Gut and Liver
https://doi.org/10.5009/gnl220308
pISSN 1976-2283 eISSN 2005-1212

Original Article

Downregulation of Heat Shock Protein 72 Contributes to Fibrostenosis in Crohn's Disease

Seung Won Kim[1,2], Jae-Young Lee[3], Han Cheol Lee[3], Jae Bum Ahn[3], Ji Hyung Kim[1], I Seul Park[1], Jae Hee Cheon[1,2], and Duk Hwan Kim[4]

[1]Department of Internal Medicine and Institute of Gastroenterology, Graduate School of Medical Science, Brain Korea 21 Project, [2]Severance Biomedical Science Institute, and [3]Department of Medicine, Yonsei University College of Medicine, Seoul, and [4]Digestive Disease Center, CHA Bundang Medical Center, CHA University, Seongnam, Korea

Article Info
Received July 18, 2022
Revised October 21, 2022
Accepted November 29, 2022
Published online February 23, 2023

Corresponding Author
Duk Hwan Kim
ORCID https://orcid.org/0000-0003-3841-5802
E-mail dhkim@cha.ac.kr

Jae Hee Cheon
ORCID https://orcid.org/0000-0002-2282-8904
E-mail GENIUSHEE@yuhs.ac

Background/Aims: Crohn's disease (CD) with recurrent inflammation can cause intestinal fibrostenosis due to dysregulated deposition of extracellular matrix. However, little is known about the pathogenesis of fibrostenosis. Here, we performed a differential proteomic analysis between normal, inflamed, and fibrostenotic specimens of patients with CD and investigated the roles of the candidate proteins in myofibroblast activation and fibrosis.

Methods: We performed two-dimensional difference gel electrophoresis and identified candidate proteins using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and orbitrap liquid chromatography-mass spectrometry. We also verified the levels of candidate proteins in clinical specimens and examined their effects on 18Co myofibroblasts and Caco-2 intestinal epithelial cells.

Results: We identified five of 30 proteins (HSP72, HSPA5, KRT8, PEPCK-M, and FABP6) differentially expressed in fibrostenotic CD. Among these proteins, the knockdown of heat shock protein 72 (HSP72) promoted the activation and wound healing of myofibroblasts. Moreover, knockdown of HSP72 induced the epithelial-mesenchymal transition of intestinal epithelial cells by reducing E-cadherin and inducing fibronectin and α-smooth muscle actin, which contribute to fibrosis.

Conclusions: HSP72 is an important mediator that regulates myofibroblasts and epithelial-mesenchymal transition in fibrosis of CD, suggesting that HSP72 can serve as a target for antifibrotic therapy. **(Gut Liver 2023;17:905-915)**

Key Words: Crohn disease; Epithelial-mesenchymal transition; Fibrostenosis; Myofibroblast; Heat-shock proteins

INTRODUCTION

Crohn's disease (CD) is a relapsing and destructive disorder of the gastrointestinal tract characterized by recurrent episodes of chronic inflammation and injury,[1-5] followed by wound healing.[6-8] These recurrent cycles of CD cause accumulation of submucosal extracellular matrix (ECM) and progress to structural fibrosis and eventually stenosis and intestinal failure.[9] Because there is no treatment other than surgery in such cases, patients with fibrostenotic CD have an increased risk of surgery.[10,11] Although fibrosis is initiated by chronic inflammation, suppression of inflammation alone is not sufficient for reversing fibrosis.[12] There is no currently approved fibrosis treatment except for that of idiopathic pulmonary fibrosis.[13] Therefore, there is an unmet need to explore targets for therapeutics and the diagnosis of intestinal fibrosis.[14]

Fibrostenotic CD is aggravated by increased inflammatory cytokines, growth factors, and other mediators produced by inflammation.[15,16] Fibrostenosis in the intestine has typical characteristics similar to those of fibrosis in other organs, displaying excess deposition of ECM compo-

nents such as collagens and fibronectins and ECM remodeling by activated myofibroblasts, α-smooth muscle actin (α-SMA)-positive mesenchymal cells, and matrix metalloproteinases.[17] Therefore, control of ECM deposition by suppression of profibrotic myofibroblasts or myofibroblast transition is a pivotal target for therapeutics and the diagnosis of fibrosis. Although the origin of myofibroblasts is unclear, fibroblasts and epithelial cells are considered the main sources. Myofibroblast activation enhances cell mobility through conformational changes of cytoskeletons and intermediates. Other than myofibroblasts, the epithelial-to-mesenchymal transition (EMT), in which epithelial cells lose their polarized phenotype, has been suggested to contribute to ECM remodeling.[18,19] The EMT is induced by downregulated epithelial proteins such as E-cadherin in concurrence with upregulated mesenchymal-related proteins, such as vimentin and α-SMA, and master transcription factors, such as TWIST and SLUG.[20] There is evidence that EMT is essential for fibrosis in various organs including the fibrostenosis in CD.[18,21]

Although fibrostenosis of CD significantly increases morbidity and mortality of patients, only a few proteins are known to affect the recurrent fibrostenotic phenotype in CD, and the molecular mechanisms of intestinal fibrosis need to be further investigated. Although there have been recent reports on the search for fibrostenosis genes using genomics,[22] there are several challenges in exploring fibrosis targets, as they do not affect protein expression or post-translational modification. Approaches using proteomics are an alternative tool to discover biomarkers that can overcome these limitations. Because targeted therapy can potentially reduce the risk of surgery, we aimed to find proteins associated with fibrostenotic CD using a proteomics approach and to elucidate the protein markers involved in crosstalk between fibrosis and inflammation including the EMT process.

MATERIALS AND METHODS

1. Patients and clinical specimens

This study for marker discovery included 14 patients with fibrostenotic CD and six healthy controls at Severance Hospital (Yonsei University). The clinical characteristics of the patients are summarized in Supplementary Table 1. The fibrostenotic disease characteristics of the patients were analyzed based on colonoscopy and/or magnetic resonance enterography. All patients underwent surgical resection of the bowel owing to medically intractable clinical complications from bowel stricture. Surgical specimens of the patients were grossly distinguished into normal sections and fibrostenotic lesions. Colonoscopic biopsy samples of inflamed terminal ileum or colon without stricture were analyzed from four patients who had undergone colonoscopy before surgery. The isolated specimens were snap-frozen in liquid nitrogen and stored at –70°C. Written informed consent was obtained from all participants enrolled in this study, and the study was approved by the Institutional Review Board of Severance Hospital, Yonsei University (IRB number: 4-2012-0302).

The detailed methods for two-dimensional difference gel electrophoresis, protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, orbitrap liquid chromatography-mass spectrometry, and immunohistochemistry are described in the Supplementary Methods.

2. Cell culture, treatment, and knockdown by RNA interference

For *in vitro* experiments, the 18Co-cell line, a human colonic subepithelial myofibroblast cell line, and Caco-2, a colon cancer epithelial cell line, were used (ATCC, Manassas, VA, USA). Cells were grown in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle medium with 1% antibiotics, with or without transforming growth factor β (TGF-β; 5 ng/mL, R&D Systems, Minneapolis, MN, USA) or tumor necrosis factor-α (10 μg/mL, R&D Systems) for the indicated times. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The small interfering RNAs (siRNAs) for *HSPA1A*, *HSPA5*, and a non-targeting control were purchased from Bioneer (Daejeon, Korea). Cells were knocked down by transfection of siRNA using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA).

The detailed methods for the wound healing assay, primer list and quantitative real-time polymerase chain reaction are described in the Supplementary Methods and Supplementary Table 2.

3. Immunofluorescence staining

Cells or tissue sections were fixed with 10% formalin (pH 7.4), permeabilized with 0.5% Triton X-100 in PBS (PBS-T), blocked with 1% BSA in PBS-T, and incubated with E-cadherin (1:500; Cell Signaling Technology, Boston, MA, USA), fibronectin (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α-SMA (1:200; Santa Cruz Biotechnology Inc.), and heat shock protein 72 (HSP72; 1:200, EnoGene Biotech, New York, NY, USA) primary antibodies or fluorescently conjugated antibodies overnight at 4°C. After incubation with primary antibodies, the cell sections were incubated with Alexa Fluor-488-, Alexa Fluor-555-, or Alexa Fluor-633-conjugated secondary an-

tibodies (Thermo Fisher Scientific, San Jose, CA, USA). Cell nuclei were counterstained with DAPI (blue) (Thermo Fisher Scientific). All images were obtained by light microscopy (Olympus BX41; Olympus Optical, Tokyo, Japan) or by confocal microscopy (Carl Zeiss LSM 700, Prenzlauer, Berlin, Germany).

4. Statistical analysis

All results are expressed as mean±standard error of the mean. Prism 5.0 software (GraphPad Inc., San Diego, CA, USA) was used for statistical analysis. The significance of differences between conditions was assessed using the Student t-test, one-way analysis of variance, or Kruskal-Wallis test. A p-value less than 0.05 was considered statistically significant.

RESULTS

1. Proteomic analysis uncovers distinct protein profiles among normal, inflamed, and fibrostenotic specimens of patients with CD

To identify proteins with differing expression levels between normal, inflammation, and fibrosis specimens, we performed two-dimensional difference gel electrophoresis analyses of the pooled colon specimens of the normal (n=7), inflamed-only (n=4), and fibrotic (n=7) regions of the same patient (Supplementary Fig. 1). Then, we screened the differentially expressed proteins in the colon tissues among tissues normal control, inflamed, and fibrotic lesion samples of CD patients. The analysis flow of this study is shown in Fig. 1, and characteristics of the patients are shown in Supplementary Table 1. We identified 56 protein spots with 2-fold or higher changes between normal, inflamed, and fibrostenotic regions of the bowel from two-dimensional difference gel electrophoresis analysis (Fig. 2A, Supplementary Table 3). Among the spots, the 18 spots that were not selected had a problem that the signal intensity deviation expressed in the two gels targeting the same tissue was relatively large. Thus, final 30 spots were selected from the preparation gels for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Of these, 12 spots with significant and meaningful human protein database results were confirmed by orbitrap liquid chromatography-mass spectrometry analysis. Finally, we identified and distinguished five spots from fibrostenotic specimens compared to normal or inflamed lesions. Of the five spots in fibrostenotic CD, three were increased in

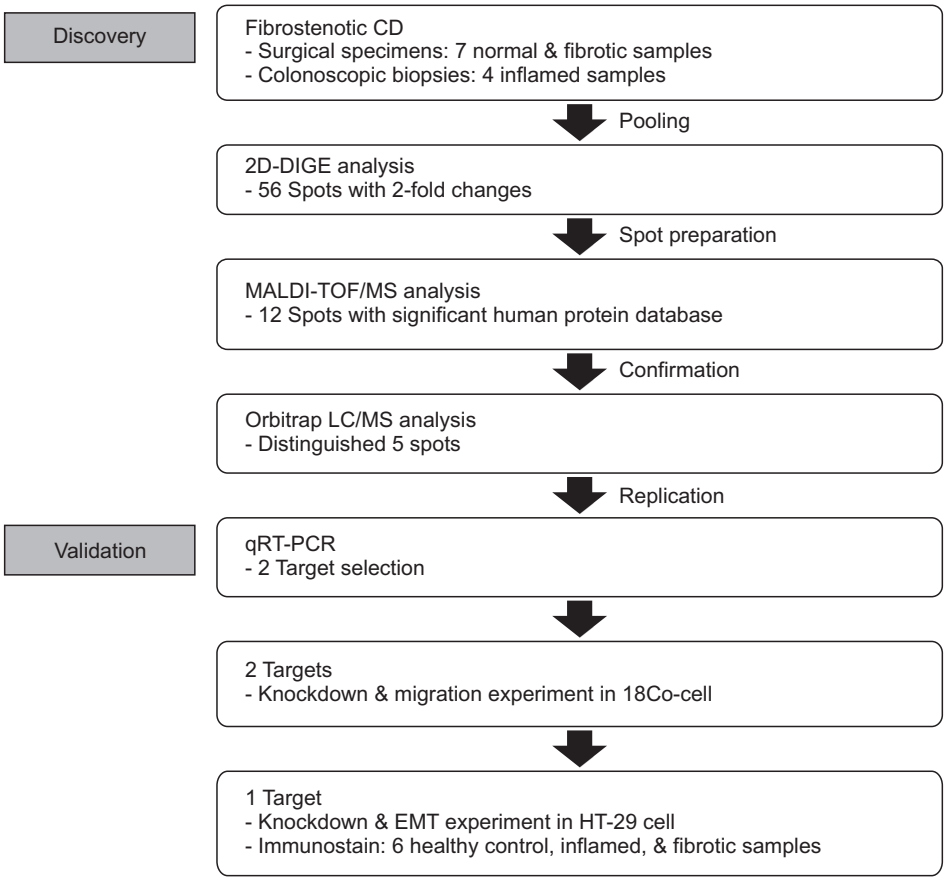

Fig. 1. Workflow of this study.
CD, Crohn's disease; 2D-DIGE, two-dimensional difference gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; LC/MS, liquid chromatography-mass spectrometry; qRT-PCR, quantitative real-time polymerase chain reaction; EMT, epithelial-to-mesenchymal transition.

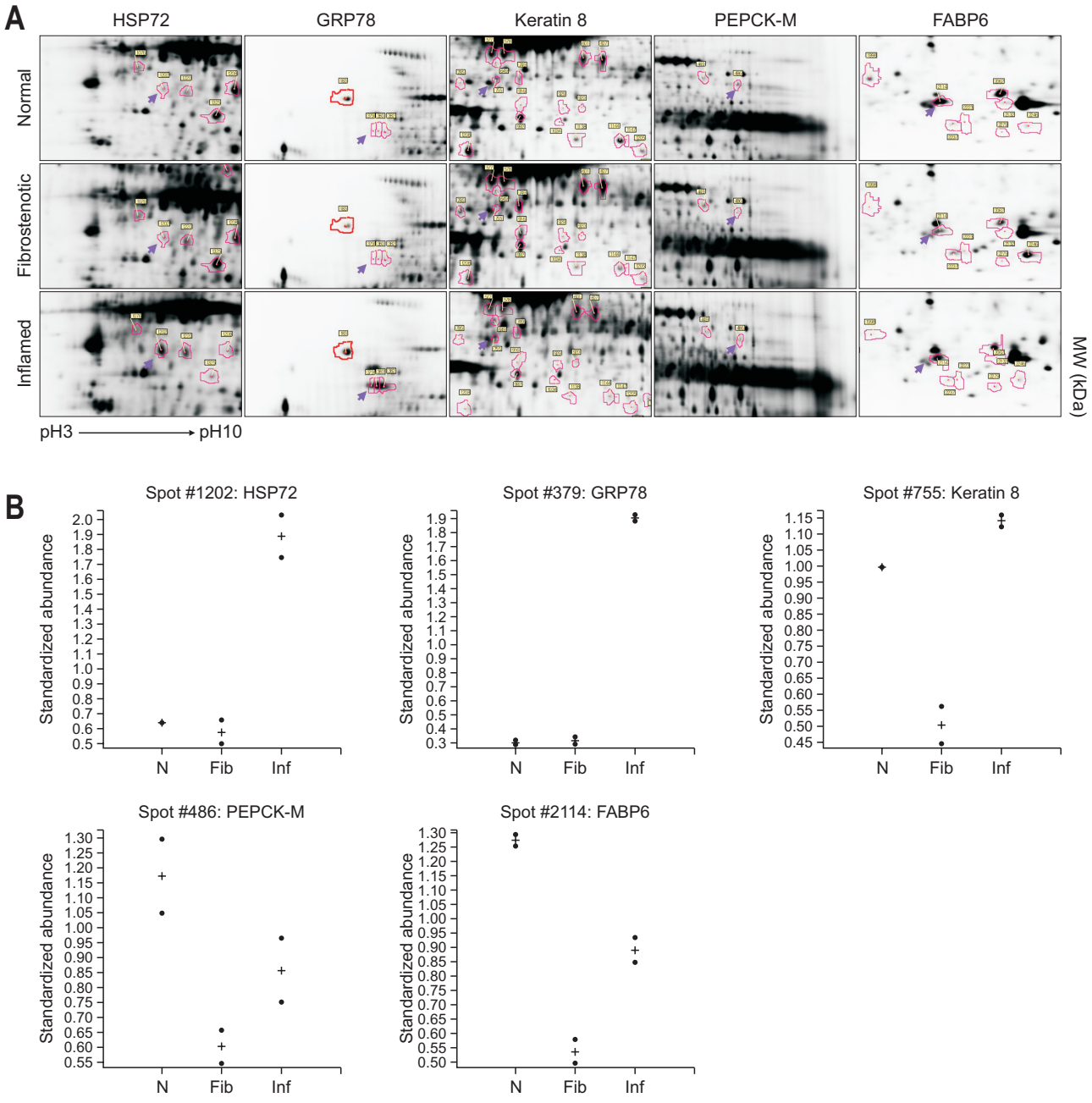

Fig. 2. Proteomic analysis reveals distinct protein profiles between normal, inflamed, and fibrostenotic specimens of patients with Crohn's disease. (A) Representative images of candidate spots in gels of 2D-DIGE using pooled colon specimens of normal (n=7), inflamed-only (n=4), and fibrostenotic (n=7) regions of the same patient. Thirty spots that were differentially expressed between inflamed and fibrostenotic regions (>2-fold) were identified using 2D-DIGE (red circles) and validated with TOF/MS. Spot number was assigned by an image analysis program, followed by 2D-DIGE. Purple arrows indicate five candidate spots for identification. (B) Identification of stenosis candidate proteins using peptide mass fingerprinting, MALDI-TOF MS, and orbitrap LC/MS. We observed a standardized abundance of spot #1202 (HSP72), spot #379 (GRP78), spot #755 (keratin 8), spot #486 (PEPCK-M), and spot #755 (FABP6). These spots were excised, identified by MALDI-TOF/MS, and confirmed by orbitrap LC/MS. The results are shown as the calculated standardized abundance from a human database (MASCOT).
N, normal region; Fib, fibrostenotic region; Inf, inflamed region; 2D-DIGE, two-dimensional difference gel electrophoresis; HSP72, heat shock protein 72; GRP78, glucose regulatory protein 78; PEPCK-M, phosphoenolpyruvate carboxykinase mitochondrial isoform; FABP6, fatty acid-binding protein 6; MALDI-TOF/MS, matrix-assisted laser desorption/ionization-time of flight/mass spectrometry; LC/MS, liquid chromatography-mass spectrometry.

inflamed CD, whereas two were decreased. The typical peptide mass fingerprinting results of the spots are shown in Fig. 2A. The spots were identified as heat shock 70 kDa protein 1A (HSPA1A, HSP72), heat shock 70 kDa protein 5 (HSPA5, GRP78), keratin 8 (KRT8), phosphoenolpyruvate carboxykinase mitochondrial isoform (PEPCK-M),

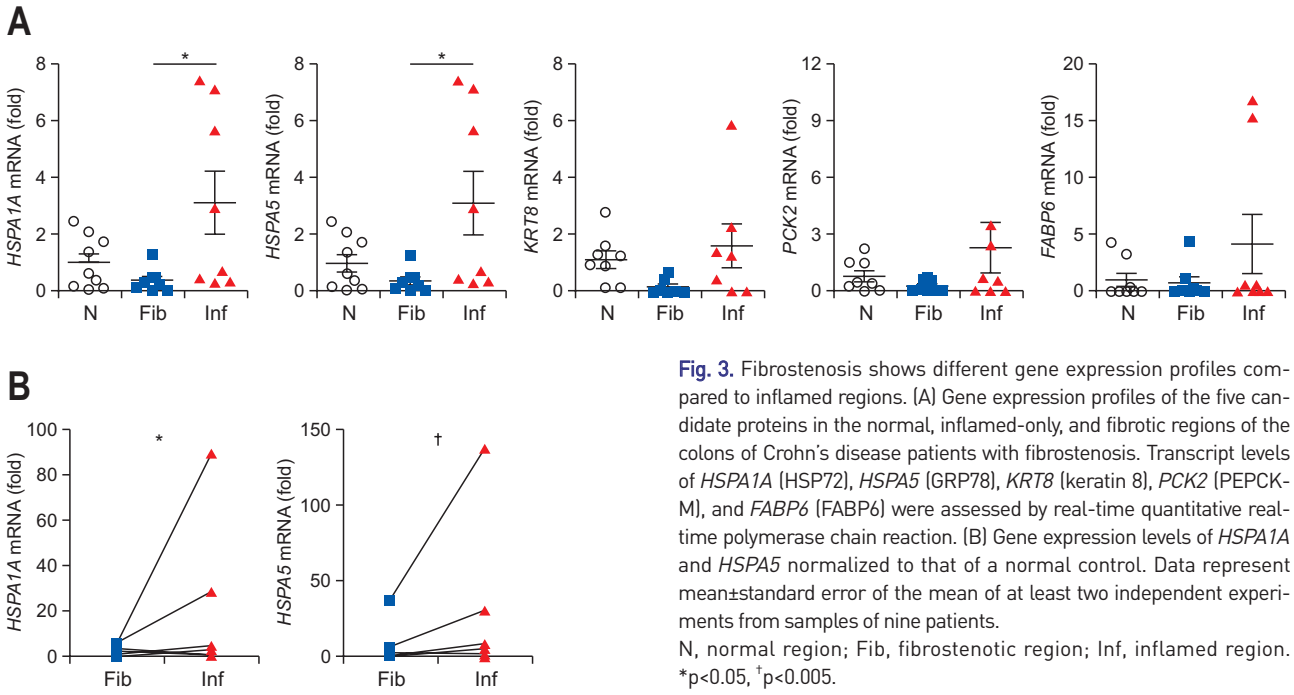

Fig. 3. Fibrostenosis shows different gene expression profiles compared to inflamed regions. (A) Gene expression profiles of the five candidate proteins in the normal, inflamed-only, and fibrotic regions of the colons of Crohn's disease patients with fibrostenosis. Transcript levels of *HSPA1A* (HSP72), *HSPA5* (GRP78), *KRT8* (keratin 8), *PCK2* (PEPCK-M), and *FABP6* (FABP6) were assessed by real-time quantitative real-time polymerase chain reaction. (B) Gene expression levels of *HSPA1A* and *HSPA5* normalized to that of a normal control. Data represent mean±standard error of the mean of at least two independent experiments from samples of nine patients.
N, normal region; Fib, fibrostenotic region; Inf, inflamed region. *p<0.05, †p<0.005.

and fatty acid-binding protein 6 (FABP6) (Supplementary Table 4). The decreased spots more highly downregulated in the transition to fibrosis were identified as PEPCK-M, a mitochondrial isoform of a gluconeogenic enzyme that converts oxaloacetate to phosphoenolpyruvate,[23] and FABP6, a fatty acid-binding protein known as a marker of tissue injury in the intestine (Fig. 2B).[24,25]

To further confirm the observed changes in transcriptional levels as fibrostenotic markers, we isolated RNA from the colon tissues of independent clinical samples and performed quantitative real-time polymerase chain reaction. Although the gene expression levels of all markers showed a trend consistent with that of the proteomic analysis, statistical analysis showed that only *HSPA1A* (HSP72) and *HSPA5* (GRP78) among the genes of the five candidate proteins were significantly reduced in fibrostenotic samples compared to inflamed tissues; the other genes had no significant differences compared to normal control or inflamed CD groups (Fig. 3).

2. HSP72 downregulation activates myofibroblasts in a fibrotic condition

Given the central role of myofibroblasts in fibrostenosis of CD, we examined whether gene silencing of *HSP72* and *HSPA5* affected myofibroblast activation. Thus, we knocked down those genes in 18Co-cells, a myofibroblast cell line, using siRNA against their genes, *HSPA1A* and *HSPA5* (the genes for HSP72 and GRP78, respectively). Interestingly, *HSPA1A* or *HSPA5* knockdown showed a compensatory increase of respective *HSPA5A* or *HSPA1* expression in each knockdown cell line (Fig. 4A). The *HSPA1A* knockdown cells exhibited markedly increased gene expression of interleukin 1-β (*IL1B*) compared to the control (Fig. 4B). In addition, *HSPA1A* or *HSPA5* knockdown cells showed increased expression of *MMP2*, a gene related to ECM remodeling, compared with control cells transfected with scramble (Fig. 4B).

Myofibroblast activation facilitates their active movement by a highly structured scaffold of actin fibers and intermediate filaments and contributes to the development of fibrosis. Fibronectin is considered one of the most potent inducers of migration.[26] Because the migration of myofibroblasts due to injury is essential for the fibrotic process, myofibroblast migration was assessed using cell migration assay, in which 18Co-cells were scratched after being transfected with siRNA. Cell migration was significantly increased in *HSPA1A* knockdown cells, but not in *HSPA5* knockdown cells compared to control cells (Fig. 4C and D), suggesting that defects in HSP72 promote myofibroblast activation.

3. HSP72 induces epithelial-mesenchymal transition of intestinal epithelial cells in fibrotic conditions

Among the fibrogenic components, subepithelial myofibroblasts play a key role, as the primary source of ECM components and the main mediators between intestinal inflammation and fibrosis. TGF-β1, which is overexpressed in the intestinal tissues in CD patients with fibrostenosis, is important in the migration of fibroblasts, transdifferentiation into myofibroblasts, and ECM formation,[11] although

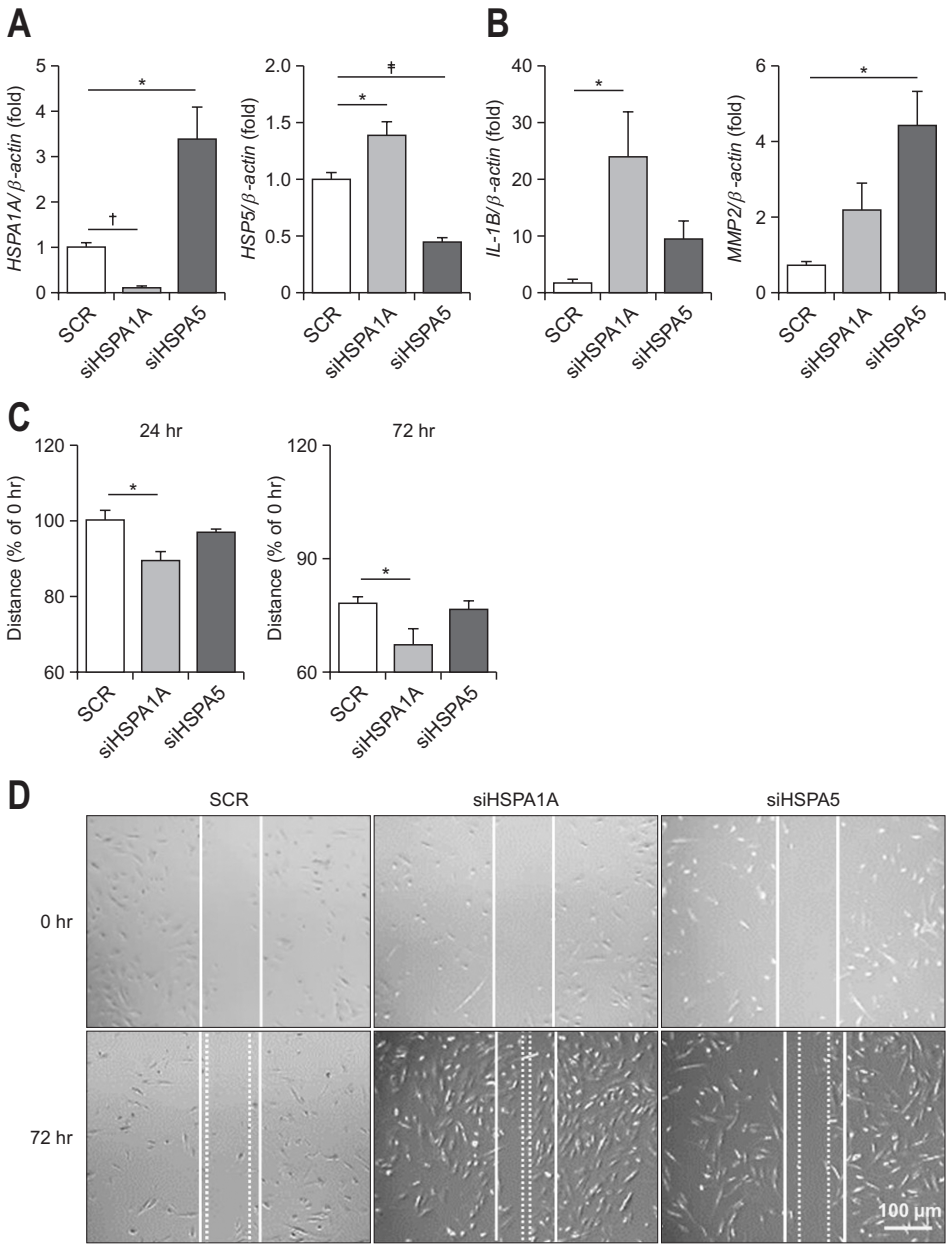

Fig. 4. Knockdown of *HSPA1A* promotes activation of myofibroblasts. (A, B) Expression of *HSPA1A* and *HSP5* (A) and inflammation-related (*IL-1B*) and ECM remodeling-related (*MMP2*) genes (B). 18Co-cells were transfected with scrambled (SCR) or *HSPA1A*- or *HSP5*-specific siRNAs (siHSPA1A and siHSP5, respectively), and gene expression was assessed by real-time quantitative real-time polymerase chain reaction 24 hours after transfection. (C, D) Effects of *HSPA1A* knockdown on 18Co-cell migration. (C) Cell migration rates at 24 and 72 hours. (D) Representative images of the cell migration assay. The white dotted line and continuous line display the border of the scratch at the indicated time and at 0 hour, respectively. The solid line and dotted line indicate the border at 0 and 72 hours. All experiments were repeated three times independently (×20). Data represent mean±standard error of the mean of duplicate samples. *p<0.05, †p<0.01, ‡p<0.005.

other growth factors and cytokines are also involved in the fibrosis.[13] TGF-β1 is also a major mediator in EMT as well as in the recruitment of fibroblasts, their transdifferentiation to myofibroblasts, and the stimulation of ECM secretion.[27] To examine whether loss of HSP72 induces EMT in epithelial cells, we knocked down *HSPA1A* in Caco-2 cells, an intestinal epithelial cell (IEC) line, and stimulated them with TGF-β1. *HSPA1A*-knockdown cells showed fibroblast-like morphology regardless of treatment with TGF-β1 (Fig. 5A). To find changes in EMT-related markers, we performed immunostaining analysis. Immunofluorescence staining revealed that E-cadherin, an epithelial cell marker, was intact in the control cells. In contrast, *HSPA1A*-knockdown cells showed drastically reduced E-cadherin expression (Fig. 5B). Of note, *HSPA1A*-knockdown cells untreated with TGF-β1 showed increased levels of mesenchymal cell markers such as fibronectin, as did α-SMA-positive cells like TGF-β1-treated cells (Fig. 5C and D), indicating EMT induction by *HSPA1A* downregulation in IECs.

One of the two types of myofibroblasts, the Cajal cell, is located in the intramuscular space between the submucosa and the muscular propria, and the other myofibroblasts are located below the epithelial cell layer of the villi and crypts.[28] Myofibroblasts are identified by the increased expression of α-SMA and intermediate filaments such as vimentin. Consistently, we found increased quantities of α-SMA-positive cells in the mucosa and submucosa of

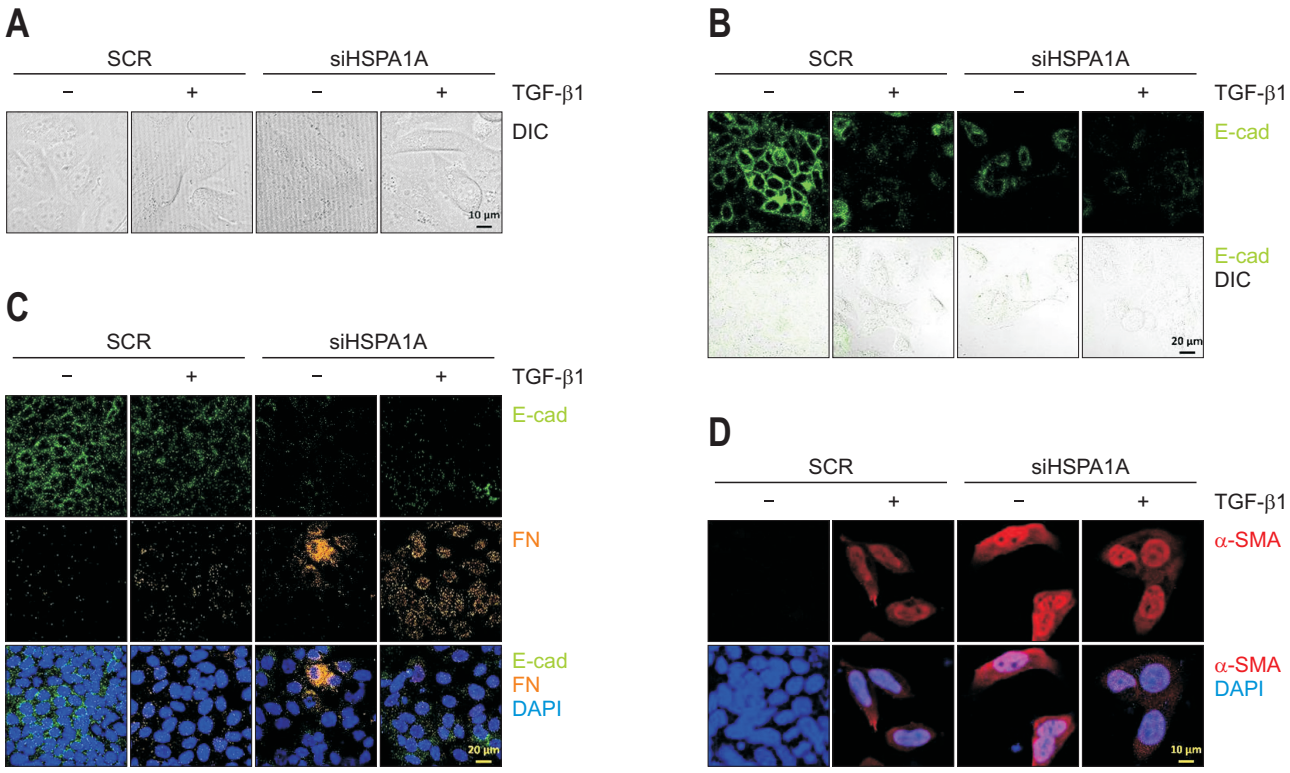

Fig. 5. Knockdown of *HSPA1A* induces the epithelial-mesenchymal transition in intestinal epithelial cells. Caco-2 cells were transfected with scrambled (SCR) or *HSPA1A*-specific siRNA (siHSPA1A) and were treated with transforming growth factor (TGF)-β1 for 48 hours. (A) Cell morphology. (B) Representative images of morphology and immunostaining for E-cadherin (E-cad, green; scale bar=20 μm). (C) Representative images of immunostaining for E-cadherin (E-cad, green) and fibronectin (FN, orange color; scale bar=20 μm). (D) Representative images of immunostaining for α-smooth muscle actin (α-SMA, red). DAPI shows nuclei (scale bar=10 μm). All experiments were repeated three times independently.
DIC, differential interference contrast.

CD patients compared to healthy controls (Fig. 6A). Next, we investigated HSP72 and α-SMA expression in normal control, inflamed, and fibrotic colons of CD patients using immunofluorescence staining. Both HSP72 and α-SMA were weakly expressed in IECs but were strongly expressed in stroma cells (Fig. 6B and C). Consistent with previous results, HSP72 was upregulated in the inflamed lesions but downregulated in the fibrotic lesions, whereas α-SMA was increased in the fibrostenotic lesions (Fig. 6B-D). Of note, a weak HSP72 signal was observed in the fibrostenotic specimens, and the signals of HSP72 and α-SMA were not overlapping, suggesting that loss of HSP72 increases myofibroblasts in fibrostenosis, as in a previous study for renal fibrosis.[29]

DISCUSSION

Because there is no reliable biomarker for fibrostenotic CD, the longer the delay of diagnosis in CD, the greater the risk of intestinal stenosis. Since the process and mechanism of fibrosis are intricate, effective targeted treatments for fibrostenotic CD have not yet been developed. Therefore, it is important to predict stenosis at an early time point.[22,30] In this context, although there have been recent reports on the search for fibrostenosis genes using genomics,[22] there has been no study investigating colonic protein profiles using a proteomics approach. Proteomic analysis can analyze all the proteins present in a sample and their differences between normal and disease groups, in particular, the role of proteins and posttranslational modification regulated by cytokines and immune responses. Due to the complexity of fibrostenotic CD and the technical interference by serological proteins,[31,32] proteomic studies for serological biomarkers including serological proteins showed conflicting research results, low-quality evidence, and no reliable biomarkers.[33] In this study, therefore, we tried to identify proteins involved in the link between inflammation and fibrosis using intestinal tissue-specific proteomics.

Direct targeting of fibrosis has not yet been approached as a therapy in CD because fibrosis is a dynamic process involving various types of cells and immune responses. Moreover, the failure of anti-inflammatory therapies in preventing fibrosis indicates that there are other specific

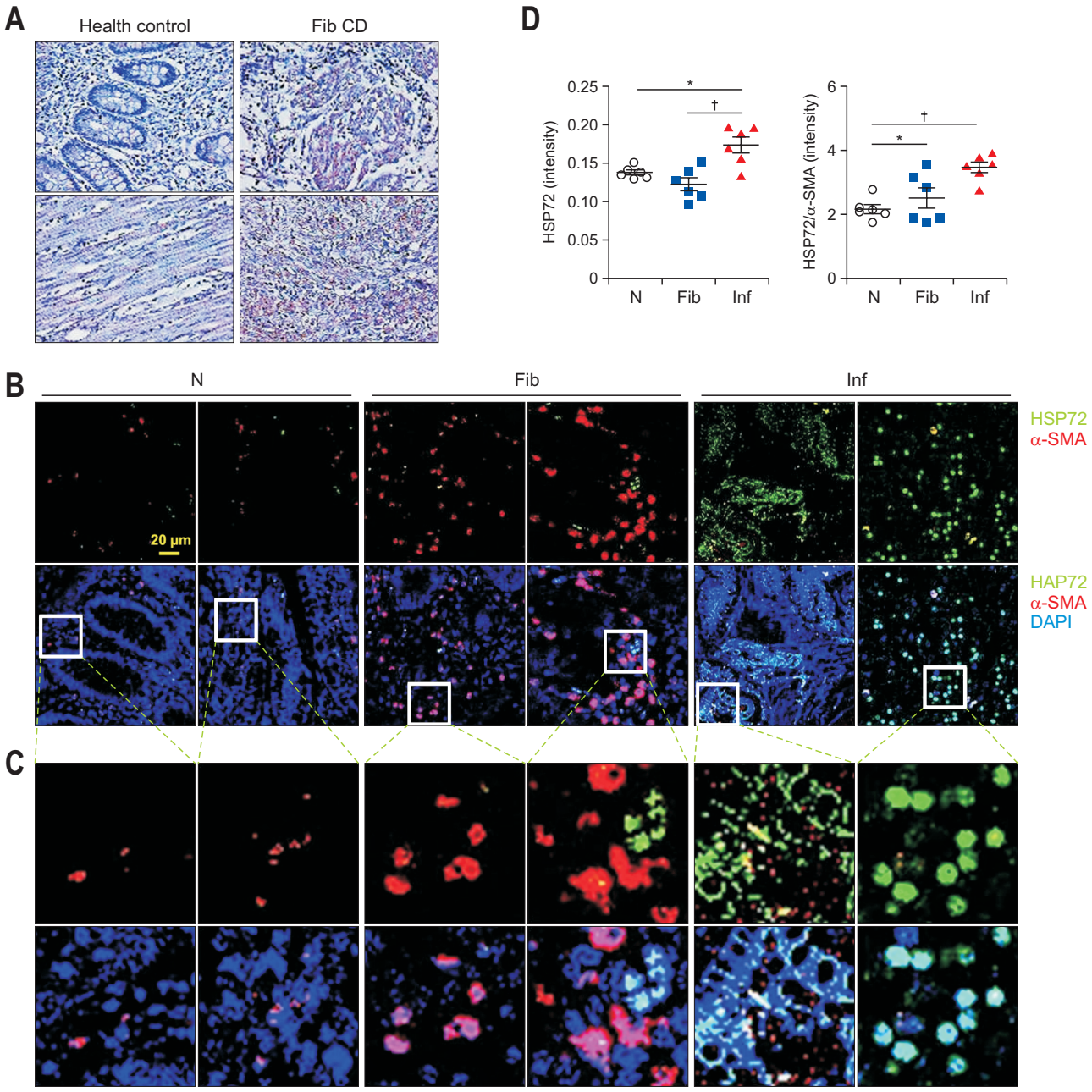

Fig. 6. Fibrostenotic regions of Crohn's disease patients with fibrostenosis showed downregulated HSP72 but upregulated markers of myofibroblast activation. (A) Representative image of immunohistochemical staining for α-smooth muscle actin (α-SMA) in colon sections of mucosa (upper panel) and submucosa (bottom panel) of a healthy control (left panel) and a Crohn's disease patient with fibrostenosis (right panel). Hematoxylin was used as a counterstain (×20). (B-D) Immunofluorescence staining for HSP72 and α-SMA in normal, inflamed-only, and fibrotic regions of the colons of Crohn's disease patients with fibrostenosis. (B, C) Representative image of immunofluorescence staining for HSP72 (green) and α-SMA (red). DAPI indicates nuclei. Original magnification: ×40. (D) Densitometry analysis (n=6). Data represent mean±standard error of the mean of six samples.
N, normal region; Fib, fibrostenotic region; Inf, inflamed region. *p<0.05, †p<0.01.

pathways involved in intestinal fibrosis.[13] This likely results in the multifactorial and complex processes of fibrosis after inflammation. Given that inflammation underlies fibrosis, there are critical changes in transition from inflammation and fibrosis. In addition, inflammation-induced factors such as TGF-β and matrix stiffness induce this transition. Thus, we focused on the proteins that changed between inflammation and fibrosis and compared paired samples from the same CD patients. We identified distinct protein profiles between inflamed and fibrotic legions which the association of these proteins with intestinal fibrosis remains unknown.

Heat shock proteins that respond to environmental stress are abundantly expressed in the intestine and highly increased in patients with inflammatory bowel disease[34] and in mouse colitis models.[35] Among them, the HSP70 subfamily is one of the most studied. GRP78, a molecular chaperone, is commonly used as an endoplasmic reticulum stress marker and is involved in a variety of inflammatory diseases including fibrosis[36] and inflammatory bowel disease.[37] GRP78 also mediates macrophage apoptosis in pulmonary fibrosis. Although it did not appear to be involved in myofibroblast activation in our study, some studies have suggested that reduced expression of GRP78 resulted in increased myofibroblast activation. Of note, HSP72 from the HSP70 subfamily is also induced by various stresses such as reactive oxygen species[38] and parasite infestation[39] in IECs. HSP72 is a multifunctional inducible molecular chaperone that is expressed in many cell types and plays a protective role in a variety of disease models.[40] It has been demonstrated that HSP72 plays a protective role in renal fibrosis by inhibiting fibroblast accumulation and TGF signaling.[29,41] It has also been reported that *HSPA1A* polymorphism and the autoantibody production of HSP72 are associated with idiopathic pulmonary fibrosis and improved outcomes, respectively.[42,43] In addition, HSP72 inhibits renal fibroblast activation by suppressing the expression of fibrotic markers such as α-SMA and fibronectin.[29,41] In agreement with these studies, we demonstrated that HSP72 is downregulated in the transition from inflammation to fibrosis. We also showed that HSP72 knockdown further promotes wound healing in 18Co-cells, suggesting that it is an important marker in the progression of fibrostenotic CD. Given that tumor necrosis factor-α level is increased in CD patients with fibrosis, HSP72 might be an important regulator of myofibroblasts in the entry phase of fibrosis and might provide protective signaling against persistent inflammatory signals.

In this study, the knockdown of HSP72 induced the EMT of IEC, increasing mobility and ECM production. Several studies have documented the EMT as the source of fibroblast production in many organs, and this has been confirmed in CD-associated intestinal fibrosis and animal models.[18,44] However, the mechanism of this process remain unclear. Supporting our results, recent studies have reported that HSP72 suppresses EMT.[45,46] We demonstrated a marked increase of α-SMA, which is a marker of myofibroblasts and functions in wound healing, in *HSPA1A*-knockdown cells as well as loss of E-cadherin. These results suggest that the EMT process of IEC caused by downregulation of HSP72 can contribute to fibrosis in CD. To identify further mechanisms, mutations in fibrostenotic CD should be analyzed using whole-genome sequencing. Moreover, further studies using large-scale samples using external cohorts and knockout mouse models for mechanism evaluation are needed to confirm our results.

In conclusion, we found that HSP72 plays an important role as a mediator by regulating myofibroblasts and EMT in fibrosis of CD, suggesting that HSP72 can serve as a target for antifibrotic therapy.

CONFLICTS OF INTEREST

J.H.C. is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (No. NRF-2016R1D1A1B03935006) and by a National Research Foundation of Korea grant funded by the Korean government (MSIT) (2020R1A2C2003638).

AUTHOR CONTRIBUTIONS

Study concept and design: D.H.K., J.H.C., S.W.K. Data acquisition: S.W.K., J.Y.L., H.C.L. Data analysis and interpretation: S.W.K., D.H.K. Drafting of the manuscript: S.W.K., D.H.K., J.H.C. Critical revision of the manuscript for important intellectual content: J.H.C. Statistical analysis: S.W.K., J.B.A. Obtained funding: D.H.K. Administrative, technical, or material support: J.H.K., I.S.P. Study supervision: D.H.K., J.H.C. Approval of final manuscript: all authors.

ORCID

Seung Won Kim https://orcid.org/0000-0002-1692-1192
Jae-Young Lee https://orcid.org/0000-0001-9131-7043
Han Cheol Lee https://orcid.org/0000-0003-1811-5175
Jae Bum Ahn https://orcid.org/0000-0002-6618-8363
Ji Hyung Kim https://orcid.org/0000-0001-5381-3628
I Seul Park https://orcid.org/0000-0001-6546-5456
Jae Hee Cheon https://orcid.org/0000-0002-2282-8904
Duk Hwan Kim https://orcid.org/0000-0003-3841-5802

SUPPLEMENTARY MATERIALS

Supplementary materials can be accessed at https://doi.org/10.5009/gnl220308.

REFERENCES

1. Mizoguchi E, Low D, Ezaki Y, Okada T. Recent updates on the basic mechanisms and pathogenesis of inflammatory bowel diseases in experimental animal models. Intest Res 2020;18:151-167.
2. Park IS, Son M, Ma HW, et al. Succinate-treated macrophages attenuate dextran sodium sulfate colitis in mice. Intest Res 2021;19:349-353.
3. Kim JM, Cheon JH. Pathogenesis and clinical perspectives of extraintestinal manifestations in inflammatory bowel diseases. Intest Res 2020;18:249-264.
4. Kim SB, Cheon JH, Park JJ, et al. Risk factors for postoperative recurrence in Korean patients with Crohn's disease. Gut Liver 2020;14:331-337.
5. Nakase H. Optimizing the use of current treatments and emerging therapeutic approaches to achieve therapeutic success in patients with inflammatory bowel disease. Gut Liver 2020;14:7-19.
6. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med 2009;361:2066-2078.
7. Simmons A. Crohn's disease: genes, viruses and microbes. Nature 2010;466:699-700.
8. Saruta M, Park DI, Kim YH, et al. Anti-MAdCAM-1 antibody (PF-00547659) for active refractory Crohn's disease in Japanese and Korean patients: the OPERA study. Intest Res 2020;18:45-55.
9. Yoo JH, Holubar S, Rieder F. Fibrostenotic strictures in Crohn's disease. Intest Res 2020;18:379-401.
10. Rieder F, Zimmermann EM, Remzi FH, Sandborn WJ. Crohn's disease complicated by strictures: a systematic review. Gut 2013;62:1072-1084.
11. Chan WP, Mourad F, Leong RW. Crohn's disease associated strictures. J Gastroenterol Hepatol 2018;33:998-1008.
12. Urban ML, Manenti L, Vaglio A. Fibrosis: a common pathway to organ injury and failure. N Engl J Med 2015;373:95-96.
13. Bettenworth D, Rieder F. Medical therapy of stricturing Crohn's disease: what the gut can learn from other organs: a systematic review. Fibrogenesis Tissue Repair 2014;7:5.
14. Imai J, Yahata T, Ichikawa H, et al. Inhibition of plasminogen activator inhibitor-1 attenuates against intestinal fibrosis in mice. Intest Res 2020;18:219-228.
15. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med 2012;18:1028-1040.
16. Di Sabatino A, Jackson CL, Pickard KM, et al. Transforming growth factor beta signalling and matrix metalloproteinases in the mucosa overlying Crohn's disease strictures. Gut 2009;58:777-789.
17. Shimshoni E, Yablecovitch D, Baram L, Dotan I, Sagi I. ECM remodelling in IBD: innocent bystander or partner in crime? The emerging role of extracellular molecular events in sustaining intestinal inflammation. Gut 2015;64:367-372.
18. Scharl M, Huber N, Lang S, Fürst A, Jehle E, Rogler G. Hallmarks of epithelial to mesenchymal transition are detectable in Crohn's disease associated intestinal fibrosis. Clin Transl Med 2015;4:1.
19. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009;119:1420-1428.
20. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol 2014;15:178-196.
21. Zhang HJ, Zhang YN, Zhou H, Guan L, Li Y, Sun MJ. IL-17A promotes initiation and development of intestinal fibrosis through EMT. Dig Dis Sci 2018;63:2898-2909.
22. Visschedijk MC, Spekhorst LM, Cheng SC, et al. Genomic and expression analyses identify a disease-modifying variant for fibrostenotic Crohn's disease. J Crohns Colitis 2018;12:582-588.
23. Méndez-Lucas A, Hyroššová P, Novellasdemunt L, Viñals F, Perales JC. Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK-M) is a pro-survival, endoplasmic reticulum (ER) stress response gene involved in tumor cell adaptation to nutrient availability. J Biol Chem 2014;289:22090-22102.
24. Derikx JP, Poeze M, van Bijnen AA, Buurman WA, Heineman E. Evidence for intestinal and liver epithelial cell injury in the early phase of sepsis. Shock 2007;28:544-548.
25. Cronk DR, Houseworth TP, Cuadrado DG, Herbert GS, McNutt PM, Azarow KS. Intestinal fatty acid binding protein (I-FABP) for the detection of strangulated mechanical small bowel obstruction. Curr Surg 2006;63:322-325.
26. Leeb SN, Vogl D, Grossmann J, et al. Autocrine fibronectin-induced migration of human colonic fibroblasts. Am J Gastroenterol 2004;99:335-340.
27. Medina C, Santos-Martinez MJ, Santana A, et al. Transforming growth factor-beta type 1 receptor (ALK5) and Smad proteins mediate TIMP-1 and collagen synthesis in experimental intestinal fibrosis. J Pathol 2011;224:461-472.
28. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. Am J Physiol 1999;277:C183-C201.
29. Zhou Y, Cao S, Li H, et al. Heat shock protein 72 antagonizes STAT3 signaling to inhibit fibroblast accumulation in renal fibrogenesis. Am J Pathol 2016;186:816-828.
30. Limketkai BN, Bayless TM. Editorial: can stenosis in ileal

Crohn's disease be prevented by current therapy? Am J Gastroenterol 2013;108:1755-1756.
31. Jacobs JM, Adkins JN, Qian WJ, et al. Utilizing human blood plasma for proteomic biomarker discovery. J Proteome Res 2005;4:1073-1085.
32. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845-867.
33. He JS, Tan JY, Li XZ, et al. Serum biomarkers of fibrostenotic Crohn's disease: where are we now? J Dig Dis 2020;21:336-341.
34. Ludwig D, Stahl M, Ibrahim ET, et al. Enhanced intestinal expression of heat shock protein 70 in patients with inflammatory bowel diseases. Dig Dis Sci 1999;44:1440-1447.
35. Otani S, Otaka M, Jin M, et al. Effect of preinduction of heat shock proteins on acetic acid-induced colitis in rats. Dig Dis Sci 1997;42:833-846.
36. Koo JH, Lee HJ, Kim W, Kim SG. Endoplasmic reticulum stress in hepatic stellate cells promotes liver fibrosis via PERK-mediated degradation of HNRNPA1 and up-regulation of SMAD2. Gastroenterology 2016;150:181-193.
37. Stengel ST, Fazio A, Lipinski S, et al. Activating transcription factor 6 mediates inflammatory signals in intestinal epithelial cells upon endoplasmic reticulum stress. Gastroenterology 2020;159:1357-1374.
38. Ohkawara T, Nishihira J, Nagashima R, Takeda H, Asaka M. Polaprezinc protects human colon cells from oxidative injury induced by hydrogen peroxide: relevant to cytoprotective heat shock proteins. World J Gastroenterol 2006;12:6178-6181.
39. Kammanadiminti SJ, Chadee K. Suppression of NF-kappaB activation by Entamoeba histolytica in intestinal epithelial cells is mediated by heat shock protein 27. J Biol Chem 2006;281:26112-26120.
40. Pockley AG. Heat shock proteins, inflammation, and cardiovascular disease. Circulation 2002;105:1012-1017.
41. Mao H, Li Z, Zhou Y, et al. HSP72 attenuates renal tubular cell apoptosis and interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol 2008;295:F202-F214.
42. Mills R, Mathur A, Nicol LM, et al. Intrapulmonary autoantibodies to HSP72 are associated with improved outcomes in IPF. J Immunol Res 2019;2019:1845128.
43. Aquino-Gálvez A, González-Ávila G, Pérez-Rodríguez M, et al. Analysis of heat shock protein 70 gene polymorphisms Mexican patients with idiopathic pulmonary fibrosis. BMC Pulm Med 2015;15:129.
44. Flier SN, Tanjore H, Kokkotou EG, Sugimoto H, Zeisberg M, Kalluri R. Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. J Biol Chem 2010;285:20202-20212.
45. Zhou Y, Mao H, Li S, et al. HSP72 inhibits Smad3 activation and nuclear translocation in renal epithelial-to-mesenchymal transition. J Am Soc Nephrol 2010;21:598-609.
46. Kasioumi P, Vrazeli P, Vezyraki P, et al. Hsp70 (HSP70A1A) downregulation enhances the metastatic ability of cancer cells. Int J Oncol 2019;54:821-832.