Aberrant expression and regulation of NR2F2 and CTNNB1 in uterine fibroids

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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...
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), secretary (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 secretary 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 (A260:A280).

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 × 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^−ΔΔCT 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 \( (n=10 \text{ proliferative} \text{ and } n=10 \text{ secretory myometrial and fibroid pairs}) \) 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 markers, 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. 2006). 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 \( (n=10 \text{ proliferative} \text{ and } n=10 \text{ secretory myometrial and fibroid pairs}) \) 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 vinemint, zSMA 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 zSMA (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, AxiosCam 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 (Warthington Biochemical Corp., Lakewood, NJ, USA) and DNase type-I (Warthington

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**Table 1** Primer sequences for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>TaqMan assay/UPL probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2F2</td>
<td>NA</td>
<td>TaqMan</td>
</tr>
<tr>
<td>CTNNB1</td>
<td>5′-gcagagttcaaggtgctga-3′</td>
<td>HS01047078_ml</td>
</tr>
<tr>
<td></td>
<td>5′-tgcggagctgaaccaatga-3′</td>
<td>UPL #31</td>
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<tr>
<td>PTCH1</td>
<td>5′-gcctcgccaggtgctga-3′</td>
<td>UPL #15</td>
</tr>
<tr>
<td>GLI1</td>
<td>5′-gattcggctctctgctgcttg-3′</td>
<td>UPL #17</td>
</tr>
<tr>
<td>PR</td>
<td>5′-tttaaacctcgcc-3′</td>
<td>UPL #11</td>
</tr>
<tr>
<td>18S</td>
<td>NA</td>
<td>TaqMan eukaryotic</td>
</tr>
<tr>
<td></td>
<td>18S rRNA endogenous control</td>
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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.

**Progestrone 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 µM) (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 µM) 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, Hocman et al. 2006, Park et al. 2011). Briefly, media were collected from SHH-N-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 transplanted 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...
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 (αSMA)/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 (Fig. 2iii). 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 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 αSMA (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 μm. 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) ± S.E.M. All other data are presented as the mean ± 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).
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. 5 i). In contrast, the mRNA expression of NR2F2 and CTNNB1 was unaltered by treatment with SHH CM in both myometrial and fibroid cells (Fig. 5 ii).

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 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
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 angioptoein-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

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**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 ± S.E.M., with significant differences denoted by *P<0.05.
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. Fibroblasts 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 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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