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Running Title: MMP3 and α5 integrin expression in human myometrium

Title: Differential expression of the metalloproteinase MMP3 and the α5 integrin subunit in human myometrium at labour

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Abstract

Extensive tissue remodelling occurs in the human myometrium before, during and after parturition. This study aim was to investigate the expression of two tissue remodelling molecules, matrix metalloproteinase 3 (MMP3) and α5 integrin (ITGA5) subunit expression in human myometrium, during pregnancy and labour. mRNA and protein were isolated from human pregnant labouring and non-labouring myometrial tissue, and also from human primary uterine smooth muscle cells. Semi-quantitative RT-PCR, real-time fluorescence RT-PCR and western blotting were subsequently performed to determine the expression levels of MMP3 and ITGA5 in the myometrial tissues during pregnancy and labour, and in the primary uterine smooth muscle cells. The expression of MMP3 and ITGA5 mRNA and protein are reported for the first time during pregnancy and labour in human myometrium. Furthermore, a significant increase in expression of *MMP3* mRNA (41-fold, $P = 0.001$), and a significant decrease in *ITGA5* mRNA expression (4-fold, $P < 0.001$) at labour, were observed. Protein expression of these two molecules also altered at labour, MMP3 expression significantly increased while ITGA5 protein expression decreased. Expression of these molecules was also observed in primary cultured human uterine smooth muscle cells. The differential expression of these two tissue remodelling molecules at labour and their detection in uterine smooth muscle cells highlights their potential importance in myometrial function during pregnancy, labour and postpartum.

Keywords: integrin/labour/matrix metalloproteinase/myometrium

Introduction

During pregnancy the uterus is transformed into a large muscular organ sufficient to accommodate the foetus, placenta and amniotic fluid. This is facilitated in part by tissue remodelling and cellular hypertrophy (Fata *et al.*, 2000). Furthermore, uterine contractility at labour involves significant remodelling to achieve the powerful synchronous contractions of labour, while to enable the postpartum uterus to return to its pre-pregnancy state, considerable extracellular matrix (ECM) degradation also occurs (Monga and Sanborn, 1995; Manase *et al.*, 2006). Amongst the major proteolytic enzymes involved in tissue remodelling are the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that degrade extracellular matrix (ECM) components (Le *et al.*, 2007; Yan and Boyd, 2007). MMP enzymatic activity is transcriptionally regulated by growth factors, hormones, and cytokines, and post-translationally controlled by tissue inhibitors of metalloproteinases (TIMPs) (Matrisian, 1992; Birkedal-Hansen *et al.*, 1992). MMP3 degrades amongst other substrates, collagen III, IV and V, proteoglycans, fibronectin, elastin, laminin, and gelatin (Sternlicht *et al.*, 1999). Furthermore, it mediates an ECM-degrading proteolytic cascade by activation of the zymogenic forms of other MMPs (e.g. MMP1, 7, 8, 9, 13) (He *et al.*, 1989; Ogata *et al.*, 1992; Knauper *et al.*, 1996). MMP3 itself is also activated, from a precursor form (pro-MMP3) by proteases, notably plasmin (Okada *et al.*, 1988).

A significant increase in the expression of MMP1, 2, 3 and 9 in placenta and foetal membranes or amniotic fluid occurs with the onset of term and preterm parturition, while MMP9 expression increases in human labouring myometrium (Vadillo-Ortega *et al.*, 1995; Maymon *et al.*, 2000; Xu *et al.*, 2002; Park *et al.*, 2003; Smith, 2007).

During pregnancy, uterine collagen content increases ~10-fold in various mammalian species (Woessner and Brewer, 1963). After delivery, this collagen content rapidly decreases due to extracellular degradation by activated collagenases (MMP7 and 13), an important process of post-partum uterine involution (Shimizu and Maekawa, 1983). MMP3 expression has been demonstrated in human perivascular tissue within the uterus, myometrium, and in myometrial smooth muscle cells (Dou *et al.*, 1997; Huppertz *et al.*, 1998; Ma and Chegini, 1999). *Mmp3* mRNA expression was found to be down-regulated in mouse myometrium during pregnancy, and up-regulated 24 hours postpartum (Salomonis *et al.*, 2005), while *Mmp3* mRNA increased during late pregnancy in rat uterine artery (Kelly *et al.*, 2003).

Integrins are divalent cation-dependent heterodimeric, transmembrane receptors that mediate cell attachment to the ECM and signal transduction from the ECM to the cell. They are composed of α and β subunits, their substrate specificity being determined by the composition of these various α and β subunits (Reddy and Mangale, 2003). The α5 and β1 integrin subunits partner to form the major fibronectin receptor (Reddy and Mangale, 2003; Robinson *et al.*, 2003). The actin cytoskeleton of the myometrial smooth muscle cell is connected to the ECM at membrane-associated dense plaques or 'focal adhesions' (Macphee and Lye, 2000). Focal adhesions consist of clusters of integrins that mediate interactions between the extra- and intra-cellular environments. The cytoplasmic regions of integrins connect with actin cytoskeletal elements and signalling components such as focal adhesion kinase (FAK), while the extracellular regions connect to specific extracellular matrix molecules such as fibronectin (Breuiller-Fouche and Germain, 2006). At the end of pregnancy progesterone withdrawal increases the attachment of myometrial smooth muscle cells to the ECM, through integrins at the focal adhesion (Lye *et al.*, 2001). This activates mitogen-

111 associated protein kinase, thus increasing contractility (Loudon *et al.*, 2004). It is
112 reported that expression of the α5β1 integrin substrate fibronectin is increased during
113 pregnancy up to labour and fibronectin is deposited around smooth muscle cells in
114 myometrium during late pregnancy (Nishinaka and Fukuda, 1991; Stewart *et al.*,
115 1995; Shynlova *et al.*, 2004; Williams *et al.*, 2005). In rat myometrium *Itga5* mRNA
116 expression also increased throughout pregnancy, while a decrease in expression was
117 noted after labour, which further decreased postpartum (Williams *et al.*, 2005).
118 ITGA5 expression has been observed in non-pregnant human myometrium (Taylor *et*
119 *al.*, 1996). However, to date, no data have been reported on the expression of ITGA5
120 in human myometrium, during pregnancy and at labour.

121 We therefore investigated the expression of MMP3 and ITGA5 mRNA and protein in
122 human pregnant myometrium at term and during labour, utilising RT-PCR and
123 western blot technologies.

124

Materials and Methods

Patient Recruitment and Tissue Collection

Patient recruitment took place in the Department of Obstetrics and Gynaecology, 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. Biopsies of myometrium were excised from the midline of the upper lip of the uterine incision, during elective (pregnant non-labouring, PNL) and intrapartum (pregnant labouring, PL) caesarean section. The criteria for inclusion in the intrapartum group were regular spontaneous uterine contractions, effacement of the cervix, and cervical dilatation >3cm prior to caesarean section. Women with malignant conditions, and those receiving exogenous hormone therapy (e.g. progestagens), were excluded from the study. Immediately upon removal, biopsies were rinsed in sterile saline, snap frozen in liquid nitrogen and stored at –80°C until RNA or protein isolation.

Cell culture

Primary uterine smooth muscle cells were obtained from Cambrex, Biowhittaker, UK, and cultured in medium 231 (Cascade Biologics, Inc. Mansfield, Nottinghamshire, NG12 5BR, UK).

RNA Extraction

Total RNA was isolated from human myometrium using TRIzol reagent (Life Technologies Ltd., Paisley, UK) (Chomczynski, 1993). Total RNA was isolated from the uterine smooth muscle cells using the RNeasy mini RNA isolation kit (Qiagen, Crawley, West Sussex, UK). RNA samples were DNase-treated with DNA-free™ DNA removal kit (Ambion, Spitfire Close, Huntingdon, Cambridgeshire, UK). RNA

concentration was determined with the Nanodrop (Nanodrop Technologies, Wilmington, USA).

Reverse Transcription

Reverse transcription was performed on RNA (500ng) 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 (Promega, Southampton Science Park, Southampton, UK), dNTPs (0.2mM) (Promega, UK) and 200U M-MLV reverse transcriptase (Promega, UK). Control RNA samples, in which no reverse transcriptase was added, were included to confirm that no genomic DNA contamination was present.

PCR

1µl of the 20µl RT reaction was then used in the subsequent PCR. PCR was performed in a final volume of 50µl 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 1.5-2% agarose gels alongside the 100bp DNA molecular weight ladder (Promega, UK) for sizing. The sequences of the PCR oligonucleotide primers were:

ITGA5 Sense 5'-CCC AGA CTT CTT TGG CTC TG-3'

Antisense 5'-GCA AGA TCT GAG CCT TGT CC-3' Accession NM_002205

MMP3 Sense 5'-CCT GCT TTG TCC TTT GAT GC-3'

Antisense 5'-TGA GTC AAT CCC TGG AAA GTC-3' (Mackenzie *et al.*, 2004)

ACTB Sense 5'-CAA CTC CAT CAT GAA GTG TGA-3'

Antisense 5'-GCC ATG CCA ATC TCA TC-3' (Accession M10277)

Real-time fluorescence PCR using ABI Prism 7000 technology

Real-time PCR was performed on a 1/125 dilution of each the 6 PNL and 6 PL myometrial cDNA in triplicate for each transcript, using the Applied Biosystems ABI Prism 7000 sequence Detection System (ABI, Foster City, USA). The PCR reactions were performed in a final volume of 25 μl containing 12.5 μl Sybr Green PCR Master Mix (ABI, USA), 5 μl diluted cDNA and 0.4 μM of each sense and antisense primer. The final volume of 25 μl was achieved using PCR grade water (Sigma-Aldrich, Dublin, 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. 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 95°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) for the respective reactions from their standard curves were normalised to the corresponding mean Ct value of the housekeeping gene, β-Actin (*ACTB*). The normalised Cts of the 6 PL and the 6 PNL myometrial tissue types (PL v PNL) were then averaged and the values obtained compared using the statistical analysis tool, the independent samples *t* test. Results were expressed as mean normalised Ct units ± the

197 standard error of the mean (SEM). A *P* value of < 0.05 was considered to be
198 statistically significant. Relative fold changes were then calculated using the
199 difference in the Ct values (x) between the pregnant at-term and the labouring
200 myometrium for each transcript, Relative fold change=2^{x}. All statistical analysis was
201 performed using the SPSS statistical package (Statistical Package for the Social
202 Sciences, v.11, SPSS Inc., Chicago, IL, USA). The sequences of the real-time PCR
203 oligonucleotide primers were:
204 *MMP3* Sense 5' AGC AAG GAC CTC GTT TTC ATT-3',
205 Antisense 5'-GTC AAT CCC TGG AAA GTC TTC A-3' (Ramon *et al.*, 2005)
206 *ITGA5* Sense 5'-GTC GGG GGC TTC AAC TTA GAC-3'
207 Antisense 5'-CCT GGC TGG CTG GTA TTA GC (Wang and Seed, 2003)
208 *ACTB* Sense 5'-GGG CAT GGG TCA GAA GGA TT-3',
209 Antisense 5'-AGT TGG TGA CGA TGC CGT G-3' Accession M10277
210

211 ***Protein isolation***

212 Human myometrial tissue or human primary uterine smooth muscle cells were
213 homogenized in Protein lysis buffer: 50mM Tris pH 7.4, 100mM NaCl, 5mM MgCl2,
214 0.1% Triton X-100, 10% glycerol with inhibitors (10 μg/ml leupeptin, 10 μg/ml
215 aprotinin, 1 mM PMSF) ice-cold buffer (Sigma-Aldrich, Ireland). Cellular debris was
216 removed by centrifugation at 10,000 × *g*, 4°C for 15 minutes. The resultant
217 supernatant was used for Western blot analysis. Protein concentrations were
218 determined using the Pierce BCA protein assay reagent kit (Promega, UK) as per the
219 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 for 60 minutes at room temperature with a or with a 1:10,000 dilution of ACTB clone number AC-15 mouse polyclonal IgG anti-human primary antibody (Sigma-Aldrich, Ireland) or 1:1,000 dilution of primary mouse anti-human MMP3 monoclonal antibody (ab17790-Abcam, Cambridge Science Park, Cambridge, UK) or ITGA5 rabbit anti-human polyclonal antibody (sc-10729, Santa Cruz Biotechnology, Inc, Heidelberg, Germany) (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 goat anti-mouse horseradish peroxidase-conjugated antibody (sc2005 Santa Cruz Biotechnology, Germany) or a 1:4,000 dilution of a swine anti-rabbit IgG horseradish peroxidase-conjugated antibody (P-0217, DakoCytomation Ltd, Cambridgeshire, 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 ACTB levels.

Protein statistical analysis

Densitometric values for ITGA5, MMP3 were determined, averaged and normalised to the corresponding ACTB values and were expressed as normalised mean densitometric units ± SEM. Independent samples t-tests were performed on the data using the statistical package SPSS for Windows version 14 (SPSS Inc., USA). A *P* value < 0.05 was considered to be statistically significant.

Results

Tissue Samples for mRNA Expression

Biopsies of myometrium were obtained at the time of elective (PNL) (n = 6) and intrapartum (PL) (n = 6) caesarean section. The reasons for elective caesarean section included previous caesarean section (n = 5) and placenta praevia (n = 1). The reasons for emergency caesarean section were face presentation (n = 3), suspected foetal distress (n = 2) and previous classical caesarean section (n = 1). The mean age of the women was 34.83 years (range, 29–41), 2 were primagravida and 11 were multigravida. All women were delivered between 37 and 42 weeks' gestation. There was no significant difference between those undergoing elective or emergency caesarean section in terms of age, gestation or parity. The non-pregnant biopsy was taken from taken from the body of the uterus of a woman undergoing a hysterectomy for menorrhagia (aged 45).

Tissue samples for protein expression

Biopsies of myometrium during pregnancy were obtained at elective (n = 3) and intrapartum (n = 3) caesarean section. The reasons for elective caesarean section included maternal request (n = 1) and previous caesarean section (n = 2). The reasons for emergency caesarean delivery were foetal distress (n = 1), failed induction (n = 1) and failure to progress (n = 1). The mean age of the women was 35.5 years (range, 30-41), 3 were primagravida and 3 were multigravida. All women were delivered between 39 and 40 weeks' gestation.

RT-PCR

RT-PCR analysis using DNA-free™ treated RNA demonstrated expression of *MMP3* both in non-labouring and labouring human myometrium, where *MMP3* expression increased at labour (Figure 1a). RT-PCR analysis demonstrated expression of *ITGA5* in non-pregnant, pregnant non-labouring and labouring human myometrium (Figure 1b). The absence of transcripts in reverse transcriptase negative reactions (RT-) confirmed that all products were RNA derived and not generated from contaminating genomic DNA. In order to determine cellular expression, RT-PCR analysis was also performed using DNA-free™ treated RNA from primary human uterine smooth muscle cells (passage 6). The expression of both *MMP3* and *ITGA5* mRNAs was evident in the uterine smooth muscle cells (Figure 2a and b).

Real-Time Fluorescence RT-PCR

Relative quantitative expression analysis was performed on human myometrium from pregnant non-labouring and labouring biopsies by real-time RT-PCR. In order to minimise any undue experimental error from sources such as pipetting inaccuracies, analyses of each gene was performed in triplicate. All labouring and non-labouring myometrial biopsies demonstrated expression of *MMP3*, *ITGA5* and *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. *ACTB* expression showed no significant difference between the different tissue types. The mean Ct values for each candidate gene were compared to the corresponding mean *ACTB* value. The mean Ct value of each gene for each patient

(PCRs were performed in triplicate) at the different pregnancy stage was normalised to that of the corresponding mean *ACTB* Ct value, and the resultant values averaged. These values, *MMP3* mean normalised Ct values (per 0.5 μg total RNA) ± SEM were: PL (n = 6) 31.8582 ± 1.08840; PNL (n = 6) 37.2167 ± 0.58237, P = 0.001. These data are graphically represented in Figure 3a. The relative fold change was calculated from the difference in the mean normalised Ct values (x) between the pregnant labouring and non-labouring myometrium, Relative fold change=2^x i.e. 37.2167-31.8582, $2^{5.3585}$. This resulted in a 41-fold up-regulation of *MMP3* mRNA expression at labour (P = 0.001) (Figure 3b).

The *ITGA5* mean normalised Ct values (per 0.5 μg total RNA) ± SEM were: PL (n = 6) 28.9333 ± 0.307; PNL (n = 6) 26.9367 ± 0.22658, P < 0.001. These data are graphically represented in Figure 4a. A statistically significant 4-fold down-regulation in *ITGA5* mRNA expression at labour was calculated from the difference in the Ct values (x) between the pregnant labouring and non-labouring myometrium (P < 0.001) (Figure 4b).

Western Blot Analysis

Western blotting demonstrated the expression of MMP3 and ITGA5 in non-labouring and labouring human myometrium and alterations in the levels of these proteins in myometrium at labour in comparison to the ACTB control (Figures 5 and 6). A single band of approximately 60 kDa was observed with the MMP3 antibody (which can detect both the pro- and active forms of MMP3), this corresponds to the size of the previously observed 59 kDa pro-MMP3 protein (Figure 5a) (Watari *et al.*, 1999; Mackenzie *et al.*, 2004). A single band of approximately 150 kDa was demonstrated

for α5 integrin (Figure 6a). An increase in MMP3 protein expression and a decrease in ITGA5 protein expression was observed at labour (Figures 5a and 6a). The ACTB protein levels did not significantly differ amongst the pregnant non-labouring, and pregnant labouring myometrial samples. Quantitative densitometric analysis was performed where protein expression was normalised to ACTB protein from the same blot, PL (n = 3) and PNL (n = 3). The averaged densitometric units normalised to (per 30 μg total protein) ± SEM for MMP3 were: PL (n = 3) 49.6667 ± 1.76383; PNL (n = 3) 38 ± 2.64575, P = 0.021. A graphical representation of these quantitative data is presented in Figure 5b. Statistical analysis revealed a significant 77% increase in MMP3 protein expression in human myometrial tissues at labour (P = 0.021).

The averaged densitometric units normalised to ACTB (per 30 μg total protein) ± SEM for ITGA5 were: PL (n = 3) 0.4033 ± 0.09404; PNL (n = 3) 0.6413 ± 0.03692, P = 0.067. A graphical representation of these quantitative data is presented in Figure 6b. The protein expression level was found to be decreased at labour, by 60%, however, the reduction was found not to be statistically significant (P = 0.067).

Western blotting confirmed expression of MMP3 and ITGA5 in primary human uterine smooth muscle cells (Figure 7a and b). A single band of approximately 60 kDa indicated the presence of pro-MMP3 protein while a faint band of approximately 47 kDa suggested activated MMP3 protein in the smooth muscle cells (Figure 7a) (Galazka *et al.*, 1996). A single band (150 kDa) was evident on the ITGA5 western which indicated the expression of this protein in the uterine smooth muscle cells (Figure 7b).

Discussion

This study investigated the expression of the ECM-related and tissue remodelling molecules, the α5 integrin subunit, ITGA5 and the matrix metalloproteinase, MMP3, at the mRNA and protein levels in human myometrium during pregnancy and labour. MMP3 expression was previously observed in non-pregnant human myometrium (Dou *et al.*, 1997); however, this is the first report of its expression in human myometrium during pregnancy and labour and its subsequent up-regulation at labour.

The expression of *MMP3* mRNA was found to be significantly increased in the human myometrium at labour. A single band of approximately 60 kDa was observed on myometrial tissue western blots, with a monoclonal antibody to both the pro-MMP and the active forms, suggesting that only the non-activated pro-MMP3 form was present. A significant increase in pro-MMP3 protein was also detected at labour. MMP3 mRNA and protein were found to be expressed in human uterine smooth muscle cells, both the pro-MMP3 (60kDa) and a weaker band of the correct size (47 kDa) was also visible on the western blot, suggesting the presence of active MMP3 protein (Galazka *et al.*, 1996). MMPs are induced in tissues that normally undergo extensive remodelling, e.g. the endometrium during the menstrual cycle, the wound environment, and in tissue responses to various inflammatory conditions (Mignatti and Rifkin, 1996; Salamonsen and Woolley, 1996; Hulboy *et al.*, 1997). Certainly substantial remodelling occurs in the myometrial environment, in the lower uterine segment during late pregnancy and labour. The ECM molecules surrounding myometrial smooth muscle cells (SMCs) include structural proteins (fibrillar collagens and elastin), substrate adhesion molecules (fibronectin, laminin, and collagen IV), and proteoglycans (Shynlova *et al.*, 2004), all of which are MMP3 substrates (Sternlicht *et al.*, 1999). The myometrial biopsies utilised in these studies

were obtained from the lower uterine segment and the increased production of pro-MMP3 at labour suggests a significant role for this metalloproteinase in the adaptation of this region for birth. Another study observed an increase in *Mmp3* gene expression 24 hours postpartum (Salomonis *et al.*, 2005). MMP3 therefore may also play a role in the extensive postpartum remodelling where ECM degradation during involution enables the uterus to return to its pre-pregnancy state. Other MMPs, MMP7 and 9 are previously known to contribute to uterine collagen extracellular degradation at this time (Shimizu and Maekawa, 1983).

MMP3 also has the ability to activate other MMP enzymes e.g. MMP1, 7, 8, 9 and 13 (some of which play important roles in cervical ripening and placental membrane degradation). This may serve an important function in the overall MMP activation in the myometrium and also perhaps in other uterine or placental tissues. The induction of MMP3 expression at labour, its effects on multiple extracellular matrix components and its role in cascade initiation all suggest a significant role for MMP3 in uterine function during this critical time. Further investigation is necessary to monitor MMP3 expression throughout pregnancy and post-partum and also to study the regulation of its expression and activation whether by prostaglandins, growth factors or cytokines. The activation of other MMPs by MMP3 also merits further investigation.

Integrins are crucial to the control of cell-ECM interactions where they serve as a link between the ECM and the cytoskeleton. Integrins also contribute to the remodelling of the uterus before parturition. Other investigators have observed an increase in rat ITGA5 thoughout pregnancy and labour which decreases postpartum (Williams *et al.*, 2005). An increase in expression of its substrate, the ECM component fibronectin,

396 was also evident throughout pregnancy (Stewart *et al.*, 1995; Nishinaka and Fukuda,
397 1991; Shynlova *et al.*, 2004). ITGA5 expression was previously demonstrated in non-
398 pregnant human myometrium (Taylor *et al.*, 1996), though to our knowledge, this is
399 the first study to report the expression of an integrin subunit, specifically ITGA5, in
400 human myometrium during pregnancy and labour, and its subsequent down-regulation
401 at labour.

402 Integrins contribute to ECM remodelling during pregnancy to facilitate the constant
403 cell growth, form links between the cell cytoskeleton and the ECM and trigger
404 signalling pathways during pregnancy and labour. The decrease of *ITGA5* mRNA in
405 human myometrium at labour onset suggests its expression levels may be returning to
406 its pre-pregnancy levels, after labour-associated contractility has been activated.

407 This report reveals for the first time MMP3 and ITGA5 expression in the human
408 myometrium during pregnancy and labour, and also their differential expression at
409 labour. These data suggests key functions for these ECM related molecules in tissue
410 remodelling in the myometrium, and highlights the importance of ECM organisation
411 in the human uterus during pregnancy and labour.

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References

Birkedal-Hansen H, Lin HY, Birkedal-Hansen B, Windsor LJ and Pierson MC (1992) Degradation of collagen fibrils by live cells: role of expression and activation of procollagenase. Matrix Suppl 1,368-374.

Breuiller-Fouche M and Germain G (2006) Gene and protein expression in the myometrium in pregnancy and labor. Reproduction 131,837-850.

Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15,532-534, 536-537.

Dou Q, Tarnuzzer RW, Williams RS, Schultz GS and Chegini N (1997) Differential expression of matrix metalloproteinases and their tissue inhibitors in leiomyomata: a mechanism for gonadotrophin releasing hormone agonist-induced tumour regression. Mol Hum Reprod 3,1005-1014.

Fata JE, Ho AT, Leco KJ, Moorehead RA and Khokha R (2000) Cellular turnover and extracellular matrix remodeling in female reproductive tissues: functions of metalloproteinases and their inhibitors. Cell Mol Life Sci 57,77-95.

Galazka G, Windsor LJ, Birkedal-Hansen H and Engler JA (1996) APMA (4-aminophenylmercuric acetate) activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide. Biochemistry 35,11221-11227.

He CS, Wilhelm SM, Pentland AP, Marmer BL, Grant GA, Eisen AZ and Goldberg GI (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc Natl Acad Sci U S A 86,2632-2636.

Hulboy DL, Rudolph LA and Matrisian LM (1997) Matrix metalloproteinases as mediators of reproductive function. Mol Hum Reprod 3,27-45.

Huppertz B, Kertschanska S, Demir AY, Frank HG and Kaufmann P (1998) Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta. Cell Tissue Res 291,133-148.

Kelly BA, Bond BC and Poston L (2003) Gestational profile of matrix metalloproteinases in rat uterine artery. Mol Hum Reprod 9,351-358.

Knauper V, Lopez-Otin C, Smith B, Knight G and Murphy G (1996) Biochemical characterization of human collagenase-3. J Biol Chem 271,1544-1550.

Le NT, Xue M, Castelnoble LA and Jackson CJ (2007) The dual personalities of matrix metalloproteinases in inflammation. Front Biosci 12,1475-1487.

Loudon JA, Sooranna SR, Bennett PR and Johnson MR (2004) Mechanical stretch of human uterine smooth muscle cells increases IL-8 mRNA expression and peptide synthesis. Mol Hum Reprod 10,895-899.

Lye SJ, Mitchell J, Nashman N, Oldenhof A, Ou R, Shynlova O and Langille L (2001) Role of mechanical signals in the onset of term and preterm labor. Front Horm Res 27,165-178.

Ma C and Chegini N (1999) Regulation of matrix metalloproteinases (MMPs) and their tissue inhibitors in human myometrial smooth muscle cells by TGF-beta1. Mol Hum Reprod 5,950-954.

Mackenzie AP, Schatz F, Krikun G, Funai EF, Kadner S and Lockwood CJ (2004) Mechanisms of abruption-induced premature rupture of the fetal membranes: Thrombin enhanced decidual matrix metalloproteinase-3 (stromelysin-1) expression. Am J Obstet Gynecol 191,1996-2001.

Macphee DJ and Lye SJ (2000) Focal adhesion signaling in the rat myometrium is abruptly terminated with the onset of labor. Endocrinology 141,274-283.

Manase K, Endo T, Chida M, Nagasawa K, Honnma H, Yamazaki K, Kitajima Y, Goto T, Kanaya M, Hayashi T *et al.* (2006) Coordinated elevation of membrane type 1-matrix metalloproteinase and matrix metalloproteinase-2 expression in rat uterus during postpartum involution. Reprod Biol Endocrinol 4,32.

Matrisian LM (1992) The matrix-degrading metalloproteinases. Bioessays 14,455-463.

Maymon E, Romero R, Pacora P, Gervasi MT, Gomez R, Edwin SS and Yoon BH (2000) Evidence of in vivo differential bioavailability of the active forms of matrix metalloproteinases 9 and 2 in parturition, spontaneous rupture of membranes, and intra-amniotic infection. Am J Obstet Gynecol 183,887-894.

Mignatti P and Rifkin DB (1996) Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein 49,117-137.

Monga M and Sanborn BM (1995) Uterine contractile activity. Introduction. Semin Perinatol 19,1-2.

Nishinaka K and Fukuda Y (1991) Changes in extracellular matrix materials in the uterine myometrium of rats during pregnancy and postparturition. Acta Pathol Jpn 41,122-132.

Ogata Y, Enghild JJ and Nagase H (1992) Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem 267,3581-3584.

Okada Y, Harris ED, Jr. and Nagase H (1988) The precursor of a metalloendopeptidase from human rheumatoid synovial fibroblasts. Purification and mechanisms of activation by endopeptidases and 4-aminophenylmercuric acetate. Biochem J 254,731-741.

Park KH, Chaiworapongsa T, Kim YM, Espinoza J, Yoshimatsu J, Edwin S, Gomez R, Yoon BH and Romero R (2003) Matrix metalloproteinase 3 in parturition, premature rupture of the membranes, and microbial invasion of the amniotic cavity. J Perinat Med 31,12-22.

Ramon L, Gilabert-Estelles J, Castello R, Gilabert J, Espana F, Romeu A, Chirivella M, Aznar J and Estelles A (2005) mRNA analysis of several components of the plasminogen activator and matrix metalloproteinase systems in endometriosis using a real-time quantitative RT-PCR assay. Hum Reprod 20,272-278.

Reddy KV and Mangale SS (2003) Integrin receptors: the dynamic modulators of endometrial function. Tissue Cell 35,260-273.

Robinson EE, Zazzali KM, Corbett SA and Foty RA (2003) Alpha5beta1 integrin mediates strong tissue cohesion. J Cell Sci 116,377-386.

Salamonsen LA and Woolley DE (1996) Matrix metalloproteinases in normal menstruation. Hum Reprod 11 Suppl 2,124-133.

Salomonis N, Cotte N, Zambon AC, Pollard KS, Vranizan K, Doniger SW, Dolganov G and Conklin BR (2005) Identifying genetic networks underlying myometrial transition to labor. Genome Biol 6,R12.

Shimizu K and Maekawa K (1983) Collagen degradation in the mouse uterus during postpartum involution: extracellular pathway. Acta Anat (Basel) 117,257-260.

Shynlova O, Mitchell JA, Tsampalieros A, Langille BL and Lye SJ (2004) Progesterone and gravidity differentially regulate expression of extracellular matrix components in the pregnant rat myometrium. Biol Reprod 70,986-992.

Smith R (2007) Parturition. N Engl J Med 356,271-283.

Sternlicht MD, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ and Werb Z (1999) The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell 98,137-146.

Stewart EA, Floor AE, Jain P and Nowak RA (1995) Increased expression of messenger RNA for collagen type I, collagen type III, and fibronectin in myometrium of pregnancy. Obstet Gynecol 86,417-422.

Taylor CV, Letarte M and Lye SJ (1996) The expression of integrins and cadherins in normal human uterus and uterine leiomyomas. Am J Obstet Gynecol 175,411-419.

Vadillo-Ortega F, Gonzalez-Avila G, Furth EE, Lei H, Muschel RJ, Stetler-Stevenson WG and Strauss JF, 3rd (1995) 92-kd type IV collagenase (matrix metalloproteinase-9) activity in human amniochorion increases with labor. Am J Pathol 146,148-156.

Wang X and Seed B (2003) Selection of oligonucleotide probes for protein coding sequences. Bioinformatics 19,796-802.

Watari M, Watari H, DiSanto ME, Chacko S, Shi GP and Strauss JF, 3rd (1999) Pro-inflammatory cytokines induce expression of matrix-metabolizing enzymes in human cervical smooth muscle cells. Am J Pathol 154,1755-1762.

Williams SJ, White BG and MacPhee DJ (2005) Expression of alpha5 integrin (Itga5) is elevated in the rat myometrium during late pregnancy and labor: implications for development of a mechanical syncytium. Biol Reprod 72,1114-1124.

Woessner JF and Brewer TH (1963) Formation and Breakdown of Collagen and Elastin in the Human Uterus During Pregnancy and Post-Partum Involution. Biochem J 89,75-82.

Xu P, Alfaidy N and Challis JR (2002) Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in human placenta and fetal membranes in relation to preterm and term labor. J Clin Endocrinol Metab 87,1353-1361.

Yan C and Boyd DD (2007) Regulation of matrix metalloproteinase gene expression. J Cell Physiol 211,19-26.

Figure Legends

Figure 1

RT-PCR amplification of (a) *MMP3* from representative samples of pregnant non-labouring (PNL lanes 1-3) and labouring (PL lanes 4-5) human myometrial mRNA and (b) *ITGA5* from representative samples of non-pregnant (NP lane 1) pregnant non-labouring (PNL lane 2) and labouring (PL lane 3) human myometrial mRNA. The corresponding β-actin RT-PCR's for each gene are indicated underneath the gel pictures. The PCR negative control (C) and the 100bp DNA ladder (Promega, UK) are indicated (M).

Figure 2

RT-PCR amplification of (a) *MMP3* (lanes 1-5) and (b) *ITGA5* (lanes 1-5) from myometrial smooth muscle cell mRNA. The PCR negative control (C) and the 100bp 100 bp ladder are indicated (M).

Figure 3

Real-time fluorescence RT-PCR results of *MMP3* mRNA expression in human pregnant labouring (PL), $n = 6$ and non-labouring (PNL), $n = 6$ human myometrium. (a) ACTB normalised Ct values were plotted against pregnancy state mRNA ± SEM (indicated with the error bars). An asterisk indicates a significance value of $P < 0.05$. (b) The relative fold change was calculated from the difference in Ct values between the two pregnancy states, PL and PNL. The significance value is indicated.

Figure 4

Real-time fluorescence RT-PCR results of *ITGA5* mRNA expression in human pregnant labouring (PL), $n = 6$ and non-labouring (PNL), $n = 6$ human myometrium. (a) ACTB normalised Ct values were plotted against pregnancy state mRNA ± SEM (indicated with the error bars). An asterisk indicates a significance value of $P < 0.05$. (b) The relative fold change was calculated from the difference in Ct values between the two pregnancy states, PL and PNL. The significance value is indicated.

Figure 5

(a) Western Blot analysis of MMP3 protein expression in pregnant labouring (PL lanes 1-3) and pregnant non-labouring human myometrium (PNL lanes 4-6). The corresponding ACTB western is presented underneath. The molecular weight in kDa is indicated.

(b) Quantitative densitometric analysis of the MMP3 western presented above. ACTB normalised densitometric units were plotted against protein isolated from each pregnancy state, PL ($n = 3$) and PNL ($n = 3$) ± SEM (indicated with the error bars). An asterisk indicates a significance value of $P < 0.05$.

Figure 6

(a) Western Blot analysis of ITGA5 protein expression in pregnant labouring (PL lanes 1-3) and pregnant non-labouring human myometrium (PNL lanes 4-6). The corresponding ACTB western is presented underneath. The molecular weight in kDa is indicated.

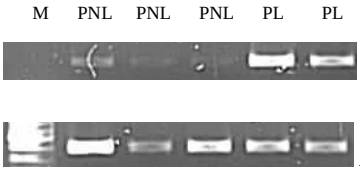
(b) Quantitative densitometric analysis of the ITGA5 western presented above. ACTB normalised densitometric units were plotted against protein isolated from each

pregnancy state, PL (n = 3) and PNL (n = 3) ± SEM (indicated with the error bars). An asterisk indicates a significance value of $P < 0.05$.

Figure 7

Western Blot analysis of (a) MMP3 (the arrows indicate both the pro- and active forms of MMP3) and (b) ITGA5 in human myometrial smooth muscle cells (lanes 1-6 both blots). Molecular weights are indicated in kDa.

Figures



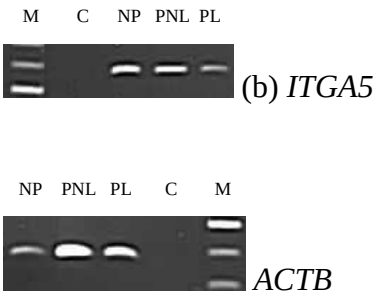
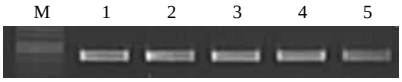
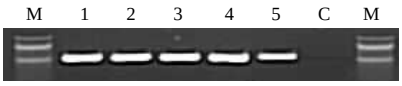


Figure 1 O'Brien et al



(a) *MMP3*



(b) *ITGA5*

Figure 2 O’Brien et al

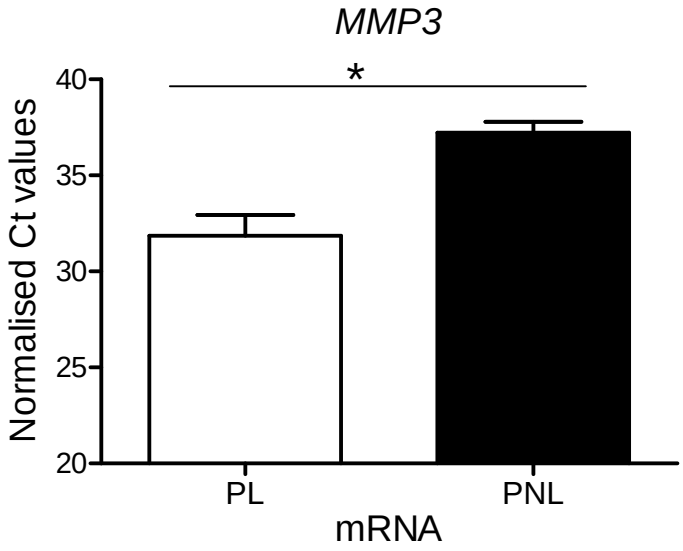


Figure 3a O'Brien et al

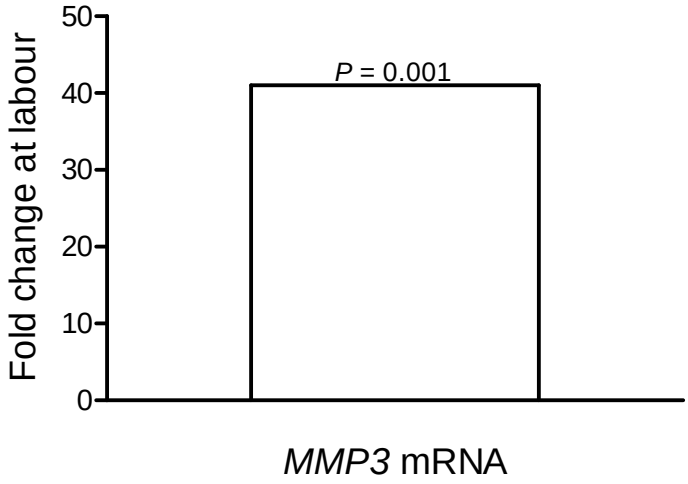


Figure 3b O'Brien et al

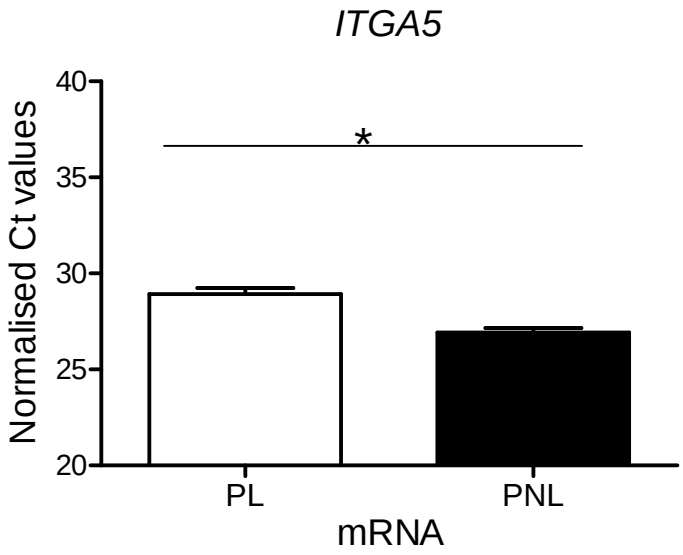


Figure 4a O'Brien et al

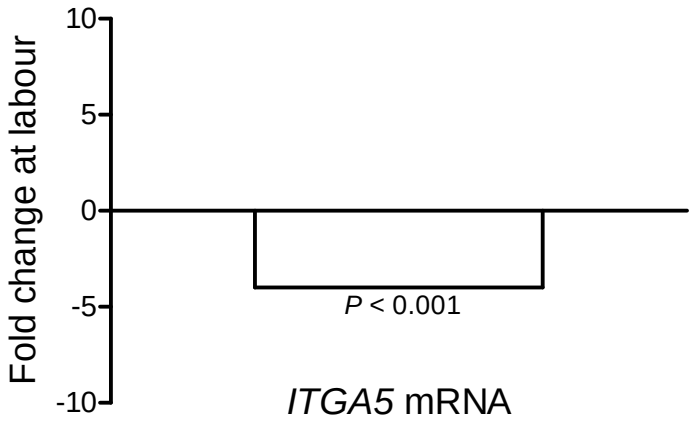
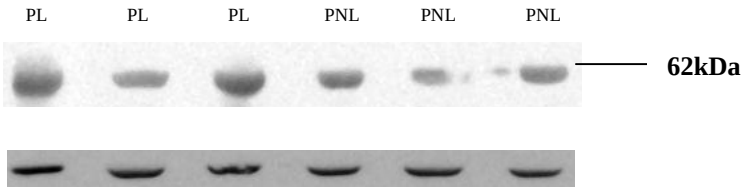
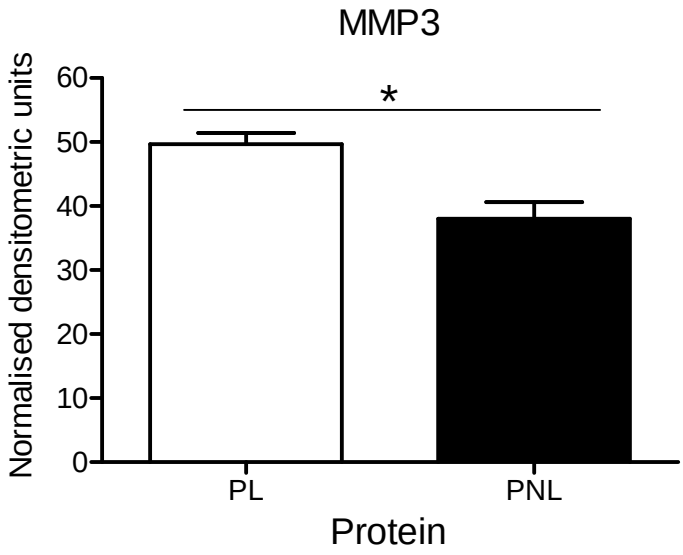


Figure 4b O'Brien et al

(a)

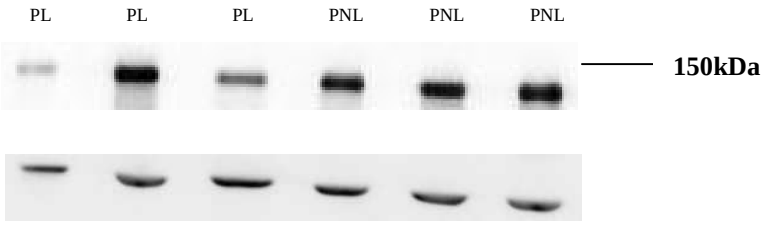


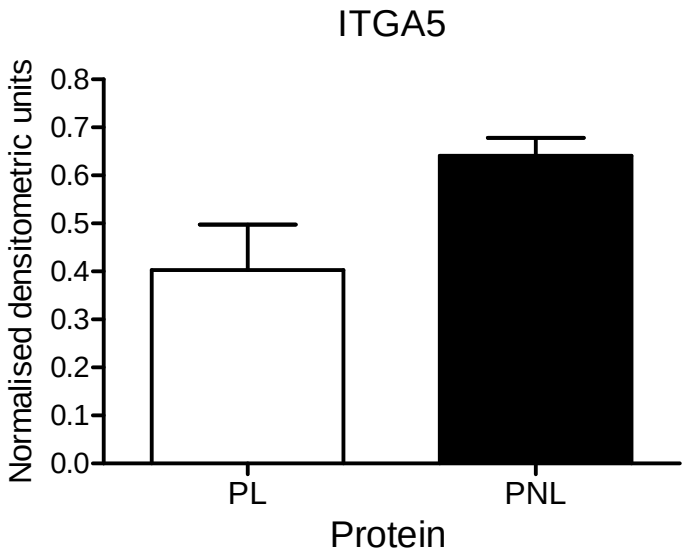


(b)

Figure 5 O'Brien et al

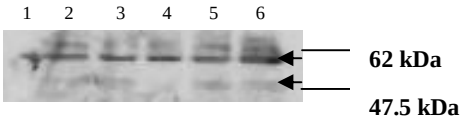
(a)



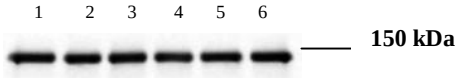


(b)

Figure 6 O'Brien et al



(a) MMP3



(b) ITGA5

Figure 7 O'Brien et al