Extracellular matrix (ECM) activates β -catenin signaling in uterine fibroids

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Abstract

Recent studies showed that genetic aberrations in the *MED12* gene, probably through the canonical WNT/ β -catenin pathway, lead to the pathogenesis of uterine fibroids. However, a comprehensive analysis of the WNT pathway in *MED12*-mutated and *MED12*-wild-type fibroids has not been performed. The objective of this study was to determine the status of the WNT pathway in human fibroids. We performed Sanger sequencing to define the *MED12* mutational status of fibroids and normal myometrium samples. qPCR arrays were carried out to determine the status of the WNT signaling pathway in *MED12*-mutated and *MED12*-wild-type fibroids. Liquid chromatography–mass spectrometry (LC–MS), Western blotting and immunohistochemistry were used to monitor the expression of β -catenin. We showed that β -catenin expression was increased in fibroids compared to the adjacent myometrium samples. However, β -catenin expression showed no correlation with *MED12* mutation status. Of all the WNT signaling components, WNT inhibitors showed the greatest differences in expression between fibroids and controls. *WIF1*, a WNT inhibitor, was identified as the most significantly upregulated gene in fibroids. We cultured primary fibroid cells on hydrogels of known stiffness to decipher the influence of biomechanical cues on β -catenin expression and revealed increased levels of β -catenin when cells were cultured on a stiffer surface. In conclusion, our data showed that β -catenin expression in fibroids occurs independently of *MED12* mutations.

Biomechanical changes upregulate β -catenin expression in fibroids, providing an attractive avenue for developing new treatments for this disease.

Reproduction (2018) **155** 61–71

Introduction

Uterine leiomyomas, also known as fibroids, are hormone-dependent benign tumors that occur in 60% of premenopausal women (Okolo 2008). The majority of these women present with symptoms including heavy or prolonged menstrual bleeding, pelvic pain and bowel and bladder dysfunction due to excessive pressure applied by the tumors (Okolo 2008, Bulun 2013). Fibroids, especially at the submucosal location, are also associated with infertility and abortion (Bulun 2013). Surgical methods such as hysterectomy and myomectomy are still considered the main treatment for this disease. Fibroids account for 35% of all hysterectomies performed in the USA, which represents a very high economic cost on health care (Cramer & Patel 1990).

Recent advances in gene sequencing approaches have revealed significant heterogeneity in fibroids. The most

common driver mutations are found in high mobility group AT-hook 2 (*HMG2*), fumarate hydratase (*FH*), mediator subunit 12 (*MED12*) and collagen type IV, alpha 5 and 6 (*COL4A5–COL4A6*) (Mehine *et al.* 2013). Among the four mutation subtypes, the most prevalent mutations are in the *MED12* gene, which contributes to 70% of the tumors (Mäkinen *et al.* 2011). *MED12* is a part of a large protein complex that mediates gene expression by assisting the communication between DNA binding transcription factors and RNA polymerase (Conaway *et al.* 2005). *Med12* was shown to be essential for early mouse development via the WNT signaling pathway (Rocha *et al.* 2010) and the mediator complex can bind directly to β -catenin via the *MED12* subunit to assist transcription (Kim *et al.* 2006). Many different groups have identified hyper-activation of WNT/ β -catenin signaling in fibroids (Bulun 2013, Zaitseva *et al.* 2013). We developed a mouse model and demonstrated

that sustained activation of β -catenin signaling drives the growth of fibroids that are histopathologically similar to human fibroids and responsive to changes in ovarian hormones (Tanwar *et al.* 2009). Treatment of human fibroid cells *in vitro* with WNT/ β -catenin inhibitors has been shown to suppress their growth and proliferation, suggesting that targeting this pathway might be an effective therapeutic strategy against this disease (Ono *et al.* 2014). Despite the significance of WNT/ β -catenin signaling in uterine leiomyogenesis, no genetic alterations affecting the WNT pathway have been identified in fibroids. Additionally, there is currently very little evidence to support that *MED12* mutations/deletions act through WNT/ β -catenin signaling to drive the process of fibroid development.

In this study, we have performed a comprehensive analysis of the entire WNT pathway in fibroids with, and without, *MED12* mutations. We examined at both the mRNA and the protein level to uncover the relationship between *MED12*, WNT/ β -catenin signaling and fibroid growth. We have also compared the status of WNT/ β -catenin signaling in fibroids within the same patient and between different patients to minimize the influence of patient-to-patient variation. Our assessment of the WNT ligands, receptors, activators, inhibitors and targets revealed that β -catenin mRNA and protein expression was upregulated in fibroids compared to the adjacent normal myometrium. In contrast to a previously published report (de Graaff *et al.* 2013), we found that the *MED12* mutation status of fibroids did not affect β -catenin expression, and the expression of WNT inhibitors, *DKK1* and *SFRP1*, was upregulated in fibroids with *MED12* mutations compared to those with normal *MED12*. We have also provided evidence that extracellular matrix (ECM), which is one of the major components of fibroids, upregulates β -catenin.

Materials and methods

Human tissue collection

Human fibroid and the adjacent myometrium samples were collected from patients who underwent hysterectomies at the John Hunter Hospital using a protocol approved by the Institutional Human Research Ethics Committee at the University of Newcastle. The participants gave written informed consent to donate their tissues for this study. For patients with multiple fibroids, small (<2 cm diameter), medium (2.1–4 cm diameter) and large (>4 cm diameter) fibroids were collected separately. Fresh samples were transported immediately to the laboratory in phosphate-buffered saline (PBS) on ice. Samples were extensively washed with PBS to remove excess blood prior to any molecular, cellular and histological analysis.

Sanger sequencing

To determine the status of *MED12* genetic aberrations in our tissue samples, we performed Sanger sequencing using

a published protocol (Halder *et al.* 2015). Genomic DNA extraction from fibroids and normal adjacent tissue samples was performed using QIAamp DNA Mini Kit (Qiagen). The DNA fragment was amplified using specific forward and reverse (F: 5'-GCCCTTTCACCTTGTCCTT-3' and R: 5'-TGTCCTATAAGTCTTCCCAACC-3') primers that targeted exon 2 of *MED12*. Following PCR amplification, the products were purified with QIAquick PCR Purification Kit (Qiagen) and quantified with NanoDrop 2000/2000c Spectrophotometer. The purified PCR products were directly sequenced using the BigDye Terminator method according to the manufacturer's instructions (Life Technologies) and the same PCR primers specific to the exon 2 of *MED12*.

Human WNT signaling pathway arrays

RNA was extracted from 32 fresh frozen tissue samples from 13 different patients using TRI Reagent solution (Life Technologies) and further purified by RNeasy MinElute Cleanup kit (Qiagen) with additional RNase-free DNase I treatment. The amount of RNA was quantified using a NanoDrop 2000/2000c Spectrophotometer. Five hundred nanograms of purified RNA were then synthesized into cDNA with the RT² First Strand Kit (Qiagen). All steps were followed as per the manufacturer's instructions. Gene expression in relation to the WNT pathway was quantified using Human WNT Signaling Pathway Plus PCR Array (Qiagen) using the 7900HT Fast Real-Time PCR System (Applied Biosystem). The fold regulation change of each gene for all tumor samples was compared to the respective normal adjacent tissue from each individual patient. Two-way ANOVA (paired *t*-test) was utilized here to identify the differential gene expression in *MED12* mutation-positive and -negative tumor samples and between the paired adjacent myometrium and fibroid tissue. Gene with *P* value <0.05 is considered significant. A heat-map showing significantly upregulated genes was generated using Glttools analysis software.

Immunohistochemistry (IHC)

IHC for β -catenin was performed using a protocol described previously (Goat *et al.* 2017). Briefly, tissues were dewaxed and incubated in a series of xylenes, ethanol and MilliQ water, followed by antigen retrieval in sodium citrate buffer (pH 6) containing 5% Tween 20 at 95°C for 20 min. Slides were cooled and washed with MilliQ water and TBS-T (tween-20) then followed by quenching with 0.3% hydrogen peroxide in absolute methanol. After few washes, tissue slides were blocked with 10% goat serum blocking solution (0.1% BSA and 0.1% triton x in TBS-T) for one hour followed by overnight incubation at 4°C with a primary anti- β -catenin antibody (1:100, Cat no. D10A8, Cell Signaling Technology). Next, tissue samples were incubated with secondary antibody, goat-anti-rabbit (1:250, Jackson Lab) for one hour. Slides were washed and treated with peroxidase-conjugated Streptavidin (1:250, Jackson Lab) and finally exposed to diaminobenzidine (DAB, Sigma) containing 0.3% hydrogen peroxide. Slides were imaged using the Aperio AT2 slide scanner (Leica Biosystems). The gain and exposure time was set constant across tissue samples.

Quantitative proteomics using isobaric tags

Fibroid samples were cut into small pieces and homogenized in ice-cold lysis buffer 0.1 M sodium carbonate, 10 mM sodium ortho-vanadate, protease inhibitor cocktail (Complete Mini, Roche) and 1 tablet of PhosStop (Roche) and homogenized using the beadbug homogenizer (Benchmark Scientific). The concentrated soluble proteins were dissolved in 12 M Urea/4 M Thiourea buffer, reduced with 10 mM of dithiothreitol, alkylated with 20 mM Iodoacetamide and digested with 50 μ g of trypsin (Promega) overnight at 37°C. After trypsin digestion, peptides were dried by SpeedVac SC100 vacuum centrifugation. Peptides were reconstituted in the dissolution buffer and processed according to the manufacturer's protocol for 4-plex iTRAQ reagent (Sigma). The peptides were labeled with the isobaric tags, incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation. The mixed peptides were resuspended in the reconstitution buffer, fractionated by strong cation exchange chromatography and loaded onto a hydrophilic interaction liquid chromatography (HILIC) column. The eluted peptides were collected into 20 fractions, desalted, vacuum-dried and stored at -20°C until analyzed by mass spectrometry (MS). MS was performed using Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific) and intact peptides, ion fragments were detected in the Orbitrap using specific perimeter resolution. Data analysis and protein identification were performed using Proteome Discoverer software package (Thermo Scientific, version 2.0.0.802) and UniProt Human reference proteome database, respectively.

Western blot

Western blot analysis method has been described in detail by us (Bajwa et al. 2016). Briefly, human leiomyoma tissue samples were homogenized in ice-cold radioimmunoprecipitation assay buffer (RIPA) supplemented with protease and phosphatase inhibitors. Equal amounts of proteins were loaded equally onto 10% Mini-PROTEAN TGX Precast Protein Gels (BioRad). Membranes were incubated with a primary anti- β -catenin antibody (1:1000, CST) and β -actin antibody (1:2000, Developmental Studies Hybridoma Bank, IA, USA) overnight at 4°C followed by incubation with HRP-conjugated secondary antibodies against mouse and rabbit IgG in room temperature for one hour. Images were captured with Fujifilm LAS-3000 Imager. The band of Western blot was quantified using NIH ImageJ plugin.

Human leiomyoma cell isolation and culture

Primary cells were isolated using a published protocol (Kim et al. 2015). Tissue samples were washed with PBS and chopped into very fine pieces followed by digestion with 0.2 mg/mL collagenase 1A, 0.2 mg/mL collagenase XI and 2 mg/mL bovine serum albumin at 37°C for 1 h. The mixture was then filtered with 0.2 μ m sterile Minisart filter and passaged in DMEM media supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum (FBS), and 200 nm L-glutamine. Cells were incubated at 37°C with 5% CO₂. Complete media was changed every alternate day and further passaged with

0.25% trypsin for five minutes when the cells reached 70% confluency. Seeding density was 60,000 cells per 10 cm² with expected 70% confluency in 72 h.

Hydrogel preparation and imaging

Polyacrylamide gel of varying stiffness was prepared as per a published protocol (Tse & Engler 2010). Briefly, acrylamide and bis-acrylamide were mixed with indicated concentration in Table 1, followed by polymerization for 15 min. Gels were then covered with 0.001 g/mL of Sulfo-SANPAH (sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate) (Sigma) and exposed directly under UV light for 10 min. After two washes, the gels were incubated overnight with neutralized 0.2 mg/mL type I collagen (Sigma) at 4°C. The isolated leiomyoma cells were plated at a density of 20,000 cells per gel and incubated at 37°C for three days prior to immunofluorescent staining and analysis. Hydrogels were fixed with 4% paraformaldehyde followed by two washes and incubation with primary monoclonal anti- β -catenin (1:100, CST) overnight at 4°C. Alexa-Fluor 488 secondary antibodies (1:250; Jackson ImmunoResearch Labs) were used for one-hour incubation at room temperature. The image was taken using Olympus DP72 microscope. Images were analyzed using ImageJ software (NIH).

Statistical analysis

GraphPad Prism 6.0 was used in this study for statistical analyses. Data were presented as mean $\pm$ S.E.M. Student's *t*-test was performed for Western blot analysis to compare β -catenin protein expression in normal myometrium and fibroid tissue. Two-way ANOVA was applied to all the qPCR analysis to compare the gene expression between normal myometrium and fibroid tissue and also between MED12 mutation-positive and -negative tissue. *P* value <0.05 was considered statistically significant.

Results

Differential expression of WNT pathway members in human uterine fibroids and adjacent normal tissue samples

The WNT signaling pathway can be divided into three major arms, namely, canonical WNT, WNT/planar cell polarity (PCP) and WNT/Ca⁺⁺ pathway. Each pathway activates a different set of downstream effectors that are responsible for different actions such as cell proliferation, planar cell polarity and calcium channel activation, respectively. To determine whether WNT

Table 1 Composition of hydrogels of varying stiffness.

	3 kPa	12 kPa	40 kPa
10 mM HEPES (μ L)	806	716	560
40% Acrylamide (μ L)	137.2	188.8	200
2% bis-acrylamide (μ L)	44.96	80	240
APS (μ L)	5	5	5
TEMED (μ L)	0.5	0.5	0.5

signaling is altered in uterine fibroids, we collected both fresh fibroids and adjacent normal tissue samples from each patient undergoing hysterectomy ($n=13$; Fig. 1A). Next, we analyzed the expression of 84 genes belonging to the WNT pathway using qPCR arrays to compare the level of their expression between fibroids ($n=19$) and the adjacent normal tissue samples ($n=12$). Genes representing different components of the WNT pathway that were significantly differentially expressed between fibroids and normal adjacent tissue samples are presented in the heat-map ($P<0.05$; Fig. 1B). Interestingly, β -catenin expression was significantly upregulated in fibroids compared to controls ($P=0.004$; Fig. 1C). To further determine the changes in different components of the WNT pathway, we separated all the genes examined in this study into WNT ligands, WNT receptors, WNT inhibitors and WNT targets based on their established roles in previous studies (Fig. 1D). Of all the WNT ligands, *WNT2B*, *WNT5B* and *WNT9A* showed the most significant downregulation in fibroid tissue compared to adjacent normal tissue samples (Fig. 1D). None of the WNT ligands expression was upregulated compared to controls (Fig. 1D). The most significant differences in WNT receptor expression was in Frizzled Class Receptor 6 (*FZD6*), Low-density Lipoprotein Receptor-Related Protein 5 (*LRP5*) and Low-density Lipoprotein Receptor-Related Protein 6 (*LRP6*) and all were downregulated in fibroids (Fig. 1D). Interestingly, the most distinctive changes in expression were found in the genes belonging to the WNT inhibitor category; *F-Bbox* and WD Repeat Domain Containing 11 (*FBXW11*), Secreted Frizzled-Related Protein 4 (*SFRP4*) and WNT Inhibitory Factor 1 (*WIF1*) were significantly upregulated in fibroids with a noticeably higher than 20-fold increase for *WIF1* compared to adjacent normal tissue samples (Fig. 1D). In contrast, expression of the WNT inhibitors: Adenomatous Polyposis Coli (*APC*), Dickkopf-Related Protein 1 (*DKK1*), *DKK3*, *SFRP3/FRZB*, *PRICKLE1*, Ras Homolog Gene Family Member A (*RHOA*) and SRY-related HMG-Box 17 (*SOX17*), were significantly decreased in fibroids compared to the adjacent normal myometrium (Fig. 1D). The WNT target, Vang-like protein 2 (*VANGL2*), was the only gene in this category that was significantly upregulated in fibroids. Conversely, expression of other WNT targets: Disabled homolog 2 (*DAB2*), *JUN*, Matrix Metalloproteinase 7 (*MMP7*), Transcription Factor 7 Like 1 (*TCF7L1*), WNT1 Inducible Signaling Pathway Protein 1 (*WISP1*), Cytochrome P450 Family 4 Subfamily V Member 2 (*CYP4V2*), Heat Shock Protein Family A (*Hsp70*) Member 12A (*HSPA12A*), was significantly downregulated in fibroids (Fig. 1D).

To investigate further whether these changes in fibroid gene expression related to any specific WNT pathway, we separated the genes based on the three main arms of the WNT pathways, which are canonical WNT, WNT/planar cell polarity (PCP), WNT/ Ca^{++} pathway,

as well as the negative regulators of the WNT pathway (Fig. 2A). Figure 2B outlines the pathway structure and the molecules involved in each path. For canonical WNT signaling, β -catenin (*CTNNB1*), *SFRP4* and *WIF1* expression were increased, whereas *DKK1*, *DKK3*, *SOX17* and *WNT2B* expression were significantly decreased (Fig. 2A). For the WNT/PCP pathway, *VANGL2* expression was highly upregulated and *APC*, *PRICKLE1*, *RHOA* and *WNT9A* expression levels were significantly reduced. For the WNT/ Ca^{++} pathway, only the ligands were significantly altered; *WNT2B*, *WNT5B* and *WNT9A* showed a significant decrease in expression in fibroid samples (Fig. 2A). Finally, in the group of negative regulators, *SFRP4* and *WIF1* were the most highly upregulated genes; whereas *DKK1*, *DKK3*, *SOX17* and *FRZB* were significantly downregulated in fibroid tissue (Fig. 2A). Among the three WNT pathway categories, most of the significantly altered genes, which were *PRICKLE1*, *VANGL2*, *WNT9A*, *DKK1*, *SFRP4* and *WIF1*, belong predominantly to the PCP pathway and negative regulator category (Fig. 2A).

Expression of the WNT pathway was downregulated in MED12 gene-mutated uterine fibroids compared to MED12 wild-type fibroids

To examine whether mutations in the *MED12* gene have any effect on WNT signaling, we investigated the expression of WNT pathway components at the RNA level using real-time PCR. Firstly, we determined the *MED12* mutation status of nineteen fibroids ($n=19$) and twelve adjacent normal myometrium ($n=12$ tissue samples) using Sanger sequencing as described by Mäkinen and coworkers (Mäkinen *et al.* 2011). We noticed that all the adjacent normal myometrium samples had wild-type *MED12*, whereas some fibroids harbored mutations in *MED12* and multiple mutational status was noted in different fibroids taken from one individual. By comparing WNT signaling pathway expression between *MED12*-mutated and wild-type fibroid samples, we found that *CTNNB1* (β -catenin) expression did not significantly change with *MED12* mutation status ($P=0.207$, $n=19$; Fig. 3A and B). Comparative analysis of *MED12* mutation-positive and *MED12* mutation-negative fibroid samples revealed significant differences in the levels of gene expression (Fig. 3C). Of all the genes, *FBXW11/BTRC*, E1A-associated protein p300 (*EP300*), *FBXW11*, *JUN*, *SOX17* and *TCF7L1* had significantly decreased expression levels in the *MED12*-mutation-positive fibroids compared to the *MED12* wild type (Fig. 3D). Conversely, expression of *SFRP1*, *WNT2*, *DKK1* and Lymphoid enhancer-binding factor (*LEF1*) was increased in those fibroids with *MED12* mutations (Fig. 3D). Intriguingly, we did not find any difference in *WNT4* expression between *MED12*-mutated and *MED12*-wild-type fibroids (Fig. 3A); this finding is in

Figure 1 Differential expression of WNT signaling components in fibroids and normal myometrium. (A) Representative pictures of multiple fibroids collected from the same patient. (B) RNA from each tissue sample was extracted and quantified by qPCR. The genes belonging to the WNT pathway that were significantly ($P < 0.05$) different in fibroid from the adjacent normal myometrium were listed in a heat-map. The panel was constructed with 19 fibroid tissue from 13 patients, samples were normalized with its own normal adjacent myometrium. (C) Comparison of β -catenin expression between normal adjacent myometrium with fibroids ($P < 0.05$). (D) Grouping of different genes into WNT ligands, receptors, activators, inhibitors and targets based on their reported functions. The expression level was normalized with paired myometrium, the relative values were used to generate the bar graph. * $P < 0.05$.

Figure 2 WNT pathway-specific alterations in fibroids and adjacent normal myometrium. (A) Grouping of genes based on the components in canonical WNT pathway, the WNT/PCP pathway, the WNT/Ca⁺⁺ pathway, and WNT signaling negative regulators. The expression level was normalized with paired myometrium, the relative values were used to generate the bar graph. **P* < 0.05. (B) Schematic diagram of three main pathways: canonical WNT signaling, Wnt/PCP, and Wnt/Ca⁺⁺ pathways.

contrast to a previous study that reported *WNT4* to be highly upregulated in *MED12*-mutated fibroids (Markowski *et al.* 2012). In opposition to the currently accepted paradigm that *MED12*-mutations act through the canonical WNT pathway to alter proliferation of fibroid cells (Markowski *et al.* 2012, Ono *et al.* 2013, 2014), we did not find any correlation between *MED12* mutation status and β -catenin expression

(Fig. 3A). Additionally, most canonical WNT pathway components did not show significant differences in expression between *MED12* mutation-positive and *MED12* mutation-negative samples (Fig. 3). This was consistent with findings by de Graaff and coworkers who suggested the involvement of pathways other than canonical WNT signaling in uterine leiomyogenesis (de Graaff *et al.* 2013). In summary, these results suggest

Figure 3 Changes in the expression of the members of the WNT signaling pathway in *MED12*-mutation-positive (*MED12*+) and negative (*MED12*-) fibroids. (A) Analysis of expression of the WNT pathway members in fibroids with and without *MED12* mutations. (B) Analysis of β -catenin expression in fibroids with *MED12*-mutations and normal *MED12* revealed no significant difference between these two groups. (C) Heat-map panel represents the genes of WNT signaling significantly ($P < 0.05$) different between *MED12*-mutation-positive and *MED12*-wild-type fibroids. All values were normalized with paired normal myometrium within each patient. (D) Graphs of normalized expression levels for the significantly altered genes of fibroids with and without *MED12*-mutations.

that genetic alterations in the *MED12* gene are unlikely to affect the WNT signaling pathway.

Proteomic analysis of uterine fibroids confirmed that β -catenin protein expression was increased in uterine fibroids compared to adjacent normal myometrium

To validate whether β -catenin expression was increased in fibroids, we used the most advanced method by comparing the proteomic status between adjacent normal myometrium and fibroids from three patients

using mass spectrometry. From each patient, we analyzed at least three fibroids and one adjacent normal tissue sample (Fig. 1). In agreement with our RNA data (Figs 1 and 2), we observed upregulation of β -catenin protein expression in fibroids compared to controls (Fig. 4A). In accordance with our array results (Fig. 3), β -catenin protein expression pattern was not correlated with *MED12*-mutation status. The proteomic data were confirmed using Western blot analysis (Fig. 4B). Quantification of protein bands revealed significant differences in β -catenin protein expression ($P = 0.0175$)

Figure 4 β-Catenin protein expression in fibroids was increased compared to the adjacent normal myometrium independent of the *MED12*-mutation status. (A) Mass spectrometry analysis of β-catenin in fibroids. Three fibroids and adjacent normal myometrial tissue samples were analyzed from each patient. (B) Equal amounts of tissue lysates from three patients were analyzed for β-catenin expression using Western blot. β-Actin was used as loading control. HEC1α cell line protein was used as a positive control for β-catenin expression. (C) Protein quantification of Western blot using NIH Image J. The intensity of β-catenin level for each patient was normalized with the corresponding control β-actin. (D) Immunohistochemistry of β-catenin. (a) Endometrium tissue used as a positive control. (b) Adjacent normal myometrium tissue, (c and d) uterine leiomyomas.

between myometrium and fibroids (Fig. 4C). The β-catenin protein expression was further validated with immunohistochemistry and showed relatively higher expression in fibroids compared to adjacent myometrium (Fig. 4Db, c and d). Human endometrium was used as a positive control for β-catenin staining (Fig. 4Da). Altogether, our protein analysis further supports our qPCR profiling whereby β-catenin appears to be upregulated at both RNA and protein levels in fibroids compared to adjacent normal myometrium.

Increase in ECM stiffness triggers the upregulation of β-catenin in fibroid cells

Our extensive analysis of the WNT pathway, both at the RNA and protein level, showed that β-catenin is overexpressed in fibroids compared to adjacent normal myometrium (Figs 1, 2, 3 and 4). However, we found no evidence for the upregulation of upstream positive regulators (WNT ligands) of β-catenin and instead, many negative regulators of WNT signaling were expressed at higher levels in fibroids compared to the adjacent normal myometrium (Figs 1, 2, 3 and 4). These findings suggest that β-catenin overexpression in fibroids does not occur through canonical WNT signaling and possibly involves alternative signaling mechanisms. To determine if an increase in the ECM stiffness leads to augmented β-catenin expression in uterine leiomyomas, we isolated primary fibroid cells and cultured these cells on hydrogels of known stiffness ($n=3$). Similar to the primary tumor sample, these cultured cells had a mutation in *MED12* (c.130G>A, p.G44S). We performed immunofluorescent staining to examine the level of β-catenin expression. The results revealed a significant increase in β-catenin expression with

increasing stiffness (Fig. 5A and B). In conclusion, these data highlight that changes in mechanical stiffness lead to increased expression of β-catenin in fibroids.

Discussion

MED12 is a subunit of the mediator complex, which mediates gene expression by communicating between transcription factors and RNA polymerase (Allen & Taatjes 2015). Mutations in *MED12* are the most common genetic alterations observed in human uterine leiomyomas, present in approximately 70% of cases (Mäkinen *et al.* 2011). Previous studies have shown *Med12* conditional knockout mice that express a common variant (c.131G>A), present with an earlier onset of uterine leiomyomas (Mittal *et al.* 2015), suggesting that this *Med12* mutation is sufficient to drive leiomyogenesis. *Med12* is essential for early mouse development because the loss of this gene, accompanied with abnormal Wnt/PCP and canonical Wnt signaling, results in embryonic defects in heart formation, axis elongation and somitogenesis (Rocha *et al.* 2010). In 2006, Kim and coworkers showed by performing immunoprecipitation that the mediator complex can bind directly to β-catenin through the *MED12* subunit (Kim *et al.* 2006). The interface between β-catenin and the *MED12* subunit was found between codon 1651 and 2086 of *MED12*. Whether the common *MED12* genetic aberrations in codon 44 affects the binding between β-catenin and the mediator complex remains unknown because the crystal structure of the *MED12* protein is yet to be characterized.

Many studies in the fibroid field have suspected the involvement of aberrant WNT signaling in the development of uterine fibroids (Bulun 2013).

Figure 5 ECM stiffness regulates β -catenin expression. Primary fibroids cells were extracted and seeded onto hydrogels with three different stiffness, 3, 12, and 40 kPa. (A) Representative immunofluorescence images of primary fibroid cells with increase stiffness. (B) Intensity quantification of immunofluorescence of β -catenin in three different hydrogel stiffness.

Indeed, over-activation of β -catenin in mouse uterine mesenchyme leads to the development of mesenchymal tumors that are similar to human uterine leiomyomas and tumors of *Med12* mouse models (Tanwar *et al.* 2009). However, no activating mutations in β -catenin (*CTNNB1*) or any other member of the WNT pathway have been identified in human fibroid patients despite reports by

many groups of the overexpression of β -catenin protein in uterine fibroids (Zaitseva *et al.* 2006, 2013). These findings have led to the hypothesis, that, somehow, the common *MED12* mutations alter WNT signaling, which in turn change the expression of β -catenin and results in leiomyoma pathogenesis (Markowski *et al.* 2012). In the present study, we examined the expression of different components of the WNT pathway in fibroids with, and without, *MED12* mutations. To minimize the influence of patient-to-patient variations, we also analyzed multiple fibroids from the same patient. Despite our extensive search using different techniques, we did not find any significant differences in the expression of β -catenin between fibroids with, or without, *MED12* mutations. Oddly, we found increased expression of DKK1 and SFRP1, known inhibitors of WNT signaling, in *MED12*-mutated fibroids compared to *MED12*-wild-type fibroids suggesting that WNT signaling might be downregulated in fibroids with *MED12* mutations. Interestingly, increased expression of SFRP1 in *MED12*-mutated fibroids was also recently reported in another study (Mehine *et al.* 2016).

ECM is a major component of uterine fibroids and excessive deposition of ECM proteins is responsible for the growth of fibroids and bulk type symptoms (Stewart *et al.* 2016). It is well established that changes in mechanical properties of tissues play a significant role in the development of organ systems, stem cell functions and pathogenesis of diseases. Du and coworkers using bone marrow mesenchymal stem cells and primary chondrocytes, presented evidence showing a concomitant increase in β -catenin levels with increasing ECM stiffness via activation of the integrin-FAK pathway, which may trigger further feedback loop in WNT signaling (Du *et al.* 2016). Uterine leiomyomas are very stiff tumors and are known to have hyperactive integrin-FAK signaling. We examined the effect of ECM stiffness on fibroid cells by culturing cells on hydrogels of known stiffness and found that β -catenin expression upregulates with increase in stiffness. These results provide a novel insight into how changes in the mechanical properties of fibroids cause changes in the expression of biochemical signals such as β -catenin.

Our comparative analysis of *MED12* mutation-positive and -negative fibroids revealed that β -catenin expression is not affected by the alterations in *MED12*. Our examination using primary fibroid cells provided evidence that ECM stiffness affects β -catenin expression. A recent study by Bloch and coworkers showed that *MED12*-mutation-positive cells progressively disappear during *in vitro* culture (Bloch *et al.* 2017). We performed Sanger sequencing on primary fibroid cells after three passages and still, able to detect the same *MED12* mutation that was present in the primary tumor. The differences in our results and results from study by Bloch and coworkers are probably because of different culture conditions (Bloch *et al.* 2017). Bloch and coworkers

have used RPMI-1640 supplemented with 20% fetal calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin for culturing fibroid cells, whereas we are using DMEM media supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum and 200 nmol/L glutamine. The lack of glutamine could have a profound effect on cell growth and survival.

One of the most surprising findings from our study was the observation that of all the WNT pathway members examined, WNT inhibitors showed the most significant difference in expression between fibroids and adjacent normal myometrium. For example, *WIF1* expression was on average 20-fold higher compared to normal adjacent myometrium. Similarly, expression of *APC*, *DKK1*, *DKK3*, *FBXW11*, *PRICKLE1*, *RHOA*, *SFRP4* and *SOX17* was significantly altered in fibroids compared to controls. Consistent with our findings, a previous study reported overexpression of *SFRP1* at both RNA and protein levels in uterine leiomyomas compared to the paired adjacent myometrium; *SFRP1* appears to exert an anti-apoptotic effect on the tumors (Fukuhara *et al.* 2002). Similarly, significantly increased levels of the WNT antagonists, *WIF1* and *SFRP1* were observed in fibroids with alterations in the *HMGA2* or *MED12* genes (Mäkinen *et al.* 2014). Collectively, these findings suggest that WNT signaling involvement in uterine leiomyogenesis is complex, and further studies are required to decipher the individual contributions of various components of the WNT pathway to the pathogenesis of this disease.

In summary, we have confirmed increased β -catenin expression in uterine leiomyomas compared to paired adjacent normal myometrium at both the post-transcriptional and translational level. We also proposed that ECM stiffness is the key to trigger upregulation of β -catenin through the integrin-FAK pathway.

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.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement

P B and P N: collected samples and clinical information. M F J and R J S: performed proteomic and mutation analysis. Y A Ko: performed qPCR arrays. Y A Ko and M A: cell culture work and analysis. Y A Ko and A M D: analysis of array data. Y A Ko and P S T: wrote the paper. P S T: designed the study, analyzed the data and provided financial support and final approval

of the manuscript. All authors approved and commented on the manuscript.

Acknowledgments

Work in the Tanwar lab was in part supported by funding from the National Health and Medical Research Council, the Australian Research Council, and the Cancer Institute NSW. P B is a recipient of the University of Newcastle Postgraduate Research Fellowship.

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Received 5 June 2017

First decision 10 July 2017

Revised manuscript received 16 September 2017

Accepted 23 October 2017