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Common fibroid-associated genes are differentially expressed in phenotypically dissimilar cell populations isolated from within human fibroids and myometrium

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Abstract

Uterine fibroids are a prevalent gynaecological condition in reproductive-aged women and are the commonest reason for hysterectomy. The cellular composition of clonal fibroids are heterogeneous, with phenotypically dissimilar cells that include smooth muscle cells (SMC), vascular SMC (VSMC) and fibroblasts. The aim of our study was to investigate genes that are commonly differentially expressed between fibroid and myometrial whole tissues in phenotypically different sub-populations of cells isolated from fibroid and myometrium. Genes to be investigated by fluorescence-activated cell sorting, quantitative real-time PCR and immunocytochemistry include transforming growth factor β (TGFB) and retinoic acid (RA) signalling families and steroid hormone receptors. We hypothesised that each cell population isolated from fibroid and myometrium would differ in the expression of fibroid-associated genes. We demonstrated that phenotypically different cellular constituents of uterine fibroids differentially express cellular RA-binding protein 2 (*CRABP2*), progesterone receptor B (*PRB*) and TGFB receptor 2 mRNA in fibroid-derived cells of VSMC and SMC phenotype. *CRABP2* mRNA was also differentially expressed in fibroblasts and VSMC sub-populations from within clonal fibroid tumours. We conclude that differential regulation of RA, TGFB and PR pathway transcription occurs in fibroid-associated SMC and -fibroblasts and that investigation of paracrine interactions between different cell types within the fibroid microenvironment provides an important new paradigm for understanding the pathophysiology of this common disease.

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Introduction

Uterine fibroids are benign tumours of the myometrium and are a prevalent gynaecological condition in reproductive-aged women (reviewed by Rice *et al.* (2012)). In Australia and globally, the commonest reason for hysterectomy is fibroids (Treloar *et al.* 1999, Rice *et al.* 2012). In the USA, uterine fibroids pose a significant cost to society, with annual direct and indirect healthcare costs estimated at up to US$34.4 billion (Cardozo *et al.* 2012). In spite of the high incidence, substantial health problems and significant economic burden, a lot remains to be learnt about the origins and pathogenesis of uterine fibroids.

In the vast majority of published studies, uterine fibroids are considered to be composed of entirely smooth muscle cells (SMC). Others and we have shown that in addition to SMC, phenotypically dissimilar cells make up a major component of uterine fibroids, including vascular SMC (VSMC) and fibroblasts (Koumas *et al.* 2001, Zaitseva *et al.* 2007, Moore *et al.* 2010, Holdsworth-Carson *et al.* 2014). It is known that the tumour microenvironment greatly impacts on tumour growth and is strongly influenced by paracrine signalling between several different interacting cells and their secretomes; critical in this process are fibroblasts (Elenbaas & Weinberg 2001). Members of the transforming growth factor β (TGFB) family participate in tumour microenvironment signalling, playing a major role in fibroblast-associated tumorigenesis. Members of the TGFB family can elicit tumour-promoting effects in a paracrine manner by triggering fibroblast differentiation and desmoplastic responses from neighbouring mesenchymal cells within a tumour (reviewed by Elenbaas & Weinberg (2001) and Polanska & Orimo (2013)). Activation of the retinoic acid (RA) pathway significantly reduces growth and extracellular matrix composition of uterine fibroid cells via regulation of the TGFB pathway (Malik *et al.* 2008). In fibroblasts,

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differential regulation can occur via the RA receptors (RAR), which are cell-type specific with distinctive receptor binding properties between different cell types (Delacroix *et al.* 2010), providing a mechanism for paracrine interaction in the tumour microenvironment. Uterine fibroids are hormonally regulated, as are cancerous tumours of the breast. Interactions between different cell types (fibroblasts and tumour cells) within breast tissues underlie oestrogen- and progesterone-dependent mechanisms of tumour cell proliferation (Haslam & Woodward 2003, Chottanapund *et al.* 2013). Therefore, the relationship and paracrine signalling between heterogeneous cells of uterine fibroids warrants consideration as a possible mechanism underlying fibroid progression, with particular attention paid to steroid hormone receptors, TGFB and RA pathways.

Despite recent evidence demonstrating cellular heterogeneity in uterine fibroids, these tumours are clonal (Holdsworth-Carson *et al.* 2014). Uterine fibroids are amplified from a single progenitor cell that differentiates into phenotypically different cells which in many ways mimic the surrounding myometrium. Previous comparisons of differential gene expression between fibroid and normal myometrium have been performed on heterogeneous cell populations from whole tissue or mixed cell cultures. While such investigations have uncovered significant pathways that distinguish fibroid from myometrium, the phenotypic heterogeneity of cells within the fibroid is not taken into consideration.

Studies describing differential gene expression between fibroid and myometrial whole tissues have repeatedly identified overlapping gene pathways that are abnormally expressed in fibroids, including genes important to the paracrine mechanisms within the local tumour microenvironment. Of relevance to this investigation, it is interesting to note that genes associated with steroid hormone regulation, the TGFB pathway and genes involved in the RA pathway make up a significant portion of genes that are differentially expressed in fibroid tissue compared with myometrium (refer to Table 1 for a brief summary; Sadan *et al.* 1987, Dou *et al.* 1996, Tsibris *et al.* 2002, Skubitz & Skubitz 2003, Weston *et al.* 2003, Shozu *et al.* 2004, Arslan *et al.* 2005, Leppert *et al.* 2006, Zaitseva *et al.* 2006, 2007, Roth *et al.* 2007, Litovkin *et al.* 2008, Lee *et al.* 2010, Moore *et al.* 2010, Davis *et al.* 2013, Yin *et al.* 2013).

The aim of this study was to investigate the genes that are differentially expressed between fibroid and myometrial whole tissues and have known roles in maintaining the tumour microenvironment, in phenotypically different sub-populations of cells isolated from fibroid and myometrium. Genes to be investigated belong to the steroid hormone receptor (oestrogen receptors (ERα/β) and progesterone receptors (PRA/B)), TGFB (TGFB1/3 and TGFB receptors (TGFBR1/2)) and RA signalling families (cellular RA-binding protein 2 (CRABP2)). We hypothesised that each cell population isolated from fibroid and myometrium will differ in the expression of fibroid-associated genes. Study of gene expression in each cellular constituent of fibroids relative to myometrium may provide important new insights into how paracrine interactions between different cell type(s) contribute to fibroid growth and development.

Materials and methods

Tissue collection

Paired myometrium and fibroid tissues were collected from women who were undergoing hysterectomy for fibroids ($n=13$, mean age 46.9 ± 1.1 years). Ethics approval for this study was obtained from the Royal Women's Hospital Human

Table 1 TGFB, steroid hormone and RA pathway genes differentially expressed in fibroid compared with myometrium tissues.

Pathway	Gene name	Increased/decreased in fibroid relative to myometrium	References
Transforming growth factor β	Transforming growth factor β (*TGFB1*)	↑/no change	Dou *et al.* (1996), Tsibris *et al.* (2002), Leppert *et al.* (2006), Moore *et al.* (2010) and Yin *et al.* (2013)
	TGFB3	↑	
	TGFB receptor 1 (*TGFBR1*)	↑	
	TGFBR2	↑/↓	
Steroid hormone	Oestrogen receptor α (*ESR1* or *ERα*)	↑	Shozu *et al.* (2004), Lee *et al.* (2010) and Yin *et al.* (2013)
	Oestrogen receptor β (*ESR2* or ERβ)	↑	
	Aromatase	↑	
	17b-Hydroxysteroid dehydrogenase	↑	
	Progesterone receptor (*PRG* or *PR*)	↑/no change	Sadan *et al.* (1987), Leppert *et al.* (2006), Zaitseva *et al.* (2006), Lee *et al.* (2010) and Yin *et al.* (2013)
Retinoic acid	Cellular retinoic acid-binding protein (*CRABP1* and *CRABP2*)	↑	Tsibris *et al.* (2002), Skubitz & Skubitz (2003), Arslan *et al.* (2005), Zaitseva *et al.* (2006, 2007) and Yin *et al.* (2013)
	Retinol dehydrogenase (*RODH*)	↑	
	Alcohol dehydrogenase 1 (*ADH1*)	↓	
	Aldehyde dehydrogenase (*ALDH1*)	↓	
	Cellular retinol-binding proteins (*CRBP1* and *CRBP2*)	↓	
	Retinoid X receptors (*RXRα* and *RXRγ*)	↓	

Research Ethics Committee and Southern Health Human Research Ethics Committee. Detailed clinical histories including menstrual cycle stage and menopause status were collected at the time of informed consent (Table 2). Women who received hormone treatment in the 3 months prior to surgery were excluded from the study.

Normal myometrium sample of at least 2 cm was taken from adjacent fibroid tissue. If more than one fibroid was present per uteri, a single sample of the largest visible fibroid was taken from the body. Patients were not stratified according to fibroid location (subserosal, submucosal or intramural); however, degenerative type fibroids (hyaline, calcification, cystic or red (haemorrhagic)) were excluded from the study. Tissues were collected in HEPES-buffered DMEM culture medium with 10% (v/v) fetal bovine serum (FBS) and antibiotic–antimycotic solution (all from Life Technologies).

Fluorescence-activated cell sorting

Single-cell suspensions of myometrium and fibroid ($n=13$ pairs) cells were established as described previously (Gargett *et al.* 2002, Zaitseva *et al.* 2006). Myometrium and fibroid cell were separated into independent cell populations using five colour fluorescence-activated cell sorting (FACS) as previously described (Holdsworth-Carson *et al.* 2014). Briefly, leucocytes, endothelial cells and dead cells were removed following identification with CD45, CD31 and 4′,6-diamidino-2-phenylindole (DAPI, for cell viability) respectively. Remaining cells were sorted based on CD90 positivity and aldehyde dehydrogenase 1 (ALDH1) using an ALDEFLUOR assay kit (StemCells Technologies, Tullamarine, VIC, Australia) and a MoFlo flow cytometer using Cyclops SUMMIT Software (version 4.1; Cytomation, Inc., Fort Collins, CO, USA). Cells were re-suspended in PBS/5% FBS (w/v) and incubated with allophycocyanin-conjugated CD90 (1:100 or 2 μg/ml, BD Biosciences, Franklin Lakes, NJ, USA), phycoerythrin cyanine 7 (PeCy7)-conjugated CD31 (1:100 or 2 μg/ml, BioLegend, San Diego, CA, USA) and PeCy5.5-conjugated CD45 (1:100, Life Technologies) for 45 min at 4 °C. Negative controls were stained with directly conjugated mouse IgG antibodies at equivalent concentrations. Cells were washed, re-suspended in ALDEFLUOR assay buffer and incubated with ALDH substrate BODIPY-aminoacetaldehyde for 45 min at 37 °C. Negative controls were pre-treated with diethylaminobenzaldehyde, a specific inhibitor of ALDH. Cells were pelleted and re-suspended in ALDEFLUOR assay buffer containing DAPI (1 μg/ml, Sigma–Aldrich) for FACS analysis. Cells were separated into three myometrium and four fibroid sub-populations based on their CD90 and ALDH positivity: $ALDH^-/CD90^-$ (A^-C^-) enriched for VSMC, $ALDH^-/CD90^+$ (A^-C^+) enriched for SMC, $ALDH^+/CD90^+$ (A^+C^+) enriched for fibroblasts and fibroid-specific $ALDH^-/CD90^{+bright}$ (A^-C^{+br}) fibroblast cells (Holdsworth-Carson *et al.* 2014).

Table 2 Patient information.

Patient	Age	Cycle stage	Menopause
1	50	Proliferative	Pre-
2	52	Proliferative	Pre-
3	46	Proliferative	Pre-
4	46	Proliferative	Pre-
5	51	Proliferative	Pre-
6	49	Proliferative	Pre-
7	44	Proliferative	Pre-
8	45	Secretory	Pre-
9	47	Secretory	Pre-
10	46	Shedding	Pre-
11	38	Inactive	Pre-
12	43	Inactive	Post-
13	52	Inactive	Post-

RNA extraction, cDNA synthesis and qRT-PCR

RNA was extracted from sorted cell sub-populations using the AllPrep DNA/RNA Micro Kit (with on-column DNase treatment; Qiagen) following the manufacturer's instructions. The number of cells ranged from 2.2×10^5 to 1.2×10^6 cells/sub-population. RNA quality and concentration was assessed using a Nanodrop u.v. spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA (5–50 ng/sample) was converted to cDNA using VILO cDNA synthesis kit (Life Technologies), diluted in DEPC-treated water (1:20) and stored at −80 °C.

All qRT-PCR experiments were performed using a ViiA7 real-time PCR instrument (384-well format; Life Technologies), Universal Probe (Roche) or TaqMan (Life Technologies) assays. The list of the primers used in this study and their average C_t values are summarised in Table 3. Four microlitres of diluted cDNA (final dilution 1:50) was used per 10 μl qRT-PCR using FastStart Universal Probe Master (Roche), in duplicate. Primers were used at 500 nM and probes at 200 nM/reaction. Amplification was performed for 40 cycles with annealing temperatures of 60 °C for all primers. Relative quantification was performed using the comparative C_t method ($2^{-\Delta\Delta C_t}$), with average C_t values of human β-2-microglobulin (*B2M*; VIC/MGB Probe, Life Technologies, catalogue number 4326319E) and ribosomal protein L13a (*RPL13A2*) used as an endogenous control to correct for differences in concentration of the starting template (see Table 4 for the relevant n numbers for each gene and each cell population). *B2M* and *RPL13A2* were chosen as housekeeping genes for this study as they were stably expressed between myometrium and fibroid cell sub-populations and demonstrated minimal variability (data not shown). The ratio of *PR*/*PRB* was calculated to represent the relative expression level of *PRA* (Vladic-Stjernholm *et al.* 2009).

Note that the expression of *ERβ* mRNA was low (Table 3); we were unable to amplify *ERβ* in >25% of samples. It has been reported previously that endothelial cells, not SMC, express *ERβ* in myometrium and fibroids (Gargett *et al.* 2002). Owing to our FACS protocol, ERβ-expressing endothelial cells were removed. Others have also had difficulties in detecting ERβ in fibroids (Yin *et al.* 2013).

Immunocytochemistry and image analysis

Selected sorted cell sub-populations were air-dried on frosted slides (sorted cells from $n=6$ paired myometrium and fibroid tissues). Cells were fixed with chilled acetone for 2 min and stored at −20 °C until ready for use. Cells were washed with PBS prior to blocking endogenous peroxidases with 0.3% (v/v)

Table 3 Primer sequences and mean C_t values.

Gene	Primer sequence	Universal probe library	Mean C_t
ESR1 (ERα)	F: ATCCACCTGATGGCCAAG R: GCTCCATGCCTTTGTTACTCA	#017	29.01
ESR2 (ERβ)	F: ATGATGGGGCTGATGTGG R: TTCTACGCATTTCCCCTCA	#050	34.50
PGR (PR)	F: TTTAAGAGGGCAATGGAAGG R: CGGATTTTATCAACGATGCAG	#011	29.72
PGR (PRB)	F: GACTGAGCTGAAGGCAAAGG R: AAACTTCAGGCAAGGTGTCC	#036	31.97
TGFB1	F: ACTACTACGCCAAGGAGGTCAC R: TGCTTGAACTTGTCATAGATTTCG	#031	28.91
TGFB3	F: GGGCTTTGGACACCAATTAC R: GGCATAGTAGCCCTTAGGTTCA	#011	29.09
TGFBR1	F: AAATTGCTCGACGATGTTCC R: CATAATAAGGCAGTTGGTAATCTTCA	#031	29.19
TGFBR2	F: TGTGGATGACCTGGCTAACA R: CTCCAAATTCATCCTGGATTCT	#050	29.41
CRABP2	F: GGGTCTCTCTAAAGGGGACTTG R: GGCTACAGGGACAAAGGGTAG	#015	29.21
RPL13A	F: TGACCAATAGGAAGAGCAACC R: AGATGCCCCACTCACAAGAT	#036	27.48

H_2O_2 in methanol (10 min at room temperature (RT)) and then serum-free protein block (Dako, Glostop, Denmark) (10 min, RT). The cells were incubated with anti-goat CRABP2 antibody (Santa Cruz Biotechnology, Inc.) diluted 1:500 (0.4 μg/ml) in 1% (w/v) BSA/PBS overnight at 4 °C. Incubation with goat IgG (Santa Cruz Biotechnology, Inc.; diluted to 0.4 μg/ml) was also performed as a negative control. The following day, cells were incubated with biotinylated anti-goat secondary antibody 1:2000 (Life Technologies; 30 min, RT). CRABP2 was visualised following incubation with ABC Vectastain Kit (Vector Laboratories, Burlingame, CA, USA; 5 min, RT) followed by 3,30′-diaminobenzidine (DAB; Sigma–Aldrich; 5 min, RT). Cells were then briefly counterstained in Harris haematoxylin (Amber Scientific, Midvale, WA, Australia).

Images were observed using a Zeiss Axioskop light microscope, AxioCam ICc 3 Zeiss camera and AxioVision System Software (release 4.6; Carl Zeiss Imaging Solutions, Munchen, Germany). Image analysis was performed using the ImmunoRatio automated image analysis program (Tuominen *et al*. 2010) as a plugin in Fiji (ImageJ; http://fiji.sc/How_to_cite_Fiji%3F) (Schindelin *et al*. 2012). Three fields of view per sorted cell sub-population were analysed. Data are described as percentage of DAB staining/total cell area.

Statistical analysis

Statistical analysis was performed using SPSS (version 20, SPSS, Inc.). Analysis of qRT-PCR data was performed using non-parametric Kruskal–Wallis and Mann–Whitney *U* tests ($P<0.05$). To reduce the chance of obtaining false-positive results as a consequence of multiple pair-wise tests, a Bonferroni's correction was also applied so that any comparison was only considered significant if $P<0.0125$. CRABP2 protein expression analysis data were normally distributed and analysed by *t*-tests ($P<0.05$).

Results

Three sub-populations of phenotypically different cells were compared between myometrial and fibroid isolates: A^-C^- VSMC (Fig. 1), A^-C^+ SMC (Fig. 2) and A^+C^+ fibroblasts (Fig. 3). Although multiple phenotypically different cell types exist in fibroids, these cells are clonal in origin (Holdsworth-Carson *et al*. 2014). Therefore, a comparison of the genes of interest between the four clonal sub-populations from fibroids (A^-C^-, A^-C^+, A^+C^+ and A^-C^{+br}) was also performed (Fig. 4).

Table 4 Number of patients included in qRT-PCR analysis.

	Number of patients included in qRT-PCR analysis						
	Myometrium derived			Fibroid derived			
	A^-C^-	A^-C^+	A^+C^+	A^-C^-	A^-C^+	A^+C^+	A^-C^+br
ESR1 (ERα)	13	13	13	13	13	12	12
ESR2 (ERβ)	9	9	12	10	11	8	8
PGR (PR)	12	13	13	13	13	12	12
PGR (PRB)	13	13	13	13	13	12	12
TGFB1	13	13	13	13	13	12	12
TGFB3	13	13	13	13	13	12	12
TGFBR1	13	13	13	13	13	12	13
TGFBR2	13	13	13	13	13	12	12
CRABP2	13	13	13	13	13	12	12
RPL13A	12	13	13	13	13	12	12

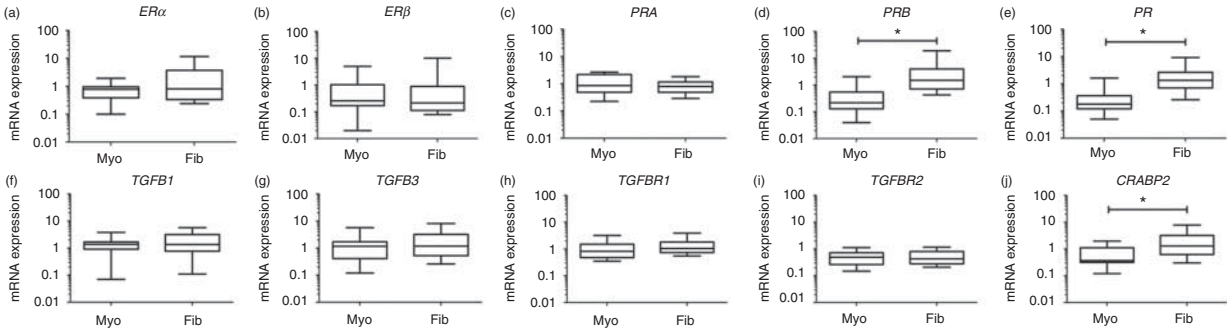

Figure 1 Gene expression in the A^-C^- vascular smooth muscle cell sub-population derived from paired myometrium (Myo) and fibroid (Fib). Relative mRNA expression is displayed for (a) *ERα*, (b) *ERβ*, (c) *PRA* (ratio of *PR:PRB*), (d) *PRB*, (e) *PR*, (f) *TGFB1*, (g) *TGFB3*, (h) *TGFBR1*, (i) *TGFBR2* and (j) *CRABP2*. Data were analysed by non-parametric Kruskal–Wallis and Mann–Whitney *U* tests, with significant difference denoted by *($P<0.0125$).

The same genes demonstrated differential mRNA expression between myometrium-derived cell populations and fibroid-derived cell populations. *PRB* and *CRABP2* mRNA were increased in fibroid-derived A^-C^- VSMC compared with myometrium-derived cells (*PRB*, $P=0.001$ and *CRABP2*, $P=0.004$; Fig. 1d and j). Similarly, *PRB* and *CRABP2* mRNA expression in A^-C^+ SMC from fibroid origin was increased relative to those derived from myometrium (*PRB*, $P=0.001$ and *CRABP2*, $P=0.026$; Fig. 2d and j). *PR* mRNA (which represents total *PRA* and *PRB* mRNA, Vladic-Stjernholm *et al.* (2009)) was similarly increased in fibroid-derived A^-C^- VSMC ($P=0.001$) (Fig. 1e) and A^-C^+ SMC ($P=0.003$; Fig. 2e) relative to myometrial cell populations. When comparing the clonal fibroid cell sub-populations with each other, *CRABP2* mRNA was significantly increased in the A^+C^+ fibroblast population compared with both A^-C^{+br} fibroblasts ($P=0.003$) and A^-C^- VSMC ($P=0.024$) (Fig. 4j).

Of the TGFB pathway genes investigated, only *TGFBR2* mRNA expression in myometrium-derived A^-C^+ SMC increased significantly when compared with fibroid A^-C^+ SMC ($P=0.029$; Fig. 2i). Expression of *TGFB1*, *TGFB3*, *TGFBR1*, *ERα*, *ERβ* and *PRA* mRNA was not different between any of the cell sub-populations isolated from myometrium or fibroid (Figs 1, 2, 3 and 4). None of the genes examined demonstrated significant changes in expression between myometrium and fibroid in A^+C^+ fibroblasts (Fig. 3).

As demonstrated in Table 2, the patients included in this study were of mixed endometrial stage (different menstrual cycle stages and menopausal status). When qRT-PCR data were stratified into more uniform groups, for example, inclusion of only proliferative phase women ($n=7$) or only pre-menopausal women ($n=11$), the differential expression of *CRABP2*, *PRB* (*PR*) and *TGFBR2* mRNA remained statistically significant (data not shown).

CRABP2 mRNA gene expression was most commonly altered between isolated cell sub-populations; therefore, CRABP2 immunocytochemistry was performed to determine whether altered expression extended to CRABP2 protein expression. Fibroid-associated A^-C^- VSMC (Fig. 5b) and A^-C^+ SMC (Fig. 5e) sub-populations displayed stronger CRABP2 immunostaining compared with their respective myometrium-derived cell populations (Fig. 5a and d). This was confirmed by quantitative image analysis, where the percentage of CRABP2-positive A^-C^- cells was significantly increased in fibroid populations compared with myometrial A^-C^- cells ($P=0.001$; Fig. 5c). Similarly, the

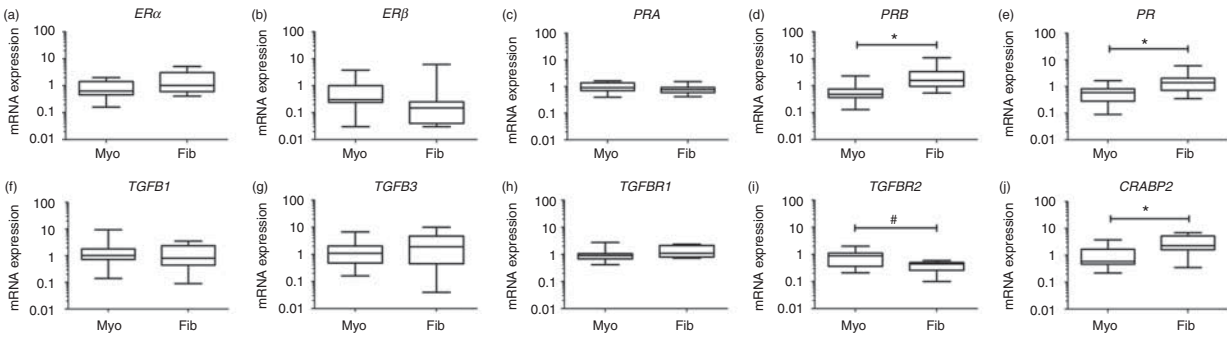

Figure 2 Gene expression in the A^-C^+ smooth muscle cell sub-population derived from paired myometrium (Myo) and fibroid (Fib). Relative mRNA expression is displayed for (a) *ERα*, (b) *ERβ*, (c) *PRA* (ratio of *PR:PRB*), (d) *PRB*, (e) *PR*, (f) *TGFB1*, (g) *TGFB3*, (h) *TGFBR1*, (i) *TGFBR2* and (j) *CRABP2*. Data were analysed by non-parametric Kruskal–Wallis and Mann–Whitney *U* tests, with significant difference denoted by *($P<0.0125$) and $^{\#}$($P<0.05$).

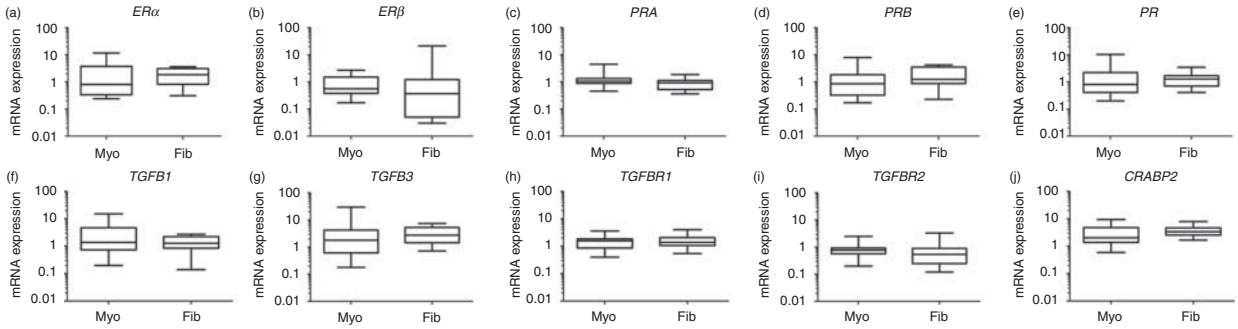

Figure 3 Gene expression in the A^+C^+ fibroblast cell sub-population derived from paired myometrium (Myo) and fibroid (Fib). Relative mRNA expression is displayed for (a) *ERα*, (b) *ERβ*, (c) *PRA* (ratio of *PR*:*PRB*), (d) *PRB*, (e) *PR*, (f) *TGFB1*, (g) *TGFB3*, (h) *TGFBR1*, (i) *TGFBR2* and (j) *CRABP2*. Data were analysed by non-parametric Kruskal–Wallis and Mann–Whitney *U* tests.

percentage of CRABP2-positive A^-C^+ SMC was significantly increased in fibroid populations relative to myometrium A^-C^+ SMC ($P=0.010$; Fig. 5f). In contrast, fibroid-derived fibroblast cell populations demonstrated greater variability in CRABP2 protein expression (Fig. 5g and h) and consequently did not differ significantly between A^+C^+ and A^-C^{+br} cell sub-populations ($P=0.227$; Fig. 5i).

Discussion

Fibroblasts are an integral cellular component of human uterine fibroids and myometrium (Koumas *et al*. 2001, Zaitseva *et al*. 2007, Moore *et al*. 2010, Holdsworth-Carson *et al*. 2014). Although fibroids are clonal, significant cellular heterogeneity exists (Holdsworth-Carson *et al*. 2014). This is the first study to recognise the heterogeneous nature of uterine fibroid and myometrial cell phenotypes and sort tissues into specific cell sub-populations (A^-C^- VSMC, A^-C^+ SMC, A^+C^+ fibroblasts and A^-C^{+br} fibroid fibroblast) prior to examining genes known to be differentially expressed in fibroids and fibroblast-associated tumorigenesis. We hypothesised that cell populations isolated from fibroid and myometrium would differ in the expression of fibroid- and fibroblast-associated genes. *CRABP2*, *PRB*

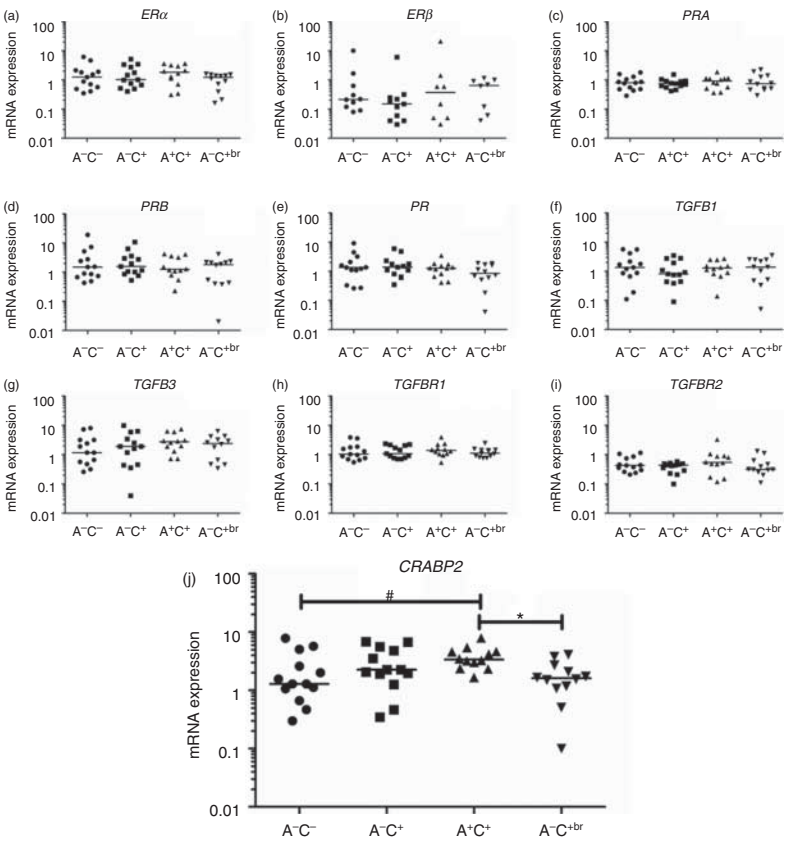

Figure 4 A comparison of the gene expression in fibroid-derived A^-C^-, A^-C^+, A^+C^+ and A^-C^{+br} cell sub-populations. Relative mRNA expression is displayed for (a) *ERα*, (b) *ERβ*, (c) *PRA* (ratio of *PR*:*PRB*), (d) *PRB*, (e) *PR*, (f) *TGFB1*, (g) *TGFB3*, (h) *TGFBR1*, (i) *TGFBR2* and (j) *CRABP2*. Data are displayed as scatter plots with medians. Data were analysed by non-parametric Kruskal–Wallis and Mann–Whitney *U* tests, with significant difference denoted by $^*(P<0.0125)$ or $^{\#}(P<0.05)$.

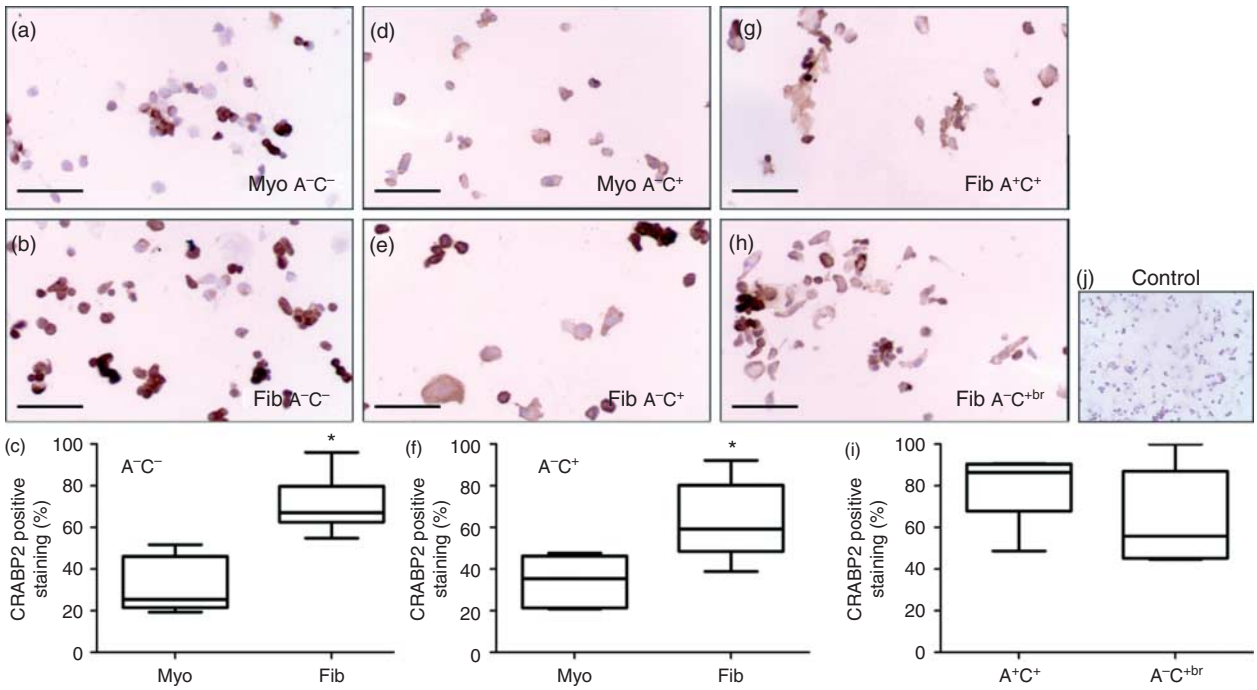

Figure 5 CRABP2 immunocytochemistry on A^-C^-, A^-C^+, A^+C^+ and A^-C^{+br} sub-populations derived from paired myometrium (Myo) and fibroid (Fib) ($n=6$ patients). Images show representative CRABP2 (brown) staining for (a) Myo A^-C^- and (b) Fib A^-C^-, (d) Myo A^-C^+ and (e) Fib A^-C^+, and (g) Fib A^+C^+ and (h) Fib A^-C^{+br}. Scale bar = 50 μm. Isotype negative control (j). CRABP2 image analysis quantification was performed on (c) Myo (pink) vs Fib (purple) A^-C^- vascular smooth muscle cells, (f) Myo (pink) vs Fib (purple) A^-C^+ smooth muscle cells and (i) Fib A^+C^+ (purple) vs Fib A^-C^{+br} (pale purple) fibroblasts cells. Data were analysed by *t*-test, with significant difference denoted by *($P<0.0125$).

(and *PR*) and *TGFBR2* gene expression was found to be different between fibroid- and myometrium-derived cell populations. *CRABP2* gene expression was also altered between clonal fibroid sub-populations, and these differences were confirmed at the protein level.

Fibroid development, symptomology, demographics and response to treatment is heterogeneous (Peddada *et al*. 2008, Davis *et al*. 2009, Zhao & Rogers 2013). This is reflected in our sample group (Table 1). We hypothesised that expression of genes known to be altered in association with fibroids would also be altered in different sub-populations of cells isolated from fibroid and myometrial tissues. This hypothesis was upheld, even in our relatively small and mixed patient population. Even when data were stratified using smaller, more homogeneous patient sets (for example, proliferative phase only, or pre-menopausal only), the differential gene expression remained statistically significant. This finding demonstrates that despite the heterogeneous phenotype of uterine fibroids, common gene pathways are dysregulated between phenotypically dissimilar cellular components of fibroids.

The RA pathway has pleiotropic effects in various processes, including cell growth, differentiation and apoptosis. CRABP2 binds with and delivers all-*trans*-RA (ATRA) to the nucleus where it then binds and activates retinoid receptors (RAR and RXR) (reviewed by Rhinn & Dollé (2012)). *CRABP2* expression has been shown to be increased in whole fibroid tissues relative to myometrial tissue (Tsibris *et al*. 2002, Arslan *et al*. 2005, Zaitseva *et al*. 2006, 2007). In this study, we demonstrate that fibroid-derived A^-C^- VSMC and A^-C^+ SMC similarly express increased levels of *CRABP2* mRNA and protein relative to their myometrium-derived equivalents. Elevated *CRABP1* and *CRABP2* mRNA expression is associated with myoblast differentiation *in vivo* and *in vitro*, implicating an important role for CRABPs in myogenesis (Tang *et al*. 2007, Jing *et al*. 2013). As for fibroid research, the concept of cellular heterogeneity and differential SMC-subset modulation is also recognised in the field of atherosclerosis (Seidel 1997, Neuville *et al*. 1999). In rats, two phenotypically distinct arterial SMC populations behave differently in response to ATRA and/or RAR/RXR agonists (Neuville *et al*. 1999). Expression of CRABP2 is associated with cells that metabolise large quantities of RA, indicating that cells with high RA requirements increase both RA synthesis and the level of CRABP2 expression, thus providing efficient delivery of RA to its cognate receptors (Noy 2000). Interestingly, extracts from human tissue show more than fourfold higher ATRA levels in uterine fibroids compared with myometrium (Tsibris *et al*. 1999); however, others have observed reduced ATRA concentrations in fibroids (Catherino & Malik 2007). ATRA has also been shown to regulate downstream transcriptional pathways in myometrial mixed cultures (Zaitseva *et al*. 2013).

RA-mediated apoptosis signalling has been identified as a canonical pathway involved with differential fibroid growth (Davis *et al*. 2013). RA signalling can also promote cell survival and proliferation via anti-apoptotic mechanisms; however, these pro-survival properties of RA are unlikely to be mediated by its cognate RAR and instead an alternative receptor, peroxisome proliferator-activated receptor β/δ, may be employed (Noy 2010). It is therefore hypothesised that fibroid VSMC and SMC act independently of myometrial sub-populations, dysregulating the RA signalling pathway via abnormal *CRABP2* expression, thus contributing to fibroid progression.

We also identified that *CRABP2* gene expression was altered in clonal sub-populations derived from within the fibroid itself, being elevated in A^+C^+ fibroblasts relative to A^-C^{+br} fibroblasts and A^-C^- VSMC. We have previously hypothesised that the four sub-populations identified in uterine fibroids are clonally amplified from a progenitor cell that differentiates into SMC, VSMC and fibroblast cells (Holdsworth-Carson *et al*. 2014). Clonal pluripotent cells, including human embryonal carcinoma NT2 cells, have the capacity to differentiate into several cell types. NT2 cells induced to differentiate with RA demonstrate increased *CRABP2* expression and it is suggested that *CRABP2* up-regulation correlates with increased RA sensitivity during differentiation (Borghi *et al*. 2003). Studies on melanoma have identified that these tumours induce restricted production of RA within the tumour microenvironment (with much higher RA concentration compared with surrounding tissues; Guo *et al*. 2012). Interestingly, only selective cells within this microenvironment were responsible for ATRA accumulation (Guo *et al*. 2012). We suggest that within the fibroid microenvironment, A^+C^+ fibroblasts are responsible for primary aberrant RA signalling via up-regulated *CRABP2* expression, and subsequent paracrine actions of A^+C^+ fibroblasts go on to mediate cell survival, proliferation and fibroid growth.

There is significant evidence in the literature supporting a progesterone- and TGFB-mediated role in the proliferation of uterine fibroids (reviewed by Rein *et al*. (1995), Ciarmela *et al*. (2011) and Kim & Sefton (2012)). *TGFBR2* gene expression was decreased in fibroid-derived A^-C^+ SMC relative to myometrium. This is in contrast to whole tissue data, where *TFGBR2* expression is increased in fibroid tissues compared with myometrial tissues (Dou *et al*. 1996). However, studies of total or partial knockout of *Tgfbr2* in mice have associated TGFBR2 loss with enhanced mammary tumour progression (Fang *et al*. 2011). Up to 70% of uterine fibroids carry a mutation in the mediator complex subunit 12 (*Med12*) gene on chromosome Xq13.1 (Mäkinen *et al*. 2011). Since individual cell populations derived from fibroids are clonal (Holdsworth-Carson *et al*. 2014), all *Med12* mutation-positive cell populations should carry the mutation. MED12 makes up part of the mediator complex and additionally interacts with several proteins including β-catenin (Wnt signalling) and GLI3 (Sonic hedgehog signalling), which others and we have identified in association with uterine fibroids (Tanwar *et al*. 2009, Mäkinen *et al*. 2011, Zaitseva *et al*. 2013). Recent studies have shown that MED12 interacts with and up-regulates TGFBR2 and TGFB signalling (Huang *et al*. 2012). Although we did not screen our samples for *Med12* mutations, the reduction in TGFBR2 expression in our A^-C^+ SMC fibroid subpopulation may reflect an inherent loss of MED12 functionality as a consequence of this common fibroid-associated gene mutation.

PRB (and *PR*) gene expression was increased in A^-C^- VSMC and A^-C^+ SMC derived from fibroid tissues compared with myometrium. Previously, studies have reported an increase in *PR* in association with whole fibroid tissues (Sadan *et al*. 1987, Lee *et al*. 2010). Elevated *PR* expression in VSCM and SMC may have anti-apoptotic properties, whereby progesterone plays a role in triggering BCL2 protein expression in human uterine fibroid cultures (Matsuo *et al*. 1997). Similar to the myometrium, breast and endometrial tissues also display cellular heterogeneity, and in these tissues, PR expression has been demonstrated to be cell-type specific (Snijders *et al*. 1992, Shyamala *et al*. 1997, Mote *et al*. 2000). In both breast and endometrium, PR is regulated by, and functions via, paracrine mechanisms in sub-sets of cells (typically epithelial; Brisken *et al*. 1998, Kurita *et al*. 2000). Emphasising the mitotic proliferative effects of PR on uterine fibroid growth, therapeutic PR antagonists including ulipristal acetate have been demonstrated to reduce fibroid size, bleeding and pain/discomfort in well-controlled pilot studies (Donnez *et al*. 2012). A^-C^+ SMC and A^-C^- VSMC sub-populations respectively are the largest populations of cells isolated from uterine fibroids (Holdsworth-Carson *et al*. 2014). Inhibition of VSMC and SMC proliferation potential by antiprogestins is a plausible approach for controlling PR effects and thus ameliorating fibroid growth.

In conclusion, we have demonstrated that phenotypically different cellular constituents of uterine fibroids differentially express genes with known involvement in fibroid pathophysiology. In particular, *CRABP2*, a component of the RA differentiation pathway, and *PRB* demonstrated altered gene expression in fibroid-derived cells of VSMC and SMC phenotype. Despite the inherent heterogeneity in our sample group, these genes demonstrated significant differences in expression in association with fibroid origin. *CRABP2* is also a potential mediator of differentiation of fibroblasts and VSMC sub-populations within clonal fibroid tumours. It is possible that RA and PR-regulated transcription may control the expression of secretory factors in the tumour microenvironment by fibroid-associated SMC and fibroblasts and affect cell proliferation through paracrine mechanisms. We conclude that differential regulation of RA, TGFB and PR pathway transcription occurs in

fibroid-associated SMC and fibroblasts and that investigation of paracrine interactions between different cell types within fibroids provides an important new paradigm for investigating the pathophysiology of this common disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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