Identification of human myometrial target genes of the c-Jun NH₂-terminal kinase (JNK) pathway: the role of activating transcription factor 2 (ATF2) and a novel spliced isoform ATF2-small

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Abstract

Activating transcription factor 2 (ATF2), a ubiquitously expressed member of the basic region leucine zipper (bZIP) family of transcription factors activated by mitogen activated protein kinase (MAPK) pathways, is important in the mediation of cellular stress responses, development and transformation. We have previously reported the differential expression of active ATF2 in the human myometrium throughout pregnancy and labour, and identified and partially characterized a novel splice variant ATF2-small (ATF2-sm). To further understand the role of these factors in the myometrium, we have used gene microarrays to define the target genes in cultured myometrial cells stably-transfected with ATF2 and ATF2-sm cDNAs. Many of the genes identified appear to have potential roles in regulating myometrial function and include proteins involved in G-protein receptor signalling, cytokine signalling, transcriptional regulation, cell-cycle control, formation of the extracellular matrix and cytoskeletal architecture. ATF2 was found to affect the expression of 204 genes; 113 being up-regulated and 91 down-regulated whereas the novel ATF2-sm factor altered the expression of 55 genes; expression was increased in 29 cases and decreased in 26. A further 25 genes affected by ATF2-sm were identified by suppression subtractive hybridisation (SSH). Notably, the genes affected by ATF2 and ATF2-sm appear to belong to discrete groups: only two genes were affected by both factors.

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Introduction

Activating transcription factor 2 (ATF2) is a ubiquitously expressed member of the basic region leucine zipper (bZIP) transcription factor family (Landschulz *et al.* 1988, Ziff EB 1990), which includes other factors such as cyclic-AMP response-element binding protein (CREB) (Hoeffler *et al.* 1988), the highly spliced cyclic-AMP response-element modulator protein (CREM) (Foulkes *et al.* 1991), and other members of the ATF family. ATF2 plays an important role in mediating cellular stress responses, development and transformation (Hai *et al.* 1989, Maekawa *et al.* 1989, Abdel-Hafiz *et al.* 1992, Karin & Hunter 1995, Reimold *et al.* 1996, Ronai *et al.* 1998, Maekawa *et al.* 1999, van Dam & Castellazzi 2001), and its trans-activation potential is enhanced via phosphorylation at amino acid residues Thr69 and Thr71 (Davis 2000, Fuchs *et al.* 2000) by the mitogen activated protein kinases (MAPK) JNK/SAPK, p38 (Derijard *et al.* 1994, Gupta *et al.* 1995, Livingstone *et al.* 1995, van Dam *et al.* 1995, Raingeaud *et al.* 1996, Read *et al.* 1997, Chang & Karin 2001, Kyriakis & Avruch 2001), and the Ras effector pathways Raf-MEK-ERK and Ral-RalGDS-Src-p38 (Ouwens *et al.* 2002). It modulates the expression

of downstream-affected genes by binding either as a homo- or hetero-dimer with other members of the bZIP family (Hai & Curran 1991) to cAMP response elements (CREs) and Activator Protein-1 (AP-1) sites within their promoter regions (Montminy *et al.* 1986, Lee *et al.* 1987, Yamamoto *et al.* 1988). Transcriptional activation is promoted via the intrinsic histone acetyl transferase (HAT) activity of ATF2 (Kawasaki *et al.* 2000), and by recruitment of co-activators such as p300/CBP (Cho *et al.* 2001). Interaction of ATF2 with p300/CBP and the basal transcriptional machinery is dependent upon phosphorylation at Ser121 within its p300 interaction domain (Kawasaki *et al.* 1998), mediated by protein kinase C α (PKC); it has been suggested that Ser121-unphosphorylated ATF2 is transcriptionally silent, necessitating the use of constitutively active ATF2 mutants in transcription studies (Steinmuller & Thiel 2003). However, we have reported highly significant trans-activation of CRE-containing reporter genes by transient transfection of an ATF2-expressing plasmid into both myometrial and COS-7 cell lines (Bailey *et al.* 2002). Furthermore, we present data here to indicate promiscuous transcriptional modification in myometrial cell lines stably transfected with ATF2 constructs.

Recently we have shown that various members of the bZIP transcription factor family including ATF2 are differentially expressed in the human myometrium during gestation and parturition (Bailey *et al.* 2002). However, in addition to the well characterized 60 000 mol wt ATF2 protein, we also reported the existence of a novel putative splice-variant designated ATF2-small (ATF2-sm) (Bailey *et al.* 2000), showing potent trans-activation properties. To our knowledge, this is the first alternatively-spliced variant of ATF2 to be identified in human tissue. Although comprising 432 bp and translating to a polypeptide of 144 amino acids with a calculated mol wt of 15 408, *in vitro* translation experiments and western blotting of human myometrial tissue homogenates consistently demonstrated an apparent mol wt of approximately 28 000; reasons for this are discussed in detail with supporting evidence in Bailey *et al.* (2002). Of particular interest was the fact that western blot profiling of the ATF2 and ATF2-sm species in pregnant non-labouring myometrium, sampled from the upper and lower uterine segments (as detailed in Fig. 1a), indicated that both isoforms were spatially expressed within the body of the uterus; ATF2 is expressed only in the lower segment, whereas there exists a gradient of expression of the ATF2-sm protein with the highest level in the upper segment. This may correlate with the known contractile and relaxatory properties of these uterine regions at term and at the onset of parturition. Although no definite exonic structure of the ATF2 gene has been reported, a predicted model has been proposed (Ensembl gene ID ENSG00000115966, Fig. 1b) comprising 12 exons and five functional domains, including a proline-rich domain (involved in protein–protein interaction), two glutamine-rich domains (involved in transcriptional trans-activation), a HAT domain and a bZIP domain involved in CRE-binding. Despite comprising only the first two and last two putative exons, and being devoid of most exons coding for functional domains, the novel ATF2-sm protein was found to possess CRE-binding activity and to trans-activate CRE-directed transcription of a reporter gene in myometrial and COS-7 cells to an equivalent degree to that of the full-length ATF2. To further characterize the roles of ATF2 and ATF2-sm in regulating myometrial gene expression during foetal maturation and parturition, we have stably transfected myometrial cell lines with plasmid constructs expressing the individual proteins, and made use of microarray, suppression subtractive hybridization (SSH) and semi-quantitative/real time RT-PCR experiments to identify downstream target genes that may be under their transcriptional control.

Materials and methods

Human myometrial tissue collection

Human myometrial tissue biopsies were collected from pregnant non-labouring (P) women at elective caesarean

section and from non-pregnant (NP) pre-menopausal women (ages 32–46 yr) during hysterectomies performed for benign gynaecological disorders such as menorrhagia or dysmenorrhoea. NP tissue samples were taken from the middle of the uterine wall, about 5–10 mm away from the endometrial or serosal surfaces: upper segment samples were taken from the corpus and lower segment samples were taken from close to the cervix, although it is generally realised that the lower uterine segment is poorly defined in the NP state. P samples were taken using laparoscopic biopsy forceps from the side of the uterus opposite the placental bed to minimize the inclusion of deciduas, again from the corpus (upper segment) and close to the cervix (lower segment). These myometrial samples were then snap frozen in liquid nitrogen and stored at -70°C . Written consent was obtained from all women and ethics approval for the study was granted by the Newcastle and North Tyneside Health Authority Ethics Committee.

Establishment of stable myometrial cell lines

Primary cultures of human myometrial cells were initiated by treating small pieces of P myometrium with collagenase solution (1 mg/ml collagenase, 0.02 mg/ml DNase, 0.2 mg/ml trypsin inhibitor, elastase and BSA fraction V, in Hank's balanced salt solution free of Ca^{+} and Mg^{2+} ions). Samples were incubated with gentle shaking for 4 h, then transferred to a new tube to which 5 ml complete medium plus 10% FCS had been added. After centrifugation at 1000 *g* for 10 min, the supernatant was decanted and discarded, the cells were resuspended in 15 ml of fresh complete medium plus 10% FCS and cells were then seeded into flasks. After incubation at 37°C for 1 h to allow fibroblast attachment, the medium, now containing primarily myometrial cells with few contaminating fibroblasts, was transferred to new flasks and incubated for 24 h at 37°C . The growth medium was then replaced to inhibit fibroblast growth, with MEM D-Valine medium (Gibco) plus 10% FCS, containing 50 U/ml of penicillin and 50 $\mu\text{g}/\text{ml}$ of streptomycin. Growth medium was replaced with fresh medium every 2–3 days, and after reaching confluence, cells were subcultured at a ratio of 1:3.

ATF2 and ATF2-sm full length plasmids were cloned as described by (Bailey *et al.* (2002). Transfections were performed upon low passage number (2 or 3) myometrial cells at 70% confluence using 4 μl Mirus *TransIT*-LT1 lipid-based transfection reagent (Cambridge Bioscience, Cambridge, UK) per 1 μg plasmid. Typically, 6 μg plasmid was used per transfection, mixed with 24 μl LT-1 reagent and 200 μl serum-free medium and incubated at room temperature (RT) for 5 min. This complex was added to the cells and incubated at 37°C for 4 h, after which time it was removed and fresh

Figure 1 (a) Spatial expression of the ATF2 and ATF2-sm transcription factors in the human myometrium during pregnancy. Western blotting of myometrial tissue extracts with an anti-ATF2 antibody revealed a post-conception switch from uniform expression to spatially differential expression of these factors. The full-length ATF2 protein was found to be present in a gradient from the lower uterine segment to the fundus; the novel ATF2-sm splice-variant, in contrast, was present in a gradient from the fundus to the lower segment. (b) Predicted functional domains of the ATF2 and ATF2-sm proteins, indicated by shading. P, proline-rich domain; Q, glutamine-rich domains involved in trans-activation; HAT, histone acetyl-transferase domain; bZIP, basic-region leucine-zipper domain.

medium (+ serum) added. Incubation at 37 °C was continued for 48 h, after which time the selective agent G418 was added to a final concentration of 80 µg/ml. The final concentration of G418 was increased to 250 µg/ml for subsequent selection and maintenance of isolated stably-transfected colonies. Transfection efficiencies were consistently above 80%.

Cell staining/immunocytochemistry

Cultured myometrial cells were stained to allow morphological examination using haematoxylin and eosin stains. Cells grown on cover-slips were rinsed with 1 × PBS, fixed in cold methanol for 10 min, washed with water and then exposed to haematoxylin for 1 min. Following a quick water wash, cells were exposed to Scott's water (0.04 M NaCO₃, 0.08 M MgSO₄) for 1 min. Another water wash was performed, then the cells were incubated in eosin stain for 2 min. After a final brief wash in water, the slips were allowed to dry and mounted in DPX resin on microscope slides for viewing.

To determine the level of fibroblast contamination in the smooth-muscle cell cultures, immunocytochemistry was performed using Fibroblast Antigen (Ab-1; Oncogene Research Products, CA, USA) (Saalbach *et al.* 1997) and the DAKO ChemMate APAAP detection kit with a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, CA, USA). Cells were cultured on cover-slips and fixed as above, then incubated in normal rabbit serum for 10 min. After a brief rinse with TBS, cells were incubated in Ab-1 primary antibody for 1 h at a 50 µl/ml dilution (10 µg/ml final concentration) at room temperature. After three 5 min rinses in 1 × TBS, cells were exposed to the secondary-link antibody for 45 min. Following three more 5 min rinses in 1 × TBS, the cells were incubated in APAAP for 20 min, followed by another round of washing in 1 × TBS. One drop of the Levamisole chromagen was then added to the cells, and colour development was monitored microscopically. Chromagenesis was halted by rinsing with water.

RNA isolation

Total RNA was isolated from approximately 1 × 10⁷ cells using TRI-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The resulting pellet was resuspended in 100 µl RNase-free H₂O and incubated at 55 °C for 10 min to aid this process. This total RNA was then applied to an RNeasy mini-column (Qiagen), and subjected to RNase-free DNase treatment using the RNase-free DNase set (Qiagen). The remainder of the manufacturer's protocol was then followed for RNA cleanup, eluting with 25 µl of RNase-free H₂O. Total RNA was quantified by spectrophotometry and used for microarray analysis or SSH.

Confirmation of ATF2/ATF2-sm expression levels in stably-transfected cell lines

Levels of ATF2/ATF2-sm expression in stably transfected cell lines were examined and compared with one another by sqRT-PCR using the Superscript one-step RT-PCR system (Invitrogen) in a total volume of 25 µl with 5% (v/v) RNaseOUT (Invitrogen) RNase inhibitor, and 100 ng total RNA template prepared as described above. Primers were as described previously by Bailey *et al.* (2002), and reaction conditions as follows: reverse transcription step=50 °C for 30 min, 94 °C for 2 min. Amplification step=30 cycles of (94 °C 15 s, 50 °C 30 s, 68 °C 1 min), followed by 68 °C for 9 min. 5 µl of each reaction was subjected to agarose gel electrophoresis and RNA samples from those colonies displaying the highest ATF2/ATF2-sm expression were selected for microarray analysis and SSH.

Affymetrix microarray analysis

Experimental design, execution, and subsequent data analysis were all carried out according to the guidelines of the Minimum Information About a Microarray Experiment (MIAME) document, developed by the Microarray Gene Expression Data Society (<http://www.mged.org/miame/>) (Brazma *et al.* 2001).

Target preparation

A total of 10 µg RNA was used for each target. Each sample was used as a basis to synthesize double-stranded cDNA, which in turn was used to synthesize biotin-labelled cRNA for hybridization to the microarray chips. The Affymetrix protocol was followed, and the steps are summarized briefly here:

cDNA synthesis

First-strand cDNA synthesis was achieved using the SuperScript Choice system (Invitrogen) and the Gene-Chip T7-Oligo(dT) primer at 42 °C for 1 h. Second strand cDNA was synthesized from this using *E.coli* DNA ligase, polymerase I and RNase H at 16 °C for 2 h.

Synthesis of biotin-labelled cRNA

This was performed according to the protocol, using the Affymetrix Enzo BioArray High Yield RNA Transcript Labelling Kit (Affymetrix, High Wycombe, Herts, UK); 20 µg of this labelled and purified cRNA was then fragmented (according to the protocol) prior to the hybridization step.

Hybridization

The hybridization cocktail was prepared according to the Affymetrix instructions, and incubated with the array at 45 °C for 16 h in rotisserie oven at 60 r.p.m.

Washing, staining and scanning

Washing and staining was performed in the Affymetrix Fluidics Station 400 according to the standard format of the single stain protocol for eukaryotic targets.

Data analysis

Data from the chip hybridizations was processed using the Affymetrix microarray suite 4.0 software. Human 133 A microarray chips were hybridized with biotin-labelled cRNA from the following myometrial cell-lines: (i) Control non-transfected primary cultures (Ctrl); two replicates (ii) Control cells stably-transfected with empty vector with no insert downstream of a CMV promoter (CMV –); three replicates (iii) Cells stably-transfected with a plasmid to express ATF2 (ATF2); three replicates (iv) Cells stably-transfected with a plasmid to express ATF2-sm (ATF2-sm); three replicates. Raw data files direct from the Affymetrix chip scanner were imported into the GeneSpring program (Silicon Genetics) for each chip. These data sets were transformed by converting all signal values below 0.01 to 0.01, then subjected to per chip normalization to the 50th percentile and per gene normalization to the median. To determine a baseline profile of gene expression with which to compare the ATF2 and ATF2-sm results, an initial comparison was made between cell-lines (i) and (ii), using data from the primary non-transfected cells as a baseline and data from the CMV – empty vector transfected cells as the experimental set. This established which genes were affected by the stable transfection of the vector, and which therefore should be excluded from the main analyses involving the ATF2 constructs. According to related literature (Mayanil *et al.* 2001, Gibellini *et al.* 2002), an appropriate cut-off point for this purpose is in the region of greater than or equal to a 1.5–2-fold change in expression; 1.5 was used in our analysis. The remaining data set following this exclusion was then used as a baseline for comparisons with ATF2 and ATF2-sm expression. This was achieved by performing a parametric one-way analysis of variance (ANOVA) on the normalized data assuming equal variances, incorporating the Benjamini and Hochberg False Discovery Rate multiple testing correction (Hochberg & Benjamini 1990) set at a rate of 0.05. The resultant list of genes differentially expressed between the two control classes was then filtered to include only those genes showing a fold change in expression of 1.5 or greater. These genes were then subtracted from the list of genes represented on the Affymetrix Human 133 A chip (22 283), resulting in the elimination of genes whose expression was altered by the process of stable transfection and selection. The remaining data set following this exclusion was then used as a final baseline for comparisons with ATF2/ATF2-sm expression. These comparisons involved much the same process;

data was normalized as above, then compared with gene expression levels in the final baseline by ANOVA using the same criteria. The results were then filtered to include only those genes whose expression altered by a fold change of two or greater.

SSH and differential screening

SSH was performed according to the published protocols using the PCR-Select cDNA subtraction kit and PCR-Select differential screening kit (BD Biosciences Clontech, Oxford, UK). Briefly, mRNA was isolated from the stably-transfected ATF2-sm cell-line using the Poly(A)Pure mRNA Isolation Kit (Ambion Europe Ltd., Huntingdon, Cambs, UK) 2 µg was used to synthesize ds-cDNA. Following RsaI digestion and ligation of adaptors to tester cDNA, two rounds of hybridization were performed with excess driver cDNA to subtract non-differentially expressed molecules, then two rounds of adaptor-directed suppression PCR to selectively amplify the cDNAs representing differentially expressed genes. These products were then cloned into the pCR4-TOPO vector (Invitrogen) to form a subtracted cDNA library, and subtracted and unsubtracted probes were synthesized from these PCR products for use in the library screening procedure. Briefly, transformed bacterial colonies from the library construction step were picked, and grown overnight in 96-well plates in LB AP⁺ medium. 1 µl of each culture was then used as a template for adaptor-directed PCR amplification of the plasmid inserts, of which 5 µl was spotted onto a Hybond N membrane. Four replicate membranes were produced for probing with forward subtracted, reverse subtracted, unsubtracted tester and unsubtracted driver probes. These probes were synthesized by random primer labelling of 75 ng of the appropriate cDNA, and hybridized to the membranes in sealed trays with shaking at 72 °C after pre-hybridizing with PerfectHyb Plus buffer (Sigma-Aldrich) containing 0.2 × SSC and 50 µl blocking solution (10 mg/ml sheared salmon sperm DNA, 0.3 mg/ml oligonucleotides corresponding to nested primers and complementary sequences). After washing and drying, the membranes were subjected to autoradiography. Clones representing differentially expressed genes were identified according to the combination of hybridization results for the four different probes summarized in the PCR-Select differential screening kit protocol, and identified by DNA sequencing.

Semi-Quantitative RT-PCR (SQ RT-PCR)

In order to support the results obtained from the microarray and SSH experiments, a selection of candidate genes were examined for differential expression as a result of the over-expression of ATF2 and/or

ATF2-sm by sqRT-PCR. The primers used for this were as follows, where F=forward primer, R=reverse primer. GAPDH: F=CTGCCGTCTAGAAAAACC, R=CCACCTTCGTTGTCATACC; MTATP6: F=TGTTCGCTTCATTTCATTGCC, R=ATGTGTTGTCTGTCAGGTAGA; CYB: F=CCCAATACGCAAAATTAA CCC, R=CGGATGCTACTTGTCCAATGA; HTMa: F=ATGGACGCCATCAAGAAGAA, R=GAAAATGTCCTACAATGTGCA; CDH2: F=GCCTCCATGTGCCGGATA, R=TCTCGGTCCAAAACAGCAAT; ZNF297b: F=AAGGCACAGGCTGAATCTGTT, R=TCTGTGCCCAGATTTGCATT; LIM: F=GAGTCACTTGTTCAGCCCTTGT, R=ACATTGTTCCGAATGGGCTT; TES: F=ATTGATGGGCTTAGGTCACGA, R=TGAGGGGGGAAAAAGAAGTG; RPL15: F=TAAGCCAAGATGGGTGCATA, R=CAAATTGACCCTGGACAACA; GPR63: F=CGCTGCCCTTCGCTTGA, R=GCAGTATCAGGTCCATTGATG; GNA15: F=CGGGCCTACTATGAGCGTC, R=ACGTAGCCCTCCTCGGTGAT; GAS1: F=CCGCCGCTCATCTGCT, R=GCCTCGGCGTACTGGTTG; MAP7: F=CGGCTCTCTCTCTCATCTGTC, R=GAACGAATGTGTGGGGCGTC; HSP70B': F=TTCCGACAACCGGCTCGTG, R=TTGTTCCCGCTCAGGTCCTT; KIF20: F=GACAGGAAGATCAGGGTTGTGTC, R=CAAAAGAGTCCTTGGGTGCTT; CALCL: F=TGAGGACTCAATTGAGTTGGGAGT, R=CCATCCATCCCAGGTTCTGTTG and GAB2: F=ACAGCCGACTTCACCGAGCTTC, R=CAGACCGGCCTGCACTCTCT

sqRT-PCR was performed using the SuperScript OneStep RT-PCR system. 50 ng DNase-treated total RNA from stably-transfected cells, or 5 ng mRNA from pooled non-pregnant myometrial tissue or pregnant non-labouring myometrial tissue was used as template according to the manufacturer's protocol, and a total of 28, 30 and 32 cycles of PCR were performed at an appropriate annealing temperature for each particular primer pair to ascertain the best quantitative signal for each target sequence following agarose gel electrophoresis (2% E-gels, Invitrogen) of the products. Image analysis and band intensities were analyzed with the Intelligent Quantifier program (Bio Image Systems Inc., Jackson, MI, USA).

Real-time RT-PCR analysis

Forward primers were designed using LUX primer design software (www.invitrogen.com) and were as follows. ATF2: GNA15=5'-GACGCCGGGCTGCTATGACCGC-3' and ATF2-sm: HSP70B'=5'-CACGATTCGACAACCGGCTCGG-3'. Reverse non-LUX labelled primers were as above for SQ RT-PCR. Reactions were performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen). Reactions were set up in 25 µl volumes

according to the manufacturer's protocol, and cycled as follows: 50 °C for 15 s; 95 °C for 2 s; then 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Commercially available LUX primers (Invitrogen) were used to amplify GAPDH as a reference gene. Melting curve analysis was performed to ensure reaction specificity, and relative quantitation was determined by standard curve analysis.

Results

Stable transfection and microarray data

Following the stable transfection and selection process, cell-lines were examined to verify that (a) they were predominantly composed of myometrial smooth-muscle cells and that fibroblast contamination had been successfully kept to a minimum, and (b) that the cells had not been in any way damaged, i.e. were in a healthy state with no gross morphological changes. Figure 2a and b shows the results of these examinations following the appropriate cell-staining procedures, indicating not only low-level or zero presence of fibroblasts in the cultures (less than 1%), but also a 'normal' morphology in the stably-transfected cell-lines when compared with the non-transfected control.

Agarose gel electrophoresis of ATF2 and ATF2-sm RT-PCR products from stably transfected cell lines revealed a wide range of expression levels between isolated clones (Fig. 2c). Total RNA was isolated and purified from those colonies showing the highest levels of expression, and amplified and labelled prior to microarray hybridization and analysis or SSH according to the methods described below. Chip Sequence file results produced by the array scanner have been submitted to the NCBI gene expression and hybridization array data repository (GEO) (The microarray data derived from chip sequence files for all hybridizations, including the cell-only control, two replicates of the empty-vector control and three replicates of each of the ATF2 and ATF2-sm experimental samples, have been submitted to the NCBI GEO (<http://www.ncbi.nlm.nih.gov/geo/>), under GEO numbers GSM17039, GSM17040, GSM 17041, GSM 17067, GSM 17068, GSM 17069, GSM 17070, GSM 17071 and GSM 17072). Following hybridization and scanning, chip data was imported into GeneSpring software for normalization and manipulation. A 'final baseline' of gene expression in cultured myometrial cells with which to compare the experimental data from the stably transfected cells was obtained as described in Materials and methods, and indicated that the expression of a total of 1317 genes (5.9% of the total) was affected significantly (greater than or equal to 1.5-fold) by the transfection and selection process, of which 879 were up-regulated and 438 down-regulated. These genes were subtracted from the 22 283 genes represented on the

microarray chip, to give a final baseline of 20 966 genes. All subsequent analyses of gene expression in the stably-transfected cell lines included data for these genes only, and the mean levels of expression in this final baseline were used as the basis for these comparisons, the outcome of which is summarized in Table 1, and presented in Fig. 3a and b for ATF2 and ATF2-sm respectively.

Over-expression of the ATF2 gene in cultured human myometrial cells by stable transfection gave rise to a total of 204 downstream genes whose expression was altered by a factor of two or more (0.97% of genes represented on the chip), 113 of which showed increased expression and 91 of which showed a decrease. Similar experiments with ATF2-sm over-expression altered the abundance of 55 mRNA species (0.26%), 29 of which were up-regulated and 26 down-regulated.

From the total of 259 genes found to be significantly altered as a result of the expression of either ATF2 factor, only two were affected by both: transient receptor potential cation channel, subfamily C, member 4 (TRPC4) which was up-regulated by both isoforms, and calcitonin receptor like (CALCRL) which was down-regulated by both. The genes in all lists were classified and grouped according to their functions as determined by the GeneSpring software; where genes belonged to more than one functional group, the results tables were manually trimmed to prevent multiple entries of these particular genes. Many of these genes are described in detail in the discussion section.

SSH of ATF2-sm stably transfected myometrial cells

Dot-blot screening of 100 transformed bacterial colonies resulting from this procedure gave rise to 25 putative differentially expressed clones, as determined from the blot patterns with probes derived from (i) forward

Figure 2 (a) Immunocytochemical staining of human myometrial and fibroblast cultured cells using anti-fibroblast specific antigen (Ab-1) mAb ASO2 (oncogene). (i) Note intense anti-ASO2 reactivity with fibroblast cells, in contrast to the light staining with myometrial cells in (ii). Negative controls, omitting the primary ASO2 mAb, are shown in (iii) and (iv) for fibroblast and myometrial cells, respectively. (b) Haematoxylin and eosin (H&E) staining of stably-transfected myometrial cells expressing (i) ATF2 and (ii) ATF2-sm. Morphology was compared with control non-transfected (iii) and empty-vector stably-transfected (iv) cells, and showed no difference. (c) RT-PCR analysis of isolated stably-transfected colonies to determine abundance of ATF2 and ATF2-sm mRNAs. Total RNA was isolated from these cell lines, and used as a template for RT-PCR of the ATF2 and ATF2-sm isoforms. Expression levels were compared with control non-transfected (-) and empty-vector stably-transfected (C) cells. Those with the highest degrees of ATF2 and ATF2-sm expression (*) were used for SSH analysis and as the three replicate RNA populations for the microarray analysis.

(a)

Gene	Fold change	Genbank
Cell death/apoptosis regulation		
TM6SF9	Lum necrosis factor receptor superfamily, member 9	4.7 1 BC008198.1
ARTS-1	type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	2.3 1 BE551138
DEFCAP	death effector filament-forming Csd-4-like apoptosis protein	2.0 1 AF220661.1
P450RAJ2	Dead apoptotic inhibitory molecule 2	4.8 1 NM_012306
Cell growth & maintenance		
FGF20	fibroblast growth factor 20	8.9 1 NM_019851
GDF1	growth differentiation factor 1	2.6 1 NM_001492
FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	4.0 1 NM_002009
P450RAJ2	cytochrome P450 retinoid metabolizing protein	4.5 1 NM_019895
GAS1	growth arrest-specific 1	40.1 1 AW611727
Signal transduction		
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	3.1 1 AF004884.1
AVP	arginine vasopressin (neurophysin II, antidiuretic hormone, diabetes insipidus, neurohypophyseal)	3.0 1 NM_000490
TACR1	tachykinin receptor 1	3.4 1 M81797.1
RGS14	regulator of G-protein signalling 14	3.7 1 AF037194.1
Developmental processes		
D21S2056E	DNA segment on chromosome 21 (unique) 2056 expressed sequence	8.2 1 NM_003683
M1S1	tumor-associated calcium signal transducer 2	3.3 1 J04152
G1OT-3	G1OT-3 for gonadotropin inducible transcription repressor-3	2.2 1 NM_016265
TLE1	transducin-like enhancer of split 1 (E1sp1) homolog, Drosophila	2.4 1 NM_005077
EFS	signal transduction protein (SH3 containing)	2.9 1 NM_005864
EED	embryonic ectoderm development	3.1 1 AF099032.1
Protein kinase cascade		
CSF5	cystatin D	13.4 1 NM_001900
STAT5A	signal transducer and activator of transcription 5A	4.1 1 NM_003152
PMAP1	phorbol-12-myristate-13-acetate-induced protein 1	2.8 1 AB57639
Physiological processes		
F2RL2	coagulation factor II (thrombin) receptor-like 2	3.2 1 NM_004011
PSG6	prolymphocyte specific beta-1-glycoprotein 6	3.0 1 M31125.1
CBCL	Cas-Bt-M (murine) ecotropic retroviral transforming sequence c	6.1 1 NM_012116
Extracellular matrix		
COL13A1	collagen, type XIII, alpha 1	8.6 1 M92171.1
	ESTs, Weakly similar to CGHU7L collagen alpha 1(I) chain precursor-human [H.sapiens]	5.8 1 BC023839
COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)	4.0 1 M81379.1
COL6A1	collagen, type VI, alpha 1	2.9 1 BE350145
COL7A1	collagen, type XVII, alpha 1	3.0 1 NM_000494
MMP20	matrix metalloproteinase 20 (enamelysin)	5.7 1 NM_004771
Cytoskeleton		
HIFK2	homeodomain interacting protein kinase 2	2.1 1 NM_022740
SLAHBP1	fuse-binding protein-interacting repressor	2.0 1 AF217197.1
MARK2	MAP/microtubule affinity-regulating kinase 2	2.9 1 NM_017490
ATP1P	AT2 receptor-interacting protein 1	2.9 1 AL096842.1
HRP1	hematopoietic PBX-interacting protein	5.4 1 BF344265
MAP7	microtubule-associated protein 7	8.3 1 T62571
MYH5	myosin, heavy polypeptide 3, skeletal muscle, embryonic	9.0 1 NM_002470
Intracellular components		
CYP3A43	cytochrome P450, subfamily IIIA, polypeptide 43	4.6 1 NM_022820
	cytochrome P450, subfamily IJ (aridonic acid epoxidase) polypeptide 2	2.9 1 NM_000775
CYP2J2	mitochondrial ribosomal protein L17	2.4 1 AK028857.1
MRPL17	mitochondrial ribosomal protein S34	2.1 1 NM_023936
MRPS34	ubiquitin fusion degradation 1-like	2.0 1 BC001049.1
UFDL1	nuclear receptor coactivator 2	2.1 1 NM_006540
NCOA2	heterogenous nuclear ribonucleoprotein A1	2.9 1 A1144007
HNRPA1	golin-67	5.1 1 AF163441.1
GOLGIN-67		
Cell cycle regulation		
CND2	cyclin D2	15.2 1 NM_001759.1
CND1	cyclin D1 (PRAD1; parathyroid adenomatosis 1)	5.5 1 Z23022.1
Enzymes (general)		
GGCX	gamma-glutamyl carboxylase	6.2 1 BE326952
NAMT1	putative N6-DNA-methyltransferase	5.1 1 NM_013240
MTFR	5,10-methyleneetetrahydrofolate reductase (NADPH)	2.9 1 NM_005957
ATP4B	ATPase, H ⁺ /K ⁺ exchanging, beta polypeptide	2.4 1 NM_000705
GLRX	glutaredoxin (thioltransferase)	2.4 1 NM_002064
HADH2	hydroxyacyl-Coenzyme A dehydrogenase, type II	2.1 1 NM_004494
NDFU6A	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6, 14kDa	2.1 1 NM_002490
HRMT1L2	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	2.0 1 NM_001536
ALDH1B1	aldehyde dehydrogenase 1 family, member B1	2.0 1 BC001619.1
REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast)	2.3 1 NM_002912
ACACA	acyl-Coenzyme A carboxylase alpha	3.4 1 BE555983
UC7B	UDP-glucosyltransferase 6 (UDP-galactose ceramide galactosyltransferase)	3.6 1 NM_003380
SARDH	sarcosine dehydrogenase	4.0 1 AF047004.1
Kinases		
CAMK2K	calcium/calmodulin-dependent protein kinase kinase 2, beta	2.5 1 AK024748.1
PTK9L	protein tyrosine kinase 9-like (A6-related protein)	2.5 1 NM_007284
LIM	LIM protein (similar to rat protein kinase C-binding enigma)	2.0 1 AA196325
PRKCI	protein kinase C, iota	2.9 1 L18964.1
Nucleic acid binding		
TCBE2	transcription elongation factor B (SII), polypeptide 2 (18kDa, elongin B)	5.2 1 A1668533
LOC44431	similar to RNA polymerase I transcription factor RRM3	5.3 1 BC008441.1
TOE1B	transcription elongation factor B (SII), polypeptide 1 (15kDa, elongin C)	2.5 1 NM_005648
GTF3A	general transcription factor IIIA	2.0 1 BE542815
RBMS	RNA binding motif protein 3	2.0 1 NM_008743
CUGBP2	CUG triplet repeat, RNA binding protein 2	11.8 1 NM_006561
Transport		
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	7.8 1 A1346187
SLC25A12	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	2.3 1 NM_003705
MRS3/4	putative mitochondrial solute carrier	5.1 1 NM_031212
SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	8.1 1 NM_003039
Molecular function		
RRP4	homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease	3.2 1 NM_014285.1
IAPP	islet amyloid polypeptide	4.2 1 NM_000415
Non-classified		
HIST1H2AG	H2A histone family, member P	13.8 1 NM_021094
HIST1H2BH	H2B histone family, member J	12.1 1 NM_003524
PCDH9	protocadherin 9	7.2 1 NM_020403
SPRR3	G protein-coupled receptor 63	5.9 1 NM_003704
GLUD1	lensin	5.9 1 NM_016571
ALOX5	arachidonate 5-lipoxygenase	4.8 1 NM_000698
GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	4.5 1 NM_002068

Gene	Fold change	Genbank
Non-classified		
OAS1	2',5'-oligoadenylate synthetase 1, 404kDa	3.8 1 NM_016816
CAMK2B	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	3.7 1 AF078803.1
PAX8	paired box gene 8	3.6 1 BC001060.1
NEFL	neurofilament, light polypeptide 68kDa	3.5 1 BF055311.1
S100A13	S100 calcium binding protein A13	3.5 1 NM_005979
SERPBNB3	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3	3.3 1 BC005224.1
PHLDA1	pleckstrin homology-like domain, family A, member 1	3.1 1 AA576961
CTSC	cathepsin C	2.9 1 NM_001814
KIR-023GB	NK-receptor	2.9 1 AF022048.1
DLRLE1A	DNA cross-link repair 1A (PSO2 homolog, S. cerevisiae)	2.8 1 D42405.1
TONDU	tondu	2.8 1 BE542323
DLX6	distal-less homeo box 6	2.7 1 NM_005222
TRPC4	transient receptor potential cation channel, subfamily C, member 4	2.7 1 NM_016179
SYNGR1	syngamin 1	2.7 1 NM_004711
CD6	CD6 antigen	2.6 1 U66146.1
RPL29	ribosomal protein L29	2.6 1 AW582267
CAV2	caveolin 2	2.6 1 AA150110
MAG	myelin associated glycoprotein	2.5 1 X98405.1
TRA@	T cell receptor alpha locus	2.4 1 BF676764
GLRX2	glutaredoxin 2	2.4 1 NM_016806
CCR4	chemokine (C-C motif) receptor 4	2.4 1 NM_005508
TRIM3	tripartite motif-containing 3	2.4 1 AH19307
KRT10; K10;	keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	2.3 1 X14487
KPP	thyroid stimulating hormone receptor	2.3 1 BE740743
TSNR	cadherin-like 22	2.3 1 AF035303.1
CD422	CGI-101 protein	2.3 1 NM_016041
F-LAN-1	estrogen receptor 2 (ER beta)	2.2 1 AB006589.1
ESR2	thymine primase exonuclease 1	2.2 1 AJ243767
dn3	thiamin pyrophosphokinase 1	2.2 1 NM_022445
TPK1	BAI1-associated protein 3	2.1 1 NM_003933
BAIAP3	hemoglobin, beta	2.1 1 AF549114.1
HBB	hemoglobin, beta	2.0 1 AV007368
JTV1	heme-regulated initiation factor 2-alpha kinase	2.0 1 NM_002788
PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	2.0 1 BC000023.1
RPS19	ribosomal protein S19	2.0 1 NM_000023.1
CPD	carboxypeptidase D	2.1 1 AA807514
RTP801	HIF-1 responsive RTP801	2.1 1 NM_019058
PLN	phospholamban	2.1 1 AW66803
ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2	2.3 1 AL162060.1
ZNF217	zinc finger protein 217	2.3 1 NM_006526
ALOX15	arachidonate 15-lipoxygenase	2.3 1 NM_001410
SCA1	spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1)	2.3 1 NM_000332
HMG20A	high-mobility group 20A	2.4 1 NM_016203
WISP1	WNT11 inducible signaling pathway protein 1	2.1 1 AB034725.1
KLRK3	killer cell lectin-like receptor subfamily C, member 3	2.1 1 NM_002261
ITSN1	intersectin 1 (SH3 domain protein)	2.1 1 AF144488.1
EDNRN	endothelin receptor type A	2.1 1 NM_001957
DKFZp564B0769	SRH rich protein	2.7 1 AL080186.1
RYR1	ryanodine receptor 1 (skeletal)	2.8 1 NM_000540
KIAA0279	cadherin, EGF-LAC seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	3.0 1 D87469
FLJ10697	zinc finger protein	3.3 1 NM_018181
EMCN	endomucin	3.3 1 NM_018242
CA9	carbonic anhydrase IX	3.3 1 NM_001216
MECP2	methyl CpG binding protein 2 (Rett syndrome)	4.2 1 AB13140
CALCR	calcitonin receptor-like	4.3 1 U14743.1
NF1	neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)	4.5 1 M60915.1
PRDM9	PR domain containing 9	4.1 1 NM_020227
PGM6B	glycoprotein M6B	4.5 1 AF016004.1
ZNF154	zinc finger protein 154 (pH-92)	5.3 1 U20464.1
CRMI1	crossinced 1	3.3 1 NM_008607
PALM	paralectin	5.5 1 NM_002579
CD1E	CD1E antigen, e polypeptide	6.3 1 AA309511
Miscellaneous		
PRO2086	PRO2086 protein	42.8 1 NM_014110
FLJ20060	hypothetical protein FLJ20060	25.5 1 NM_017645
PRO2796	PRO2796 (Homo sapiens), mRNA sequence	16.4 1 NM_018633
MGC3731	hypothetical protein MGC3731	14.4 1 NM_020133
FLJ12525	hypothetical protein FLJ12525	7.7 1 NM_031206
ESTs	ESTs	6.7 1 AA018167
FLJ21135	hypothetical protein FLJ21135	6.6 1 NM_024866
FLJ23537	hypothetical protein FLJ23537	4.6 1 NM_024889
DKFZp44P2435	Homo sapiens mRNA: cDNA DKFZp44P2435 (from clone DKFZp44P2435), mRNA sequence	4.2 1 AL117520.1
BRACE2	Homo sapiens mRNA full length insert cDNA clone EUROMIMAGE 29534, mRNA sequence	4.0 1 AL109707.1
E2IG2	Homo sapiens cDNA FLJ33407 fs, clone BRACE210535, mRNA sequence	3.2 1 BF223679
ANXA2P2	annexin A2 pseudogene 2	3.0 1 NM_018656
FLJ13615	hypothetical protein FLJ13615	2.8 1 M62996.1
FLJ13615	Homo sapiens cDNA FLJ14102 fs, clone MAMMA1000940, mRNA sequence	2.7 1 AF317887.1
PTD015	PTD015 protein	2.6 1 AU147528
HSPC155	HSPC155	2.5 1 BC002752.1
DM1	similar to S. pombe dm1+	2.4 1 NM_016406
ESTs	Type II AF10 protein (Homo sapiens), mRNA sequence	2.3 1 NM_006701
HT021	HT021	2.3 1 AF050603
FLJ23469	hypothetical protein FLJ23469	2.3 1 AA725078
FLJ21736	hypothetical protein FLJ21736	2.2 1 NM_020685
FLJ20452	hypothetical protein FLJ20452	2.1 1 NM_024710
DKG3	DKG-like 5-6 (Homo sapiens), mRNA sequence	2.1 1 BE313890
FLJ21665	hypothetical protein FLJ21665	2.1 1 NM_015882
EZH2	enhancer of zeste homolog 2 (Drosophila)	2.1 1 NM_004456
LANCL2	Homo sapiens cDNA FLJ13633 fs, clone THYRO100676, mRNA sequence	2.1 1 AK023895.1
LANCL2	lanC lantibiotic synthetase component C-like 2 (bacterial)	2.0 1 NM_018697
LOC55580	Homo sapiens mRNA: cDNA DKFZp566F1822 (from clone DKFZp566F1822), mRNA sequence	2.0 1 NM_018697
COPS6	COP9 subunit 6 (MOV34 homolog, 34 kD)	2.0 1 NM_006833
FLJ22582	hypothetical protein FLJ22582	2.0 1 NM_025045
TEX14	testis expressed sequence 14	2.1 1 NM_031272
FLJ20986	hypothetical protein FLJ20986	2.1 1 BF218804
FKSG44	hypothetical protein FKSG44	2.1 1 AF334946.1
KIAA1046	KIAA1046	2.1 1 NM_014928
C6orf37	chromosome 6 open reading frame 37	2.2 1 AL078599
FLJ20037	Type II AF10 protein (Homo sapiens), mRNA sequence	2.2 1 AF069338.1
CUTL1	Homo sapiens mRNA: cDNA DKFZp566F1822 (from clone DKFZp566F1822), mRNA sequence	2.2 1 AA195936
CUTL1	cut-like 1, CCAAT displacement protein (Drosophila)	2.2 1 BE046521
LOC55580	Homo sapiens cDNA FLJ11918 fs, clone HEMBB1000272, mRNA sequence	2.2 1 AK021980.1
LOC55580	ESTs	2.4 1 NM_016166.1
LOC55580	H.sapiens mRNA for 5'UTR for unknown protein (clone ICRFP507C00882), mRNA sequence	2.4 1 AL567302
LOC55580	hypothetical protein LOC55580	2.6 1 NM_017571
LOC55580	Homo sapiens mRNA: cDNA DKFZp564I153 (from clone DKFZp564I153), mRNA sequence	2.6 1 AL049278.1
LOC55580	Homo sapiens cDNA FLJ12683 fs, clone NT2RM4002457, mRNA sequence	2.8 1 AA827892
BDG-29	BDG-29 protein	3.1 1 AL117532.1
FLJ12089	hypothetical protein FLJ12089	3.1 1 NM_024552
DC42	hypothetical protein DC42	3.2 1 NM_030921
C8orf1	chromosome 8 open reading frame 1	3.7 1 BF057438
KIAA1237	KIAA1237 protein	3.7 1 AA121502
KIAA1102	KIAA1102 protein	4.2 1 AK027231.1
ORF1	ORF1, mRNA sequence	4.8 1 AU134977
KIAA0217	KIAA0217 protein	4.8 1 AW626868
KIAA0227	KIAA0227 protein	5.7 1 D86980.1
EZH1	enhancer of zeste homolog 1 (Drosophila)	6.1 1 AB02386.1
ANKH	ankyrin-like, progressive homolog (mouse)	6.1 1 NM_019847
KIAA0924	KIAA0924 protein	8.1 1 NM_014897
DN7	D15F37 (pseudogene)	19.4 1 AB02391.2

(b)

Gene	Fold change	Genbank
Cell growth & maintenance		
HSPA6	heat shock 70kDa protein 6 (HSP70B)	5.6 ↑ NM_002155,1
Signal transduction/integrin receptor signalling		
DAAAI	dishevelled associated activator of morphogenesis 1	2.1 ↑ AK021890,1
ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	2.4 ↑ NM_002204
BMPT1B	bone morphogenetic protein receptor, type IB	7.0 ↓ D89675,1
Intracellular signalling		
NME1	non-metastatic cells 1, protein (NM23A) expressed in	2.2 ↑ NM_000269
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	2.6 ↓ NM_004476
Intracellular		
KIF20A	RAB6 interacting, kinesin-like (rakibnesin6)	14.1 ↑ NM_005733
NCF2	neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	2.6 ↓ BC001606,1
CORO1A	coronin, actin binding protein, 1A	4.5 ↓ U34690,1
Cell cycle regulator		
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.9 ↓ N33167
Chaperone		
TBCE	tubulin-specific chaperone e	2.9 ↑ NM_003193
Enzymes (general)		
POMT2	putative protein O-mannosyltransferase	5.4 ↑ NM_013382
DHFR	dihydrofolate reductase	3.4 ↓ BC003584,1
NQO2	NAD(P)H dehydrogenase, quinone 2	3.3 ↑ NM_000904
UBE2G2	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)	3.0 ↑ BC001738,1
Transcription factors		
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	4.4 ↑ X52078,1
NFX1	nuclear transcription factor, X-box binding 1	3.0 ↓ AF332009,1
Transport		
SLC34A1	solute carrier family 34 (sodium phosphate), member 1	2.0 ↓ NM_003052
Non-classified		
TRPC4	transient receptor potential cation channel, subfamily C, member 4	6.2 ↑ NM_016179
LOC63920	transposon-derived Buster3 transposase-like	5.4 ↑ NM_022090
LAMB3	laminin, beta 3	3.6 ↑ L25541,1
TBR1	T-box, brain, 1	3.3 ↑ NM_006593
PCDH8	protocadherin beta 8	3.0 ↑ NM_019120
CKNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	3.0 ↑ NM_021614
PRO1386	PRO1386 protein	2.8 ↑ NM_031269
MUC8	mucin 8, tracheobronchial	2.7 ↑ U14383,1
CLCNKA	chloride channel Ka	2.6 ↑ NM_004070
TMEM4	transmembrane protein 4	2.6 ↑ BC001027,1
RIL	LIM domain protein	2.4 ↑ AF153882,1
PCDHGA11	protocadherin gamma subfamily A, 11	2.3 ↑ AF152905,1
PCDHGA8	protocadherin gamma subfamily A, 8	2.0 ↓ AB002325,1
KRT4	keratin 4	2.0 ↓ BE612352
ZFH4	zinc finger homeodomain 4	2.2 ↓ NM_024721
BRD3	bromodomain containing 3	2.2 ↓ NM_007371
SPHK2	sphingosine kinase 2	2.4 ↓ AF245447,1
GAB2	GRB2-associated binding protein 2	2.4 ↓ NM_012296
ZNF215	zinc finger protein 215	3.3 ↓ NM_013250
ITPR1	inositol 1,4,5-trisphosphate receptor, type 1	3.6 ↓ L38019,1
CALCRL	calcitonin receptor-like	4.0 ↓ U17473,1
PER2	period homolog 2 (Drosophila)	4.2 ↓ NM_003894
TNRC9	trinucleotide repeat containing 9	6.2 ↓ AK025084,1
Miscellaneous		
KIAA0220	KIAA0220 protein	8.4 ↑ AK023827,1
FLJ30002	Homo sapiens cDNA FLJ13754 fis, clone PLACE3000362, mRNA sequence	3.0 ↑ AK023816,1
	hypothetical protein FLJ30002	2.6 ↓ AL520975
	Homo sapiens cDNA: FLJ21690 fis, clone COL09538, mRNA sequence	2.5 ↓ AK025343,1
PRO1575	PRO1575 protein	2.5 ↑ NM_014092
FLJ20186	hypothetical protein FLJ20186	2.3 ↑ NM_017702
	Homo sapiens clone IMAGE:205688, mRNA sequence	2.3 ↑ AF339787,1
KIAA0977	KIAA0977 protein	2.0 ↓ BF002844
	Unknown (protein for MGC:13137) [Homo sapiens], mRNA sequence	2.2 ↓ BC006361,1
DKFZP564O0423	DKFZP564O0423 protein	2.5 ↓ BF112171
FLJ11939	hypothetical protein FLJ11939	2.6 ↓ AF525402
KIAA0323	KIAA0323 protein	3.0 ↓ AF075450
LOC91316	Homo sapiens cDNA FLJ14312 fis, clone PLACE3000322, mRNA sequence	4.0 ↓ AU158566
KIAA0652	KIAA0652 gene product	5.3 ↓ BC001331,1

Figure 3 (a) Total RNA samples were isolated from stably-transfected cell-lines expressing the ATF2 gene, and hybridized to Affymetrix Human 133 A microarray chips as described in Materials and methods. Following scanning and data analysis, those genes found to be up- or down-regulated by 2-fold or greater in comparison to baseline expression were classified according to their function by GeneSpring software, and are listed here. (b) Total RNA samples were isolated from stably-transfected cell-lines expressing the ATF2-sm isoform, and hybridized to Affymetrix Human 133 A microarray chips as described in Materials and methods. Following scanning and data analysis, those genes found to be up- or down-regulated by 2-fold or greater in comparison to baseline expression were classified according to their function by GeneSpring software, and are listed here.

subtracted, (ii) reverse subtracted, (iii) unsubtracted driver and (iv) unsubtracted tester cDNAs (Fig. 4). These genes were identified by sequencing of the clones represented by the colonies on the dot-blots, and are listed in Table 2.

Semi-quantitative and realtime RT-PCR

The validity of the results from the microarray and SSH experiments was examined by performing a panel of semi-quantitative RT-PCR reactions. Four randomly-selected genes were chosen from the results of the ATF2 and ATF2-sm microarray experiments for each factor and, by semi-quantitative RT-PCR, their mRNA levels assessed in control cultured myometrial cells containing empty vector, the stably-transfected myometrial cell-line used for the array hybridization, pooled non-pregnant (NP) tissue and pooled pregnant non-labouring (P) tissue ($n=6$; Fig. 5). In each case, the level of expression in stably-transfected cells compared with control cells reflected the results from the microarrays: those genes found to be up- or down-regulated by expression of ATF2 and ATF2-sm by microarray analysis also showed increased or decreased expression respectively in the stable cell-lines compared with control cells by RT-PCR. In addition, the expression of these genes was found to be significantly altered between the pooled NP and P tissue, reflecting the *in vivo* biological situation. Of the eight genes examined, four were expressed at a higher level in NP tissue and four at a higher level in P tissue. Real-time RT-PCR analysis of two of these genes, GNA15 and HSP70B', also indicated significant up-regulation of their expression in stable cell-lines in concordance with the microarray data, and also increased expression in NP tissue compared with P tissue reflecting the *in vivo* situation.

Confirmation of differential expression *in vivo* of eight randomly selected genes from the SSH results was obtained by semi-quantitative RT-PCR using total RNA isolated from pooled ($n=6$) NP and P myometrial tissue samples (see Materials and methods); representative products are shown in Fig. 6. Of the eight genes tested, all were found to be at a higher level in NP tissue when compared with P, possibly reflecting the *in vivo* condition.

Discussion

Using microarray analysis, SSH techniques, and semi-quantitative/realtime RT-PCR, comparing gene expression in cultured human myometrial cells stably transfected with expression constructs of ATF2 proteins with similar, empty-vector transfected control cell lines and pooled non-pregnant and pregnant myometrial tissue, we have identified a significant number of genes that are regulated by the ATF2 and novel ATF2-sm

Table 1 Number of genes affected by stable expression of ATF2 and ATF2-sm in cultured myometrial cells according to data from Affymetrix Human 133 A microarray chips 5

	No. of downstream genes affected	% of affected genes per chip	No. of up-regulated genes	No. of down-regulated genes
Stably-transfected gene				
ATF2-fl	204	0.97	113	91
ATF2-sm	55	0.26	29	26

transcription factors. The expression of 204 genes was altered by the full-length ATF2 isoform, representing just under 1% of the genes represented on the microarray chip; the recently identified and apparently novel ATF2-sm isoform, encoded by the first two and last two putative exons of the 12-exon ATF2 gene, altered the expression of 55 downstream target genes corresponding to 0.26% of genes on the chip. Screening of an SSH library identified a further set of 25 genes regulated by ATF2-sm.

The results revealed a diverse range of gene categories in terms of biological processes, cellular components and molecular functions that are regulated by these bZIP factors. A small selection of these genes is detailed here, in terms of their patterns of expression in the presence of the ATF2 bZIP transcription factors and how these relate to myometrial function during gestation and labour.

TNFR9 was observed to be up-regulated by ATF2. Genes from the tumour necrosis factor receptor superfamily (TNFR) have significant roles in the myometrium due to the importance of TNF α in gestation; this is expressed by myometrial stromal cells during pregnancy, although the major source in the myometrium is thought to be macrophages and other leukocytes (Yelavarthi *et al.* 1991, Tabibzadeh *et al.* 1994, Young *et al.* 2002). It stimulates the production of arachadonic acid and prostaglandins (in particular the stimulatory PGF1 α) (Pollard & Mitchell 1996a, b, Hertelendy *et al.* 2002); up-regulates matrix metalloproteinase-9 (MMP-9), which is involved in tissue remodelling of the myometrium during labour (Roh *et al.* 2000); increases adenyl cyclase (AC) activity in myometrial cells (Gogarten *et al.* 2003); and may prepare the myometrium for labour by regulating the cyclic-ADP-ribose-signalling (cADPR) pathway which controls the development of intracellular Ca²⁺ transients (Barata *et al.* 2004).

ATF2-sm increased the expression of the heat shock 70 kDa protein (HSPA6/HSP70B'); heat shock proteins form associations with steroid receptors, including those for oestrogen and progesterone, and modulate their functions (Komatsu *et al.* 1997). HSPs 70 and 90 have been shown to increase dramatically during labour in

the ovine myometrium, and possibly inhibit progesterone receptors and activate oestrogen receptors (Wu *et al.* 1996).

In the signal transduction/integrin receptor signalling class, ATF2 caused an increase in expression of the calcium channel, voltage dependent, P/Q type, α 1A subunit (CACNA1A), and a decrease in the expression of the regulator of G-protein signalling 14 (RGS14). With regard to CACNA1A, the importance of the regulation of calcium homeostasis in the myometrium and its relation to uterine contractility is self-evident. RGS proteins interact with the G α q and G α i proteins to accelerate GTPase activity and RGS2, for example, is known to increase in cultured human myometrial cells in response to oxytocin via an increase in intracellular Ca²⁺ concentration, and also increases during pregnancy and decreases just prior to labour in rat myometrium in response to progesterone levels (Park *et al.* 2002, Suarez *et al.* 2003).

The expression of various integrins and cadherins are known to alter in the human uterus during pregnancy (Brackin *et al.* 2002, Charpigny *et al.* 2003); for example, ICAM-1 has been found to be up-regulated in the myometrium 10.5-fold during labour (Ledingham *et al.* 2001), and E-selectin (SELE; endothelial adhesion molecule 1) has been shown to increase during labour (Thomson *et al.* 1999). In this respect ATF2-sm down-regulated expression of integrin α 3 (ITGA3).

Among the other functional classes of genes affected by ATF2 and ATF2-sm over-expression were the following: gonadotropin inducible transcription repressor-3 (GIOT-3) was down-regulated by ATF2, which may lead to an increase in its expression in the transition from the non-pregnant to the pregnant state as ATF2 expression decreases. This could also be augmented by the increase in myometrial hCG levels during pregnancy. The gene expression of four collagen types was altered by ATF2; XIII α 1 and IV α 3 were increased, whereas VI α 1 and XVII α 1 were decreased. Collagen is important in the fibrillar network of the uterus, where it provides mechanical support for its structural components and a favourable milieu for their activities (Goranova *et al.* 1993). Fibrils have been shown to reorganize in response to the hormonal changes that

Forward Subtraction

Reverse Subtraction

Unsubtracted Tester

Unsubtracted Driver

Figure 4 SSH results revealed differentially expressed genes in ATF2-sm stably transfected cells. Briefly, libraries of putative differentially expressed genes were constructed following the subtraction procedure, and transformed into bacterial cells for amplification. The inserts in the library plasmids were then amplified from these bacterial cultures by PCR, and spotted onto membranes for screening with forward subtracted, reverse subtracted, unsubtracted driver and unsubtracted tester probes. Examples of the resultant blots are shown here. The likelihood of differential expression of any particular clone was indicated by the pattern and intensity of the signals from the four blots; three instances are shown here, where $\blacklozenge$ = probably differentially expressed; $\whitearrow$ = probably differentially expressed if intensity difference between forward subtraction and others is high; $\whitearrow$ = not differentially expressed. Boxed area = negative controls.

take place during pregnancy, and also to be degraded by myofibroblastic interstitial cells in preparation for the labouring and postpartum stages to allow the development of elastic fibres and basement membranes (Nishinaka & Fukuda 1991). Three forms of collagen

type VI (one of which was down-regulated by ATF2 here) have been shown to be present at high levels in murine myometrium during pregnancy (Dziadek *et al.* 1995). In addition, ATF2 caused a decrease in the expression of the MMP-20. The expression of MMPs in

Table 2 Putative differentially expressed genes in ATF2-sm stably-transfected myometrial cells, as revealed by SSH

Gene symbol	Gene description	Accession No.
CYB	Cytochrome b	AF346979
SF3B1	Splicing factor 3b (subunit 1)	NM_012433
ACTA1	Actin α 1	XM_001869
NACA	Nascent polypeptide associated complex	NM_005594
BZW1/BZAP45	Basic leucine Zipper & W domains 1	NM_014670
bTMalpha/TPM1	Tropomyosin alpha	NM_000366
RPL9	Ribosomal protein L9	U009953
ATP5O	O subunit of mitochondrial ATP synthase	NM_001697
LIM	LIM protein (similar to rat PKC binding enigma)	BC008741
MTATP6	ATP synthase 6	AF347008
TES	Testis derived transcript	NM_152829
COL4A1	Collagen type IV alpha 1	NM_001845
MYBPC1	Myosin-binding protein C (slow type)	XM_017524
FLJ32432	Similar to human Nebulin (NEB) gene	AK056994
VPS33B	Vacuolar protein sorting 33B	NM_018668
CAP350	Centrosome associated protein 350	NM_014810
CDH2	Cadherin2, type I/N-Cadherin	NM_001792
RPL15	Ribosomal protein L15	NM_002948
CITED2	CBP/p300-interacting transactivator	NM_006079
FACL4	Fatty acid co-enzyme A ligase, long chain 4	NM_004458
SCA2	Spinocerebellar ataxia 2	NM_002973
HRF/TCTP	Histamine-releasing factor (tumour protein); aka Fortilin	NM_003295
ZNF297B	ZNF protein, aka ZNF-X	NM_014007
HNRPDL	Heterologous nuclear ribonucleoprotein D-like	NM_031372
ANXA2	Annexin A2, aka lipocortin II, calpactin I	BC023990

the myometrium is regulated by TGF- β 1 (Ma & Chegini 1999), and is absolutely dependent on the presence of serotonin, which is induced by IL-1 α (Dumin *et al.* 1998).

Protein kinase C (PKC), involved in the mediation of endothelin-induced uterine contractions (Oriji & Keiser 1996) and the regulation of oxytocin-mediated myometrial contractions (Phillippe 1994), is of critical importance in the myometrium. It acts to promote a contractile state by reducing the activity of adenylyl cyclase and therefore cAMP accumulation (Grammatopoulos *et al.* 1996, Grammatopoulos & Hillhouse 1999), and by reducing guanylyl cyclase activity and therefore cGMP accumulation, to neutralize relaxatory pathways. ATF2, however, caused a decrease in expression of the ι (iota) isoform; this may be involved in cytokine-induced NF-kappa B activation (Anthonsen *et al.* 2001).

G protein coupled receptors (GPCRs) comprise the largest family of receptors in the uterus; they can be stimulatory or inhibitory, and their interaction with heterotrimeric GTP-binding proteins enables them to induce the hydrolyzation of GTP and to modulate the activity of a wide variety of enzymes and ion channels that affect uterine contractility. Some GPCRs are coupled to phospholipase C, which generates the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. These act to stimulate contractions via the

mobilization of calcium in the sarcoplasmic reticulum in the case of IP₃, and in the case of diacylglycerol to activate PKC and the MAPK cascades (Bernal 2003). Other GPCRs are coupled to adenylyl cyclase, which promotes the accumulation of cAMP which acts to promote uterine relaxation via the inactivation of myosin light-chain kinase (MLCK) and the action of the cAMP-dependent bZIP transcription factors. In addition, uterine contractility can also be enhanced via the activation of Rho GTPases, and the subsequent action of Rho kinases to potentiate the effect of MLCK (Moore *et al.* 2000, Moran *et al.* 2002). GPCR63 was up-regulated by ATF2.

S100 calcium-binding protein A13 (S100A13) has been shown to mediate the stress-induced release of IL-1 α (Mandinova *et al.* 2003); this interleukin induces the expression of serotonin in the uterus, which in turn allows the expression of MMPs in myometrial cells (Dumin *et al.* 1998). The importance of collagen and MMPs in the uterus has already been discussed; S100A13 was up-regulated by ATF2.

The gene for caveolin 2 (CAV2) was up-regulated by ATF2; caveolin expression in the myometrium of the pregnant rat is known to be suppressed in the first half of pregnancy, after which it increases up to delivery, increasing the number of caveolae present in myometrial cells (Turi *et al.* 2001). This has been shown to be dependent upon levels of oestrogen and progesterone.

Gene	Discovered in stably-transfected cells expressing:	Microarray result:	Quantitative RT-PCRs		Semi-Quantitative RT-PCRs			
			Stable cells/control cells	NP tissue/P tissue	Empty vector control cells	Stably-transfected cells	NP	P
GPR63	ATF2	5.9 ↑						
GNA15	ATF2	4.5 ↑	4.4 ±0.39 ↑	8.3 ±0.59 ↑				
GAS1	ATF2	40.1 ↓						
MAP7	ATF2	8.3 ↓						
HSP70B'	ATF2-sm	5.6 ↑	2.3 ±0.3 ↑	2.0 ±0.61 ↑				
KIF20	ATF2-sm	14.1 ↑						
CALCRL	ATF2-sm	4.0 ↓						
GAB2	ATF2-sm	2.4 ↓						

Figure 5 Semi-quantitative and quantitative RT-PCR was performed to verify differential expression of a random selection of genes revealed in the microarray analyses. Comparisons were made between (a) total RNA templates isolated from control empty vector stably-transfected cell lines and cell lines stably transfected with ATF2 and ATF2-sm constructs, and (b) mRNA templates isolated from pooled ($n=6$) NP and P myometrial tissue samples. 5 ng of mRNA or 50 ng of total RNA was used as template in RT-PCR reactions using the SuperScript OneStep RT-PCR system (Invitrogen) for 28, 30 and 32 PCR cycles, and the linear-range products were analyzed by 2% agarose gel electrophoresis, as shown above. In each case, the level of expression in stably-transfected cells compared with control cells reflected the results from the microarrays: those genes found to be up- or down-regulated by expression of ATF2 and ATF2-sm by microarray analysis also showed increased or decreased expression, respectively, in the stable cell-lines compared with control cells by RT-PCR. In addition, the expression of these genes was found to be significantly altered between the pooled NP and P tissue, reflecting the *in vivo* biological situation. Of the eight genes examined, four were expressed at a higher level in NP tissue and four at a higher level in P tissue. Real-time RT-PCR analysis of two of these genes, GNA15 and HSP70B', also indicated significant up-regulation of their expression in stable cell-lines in concordance with the microarray data, and also increased expression in NP tissue compared with P tissue reflecting the *in vivo* situation.

There is mounting evidence that cytokines are critically important in the modulation of uterine activity and that the regulation of parturition is, to a large degree, an inflammatory process. A rise in pro-inflammatory cytokines and the infiltration of leukocytes into the uterine tissues at labour are well documented. They are known to be largely derived from the placenta and extra-placental membranes (gestational tissues) (Bowen *et al.* 2002), although the cytokine-inducible expression of interleukins in uterine smooth muscle cells suggests that the myometrium itself may be a significant contributor of inflammatory mediators (Helmer *et al.* 2002). The chemokine (C-C motif) receptor 4 (CCR4) was up-regulated by ATF2.

Endothelin receptors were affected by ATF2; receptor type A, which is preferentially expressed in the upper segment of the uterus and thought to exert a stimulatory effect with regard to contractility, was found to be down-regulated.

Any variance in the expression of oestrogen receptors will, taking into account the importance of the hormone and its wide ranging effects upon other factors involved in the control of uterine gene expression and contractility, exert promiscuous and far reaching effects. It is therefore interesting to note the increased

expression of oestrogen receptor 2 (ESR2/ESR-beta) with over-expression of ATF2, and to compare this to the detected increase in expression of the alternative oestrogen receptor 1 (ESR1/ESR-alpha) associated with over-expression of the ATF2-related bZIP transcription factor CREB in myometrial cells (Bailey *et al.* 2004), which has been found to be prevalent in the non-pregnant myometrium but almost absent in pregnant non-labouring and spontaneous labouring tissue (Bailey *et al.* 2000). Interestingly, a significant minority of genes found to be affected by the over-expression of ATF2 and ATF2-sm in myometrial cells in this study are also affected by CREB (Bailey *et al.* 2004): 25% of the genes affected by ATF2, and 38% of the genes affected by ATF2-sm.

An overlap also exists between the groups of genes affected by ATF2 and ATF2-sm. This is represented in the form of a Venn diagram in Fig. 7. The number of genes commonly affected by the two ATF2 proteins amounts to only two, or just under 1% and just over 3% of genes affected by ATF2 and ATF2-sm respectively. However, these two genes may well be of great importance with regard to myometrial contractility; one, TRPC4 encodes a component of store-operated calcium entry (SOCE) channels that play an important role in

Figure 6 Semi-quantitative RT-PCRs of selected differentially expressed candidate genes from the SSH screen of ATF2-sm stably-transfected cells. One-tube RT-PCR was performed using total RNA isolated from NP and P lower segment myometrial tissue samples as template, using 28, 30 and 32 PCR cycles to obtain a representative band from the linear amplification range after agarose gel electrophoresis. Bands were visualized using a gel documentation system at equivalent integrations, and scans of these are shown above. In each case, candidate positive genes from the SSH screen, i.e. those up-regulated in ATF2-sm stably-transfected cells, were found to be more highly expressed in the NP myometrium.

myometrial calcium homeostasis (Dalrymple *et al.* 2002), and was up-regulated by both ATF2 isoforms. The other, CALCRL is related to the CGRP, which induces dose-dependent relaxation in spontaneously contracting myometrium from pregnant women, an effect that is diminished in myometrium obtained from patients during labour and in the non-pregnant states (Dong *et al.* 1999). This gene was down-regulated by both ATF2 isoforms.

Our previous results indicate that *in vivo*, at some point during pregnancy, expression of ATF2 is down-regulated in the myometrium whereas ATF2-sm becomes spatially expressed with greater levels detected in the fundus compared with the lower uterine segment (Bailey *et al.* 2000). These events may consequently have major effects on the genes observed to be regulated by

these proteins in this study. It seems increasingly clear that the uterus can almost be regarded as two separate organs as term approaches, with the upper segment becoming highly contractile to exert downward pressure on the foetus at parturition, and the lower segment dilating to allow passage of the foetus through the birth canal. Plainly, such juxtaposing functions in the same organ must require global differences in gene expression, and the spatial expression of ATF2-sm in different regions of the uterus may have a role in this process. Regarding this spatial expression, a number of other genes have also been shown to be expressed spatially in the uterus; these include the oxytocin receptor (OTR) (Fuchs *et al.* 1984), connexin-43 and cyclo-oxygenase-2 (COX-2) (Sparey *et al.* 1999).

The spatio-temporal expression of ATF2 bZIP transcription factors raises the concern of their modes of action; not only are these factors responsive to different stimuli and activated by different pathways, but their dimerization codes bring into play several promoter elements in the downstream genes affected by them. Although the ATF2 and ATF2-sm proteins, in common with the related CREB/CREM factors, are substrates for PKA-mediated phosphorylation *in vitro*, the ATF2 proteins are insensitive to cAMP/PKA stimulation, and instead are a major target of MAPK cascades, upon which it depends for its activation. These MAPK cascades, which are instigated for example in response to the presence of inflammatory cytokines (well-characterized modulators of uterine gene expression), are classified into three subfamilies; the p38 cascade, the c-Jun amino-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) cascade, and the extracellular signal-regulated protein kinase (ERK) cascade. All three of these cascades act in response to a variety of stresses to cause the phosphorylation and activation of ATF2 and increase its transcriptional activity, and the type of cascade involved is dependent upon the type of stimulatory factor (Hayakawa *et al.* 2003). The latter acts through the binding of mitogens or growth factors at the cell membrane, recruiting the Ras GTPase which activates the Raf Ser/Thr kinase. This in turn activates MAPK/ERK kinase (MEK) which phosphorylates and activates ERK1 and ERK2. These factors translocate to the nucleus where they activate a range of transcription factors that control cellular processes such as growth and differentiation, including ATF2. With regard to dimerization, ATF2 for example forms dimers with NF-κB and c-Jun; such dimers have greatly altered promoter motif binding properties (Wagner & Green 1994), and variable affinities to CRE contexts. ATF2:c-Jun heterodimers have a great affinity for AP1 sites compared with CREs, and the two constituent proteins may show either an antagonistic relationship to one another, or be present in certain tissues at inversely proportional levels. We have previously shown that the ATF2-sm protein also

Figure 7 The ATF2 and ATF2-sm factors regulate highly discrete groups of genes; only two were regulated by both proteins. However, these two genes may well be of great importance with regard to myometrial contractility; one, TRPC4, encodes a component of store-operated calcium entry (SOCE) channels that play an important role in myometrial calcium homeostasis, and was up-regulated by both ATF2 isoforms. The other, CALCRL, is related to the CGRP, which induces dose-dependent relaxation in spontaneously contracting myometrium from pregnant women, an effect that is diminished in myometrium obtained from patients during labour and in the non-pregnant states. This gene was down-regulated by both ATF2 isoforms.

heterodimerizes with c-Jun in the myometrium, though the consequences of this are not presently known (Bailey *et al.* 2002).

In support of our hypothesis that ATF2/ATF2-sm proteins may play an important role involved in modulating myometrial gene expression and functionality, we have made an effort to link genes affected by these proteins to what is known of the *in vivo* physiology of the uterus during gestation and labour.

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References

- Abdel-Hafiz HA, Heasley LE, Kyriakis JM, Avruch J, Kroll DJ, Johnson GL & Hoeffler JP 1992 Activating transcription factor-2 DNA-binding activity is stimulated by phosphorylation catalyzed by p42 and p54 microtubule-associated protein kinases. *Molecular Endocrinology* **6** 2079–2089.
- Anthonsen MW, Andersen S, Solhaug A & Johansen B 2001 Atypical lambda/iota PKC conveys 5-lipoxygenase/leukotriene B4-mediated cross-talk between phospholipase A2s regulating NF-kappa B activation in response to tumor necrosis factor-alpha and interleukin-1 beta. *Journal of Biological Chemistry* **276** 35344–35351.
- Bailey J, Sparey C, Phillips RJ, Gilmore K, Robson SC, Dunlop W & Europe-Finner GN 2000 Expression of the cyclic AMP-dependent transcription factors, CREB, CREM and ATF2, in the human myometrium during pregnancy and labour. *Molecular Human Reproduction* **6** 648–660.
- Bailey J, Phillips RJ, Pollard AJ, Gilmore K, Robson SC & Europe-Finner GN 2002 Characterization and functional analysis of cAMP response element modulator protein and activating transcription factor 2 (ATF2) isoforms in the human myometrium during pregnancy and labor: identification of a novel ATF2 species with potent transactivation properties. *Journal of Clinical Endocrinology and Metabolism* **87** 1717–1728.
- Bailey J, Tyson-Capper (née Pollard) A, Gilmore K, Robson SC & Europe-Finner GN 2004 Identification of human myometrial target genes of the CAMP pathway: the role of CAMP-response element binding (CREB) and modulator (CREM α and CREM $\tau_2\alpha$) proteins. *Journal of Molecular Endocrinology* **34** 1–17 (see this issue).
- Barata H, Thompson M, Zielinska W, Han YS, Mantilla CB, Prakash YS, Feitoza S, Sieck G & Chini EN 2004 The role of cyclic-ADP-ribose-signaling pathway in oxytocin-induced Ca2+ transients in human myometrium cells. *Endocrinology* **145** 881–889.
- Bernal AL 2003 Mechanisms of labour—biochemical aspects. *British Journal of Obstetrics and Gynaecology* **110** 39–45.
- Bowen JM, Chamley L, Keelan JA & Mitchell MD 2002 Cytokines of the placenta and extra-placental membranes: roles and regulation during human pregnancy and parturition. *Placenta* **23** 257–273.
- Brackin MN, Cruse JM, Lewis RE, Hines RS, Stoppie JA & Cowan BD 2002 Quantitative analysis of adhesion molecules on cellular constituents of the human uterine microenvironment under the influence of estrogen and progesterone. *Experimental and Molecular Pathology* **72** 91–114.
- Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J & Vingron M 2001 Minimum information about a microarray experiment (MIAME)—toward standards for microarray data. *Nature Genetics* **29** 365–371.
- Chang L & Karin M 2001 Mammalian MAP kinase signalling cascades. *Nature* **410** 37–40.
- Chapigny G, Leroy MJ, Breuiller-Fouche M, Tanfin Z, Mhaouty-Kodja S, Robin P, Leiber D, Cohen-Tannoudji J, Cabrol D, Barberis C & Germain G 2003 A functional genomic study to identify differential gene expression in the preterm and term human myometrium. *Biology of Reproduction* **68** 2289–2296.
- Cho SG, Bhounik A, Broday L, Ivanov V, Rosenstein B & Ronai Z 2001 TIP49b, a regulator of activating transcription factor 2

- response to stress and DNA damage. *Molecular and Cellular Biology* **21** 8398–8413.
- Dalrymple A, Slater DM, Beech D, Poston L & Tribe RM 2002 Molecular identification and localization of Trp homologues, putative calcium channels, in pregnant human uterus. *Molecular Human Reproduction* **8** 946–951.
- Davis RJ 2000 Signal transduction by the JNK group of MAP kinases. *Cell* **103** 239–252.
- Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M & Davis RJ 1994 JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76** 1025–1037.
- Dong YL, Fang L, Kondapaka S, Gangula PR, Wimalawansa SJ & Yallampalli C 1999 Involvement of calcitonin gene-related peptide in the modulation of human myometrial contractility during pregnancy. *Journal of Clinical Investigation* **104** 559–565.
- Dumin J, Wilcox BD, Otterness I, Melendez JA, Huang C & Jeffrey JJ 1998 Serotonin-mediated production of interstitial collagenase by uterine smooth muscle cells requires interleukin-1 alpha, but not interleukin-1 beta. *Journal of Biological Chemistry* **273** 25488–25494.
- Dziadek M, Darling P, Zhang RZ, Pan TC, Tillet E, Timpl R & Chu ML 1995 Expression of collagen alpha 1(VI), alpha 2(VI), and alpha 3(VI) chains in the pregnant mouse uterus. *Biology of Reproduction* **52** 885–894.
- Foulkes NS, Borrelli E & Sassone-Corsi P 1991 CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64** 739–749.
- Fuchs A, Fuchs F, Husslein P & Soloff M 1984 Oxytocin receptors in the human uterus during pregnancy and parturition. *American Journal of Obstetrics and Gynecology* **150** 734–741.
- Fuchs SY, Tappin I & Ronai Z 2000 Stability of the ATF2 transcription factor is regulated by phosphorylation and dephosphorylation. *Journal of Biological Chemistry* **275** 12560–12564.
- Gibellini D, Re MC, La Placa M & Zauli G 2002 Differentially expressed genes in HIV-1 tat-expressing CD4(+) T-cell line. *Virus Research* **90** 337–345.
- Gogarten W, Lindeman KS, Hirshman CA & Emala CW 2003 Tumor necrosis factor alpha stimulates adenylyl cyclase activity in human myometrial cells. *Biology of Reproduction* **68** 751–757.
- Goranova V, Vizza E & Motta PM 1993 Collagen fibrillar network in estrous and hCG-stimulated rabbit uterus: a SEM study after NaOH maceration. *Archives of Histology and Cytology* **56** 231–241.
- Grammatopoulos D & Hillhouse E 1999 Role of corticotropin-releasing hormone in onset of labour. *Lancet* **354** 1546–1549.
- Grammatopoulos D, Stirrat GM, Williams SA & Hillhouse EW 1996 The biological activity of the corticotropin-releasing hormone receptor-adenylyl cyclase complex in human myometrium is reduced at the end of pregnancy. *Journal of Clinical Endocrinology and Metabolism* **81** 745–751.
- Gupta S, Campbell D, Derijard B & Davis RJ 1995 Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267** 389–393.
- Hai T & Curran T 1991 Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *PNAS* **88** 3720–3724.
- Hai T, Liu F, Coukos WJ & Green MR 1989 Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes and Development* **3** 2083–2090. [published erratum, 1990 **4** 682]
- Hayakawa J, Depatie C, Ohmichi M & Mercola D 2003 The activation of c-Jun NH2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *Journal of Biological Chemistry* **278** 20582–20592.
- Helmer H, Tretzmüller U, Brunbauer M, Kaider A, Husslein P & Knöfler M 2002 Production of oxytocin receptor and cytokines in primary uterine smooth muscle cells cultivated under inflammatory conditions. *Journal of the Society for Gynecologic Investigation* **9** 15–21.
- Hertelendy F, Molnar M & Romero R 2002 Interferon gamma antagonizes interleukin-1 beta-induced cyclooxygenase-2 expression and prostaglandin E production in human myometrial cells. *Journal of the Society for Gynecologic Investigation* **9** 215–219.
- Hochberg Y & Benjamini Y 1990 More powerful procedures for multiple significance testing. *Statistics in Medicine* **9** 811–818.
- Hoeffler JP, Meyer TE, Yun Y, Jameson JL & Habener JF 1988 Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* **242** 1430–1433.
- Karin M & Hunter T 1995 Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Current Biology* **5** 747–757.
- Kawasaki H, Song J, Eckner R, Ugai H, Chiu R, Taira K, Shi Y, Jones N & Yokoyama KK 1998 p300 and ATF-2 are components of the DRF complex, which regulates retinoic acid- and E1A-mediated transcription of the c-jun gene in F9 cells. *Genes and Development* **12** 233–245.
- Kawasaki H, Schiltz L, Chiu R, Itakura K, Taira K, Nakatani Y & Yokoyama KK 2000 ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405** 195–200.
- Komatsu T, Konishi I, Fukumoto M, Nanbu K, Koshiyama M, Mandai M & Mori T 1997 Messenger ribonucleic acid expression of heat shock proteins HSP70 and HSP90 in human endometrium and myometrium during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **82** 1385–1389.
- Kyriakis JM & Avruch J 2001 Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiological Reviews* **81** 807–869.
- Landschulz WH, Johnson PF & McKnight SL 1988 The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240** 1759–1764.
- Ledingham MA, Thomson AJ, Jordan F, Young A, Crawford M & Norman JE 2001 Cell adhesion molecule expression in the cervix and myometrium during pregnancy and parturition. *Obstetrics and Gynecology* **97** 235–242.
- Lee KA, Hai TY, SivaRaman L, Thimmappaya B, Hurst HC, Jones NC & Green MR 1987 A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *PNAS* **84** 8355–8359.
- Livingstone C, Patel G & Jones N 1995 ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO Journal* **14** 1785–1797.
- Ma C & Chegini N 1999 Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-beta1. *Molecular Human Reproduction* **5** 950–954.
- Maekawa T, Sakura H, Kanei-Ishii C, Sudo T, Yoshimura T, Fujisawa J, Yoshida M & Ishii S 1989 Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO Journal* **8** 2023–2028.
- Maekawa T, Bernier F, Sato M, Nomura S, Singh M, Inoue Y, Tokunaga T, Imai H, Yokoyama M, Reimold A, Glimcher LH & Ishii S 1999 Mouse ATF-2 null mutants display features of a severe type of meconium aspiration syndrome. *Journal of Biological Chemistry* **274** 17813–17819.
- Mandino A, Soldi R, Graziani I, Bagala C, Bellum S, Landriscina M, Tarantini F, Prudovsky I & Maciag T 2003 S100A13 mediates the copper-dependent stress-induced release of IL-1 alpha from both human U937 and murine NIH 3T3 cells. *Journal of Cell Science* **116** 2687–2696.
- Mayanil CS, George D, Freilich L, Miljan EJ, Mania-Farnell B, McLone DG & Bremer EG 2001 Microarray analysis detects novel Pax3 downstream target genes. *Journal of Biological Chemistry* **276** 49299–49309.

- Montminy MR, Sevarino KA, Wagner JA, Mandel G & Goodman RH 1986 Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *PNAS* **83** 6682–6686.
- Moore F, Da Silva C, Wilde JI, Smarason A, Watson SP & Lopez Bernal A 2000 Up-regulation of p21- and RhoA-activated protein kinases in human pregnant myometrium. *Biochemical and Biophysical Research Communications* **269** 322–326.
- Moran CJ, Friel AM, Smith TJ, Cairns M & Morrison JJ 2002 Expression and modulation of Rho kinase in human pregnant myometrium. *Human Reproduction* **8** 196–200.
- Nishinaka K & Fukuda Y 1991 Changes in extracellular matrix materials in the uterine myometrium of rats during pregnancy and postparturition. *Acta Pathologica Japonica* **41** 122–132.
- Orij G & Keiser HR 1996 Action of protein kinase C in endothelin-induced contractions in rat aortic rings. *American Journal of Physiology* **271** 398–404.
- Ouwens DM, de Ruiter ND, van der Zon GC, Carter AP, Schouten J, van der Burgt C, Kooistra K, Bos JL, Maassen JA & van Dam H 2002 Growth factors can activate ATF2 via a two-step mechanism: phosphorylation of Thr71 through the Ras-MEK-ERK pathway and of Thr69 through RalGDS-Src-p38. *EMBO Journal* **21** 3782–3793.
- Park ES, Echeteu CO, Soloff S & Soloff MS 2002 Oxytocin stimulation of RGS2 mRNA expression in cultured human myometrial cells. *American Journal of Physiology Endocrinology and Metabolism* **282** 580–584.
- Phillippe M 1994 Protein kinase C, an inhibitor of oxytocin-stimulated phasic myometrial contractions. *Biology of Reproduction* **50** 855–859.
- Pollard JK & Mitchell MD 1996a Effects of gestational age on prostaglandin production and its regulation in human myometrial cells. *Journal of Maternal and Fetal Medicine* **5** 93–98.
- Pollard JK & Mitchell MD 1996b Intrauterine infection and the effects of inflammatory mediators on prostaglandin production by myometrial cells from pregnant women. *American Journal of Obstetrics and Gynecology* **174** 682–686.
- Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B & Davis RJ 1996 MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Molecular and Cellular Biology* **16** 1247–1255.
- Read MA, Whitley MZ, Gupta S, Pierce JW, Best J, Davis RJ & Collins T 1997 Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. *Journal of Biological Chemistry* **272** 2753–2761.
- Reimold AM, Grusby MJ, Kosaras B, Fries JW, Mori R, Maniwa S, Clauss IM, Collins T, Sidman RL, Glimcher MJ & Glimcher LH 1996 Chondrodysplasia and neurological abnormalities in ATF2-deficient mice. *Nature* **379** 262–265.
- Roh CR, Oh WJ, Yoon BK & Lee JH 2000 Up-regulation of matrix metalloproteinase-9 in human myometrium during labour: a cytokine-mediated process in uterine smooth muscle cells. *Molecular Human Reproduction* **6** 96–102.
- Ronai Z, Yang YM, Fuchs SY, Adler V, Sardana M & Herlyn M 1998 ATF2 confers radiation resistance to human melanoma cells. *Oncogene* **16** 523–531.
- Saalbach A, Aust G, Haustein UF, Herrmann K & Anderegg U 1997 The fibroblast-specific MAb AS02: a novel tool for detection and elimination of human fibroblasts. *Cell and Tissue Research* **290** 593–599.
- Sparey C, Robson SC, Bailey J, Lyall F & Europe-Finner GN 1999 The differential expression of myometrial connexin-43, cyclooxygenase-1 and -2, and Gs alpha proteins in the upper and lower segments of the human uterus during pregnancy and labor. *Journal of Clinical Endocrinology and Metabolism* **84** 1705–1710.
- Steinmuller L & Thiel G 2003 Regulation of gene transcription by a constitutively active mutant of activating transcription factor 2 (ATF2). *Biological Chemistry* **384** 667–672.
- Suarez VR, Park ES, Hankins GD & Soloff MS 2003 Expression of regulator of G protein signaling-2 in rat myometrium during pregnancy and parturition. *American Journal of Obstetrics and Gynecology* **188** 973–977.
- Tabibzadeh S, Kong QF & Babaknia A 1994 Expression of adhesion molecules in human endometrial vasculature throughout the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism* **79** 1024–1032.
- Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, Greer IA & Norman JE 1999 Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Human Reproduction* **14** 229–236.
- Turi A, Kiss AL & Mullner N 2001 Estrogen downregulates the number of caveolae and the level of caveolin in uterine smooth muscle. *Cell Biology International* **25** 785–794.
- van Dam H & Castellazzi M 2001 Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene* **20** 2453–2464.
- van Dam H, Wilhelm D, Herr I, Steffen A, Herrlich P & Angel P 1995 ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO Journal* **14** 1798–1811.
- Wagner S & Green MR 1994 DNA-binding domains: targets for viral and cellular regulators. *Current Opinion in Cell Biology* **6** 410–414.
- Wu WX, Derks JB, Zhang Q & Nathanielsz PW 1996 Changes in heat shock protein-90 and -70 messenger ribonucleic acid in uterine tissues of the ewe in relation to parturition and regulation by estradiol and progesterone. *Endocrinology* **137** 5685–5693.
- Yamamoto KK, Gonzalez GA, Biggs WD & Montminy MR 1988 Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334** 494–498.
- Yelavarthi KK, Chen HL, Yang YP, Cowley BD Jr, Fishback JL & Hunt JS 1991 Tumor necrosis factor-alpha mRNA and protein in rat uterine and placental cells. *Journal of Immunology* **146** 3840–3848.
- Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA & Norman JE 2002 Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. *Biology of Reproduction* **66** 445–449.
- Ziff EB 1990 Transcription factors: a new family gathers at the cAMP response site. *Trends in Genetics* **6** 69–72.

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