGAAGGCAG	GACGGTACTTCTGGGCCTCT
PLCD4	NM_032726	TCAGTGGTCAGCTGGTCTTG	CACAGCTCACAGACACAGGAA
GAPDH	NM_002046	TTGAGGTCAATGAAGGGGTC	GAAGGTGAAGGTCGGAGTCA

Table 2. Top 20 genes with highest fold changes of expression following P4/E2 treatment.

Gene Symbol	Fold Changes[1]	p-Value	RefSeq ID	Operon ID
ALDH6A1	40.01	3.50E-02	NM_005589	H200009618
COL5A3	27.35	1.95E-02	NM_015719	opHsV0400006290
UGT1A9	18.82	2.46E-02	NM_021027	H300001271
RANBP3L	16.05	1.58E-02	NP_659437	H200020602
NXF5	13.05	6.80E-03	NM_033154	opHsV0400005823
DNAH8	10.24	2.62E-02	-	opHsV0400001435
MIS12	9.75	3.79E-02	NM_024039	H300014781
NCOA7	9.65	3.48E-02	-	H300012462
CUL1	9.59	2.98E-02	NM_003592	H300017702
RALGPS1	9.53	3.47E-02	-	H300015887
GAS8	-64.89	7.74E-04	NM_001481	H300015231
TTPA	-19.34	9.65E-04	NM_000370	H200005658
SHISA6	-18.75	1.94E-02	NP_997269	opHsV0400006883
TYBN	-17.28	7.47E-03	-	opHsV0400000537
ABCA13	-14.09	2.66E-02	NM_152701	H300009369
EFNA5	-13.92	1.38E-02	NM_001962	opHsV0400002585
LYG2	-13.61	2.69E-02	NM_175735	opHsV0400002540
DMRTB1	-11.51	2.31E-02	NM_033067	H200012954
HKDC1	-9.83	2.10E-02	NM_025130	H200009369
NPY	-9.75	3.85E-02	NM_000905	opHsV0400000153

[1]Negative value represents downregulation.

Table 3. Top 20 genes with highest fold change in expression following RU486 addition to P4/E2 treated cells.

Gene Symbol	Fold Changes [1]	p-Value	RefSeq ID	Operon ID
TTPA	30.83	2.14E-04	NM_000370	H200005658
DHRS7C	23.28	9.86E-04	NM_001105571	opHsV0400001964
HMX3	15.00	5.69E-03	NM_001105574.1	opHsV0400007372
SV2B	14.76	4.03E-02	NM_014848	opHsV0400006274
OR5H2	14.13	3.46E-02	NM_001005482	H300007776
NPY	13.20	4.37E-02	NM_000905	opHsV0400000153
FAM78A	12.94	5.68E-03	NM_033387	opHsV0400000177
SHISA6	12.83	4.66E-02	NM_207386	opHsV0400006883
LEPR/LEPROT	12.64	1.01E-03	NM_001198683	opHsV0400003795
CPO	12.58	1.21E-02	NM_173077	H300003209
PLEKHA2	-20.39	2.71E-02	XM_496973	H300012792
TXNRD2	-15.28	1.85E-02	NG_011835	opHsV0400005683
OSBPL1A	-15.16	1.83E-02	NG_029432	opHsV0400002980
HIST1H4A	-13.30	3.51E-02	NM_003538	H300001107
FCHO2	-13.17	1.77E-02	XM_291142	H300022280
EMR3	-13.11	3.09E-02	NM_032571	opHsV0400005026
C15orf27	-12.14	3.02E-02	NM_152335	H200021222
TBC1D7	-10.90	3.55E-03	NM_001143964	H300011765
NDUFA10	-10.90	2.26E-02	-	H300016572
RPL27	-10.29	2.14E-04	NM_000988	opHsV0400000214

[1]Negative value represents downregulation.

Table 4.

Statistically overrepresented transcription factor binding sites in hUtSMCs of genes significantly regulated under different hormonal combinations

E2 alone

TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
Gata1	GATA	65	[1]**11.8**	**5.2**	5.7E-03
Nkx2-5	Homeo	72	**11.5**	4.0	1.8E-02
NFATC2	Rel	66	**10.8**	4.1	1.7E-02
ARID3A	Arid	67	**10.6**	3.0	5.1E-02
NR1H2::RXRA	Hormone-nuclear Receptor	2	**10.1**	2.9	5.3E-02

P4 alone

TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
SP1	BetaBetaAlpha-zinc finger	49	**14.7**	0.6	5.5E-01
NF-kappaB	Rel	42	**14.0**	3.2	4.0E-02
Pax4	Homeo	2	**11.9**	2.6	7.5E-02
RELA	Rel	41	**10.5**	**5.4**	4.5E-03
NFKB1	Rel	22	**10.3**	2.9	5.8E-02
NR3C1	Hormone-nuclear Receptor	17	**10.0**	3.9	2.1E-02

P4/E2

[2]Cluster 1 [3]310

TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
Nkx2-5	Homeo	202	**31.6**	**14.2**	7.0E-07
Foxd3	Forkhead	143	**30.4**	**9.7**	6.3E-05
ARID3A	Arid	193	**27.1**	**13.1**	2.0E-06
Pdx1	Homeo	191	**25.7**	**15.3**	2.2E-07
HOXA5	Homeo	198	**25.1**	**7.8**	4.1E-04
SRY	High Mobility Group	172	**23.3**	**8.0**	3.3E-04
FOXI1	Forkhead	145	**22.7**	**9.1**	1.2E-04
Nobox	Homeo	171	**21.8**	**13.6**	1.2E-06
NKX3-1	Homeo	152	**20.1**	**7.8**	4.0E-04
Prrx2	Homeo	183	**19.6**	**12.5**	3.8E-06

Cluster 2	207				
TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
Pax4	Homeo	3	**13.8**	3.4	3.2E-02
Tal1::Gata1	Helix-Loop-Helix	44	**10.5**	2.0	1.4E-01
Klf4	BetaBetaAlpha-zinc finger	101	8.7	**6.4**	1.6E-03
PPARG::RXRA	Hormone-nuclear Receptor	52	7.8	**5.7**	3.5E-03

[2]**Cluster 3**	193				
TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
FOXI1	Forkhead	73	**20.7**	3.1	4.6E-02
HOXA5	Homeo	107	**20.4**	4.6	1.0E-02
SRY	High Mobility Group	90	**18.9**	3.4	3.2E-02
Nkx2-5	Homeo	98	**18.7**	2.1	1.2E-01
ARID3A	Arid	92	**18.1**	1.7	1.8E-01
Pdx1	Homeo	85	**17.1**	0.9	4.2E-01
NFATC2	Rel	91	**16.4**	2.9	5.5E-02
Prrx2	Homeo	89	**16.3**	2.4	8.9E-02
FOXO3	Forkhead	81	**15.8**	2.3	1.0E-01
Sox5	High Mobility Group	79	**15.0**	2.6	7.8E-02

[4]**Cluster 4**	60				
TF	Family	Target gene hits	Z-score	Fisher score/p-Value	
FOXA1	Forkhead	36	-4.0	**8.3**	2.6E-04
HNF1A	Homeo	14	5.6	**7.2**	7.3E-04
YY1	BetaBetaAlpha-zinc finger	40	0.5	**6.2**	2.1E-03
NFATC2	Rel	37	-0. 7	**6.1**	2.2E-03
Nkx3-2	Homeo	37	-1.6	**5.7**	3.3E-03
ARID3A	Arid	38	2.0	**5.7**	3.3E-03
RORA_2	Hormone-nuclear Receptor	14	6.4	**5.7**	3.4E-03
Foxd3	Forkhead	30	1.1	**5.5**	4.0E-03
Lhx3	Homeo	19	5.5	**5.5**	4.1E-03
Prrx2	Homeo	36	2.9	**5.3**	5.1E-03

[1]Motifs are considered significant if the Z-score is above 10 or the Fisher score is above 5.
[2]Only the top 10 transcription factors are shown for clusters 1 and 3
[3]Number of genes in cluster
[4]Cluster 4 is small (60 genes) and Z-scores are poor. These are included on the basis of the Fisher scores only