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1 **Running Title:** MMP3 and $\alpha 5$ integrin expression in human myometrium

2

3 **Title: Differential expression of the metalloproteinase MMP3 and the $\alpha 5$**
4 **integrin subunit in human myometrium at labour**

5

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38 Abstract

39

40 Extensive tissue remodelling occurs in the human myometrium before, during and
41 after parturition. This study aim was to investigate the expression of two tissue
42 remodelling molecules, matrix metalloproteinase 3 (MMP3) and $\alpha 5$ integrin (ITGA5)
43 subunit expression in human myometrium, during pregnancy and labour. mRNA and
44 protein were isolated from human pregnant labouring and non-labouring myometrial
45 tissue, and also from human primary uterine smooth muscle cells. Semi-quantitative
46 RT-PCR, real-time fluorescence RT-PCR and western blotting were subsequently
47 performed to determine the expression levels of MMP3 and ITGA5 in the myometrial
48 tissues during pregnancy and labour, and in the primary uterine smooth muscle cells.
49 The expression of MMP3 and ITGA5 mRNA and protein are reported for the first
50 time during pregnancy and labour in human myometrium. Furthermore, a significant
51 increase in expression of *MMP3* mRNA (41-fold, $P = 0.001$), and a significant
52 decrease in *ITGA5* mRNA expression (4-fold, $P < 0.001$) at labour, were observed.
53 Protein expression of these two molecules also altered at labour, MMP3 expression
54 significantly increased while ITGA5 protein expression decreased. Expression of
55 these molecules was also observed in primary cultured human uterine smooth muscle
56 cells. The differential expression of these two tissue remodelling molecules at labour
57 and their detection in uterine smooth muscle cells highlights their potential
58 importance in myometrial function during pregnancy, labour and postpartum.

59

60

61 **Keywords:** integrin/labour/matrix metalloproteinase/myometrium

62 **Introduction**

63 During pregnancy the uterus is transformed into a large muscular organ sufficient to
64 accommodate the foetus, placenta and amniotic fluid. This is facilitated in part by
65 tissue remodelling and cellular hypertrophy (Fata *et al.*, 2000). Furthermore, uterine
66 contractility at labour involves significant remodelling to achieve the powerful
67 synchronous contractions of labour, while to enable the postpartum uterus to return to
68 its pre-pregnancy state, considerable extracellular matrix (ECM) degradation also
69 occurs (Monga and Sanborn, 1995; Manase *et al.*, 2006). Amongst the major
70 proteolytic enzymes involved in tissue remodelling are the matrix metalloproteinases
71 (MMPs), a family of zinc-dependent endopeptidases that degrade extracellular matrix
72 (ECM) components (Le *et al.*, 2007; Yan and Boyd, 2007). MMP enzymatic activity
73 is transcriptionally regulated by growth factors, hormones, and cytokines, and post-
74 translationally controlled by tissue inhibitors of metalloproteinases (TIMPs)
75 (Matrisian, 1992; Birkedal-Hansen *et al.*, 1992). MMP3 degrades amongst other
76 substrates, collagen III, IV and V, proteoglycans, fibronectin, elastin, laminin, and
77 gelatin (Sternlicht *et al.*, 1999). Furthermore, it mediates an ECM-degrading
78 proteolytic cascade by activation of the zymogenic forms of other MMPs (e.g. MMP1,
79 7, 8, 9, 13) (He *et al.*, 1989; Ogata *et al.*, 1992; Knauper *et al.*, 1996). MMP3 itself is
80 also activated, from a precursor form (pro-MMP3) by proteases, notably plasmin
81 (Okada *et al.*, 1988).

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

86 During pregnancy, uterine collagen content increases ~10-fold in various mammalian
87 species (Woessner and Brewer, 1963). After delivery, this collagen content rapidly
88 decreases due to extracellular degradation by activated collagenases (MMP7 and 13),
89 an important process of post-partum uterine involution (Shimizu and Maekawa,
90 1983). MMP3 expression has been demonstrated in human perivascular tissue within
91 the uterus, myometrium, and in myometrial smooth muscle cells (Dou *et al.*, 1997;
92 Huppertz *et al.*, 1998; Ma and Chegini, 1999). *Mmp3* mRNA expression was found
93 to be down-regulated in mouse myometrium during pregnancy, and up-regulated 24
94 hours postpartum (Salomonis *et al.*, 2005), while *Mmp3* mRNA increased during late
95 pregnancy in rat uterine artery (Kelly *et al.*, 2003).

96 Integrins are divalent cation-dependent heterodimeric, transmembrane receptors that
97 mediate cell attachment to the ECM and signal transduction from the ECM to the cell.
98 They are composed of α and β subunits, their substrate specificity being determined
99 by the composition of these various α and β subunits (Reddy and Mangale, 2003).
100 The $\alpha5$ and $\beta1$ integrin subunits partner to form the major fibronectin receptor (Reddy
101 and Mangale, 2003; Robinson *et al.*, 2003). The actin cytoskeleton of the myometrial
102 smooth muscle cell is connected to the ECM at membrane-associated dense plaques or
103 'focal adhesions' (Macphee and Lye, 2000). Focal adhesions consist of clusters of
104 integrins that mediate interactions between the extra- and intra-cellular environments.
105 The cytoplasmic regions of integrins connect with actin cytoskeletal elements and
106 signalling components such as focal adhesion kinase (FAK), while the extracellular
107 regions connect to specific extracellular matrix molecules such as fibronectin
108 (Breuiller-Fouche and Germain, 2006). At the end of pregnancy progesterone
109 withdrawal increases the attachment of myometrial smooth muscle cells to the ECM,
110 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 $\alpha 5\beta 1$ 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

125 **Materials and Methods**

126 *Patient Recruitment and Tissue Collection*

127 Patient recruitment took place in the Department of Obstetrics and Gynaecology,
128 University College Hospital Galway (UCHG), Ireland. The study was approved by
129 the Research Ethics committee, UCHG, and recruitment was carried out by provision
130 of information sheets and obtaining written informed consent. Biopsies of
131 myometrium were excised from the midline of the upper lip of the uterine incision,
132 during elective (pregnant non-labouring, PNL) and intrapartum (pregnant labouring,
133 PL) caesarean section. The criteria for inclusion in the intrapartum group were
134 regular spontaneous uterine contractions, effacement of the cervix, and cervical
135 dilatation >3cm prior to caesarean section. Women with malignant conditions, and
136 those receiving exogenous hormone therapy (e.g. progestagens), were excluded from
137 the study. Immediately upon removal, biopsies were rinsed in sterile saline, snap
138 frozen in liquid nitrogen and stored at -80°C until RNA or protein isolation.

139 *Cell culture*

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

143 *RNA Extraction*

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

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

151 ***Reverse Transcription***

152 Reverse transcription was performed on RNA (500ng) at 42°C for 60 minutes in a
153 reaction volume of 20µl containing the following: oligo dT primer (500ng), Moloney
154 murine leukaemia virus (M-MLV) reverse transcription buffer (Promega,
155 Southampton Science Park, Southampton, UK), dNTPs (0.2mM) (Promega, UK) and
156 200U M-MLV reverse transcriptase (Promega, UK). Control RNA samples, in which
157 no reverse transcriptase was added, were included to confirm that no genomic DNA
158 contamination was present.

159

160 ***PCR***

161 1µl of the 20µl RT reaction was then used in the subsequent PCR. PCR was
162 performed in a final volume of 50µl 1.25U Taq DNA polymerase (Bioline Ltd.
163 London, UK), 0.2mM dNTPs and 0.2µM of each sense and antisense primer. cDNA
164 amplification was carried out by an initial denaturation step of 5 minutes at 95°C
165 followed by 28-40 cycles of denaturation at 94°C for 1 min, annealing at 55-60°C for
166 1min and elongation at 72°C for 30s-1 min, followed by a final extension step at 72°C
167 for 10 minutes. 10µl of each PCR product was then separated by gel electrophoresis
168 on 1.5-2% agarose gels alongside the 100bp DNA molecular weight ladder (Promega,
169 UK) for sizing. The sequences of the PCR oligonucleotide primers were:

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

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

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

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

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

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

176

177 ***Real-time fluorescence PCR using ABI Prism 7000 technology***

178 Real-time PCR was performed on a 1/125 dilution of each the 6 PNL and 6 PL

179 myometrial cDNA in triplicate for each transcript, using the Applied Biosystems ABI

180 Prism 7000 sequence Detection System (ABI, Foster City, USA). The PCR reactions

181 were performed in a final volume of 25 μ l containing 12.5 μ l Sybr Green PCR Master

182 Mix (ABI, USA), 5 μ l diluted cDNA and 0.4 μ M of each sense and antisense primer.

183 The final volume of 25 μ l was achieved using PCR grade water (Sigma-Aldrich,

184 Dublin, Ireland). cDNA amplification was performed by an initial step of 50°C for 2

185 minutes an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of

186 denaturation at 95°C for 15 seconds, annealing at 60°C and elongation at 72°C for 30

187 seconds each. Fluorescence data was acquired at the end of each PCR cycle. Melting

188 curve analysis was performed by an initial denaturation step of 95°C for 15 seconds,

189 cooling to 60°C for 10 seconds, and 95°C for 15 seconds. Fluorescence was

190 measured continually during the melting curve cycle.

191 The mean Cycle threshold (Ct) of each gene for every patient (performed in triplicate)

192 for the respective reactions from their standard curves were normalised to the

193 corresponding mean Ct value of the housekeeping gene, β -Actin (*ACTB*). The

194 normalised Cts of the 6 PL and the 6 PNL myometrial tissue types (PL v PNL) were

195 then averaged and the values obtained compared using the statistical analysis tool, the

196 independent samples *t* test. Results were expressed as mean normalised Ct units \pm 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.

220 ***Western blot analysis***

221 Protein samples (30 µg) were resolved by electrophoresis on 7.5-12% SDS
222 polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories
223 Hercules, USA) and electroblotted. Membranes were blocked with phosphate-
224 buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, Ireland) and 5%
225 low-fat milk powder (Dawn Dairies, Westmeath, Ireland). Blots were either
226 incubated for 60 minutes at room temperature with a or with a 1:10,000 dilution of
227 ACTB clone number AC-15 mouse polyclonal IgG anti-human primary antibody
228 (Sigma-Aldrich, Ireland) or 1:1,000 dilution of primary mouse anti-human MMP3
229 monoclonal antibody (ab17790-Abcam, Cambridge Science Park, Cambridge, UK) or
230 ITGA5 rabbit anti-human polyclonal antibody (sc-10729, Santa Cruz Biotechnology,
231 Inc, Heidelberg, Germany) (0.1% NaN₃) in 1XPBS containing 3% bovine serum
232 albumin and 0.03% Tween 20 for 1 hour at room temperature or overnight at 4°C.
233 Blots were then washed and incubated for 1 hour at room temperature in a 1:4,000
234 dilution of a goat anti-mouse horseradish peroxidase-conjugated antibody (sc2005
235 Santa Cruz Biotechnology, Germany) or a 1:4,000 dilution of a swine anti-rabbit IgG
236 horseradish peroxidase-conjugated antibody (P-0217, DakoCytomation Ltd,
237 Cambridgeshire, UK) containing 1XPBS, 5% low-fat milk powder (Dawn Dairies,
238 Ireland) and 0.05% Tween 20 for 1 hour at room temperature. HRP Bound secondary
239 antibody was detected with HRP substrate using the Pierce West-Pico or the Super
240 Signal West Dura chemiluminescence detection kits as per the manufacturers'
241 protocols (Promega, UK). The membranes were scanned with the fluorescence
242 imager (FluorchemTM 8900, Alpha Innotech Corporation, San Leandro, California,
243 USA) and AlphaEaseFC software was used to detect the signal, the image was

244 processed and protein expression levels were determined by densitometric analysis
245 compared to ACTB levels.

246 *Protein statistical analysis*

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

252 **Results**

253 *Tissue Samples for mRNA Expression*

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

265 *Tissue samples for protein expression*

266 Biopsies of myometrium during pregnancy were obtained at elective ($n = 3$) and
267 intrapartum ($n = 3$) caesarean section. The reasons for elective caesarean section
268 included maternal request ($n = 1$) and previous caesarean section ($n = 2$). The reasons
269 for emergency caesarean delivery were foetal distress ($n = 1$), failed induction ($n = 1$)
270 and failure to progress ($n = 1$). The mean age of the women was 35.5 years (range,
271 30-41), 3 were primagravida and 3 were multigravida. All women were delivered
272 between 39 and 40 weeks' gestation.

273

274 RT-PCR

275 RT-PCR analysis using DNA-free™ treated RNA demonstrated expression of *MMP3*
276 both in non-labouring and labouring human myometrium, where *MMP3* expression
277 increased at labour (Figure 1a). RT-PCR analysis demonstrated expression of *ITGA5*
278 in non-pregnant, pregnant non-labouring and labouring human myometrium (Figure
279 1b). The absence of transcripts in reverse transcriptase negative reactions (RT-)
280 confirmed that all products were RNA derived and not generated from contaminating
281 genomic DNA. In order to determine cellular expression, RT-PCR analysis was also
282 performed using DNA-free™ treated RNA from primary human uterine smooth
283 muscle cells (passage 6). The expression of both *MMP3* and *ITGA5* mRNAs was
284 evident in the uterine smooth muscle cells (Figure 2a and b).

285

286 Real-Time Fluorescence RT-PCR

287 Relative quantitative expression analysis was performed on human myometrium from
288 pregnant non-labouring and labouring biopsies by real-time RT-PCR. In order to
289 minimise any undue experimental error from sources such as pipetting inaccuracies,
290 analyses of each gene was performed in triplicate. All labouring and non-labouring
291 myometrial biopsies demonstrated expression of *MMP3*, *ITGA5* and *ACTB* mRNA.
292 RT-PCR product specificity was confirmed using melting curve analysis.
293 Amplification curve crossing points were determined for each gene generated within
294 the initial phase of exponential amplification, per 0.5 µg total RNA in the tissues
295 studied. *ACTB* expression showed no significant difference between the different
296 tissue types. The mean Ct values for each candidate gene were compared to the
297 corresponding mean *ACTB* value. The mean Ct value of each gene for each patient

298 (PCRs were performed in triplicate) at the different pregnancy stage was normalised
299 to that of the corresponding mean *ACTB* Ct value, and the resultant values averaged.

300 These values, *MMP3* mean normalised Ct values (per 0.5 µg total RNA) ± SEM were:
301 PL ($n = 6$) 31.8582 ± 1.08840 ; PNL ($n = 6$) 37.2167 ± 0.58237 , $P = 0.001$. These
302 data are graphically represented in Figure 3a. The relative fold change was calculated
303 from the difference in the mean normalised Ct values (x) between the pregnant
304 labouring and non-labouring myometrium, Relative fold change= 2^x i.e. $37.2167-$
305 31.8582 , $2^{5.3585}$. This resulted in a 41-fold up-regulation of *MMP3* mRNA expression
306 at labour ($P = 0.001$) (Figure 3b).

307

308 The *ITGA5* mean normalised Ct values (per 0.5 µg total RNA) ± SEM were: PL ($n =$
309 6) 28.9333 ± 0.307 ; PNL ($n = 6$) 26.9367 ± 0.22658 , $P < 0.001$. These data are
310 graphically represented in Figure 4a. A statistically significant 4-fold down-
311 regulation in *ITGA5* mRNA expression at labour was calculated from the difference in
312 the Ct values (x) between the pregnant labouring and non-labouring myometrium ($P <$
313 0.001) (Figure 4b).

314

315 **Western Blot Analysis**

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

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

333

334 The averaged densitometric units normalised to ACTB (per 30 μg total protein) \pm
335 SEM for ITGA5 were: PL ($n = 3$) 0.4033 ± 0.09404 ; PNL ($n = 3$) 0.6413 ± 0.03692 , P
336 $= 0.067$. A graphical representation of these quantitative data is presented in Figure
337 6b. The protein expression level was found to be decreased at labour, by 60%,
338 however, the reduction was found not to be statistically significant ($P = 0.067$).

339

340 Western blotting confirmed expression of MMP3 and ITGA5 in primary human
341 uterine smooth muscle cells (Figure 7a and b). A single band of approximately 60
342 kDa indicated the presence of pro-MMP3 protein while a faint band of approximately
343 47 kDa suggested activated MMP3 protein in the smooth muscle cells (Figure 7a)
344 (Galazka *et al.*, 1996). A single band (150 kDa) was evident on the ITGA5 western
345 which indicated the expression of this protein in the uterine smooth muscle cells
346 (Figure 7b).

347 Discussion

348 This study investigated the expression of the ECM-related and tissue remodelling
349 molecules, the $\alpha 5$ integrin subunit, ITGA5 and the matrix metalloproteinase, MMP3,
350 at the mRNA and protein levels in human myometrium during pregnancy and labour.
351 MMP3 expression was previously observed in non-pregnant human myometrium
352 (Dou *et al.*, 1997); however, this is the first report of its expression in human
353 myometrium during pregnancy and labour and its subsequent up-regulation at labour.

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

372 were obtained from the lower uterine segment and the increased production of pro-
373 MMP3 at labour suggests a significant role for this metalloproteinase in the adaptation
374 of this region for birth. Another study observed an increase in *Mmp3* gene expression
375 24 hours postpartum (Salomonis *et al.*, 2005). MMP3 therefore may also play a role
376 in the extensive postpartum remodelling where ECM degradation during involution
377 enables the uterus to return to its pre-pregnancy state. Other MMPs, MMP7 and 9 are
378 previously known to contribute to uterine collagen extracellular degradation at this
379 time (Shimizu and Maekawa, 1983).

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

391 Integrins are crucial to the control of cell-ECM interactions where they serve as a link
392 between the ECM and the cytoskeleton. Integrins also contribute to the remodelling
393 of the uterus before parturition. Other investigators have observed an increase in rat
394 ITGA5 throughout pregnancy and labour which decreases postpartum (Williams *et al.*,
395 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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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 \pm 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 \pm 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 ($n = 3$) \pm 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.

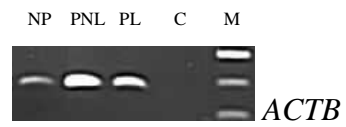
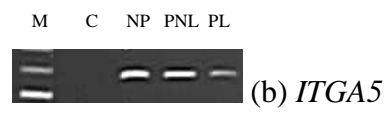
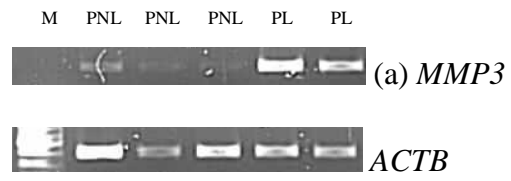
(b) Quantitative densitometric analysis of the *ITGA5* western presented above. ACTB normalised densitometric units were plotted against protein isolated from each

pregnancy state, PL ($n = 3$) and PNL ($n = 3$) \pm 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**

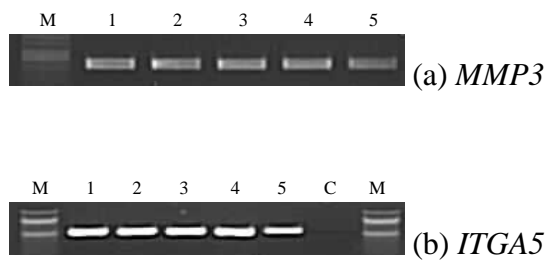


Figure 2 O'Brien et al

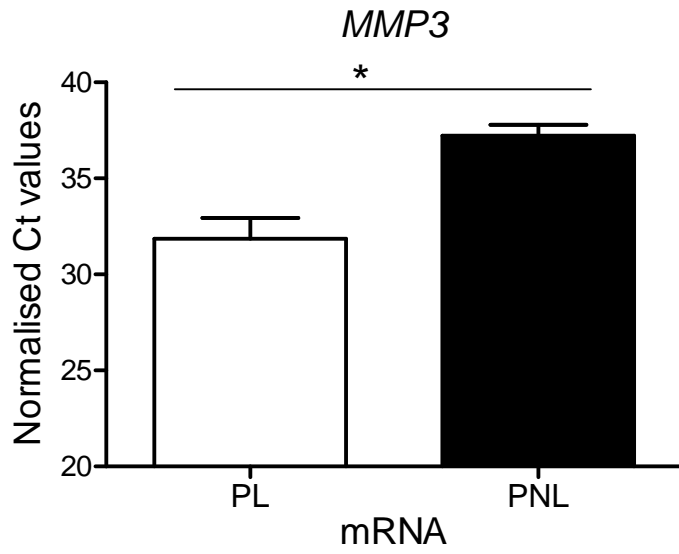


Figure 3a O'Brien et al

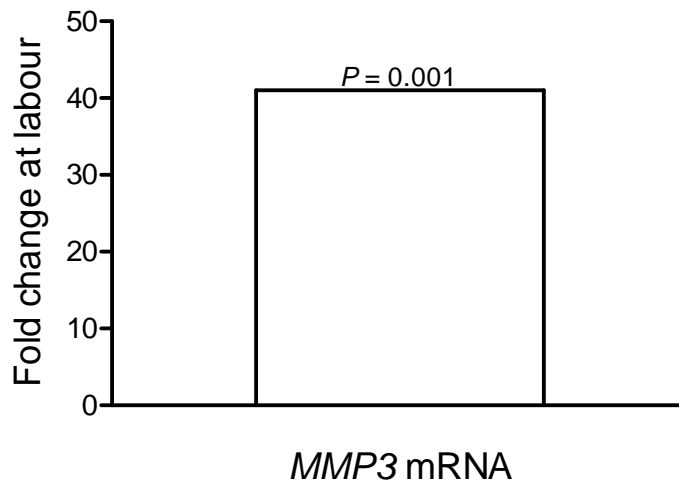


Figure 3b O'Brien et al

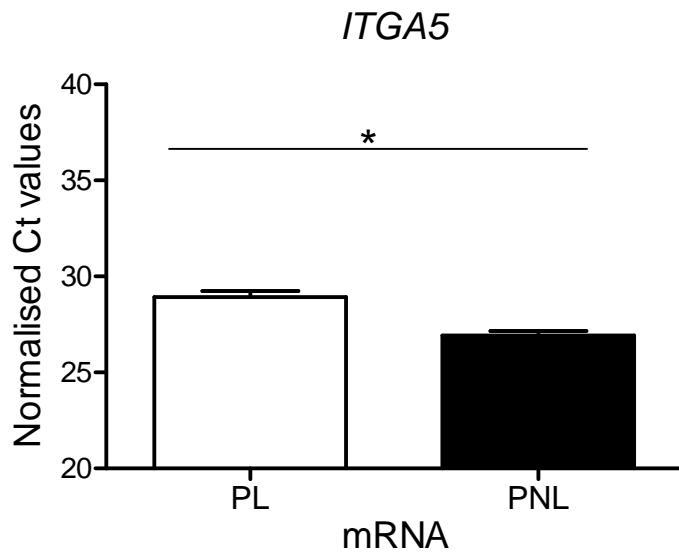


Figure 4a O'Brien et al

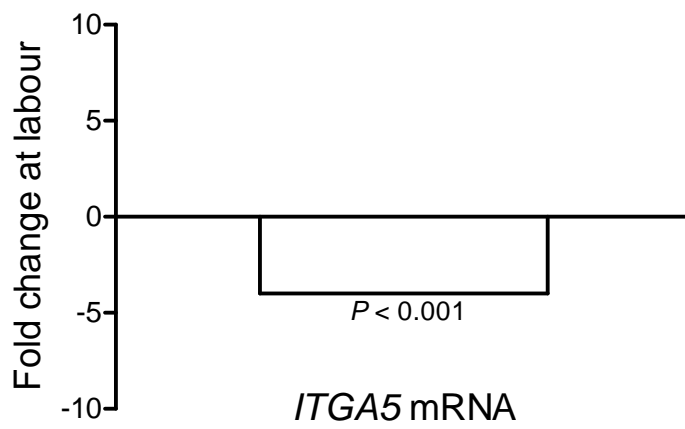


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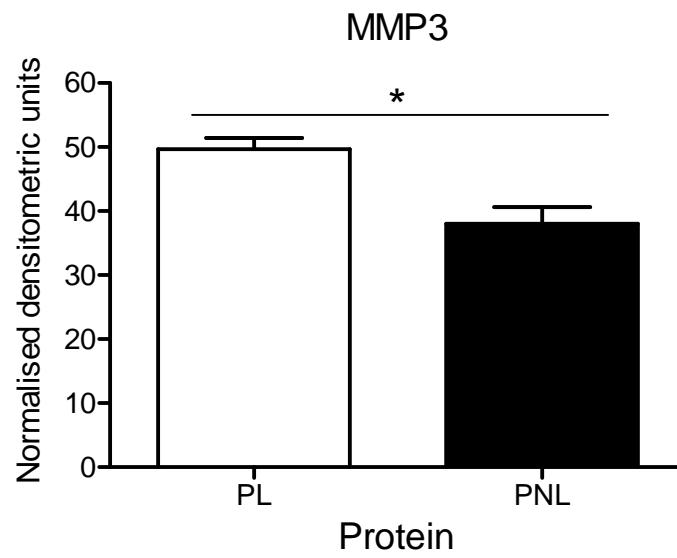
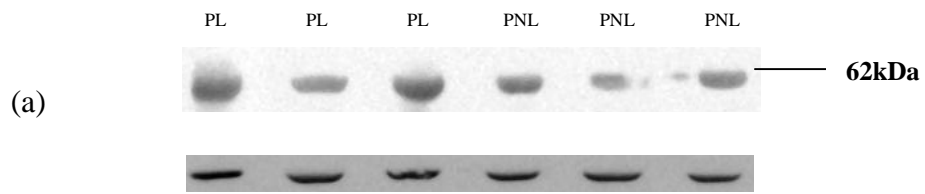
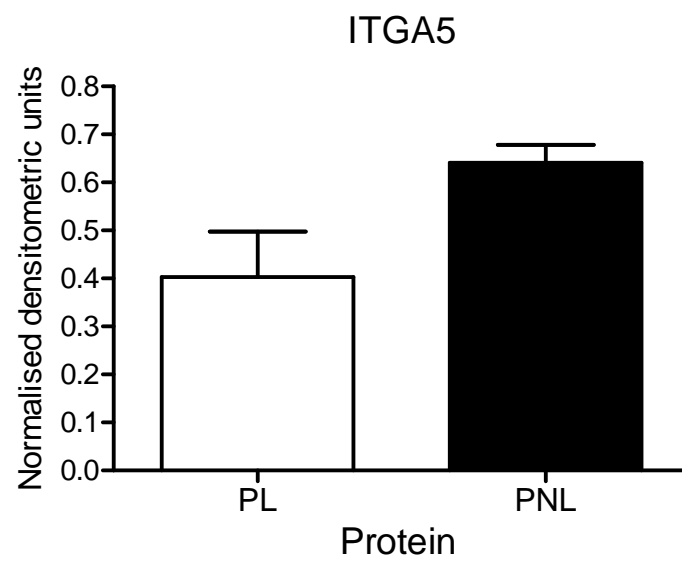
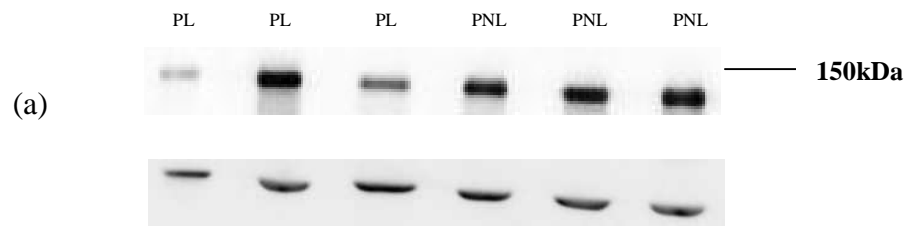
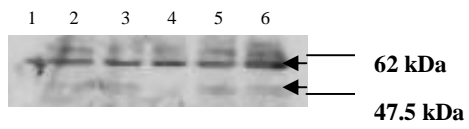


Figure 5 O'Brien et al

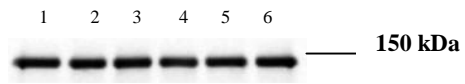


(b)

Figure 6 O'Brien et al



(a) MMP3



(b) ITGA5

Figure 7 O'Brien et al