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Expression of gastrin-releasing peptide is increased by prolonged stretch of human myometrium, and antagonists of its receptor inhibit contractility

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Key points

- Increased uterine stretch appears to increase the risk of preterm labour, but the mechanism by which this might occur is unknown.
- Gastrin-releasing peptide (GRP) mRNA levels are increased by stretch of myometrial explants and incubation of stretched explants in GRP antagonists can decrease their contractility.
- GRP may be a target for novel therapies to decrease the risk of preterm labour in women with multiple pregnancies.

Abstract Increased uterine stretch appears to increase the risk of preterm labour, but the mechanism is unknown. The aim of this study was to identify factors that mediate the effect of stretch on human myometrium. Myometrial explants, prepared from biopsies obtained at elective caesarean delivery, were either studied acutely, or were maintained in prolonged culture (up to 65 h) under tension with either a 0.6 g or a 2.4 g mass, and compared using *in vitro* contractility, whole genome array, and qRT-PCR. Tissue held at tonic stretch with the 2.4 g mass for either 24 or 65 h showed increased potassium chloride (KCl)-induced and oxytocin-induced contractility compared with that held with the 0.6 g mass. Gene array identified 62 differentially expressed transcripts after 65 h exposure to increased stretch. Two probes for gastrin-releasing peptide (GRP), a known stimulatory agonist of smooth muscle, were among the top five up-regulated by stretch (3.4-fold and 2.0-fold). Up-regulation of GRP mRNA by stretch was confirmed in a separate series of 10 samples using quantitative RT-PCR (qRT-PCR) (2.8-fold, $P = 0.01$). GRP stimulated contractions acutely when added to freshly obtained myometrial strips in 2 out of 9 cases, but Western blot demonstrated expression of the GRP receptor in 9 out of a further 9 cases. Prolonged incubation of stretched explants in the GRP antagonists PD-176252 or RC-3095 (65 and 24 h, respectively) significantly reduced KCl- and oxytocin-induced contractility. Tonic stretch of human myometrium increases contractility and stimulates the expression of a known smooth muscle stimulatory agonist, GRP. Incubation of myometrium with GRP receptor antagonists attenuates the effect of stretch. GRP may be a target for novel therapies to reduce the risk of preterm birth in multiple pregnancy.

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Abbreviations GRP, gastrin releasing peptide.

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Introduction

Pre-term birth is a major public health problem, occurring in 5–15% of all pregnancies, but being responsible for at least 60% of all neonatal deaths (Rush *et al.* 1976; Goldenberg & Culhane, 2003). In addition, very pre-term birth is associated with considerable long-term morbidity (Arias & Tomich, 1982; Keirse, 1995) and emotional stresses in families (Challis *et al.* 2000), and the management of its effects are expensive, estimated to be £3 billion annually in the UK alone (Mangham *et al.* 2009). Approximately 12% of preterm births occur in twin pregnancies, despite the fact that these account for only 2% of all pregnancies (Gardner *et al.* 1995). The association is largely due to increased rates of spontaneous preterm birth in multiple pregnancy, which is in turn thought to be secondary to a direct effect of uterine stretch to stimulate myometrial contractility (Newman *et al.* 2006). However, the mechanism by which myometrial stretch might have functional effects on contractility remains unclear.

A number of model systems have been utilized to study the effect of stretch on myometrial contractility. In animals with two uterine horns (e.g. rat), comparisons have been made between a gravid and non-gravid horn and studies have also used mechanical devices to stretch one uterine horn (Ou *et al.* 2000). However, the fact that these studies involve non-human tissue is a limitation for translating findings into clinical interventions. Studies using human myometrium have generally involved working with isolated and cultured myocytes grown onto flexible-bottom culture plates and subsequently subjected to stretch for a number of hours. Whilst such work has shown stretch to cause changes in mRNA and protein, for example those of cyclooxygenase-2 (Sooranna *et al.* 2004), this technique has the major limitation of using myometrial cells in the absence of their extracellular matrix, which is known to be regulated at the time of labour (Shynlova *et al.* 2004) and which would be expected to play a major role in mechanotransduction in the tissue (Ingber, 2006). In the present study, we used a previously developed myometrial explant model (modified from that of Young & Zhang (2004)) to study the effects of prolonged stretch of human myometrium.

Methods

Tissue collection

Human myometrial samples were obtained from non-labouring patients, undergoing routine elective caesarean section, at 38–40 weeks of pregnancy, as previously described (Cordeaux *et al.* 2010). The study was approved by the Cambridgeshire Research Ethics Committee and conformed to the *Declaration of Helsinki*, and all patients gave their informed, written consent to participate. Indications for caesarean section included breech presentation and prior caesarean section but excluded multiple gestation.

Myometrium explant culture

Strips were produced and maintained in culture using the method previously described (Cordeaux *et al.* 2010). Strips were suspended under either low tension (0.6 g mass) or high tension (2.4 g mass). We performed preliminary experiments to determine the conditions under which myometrial stretch would most consistently affect contractility; at 0.6 g tension strips exhibited only a small length change (approximately 10% increase in length) and at 2.4 g they were stretched to approximately 150% of original length and displayed increased contractility, whereas at 1.2 g tension the increase in contractility was less consistent and at 3.6 g the strips regularly tore. All strips were of approximately the same length (12 mm) with the points of attachment for suspension being approximately 2 mm from each end of the strip. Following either 24 or 65 h incubation (37°C, humidified, 5% CO_2 incubator), strips were either transferred to an eight-chamber organ bath for isometric tension studies, snap-frozen in liquid nitrogen for either RNA studies or Western blotting, or fixed in formalin for immunohistochemistry. All comparisons were of paired samples from the same patient. In experiments where drugs were added, paired samples had appropriate vehicle treatment; bovine serum albumin for GRP (0.001% final concentration), dimethyl sulphoxide for PD-176252 (0.5% final concentration) and distilled water for RC-3095 (1% final concentration).

Isometric tension measurements

Following explant culture, myometrial contractility was studied using an eight-chamber Radnoti tissue/organ bath system as previously described (Cordeaux *et al.* 2009). The previously described protocol, where tension was initially set at 2 g for all strips, was used to obtain a response to KCl and a cumulative concentration–response curve to oxytocin (Cordeaux *et al.* 2010). For studies on the acute effect of GRP or antagonists, myometrium was dissected as described above and mounted directly into the organ bath. Washes, tension resets and KCl stimulation were performed as in the experiments above and the tissue was then rested for 45 min to allow spontaneous contractions to develop.

Data analysis

Isometric tension was recorded and analysed using Powerlab Chart software (v. 5.5.6, ADInstruments, Oxford, UK). Further analysis was performed using Prism

5 (GraphPad Software, La Jolla, California, USA). For analysis of contractility after explant culture, maximal responses to KCl and oxytocin (measured in g) were normalized to strip weight (also measured in g) to produce a normalized response and the fold change with stretch (high tension compared with low tension) calculated. Similarly, all responses following incubation in the presence of GRP or antagonists were expressed as a fold change compared to the responses obtained in vehicle alone. Responses were calculated as previously described (Cordeaux *et al.* 2010). pEC_{50} values were calculated using analysis of the area under the curve for each concentration of oxytocin as previously described (Cordeaux *et al.* 2009). Only data sets where an R^2 value of ≥ 0.9 for each treatment could be obtained were used in further paired analysis. For the analysis of the acute effects of GRP or antagonists, contractility was quantified as the area under the curve (AUC) calculated using the 'integral over minimum' function in the Chart software. AUC calculated in the 30 min following addition of the agent was expressed as a fold change compared to that observed in the previous 30 min.

For all contractility experiments, the Kolmogorov–Smirnov test was used to determine if the data were normally distributed. If the data were normally distributed, they were expressed as mean [95% confidence interval] and Student's *t* test was used to test for significance. If the data were not normally distributed, they were expressed as median [inter-quartile range] and Wilcoxon's signed rank test employed. The *n* in the text refers to the number of independent experiments performed, using tissue from separate donors.

Whole genome microarray

Following culture (65 h, low or high tension), myometrial tissue samples for microarray and quantitative RT-PCR were trimmed (to remove tissue that had not been incubated under tension), snap-frozen in liquid nitrogen and stored at −80°C until further use. Total RNA was isolated, quantified, quality assessed, labelled and hybridized to Illumina Human-8 v3 beadchip arrays (United Kingdom, Little Chesterford, Essex, UK) as previously described (Cordeaux *et al.* 2010). Resulting data were processed and significantly regulated transcripts identified as previously described (Cordeaux *et al.* 2010). Array data have been deposited in the online data repository GEO (Gene Expression Omnibus, series accession number GSE31329).

Validation of transcript changes by qRT-PCR

Validation of differential levels of transcripts was performed using qRT-PCR in biological replicates. RNA was prepared from myometrial tissue samples, as described above, using myometrium from a second group of 10 independent donors. cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) and random hexamers and compared with 'No-RT' controls. The gene expression assay used for GRP was Hs01107047˙m1 (Applied Biosystems, Foster City, CA, USA), in which the primers are close to the region interrogated by the microarray. The signal obtained for GRP mRNA was normalized using the median of the three most stable housekeeping genes (peptidylprolyl isomerize A (cyclophilin A; Hs99999904_m1), glucuronidase-β (Hs99999908_m1) and β2-microglobulin (Hs00984230_m1)) using a geometric averaging technique (Vandesompele *et al.* 2002), to obtain ΔC_t values. To report fold changes, $\Delta\Delta C_t$ values were derived and the medians (and inter-quartile ranges) calculated, where $\Delta\Delta C_t = \Delta C_t$ (high tension sample) – ΔC_t (low tension sample), and fold change is derived as $2^{-\Delta\Delta C_t}$.

Immunohistochemistry for GRP

Myometrial explants were fixed in 10% formalin (4 h, room temperature). They were subsequently washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and stored in 75% ethanol at 4°C until further processing. Samples were embedded in paraffin wax and 5 μm sections cut. After deparaffination in xylene and rehydration using graduated ethanols, endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in methanol (10 min, room temperature). Antigen retrieval involved treatment in a pressure cooker (1 min, sodium citrate 0.01 M, pH 6.0) and non-specific binding was blocked by incubation with 20% goat serum in Tris-buffered saline (150 mM NaCl, 2 mM KCl, 25 mM Tris, pH 7.4) (10 min, room temperature). Incubation with primary antibody (1:1000 rabbit anti-GRP, ab22623, Abcam plc, Cambridge, UK) or negative control (1:1000 rabbit serum) in 5% goat serum in TBS (1 h, room temperature) was followed by washing and then incubation with secondary antibody (1:400 in TBS biotinylated goat anti-rabbit, Dako UK Ltd, Ely, Cambridgeshire, UK) (1 h, room temperature). Immunostaining was subsequently visualized using the Vectastain ABC kit (Vector Laboratories Ltd, Peterborough, UK) (30 min, room temperature) and diaminobenzidine reagent (Sigma-Aldrich Company Ltd, Gillingham, UK). Slides were counterstained using 50% haematoxylin and dehydrated using graduated ethanols and xylene, prior to mounting with Depex.

Immunostaining was assessed on a semi-quantitative basis by using the modified H-scoring system (Ravn *et al.* 1993), which includes an estimate of the fraction (%) of cells stained: 0 = 0–9%, 1 = 10–39%, 2 = 40–69%, 3 = 70–89% and 4 = 90–100%; and the staining intensity

(I): 0 = no staining, 1 = weak but definite staining, 2 = moderate staining, 3 = pronounced staining and 4 = intense staining. The H-score is then calculated by multiplication of the assessments of % and I. In order to avoid bias, five high power images (40×) of each section were independently assessed by two observers who were blinded to the identity of each set of images. The average score (of the two observers) for each image was calculated and then the median score for each tissue section was calculated. The first image was taken at the centre of each tissue section and another four images then taken adjacent to this image in perpendicular directions. The images were obtained by a third person, equally blinded to the identity of the samples. An average score for each tissue section was obtained and used for subsequent statistical analysis.

Western blotting for GRP receptor

Tissue homogenates were prepared, and proteins separated and transferred to polyvinyl difluoride membranes as previously described (Cordeaux *et al.* 2009). After transfer, membranes were incubated in 5% milk/Tris-buffered saline with Tween 20 (milk/TBST) solution for 2 h at room temperature to reduce non-specific binding. Membranes were incubated with primary antibody (1:1000 ab39883, Abcam plc) in milk/TBST solution at 4°C overnight. Membranes were washed in TBST solution (12 × 5 min washes), before incubation with 1:2000 goat anti-rabbit horseradish peroxidase-conjugated antibody (Dako, Glostrup, Denmark) in milk/TBST solution (1 h, room temperature). After a further series of washes with TBST solution (as above), membranes were incubated with Enhanced Chemiluminescence Plus detection reagents (GE Healthcare Life Sciences, Little Chalfont, UK) (5 min, room temperature) before being exposed to Hyperfilm-ECL X-ray film (GE Healthcare Life Sciences) for up to 5 min.

Results

Effect of stretch on myometrial contractility

Tissue held under tension with a 2.4 g mass showed significantly increased maximum normalized response to KCl (50 mM) and oxytocin (up to 100 nM) when compared to tissue held under tension with a 0.6 g mass after both 24 h and 65 h (Fig. 1). The magnitude of increase did not differ significantly comparing 24 h and 65 h ($P > 0.05$). Spontaneous activity was also significantly increased by such stretch after both 24 h and 65 h (24 h incubation: 1.734 [IQ range: 1.20–2.29] fold change, $P = 0.006$, $n = 14$; 65 h incubation: 2.138 [IQ range: 0.96–2.82] fold change, $P = 0.04$, $n = 13$). Maximum area under the curve in response to oxytocin was also increased by stretch after both 24 h and 65 h (24 h incubation: 1.67 [95% CI: 1.31–2.23] fold change, $P = 0.032$, $n = 14$; 65 h incubation: 2.50 [95% CI: 1.77–3.23] fold change, $P = 0.0008$, $n = 13$). There was no statistically significant effect of stretch on the pEC_{50} to oxytocin (24 h incubation: low tension −8.60 [IQ range: −8.82 to −8.03] *versus* high tension −9.08 [−9.21 to −8.49], $P = 0.31$, $n = 5$; 65 h incubation: low tension −9.05 [−9.38 to −8.82] *versus* high tension −8.76 [−9.08 to −8.70], $P = 0.41$, $n = 13$).

Whole genome microarray and validation

Microarray analysis was performed using nine sets of paired myometrial explants incubated for 65 h under low or high tension. All samples were hybridized at the same time to minimize batch effects and data were analysed as paired samples.

The variability in hybridization signal was probe set dependent, with the coefficients of variation among the ∼24,000 transcripts interrogated ranging from 0.017 to 2.66 and 0.014 to 2.59 (for the entire data set) in the low and high tension samples, respectively (and ranging from 0.139 to 1.112 and 0.125 to 0.946 for the regulated transcripts).

The levels of 62 transcripts were significantly different comparing low and high tension incubated explants (30 down-regulated by increased stretch and 32 up-regulated, 29 and 30 unique genes respectively, see Tables 1 and 2). Two of the top five up-regulated signals were from probes to GRP mRNA (median fold increase with stretch 3.39 and 1.98). qRT-PCR confirmed a similar significant change (2.84-fold) in GPR transcripts in biological replicates obtained from a separate group of 10 women (Fig. 2).

Immunohistochemistry for GRP

The expression of GRP in myometrium was quantified using immunohistochemistry. Stretch increased the median H-score in five out of six pairs of myometrial samples (Fig. 3). The mean value for all six samples increased from 7.5 (95% CI: 0.6–14.4) to 11.6 (6.5–16.6), although this did not reach statistical significance ($P = 0.31$). The lack of statistical significance was due to a single sample that had high levels of GRP expression (H score 20) under low tension and showed no increase under high tension.

Effects of acute treatment with GRP

Of nine strips exposed acutely to GRP, two demonstrated unambiguous contractile responses following acute exposure to GRP and there was no comparable change in activity in either of the vehicle controls (Fig. 4). We then sought to determine whether GRP may

potentiate the effects of other agonists. We obtained concentration–response curves to the prostanoid EP1/3 receptor agonist, sulprostone, in myometrium pre-relaxed with forskolin as previously described (Cordeaux *et al.* 2008), in the presence and absence of GRP. The presence of GRP had no significant effect on the response to sulprostone in terms of maximum contractile response (10 nM GRP: 1.24 [95% CI: 0.85–1.63] fold change, $P = 0.17$; 100 nM GRP: 0.93 [0.58–1.28] fold change, $P = 0.65$; 1000 nM GRP: 0.96 [0.64–1.28] fold change, $P = 0.78$, $n = 7$ for all analyses) or pEC_{50} (control: −8.54 [95% CI −9.05 to −8.03]; 10 nM GRP: −8.72 [−9.59 to −7.85],

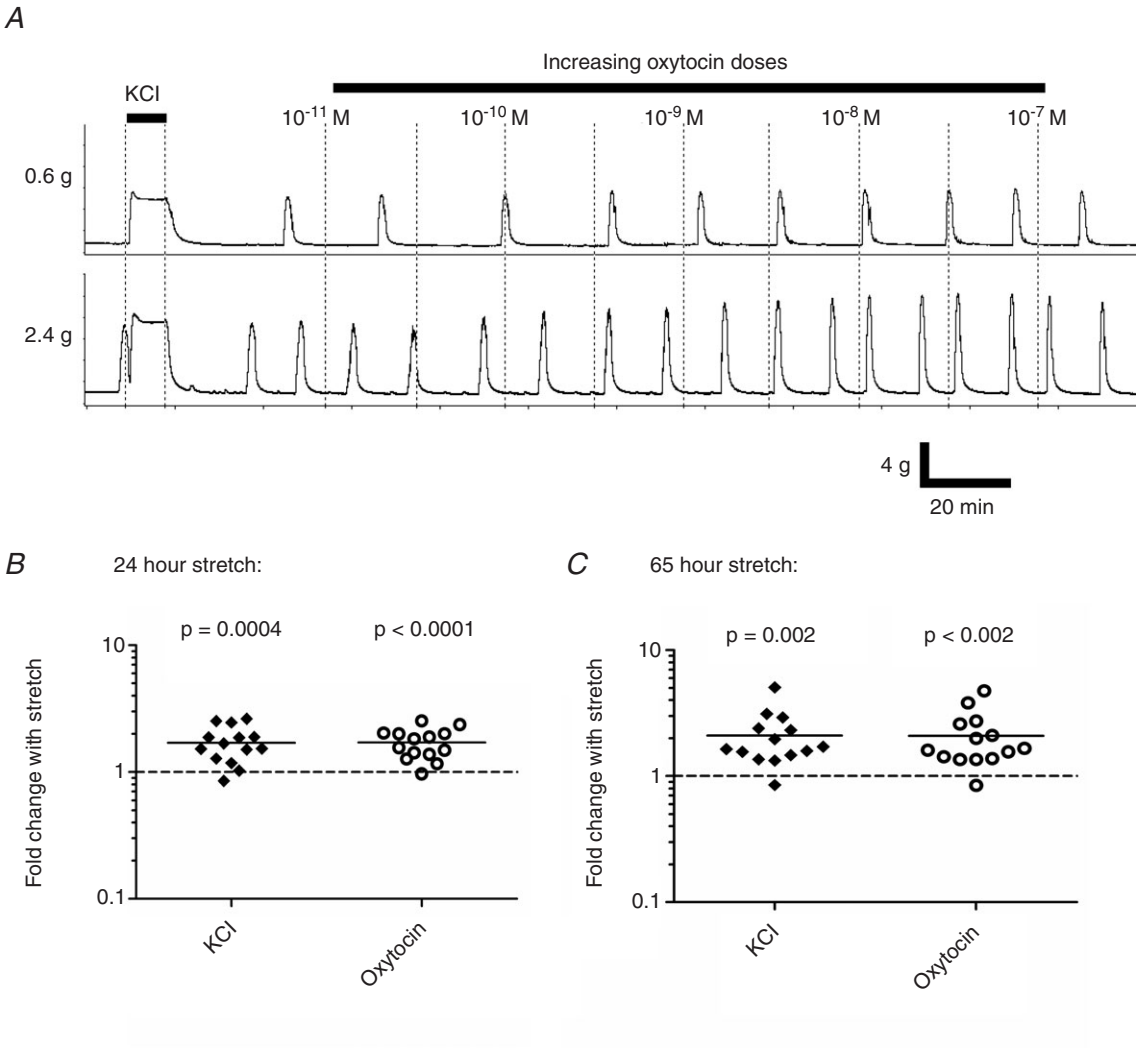

Figure 1. The effect of stretch on maximal contractile responses to KCl and oxytocin in human pregnant myometrium incubated for either 24 or 65 h
Strips of myometrium were incubated under either low tension (0.6 g mass) or high tension (2.4 g mass) for either 24 h or 65 h, and isometric tension measurements obtained, as described in Methods. *A*, a representative set of traces (in which the fold change in maximal KCl response with stretch is closest to median value) showing the effect of differing incubating tensions for 65 h upon the contractile responses to both KCl and oxytocin. All myometrial strips were of similar weight and although only alternate doses of oxytocin are labelled above, half-log doses were administered at the times indicated by the dashed lines. Maximum responses, to either 50 mM KCl or increasing concentrations of oxytocin (up to 100 nM), were expressed relative to the wet weight of each strip to produce a normalized response. *B* and *C*, fold changes with stretch (data for tissue incubated with a 2.4 g mass divided by that obtained for tissue incubated with a 0.6 g mass) for each myometrial biopsy studied, with duplicates strips at low and high stretch analysed. Data were compared using Student's paired *t* test (bars indicate mean fold change with stretch, $n = 14$ for each time point). Following both 24 h and 65 h incubation, stretch increased the response to KCl and oxytocin (24 h, KCl: 1.70 [95% CI: 1.38–2.02] fold change; 24 h, oxytocin: 1.70 [1.44–1.97] fold change; 65 h KCl: 2.09 [1.47–2.70] fold change; 65 h, oxytocin: 2.08 [1.46–2.70] fold change. $n = 14$ for all experiments.

Table 1. Genes significantly down-regulated by stretch

Illumina ID	Gene symbol	Gene name	Median fold change, down
ILMN_1758895	CTSK	Cathepsin K	1.670
ILMN_2167758	CILP	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	1.431
ILMN_2167805	LUM	Lumican	1.428
ILMN_1743620	RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	1.343
ILMN_1753312	PLXDC2	Plexin domain containing 2	1.338
ILMN_1766261	SLC2A12	Solute carrier family 2 (facilitated glucose transporter), member 12	1.314
ILMN_1722898	SFRP2	Secreted frizzled-related protein 2	1.271
ILMN_1691410	BAMBI	BMP and activin membrane-bound inhibitor homolog (*Xenopus laevis*)	1.263
ILMN_1715603	IL23A	Interleukin 23, α subunit p19	1.262
ILMN_1656057	PLAU	Plasminogen activator, urokinase	1.253
ILMN_1682099	TNFAIP8L3	Tumour necrosis factor, α-induced protein 8-like 3	1.249
ILMN_2412336	AKR1C2	Aldo-keto reductase family 1, member C2	1.243
ILMN_1685608	NPTX2	Neuronal pentraxin II	1.243
ILMN_2302757	FCGBP	Fc fragment of IgG binding protein	1.239
ILMN_1794492	HOXC6	Homeobox C6	1.238
ILMN_1680339	PDGFRL	Platelet-derived growth factor receptor-like	1.235
ILMN_1800091	RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	1.229
ILMN_1769580	PRSS35	Protease, serine, 35	1.226
ILMN_1805216	GPC6	Glypican 6	1.225
ILMN_2242937	ARSB	Arylsulfatase B	1.220
ILMN_1740407	CHSY3	Chondroitin sulfate synthase 3	1.214
ILMN_1803213	MXRA5	Matrix-remodelling associated 5	1.213
ILMN_1725387	TMEM200A	Transmembrane protein 200A	1.213
ILMN_1806787	CSDC2	Cold shock domain containing C2, RNA binding	1.212
ILMN_1784532	COL22A1	Collagen, type XXII, alpha 1	1.210
ILMN_2347592	NMB	Neuromedin B	1.209
ILMN_1748751	NLF2	nuclear localized factor 2	1.209
ILMN_1695604	DCBLD1	Discoidin, CUB and LCCL domain containing 1	1.208
ILMN_2347145	DCN	Decorin	1.207
ILMN_1672148	AKR1B10	Aldo-keto reductase family 1, member B10 (aldose reductase)	1.205

Significantly regulated transcripts were identified as previously described (Cordeaux *et al.* 2010), namely using Cyber-T and RankProduct analysis with criteria of $P < 0.001$; ppde > 0.99 and $P < 0.001$; pfp < 0.01, respectively in which ppde is the posterior probability of differential expression, i.e. the probability that a gene at a given P value is differentially expressed, and pfp is the percentage of false positives and an additional criterion of an absolute fold change of at least 1.2.

$P = 0.97$; 100 nM GRP: −8.56 [−9.14 to −7.98], $P = 0.53$; 1000 nM GRP: −8.67 [−9.39 to −7.94], $P = 0.48$, $n = 7$ for all analyses).

Effect of prolonged treatment with GRP

In order to determine whether prolonged exposure to high levels of GRP had an effect on myometrial contractility, we incubated myometrial explants for 24 h under low tension in GRP or its vehicle. GRP had no effect on the maximum response to KCl or oxytocin at 1 nM, 10 nM or 100 nM (1 nM, KCl: 0.92 [IQR: 0.83–1.42] fold change, $P = 1.0$; 1 nM, oxytocin: 0.96 [0.76–1.37] fold change, $P = 1.0$; 10 nM, KCl: 0.94 [0.81–1.50] fold change, $P = 0.84$; 10 nM, oxytocin: 1.01 [0.91–1.54] fold change, $P = 0.56$; 100 nM, KCl: 0.98 [0.65–1.25] fold change, $P = 0.84$; 100 nM, oxytocin: 1.05 [0.77–1.31] fold change, $P = 0.69$, $n = 6$ for all analyses).

Effect of prolonged exposure to GRP antagonists on prolonged exposure to stretch

We next sought to determine whether blocking the effects of endogenous GRP might prevent stretch induced stimulation of myometrial contractility using the high affinity non-peptide antagonist of the GRP receptor, PD-176252. Incubation in 10 μM PD-176252 for 65 h caused significant decreases in maximum responses to

Table 2. Genes significantly up-regulated by stretch

Illumina ID	Gene symbol	Gene name	Median fold change, up
ILMN_1781256	LEFTY2	left-right determination factor 2	4.756
ILMN_2413323	GRP	Gastrin-releasing peptide	3.392
ILMN_2161577	CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	2.060
ILMN_1777199	GRP	Gastrin-releasing peptide	1.984
ILMN_1677505	CCL21	Chemokine (C-C motif) ligand 21	1.676
ILMN_2108735	EEF1A2	Eukaryotic translation elongation factor 1 α 2	1.668
ILMN_1660114	MMRN1	Multimerin 1	1.548
ILMN_1695880	LOX	Lysyl oxidase	1.540
ILMN_2242900	IL1RL1	Interleukin 1 receptor-like 1	1.518
ILMN_1715991	SDPR	Serum deprivation response (phosphatidylserine binding protein)	1.437
ILMN_1811387	TFF3	Trefoil factor 3 (intestinal)	1.427
ILMN_1692938	PSAT1	Phosphoserine aminotransferase 1	1.420
ILMN_1796417	ASNS	Asparagine synthetase	1.407
ILMN_2049672	TMEM16C	Transmembrane protein 16C	1.362
ILMN_1758315	SLC9A9	Solute carrier family 9 (sodium/hydrogen exchanger), member 9	1.344
ILMN_2333367	FKBP1A	FK506 binding protein 1A, 12 kDa	1.337
ILMN_2361862	VLDLR	Very low density lipoprotein receptor	1.320
ILMN_2398107	ASNS	Asparagine synthetase	1.316
ILMN_1666206	GSDML	Gasdermin-like	1.309
ILMN_1655913	NUCB2	Nucleobindin 2	1.302
ILMN_1670130	ARID3A	AT rich interactive domain 3A (BRIGHT-like)	1.291
ILMN_1765641	SEMA3A	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	1.275
ILMN_1683859	SLC7A1	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.274
ILMN_1773964	H1FX	H1 histone family, member X	1.260
ILMN_1770015	KRT33B	Keratin 33B	1.258
ILMN_2323944	FAM110A	Family with sequence similarity 110, member A	1.249
ILMN_2208802	NPIP	Nuclear pore complex interacting protein	1.243
ILMN_1760990	SH3GL3	SH3-domain GRB2-like 3	1.227
ILMN_1807833	HM13	Histocompatibility (minor) 13	1.223
ILMN_1763587	TNMD	Tenomodulin	1.211
ILMN_1765212	LARP2	La ribonucleoprotein domain family, member 2	1.209
ILMN_2339028	PKD1	Polycystic kidney disease 1 (autosomal dominant)	1.202

Significantly regulated transcripts were identified as previously described for Table 1.

both KCl and oxytocin at both incubating tensions examined (Fig. 5). There was also a trend to reduced spontaneous activity, but this was only statistically significant at low tension (low tension: 0.58 [95% CI: 0.29–0.87] fold change, $P = 0.01$, $n = 6$; high tension: 0.74 [95% CI: 0.18–1.30] fold change, $P = 0.28$, $n = 6$). Lower doses of PD-176252 (tested at high incubating tension) had no significant effects upon maximum responses to either KCl or oxytocin or upon spontaneous activity (all $P > 0.05$).

In order to assess the consistency of the effect of GRP receptor blockade, we also evaluated a structurally unrelated (peptide) GRP receptor antagonist, RC-3095. Incubation of myometrial explants with 1 μM RC-3095 also significantly reduced mean maximum response to both KCl and oxytocin in high tension, but had no significant effect when incubation was at the lower tension (Fig. 6). There was no apparent effect upon spontaneous activity (low tension: 1.00 [IQR: 0.80–1.46] fold change, $P = 0.85$, $n = 10$; high tension: 0.96 [IQR: 0.60–1.96] fold change, $P = 0.85$, $n = 10$).

Effect of acute exposure to GRP antagonists on prolonged exposure to stretch

We sought to determine whether the increased contractility observed in explants exposed to prolonged stretch

could be blocked acutely by exposure to a GRP antagonist. Administration of RC-3095 to spontaneously contracting myometrial strips after incubation for 3 days at high tension had no significant effect upon myometrial contractility at any of the doses examined (10 nM RC-3095: 0.95 [95% CI: 0.77–1.13] fold change, $P = 0.53$; 100 nM RC-3095: 0.98 [0.82–1.14] fold change, $P = 0.84$; 1000 nM RC-3095: 0.99 [0.81–1.18] fold change, $P = 0.97$, $n = 6$ for all analyses).

Western blot for GRP receptor

We also sought to confirm that the GRP receptor (GRPR) was present in human myometrium using Western blot analysis. We studied samples from nine women who had elective caesarean section at term. GRPR was detected as a single, diffuse band at approximately 55–65 kDa molecular mass (Fig. 7). Although the predicted mass of the protein is 43 kDa, the band detected is consistent with previous reports (Kris *et al.* 1987; Williams & Schonbrunn, 1994) and the difference between the expected and observed molecular weight is most likely explained by glycosylation of the protein (Kusui *et al.* 1994; Benya *et al.* 2000).

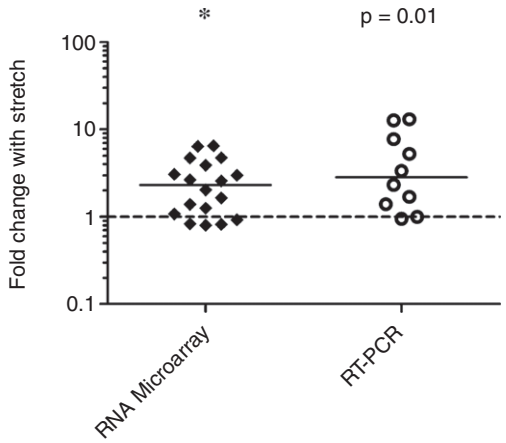

Figure 2. The effect of stretch on mRNA levels for gastrin-releasing peptide (GRP) in human pregnant myometrium incubated for 65 h
Strips of myometrium were incubated under either low tension (0.6 g mass) or high tension (2.4 g mass) for 65 h. GRP mRNA levels were measured using either RNA microarray or qRT-PCR ($n = 9$, but 18 measurements for RNA microarray as there are two probe sets on the array which hybridize to the target mRNA and $n = 10$ for qRT-PCR). Fold changes with increasing tension (data for tissue incubated with a 2.4 g mass divided by that obtained for tissue incubated with a 0.6 g mass) were calculated in each case. Bars indicate medians; *significant result as described in methods section for RNA microarray. The median fold change using qRT-PCR was 2.84 (IQR: 1.29–9.00).

Discussion

Studying the effect of myometrial stretch is clinically important, chiefly through the association between multiple pregnancy and the risk of spontaneous preterm birth. The mechanisms leading to spontaneous preterm birth in multiple pregnancy appear to be different from the mechanisms leading to this outcome in high risk singleton gestations, as the latter is significantly reduced by progestogens (da Fonseca *et al.* 2003; Meis *et al.* 2003; Fonseca *et al.* 2007), whereas these compounds have no effect in multiple pregnancy (Rouse *et al.* 2007; Caritis *et al.* 2009; Norman *et al.* 2009). Understanding the mechanism whereby stretch of the myometrium increases contractility could allow identification of targets for novel therapies for multiple pregnancy. However, work in this area is limited by weak model systems, as discussed above. We therefore developed a model using human myometrial explants to study the effect of stretch, which, despite having limitations (such as the potential for tissue changes on being removed from the body), does potentially offer advantages over other model systems. The most important characteristic of these explants is that they can be maintained over a period of days. This allows study of prolonged exposure to a given stimulus, such as the control of gene expression by progestogens or, in this case, prolonged stretch. Using this model system, we could recapitulate *in vitro* the clinically recognized effect of stretch to stimulate myometrial contractility. Moreover, these explants yield high quality mRNA which then allowed analysis of gene expression using whole genome microarray. We found that the mRNA encoding GRP, a known stimulator of smooth muscle contractility, was up-regulated by stretch and we validated this finding in biological replicates. All six myometrial samples studied using immunohistochemistry confirmed the presence of GRP. Five out of six samples tested demonstrated increased expression of GRP at the peptide level in response to stretch, but the numbers were too small to reach statistical significance. Although the majority of strips failed to respond to acute exposure to GRP, we used Western blot to confirm that the receptor for GRP is present in human myometrium. Finally, we demonstrated that two separate GRP receptor antagonists inhibited the contractility of human myometrial explants. We hypothesize that GRP mediates the pro-contractile effect of prolonged exposure of human myometrium to increased stretch. We speculate that pharmacological blockade of the GRP receptor has potential as a treatment to reduce the risk of preterm birth in multiple pregnancies.

Physiological roles of GRP

GRP is one of three identified mammalian homologues of bombesin, which was originally isolated from the *Bombina*

bombina frog (Anastasi *et al.* 1971). GRP has been shown to promote contraction in a large number of smooth muscles in a variety of species. Isolated guinea-pig stomach cells contract upon treatment with GRP (Chijiiwa *et al.* 1991; Severi *et al.* 1991) and contraction subsequent to GRP administration has been shown in strips of human muscle obtained from the stomach and duodenum (Pogrzeba *et al.* 1991) and the ileocaecal region (Vadokas *et al.* 1997). Previous studies have indicated that GRP increases contractility in rat uterus (Amiot *et al.* 1993), but this is the first study to examine its actions in the human uterus. Bombesin-like peptides have also been shown to stimulate growth of the gastrointestinal mucosa and pancreas (Patel *et al.* 2006) and a mitogenic role in tumour growth was suggested when it was shown that a monoclonal antibody to GRP was able to inhibit the growth of small cell lung cancer cells *in vitro* and of xenografts *in vivo* (Cuttitta *et al.* 1985). GRP immunoreactivity has been shown in a number of different cancers (Sessa *et al.* 1990; Vangsted *et al.* 1991; Carroll *et al.* 1999; Constantinides *et al.* 2003; Scott *et al.* 2004) and GRP antagonists have been shown to inhibit the proliferation of a number of cell lines derived from these types of tumours *in vitro* and also their growth as xenografts (reviewed in Patel *et al.* 2006).

Interpretation of contractility data

We demonstrated a clear contractile response to acute exposure to GRP in 2 of 9 samples studied. This finding confirms that the human uterus, like the rat uterus, can respond to this agonist. The inconsistent nature of the responses was not unexpected. It is frequently observed that human myometrium demonstrates considerable inter-patient variability in terms of contractile response to agonists (Gullam *et al.* 2009), which may in turn reflect variation between different women in the formation of the lower segment and their proximity to spontaneous labour. We considered the possibility that GRP may act to potentiate the response to other stimuli. However, we found no effect of GRP on responses to sulprostone after acute GRP treatment. We also considered the possibility that the increased contractility of the tissues which had been stretched was due to high levels of GRP being released and causing direct stimulation of contractility. We added GRP receptor antagonist to tissues which had been stretched for 3 days. If the increased contractility was due to immediate effects of high endogenous levels of GRP we would have expected the antagonist to have an acute effect to reduce contractility. However, acute administration of GRP antagonist had no acute effect on the spontaneous

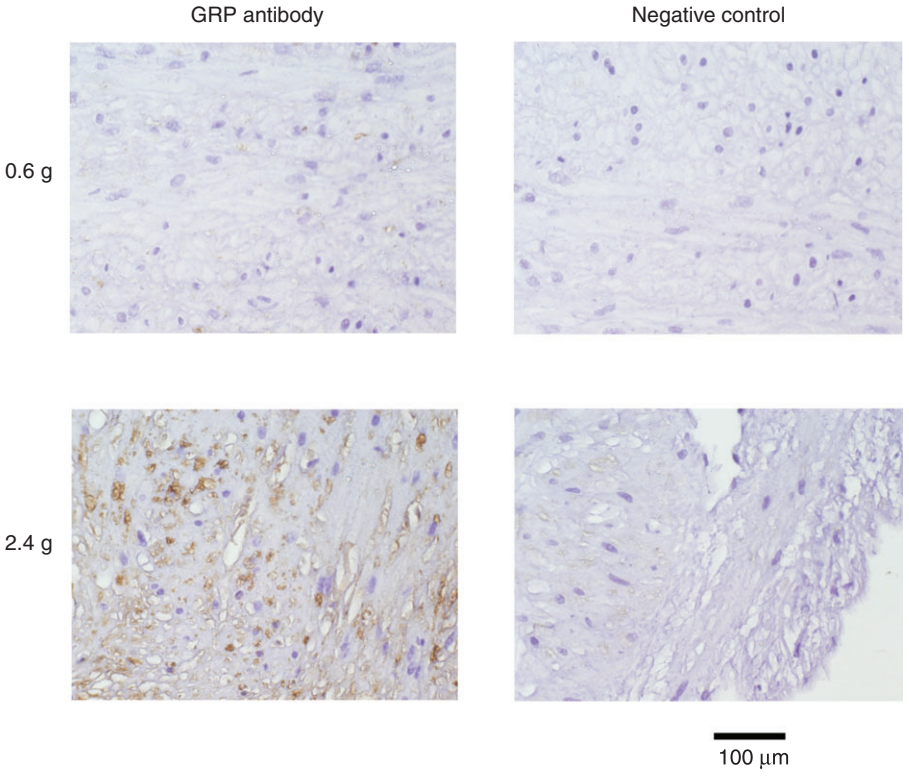

Figure 3. The effect of stretch upon GRP immunoreactivity
Representative images of immunohistochemistry for GRP performed upon a pair of myometrial explants from the same patient incubated under either low tension (0.6 g mass) or high tension (2.4 g mass) for 65 h. The median H-score change with stretch for this pair of explants was closest to the median of the six pairs of explants in which this immunohistochemistry was performed.

activity of myometrial strips which had been exposed to stretch.

We next considered the possibility that prolonged exposure to stretch may have led to prolonged elevated GRP levels, which in turn might have exerted some trophic effect on myometrium. We incubated strips for 24 h in up to 100 nM GRP but found that this had no effect on contractility. However, GRP is a peptide and we could not be confident that any GRP added would be present in the appropriate concentration for the full 24 h period. For example, in two of three small cell lung cancer cell lines, GRP appeared to be rapidly degraded and its half-life in plasma was found to be approximately 30 min (Vangsted & Schwartz, 1990). Further investigation of the effect of long term exposure to elevated levels of GRP in human myometrial explants would be facilitated by the development of stable receptor agonists.

Hence, we next explored the effect of GRP receptor antagonists. Due to concerns around the stability of peptides in our system, we performed initial experiments using PD-176252, a high affinity non-peptide antagonist. As we speculated that the increased contractility associated with stretch was due a trophic effect of prolonged increased endogenous GRP, the prediction was that the stimulatory effect of stretch on contractility would be blocked by prolonged incubation with GRP receptor antagonists. We found that that PD-176252 profoundly reduced the contractility of myometrial strips when incubation was maintained for 65 h. However, this was observed both for the stretched and non-stretched tissue. This finding could reflect an important role for endogenous GRP in myometrium irrespective of the degree of stretch. Alternatively, it could reflect a non-specific effect of the drug. Hence, we studied another GRP receptor antagonist, RC-3095. As this was a peptide, we studied it over a shorter period of incubation (we had already demonstrated that exposure to 24 h of stretch had a similar effect on contractility as 65 h). The findings with RC-3095 were consistent with our prediction: the drug had no effect on contractility in conditions of low stretch but was associated with reduced contractility in myometrial strips exposed to increased stretch. Finally, we used Western blot analysis to confirm that the GRP receptor was present in human myometrium.

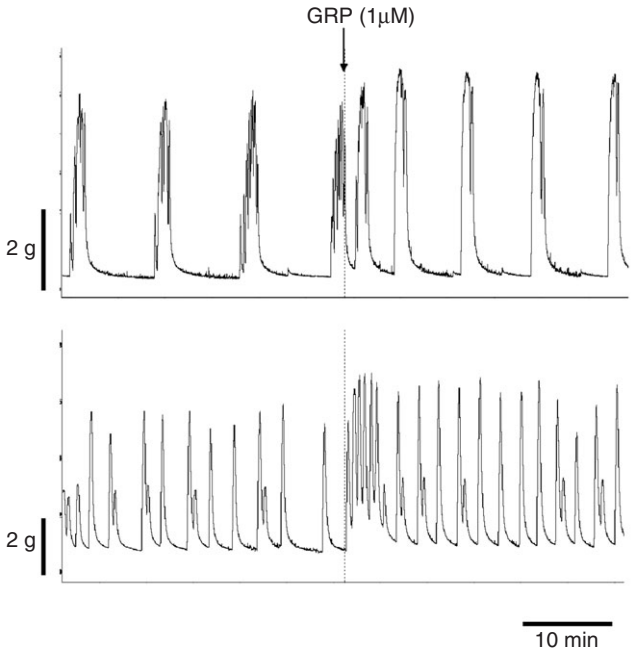

Figure 4. Stimulation of contractility of pregnant human myometrium by GRP (1 μM)
Traces show responses to 1 μM GRP in single representative strips of myometrium (of duplicates) obtained from two separate patients. Relative to spontaneous activity observed in the 30 min prior to agonist addition (left hand section), in the 30 min following GRP addition, activity was increased 1.51-fold (upper panel) and 1.78-fold (lower panel).

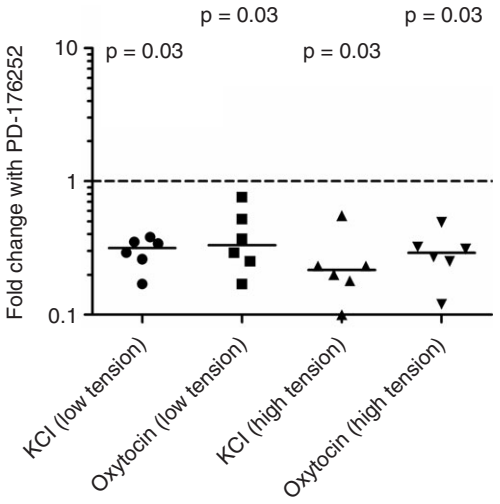

Figure 5. The effect of prolonged PD-176252 on maximal contractile responses to KCl and oxytocin in human pregnant myometrium incubated under low or high tension for 65 h
Strips of myometrium were incubated under either low tension (0.6 g mass) or high tension (2.4 g mass) for 65 h in the presence of either vehicle (0.5% final concentration of dimethyl sulfoxide) alone or with PD-176252 (10 μM) dissolved in vehicle and isometric tension measurements obtained, as described in Methods. Maximum responses to either 50 mM KCl or increasing concentrations of oxytocin (up to 100 nM), were expressed relative to the wet weight of each strip to produce a normalized response. Data shown are the fold changes with PD-176252 for each myometrial biopsy ($n = 6$) studied, with duplicate strips at low and high stretch analysed (bars indicate medians). Wilcoxon's signed rank test was used in each case to determine if there was a significant change in response with PD-176252. When incubated under low and high tension, PD-176252 reduced the median maximum response to KCl and oxytocin (low tension, KCl: 0.31 [IQR: 0.23–0.35] fold change; low tension, oxytocin 0.21 [0.16–0.30] fold change; high tension, KCl: 0.33 [0.22–0.58] fold change; high tension, oxytocin: 0.29 [0.21–0.36] fold change).

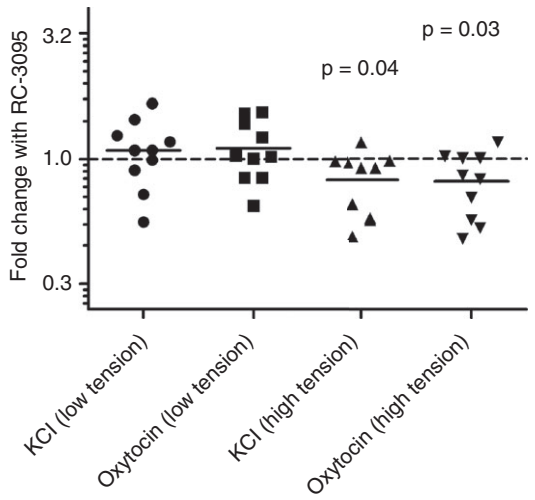

Figure 6. The effect of prolonged RC-3095 on maximal contractile responses to KCl and oxytocin in human pregnant myometrium incubated under low or high tension for 24 h
Strips of myometrium were incubated under either low tension (0.6 g mass) or high tension (2.4 g mass) for 24 h in the presence of either vehicle (0.1% final concentration of distilled water) alone or with RC-3095 (1 μM) dissolved in distilled water and isometric tension measurements obtained, as described in Methods. Maximum responses to either 50 mM KCl or increasing concentrations of oxytocin (up to 100 nM) were expressed relative to the wet weight of each strip to produce a normalized response. Data shown are the fold changes with RC-3095 for each myometrial biopsy ($n = 10$) studied, with duplicate strips at low and high stretch analysed (bars indicate means). Data were compared using Student's paired *t* test. At high tension, RC-3095 reduced the mean maximum response to KCl and oxytocin (KCl: 0.82 [95% CI: 0.66–0.98] fold change; oxytocin: 0.79 [0.61–0.98] fold change), whereas at low tension there was no change in maximal response to KCl or oxytocin (KCl: 1.08 [0.85–1.31] fold change, $P = 0.44$; oxytocin: 1.13 [0.90–1.36] fold change, $P = 0.22$).

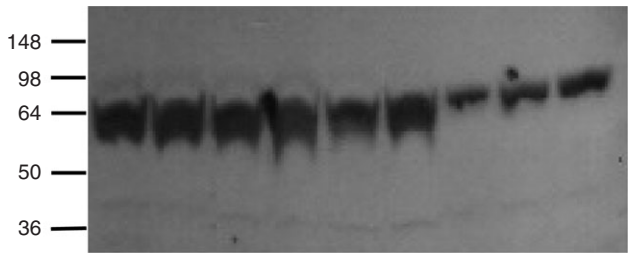

Figure 7. Immunodetection of human GRP receptor by Western blotting
Protein samples were prepared from myometrium obtained from nine separate women, and subjected to SDS-PAGE and Western blotting as described in Methods. The GRP receptor-specific antibody detected a diffuse protein at approximately 55–65 kDa in all samples. Approximate molecular mass was determined using SeeBlue plus 2 protein standards.

We found that 9 out of 9 samples studied using Western blot had a band of appropriate size.

Conclusion

We used an explant system to study the effect of stretch on human myometrium and found that increased stretch of human myometrium increased contractility, consistent with previous clinical and laboratory studies. Gene array identified the mRNA encoding GRP, a known smooth muscle stimulatory agonist, as one of the most up-regulated transcripts among a small number of genes which were up-regulated by stretch. We confirmed stretch-induced up-regulation of GRP mRNA using qRT-PCR in biological replicates. We demonstrated acute effects of GRP in some samples, but expression of the GRP receptor in all samples studied. Furthermore, prolonged incubation in two different GRP antagonists reduced myometrial contractility. We speculate that GRP may be a mediator of the increased myometrial contractility induced by stretch and that its receptor is a target for novel therapies to reduce the risk of preterm birth in multiple pregnancy.

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Author contributions

All authors were involved in conception and design of the experiments, collection, analysis and interpretation of the data and drafting the article and revising it critically for important intellectual content. The work was performed in the Department of Obstetrics and Gynaecology, University of Cambridge. All authors approved the final version of the manuscript.

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