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Title: Up-regulation of *PSCDBP*, *TLR2*, *TWIST1*, *FLJ35382*, *EDNRB* and *RGS12* gene expression in human myometrium at labor

Short title: *PSCDBP*, *TLR2*, *TWIST1*, *FLJ35382*, *EDNRB* and *RGS12* myometrial gene expression

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ABSTRACT

The regulatory mechanisms underlying myometrial smooth muscle contractility during labor are poorly understood. We therefore investigated the transcriptional profile of the changes that occur in the human myometrium at term pregnancy in comparison to that at labor. Microarray technology was utilized to identify differentially expressed genes in human myometrium at labor. Real-time fluorescence RT-PCR was subsequently performed to verify the microarray data. Semi-quantitative RT-PCR, Western blotting and microscopy methodologies were also utilized. Certain novel genes were found to be up-regulated in human myometrium at labor. Of these, *PSCDBP*, *TLR2*, *TWIST1*, *FLJ35382* and *RGS12* have not been previously characterized or identified in human myometrium. *EDNRB* is the other novel labor associated gene whose reported expression is also up-regulated at labor. All six genes were expressed on human myometrial smooth muscle cells. These novel up-regulated genes are involved in multiple pathways which may be associated with a variety of cellular processes including inflammation, transcriptional regulation and intracellular signaling.

Keywords: myometrium, labor, gene expression

INTRODUCTION

During human labor, the uterus undergoes dramatic changes in its contractile activity, whereby the myometrium is transformed from a state of relative quiescence to one of maximal contractile activity.[1] The regulatory mechanisms underlying uterine smooth muscle contractility during labor are poorly understood. Information relating to these mechanisms is essential to understand the underlying etiology of disorders associated with human parturition such as preterm labor. Preterm labor affects 5–10% of all pregnancies and accounts for 70–75% of early neonatal morbidity and mortality.[2]

Recently, functional genomics tools including microarrays have been employed in attempts to further elucidate the mechanisms regulating myometrial contractility during labor.[3-7] These studies have revealed a complex picture involving multiple pathways associated with a variety of cellular processes including transcriptional regulation, intracellular signaling and cytoskeletal rearrangement.

To further elucidate the processes occurring in the human myometrium during normal labor and consequently preterm labor we investigated transcriptional expression in this tissue, at term pregnancy and at labor.

MATERIALS AND METHODS

Patient Recruitment and Tissue Collection

Patient recruitment took place in the Department of Obstetrics and Gynecology, University College Hospital Galway (UCHG), Ireland. The study was approved by the Research Ethics committee, UCHG, and recruitment was carried out by provision of information sheets and obtaining written informed consent. All biopsies of myometrium at term delivery, were excised

from the midline of the upper lip of the uterine incision, during elective (non-laboring, NL) and intrapartum (laboring, L) cesarean section. Myometrial samples were carefully dissected to minimize decidual inclusion. Women who had received prostaglandins or oxytocin for either induction or augmentation of labor were excluded from the study. The criteria for inclusion in the intrapartum group were regular spontaneous uterine contractions, effacement of the cervix, and cervical dilatation >3cm prior to cesarean section. Women with malignant conditions, and those receiving exogenous hormone therapy (e.g. progestagens), were excluded from the study. There was no evidence of chorioamnionitis in any of the subjects recruited for this study. Immediately upon removal, biopsies were rinsed in sterile saline and used directly for primary cell preparation or snap frozen in liquid nitrogen and stored at –80°C until RNA isolation.

Myometrial Cell Isolation and Culture

Myometrial tissue samples were minced (finely and any fibrous tissue removed) and digested in sterile filtered Dulbecco Modified Eagle medium (minus calf serum) containing 1mg/ml collagenase type IA and 1 mg/ml collagenase type XI and 0.1% BSA (Sigma-Aldrich, Dublin, Ireland) for 45 minutes. The resulting suspension was vortexed and the non-dispersed tissue fragments were separated by filtration of the mixture through sterile gauze layers and individual cells were then collected by centrifugation at 400g for 10 minutes. Cells were then washed and centrifuged 2 to 3 times in sterile PBS. After washing cells were cultured in SGM-2 medium (Cambrex, Biowhittaker UK Ltd., Wokingham, Berkshire, UK) at 37°C and 5% CO_2. Cells were sub-cultivated with trypsin/EDTA at a 1:2 or 1:3 split after reaching confluence.

Myometrial smooth muscle cells subpassaged from primary cells in culture, were characterized for mRNA expression of calponin, and estrogen receptor α and for SMα actin mRNA and protein expression.

RNA Extraction

Total RNA was isolated from human myometrium using TRIzol reagent (Life Technologies Ltd., UK).[8] Total RNA was isolated from the myometrial smooth muscle cells (passage 6) using the RNeasy mini RNA isolation kit (Qiagen, Crawley, West Sussex, UK). To eliminate any residual contaminating genomic DNA all RNA samples were DNase-treated with a DNA removal kit (Ambion, Spitfire Close Huntingdon, Cambridgeshire, UK). The RNA samples were then denatured at 65°C for 10 minutes. RNA concentration was determined by absorbance at A_{260} and also using the Ribogreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA) or with the Nanodrop (Nanodrop Technologies, Wilmington, USA).

Microarray Experiments

The concentration and quality of the total RNA were assessed by spectrophotometry (Nanodrop, Nanodrop Technologies, USA) and Bioanalyzer (Agilent, Santa Clara, California, USA). Reverse Transcription-In vitro transcription (RT-IVT) digoxigenin (DIG) labeling was performed on 0.5 μg total RNA in accordance to the Applied Biosystems Chemiluminescent RT-IVT labeling protocol (ABI, Foster City, USA). All samples were analyzed separately without pooling of extracted RNA. QC procedures (Nanodrop and Agilent bioanalyzer) were carried out on the cRNA samples to confirm the quality and quantity of the cRNA. The 6 DIG labeled cRNA samples were fragmented and subsequently prepared for hybridization to Applied Biosystems Genome Survey Microarray (version 2) 32,878 probes for 29,098 genes, for 16

hours, 3 chips, NL and 3 chips, L Following hybridization the arrays were stained using the Applied Biosystems Chemiluminescence detection kit, with an anti-DIG antibody-Alkaline Phosphatase conjugate. Interaction of alkaline phosphatase, enhancer and chemiluminescent substrate produced light with an emission maximum of 458 nm. The arrays were then scanned using the Applied Biosystems 1700 Chemiluminescent Microarray Ananlyser. (ABI) (Geneservice, Cambridge, UK). Spotfire DecisionSite for Functional Genomics (Tibco Software Inc. Palo Alto, USA), Bioconductor (www.bioconductor.org), Panther (ABI, USA), Genomatix Bibliosphere Pathway (Genomatix Software GmbH, Munich, Germany) analysis tools were utilized for data analysis. The signal intensity for each gene across the 6 samples was determined and signal/noise calculated, standard deviation, co-efficient of variance, z-scores and quantiles were also computed. Normalized signal for each of the 3 NL and 3 L samples were averaged, the [log2 (L(Average)/NL(Average))] calculated and fold change determined. The Spotfire profile ANOVA algorithm was used to determine the p-values where values less than 0.05 were considered significant.

Reverse Transcription

RNA (500ng - DNase I treated) was reverse transcribed into complementary DNA (cDNA) for use as a template for Polymerase Chain Reaction (PCR). Reverse transcription was performed at 42°C for 60 minutes in a reaction volume of 20µl containing the following: oligo dT primer (500ng), Moloney murine leukaemia virus (M-MLV) reverse transcription buffer (50mmol/L Tris-HCl pH 8.3, 75mM KCl, 3mmol/L $MgCl_2$,10mmol/L dithiothreitol (DTT) (Promega, Southampton Science Park, Southampton, UK), diethylpyrocarbonate (DEPC) treated water (Sigma-Aldrich, Ireland), deoxyribonucleotide triphosphates (dNTPs) (0.2mmol/L) (Promega, UK) and 200U M-MLV reverse transcriptase (Promega, UK). Reverse transcriptase activity was

stopped by heating samples at 65°C for 10 minutes. Control RNA samples, in which no reverse transcriptase was added, were included to confirm that no genomic DNA contamination was present.

Real-time fluorescence PCR using ABI Prism 7000 technology

Real-time PCR was performed on a 1/125 dilution of each the 7 NL and 6 L myometrial cDNA in triplicate for each transcript, using the Applied Biosystems ABI Prism 7000 sequence Detection System (ABI, USA). The PCR reactions were performed in a final volume of 25µl containing 12.5µl Sybr Green PCR Master Mix (ABI, USA), 5µl diluted cDNA and 0.4µM of each sense and antisense primer. The final volume of 25µl was achieved using PCR grade water (Sigma-Aldrich, Ireland). cDNA amplification was performed by an initial step of 50°C for 2 minutes an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C and elongation at 72°C for 30 seconds each. The sequences of the oligonucleotide primers for each gene are presented in Table 1. Fluorescence data was acquired at the end of each PCR cycle. Melting curve analysis was performed by an initial denaturation step of 95°C for 15 seconds, cooling to 60°C for 10 seconds, and 72°C for 15 seconds. Fluorescence was measured continually during the melting curve cycle. The mean Cycle Threshold (Ct) of each gene for every patient (performed in triplicate) from their standard curves was normalized to the corresponding mean Ct value of β–Actin (ACTB), *Gene* Ct values/*ACTB* Ct values). β-Actin is a housekeeping gene, it is constitutively expressed and is used to normalise mRNA levels between different samples. The normalized Ct values of the 7 NL and the 6 L myometrial tissue types (NL v L) were analyzed using the independent samples t test. Results were expressed as mean normalized Ct units ± the standard

error of the mean (SEM). A P value of < 0.05 was considered to be statistically significant. Relative fold changes were then calculated using the difference in the mean normalized Ct values (x) between the pregnant at-term and the laboring myometrium for each transcript, Relative fold change=2^x. All statistical analysis was performed using the SPSS statistical package (Statistical Package for the Social Sciences, v.11, SPSS Inc., Chicago, IL, USA).

PCR

1µl of the 20µl RT reaction was then used in the subsequent PCR. PCR was performed in a final volume of 50µl containing 1.5mmol/L MgCl2, 20mmol/L Tris-HCl, 50mmol/L KCl pH8.3, 1.25U Taq DNA polymerase (Bioline Ltd. London, UK), 0.2mM dNTPs and 0.2µM of each sense and antisense primer. cDNA amplification was carried out by an initial denaturation step of 5 minutes at 95°C followed by 28-40 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for 1min and elongation at 72°C for 30s-1 min, followed by a final extension step at 72°C for 10 minutes. 10µl of each PCR product was then separated by gel electrophoresis on 1-1.5% agarose gels. Products were separated alongside a 100bp DNA molecular weight ladder (Promega, UK) for sizing.

Protein isolation

Human myometrial tissue or human primary uterine smooth muscle cells were homogenized in Protein lysis buffer: 50mM Tris pH 7.4, 100mM NaCl, 5mM MgCl2, 0.1% Triton X-100, 10% glycerol with inhibitors (10µg/ml leupeptin, 10µg/ml aprotinin, 1mM PMSF) ice-cold buffer (Sigma-Aldrich, Ireland). Cellular debris was removed by centrifugation at 10,000 × *g*, 4°C for 15 minutes. The resultant supernatant was used for Western blot analysis. Protein

concentrations were determined using the Pierce BCA protein assay reagent kit (Promega, UK) as per the manufacturer's protocol, with bovine serum albumin as a standard.

Western blot analysis

Protein samples (30 μg) were resolved by electrophoresis on 7.5-12% SDS polyacrylamide gel electropheresis gels (Bio-Rad Labarotories, Hercules, USA) and electroblotted. Membranes were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, Ireland) and 5% low-fat milk powder (Dawn Dairies, Westmeath, Ireland). Blots were either incubated with a 1:500 dilution of primary rabbit anti-human TWIST H-31 polyclonal antibody (sc-15393) or 1:400 dilution of primary goat anti-human RGS12 A-14 polyclonal antibody (sc-17740) (Santa Cruz Biotechnology, Inc, Heidelberg, Germany) or 1:5,000 dilution of β-Actin (ACTB) clone number AC-15 mouse polyclonal IgG anti-human primary antibody (Sigma-Aldrich, Ireland) (0.1% NaN_3) in 1XPBS containing 3% bovine serum albumin and 0.03% Tween 20 for 1 hour at room temperature or overnight at 4°C. Blots were then washed and incubated for 1 hour at room temperature in a 1:4,000 dilution of a swine anti-rabbit IgG horseradish peroxidase-conjugated antibody (P-0217, DakoCytomation Ltd, Cambridgeshire, UK) or a rabbit anti-goat horseradish peroxidase-conjugated antibody (P-0160, DakoCytomation Ltd, UK) containing 1XPBS, 5% low-fat milk powder (Dawn Dairies, Ireland) and 0.05% Tween 20 for 1 hour at room temperature. HRP Bound secondary antibody was detected with HRP substrate using the Pierce West-Pico or the Super Signal West Dura chemiluminescence detectection kits as per the manufacturers' protocols (Promega, UK). The membranes were scanned with the fluorescence imager (FluorchemTM 8900, Alpha Innotech Corporation, San Leandro, California, USA) and AlphaEaseFC software was used to detect the signal, the image

was processed and protein expression levels were determined by densitometric analysis compared to corresponding levels of the housekeeping protein, β-Actin (ACTB).

Immunofluorescence Microscopy

Primary human myometrial smooth muscle cells (passage 5) were cultivated on LabTekII 8 well chamber slides (Nalge Nunc Int., Naperville, IL, USA) overnight. The samples were fixed in 4% paraformaldehyde for 30 minutes at room temperature and blocked in 1X PBS, 0.01% Triton X-100 and 5% donkey serum. Cells were subsequently incubated with primary antibody, either a 1:25 or 1:50 dilution of PSCDBP (PSCDBP) (ab2247) (Abcam Cambridge, UK), RGS12 A-14 (sc-17740) or TLR2 (N-17 sc8689) goat polyclonal IgG anti-human primary antibodies (Santa Cruz, Germany) in blocking solution, overnight at 4°C. Samples were rinsed in 1XPBS 3 times and incubated with a 1:400 dilution of Alexa Fluor 488 donkey anti-goat IgG (A11055) (Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature and then rinsed in PBS. Control cells were incubated with the secondary antibody alone. After washing the coverslips were then mounted on glass slides with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame CA, USA). Fluorescent images were obtained using the Laser Scanning Microscope LSM 510 (confocal microscope) (Carl Zeiss AG, Strasse 22, Oberkocken, Germany) and/or the DP70 fluorescence microscope (Olympus, Tokyo, Japan).

Immunogold Transmission Electron Microscopy

Fresh myometrial biopsy from an elective cesarean section underwent primary fixation in 2.5% glutaraldehyde/2% paraformaldehyde, was sectioned into smaller segments, secondary fixation was in osmium tetroxide. The samples were dehydrated in alcohol and then placed in epoxypropane and gradually introduced to 100% TAAB resin. The samples in resin were poured

into beam capsule moulds and placed in a 60°C oven overnight to polymerise. Ultrathin section were (60-90nm) were cut from them on the Leica EM FC6 ultramicrotome (Leica, 2345 Bannockburn, IL, USA). The ultrathin sections were then mounted on nickel grids and incubated with goat polyclonal IgG anti-human primary antibody, RGS12 (A-14 sc17740 Santa Cruz, Germany) overnight. Grids were washed and then incubated in EM rabbit anti-goat IgG (H+L) 10nm gold particle conjugated secondary antibody (EM-RGHA10-Agar Scientific, Cambridge Road, Essex, UK) for 1 hour. The negative control was incubated without primary antibody. They were stained with uranyl acetate and lead citrate. Visualisation was performed using a Hitachi transmission electron microscope (Hitachi High Technology, Minato-ku, Tokyo, Japan).

RESULTS

Tissue Samples for Microarray and mRNA Expression

For the microarray experiment, biopsies of myometrium were obtained at the time of elective NL (n = 3) and intrapartum L (n = 3) cesarean section. The reason for elective cesarean section included previous cesarean section in all cases. The reasons for emergency cesarean section were face presentation (n = 1), previous classical cesarean section (n = 1) and nonreassuring fetal testing (n = 1). The mean age of the NL women was 33.9 years and 34.2 for L. All women were multigravida and delivered between 38 and 40.7 weeks' gestation.

For real-time RT-PCR confirmation analysis, biopsies of myometrium were obtained at the time of elective (n = 7) and intrapartum (n = 6) cesarean section. The reasons for elective cesarean section included previous cesarean section (n = 6) and placenta previa (n = 1). The reasons for emergency cesarean section were face presentation (n = 4), non-reassuring fetal testing (n = 1)

and previous classical cesarean section (n = 1). The mean age of each group was NL, 34.4 and L, 34.3 and the overall mean age was 34.35 years (range, 29–41), 2 of whom were primagravida and 11 multigravida. All women were delivered between 37 and 40.7 weeks' gestation. Patient demographic information is presented in Table 2.

Microarray Analysis

Microarray analysis of the total RNA from three pregnant human non-laboring myometrial biopsies and three laboring myometrial biopsies resulted in the differential expression of 698 genes, $P < 0.05$, and 105 genes, $P < 0.01$. In addition to a number of genes, known from previous investigations to be up-regulated in human laboring myometrium, including *IL-6* [9], phospholipase A2 [10], *CCL2* [11] *TNFRS11B* and *EGR1*[6], *HLF* and *TAGLN2*[7], a number of novel genes, which had not been previously reported to be expressed in the myometrium, were found to be up-regulated in the laboring tissues according to the microarray data. These included pleckstrin homology, Sec 7 and coiled coil domains, binding protein (*PSCDBP*), endothelin-β receptor (*EDNRB*), toll-like receptor 2 (*TLR2*), *TWIST1* and regulator of G-protein signaling-12 (*RGS12*), and unidentified genes, including *FLJ35382*. These six novel genes were chosen for further analysis.

RT-PCR

RT-PCR analysis using DNA-free™ treated (Ambion, UK) RNA demonstrated expression of *PSCDBP*, *EDNRB*, *TLR2*, *FLJ35382*, *TWIST1*, and *RGS12* and β-Actin (*ACTB*) both in non-laboring and laboring human myometrium (data not shown). The absence of transcripts in reverse transcriptase negative reactions (RT-) confirmed that all products were RNA derived and not generated from contaminating genomic DNA.

Real-Time Fluorescence RT-PCR

Relative quantitative expression analysis was performed by real-time RT-PCR. In order to minimize any undue experimental error from sources such as pipetting inaccuracies, analysis of each gene was performed in triplicate. All non-laboring and laboring myometrial biopsies demonstrated expression of *PSCDBP*, *EDNRB*, *TLR2*, *FLJ35382*, *TWIST1* and *RGS12* and β-Actin (*ACTB*) mRNA. RT-PCR product specificity was confirmed using melting curve analysis. Amplification curve crossing points were determined for each gene generated within the initial phase of exponential amplification, per 0.5 μg total RNA in the tissues studied. The mean Ct values for each transcript normalized to β-Actin (per 0.5 μg total RNA), were then averaged and values determined for both laboring (n = 6) and non-laboring myometrium (n = 7). The mean β-Actin normalized Ct values for NL and L ± SEM, respectively for the 6 genes were: *FLJ36382* 31.93 ± 0.54, 28.44 ± 0.56; *PSCDBP* 31.75 ± 0.64, 28.44 ± 0.74, *TLR2* 32.74 ± 0.67, 29.8 ± 0.76, *TWIST1* 28.98 ± 0.48, 26.46 ± 0.26, *EDNRB* 31.47 ±0.65, 28.97 ± 0.25, *RGS12* 36.64 ± 0.45, 34.265 ± 0.53 which is graphically represented in Figure 1a. Individual gene expression levels showed significant difference between laboring and non-laboring human myometrium ($P < 0.05$). Relative fold changes were then calculated using the difference in the Ct values (x) between the laboring and non-laboring myometrium for each transcript, Relative fold change=2^x. *FLJ35382* showed the greatest relative fold change by real-time RT-PCR at labor (11.3 fold up-regulation) in comparison to the non-laboring at-term myometrium. *PSCDBP*, *TLR2*, *TWIST1*, *EDNRB* and *RGS12* showed a 9.89, 7.59, 5.74, 5.67 and 5.18 fold, respectively, relative increase in mRNA expression at labor. A summary of the fold changes observed using real-time RT-PCR, and the associated *P*-values, is shown in Figure 1b.

Human myometrial smooth muscle cell RT-PCR

In order to determine cellular expression, RT-PCR analysis was performed using DNA-free™ treated RNA from primary human myometrial smooth muscle cells (passage 6). *RGS12*, *PSCDBP*, *FLJ35382*, *TWIST1*, *EDNRB* and *TLR2* mRNA expression was demonstrated in the myometrial cells (Figure 2a-f).

Human myometrial smooth muscle cell Western Blotting

Western blotting was performed on human myometrial smooth muscle cells (passage 6) using antibodies to TWIST1 and RGS12. Protein bands of the expected sizes were observed on the myometrial cell blots, 28-30kDa for TWIST1 and a 76-80 kDa band for RGS12 (Figure 3a and b).

Immunofluorescence microscopy

Immunolabeling fluorescence microscopy was used to determine the localization of PSCDBP, TLR2 and RGS12 on human primary uterine smooth muscle cells, passage number 5. PSCDBP localized to the cytoplasm of the cell, mainly surrounding the nucleus (Figure 4a-c). RGS12 appeared to localize to the cytoplasm of the uterine smooth muscle cells (Figure 4d). TLR2 protein was expressed in the cytoplasm and possibly also on the uterine smooth muscle cell membrane (Figure 4e-f).

Immunogold Transmission Electron Microscopy

Electron micrographs of pregnant non-laboring human uterine smooth muscle are indicated in Figure 5 where RGS12 was immunologically detected in human myometrium isolated from pregnant non-laboring myometrium by immunogold transmission electron microscopy, where it

mostly appeared to localize close to, or in vacuoles of the smooth muscle cell cytoplasm (Figure 5a and b).

DISCUSSION

This study compared gene expression levels in the human myometrium at term pregnancy and at labor. While it has been previously established that there is differential spatial regulation in this tissue we concentrated on expression changes in the lower uterine segment.[7, 12, 13] Digoxigenin (DIG) labeled mRNA isolated from the lower uterine segment of 3 NL and 3 L women was hybridized separately to over 29,000 genes, represented by 60mer oligonucleotide probes. The resultant signal was detected with anti-DIG antibody Alkaline Phosphatase conjugate and slides were scanned with a chemiluminescent microarray analyzer incorporating a charge-coupled device (CCD) camera. Another microarray experiment by Havelock et al. compared gene expression in fundal laboring and non-laboring human myometrium using 4 pooled samples of each, or with one sample from each group, with 9182 cDNA probes and fluorescence detection. Of the up-regulated human myometrial fundal genes identified by this study two *TNFRS11B* and *EGR1*, were identified to be up-regulated by 10.1 (P = 0.048) and 9.8-fold (P = 0.004) respectively, in the lower uterine segment of the myometrium in our study.[6] Esplin et al. also examined differential gene expression in lower uterine segment myometrium, laboring (n = 5) and non-laboring (n = 5), using cloned 6912 cDNA microarrays and fluorescence detection, where 56 genes were determined to be differentially expressed at labor.[5] Of the genes identified by this study one, *CCl2* was also identified to up-regulated (12-fold, P = 0.038) in the lower uterine segment of the myometrium by our investigation.[11] Finally Bukowski et al. investigated gene expression changes, without pooling, in the uterine fundus, lower segment and cervix, all

from the same patients, prior to the onset of labor (n = 6) or at labor (n = 7) using 25mer oligonucleotide microarrays of 12,626 genes probed with biotin labeled RNA and scanned with a confocal microscope. Among the human myometrium lower uterine segment genes identified by this investigation some were also identified by our study, including *HLF* and *TAGLN2*, both of which were found to be up-regulated, 2.1 (P = 0.0005) and 1.4-fold (P = 0.01) respectively, at labor, while *CAMKK2* was down-regulated (1.4-fold, P = 0.01) at labor.[7] While the above experiments use different microarray slides, detection systems, data analyses, patient numbers and myometrial samples there is some overlap in gene expression patterns. Our experiment however, investigates expression of a larger number of genes with longer probe lengths than previous oligonucleotide microarray experiments, with DIG labeled antibody and CCD camera detection. A number of genes from previously published northern or RT-PCR data including up-regulated human *IL6* and *PLA2G2A* and rat *junb* gene expression at labor, were also identified by our study.[9, 10, 14]

In this study, we have shown for the first time the expression of *PSCDBP*, *TLR2*, *TWIST1*, *FLJ35382* and *RGS12* genes within the human myometrium, and also up-regulation of their expression and that of EDNRB, in human myometrium at labor. *PSCDBP* is an intracellular scaffold protein that has been implicated in intercellular adhesion of lymphoid cells by regulating integrin deactivation and cytoskeletal rearrangements.[15, 16] Although *Pscdbp* mRNA has been shown to be up-regulated by estrogen in mouse uterus[17] no information regarding the expression or function of *PSCDBP* in the human reproductive system is currently available. *PSCDBP* transcription is up-regulated by cytokines, including IL-2 and IL-12, in cultured lymphocytes,[15] and roles in leukocyte trafficking, especially in response to pro-inflammatory cytokines in stress conditions, and T cell receptor mediated signaling have also been proposed.[18, 19] Our data

showed a 10-fold up-regulation of *PSCDBP* mRNA expression in the human myometrium at labor. PSCDBP expression was localized to myometrial smooth muscle cells for the first time. In myometrial smooth muscle cells, PSCDBP appears to localize in a vesicular manner within the cytoplasm and about the nuclear periphery. While the exact function of *PSCDBP* in the myometrium remains to be elucidated, our findings suggest the possible involvement of PSCDBP in the signal transduction mechanisms associated with labor.

Spontaneous labor at term and preterm delivery with histological chorioamnionitis, regardless of membrane status, is associated with an increased mRNA expression of the toll-like receptor family members, *TLR2* and *TLR4* in the chorioamniotic membranes.[20] Murine models of inflammation-induced preterm birth have demonstrated an up-regulation of *Tlr2* in the uterus and also its activation has resulted in induction of preterm delivery.[21, 22] Furthermore, the expression of *Tlr2* increased in the mouse uterus throughout normal gestation, to labor and postpartum.[23] While *TLR2* mRNA was previously reported in human uterus[24], our findings have demonstrated a 7.59 fold up-regulation of *TLR2* expression in the human myometrium at labor. We have also displayed *TLR2* expression in myometrial smooth muscle cells where the protein appeared to localize to the cytoplasm and possibly also to the cell membrane. As all the patients included in this study delivered at term and showed no evidence of chorioamnionitis, it is possible that TLR2 may play a role in the process of non-infection related normal labor.

TWIST1 is a highly conserved transcription factor that belongs to the family of basic helix-loop-helix (bHLH) proteins and has been implicated in the differentiation of multiple cell lineages including muscle and cartilage.[25] Germ-line mutations in the coding sequence of the human *TWIST1* gene, have been identified in Saethre-Chotzen syndrome and aberrant TWIST1

expression has also been observed in diverse types of cancers.[26, 27] *TWIST* expression is activated by TNFα and IL-1β, via activation of NF-κB. TWIST1 binds to E boxes in the promoters of several NFκB- dependent inflammatory cytokines, such as *Tnfα*, and *Il1β*, and suppresses cytokine production by blocking NF-κB dependent transcriptional activation, thus completing a negative feedback loop.[28] TWIST1 also mediates suppression of inflammation by type I interferons, by repression of the *Tnfα* promoter.[29] TWIST1 also plays an important role in regulating smooth muscle cell differentiation and phenotypic modulation by binding to E-boxes in the promoter regions of several genes including smooth muscle alpha actin.[30] Significant up-regulation of *TWIST1* mRNA was demonstrated in human myometrium at term labor while TWIST1 protein expression was observed in human myometrial smooth muscle cells. The role of TWIST1 in the human uterus remains to be fully elucidated however it may be involved in the control of inflammatory processes at labor either by suppression of cytokine signaling and/or regulation of known labor associated genes.

FLJ35382 is a hypothetical protein, which shows significant homology to splicing factor, SF3a60, part of the 17S U2 small nuclear ribonucleoprotein. In our study, we identified *FLJ35382* as being differentially expressed in labor, generating an 11.3 fold change in the human myometrium at labor. Further investigations however, are required to elucidate the role of this gene in the myometrium, whether it is involved in splicing or other pathways.

EDNRB is a G-protein coupled heptahelical endothelin-1 (EDN1) receptor. EDN1 a known mediator of human myometrial contraction *in-vitro* belongs to a family of three 21-amino acid isopeptides. The other EDN1 receptor, EDNRA is selective for EDN1,[31] whereas EDNRB exhibits similar affinities for all three EDN isopeptides.[32] Although both endothelin receptors have been identified in the human myometrium,[33] it is thought that only the EDNRA receptor

mediates the contractile effect of EDN1 both *in-vivo* and *in-vitro*.[34-36] EDNRB binding was decreased in rat placenta and uterus as a result of hypoxia,[37] and under inflammatory conditions, the major endothelin receptor subtype expressed in myometrial cells shifts from EDNRA to EDNRB, with a concomitant decrease in EDN1 release, leading to a loss of EDN1 induced myometrial cell contraction.[38] Interestingly, we found for the first time a significant up-regulation of *EDNRB* during normal labor, which may suggest a role for EDNRB in non-infection, non-hypoxic associated human labor.

RGS12 is a member of the regulator of G-protein signaling (RGS) group of proteins which are involved in G-protein coupled receptor signaling and a wide variety of other functions, enabled by the variety of RGS protein structure and the ability to interact with other cellular molecules. RGS12 interacts with both N-type and Ca(v)2.2 Ca^{2+} channels and also binds a C-terminal motif found in proteins such as the IL-8 receptor.[39-41] We observed a > 5-fold change in *RGS12* mRNA expression between non-laboring and laboring myometrium. Western blotting demonstrated expression of RGS12 in human myometrial smooth muscle cells and immunofluorescence microscopy localized it to the cell cytoplasm. Furthermore, immunogold labeling TEM of pregnant myometrium established that RGS12 was in close proximity to, or in vacuoles of the smooth muscle cells. To our knowledge this is the first study to identify RGS12 in the human myometrium and to demonstrate the up-regulation of an *RGS* mRNA during labor. The diversity of RGS protein structure clearly underlies a complex and broad range of physiological roles for this family, and particularly RGS12, in the myometrium during labor.

In conclusion, our data has identified the expression of several genes for the first time in the human myometrium and has demonstrated their and others' up-regulation in the tissue during

labor. While little data exists pertaining to these genes in the human reproductive setting, nonetheless, their identification provides novel avenues for future study with the aim of elucidating the underlying molecular mechanisms driving human labor.

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Figure Legends

Figure 1

(a) Graphical representations of real-time fluorescence RT-PCR results of β-Actin normalized Ct values plotted against myometrial pregnancy state (NL n = 7, L n = 6) for each of the genes ± SEM (indicated by the error bars): *FLJ35382*, *PSCDBP*, *TLR2*, *TWIST1*, *EDNRB* and *RGS12*. Lower L Ct values correspond to increased levels of mRNA expression for each gene at L, in comparison to NL.

(b) A summary of the fold changes for each gene at labor in comparison to the non-laboring state in human myometrium, normalized to β-Actin, from real-time fluorescence RT-PCR analyses. Fold change at labor is plotted against gene name: *FLJ35382*, *PSCDBP*, *TLR2*, *TWIST1*, *EDNRB* and *RGS12*, in descending order of fold change, from left to right. The corresponding *P* values are indicated.

Figure 2

Representative gel pictures of the RT-PCR amplification of (a) *RGS12* (lanes 1-4), (b) *PSCDBP* (lanes 1-5), (c) *FLJ35382* (lanes 1-3), (d) *TWIST1* (lanes 1-4), (e) *EDNRB* (lanes 1-2), and (f) *TLR2* (lane 1) from human myometrial smooth muscle cell RNA. The size of each PCR product is indicated and the DNA marker in each gel is a 100bp ladder (M). The PCR water negative control is indicated (C).

Figure 3

Western blots of (a) TWIST1 and (b) RGS12 protein expression in human myometrial smooth muscle cells. The corresponding ACTB western blot is presented underneath. Molecular weights are indicated in kDa. Arrows indicate protein bands of interest.

Figure 4

Fluorescent immunolocalization of (a-c) PSCDBP, (d) RGS12 and (e-f) TLR2 in human myometrial smooth muscle cells after incubation with primary goat anti-human antibodies and green AlexaFluor488 donkey anti-goat secondary antibodies. No staining was evident after incubation with anti-goat secondary in the absence of primary antibody (g). The original magnification was X40.

Figure 5

Immunogold labeling transmission electron microscopy localization of (a-b) RGS12 in human pregnant myometrial tissue. No staining was evident after incubation with anti-gold secondary antibody in the absence of primary antibody (c). RGS12 immunogold labeled particles are evident as black dots (some of which are indicated with arrows) in the cytoplasm. The cell organelles are visible due to uranyl acetate and lead citrate staining.

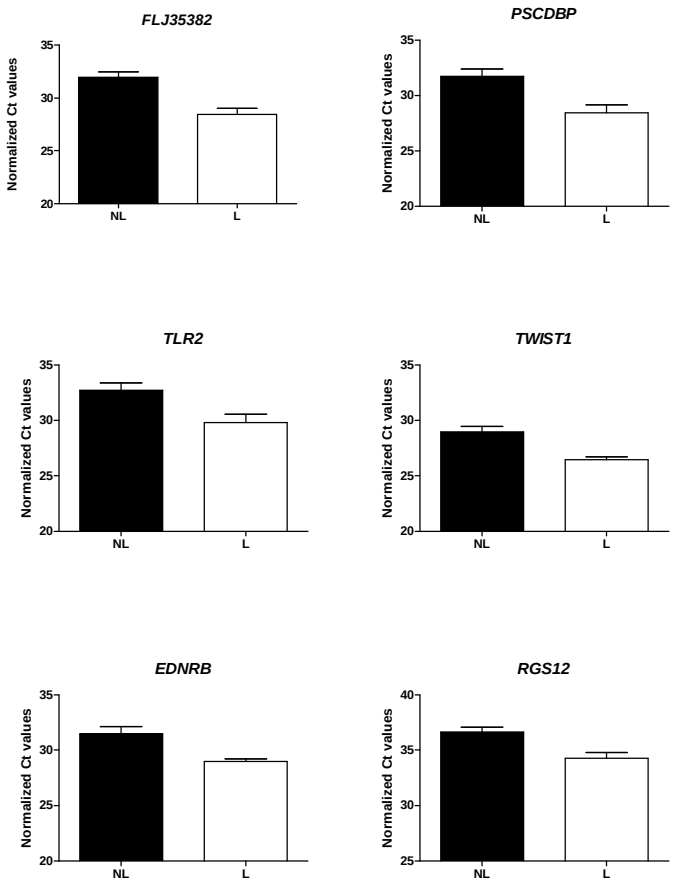
Tables

Table 1: Real-Time fluorescence RT-PCR Oligonucleotide Primer Sequences

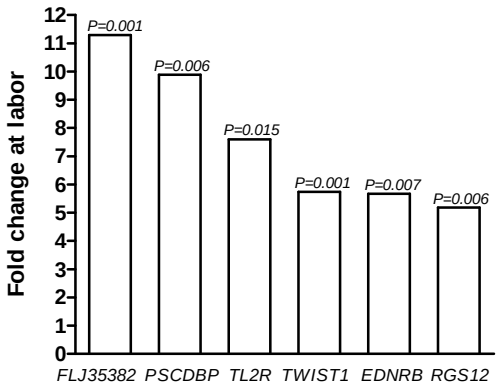
Forward oligonucleotide primer	**Reverse oligonucleotide primer**
FLJ35382 Sense 5'-CGGAAACGGCAATGGCCTA-3' Primary Gene ID hCG32786	Antisense 5'-TGAACTATCAGAGTTGGACCCTT-3'
PSCDBP Sense 5'-ATGGCTACCAGACGTGTGTG-3' Accession NM_004288	Antisense 5'-GTGTTACTGATGCTCCGGTTC-3'
RGS12 Sense 5'-TGTGCTTAGCTGCTTGTGTG-3' Accession NM_002926	Antisense 5'-TTCCCTTGGAGCCATATTTC-3'
β-Actin Sense 5'-GGGCATGGGTCAGAAGGATT-3' Accession M10277	Antisense 5'-AGTTGGTGACGATGCCGTG-3'
TLR2 Sense 5'-GCCTCTCCAAGGAAGAATCC-3'[42]	Antisense 5'-TCCTGTTGTTGGACAGGTCA-3'
TWIST1 Sense 5'-CACTGAAAGGAAAGGCATCA-3'[43]	Antisense 5'-GGCCAGTTTGATCCCAGTAT-3'
EDNRB Sense 5'-GCCAAGGACCCATCGAGAT-3'[44]	Antisense 5'-GAAGTGTGGAGTTCCCGATGAT-3'

Table 2: Patient Demographics

Pregnancy State	**NL (n = 7)**	**L (n = 6)**
Mean Age at Delivery (years) +/- SEM *Range*	34.4 ± 1.4 30-41	34.3 ± 2.0 29-41
Parity	0: 1 woman 1: 4 women ≥2: 2 women	0: 1 woman 1: 3 women ≥2: 2 women
Mean Gestation Length (weeks) +/- SEM *Range*	38.9 ± 0.1 38-39	38.8 ± 0.7 37-40.7
Mean Birth-weight (kg) +/-SEM *Range*	3.7 ± 0.2 3.4-4.6	3.5 ± 0.1 3.3-4.1



(a)



(b) Figure 1 O'Brien et al.

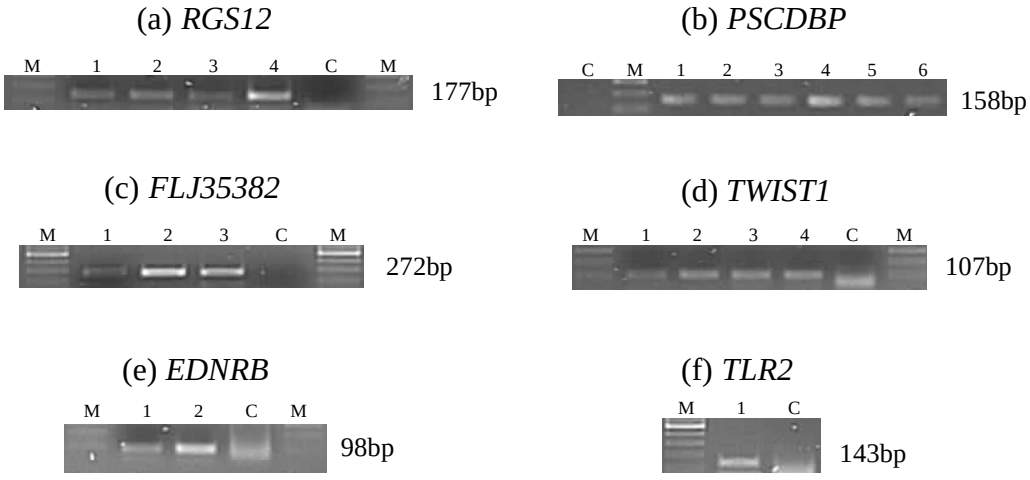


Figure 2 O'Brien et al.

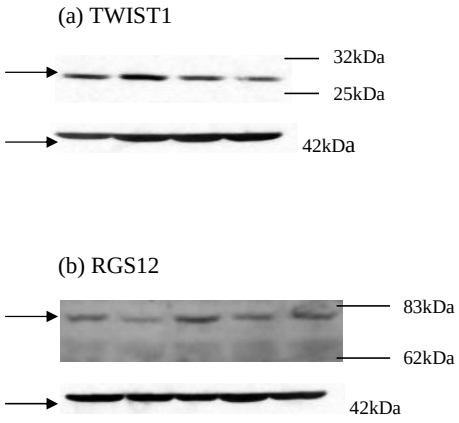
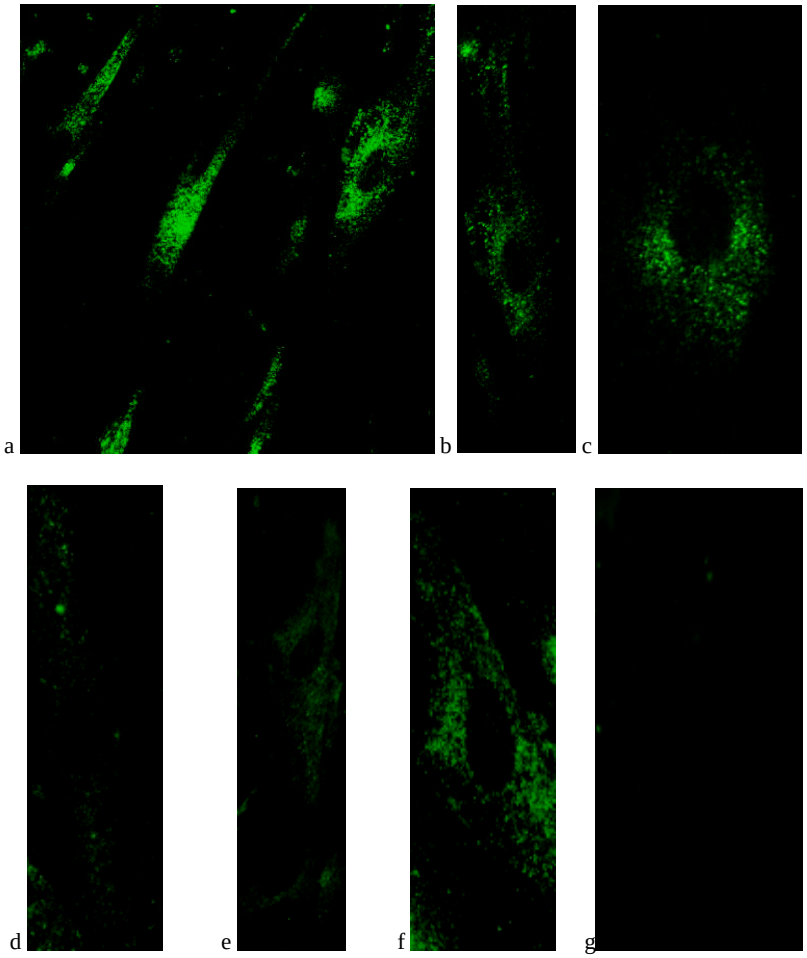


Figure 3 O'Brien et al.



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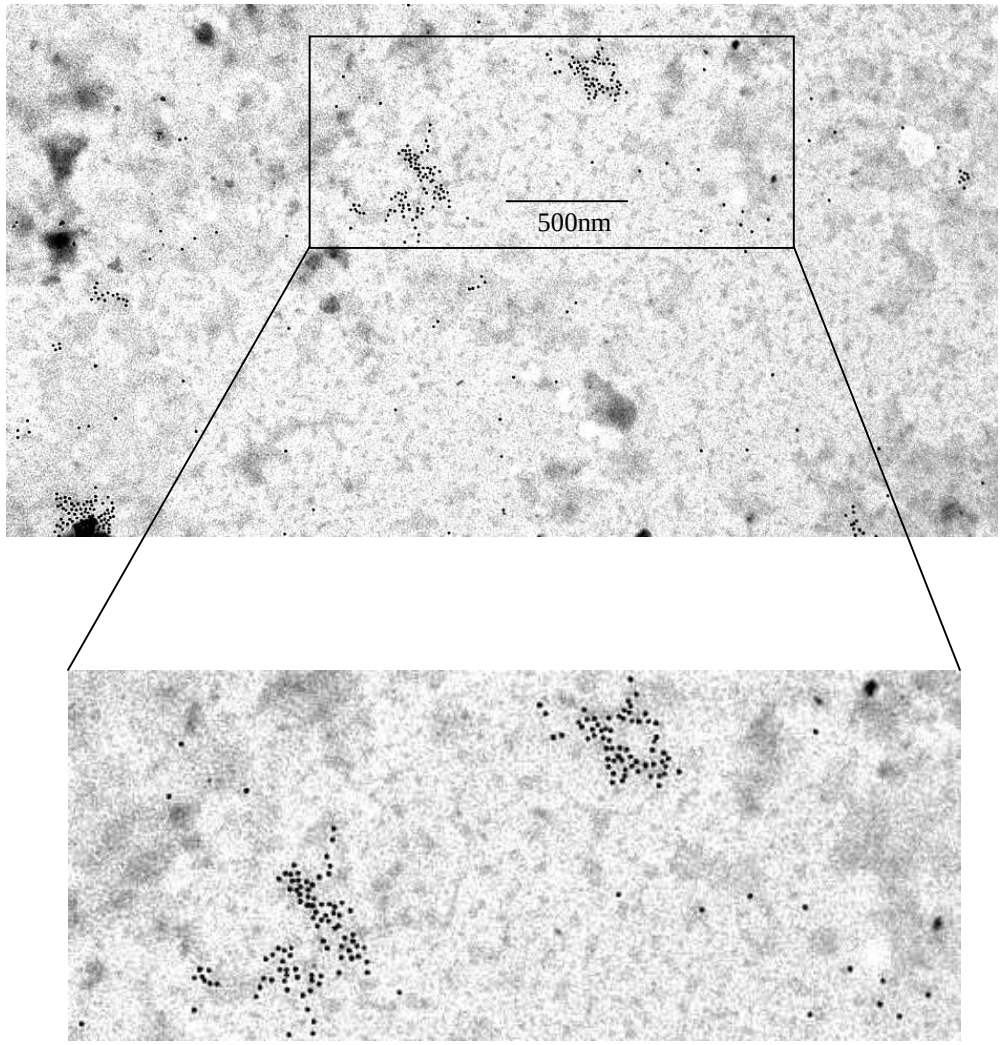

Figure 5a O'Brien et al.

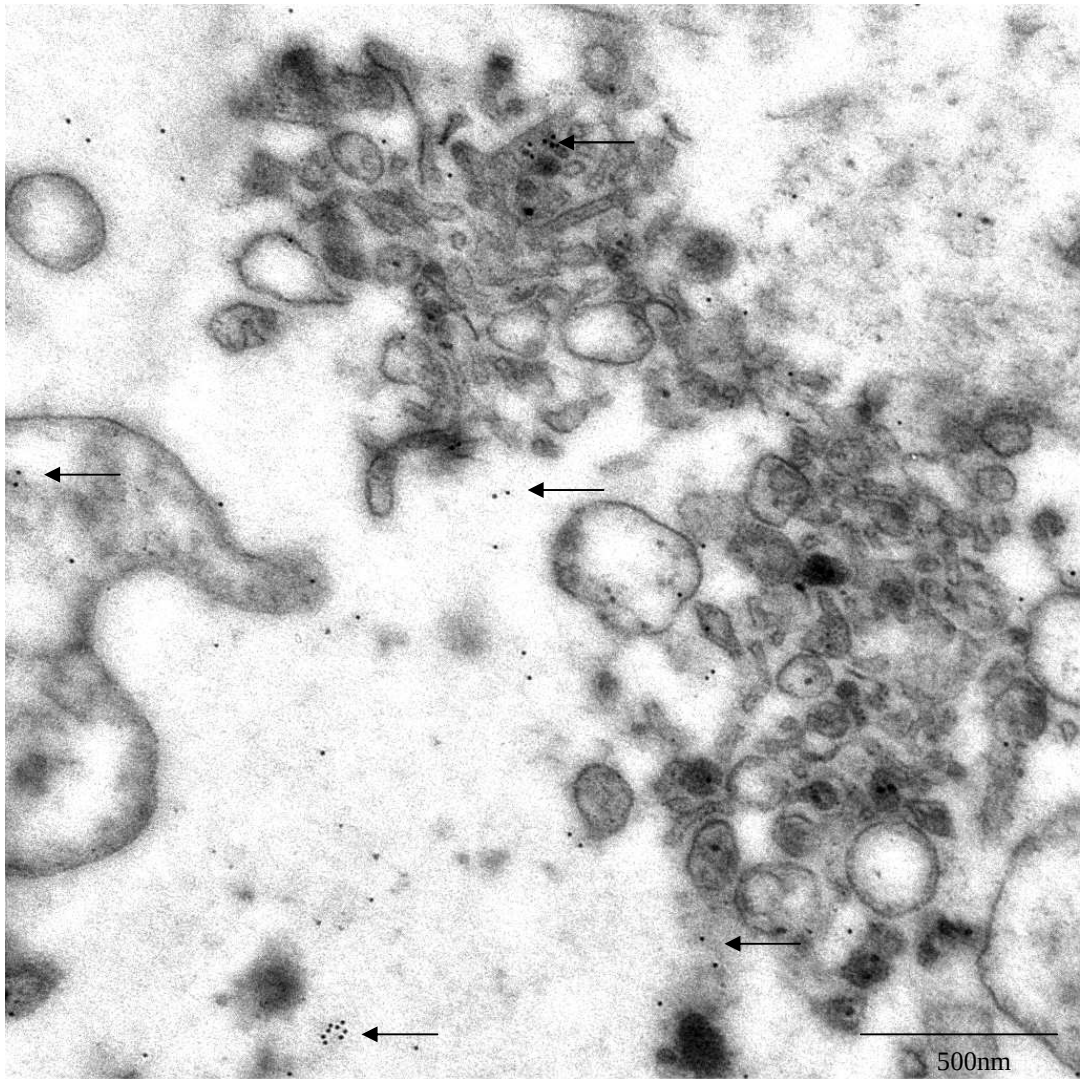

Figure 5b O’Brien et al.

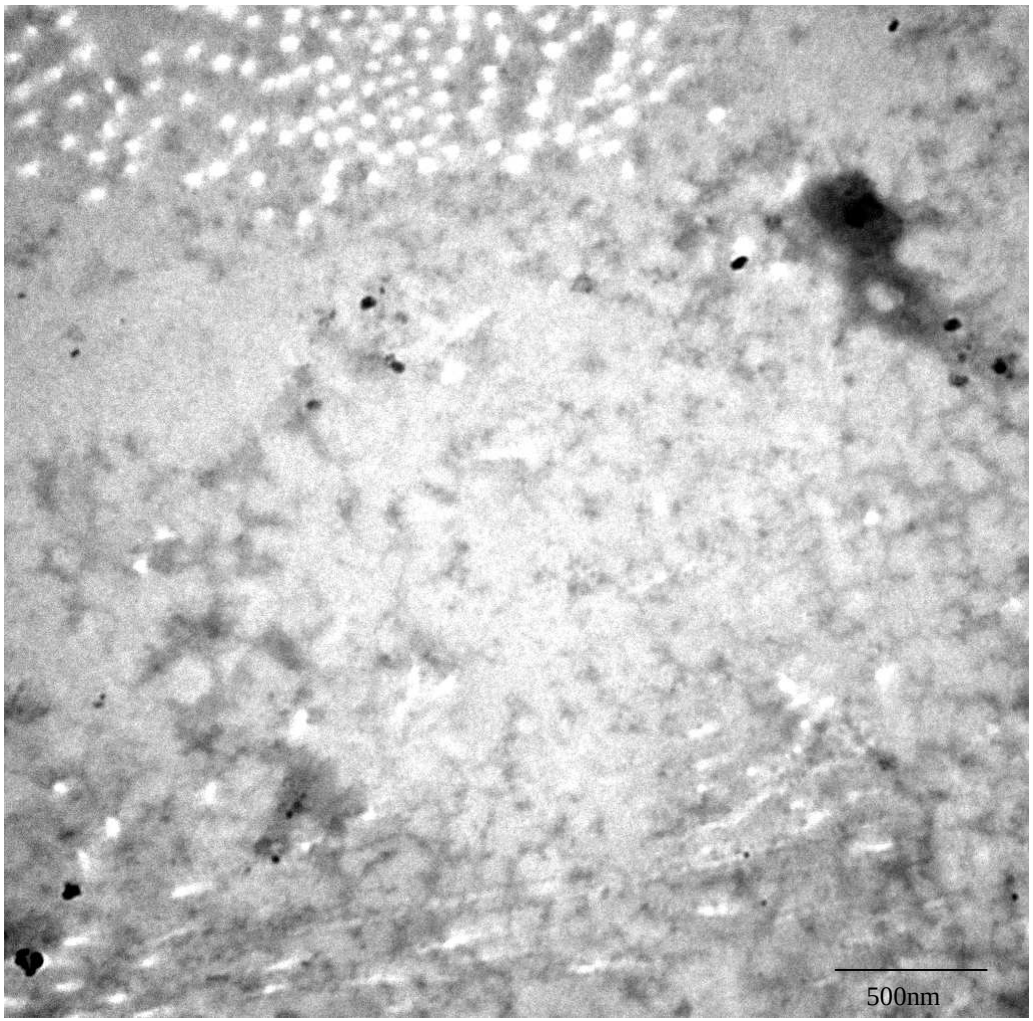

Figure 5c O’Brien et al.