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Cadherin-11 Is a Regulator of Intestinal Fibrosis

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Abstract

Background and Aims: Although the mechanisms underlying the formation of intestinal fibrostrictures in Crohn's disease [CD] are not fully understood, activation of fibroblasts and excessive collagen deposition are supposed to contribute to the development of such complications. Here, we investigated the role of cadherin-11 [CDH-11], a fibroblast-derived protein that induces collagen production in various organs, in intestinal fibrosis.

Methods: CDH-11 expression was evaluated in inflammatory [I] and fibrostricturing [FS] CD mucosal samples, ulcerative colitis [UC] mucosal samples, and ileal and colonic control samples, by real-time polymerase chain reaction, western blotting, and immunohistochemistry. CDH-11 expression was evaluated in normal and in CD intestinal fibroblasts stimulated with inflammatory/fibrogenic cytokines. FS CD fibroblasts were cultured either with a specific CDH-11 antisense oligonucleotide [AS], or activating CDH-11 fusion protein and activation of RhoA/ROCK, and TGF-β pathways and collagen production were evaluated by western blotting. Finally, we assessed the susceptibility of CDH-11-knockout [KO] mice to colitis-induced intestinal fibrosis.

Results: CDH-11 RNA and protein expression were increased in both CD and UC as compared with controls. In CD, the greater expression of CDH-11 was seen in FS samples. Stimulation of fibroblasts with TNF-α, interleukin [IL]-6, IFN-γ, IL-13, and IL-1β enhanced CDH-11 expression. Knockdown of CDH-11 in FS CD fibroblasts impaired RhoA/ROCK/TGF-β signalling and reduced collagen synthesis, whereas activation of CDH-11 increased collagen secretion. CDH-11 KO mice were largely protected from intestinal fibrosis.

Conclusions: Data show that CDH-11 expression is up-regulated in inflammatory bowel disease [IBD] and suggest a role for this protein in the control of intestinal fibrosis.

Key Words: CDH-11, IBD, RhoA/ROCK pathway, TGF-β pathway

1. Introduction

Inflammatory bowel diseases [IBD], consisting of Crohn's disease [CD] and ulcerative colitis [UC], are chronic inflammatory conditions affecting the gastrointestinal tract.[1] In both IBD, the disease is clinically marked by periods of flare-ups [e.g. bloody diarrhoea, weight loss and growth failure, debilitating abdominal pain, fatigue], which are treated with conventional drugs [e.g. mesalamine, corticosteroids], and/or immunosuppressors, and/or biologics.[2] However, pharmacological treatment is not sufficient to halt the tissue-damaging inflammatory process. In these cases, as well as in patients developing local

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complications, intestinal surgery may become mandatory.[3] Intestinal fibrostrictures are the most common complications of CD, affecting more than one-third of the patients within 10 years of disease onset, and their development results in endoscopic or surgical treatment in more than two-thirds of patients at least once during their lifetime.[4,5] CD patients can also undergo multiple endoscopic or surgical treatments for symptomatic fibrostrictures over their lifetime.[6,7] Although the pathogenesis of CD-associated fibrostrictures is not fully understood, accumulating evidence suggests that fibrostrictures develop in genetically predisposed patients as a result of chronic activity of mucosal inflammatory cells, which stimulate excessive deposition of extracellular matrix [ECM] proteins by fibroblasts.[8,9] In the late stages of the disease, inflammation may be no longer required to sustain fibrogenesis, since fibroblasts can secrete various cytokines [e.g. TGF-β, IL-1β, and IL-6], which target adjacent stromal cells and activate multiple signalling pathways [e.g. Smad, activin receptor-like kinases, and Stat3], with the downstream effect of increasing further ECM deposition and perpetuating the fibrotic process.[10–13] To date no specific intestinal anti-fibrotic therapy exists, nor has any immunosuppressive or biologic drug been shown to prevent stricture formation. Therefore, the identification of molecules involved in the development and progression of fibrostrictures could help design novel antifibrotic drugs.

Cadherins are a family of adhesion molecules that mediate homophilic, calcium-dependent cellular adhesion by binding a cadherin of the same type on an adjacent cell.[14,15] Cadherin-11 [CDH-11], a type II cadherin, is expressed in osteoblasts,[16] mesenchymal cells,[17] and epithelial cells undergoing epithelial-mesenchymal transition.[18,19] Overexpression of CDH-11 has been documented in multiple fibroblast subtypes in the derma and lung, and preliminary evidence suggests the involvement of this protein in the development of fibrotic tissue in systemic sclerosis, idiopathic pulmonary fibrosis, and liver and kidney fibrosis.[20–27] Furthermore, in rheumatoid arthritis [RA], CDH-11 stimulates synovial fibroblasts to produce inflammatory cytokines and matrix metalloproteinase and, in experimental models of RA, neutralisation of CDH-11 function significantly reduces inflammation.[28–31] Additional studies suggest that CDH-11 could contribute to collagen deposition following injury, by triggering the RhoA/Rho/Rho-associated coiled-coil forming protein kinase [ROCK] and TGF-β pathways, and consequent activation of transcription factors necessary for collagen production, such as myocardin-related transcription factor-A [MRTF-A] and myocardin [MYOCD].[32,33]

Genome-wide expression analysis by microarray has previously documented up-regulation of CDH-11 RNA in inflamed colonic biopsies of IBD patients compared with healthy controls, even though the functional relevance of such data remains unclear.[34,35] In the present study, we investigated the expression and role of CDH-11 in intestinal fibrosis.

2. Materials and Methods

2.1. Patients and samples

Biopsy samples were taken from inflamed mucosa of 23 patients with active UC and 13 patients with active colonic CD, undergoing colonoscopy for a clinically active disease at the Gastrointestinal Unit of Tor Vergata University Hospital [Rome, Italy]. Paired biopsies were also taken from inflamed and uninflamed mucosa of 2 patients with ileocolonic CD and 6 patients with UC. Seven patients [three colonic CD and four UC] were taking no drug and biopsies were collected at the time of initial diagnosis, whereas the remaining patients were receiving therapy [i.e. mesalamine, steroids, or immunosuppressives]. Additionally, surgical specimens were taken from 3 patients with UC and 14 patients with inflammatory CD [I CD] undergoing surgery for a chronic active disease poorly responsive to medical treatment, and from 21 patients with fibrostricturing CD [FS CD] undergoing surgery for such a complication at Tor Vergata University Hospital. Clinical characteristics of IBD patients are shown in Table 1. Controls [CTR] included biopsies taken from unaffected colonic mucosa of 17 healthy subjects undergoing colonoscopy for colon cancer screening. Additional CTR were mucosal specimens taken from macroscopically and microscopically unaffected areas of colon in 23 patients undergoing surgery for colon cancer. Each patient who took part in the study gave written informed consent and the study protocol was approved by the local Ethics Committee [Tor Vergata University Hospital, Rome; protocol number:154/12].

2.2. Isolation and culture of intestinal fibroblasts

All the reagents were purchased from Sigma-Aldrich [Milan, Italy] unless otherwise specified. Intestinal fibroblasts were isolated from I CD, FS CD specimens and control mucosal samples, and were phenotypically characterised as described elsewhere.[36] Depending on the studies, fibroblasts were used either freshly isolated [for assessing CDH-11 expression in basal conditions] or between passages 3 and 8. Fibroblasts were maintained in 75 cm^2 plastic flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing high glucose with ultra glutamine and supplemented with 10% fetal bovine serum [FBS], 1% of penicillin [100 U/ml], streptomycin [100 μg/ml] solution, and 1% of non-essential amino acids [all from Lonza, Verviers, Belgium]. To determine whether CDH-11 expression is regulated by inflammatory/fibrogenic cytokines, 5 x 10^4 normal, I CD, FS CD fibroblasts were plated into each well of a 12-well plate, left to adhere for 24 h, then starved for 6 h, and finally either left unstimulated or stimulated with TNF-α [20 ng/ml, R&D Systems, Minneapolis, MN], IL-6 [50 ng/ml, Peprotech Ec Ltd, London], IFN-γ [100 ng/ml, Peprotech Ec Ltd,], IL-13 [20 ngml,

Table 1. Clinical characteristics of Crohn's disease patients and ulcerative colitis patients.

	CD	UC
	n = 50	*n* = 32
Gender, male: *n* [%]	27 [54]	20 [63]
Age: median [range]	38 [23–82]	49 [24–80]
CD location: *n* [%]		
Terminal ileum	17 [34]	
Pre-anastomotic ileum	10 [20]	
Ileocolon	10 [20]	
Colon	13 [26]	
UC extent: *n* [%]		
Distal colitis		9 [28.1]
Left-sided colitis		14 [43.7]
Extensive colitis		9 [28.1]
Current therapy: *n* [%]		
None	5 [10]	4 [12.5]
Systemic CS	12 [24]	11 [34.4]
Mesalamine	13 [26]	13 [40.6]
ISS	7 [14]	0 [0]
Anti-TNF	5 [10]	1 [3.1]
CS + ISS	4 [8]	1 [3.1]
CS + Anti-TNF	4 [8]	2 [6.3]

CD, Crohn's disease; UC, ulcerative colitis; CS, corticosteroids; ISS, immunosuppressors; TNF, tumour necrosis factor.

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R&D Systems], IL-1β [20 ng/ml, Peprotech Ec Ltd], IL-34 [50 ng/ml, R&D Systems], and TGF-β1 [2 ng/ml, R&D Systems], in fresh medium containing 0.05% bovine serum albumin. After 24 h, cells were collected and total proteins were extracted to analyse CDH-11 by western blotting. In additional experiments, 5 x 10^4 FS CD fibroblasts were plated into each well of a 12-well plate, left to adhere for 24 h, starved for 6 h and then either left untreated or transfected with a specific CDH-11 antisense oligonucleotide [AS] or scrambled AS [Src AS] [both used 2 µg/ml, Exiqon, Woburn, USA, product code: AS 300600, Src AS:300610] for 24–48 h, using Opti-MEM medium and Lipofectamine 3000 reagent according to the manufacturer's instructions [both from Life Technologies, Milan, Italy]. The efficiency of the transfection was determined by western blotting. Cell-free supernatants were analysed by Sircol Collagen Assay Kit for collagen content after 48 h [Biocolor Ltd, Belfast, UK].

2.3. CDH-11-Fc-stimulated fibroblasts

Non-tissue culture 12-well plates were coated with mouse anti-human FCγ-specific IgG1 [10 µg/ml, R&D Systems,] in attachment buffer overnight at 4°C as described previously.[32,37] Recombinant human CDH-11-Fc chimera [1 µg/cm^2, R&D Systems] or human control Fc [R&D Systems] were then added in binding buffer for 1 h at 37°C. For these studies, we used 3 x 10^4 cells per well in order to appreciate CDH-11 driven biological changes. After 2–48 h, cells and cell-free supernatants were harvested. Cells were used for protein and gene expression analysis, and cell-free supernatants were analysed for collagen and matrix metalloproteinase [MMP] expression.

2.4. Real-time polymerase chain reaction

A constant amount of RNA [0.5 µg/sample] was retrotranscribed into complementary DNA [cDNA], and then 1 µl of cDNA/sample was amplified using the following conditions: denaturation 1 min at 95°C; annealing 30 s at 58°C for CDH-11 and MRTF-A, at 59°C for MYOCD, at 60°C for COL1A1, COL3A1, α-SMA and β-actin, followed by 30 s of extension at 72°C. Primer sequences: CDH-11: forward, 5´-CAACCGAGATAACACAGCAG-3´ and reverse, 5´- ATGGGCAGAAGGTACAAGTC -3´; MRTF-A: forward, 5'- GGAGCTGGTGGAGAAGAAC-3' and reverse, 5´-CTGTGGAGTCGGTGATGAG-3´; MYOCD: forward, 5´-CCTGTCCAACGGCTTCTAC-3 and reverse, 5´-CGTGTGCTCCTGAGTTCTG-3´, COL1A1: forward, 5´-GGACACAGAGGTTTCAGTGG-3´, and reverse, 5´-GGTGACTTTGGAGACACAGG-3´, COL3A1: forward, 5´-GGAGAATGTTGTGCAGTTTGC–3´ and reverse, 5´-CGTTTGACGTGTTGTAAGAGG-3´ α-SMA: forward, 5´-TCTGGAGATGGTGTCACCCA-3´ and reverse, 3´-ACCCACTGTGGTAGAGGTCT-5'; β-actin: forward, 5'-AAGATGACCCAGATCATGTTTGAGACC-3' and reverse, 5'-AGCCAGTCCAGACGCAGGAT-3'. mRNA expression was calculated relative to the housekeeping β-actin gene on the base of the ΔΔCt algorithm.

2.5. Total protein extraction and western blotting

Fibroblasts and human and murine colonic samples were lysed on ice in buffer containing 10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.2 mM EGTA, and 0.5% Nonidet P40 supplemented with 1 mM dithiothreitol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 1 mM NaF. Lysates were clarified by centrifugation at 4°C for 30 min, and separated on 10% sodium dodecyl sulphate-poliacrilamide gel electrophoresis. CDH-11, COL1A1, COL3A1, p-MYPT-1, MRTF-A, MYOCD, Smad7, and p-Smad3 were detected using the following antibodies: mouse anti-human CDH-11 [final dilution 1:500, ThermoFisher Scientific, Langenselbold, Germany], rabbit anti-human COL1A1 [final dilution 1:1000, Novus Biological Italy], rabbit anti-human COL3A1 [final dilution 1:1000, Novus Biological], rabbit anti-human/mouse p-MYPT-1 [final dilution 1:1000, Abcam Cambridge, UK], rabbit anti-human/mouse MRTF-A [1:1000, Abcam Cambridge, UK], rabbit anti-human/mouse MYOCD [final dilution 1:1000, Abcam Cambridge, UK], rabbit anti-human/mouse p-Smad3 [final dilution 1:1000, Abcam Cambridge, UK], and mouse anti-human/mouse Smad7 [final dilution 1:1000, R&D Systems], respectively, followed by horseradish peroxidise-conjugated secondary IgG monoclonal antibodies [all used at final dilution 1:20000, Dako, Milan, Italy]. The reaction was detected with a sensitive enhanced chemiluminescence kit [Pierce, Rockford, IL]. After the analysis, blots were stripped and incubated with the following internal loading controls: rabbit anti-human vinculin [final dilution 1:5000, Abcam], and mouse anti-human-mouse β-actin [final dilution 1:5000, Sigma-Aldrich]. Computer-assisted scanning densitometry [Image-Lab 5.2.1, Bio-Rad Laboratories, Milan, Italy] was used to analyse the intensity of the immunoreactive bands.

MMP-1 and MMP-3 were evaluated in cell-free supernatants of FS CD fibroblasts by western blotting using mouse anti-human MMP-1 and anti-MMP-3 antibodies [1 mg/ml, R&D Systems].

2.6. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded ileal sections of CTR, I CD patients, FS CD patients, and UC colonic sections. The sections were deparaffinised and dehydrated through xylene and ethanol, and the antigen retrieval was performed in Tris EDTA citrate buffer [pH 7.8] in a thermostatic bath at 98°C [Dako] for 30 min. Immunohistochemical staining was performed using a rabbit monoclonal antibody directed against human CDH-11 [final dilution 1:60, Novus Biological] incubated at room temperature [RT] for 1 h, followed by a biotin-free HRP-polymer detection technology with 3,3'diaminobenzidine [DAB] as a chromogen [MACH 4 Universal HRP-Polymer Kit, Biocare Medical]. The sections were counterstained with haematoxylin, dehydrated, and mounted. Isotype control IgG-stained sections were prepared under identical immunohistochemical conditions as described above, replacing the primary antibody with a purified rabbit normal IgG control antibody [R&D Systems].

2.7. Immunofluorescence

Immunofluorescence was performed on frozen sections of ileal mucosal samples taken from, CTR, I CD, and FS CD patients. Samples were embedded in a cryostat mounting medium [Neg–50 Frozen Section Medium, Thermo Scientific], snap-frozen, and stored at -80°C. Sections [6 µm thick] were mounted onto superfrost glass slides and fixed in 4% paraformaldehyde [PFA] for 10 min at 4°C. Slides were washed three times with PBS, treated with 0.1% Triton X-100 for 20 min at RT. Blocking procedure was performed with 10% normal goat serum in PBS solution for 1 h at room temperature. Slides were then incubated overnight at 4°C with mouse anti-human CDH-11 [final diluition 1:50, ThermoFisher Scientific] and rabbit anti human α-SMA [final dilution 1:50, ThermoFisher Scientific]. After washing three times with PBS, slides were incubated for 1 h

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at room temperature with specific secondary antibodies coupled with Alexa Fluor Dyes [final dilution 1:2000, Invitrogen, Milan, Italy]. Coverslips were mounted on glass slides using ProLong Gold antifade reagent with DAPI [Invitrogen] to counterstain the DNA. Samples were analysed with a Leica DMI 4000 B fluorescence microscope [Leica, Wetzlar, Germany].

2.8. Acute trinitrobenzene-sulphonic acid [TNBS]-driven colitis and TNBS-induced colitis-associated colonic fibrosis

CDH-11 wild-type [WT] and CDH-11 knockout [KO] [CDH-11tm1Mta/HensJ] mice [8 weeks of age] were purchased from the Jackson Laboratory [Calco, Lecco, Italy] and hosted at the Plaisant animal facility [Rome, Italy]. All animal experiments were approved by the local Institutional Animal Care and Use Committee [authorization no.: 545/2016-PR, protocol no.: DE 15B.19 EXT 1] and in compliance with European rules [2010/63/UE]. For induction of acute colitis, mice were firstly pre-sensitised with trinitrobenzene-sulphonic acid [TNBS] and, after 8 days, TNBS [6 mg in 50% ethanol, 150 µl/mouse] was administered through a 3.5F catheter inserted into the rectum under light anaesthesia, as previously described.[38] Littermate control mice received only the vehicle. Mice were sacrificed at Day 5 after TNBS administration, to evaluate intestinal inflammation. Colonic sections were stained with haematoxylin and eosin [H&E], and the degree of inflammation was evaluated according the method described elsewhere.[39] In particular, the degree of intestinal inflammation was evaluated by summing the scores relative to epithelial damage [0, normal; 1, focal loss of goblet cells; 2, diffuse loss of goblet cells; 3, focal loss of crypts; 4, diffuse loss of crypts] and immune infiltrate [0, no immune infiltrate; 1, immune infiltrate around bases of crypts; 2, diffuse immune infiltrate along the layer of muscularis mucosae; 3, immune infiltrate in the mucosal layer; 4, immune infiltrate in both mucosal and submucosal layers].[39] Colitis-induced fibrosis was induced in mice by weekly intrarectal administration of TNBS under light anaesthesia, as previously described.[40] Each mouse received an incremental dose of TNBS over a period of 6 weeks. At Weeks 1 and 2, mice received 0.5 mg of TNBS in 30% ethanol; at Weeks 3 and 4, mice received 0.75 mg of TNBS in 45% ethanol; and at Weeks 5 and 6, mice received 1 mg of TNBS in 45% ethanol. Littermate control mice received only the vehicle. Animals were monitored daily and examined for signs of colitis including body weight loss, diarrhoea, and rectal bleeding and prolapse, and were killed 3 days after the last TNBS administration. Moreover, colonic swiss-roll sections were stained with Sirius Red Pricate and Masson's trichrome with aniline blue, according the manufacturer's instructions [Bio-Optica, Milan, Italy]. The degree of fibrosis on Masson's trichrome-stained sections was evaluated by a score system ranging from 0 to 3 on the basis of density and extent of trichrome-positive connective tissue staining and disruption of tissue architecture [0, normal; 1, mild; 2, moderate 3, severe], as previously described.[40]

2.9 Sircol assay

Total collagen was measured in fibroblast supernatants and mouse colonic samples by Sircol Collagen Assay Kit, in accordance with the manufacturer's instructions.

2.10 Statistical analysis

Differences between groups were compared using Student's t test and the Mann–Whitney U test. All the analyses were performed using Graph-Pad 5 software.

3. Results

3.1. CDH-11 expression is increased in the gut of patients with inflammatory bowel disease

To investigate whether CDH-11 expression is increased in IBD, RNA transcripts for CDH-11 were evaluated in colonic biopsies of IBD patients and CTR by real-time PCR. CDH-11 RNA transcripts were increased in inflamed colon of both UC patients and CD patients as compared with CTR [Figure 1A]. Analysis of CDH-11 in paired biopsies taken from involved and uninvolved areas of IBD patients showed that CDH-11 RNA expression was more pronounced in areas with active inflammation [Figure 1B]. Moreover, in IBD, CDH-11 RNA expression did not differ between patients receiving no therapy and those receiving drugs [Figure 1C]. Analysis of total proteins extracted from colonic mucosal samples by western blotting confirmed overexpression of CDH-11 in both IBD [Figure 1D]. Next, we assessed CDH-11 RNA transcripts and protein expression in ileal mucosal samples of CTR, I CD patients, and FS CD patients. In line with the above results, CDH-11 RNA and protein expression was significantly increased in I CD samples as compared with CTR [Figure 1E, F]. Notably, FS CD samples exhibited a more pronounced expression of CDH-11 RNA and protein as compared with I CD and ileal CTR samples [Figure 1E, F]. Immunohistochemical analysis confirmed the enhanced expression of CDH-11 in IBD and showed that, in FS CD, stromal cell expressed CDH-11 [Figure 1G]. By immunofluorescence, we also showed that CDH-11 co-localised with α-SMA in FS and I CD samples [Supplementary Figures 1 and 2, available as Supplementary data at *ECCO-JCC* online] even though staining was faint in I CD samples. In contrast, both CDH-11 and α-SMA were barely detectable in ileal control samples [Supplementary Figure 2]. In line with the above data, enhanced CDH-11 RNA expression was seen in fibroblasts isolated from ileal samples of FS CD patients as compared with control fibroblasts [Figure 1H].

3.2 CDH-11 expression is induced by fibrogenic cytokines in normal fibroblasts

The fact that CDH-11 is strongly expressed in FS CD samples prompted us to examine whether its production by stromal cells is regulated by inflammatory/fibrogenic cytokines.[41–46] Normal fibroblasts were stimulated with TNF-α, IL-6, IFN-γ, IL-13, IL-1β, IL-34, and TGF-β1, and CDH-11 protein was analysed by western blotting. CDH-11 production was enhanced by TNF-α, IL-6, IFN-γ, IL-13, and IL-1β, whereas IL-34 and TGF-β1 did not alter CDH-11 expression [Figure 2]. Similar results were seen when fibroblasts isolated from I CD and FS CD samples were stimulated with the above cytokines [Supplementary Figure 3, available as Supplementary data at *ECCO-JCC* online].

3.3. CDH-11 activates RhoA/ROCK and TGF-β signalling pathways and induces collagen production in Crohn's disease fibroblasts

The demonstration that CDH-11 is mainly expressed in FS CD samples, and that stromal cells are major producers of CDH-11,[32] prompted us to investigate whether CDH-11 regulates collagen production in the gut. To this end, CD fibroblasts isolated from fibrotic specimens were treated with a specific CDH-11 AS. Knockdown of CDH-11 [Figure 3A] led to a significant reduction of COL1A1 and COL3A1 RNA transcripts without affecting α-SMA RNA expression [Figure 3B–D]. Consistently, CDH-11 AS decreased COL1A1 and COL3A1 protein expression [Figure 3E]. Quantification of

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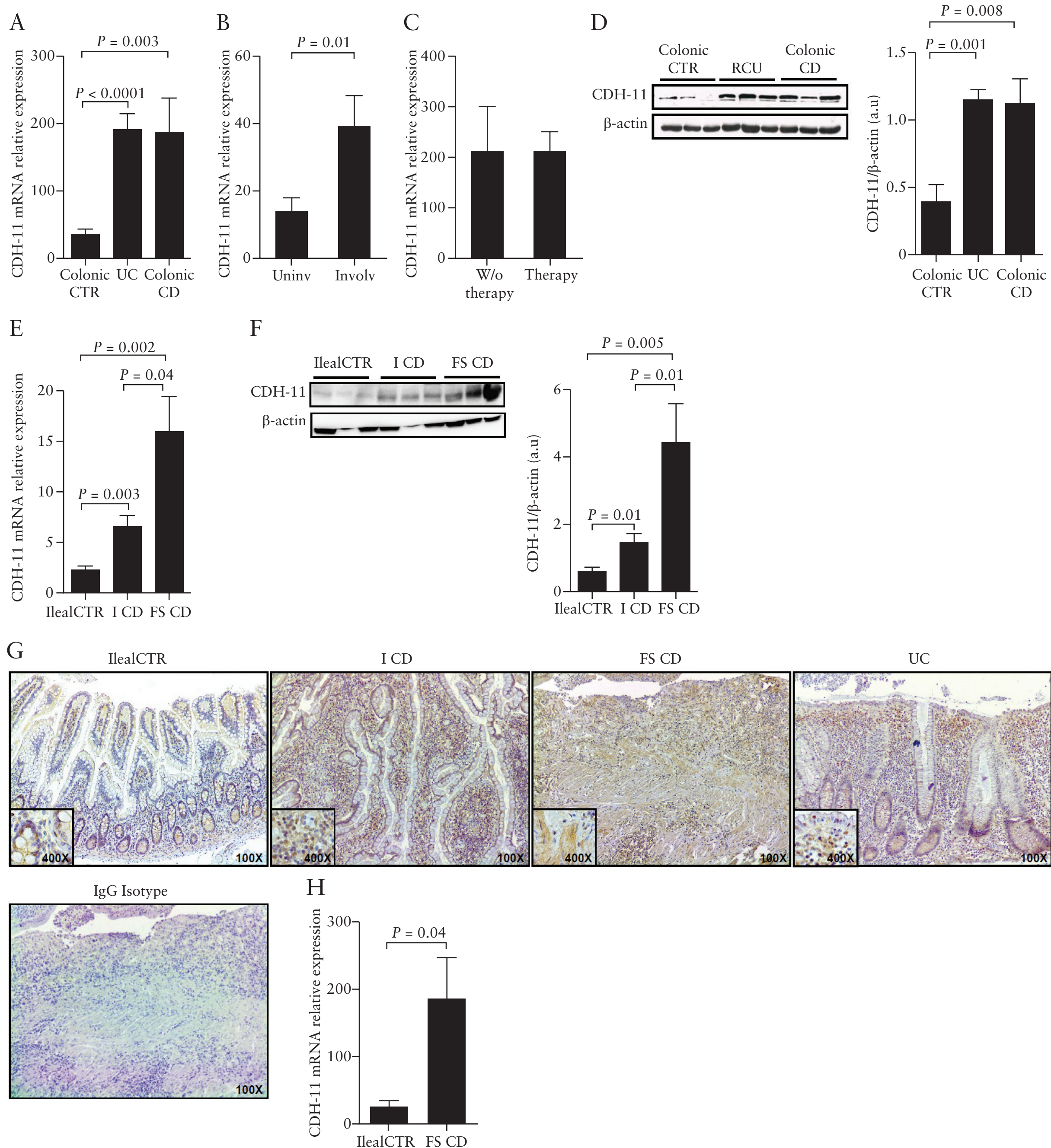

Figure 1. Cadherin [CDH]-11 RNA transcripts and protein are increased in inflammatory bowel disease [IBD]. A. Cadherin-11 [CDH-11] mRNA expression was evaluated in colonic biopsies taken from 17 controls [Colonic CTR], 23 patients with active ulcerative colitis [UC] and 13 patients with active colonic Crohn's disease [Colonic CD] by real-time PCR and levels were normalized to β-actin. B. Paired biopsies taken from the involved and uninvolved mucosa of 8 IBD patients were analyzed for CDH-11 RNA expression by real-time PCR. Levels are normalized to β-actin and data indicate mean ± SEM of all samples. C. CDH-11 RNA transcripts were assessed in biopsies taken from 7 IBD patients [4UC, 3 CD] receiving no therapy and 29 IBD patients [19 UC, 10 CD] on therapy by real-time PCR. Levels are normalized to β-actin and date indicate mean ± SEM of all samples. D. Representative Western blots showing CDH-11 and β-actin in total proteins extracted from mucosal samples of 3 colonic CTR, 3 UC patients and 3 colonic CD patients. Right panel shows the quantitative analysis of CDH-11/β-actin ratio in mucosal samples taken from 6 colonic CTR, 6 UC patients and 6 colonic CD patients as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all samples. E. CDH-11 mRNA expression was evaluated in ileal mucosal samples taken from 4 controls [Ileal CTR], 5 patients with inflammatory CD [I CD] and 5 patients with fibrostricturing CD [FS CD] by real-time PCR. Levels are normalized to β-actin and date indicate mean ± SEM of all samples. F. Representative Western blots showing CDH-11 and β-actin in total proteins extracted from mucosal samples of 3 ileal CTR, 3 I CD patients and 3 FS CD patients. Right panel shows the quantitative analysis of CDH-11/β-actin ratio in mucosal samples taken from 6 leal CTR, 6 I CD patients and 6 FS CD patients as measured by densitometry scanning of Western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all samples. G. Representative photomicrographs [100X original magnification] of CDH-11-stained paraffin-embedded sections of surgical samples taken from 1 ileal CTR, 1 patient with I CD, 1 patient with FS CD and 1 patient with UC. The figure is representative of separate experiments in which sections taken from 4 ileal CTR, 3 patients with ileal I CD, 5 patients with FS CD and 3 patients with UC were analyzed for CDH-11. Isotype control antibody-stained FS CD sections is also shown. Inserts show higher magnification [400X] images. H. CDH-11 mRNA expression was evaluated in fibroblasts isolated from ileal mucosal samples of 4 CTR and 4 patients with FS CD by real-time PCR. Levels are normalized to β-actin and data indicate mean ± SEM of all samples. PCR, polymerase chain reaction; SEM, standard error of the mean.

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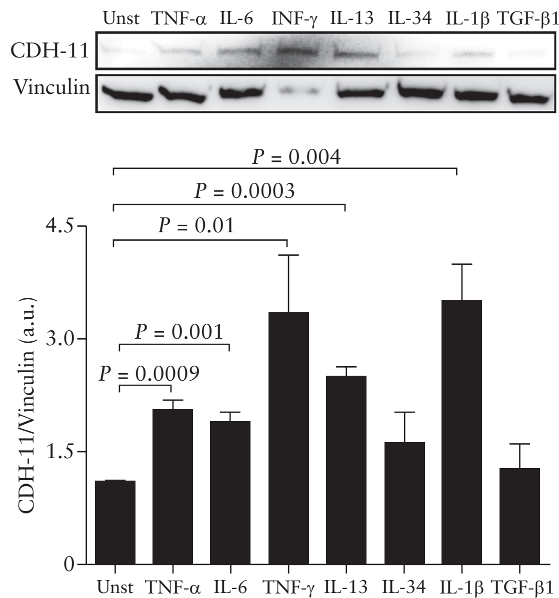

Figure 2. CDH-11 expression is induced by profibrogenic cytokines in normal fibroblasts. Representative western blots showing CDH-11 and vinculin in total proteins extracted from control fibroblasts isolated and treated with or without [unstimulated = Unst] cytokines for 24 h. Bottom panel shows the quantitative analysis of CDH-11/vinculin ratio in fibroblast protein extracts. Values are expressed in arbitrary units [a.u.] and indicate mean ± standard error of the mean [SEM] of six independent experiments.

soluble forms of collagen in the supernatants of fibroblast cultures confirmed that CDH-11 knockdown was associated with significant reduction of collagen synthesis [Figure 3F].

Studies in CDH-11 deficient mice have recently shown that CDH-11 controls mechanical strength and collagen and elastin content in smooth muscle- and myofibroblast-containing tissues, such as aorta, bladder, and skin.[32] Analysis of the basic mechanisms by which CDH-11 regulates stromal cell function showed that CDH-11 activates the TGF-β and RhoA/ROCK pathways, thus leading to the induction of MRTF-A and MYOCD, two transcription factors that up-regulate collagen synthesis.[32,33,47,48] Therefore, we next examined whether CDH-11 AS-mediated collagen production inhibition was paralleled by changes in RhoA/ROCK and TGF-β pathways. Treatment of FS CD fibroblasts with CDH-11 AS associated with reduced phosphorylation of MYPT-1, a downstream event in the RhoA/ROCK pathway activation,[49] and Smad3, a protein involved in the TGF-β-driven signalling[50] [Figure 4A]. CDH-11 AS increased Smad7, a negative regulator of TGF-β/Smad3 signalling.[50] Consistently, cells treated with CDH-11 AS exhibited reduced RNA and protein expression of MRTF-A and MYOCD [Figure 4B, C]. To further confirm the inducing effect of CDH-11 on collagen synthesis, we treated CD fibroblasts with recombinant human CDH-11-Fc in order to trigger CDH-11 mediated signalling. Since both p-MYPT-1 and p-Smad3 are highly expressed in unstimulated cells, the subsequent western blotting studies were conducted using reduced amounts of proteins, in order to appreciate any up-regulation following CDH-11 activation. As shown in Figure 5A, engagement of CDH-11 increased phosphorylation of MYPT-1 and Smad3. In line with this, CDH-11 activation increased expression of MRTF-A, MYOCD, COL1A1, COL3A1 RNA, protein expression, and soluble forms of collagen without affecting α-SMA RNA expression [Figure 5B–G]. However, in contrast to previous studies,[51,52] CDH-11 activation in FS CD fibroblasts did not alter production of MMP-1 and MMP-3 [Supplementary Figure 4, available as Supplementary data at *ECCO-JCC* online], two enzymes involved in the control of ECM deposition.[53,54]

3.4. Intestinal fibrosis is decreased in CDH-11 knockout mice

To translate the *in vitro* data to *in vivo*, we used a murine model of colitis-associated fibrosis induced by repeated rectal administration of low doses of TNBS.[40] Histological analysis of colonic swiss-roll sections and histological score analysis showed that both WT and CDH-11 KO mice developed colitis following TNBS administration, with no significant difference between the groups [Supplementary Figure 5, available as Supplementary data at *ECCO-JCC* online]. Similar results were seen when TNBS-driven acute colitis was induced in WT and CDH-11 mice [Supplementary Figure 6, available as Supplementary data at *ECCO-JCC* online]. Masson's trichrome staining and the histological fibrotic score revealed that both WT and CDH-11 KO mice developed intestinal fibrosis following TNBS administration, but a reduced amount of collagen deposition in the subepithelial and serosal areas was seen in CDH-11 KO mice as compared with WT mice after TNBS administration [Figure 6A]. These data were confirmed by Sirius Red Picrate staining [Figure 6B]. Moreover, analysis of soluble forms of collagen in mouse colon explants confirmed that CDH-11 KO mice have a significant reduction of intestinal collagen production compared with WT mice after TNBS administration [Figure 6C]. Western blotting analysis of total proteins extracted from colonic tissue samples of wild-type and CDH-11 KO mice following TNBS treatment showed a reduction expression of p-MYPT-1, p-Smad3, MRTF-A, and MYOCD in CDH-11 KO mice [Figure 7]. In contrast to the finding documented in FS CD fibroblasts following CDH-11 knockdown showing up-regulation of Smad7, Smad7 expression did not differ between CDH-11 KO and WT mice [Figure 7]. This finding could rely on the fact that, in inflamed intestine, Smad7 is over-produced by multiple mucosal cell types other than fibroblasts, and CDH-11 deficiency did not affect the course of colitis [Supplementary Figures 5, 6].

4. Discussion

Up-regulation of CDH-11 gene expression has been recently documented in IBD tissue through genome-wide studies[34,35] but the role of this protein in the pathogenesis of these disorders remains unknown. Here we assessed the expression and role of CDH-11 in IBD. By using several molecular techniques, we initially showed that CDH-11 expression was enhanced in inflamed colon of patients with UC and patients with CD as compared with normal controls, and this occurred at both RNA and protein levels. In IBD, CDH-11 was produced at greater level in inflamed areas, and such production was not influenced by current therapy, suggesting that induction of CDH-11 is secondary to the ongoing mucosal inflammation. When CD patients were stratified taking into account their phenotype, a more pronounced expression of CDH-11 was seen in FS samples. These data confirm and expand on results of previous studies documenting enhanced induction of CDH-11 in fibrotic tissues in many organs, including lung, liver, and kidney.[20,24,26,31] By immunostaining, we were also able to document marked positivity of CDH-11 in the stromal compartment of FS CD samples. Consistently, CDH-11 expression was increased in fibroblasts isolated from FS CD samples as compared with control cells.

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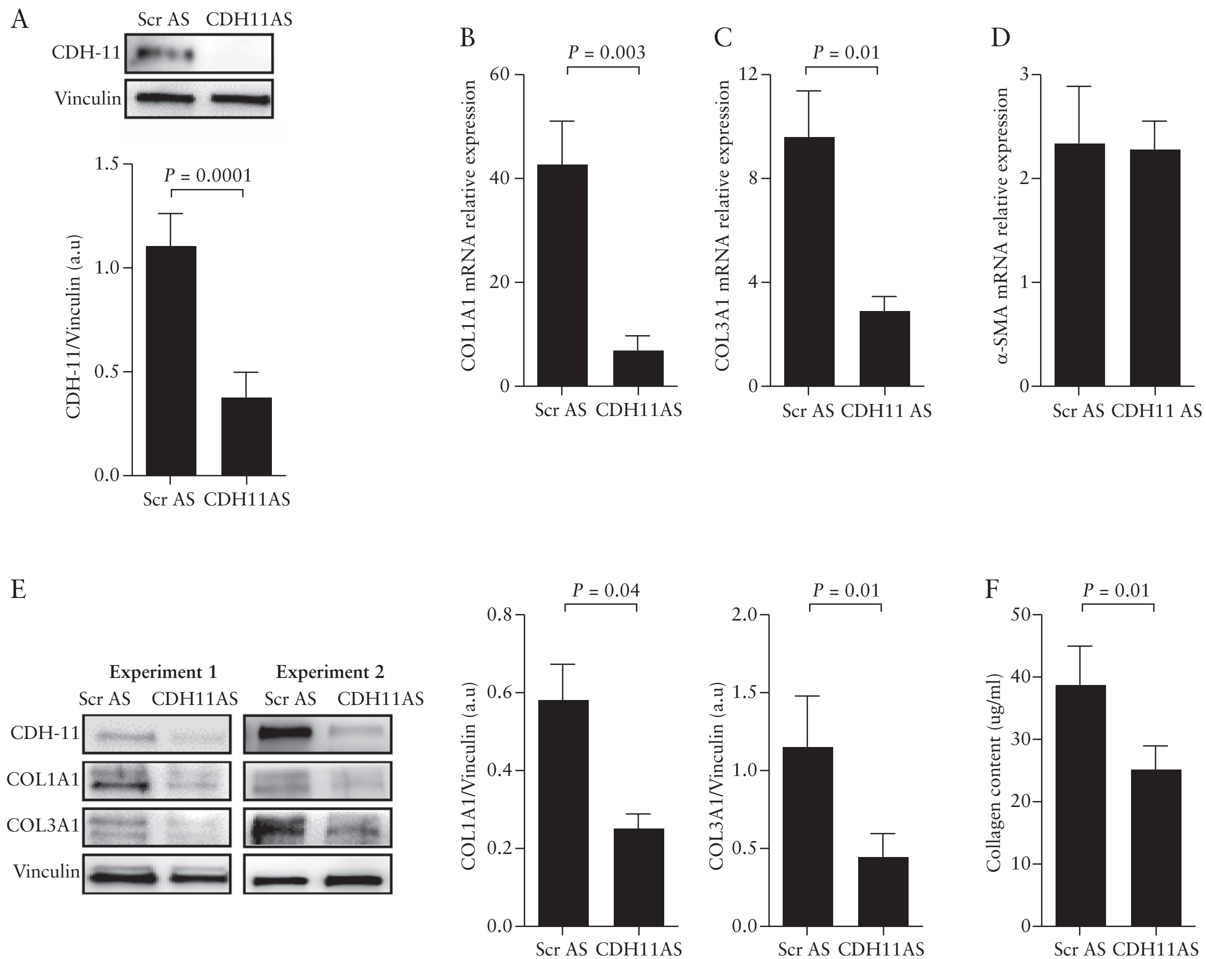

Figure 3. Knockdown of CDH-11 reduces collagen production in fibrostricturing CD fibroblasts. A. Fibrostricturing CD [FS CD] fibroblasts were transfected with scrambled negative control oligonucleotide [SCR AS] or CDH-11 antisense oligonucleotide [CDH11AS] for 24 h. CDH-11 and vinculin were analysed by western blotting. One of five independent experiments is shown. Bottom panel shows the quantitative analysis of CDH-11/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. B-D. FS CD fibroblasts were transfected with either SCR AS or CDH11AS for 24 h, and COL1A1 [B], COL3A1 [C], and α-ASMA [D] RNA transcripts were analysed by real-time PCR. Levels are normalised to β-actin. Data indicate mean ± SEM of five independent experiments. E. FS CD fibroblasts were transfected with either SCR AS or CDH11AS for 24 h, and CDH-11, COL1A1, COL3A1, and vinculin were analysed by western blotting. Two of five independent experiments are shown. Right panels show the quantitative analysis of the COL1A1/vinculin ratio and COL3A1/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. F. FS CD fibroblasts were transfected with either SCR AS or CDH11AS for 24 h, and total content of collagen was analysed by a colorimetric assay in supernatants. Data indicate mean ± SEM of three independent experiments. CD, Crohn's disease; PCR, polymerase chain reaction; SEM, standard error of the mean.

Circumstantial evidence indicates that, during intestinal fibrogenesis, fibroblast function is tightly controlled by both autocrine and paracrine mechanisms. In particular, many immune cells infiltrating the inflamed gut produce cytokines that stimulate adjacent fibroblasts to make collagen and other mediators of the fibrogenetic process.[13] Therefore, we investigated whether inflammatory/fibrogenic cytokines, which are produced in excess in IBD, enhanced CDH-11 expression in normal, I CD, and FS CD intestinal fibroblasts. Among the cytokines evaluated, both antigen-presenting cell-derived cytokines, such as TNF-α, IL-6, and IL-1β, and T cell-derived cytokines, such as IFN-γ and IL-13, increased CDH-11 expression, whereas no significant change in CDH-11 protein synthesis was seen when cells were stimulated with IL-34 or TGF-β1. Studies are now ongoing to assess the basic mechanism by which the above cytokines regulate CDH-11. Nonetheless, these results suggest the hypothesis that the local inflammatory milieu is crucial for inducing CDH-11 in intestine, as reported in rheumatoid arthritis.[28,31]

To mechanistically prove the contribution of CDH-11 in intestinal fibrosis, we inhibited CDH-11 in FS CD fibroblasts with a specific AS. Knockdown of CDH-11 was accompanied by a significant reduction of collagen transcripts and protein. Analysis of signalling pathways, which are known to mediate the CDH11-inducing effect of collagen, revealed that CDH-11 knockdown associated with disruption of RhoA/ROCK and TGF-β signalling pathways.[32] This was evidenced by reduced phosphorylation of MYPT-1 and Smad3 and diminished expression of MRTF-A and MYOCD, two transcription factors that interact with serum response factor [SRF] and bind on CArGbox regulatory elements, with the down-stream effect of inducing the transcription of SRF-responsive genes involved in fibrosis.[55–58] The above data were confirmed by CDH-11 engagement experiments showing that CDH-11 activation in FS CD fibroblasts enhanced collagen synthesis without affecting expression of α-SMA, a marker of activation of myofibroblasts. This finding did not come as a surprise for us, as previous studies showed that CDH-11 regulates collagen production

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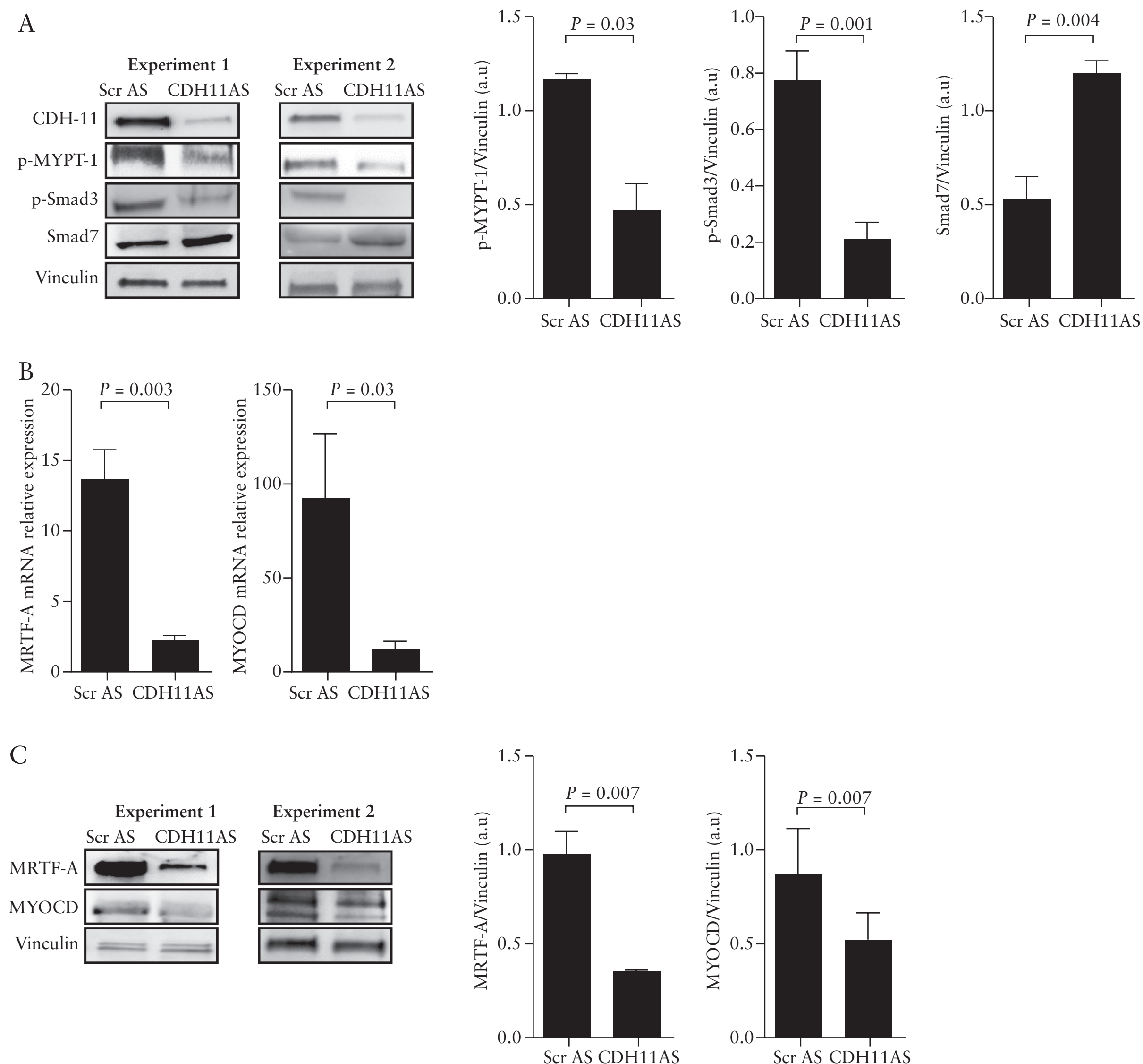

Figure 4. Knockdown of CDH-11 reduces phosphorylation of both MYPT-1 and Smad3 in fibrostricturing CD fibroblasts. A. Fibrostricturing CD [FS CD] fibroblasts were transfected with either SCR AS or CDH11AS for 24 h, and CDH-11, p-MYPT-1, p-Smad3, Smad7, and vinculin were analysed by western blotting. Two of three independent experiments are shown. Right panels show the quantitative analysis of the p-MYPT-1/vinculin ratio, p-Smad3/vinculin ratio, and Smad7/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. B. FS CD fibroblasts were transfected with either SCR AS or CDH-11AS as described above, and MRTF-A and MYOCD RNA transcripts were analysed by real-time PCR. Levels are normalised to β-actin. Data indicate mean ± SEM of five independent experiments. C. FS CD fibroblasts were transfected with either SCR AS or CDH11AS as described above, and MRTF-A, MYOCD, and vinculin were analysed by western blotting. Two of three independent experiments are shown. Right panels show the quantitative analysis of the MRTF-A/vinculin ratio and MYOCD/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of estern blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. CD, Crohn's disease; PCR, polymerase chain reaction; SEM, standard error of the mean.

but not α-SMA expression in other systems. For instance, Lodyga *et al.* showed that engagement of CDH-11 on myofibroblasts by the human CDH-11 fusion protein did not affect α-SMA amounts.[27] In this context, it is also noteworthy that α-SMA is an inconsistent marker of collagen-producing fibroblasts in experimental models of organ fibrosis[59] and, therefore, it was logical to expect reduction of collagen but not of α-SMA following CDH-11 inhibition.

The CDH11-mediated positive regulation of collagen production in the intestine was then supported by *in vivo* studies, which demonstrated that CDH-11 KO mice produced less collagen and developed a decreased colitis-driven intestinal fibrosis as compared with controls. Altogether, our findings are in agreement with those generated in murine models of skin, lung, and liver fibrosis, in which CDH-11 deficiency was associated with a significant decrease of collagen deposition and fibrosis.[20,24,25] Loss of CDH-11 did not affect the development of TNBS-induced colitis, supporting further the direct effect of CDH-11 on intestinal fibrosis. These later results conflict with those generated in the K/BxN serum transfer mouse model of arthritis, in which CDH-11 deficient mice developed approximately 50% less joint inflammation compared with wild-type mice and were almost completely protected from cartilage erosions, despite ongoing bone erosion.[30] It is thus tempting to speculate that, depending on the context analysed, CDH-11 can promote activation of signals that ultimately propagate tissue-damaging inflammatory responses.

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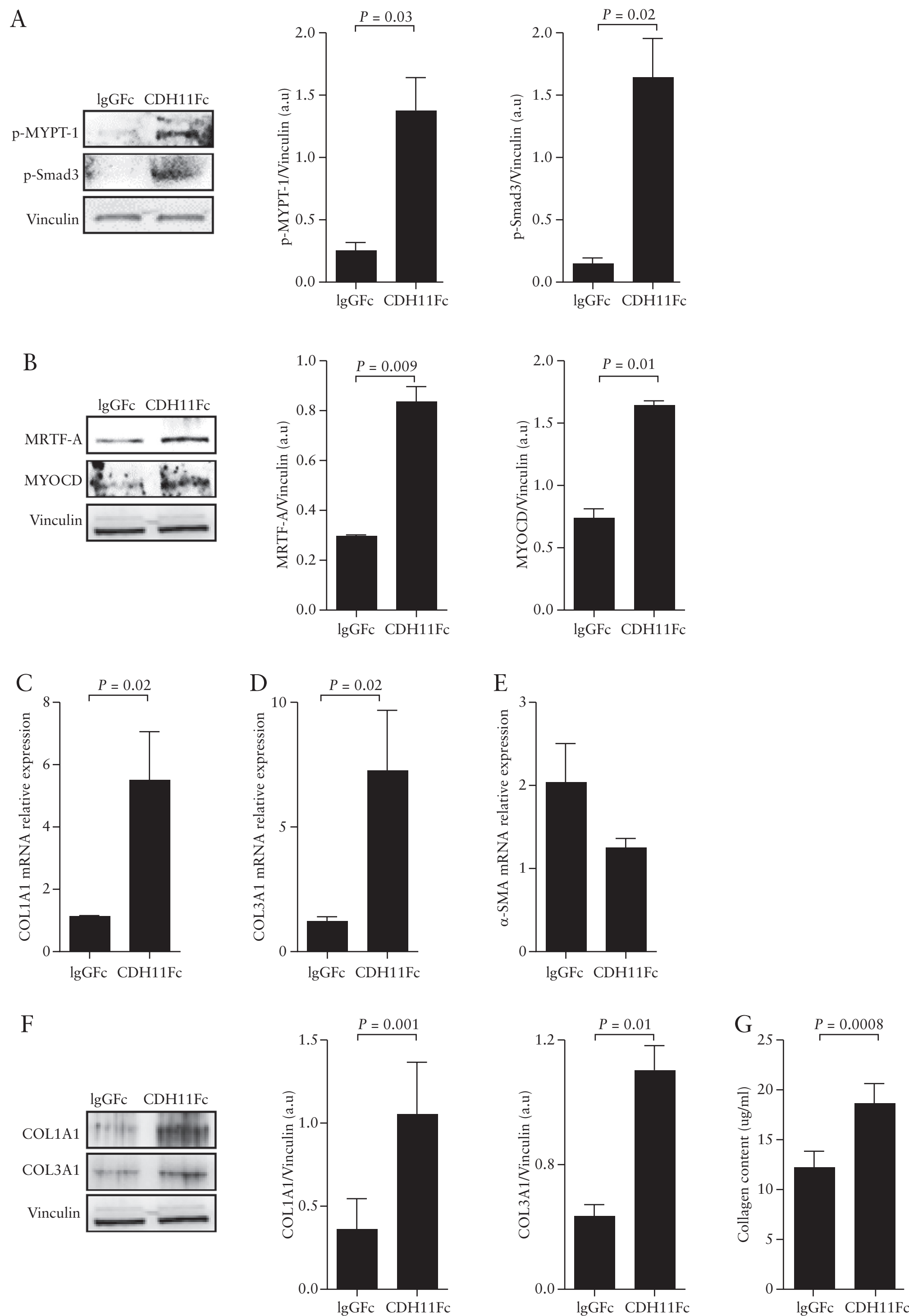

Figure 5. CDH-11 activation induces collagen production. A. Fibrostricturing [FS] CD fibroblasts were plated on CDH-11-Fc [CDH11Fc]- or IgG-Fc [IgGFc]-coated culture plates for 2 h, and p-MYPT-1, p-Smad3, and vinculin were analysed by western blotting. One of three independent experiments is shown. Right panels show the quantitative analysis of the p-MYPT-1/vinculin ratio and p-Smad3/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. B. FS CD fibroblasts were treated as described above for 24 h, and MRTF-A, MYOCD, and vinculin were analysed by western blotting. One of three independent experiments is shown. Right panels show the quantitative analysis of the MRTF-A/vinculin ratio and MYOCD/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. C-E. FS CD fibroblasts were treated as described above for 24 h, and COL1A1 [C], COL3A1 [D], and α-SMA [E] RNA transcripts were analysed by real-time PCR. Levels are normalised to β-actin. Data indicate mean ± SEM of three independent experiments. F. FS CD fibroblasts were treated as described above for 24 h, and COL1A1, COL3A1, and vinculin were analysed by western blotting. One of three independent experiments is shown. Right panels show the quantitative analysis of the COL1A1/vinculin ratio and COL3A1/vinculin ratio in FS CD fibroblast protein extracts as measured by densitometry scanning of western blots. Values are expressed in arbitrary units [a.u.] and indicate mean ± SEM of all experiments. G. Total content of collagen in supernatants of FS CD fibroblasts, treated as above described for 48 h, was analysed by a colorimetric assay. Data indicate mean ± SEM of three independent experiments. CD, Crohn's disease; PCR, polymerase chain reaction; SEM, standard error of the mean.

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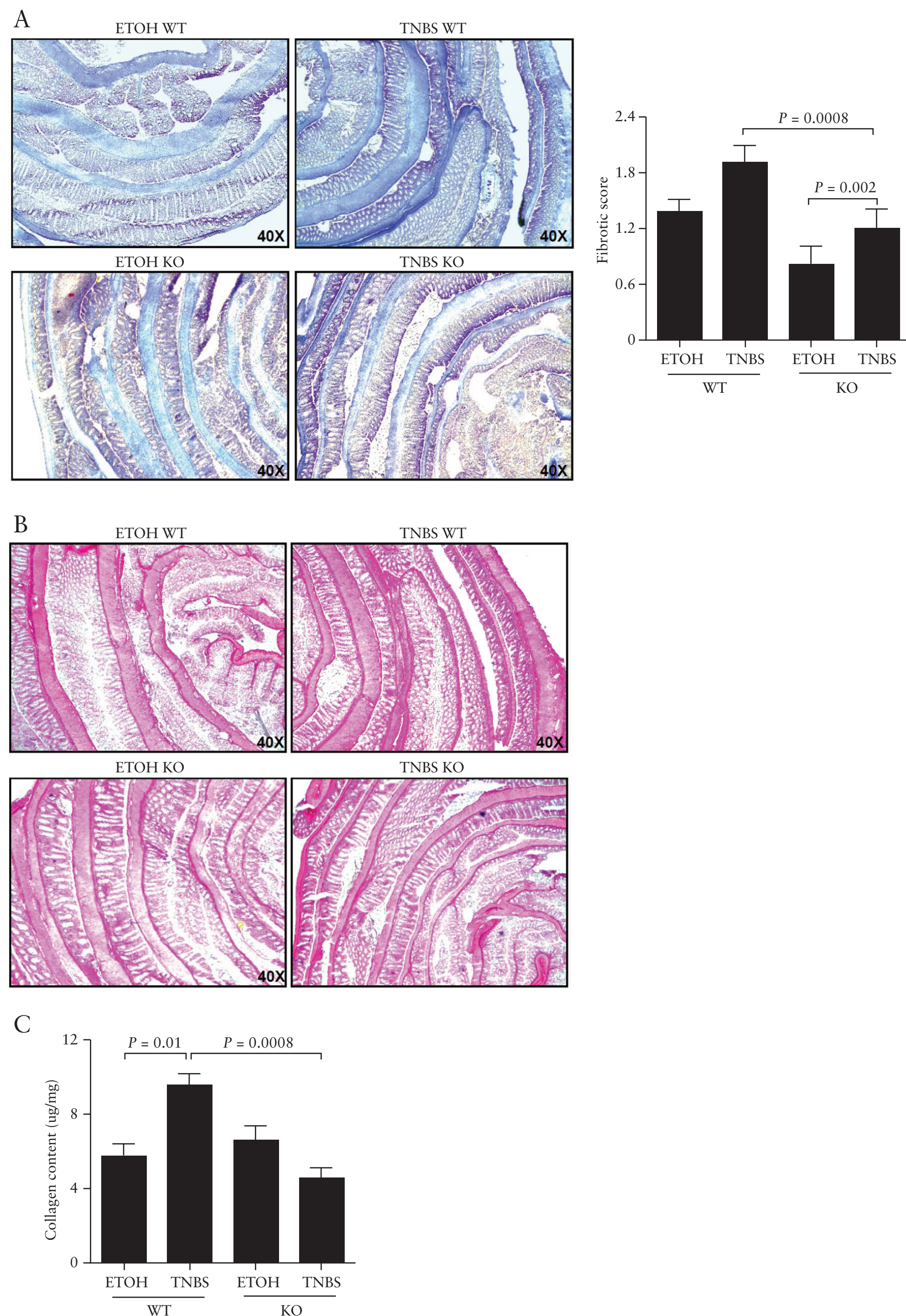

Figure 6. TNBS-induced intestinal fibrosis is reduced in CDH-11 knockout mice. A. Representative photomicrographs [40x original magnification] of colonic swiss-roll sections taken from ETOH- or TNBS-treated CDH-11 wild-type [WT] mice or CDH-11 knockout [KO] mice and stained with Masson's trichrome. Right panel shows the quantitative analysis of fibrotic score of intestinal sections taken from five WT mice and five CDH-11 KO mice treated with ETOH, and five WT mice and five CDH-11 KO mice treated with TNBS. Data indicate mean ± standard error of the mean [SEM] of all samples. B. Representative photomicrographs [40x original magnification] of colonic swiss-roll sections taken from ETOH- or TNBS-treated WT mice and CDH-11-KO mice treated as above, and stained with Picro Sirius Red. C. Soluble collagen measured by Sircol assay in extracts of colon samples taken from mice treated as above, analysed. Data indicate mean ±SEM of all samples.

The demonstration that CDH-11 knockdown in CD fibroblasts reduces collagen production, and up-regulates Smad7, could appear to conflict with our previous study showing the efficacy of an oral Smad7 antisense oligonucleotide on TNBS-driven colitis-induced fibrosis.[60] In this context however, it is noteworthy that Smad7 was induced at early stages of TNBS colitis, and mice given Smad7 antisense oligonucleotide exhibited marked amelioration of the ongoing colitis, which was accompanied by significant reduction of colonic fibrosis. This suggests that the effect of Smad7 antisense oligonucleotide on intestinal fibrosis is mainly due to the suppression of colitis.

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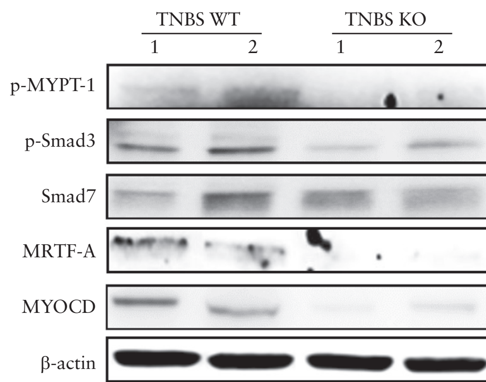

Figure 7. RhoA/ROCK and TGF-β signalling pathways are reduced in CDH-11 knockout [KO] mice with TNBS-induced intestinal fibrosis. Representative western blots showing p-MYPT-1, p-Smad3, Smad7, MRTF-A, MYOCD, and β-actin in total proteins extracted from colonic samples of two wild-type [WT] and two CDH-11-KO mice treated with TNBS. One of two experiments analysing five mice per group is shown.

The functional relevance of Smad7 up-regulation in fibroblasts following CDH-11 knockdown remains to be determined, even though we do not exclude the possibility that, in fibroblasts, Smad7 can control additional biological functions, such as cell growth and survival, perhaps through a TGF-β1 independent mechanism, as shown in other non-immune cell types.[61]

In conclusion, our study shows that CDH-11 is highly produced in inflamed tissue of IBD patients, and suggests a key role for this protein in positively regulating collagen production and intestinal fibrosis.

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Conflict of Interest

GM has served as an advisory board member for Abbvie. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

EF performed experiments, analysed data, wrote manuscript; IM, FL, AR, VD, DDF, AC, AO performed experiments; PG, SDC, GS, ADS contributed reagents; GM designed experiments and wrote the manuscript.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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