Aberrant expression and regulation of NR2F2 and CTNNB1 in uterine fibroids

Marina Zaitseva, Sarah J Holdsworth-Carson, Luke Waldrip¹, Julia Nevzorova, Luciano Martelotto², Beverley J Vollenhoven^{1,3} and Peter A W Rogers

Department of Obstetrics and Gynaecology, Royal Women's Hospital, University of Melbourne, Level 7, 20 Flemington Road, Parkville, Victoria 3052, Australia, ¹Department of Obstetrics and Gynaecology, Monash Medical Centre, Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia, ²Department of Pathology, Memorial Sloan-Kettering Cancer Center, Memorial Hospital, 1275 York Avenue, New York, New York 10065, USA and ³Monash Institute of Medical Research, Monash University, 27-31 Wright Street, Clayton, Victoria 3168, Australia

Correspondence should be addressed to P A W Rogers; Email: parogers@unimelb.edu.au

Abstract

Uterine fibroids are the most common benign tumour afflicting women of reproductive age. Despite the large healthcare burden caused by fibroids, there is only limited understanding of the molecular mechanisms that drive fibroid pathophysiology. Although a large number of genes are differentially expressed in fibroids compared with myometrium, it is likely that most of these differences are a consequence of the fibroid presence and are not causal. The aim of this study was to investigate the expression and regulation of NR2F2 and CTNNB1 based on their potential causal role in uterine fibroid pathophysiology. We used real-time quantitative RT-PCR, western blotting and immunohistochemistry to describe the expression of NR2F2 and CTNNB1 in matched human uterine fibroid and myometrial tissues. Primary myometrial and fibroid smooth muscle cell cultures were treated with progesterone and/or retinoic acid (RA) and sonic hedgehog (SHH) conditioned media to investigate regulatory pathways for these proteins. We showed that NR2F2 and CTNNB1 are aberrantly expressed in fibroid tissue compared with matched myometrium, with strong blood vessel-specific localisation. Although the SHH pathway was shown to be active in myometrial and fibroid primary cultures, it did not regulate NR2F2 or CTNNB1 mRNA expression. However, progesterone and RA combined regulated NR2F2 mRNA, but not CTNNB1, in myometrial but not fibroid primary cultures. In conclusion, we demonstrate aberrant expression and regulation of NR2F2 and CTNNB1 in uterine fibroids compared with normal myometrium, consistent with the hypothesis that these factors may play a causal role uterine fibroid development.

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Introduction

Uterine fibroids, or leiomyoma, are the most common benign tumour afflicting women of reproductive age (Okolo 2008, Wechter *et al.* 2011). While many women with uterine fibroids are asymptomatic, 35–50% of women experience fibroid-related symptoms, including heavy, painful periods, anaemia, frequent urination, abdominal swelling, pressure pains and pregnancy complications (Day Baird *et al.* 2003). Currently, uterine fibroids are the single-most common reason for hysterectomy in developed countries (Treloar *et al.* 1999, Farquhar & Steiner 2002, Garry 2005), and despite their large healthcare burden, there is limited understanding of the molecular mechanisms that drive fibroid pathophysiology. Gene profiling studies by our laboratory and others have identified a large number of genes with altered expression in fibroid tumours compared with myometrium (Tsibris *et al.* 2002, Skubitz & Skubitz 2003,

Arslan *et al.* 2005, Luo *et al.* 2005a, 2005b, Zaitseva *et al.* 2006, Dimitrova *et al.* 2009). However, it is hypothesised that the majority of these genes are differentially expressed as a consequence of the changed microenvironment caused by the fibroids' presence and are not causal to fibroid development.

This study focuses on two genes: CTNNB1 (β -catenin) and chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, known as NR2F2). Our previous studies report that CTNNB1 mRNA expression is increased in fibroids; conversely, NR2F2 mRNA is decreased in association with fibroids (Zaitseva *et al.* 2006, 2008). Importantly, circumstantial evidence exists, which supports a potential causal role for NR2F2 and CTNNB1 in fibroid development. CTNNB1 plays a structural role in cadherin junction cell–cell adhesion and is also an essential transcriptional co-activator component of the canonical Wnt signalling pathway. In uterine biology, CTNNB1 plays roles in menstruation

(Nei *et al.* 1999) and implantation (Mohamed *et al.* 2005, Jeong *et al.* 2009) and has been linked to endometrial hyperplasia and cancer (Fukuchi *et al.* 1998, Nei *et al.* 1999, Jeong & McMahon 2005). Suggestive of a causative role in uterine fibroid development is the observation that sustained *Ctnnb1* expression in transgenic mice results in uterine mesenchymal tumours, which are histologically similar to human uterine leiomyoma (Tanwar *et al.* 2009). *NR2F2* belongs to the steroid/thyroid hormone receptor superfamily and plays important roles in development, including cell differentiation, cell cycle and migration, and angiogenesis (see review Boudot *et al.* (2011)). It has been suggested that *NR2F2* has a role in cancer development and epithelial–mesenchymal–transition (EMT; Boudot *et al.* 2011). *NR2F2* is expressed in the endometrium and myometrium (Chu *et al.* 1998, Takamoto *et al.* 2005) where it is involved in regulation of implantation/decidualisation (Takamoto *et al.* 2005, Kurihara *et al.* 2007, Lee *et al.* 2010) and placentation (Petit *et al.* 2007, Hubert *et al.* 2010). Conditional knockout of *Nr2f2* in mouse uteri results in stunted uterine growth and disorganised myometrial smooth muscle layers (Petit *et al.* 2007). Therefore, based on this cumulative evidence, the first aim of this study was to more fully define the expression profiles and localisation of *CTNNB1* and *NR2F2* in human uterine fibroid and myometrial tissues.

CTNNB1 and *NR2F2* share many common regulatory factors; with particular relevance to uterine fibroids are retinoic acid (RA), progesterone and sonic hedgehog (SHH) ligands. RA has implications in uterine fibroid pathology (Arslan *et al.* 2005, Zaitseva *et al.* 2007), and *CTNNB1* is a RA-responsive gene (Byers *et al.* 1996). By interacting with RA receptor (RAR) and retinoid X receptor (RXR), *NR2F2* negatively regulates RA signalling (reviewed by Boudot *et al.* (2011)). Progesterone regulates Wnt/*CTNNB1* signalling in the uterus via the Wnt inhibitor Dickkopf-1 (DKK1; Tulac *et al.* 2006, Wang *et al.* 2010). Acting through its receptor (PR), progesterone increases uterine expression of HH protein, which in turn regulates *NR2F2* expression (Lee *et al.* 2006b, Kurihara *et al.* 2007, Simon *et al.* 2009a). HH is crucial to normal and abnormal uterine (and endometrial) development and is also regulated by progesterone (Feng *et al.* 2007, Kim *et al.* 2009, Wei *et al.* 2010, Franco & Yao 2012). Currently, however, data are lacking on the potential roles of progesterone, RA and/or SHH regulatory molecules in controlling *CTNNB1* and/or *NR2F2* in human myometrium and fibroid tissues. Thus, the second aim of this study was to investigate *in vitro* regulation of *CTNNB1* and *NR2F2* in human myometrial and fibroid primary cells by progesterone, RA and SHH.

Epidemiological data shows that uterine fibroids are one of the most significant gynaecological conditions affecting reproductive-aged women today

(Wechter *et al.* 2011). We hypothesise that *CTNNB1* and *NR2F2* have causal roles in the development of uterine fibroids. This study investigated the expression and regulation of *CTNNB1* and *NR2F2* in uterine fibroids and myometrium with the overall goal to achieve a better understanding of the molecular mechanisms that drive fibroid initiation and growth.

Materials and Methods

Tissue collection

Human myometrial and fibroid tissues were obtained from premenopausal women who had not been on hormonal therapy for at least 3 months before collection and were undergoing hysterectomy for fibroids ($n=57$, mean age 46.4, range 35–56 years). Informed consent was obtained from each patient with approval from the Royal Women's Hospital Human Research Ethics Committee or Monash Medical Centre Human Research Ethics Committee. Detailed clinical menstrual history was obtained at the time of consent and cycle stage was confirmed by pathology (Noyes *et al.* 1950). Patient endometrium was classified as proliferative ($n=31$), secretory ($n=23$) or inactive ($n=2$). Normal myometrium was taken at least 2 cm from adjacent fibroid tissue. When more than one fibroid was identified in a uterus, a sample was taken from the body of the largest fibroid. Patients were not stratified according to fibroid location (subserosal, submucosal or intramural); however, degenerative-type fibroids (hyaline, calcification, cystic or red (hemorrhagic)) were excluded from the study. Myometrial and fibroid tissues were immediately snap frozen and stored at -80°C , fixed in formalin or collected in HEPES-buffered M199-culture medium with 10% (v/v) FCS and antibiotic–antimycotic liquid (all reagents from Life Technologies) and stored overnight at 4°C before being processed for primary cell culture.

RNA extraction and RT-qPCR

RNA was extracted from primary cells or frozen tissue ($n=10$ proliferative and $n=10$ secretory myometrial and fibroid pairs) in 1 ml TRIzol reagent followed by DNase treatment as per manufacturer's instructions (both from Life Technologies). Frozen tissue samples were homogenised using a PowerLyzer 24 bench top bead-based homogeniser for 3×35 s bursts (MO BIO Laboratories, Inc., Carlsbad, CA, USA). RNA was ethanol precipitated, resuspended in RNase-free water and stored at -80°C . Concentration and quality of RNA was assessed with a Nanodrop u.v. spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia) using an absorbance ratio of 260:280 nm ($A_{260:280}$).

RNA was converted to cDNA using High Capacity cDNA RT Kit with RNase inhibitor according to the manufacturer's instructions (Life Technologies). Briefly, 1 μg total RNA was incubated with 4 mM dNTPs, 2 μl random primers, 2 μl $10\times$ RT buffer, 20 U RNase inhibitor and 50 U MultiScribe Reverse Transcriptase for 10 min at 25°C , followed by 120 min at 37°C and 5 min at 85°C in 20 μl volume. All RT-qPCR experiments were performed using a LightCycler 480 real-time PCR machine (Roche), LightCycler 480 Probe Master (Roche)

using TaqMan (Life Technologies) or Universal Probe (Roche) assays (see Table 1 for primer details). Relative quantification was performed using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001), with 18S rRNA used as an endogenous control to correct for differences in concentration of the starting template.

Protein extraction and western blotting

Whole cell protein was extracted from frozen myometrium and fibroid tissue ($n=10$ proliferative and $n=10$ secretory myometrial and fibroid pairs) via homogenisation in RIPA buffer plus phosphatase inhibitor cocktail 2 (1:200 dilution) and protease inhibitor cocktail (1:1000 dilution) (all from Sigma–Aldrich). Tissue homogenisation conditions are same as described earlier. Protein concentrations were determined using the Pierce BCA Protein Assay following the manufacturer's instructions (Thermo Fisher Scientific).

Protein samples (20 µg) were resolved on 10% NuPAGE Bis–Tris polyacrylamide gels (Life Technologies) at 150 V for 60 min and transferred onto nitrocellulose membrane (Bio-Rad Laboratories) at 30 V for 60 min using Life Technologies's XCell II blot module. Membranes were blocked with 5% (w/v) skim milk powder in Tris-buffered saline plus 0.1% v/v Tween 20 (TBS-T) for 1 h at RT. Primary antibody incubations occurred at 4 °C overnight in 2% (w/v) skim milk powder in TBS-T. Dilutions were 1:1000 for mouse monoclonal anti-NR2F2 (Clone H7147) (Abcam, Cambridge, UK) and mouse monoclonal anti-CTNNB1 (Clone 14; BD Biosciences, Sparks, MD, USA) and 1:10 000 for mouse monoclonal anti-β-actin (Sigma–Aldrich). HRP-conjugated goat anti-mouse secondary antibody (Life Technologies) was diluted 1:4000 for NR2F2 and CTNNB1 blots and 1:7500 for β-actin blots in 2% (w/v) skim milk powder in TBS-T and incubated for 1 h at RT. ECL Prime Western blotting detection reagent (GE Healthcare, Uppsala, Sweden) was for signal detection and was captured using an ImageQuant LAS 4000 (GE Healthcare). Densitometry values were measured using Multi Gauge V3.0 Software (Fujifilm, Brookvale, NSW, Australia). Protein molecular weight marker, SeeBlue Plus2 (Life Technologies), was loaded (5 µl/gel) in order to determine band molecular weights. β-Actin was detected at 42 kDa, NR2F2 at 46 kDa and native CTNNB1 at 85 kDa. CTNNB1 degradation products were also detected between 70 and 55 kDa (Tesco *et al.* 1998). NR2F2 and

CTNNB1 immunoblots were normalised using β-actin with values displayed as mean optical densities (OD/mm²).

Immunohistochemistry

Paraffin-embedded myometrial and fibroid tissues ($n=10$ proliferative and $n=10$ secretory myometrial and fibroid pairs) were cut into 3 µm sections, mounted on saline-coated slides, dewaxed and dehydrated. Sections underwent antigen retrieval (boiling citrate buffer (pH 6.0) for 15 min) and endogenous peroxidases were blocked with 3% (v/v) H₂O₂ in methanol (10 min at RT). Sections were blocked with serum-free protein block (Dako, Glostrup, Denmark; 10 min at RT) and incubated with mouse monoclonal NR2F2 Clone H7147 (2 µg/ml) (Abcam) or mouse monoclonal CTNNB1 Clone 14 (0.5 µg/ml; BD Biosciences) overnight at 4 °C. Mouse IgG1 or IgG2a (Dako) at equivalent concentrations were used as negative controls. Sections were incubated with mouse EnVision+ System-HRP (Dako) (30 min at RT) followed by 3,3'-diaminobenzidine (Sigma–Aldrich) for 5 min at RT. Sections were counterstained with Harris haematoxylin (Amber Scientific, Midvale, WA, Australia).

Double IHC was performed for NR2F2/CTNNB1 and for NR2F2 or CTNNB1 with vimentin, αSMA and CD31. Dewaxed and rehydrated sections were first incubated overnight at 4 °C with mouse anti-human vimentin clone V9 (0.14 µg/ml; Life Technologies), mouse anti-human αSMA (0.0875 µg/ml; Dako) and mouse anti-human CD31 clone JC70A (0.86 µg/ml; Dako). Mouse IgG (Dako) at equivalent concentrations was used as a negative control for each step. For CD31 only, sections underwent antigen retrieval (as described earlier) immediately following dewaxing and rehydration. Sections were incubated with LSAB+ alkaline phosphatase kit, as per the manufacturer's instructions (Dako), followed by visualisation with vector blue (Vector Laboratories, Burlingame, CA, USA). Immunostaining for either NR2F2 or CTNNB1 followed (as described earlier). For NR2F2/CTNNB1 double IHC, NR2F2 staining preceded CTNNB1 staining. No counterstaining was used for double IHC.

Slides were mounted with aqueous mounting media (Dako). 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). Immunostaining for NR2F2 and CTNNB1 was semiquantitatively assessed in different tissue compartments (myometrium, myometrial blood vessels and fibroid and fibroid blood vessels) by three independent observers. At least four fields of view per sample were assessed. Staining was graded from 0 to 3 (with 0.5 increments), where 0 was no staining and 3 was an intense staining.

Primary myometrial and fibroid culture

Primary cultures of myometrium and fibroid SMC were established as described previously (Gargett *et al.* 2002, Zaitseva *et al.* 2006, 2008). Briefly, myometrial and fibroid tissues were finely chopped and dissociated with multiple rounds of collagenase type-2 (Worthington Biochemical Corp., Lakewood, NJ, USA) and DNase type-I (Worthington

Table 1 Primer sequences for RT-qPCR.

Gene	Primer sequence	TaqMan assay/UPL probe
NR2F2	NA	TaqMan HS01047078_ml
CTNNB1	5'-gcagagtctgaaggtgcta-3' 5'-tctgtcaggtagaagctctaaagc-3'	UPL #31
PTCH1	5'-aggatacaacccaatgcag-3' 5'-caggctctgaacctctgga-3'	UPL #15
GLI1	5'-ggattaaaagcagcgaaacctc-3' 5'-cacgactactctgctctcaa-3'	UPL #17
PR	5'-tttaagagggaatggaagg-3' 5'-cggattttatcaacgatgcag-3'	UPL #11
18S	NA	TaqMan eukaryotic 18S rRNA endogenous control

Biochemical Corp.), followed by a short trypsin digestion (Life Technologies) until single-cell suspensions were generated. Cells were seeded into six-well culture plates and grown to confluence (~7 days) in DMEM/10% FCS/antibiotics culture media in the absence of exogenous hormones. All primary cell culture experiments were performed at P0.

Progesterone and RA study

To investigate the effect of progesterone and/or all-trans RA on *NR2F2* and *CTNNB1* mRNA expression, primary cultures of myometrial and fibroid SMC ($n=5$) were grown to confluence and serum starved for 48 h in phenol red-free DMEM with 1% charcoal-stripped FCS (Ch-FCS; Sigma-Aldrich). Cells were treated with progesterone (100 nM) and/or all-trans RA (1 μ M) (both from Sigma-Aldrich) or vehicle control (ethanol) in DMEM/1% Ch-FCS and antibiotics for 24 h (Fahnenstich *et al.* 2003, Zaitseva *et al.* 2008). RA (1 μ M) has been shown to increase RA-responsive gene expression in myometrial and fibroid culture (Zaitseva *et al.* 2008). No cycle stage-associated differences were observed following culture and therefore women included in this study were from mixed cycle stage ($n=3$ proliferative, $n=1$ secretory and $n=1$ inactive endometrium). Following treatment, cells were collected in TRIzol (Life Technologies) for RNA extraction.

SHH study

To investigate a role for SHH in *NR2F2* and *CTNNB1* regulation, primary cultures of myometrial and fibroid SMC ($n=6$) were grown to confluence, serum-starved (as described earlier) and treated with SHH conditioned media (CM) in DMEM/1% FCS for 24 h (Park *et al.* 2011). SHH CM was prepared as described previously (Ingram *et al.* 2002, Hochman *et al.* 2006, Park *et al.* 2011). Briefly, media were collected from SHHN-923 cells, which were transfected to secrete active SHH (N-terminal domain) in a pMT21 (pSHH-N-PMT21) plasmid. Myometrial and fibroid control SMC were treated with CM collected from cells transfected with an empty vector. Following treatment, cells were collected in TRIzol (Life Technologies) for RNA extraction.

Statistical analysis

All data were analysed using Graph Pad Prism Software (version 5, GraphPad Software, La Jolla, CA, USA). All data were tested for Gaussian distribution using the D'Agostino and Pearson omnibus normality test. Based on the normality test, parametric (repeated measures ANOVA followed by Tukey's *post hoc* test or paired *t*-test) or non-parametric (Friedman test with *post hoc* Dunn's test or Wilcoxon's signed rank matched pairs test) tests were utilised.

Results

NR2F2 is differentially expressed during the secretory phase

We investigated *NR2F2* mRNA in myometrium and fibroids using real-time quantitative RT-PCR (RT-qPCR).

In agreement with our previous microarray data (Zaitseva *et al.* 2006, 2008), *NR2F2* mRNA was down-regulated in fibroids compared with myometrium (Fig. 1i). The fibroid-associated reduction in *NR2F2* mRNA was only observed in secretory stage of the menstrual cycle, not during the proliferative phase (Fig. 1i; $P=0.002$). Western blot analysis and immunohistochemistry (IHC) were undertaken to characterise expression and localisation of NR2F2 protein in myometrium and fibroids. Western blotting for NR2F2 detected a 45 kDa band in myometrial and fibroid whole-tissue extracts; however, unlike our mRNA data, densitometry analysis found that there was no significant difference in NR2F2 protein expression (Fig. 1ii). In contrast, significant differences in protein expression were identified following NR2F2 IHC. NR2F2 protein was nuclear and was localised to myometrial and fibroid smooth muscle cells (SMC), perivascular cells, endothelial cells and connective tissue (Fig. 1iv). Semiquantitative scoring determined that significantly stronger NR2F2 immunostaining (approximately twofold increase) was found around blood vessels in both myometrium and fibroid tissues compared with SMC (Fig. 1iii; $P<0.01$). However, with respect to blood vessel staining, there was no difference between myometrium and fibroid tissue. Like *NR2F2* mRNA, myometrial SMC displayed significantly stronger NR2F2 expression compared with fibroid SMC during the secretory phase (Fig. 1iii; $P<0.05$). In addition, NR2F2 immunostaining in secretory phase myometrial SMC was also significantly increased compared with myometrial SMC in the proliferative stage (Fig. 1iii; $P<0.001$). There was no difference in NR2F2 immunostaining between myometrial and fibroid SMC in the proliferative phase (Fig. 1iii).

CTNNB1 is differentially expressed during the proliferative and secretory phases of the menstrual cycle

CTNNB1 expression was also examined using RT-qPCR, western blotting and IHC. *CTNNB1* mRNA expression was elevated in fibroids compared with matched myometrium (Fig. 2i), confirming our previous findings (Zaitseva *et al.* 2006). More specifically, *CTNNB1* mRNA expression was significantly increased in fibroid tissue compared with myometrium in the proliferative phase ($P=0.04$) and compared with secretory-phase fibroid tissue ($P=0.02$) (Fig. 2i). *CTNNB1* mRNA was unchanged in the myometrium during the menstrual cycle. Western blotting for CTNNB1 detected several bands between 85 and 55 kDa (Fig. 2ii). The 85 kDa band represents the full-length native protein, while the smaller molecular weight bands represent degradation fragments (Tesco *et al.* 1998). CTNNB1 (85 kDa) expression was significantly elevated in fibroids compared with normal myometrium in the secretory

Figure 1 Differential NR2F2 expression in uterine myometrium and fibroids. Relative expression of NR2F2 mRNA (i) and protein (ii) in uterine fibroids (F) compared with matched normal myometrium (M) tissue. IHC (iv) for NR2F2 was performed on paired F and M samples. M and F were grouped according to proliferative and secretory phases of the menstrual cycle with $n=10$ paired M and F samples included per phase. IHC micrographs are representative only and do not depict cycle stage. Single NR2F2 myometrial (a) and fibroid (b) staining (brown). Double IHC for NR2F2 (brown) plus vimentin (Vim; blue) for M (c) and F (d), NR2F2 (blue) and α SMA (brown) for M (e) and F (f), and NR2F2 (brown) and CD31 (endothelial marker; blue) for M (g) and F (h). Note the distinctive strong NR2F2 staining in cells surrounding blood vessels. Scale bar = 20 μ m. Semiquantitative scoring of NR2F2 protein staining intensity is displayed for M and F smooth muscle cells/stroma and also for MBV and FBV (iii). Scoring were performed on $n=10$ paired M and F samples from the proliferative phase and $n=12$ paired M and F samples from the secretory phases of the menstrual cycle. Relative protein expression of NR2F2 is displayed as the mean optical density (OD/mm²) $\pm$ S.E.M. All other data are presented as the mean $\pm$ S.E.M., with statistical difference denoted by *** $P<0.001$, ** $P<0.01$ and * $P<0.05$.

phase of the menstrual cycle ($P<0.05$; Fig. 2ii). Although CTNNB1 expression was higher in fibroids compared with myometrium during the proliferative phase, this failed to reach significance (Fig. 2ii).

CTNNB1 immunostaining was diffuse throughout the cytoplasm and cell surface, with weak to moderate intensity in myometrial and fibroid SMC (Fig. 2iv). Some nuclear staining was present in some cells. Similar to NR2F2, stronger staining was observed around some but not all blood vessels (Fig. 2iii and iv). CTNNB1 vascular staining was associated with CD31-positive (endothelial) cells and α smooth muscle actin (α SMA)/vimentin-positive vascular SMC/pericytes (Fig. 2iv c, d, e, f, g and h). In the myometrium, but not fibroid, CTNNB1 immunostaining was significantly increased in association with blood vessels regardless of cycle stage

(Fig. 2iii). Furthermore, fibroid-associated blood vessel CTNNB1 staining was also significantly stronger compared with myometrial SMC (twofold increase) in both proliferative and secretory phases (Fig. 2iii). Semiquantitative scoring demonstrated weaker CTNNB1 staining in myometrial SMC compared with fibroid tissue (SMC and blood vessels) in both proliferative and secretory phases, with some samples appearing nearly negative (Fig. 2iii and iv).

Double IHC for NR2F2 and CTNNB1 demonstrated that the two proteins are co-localised to the same cell types, but mostly within different sub-cellular compartments (Fig. 3). NR2F2 was localised to the nucleus, whereas CTNNB1 protein was found within the cytoplasm, cellular membrane and occasionally on the nucleus. When nuclear co-localisation of NR2F2 and

Figure 2 Differential *CTNNB1* expression in uterine myometrium and fibroids. Relative expression of *CTNNB1* mRNA (i) and protein (ii) in uterine fibroids (F) compared with matched normal myometrium (M) tissue. IHC (iv) for *CTNNB1* was performed on paired F and M samples. M and F were grouped according to proliferative and secretory phases of the menstrual cycle with $n=10$ paired M and F samples included per phase. IHC micrographs are representative only and do not depict cycle stage. Single CTNNB1 myometrial (a) and fibroid (b) staining (brown). Double IHC for *CTNNB1* (brown) plus vimentin (Vim; blue) for M (c) and F (d), *CTNNB1* (blue) and α SMA (brown) for M (e) and F (f) and *CTNNB1* (brown) and CD31 (endothelial marker; blue) for M (g) and F (h). Semiquantitative scoring of *CTNNB1* protein staining intensity is displayed for M and F smooth muscle cells/stroma and also for MBV and FBV (iii). Scoring were performed on $n=12$ paired M and F samples from the proliferative phase and $n=11$ paired M and F samples from the secretory phases of the menstrual cycle. Relative protein expression of *CTNNB1* is displayed as the mean optical density (OD/mm²) $\pm$ S.E.M. All other data are presented as the mean $\pm$ S.E.M., with statistical difference denoted by *** $P<0.001$, ** $P<0.01$ and * $P<0.05$.

CTNNB1 was observed, it was limited to strongly stained perivascular cells (Fig. 3, arrows).

Progesterone and RA in combination regulate *NR2F2* mRNA expression in myometrial cells

We investigated whether progesterone, RA or combined progesterone+RA treatment could regulate *NR2F2* and *CTNNB1* mRNA expression in myometrial and fibroid primary cells (P0). Following culture and treatment, cycle stage-associated differences in *NR2F2* and *CTNNB1* gene expression were no longer observed (data not shown), and therefore, women included in primary culture experiments in the remainder of this investigation were from mixed cycle stage. *PR* mRNA expression was confirmed in P0 myometrial and fibroid cells (relative *PR* mRNA expression in myometrial cells

1.25 ± 0.21 (range 0.26–2.27) and fibroid cells 2.68 ± 0.58 (range 0.57–4.58)). When progesterone and RA were combined, *NR2F2* mRNA expression was significantly down-regulated in myometrial cells compared with controls ($P<0.05$) and compared with myometrial cells treated with progesterone alone ($P<0.05$) (Fig. 4i). RA or progesterone treatment alone did not significantly alter myometrial *NR2F2* mRNA expression compared with controls. Furthermore, progesterone and/or RA exposure did not affect *NR2F2* mRNA expression in fibroid cells (Fig. 4i). Similarly, expression of *CTNNB1* was not significantly altered by progesterone, RA or combined progesterone+RA treatment in both cell types (Fig. 4ii). There was a large degree of *NR2F2* and *CTNNB1* gene expression variability in response to progesterone/RA between myometrial, but not fibroid, samples (Fig. 4i and ii).

Figure 3 Co-localisation of NR2F2 and CTNNB1 in myometrial and fibroid tissues. Double IHC for NR2F2 (blue) and CTNNB1 (brown) was performed on $n=10$ paired myometrium and fibroid samples. Scale bar = 10 μm . Arrows indicate NR2F2 and CTNNB1 co-localised to the nucleus.

SHH does not regulate NR2F2 or CTNNB1 expression in myometrial and fibroid cells

We also investigated whether the SHH pathway was involved in regulating NR2F2 and CTNNB1 mRNA expression in myometrial and fibroid primary cells (P0). To confirm that the SHH pathway was active in myometrial and fibroid primary cell cultures, we examined the mRNA expression of two SHH-responsive genes, glioma-associated oncogene homologue 1 (*GLI1*) and Patched1 (*PTCH1*), following treatment with SHH CM. In support of an active SHH pathway in myometrial and fibroid cultured cells, both *GLI1* and *PTCH1* genes were significantly up-regulated after SHH CM treatment in both cell types ($P<0.05$; Fig. 5i). In contrast, the mRNA expression of NR2F2 and CTNNB1 was unaltered by treatment with SHH CM in both myometrial and fibroid cells (Fig. 5ii).

Discussion

Although a large number of genes are differentially expressed in fibroids compared with myometrium (Tsibris *et al.* 2002, Skubitz & Skubitz 2003, Arslan *et al.* 2005, Luo *et al.* 2005a, 2005b, Zaitseva *et al.* 2006, Dimitrova *et al.* 2009), it is likely that most of these changes occur as a consequence of the fibroids' presence and do not cause fibroid development. We have targeted NR2F2 and CTNNB1 in this study based on their significant potential for a causal role in uterine fibroid pathophysiology (Petit *et al.* 2007, Tanwar *et al.* 2009). We report that NR2F2 and CTNNB1 levels are altered in human uterine fibroids and that both proteins display strong blood vessel localisation in myometrial and fibroid tissues. We show that in cultured myometrial cells, NR2F2 mRNA expression is reduced by progesterone/RA treatment. We also demonstrate that while the HH pathway is active in primary myometrial and fibroid cells, SHH does not regulate NR2F2 or CTNNB1 mRNA expression.

This study demonstrated decreased expression of NR2F2 in uterine fibroids, consistent with our previous

work (Zaitseva *et al.* 2008). Fibroid-associated decrease in NR2F2 expression was evident at the mRNA and protein level (statistically significant for IHC, but not western blotting). There are several reasons why NR2F2 mRNA and protein levels may not match, including differences in turnover dynamics, post-translational or -transcriptional regulation and targeted degradation. Reduced NR2F2 expression was specific to the secretory

Figure 4 Progesterone and RA regulate NR2F2 gene expression in myometrial but not fibroid primary culture. Primary cultures of paired M and F cells (P0) were treated for 24 h with vehicle control, progesterone, RA or progesterone + RA ($n=5$). Relative mRNA expression of NR2F2 (i) and CTNNB1 (ii) is displayed for M and F cultures. Data are displayed as mean $\pm$ s.e.m., with significant differences denoted by $*P<0.05$.

Figure 5 SHH pathway does not regulate *NR2F2* or *CTNNB1* mRNA expression. Primary cultures of paired M and F cells (P0) were treated for 24 h with SHH incubation media ($n=6$). Relative mRNA expression of specific SHH target genes, *PTCH1* and *GLI1* (i), is displayed for M and F +/– SHH incubation media. Relative mRNA expression of *NR2F2* and *CTNNB1* (ii) is also displayed for M and F +/– SHH incubation media. Data are displayed as mean $\pm$ S.E.M., with significant differences denoted by $*P<0.05$.

stage of the menstrual cycle. Menstrual cycle stage-associated expression of *NR2F2* has not been reported previously in the uterus. However, granulosa cells from normal cycling ovaries also display cycle stage-specific *NR2F2* expression, with highest expression occurring in dominant follicles (Sato *et al.* 2003). Altered *NR2F2* expression has been demonstrated in several cancers, including lymphoma (Enjuanes *et al.* 2011), leukaemia (Bullinger *et al.* 2010), breast cancer (Tommasi *et al.* 2009) and salivary gland carcinoma (Bell *et al.* 2011). Ablation of *NR2F2* in mice directs the plasticity of mesenchymal cells towards the myoblast lineage (resulting in increased muscle mass; Xie *et al.* 2011); these observations support the notion that tumour-related down-regulation of *NR2F2* expression could play a role in fibroid growth as uterine fibroids are mesenchymally derived tumours.

With respect to *CTNNB1*, we observed increased protein and mRNA expression in fibroids compared with paired myometrium. Cycle stage differences in *CTNNB1* expression were observed for mRNA (proliferative stage)

and protein (western blot; secretory stage); however, IHC demonstrated increased β -catenin expression in fibroids during both proliferative and secretory phases, relative to myometrium. A fibroid-associated increase in *CTNNB1* confirms microarray data by us and others (Luo *et al.* 2005a, Zaitseva *et al.* 2006). Aberrant expression/mutations of *CTNNB1* leading to increased nuclear localisation, and enhanced Wnt signalling, have been observed in endometrial carcinoma (Fukuchi *et al.* 1998, Saegusa & Okayasu 2001, Moreno-Bueno *et al.* 2002, Kim *et al.* 2009). Non-uterine smooth muscle tumours similarly demonstrate increased *CTNNB1* expression and nuclear translocation during proliferation or predisposing tumour development (Gosens *et al.* 2010, van Veelen *et al.* 2011). While we did not analyse differences in nuclear expression of *CTNNB1*, we did observe nuclear localisation in both myometrium and fibroids. Compelling evidence for a role of *CTNNB1* in the development of uterine fibroids comes from a mouse model with constitutive activation of *CTNNB1* (Tanwar *et al.* 2009). These mice develop uterine smooth muscle tumours resembling fibroids and endometrial stromal sarcoma-like lesions with 100% penetrance (Tanwar *et al.* 2009). Evidence from this mouse model in combination with the data generated in our study strongly implicate an up-regulation of *CTNNB1* in having a central role in fibroid development.

Both *NR2F2* and *CTNNB1* demonstrated increased blood vessel-specific expression patterns in myometrium and fibroid tissues. Fibroids commonly have altered vasculature suggestive of impaired angiogenesis (reviewed by Fleischer *et al.* (2008)), including reduced vascular density and a lack of muscular layers surrounding vessels (Casey *et al.* 2000, Aitken *et al.* 2006). In mice, full or partial deletion of *NR2F2* or *CTNNB1* is lethal due to malformations of the large vessels and microvasculature (Pereira *et al.* 1999, Cattelino *et al.* 2003, Dejana 2010). We propose that the decreased expression of *NR2F2* in fibroids may lead to altered expression of angiogenic factors, such as angiopoietin-1 (Qin *et al.* 2010, Jeansson *et al.* 2011, Saharinen & Alitalo 2011), which may contribute to the altered and immature vasculature of uterine fibroids. *CTNNB1* is also fundamental to maintenance of normal vasculature (Cattelino *et al.* 2003). Quiescent blood vessels maintain low basal Wnt/*CTNNB1* signalling; however, in the presence of disease, *CTNNB1* signalling is up-regulated (reviewed by van de Schans *et al.* (2008)). Therefore, both *NR2F2* and *CTNNB1* have the capacity to regulate genes involved in blood vessel maintenance. Given the strong expression of *NR2F2* and *CTNNB1* in myometrial and fibroid vasculature, it is possible that these proteins may play a role in the altered phenotype of fibroid blood vessels.

We examined potential regulators of *NR2F2* and *CTNNB1* expression *in vitro* and report that treatment with progesterone and RA (in combination) significantly

decreases *NR2F2* expression in myometrial but not fibroid primary cells. While the precise mechanisms are unclear, fibroid cells demonstrate an inability to interact with or recognise progesterone and RA and respond like normal adjacent myometrium. *PR* expression reduces in myometrial and fibroid cultures; however, as demonstrated previously (Zaitseva *et al.* 2006), we confirmed that *PR* is expressed in measurable amounts in P0 myometrium and fibroid cell. There is also evidence from human myometrial primary and explant cultures that *PR* functionality is retained *in vitro* (Cermik *et al.* 2001, Dong *et al.* 2003). In contrast, *CTNNB1* expression in myometrial and fibroid cells were not affected by progesterone and/or RA treatments. Several studies report that members of the Wnt signalling pathway, but not *CTNNB1* itself, are regulated by progesterone in human endometrial cells (Tulac *et al.* 2006, Catalano *et al.* 2007, Kane *et al.* 2008). Therefore, the progesterone-associated effects on the *CTNNB1* pathway in the endometrium may be cell-type specific and not affect myometrial cells. *In vivo* we saw secretory-stage differential regulation of *NR2F2* expression, when progesterone levels are physiologically highest; however, in culture, progesterone only affected *NR2F2* expression in the presence of RA. We have previously demonstrated that components of the RA pathway are significantly altered in fibroids (Zaitseva *et al.* 2007) and that many of the genes that are regulated by RA are differentially expressed between myometrium and fibroids (Zaitseva *et al.* 2008). *NR2F2* is an orphan receptor, and in an attempt to identify its endogenous ligand, Kruse *et al.* (2008) showed that high concentrations of RA (10–100 times physiological levels) up-regulated *NR2F2* transcriptional activity. Fibroids are reported to have higher levels of all-trans RA compared with myometrium (Tsibris *et al.* 1999). *NR2F2* also directly interacts with RARs and effects the expression of target genes via the RA pathway (Pinaire *et al.* 2000, Hubert *et al.* 2010, Prahalad *et al.* 2010, Vilhais-Neto *et al.* 2010). Alone, progesterone has been found to have little effect on *NR2F2* activity (Kruse *et al.* 2008). Indirectly, however, *NR2F2* expression can be modulated by progesterone in the mouse uterus, through paracrine mechanisms involving epithelial cells and the HH pathway (Lee *et al.* 2006b, Simon *et al.* 2009b). Upstream regulators of *NR2F2* expression must also be considered, including brahma-related gene 1 (*SMARCA4* (*BRG1*)), which is a tumour suppressor that normally promotes *NR2F2* expression (Davis *et al.* 2013) and is induced by synthetic retinoids (Xu *et al.* 2010). Interestingly, studies on *SMARCA4* conditional homozygous mutants show that these mice develop uterine tumours (Serber *et al.* 2012). Overall, our findings raise the possibility that decreased levels of *NR2F2*, as a consequence of interacting progesterone and RA, may contribute to aberrant RA signalling in uterine fibroids.

To the best of our knowledge, we report for the first time that the HH pathway is functional in uterine fibroids and myometrium. The HH pathway is important in many normal and abnormal cellular functions, including embryonic development, stem cell proliferation and cancer genesis (reviewed by Varjosalo & Taipale (2008), Choi *et al.* (2011), Harris *et al.* (2011) and Park *et al.* (2011)). The HH pathway is also crucial to the development of reproductive tract (Franco & Yao 2012), uterine function (Takamoto *et al.* 2005, Lee *et al.* 2006a, Petit *et al.* 2007) and uterine malignancies (Feng *et al.* 2007, Kim *et al.* 2009). Despite evidence for a role of HH in the uterus, and our results demonstrate that the HH pathway is active in cultured myometrial and fibroid cells; *NR2F2* and *CTNNB1* mRNA expressions were not affected by SHH treatment. Previously, *NR2F2* was found to be induced by HH in uterine stroma in mice (Matsumoto *et al.* 2002, Simon *et al.* 2009b) and a SHH response element has been identified in the *NR2F2* promoter (Krishnan *et al.* 1997). Conversely, another study failed to identify *NR2F2* as a SHH target in pluripotent mesenchymal cells using microarray technology (Ingram *et al.* 2002). Also using mouse models, SHH (and Gli3) have been implicated in Wnt/*CTNNB1* pathway activation and formation of the genital tract (Ulloa *et al.* 2007, Miyagawa *et al.* 2009). In the endometrium, the HH pathway functions via epithelial–mesenchymal cross talk (Matsumoto *et al.* 2002, Simon *et al.* 2009b). Our results suggest that mechanisms of HH signalling differ between endometrium and myometrium and that *NR2F2* and *CTNNB1* are not HH-activated targets in myometrium and fibroids.

Both *NR2F2* and *CTNNB1* have been implicated in tumour progression via EMT mechanisms. For example, aberrant expression of *NR2F2* has been associated with induction of dedifferentiation, cell migration and angiogenesis (reviewed by Boudot *et al.* (2011)), while *CTNNB1* is considered to be an early signalling molecule in EMT (Zhou *et al.* 2012). In uterine disease, EMT is mainly associated with endometrial disorders including adenomyosis (Chen *et al.* 2010) and endometrial cancer (Montserrat *et al.* 2012). In renal fibrogenesis, more than one third of disease-related fibroblasts come from epithelia involved in EMT (Kalluri & Neilson 2003). We have shown that fibroblasts constitute a major cellular component of uterine fibroids (Zaitseva *et al.* 2007; SJ Holdsworth-Carson, M Zaitseva, BJ Vollenhoven & PAW Rogers, unpublished observations). Therefore, we suggest that *NR2F2* and *CTNNB1* may play roles in EMT-mediated development of some uterine fibroids, in particular fibroid fibroblasts.

In conclusion, we demonstrate aberrant expression of *NR2F2* and *CTNNB1* in uterine fibroids compared with normal myometrium. In particular, we identified that these proteins display strong blood vessel-specific localisation, indicating that they may play a role in angiogenesis or vessel homeostasis, a process that is

often abnormal in uterine fibroids. With respect to their regulation, we show that the HH pathway does not regulate *NR2F2* or *CTNNB1* expression, while *NR2F2* expression was regulated by combined RA and progesterone, two factors with important roles in uterine fibroid pathology. Further work is required to fully clarify the roles of *NR2F2* and *CTNNB1* in fibroid development. However, our work shows that these proteins exhibit differential expression and dysregulation in association with fibroids, and given that both *NR2F2* and *CTNNB1* are critical to many aspects of uterine biology, these genes are worthy of further consideration as potential causal factors in uterine fibroid development.

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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