

New endoscopic approach of anti-fibrotic therapy for inflammatory bowel disease

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Abstract: Fibrosis continues to be paid a great attention in not only basic research but also clinical practice, especially for the development of novel therapeutics in various fibrotic diseases. However, there remain several obstacles to translation in developing anti-fibrosis therapy. The present review documents our translational practice from target discovery to first-in-patient studies in the development of anti-fibrosis therapy for inflammatory bowel disease (IBD). First topic is a target selection. We have focused on the target that has an ability to regulate multifactorial cascades of fibrosis. Carbohydrate sulfotransferase 15 (CHST15) synthesizes matrix proteoglycan that regulates various pathogenic mediators and contributes to tissue remodeling during injury. Small interfering RNA (siRNA) targeting CHST15 inhibited activation of fibroblasts *in vitro* and reduced fibrosis *in vivo*. Second topic is a clinically feasible application. We established a safe and novel pancolonial delivery of siRNA, which is achieved by direct injection to extracellular matrix (ECM) through endoscope. Third topic is an endpoint for both nonclinical and clinical studies. We have focused on tissue-specific findings for co-existence of fibrosis in ulcerative lesions in IBD and investigated whether the balance of mucosal healing (MH) and fibrosis, which is evaluated by endoscopy and histology respectively, can be used for study endpoints. Phase 1 clinical trial of STNM01, a synthesized CHST15 siRNA, by a single dose endoscopic submucosal injection for non-healer patients with Crohn's disease showed high rates of MH. Analyses of biopsy specimens revealed that STNM01 reduced CHST15 expression at local lesions, repressed pre-existing fibrosis and repaired the damaged crypts. Thus, blockade of multifactorial modulator CHST15 in ECM showed a potential to treat tissue remodeling and skew fibrosis toward mucosal repair. Our practice suggests that target- and tissue-specific findings-based strategy would be a key to translation in developing anti-fibrosis therapy.

Keywords: Crohn's disease (CD); carbohydrate sulfotransferase 15 (CHST15); siRNA; fibrosis; mucosal healing (MH)

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Introduction

Inflammatory bowel disease (IBD), comprised of Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, progressive, destructive disease of the gastrointestinal tract characterized by the presence of extensive ulceration and

mucosal inflammation in the gut. Despite a great progress in modern anti-inflammatory therapies including immunosuppressants and biologics, intestinal fibrosis is a frequent complication in the natural history of IBD, as up to one-third of CD patients and about 5% of UC patients develop

strictures in the clinical course of the disease (1-12). In CD, chronic inflammation induces transmural damage which causes accumulation of extracellular matrix (ECM) and expansion of mesenchymal cells, finally leading to intestinal strictures. In UC, increased amount of collagen and thickening of the muscularis mucosa have been identified in the colon, with consequent shortening and increased rigidity of the colon. This implies that control of intestinal inflammation alone does not necessarily affect the associated fibrotic process. There are no standard anti-fibrotic medical treatments for IBD, and dilation of intestinal strictures with endoscopy or surgical treatment plays an important role in managing the strictures of IBD (3-10). Therefore, treatment goals of IBD include not only symptom control alone but prevention of intestinal fibrosis with structural bowel damage, that is, bowel tissue remodeling (11,12).

In response to inflammation-driven bowel damage, “fibrotic healing” occurs instead of adequate mucosal repair, leading to co-existence of mucosal defect like ulcer and fibrosis at local sites. In clinical practice, refractory ulcers often associated with fibrosis not only in drug-resistant peptic ulcer and esophageal ulcer post submucosal dissection but also in IBD (3,4,13,14). Co-existence of ulcer and fibrosis is thus characterized by “tissue remodeling” in the gut and is one of the causes of difficulty to treat local lesions in IBD. Since multifactorial process is involved in tissue remodeling, therapeutic approaches against single mediator alone would not be sufficient to normalize fibrotic lesions (3,4,14,15). Integrated anti-fibrotic/tissue remodeling strategy that simultaneously targets inflammatory mediators, profibrotic factors and tissue intrinsic changes is recommended as the most successful way to treat this highly complex and difficult-to-manage pathology (15). We therefore focused on ECM molecules that modulate the function of multiple mediators at sites of injury and hypothesized that blockade of certain ECM molecule could normalize the fibrotic architecture en bloc.

Carbohydrate sulfotransferase 15 (CHST15) and chondroitin sulfate-E (CS-E) in tissue remodeling

Mechanism of CHST15/CS-E-mediated fibrosis

CHST15, formerly known as N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase (GalNAc4S-6ST), is a type II transmembrane Golgi protein that biosynthesizes highly sulfated disaccharide units (E-units) of chondroitin sulfate (CS), which binds to various functional proteins and

pathogenic microorganisms (16-18). CHST15/CS-E axis is reported to be involved in fibrosis through two major mechanisms; activation of fibroblasts and formation of collagen fibrils (*Figure 1*). CS-E acts as the co-receptor for Wnt for signal transduction of Wnt/Frizzled pathway of fibroblasts. CS-E acts as the direct receptor for receptor for advanced glycation end product (RAGE) on fibroblasts, suggesting that CS-E directly stimulates RAGE-mediated fibrotic pathway (16). CS-E is also reported to bind CD44, chemokines like MCP-1/CCL2 and SDF-1/CXCL12 and growth factors like PDGF and TGF- β indicating the involvement in adhesion, migration and proliferation of fibroblasts (17). In addition, CS-E was shown to enhance fibril formations of collagen as well as A β (19,20). At the same time, CS proteoglycan was shown to inhibit proteolytic degradation of collagen and A β (19,20). CS-E binds type V collagen, which is increased in submucosa of CD patients (21-24), suggesting that matrix CS-E strengthens CS-E-collagen interaction thereby solid fibrotic formation which is an obstacle of tissue repair.

Mode of action of CHST15 inhibitor STNM01 in fibrosis

STNM01 is a synthesized double stranded RNA oligonucleotide that selectively inhibits the expression of CHST15 gene (25,26). STNM01 (or experimental grade of CHST15 siRNA with the same sequence; hereafter we refer both as “STNM01”) suppressed CHST15 mRNA in human colon and lung fibroblast cell lines *in vitro* and reduced the expression of CHST15 mRNA in the colon of mouse DSS colitis (25), the esophagus of pig esophageal stricture post ESD (27), the lung of mouse pulmonary fibrosis (28) and the heart of rat myocarditis (29). CHST15 protein was also reduced by STNM01 in *in vivo* disease models. STNM01 reduced the amount of CS/sulfated Glycosaminoglycan (GAG) in the human colon fibroblast and in *in vivo* disease models. The amount of E-unit of CS/sulfated GAG chain was selectively reduced in the human colon fibroblast (25).

STNM01 reduced the mRNA expressions of vimentin, α -SMA and Wnt3 by TGF- β -stimulated human fibroblast cell line as well as human colon cancer cell line, indicating that STNM01 inhibits the activation of fibroblasts from both resident fibroblasts and epithelial cells through epithelial mesenchymal transition (EMT) (25). STNM01 enhanced the mRNA expressions of E-cadherin and TGF- β induced EMT-counteracting molecule BMP-7, suggesting EMT-inhibiting effect (25). Reduced production of IL-6, MCP-1/CCL2 and collagen was observed in fibroblast

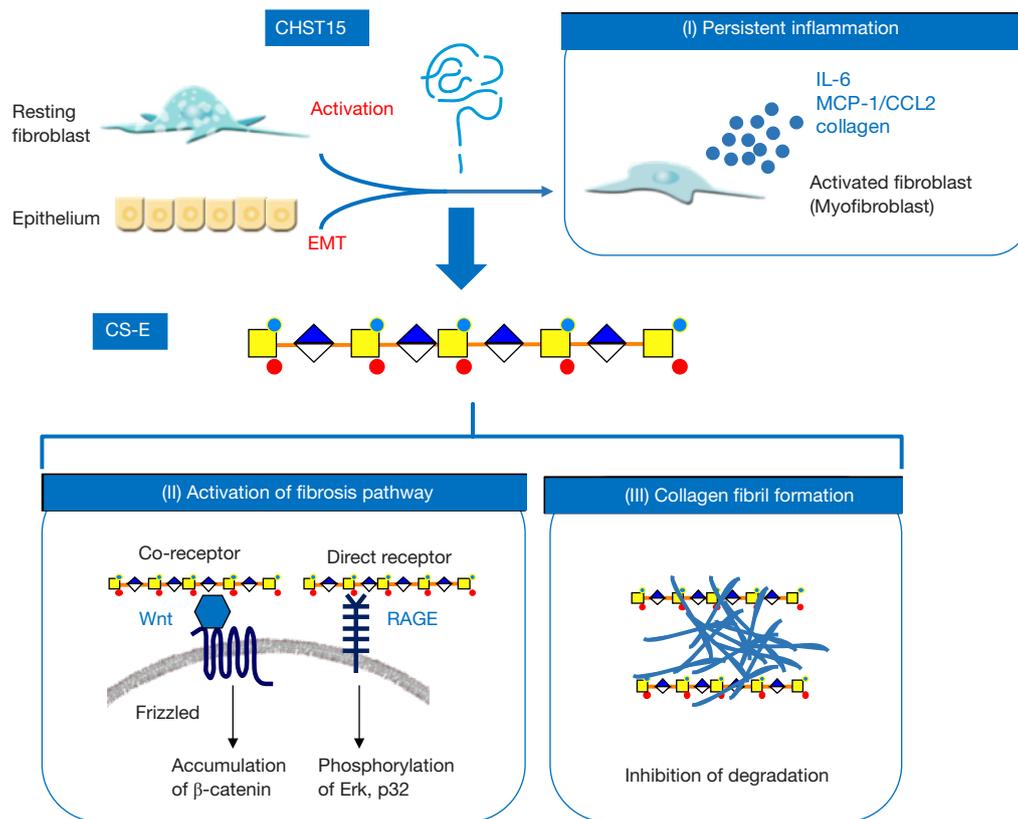


Figure 1 Overview of the MOA of CHST15/CS-E and STNM01 in tissue remodeling in IBD. Currently, 3 mechanisms are involved in tissue remodeling: (I) Persistent inflammation by CHST15. CHST15 (mRNA and protein) is induced during the activation of fibroblasts both from resident (or recruited) fibroblasts and epithelial cells (EMT). STNM01 inhibits the activation process, leading to reduced productions of IL-6, MCP-1/CCL2 and collagen by activated fibroblasts (myofibroblasts). (II) Activation of fibrosis pathway by CS-E. CS-E acts as a co-receptor for Wnt ligand and a direct receptor for RAGE, both leading to activation of fibrosis signaling pathways. (III) Collagen fibril formation by CS-E. CS-E directly augments collagen fibril formation and inhibits its degradation, leading to intractable fibrosis. STNM01 inhibits CS-E biosynthesis into ECM. STNM01 represses the excessive collagen deposition and promotes mucosal healing in clinical studies, indicating anti-tissue remodeling effect *in vivo* and in patients. CCL2, CC chemokine ligand 2; MCP-1, monocyte chemoattractant protein-1; EMT, epithelial mesenchymal transition; MOA, mechanism of action; CHST15, carbohydrate sulfotransferase 15; CS-E, chondroitin sulfate-E; RAGE, receptor for advanced glycation end product.

cell lines by the treatment with STNM01. STNM01 also reduced the accumulation of fibroblast as well as the expression of α -SMA in *in vivo* disease models, supporting its inhibitory effect on fibroblasts. Interestingly, STNM01 reduced the expression of Loxl2, an enzyme that catalyzes the collagen cross-linking, suggesting an involvement in regulating fibrinolytic pathway (28).

Since STNM01 possess dual targets, CHST15 and its specific product CS-E, the mode of action in IBD is summarized in *Figure 1*. In response to tissue injury, CHST15 is induced by activated fibroblasts from resident/

recruited fibroblasts or through EMT. Activated fibroblasts/myofibroblasts produce IL-6, MCP-1, collagen and CS-E into ECM. Matrix CS-E directly binds to RAGE or acts as a co-receptor for Wnt/Frizzled on fibroblasts, then further stimulates fibrosis pathway in an autocrine or paracrine manner. Matrix CS-E directly promotes collagen fibrils and also inhibits degradation of established fibrils, leading to the inhibition of fibrinolytic property. STNM01 therefore could shut down the vicious cycle of fibrogenesis. In addition, as excessive fibrils would be an obstacle to epithelial healing, restoration or promotion of fibrinolytic

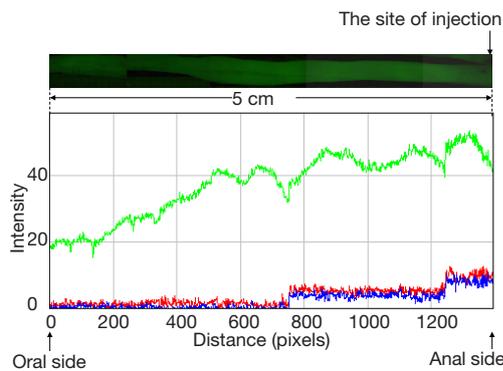


Figure 2 Intensity of fluorescent-labeled siRNA after single, one site-injection to rectum in mice. (A) Stereoscopic microscopy of normal mouse colon after single injection of FAM-labeled siRNA. The siRNA was injected at one-site in rectum near anal verge; (B) intensity of fluorescence signal at the corresponding location by ImageJ software. The intensities of Red, Green and Blue are plotted. The intensity of FAM-labeled siRNA was indicated as Green. The intensities of Red and Blue channels are also shown as background.

activity is prerequisite for epithelial regeneration. STNM01 would enhance the degradation of established collagen fibrils, and this also contribute to treat tissue remodeling.

A novel pancolonic delivery as a translatable application of oligonucleotide

Endoscopic submucosal injection

To translate therapeutic potential of oligonucleotide into clinical practice, it is important to increase targeting and retention of siRNA at sites of injury in patients. We have established a novel pancolonic delivery in mice by an approach utilizing submucosal injection through endoscopy (25). Only one site-injection enables siRNA to rapidly spread in a circumferential manner and at the same time, to diffuse from rectum to cecum in mice (25). Imaging analysis of the intensity of fluorescence signal in stereoscopic microscopy was performed after one-site injection of green fluorescence-labeled siRNA in normal mouse colon (Figure 2). The average intensity, which was indicated as the intensity of Green channel, at 5 cm (oral side) away from the site of injection (anal side) was approximately 40% of that at the site of injection (Figure 2). This suggested that the amount of siRNA at 5 cm away from the site of injection was also approximately 40%

at the injection site. Thus, if the injection is performed at 10 cm-interval, the theoretical amount of siRNA at the middle position (5 cm from the injection sites) will be 80% in total. We also demonstrated previously that injected siRNA could retain within matrix space and to be incorporated into cells in a sustained manner (25). Our discovery indicates that the direct injection of siRNA into ECM without facing bloodstream is the most important key technique to achieve successful retention of siRNA and continuous supply to cells from matrix fiber.

Clinical application for IBD and evidence of target CHST15 reduction in patients

Given the novel pancolonic siRNA delivery, we next tried to translate it to clinical study for IBD. For CD, we took an approach to focal injection as most CD patients have localized ulcers and lesions (26). In accordance with “lifting-up” technique before endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) against localized gastrointestinal caners, STNM01 was submucosally administered to surround the periphery of the target, localized lesion (Figure 3) in a phase 1 first-in-patient clinical trial (26). There were 8 total injection sites. The volume of injection was 1 mL/site per injection, thus 8 mL/one lesion in total (Figure 3). The effect of the target CHST15 reduction was also evaluated by immunological staining of biopsy specimens from the edge of the target ulcer. The grade of CHST15 staining was scored (26) and the % reduction of CHST15-positive score was evaluated at day 30 from the baseline (Figure 4). Compared to the placebo group who did not show the reduction of CHST15, the STNM01-treatment groups showed the significant reductions at day 30 after single injection of STNM01 (Figure 4). Of these, the highest dose of STNM01 (250 nM) showed the superior silencing efficiency (Figure 4), indicating that CHST15 immunostaining is a good method to identify the drug efficiency in patients. There was no drug-related side effect as well as inflammation at the injection sites, indicating that the method is safe as expected.

Distinct from CD, UC depicts a continuous lesion from rectum. We have therefore conducted distinct injection model for UC in a phase 2a study. STNM01 is evenly administered in a 10 cm-interval (Figure 3) based on the pancolonic delivery observed in mice (Figure 2). There were total 8 site injection sites. Two site-injections (upper and bottom sites) at 35, 25, 15 and 5 cm from anal verge for

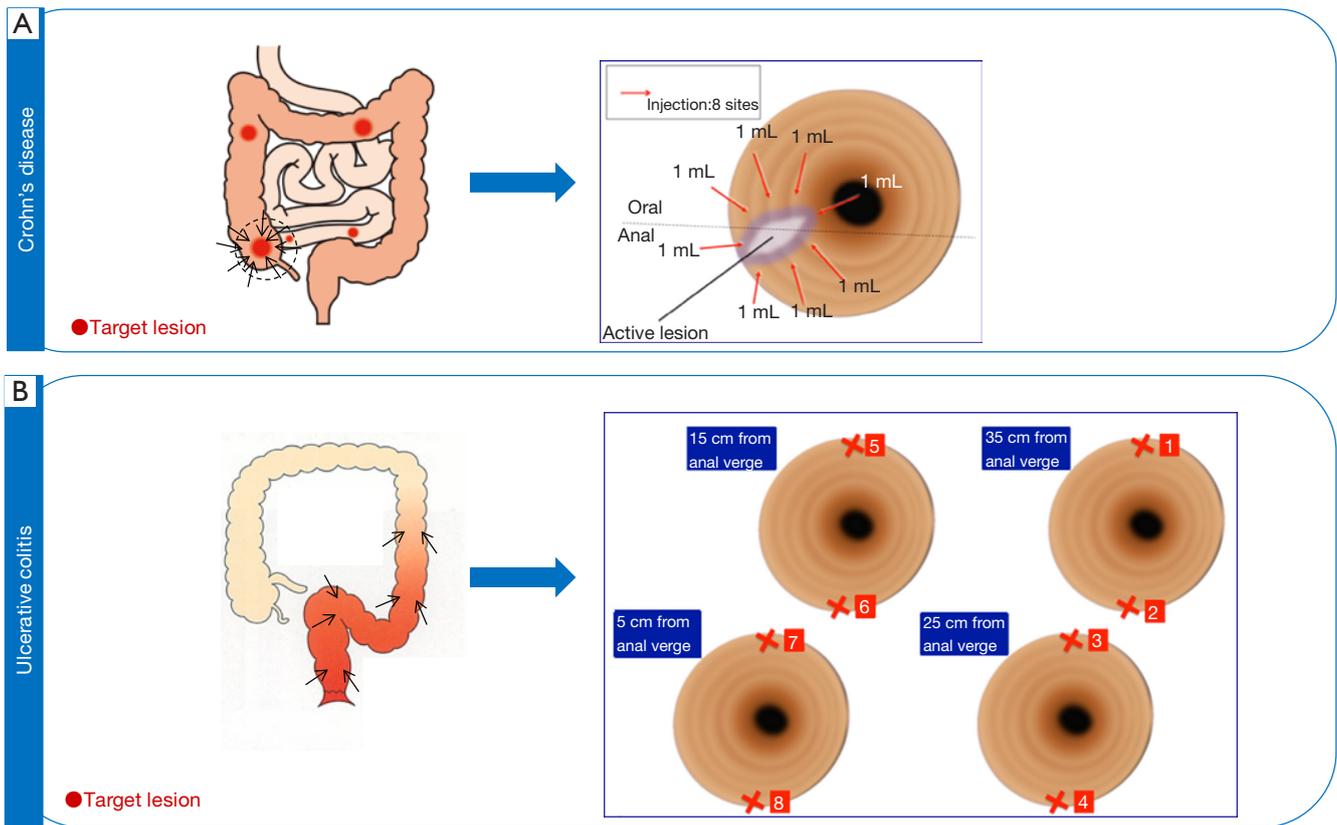


Figure 3 Clinical application of new endoscopic therapy for IBD. (A) For localized lesion(s) like Crohn’s disease (CD); (B) for continuous lesion like ulcerative colitis (UC).

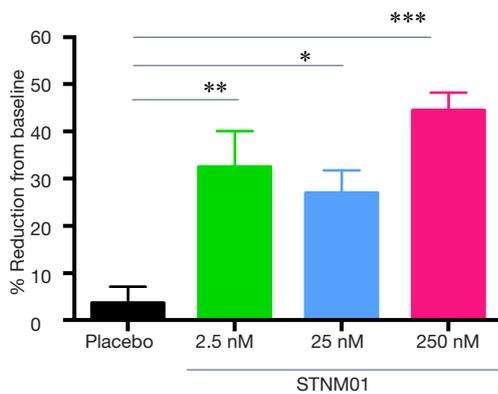


Figure 4 Evidence of target CHST15 reduction in target lesions by STNM01. % reduction of CHST15 at day 30 from baseline was shown in 3 doses of STNM01 and placebo. % reduction was calculated by CHST15-immunostaining score of biopsy specimens from patients (26). ***, P<0.001; **, P<0.01; *, P<0.05. One-way ANNOVA multiple comparison test.

left-sided colitis were performed. The volume of injection was 2 mL/site per injection, thus 16 mL/left-side colon in total (Figure 3). Theoretically, at least 40 cm length of colon can be fully covered by this approach. The results will be presented soon.

Endoscopic MH and histological fibrosis as efficacy measures

Induction of quick MH response by STNM01 with anti-fibrotic action

Phase 1 first-in-patient trial was conducted for patients with CD who do not respond to conventional treatment including biologics (hereafter, “non-healer”) and endoscopy as well as histology were investigated as secondary endpoints (26). Figure 5 shows a typical patient depicting deep ulcer despite intensive treatment with infliximab for

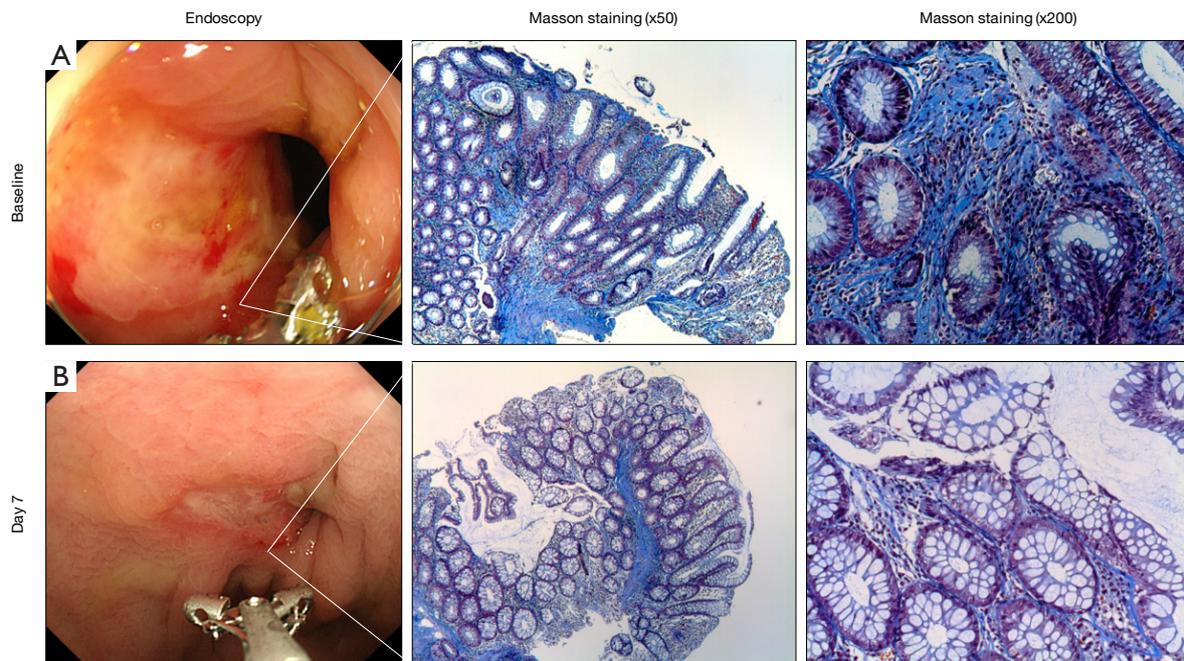


Figure 5 Endoscopic and corresponding histological findings of refractory ulcer before and 7 days after treatment with STNM01. (A) Baseline; (B) day 7 after single injection with STNM0.

2 years. Biopsies were performed from the edge of ulceration or the center of scar during the study period. Masson trichrome staining revealed that the existence of severe fibrosis, inflammation and crypt destruction at baseline, indicating infliximab refractory (*Figure 5A*). Surprisingly, only 7 days after single injection with STNM01, the refractory ulcer reduced dramatically and red regenerative mucosa appeared surrounding healing ulcer (*Figure 5*, lower left panel). The biopsy forceps are used for estimating the size of ulcer and clearly demonstrated that the size of ulcer exhibited over 50% reduction in diameter 7 days after injection. Histology of biopsy specimens demonstrated that the repression of fibrosis, reduction of inflammatory infiltrate and re-appearance of goblet cells with fine crypt architecture, supporting the endoscopic findings of the regenerative mucosa (*Figure 5B*).

Figure 6 showed histology of 3 patients who received STNM01 at another clinical site. Case 1 was an early CD patient as the disease duration was 1 year (y1; *Figure 6*, left panels). The patient received 5-ASA and corticosteroid but depicted drug-resistant ulcer, then was enrolled in the study before using biologics [biologics naive]. The histology showed that inflammation and crypt destruction were relatively weak but fibrosis was evident, indicating that fibrotic reaction already occurred in such an early disease phase and was uncontrollable by 5-ASA and corticosteroid

alone. At day 30, however, the histology was almost normalized with repression of fibrosis by STNM01. Case 2 was infliximab-refractory patient with disease duration of 15 years (y15; *Figure 6*, middle panels). Diffuse inflammation within fibrotic space and crypt destruction were evident at baseline, supporting infliximab-refractory. At day 30, the patient showed reduced fibrosis/inflammation and improved crypt architecture including re-appearance of goblet cells. Case 3 was infliximab/adalimumab-double failure with disease duration of 8 years (y8; *Figure 6*, right panels). At baseline, heavily inflammation with more fibrotic space and severer crypt destruction were observed. At day 30, fibrosis was clearly repressed and crypt was also repaired although minor focal inflammation still existed. All patients here also showed clear endoscopic MH, suggesting that repression of histological fibrosis was predicted by endoscopic MH.

Mechanism why anti-fibrotic drug STNM01 induces MH in refractory CD patients

Tissue remodeling consists of (I) failure to eliminate the inciting factors, (II) obstacle to mucosal repair and (III) persistent activation of inflammatory cells and fibroblasts (15). Collagen fibril in fibrotic tissue is an obstacle to epithelial regeneration/MH. As prerequisite for epithelial

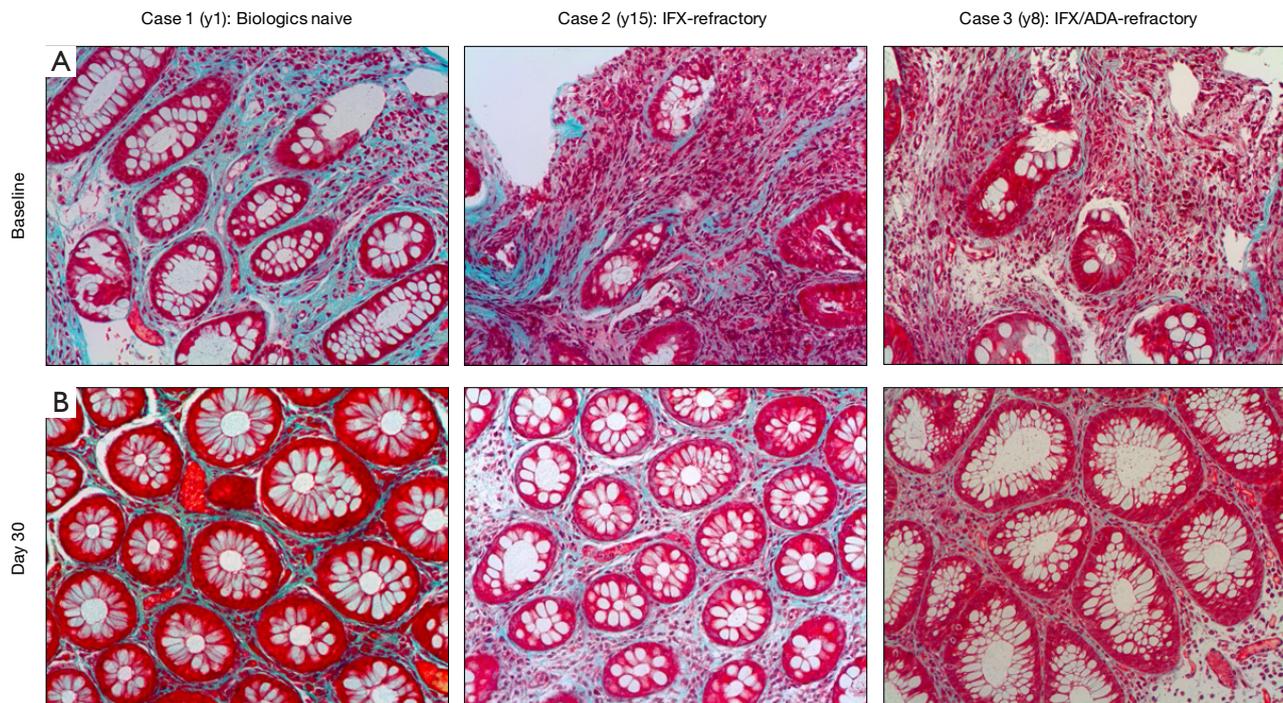


Figure 6 Histological findings of refractory ulcer before and 30 days after treatment with STNM01. (A) Baseline; (B) day 10 after single injection with STNM0. Masson Trichrome staining, $\times 200$. y, year (duration of disease); IFX, infliximab; ADA, Adalimumab.

regeneration, the blockade of CS-E by STNM01 could enhance the degradation of established collagen fibril and restore fibrinolytic activity. In addition, STNM01 inhibits further fibrosis pathways, thus could shutdown malignant cycle of fibrogenesis. Whether STNM01 directly acts on the epithelial cells to promote their proliferation or not was not established yet. But STNM01 increases BMP-7 which counteracts to TGF- β -mediated EMT. This also contributes to enhancing MH activity. Although STNM01 showed reduced inflammatory infiltrates in patients and in animal models, it is also still unclear whether anti-inflammatory lead-out by STNM01 was secondary to anti-fibrotic action or due to other distinct mechanism of action. The effects of STNM01 on epithelium and inflammation should be investigated further in order to clarify the mechanism of its integrated anti-tissue remodeling activity.

One question arose whether repression of fibrosis may increase the risk of perforation. In animal models, STNM01 did not affect normal fibroblasts and tissue architecture. Major activity of STNM01 is on activated fibroblasts but not on normal fibroblasts that do not show higher level of CHST15. Important information was achieved from pig model of esophageal stricture after ESD which

is characterized by both fibrosis and atrophic change of muscularis proper (27). STNM01 showed no muscle layer defect, supporting the low risk of perforation or fistula. Interestingly, STNM01 possess rather protective effect to muscle layer. Indeed, there was no sign of perforation and fistula in phase 1 study and its long-term observation over 1 year (26). Thus, anatomy-based, appropriate anti-fibrosis therapy does not increase the risk of perforation or fistula because it does not impact on muscle layer.

Future perspective

Fibrosis in IBD is a largely unresolved clinical problem, but no anti-fibrotic therapies are available at present. Multiple obstacles have hampered the progress of developing novel anti-fibrotic drugs in IBD. To overcome these obstacles, we have conducted a series of studies. To summarize, our research road map is shown in *Figure 7*. We have identified a new target CHST15 as a key pathogenic factor that has a potential to regulate multi-factors in tissue remodeling. To increase the specificity, we selected siRNA as a feasible compound and took suitable application for siRNA delivery. This is a new endoscopic submucosal injection possessing

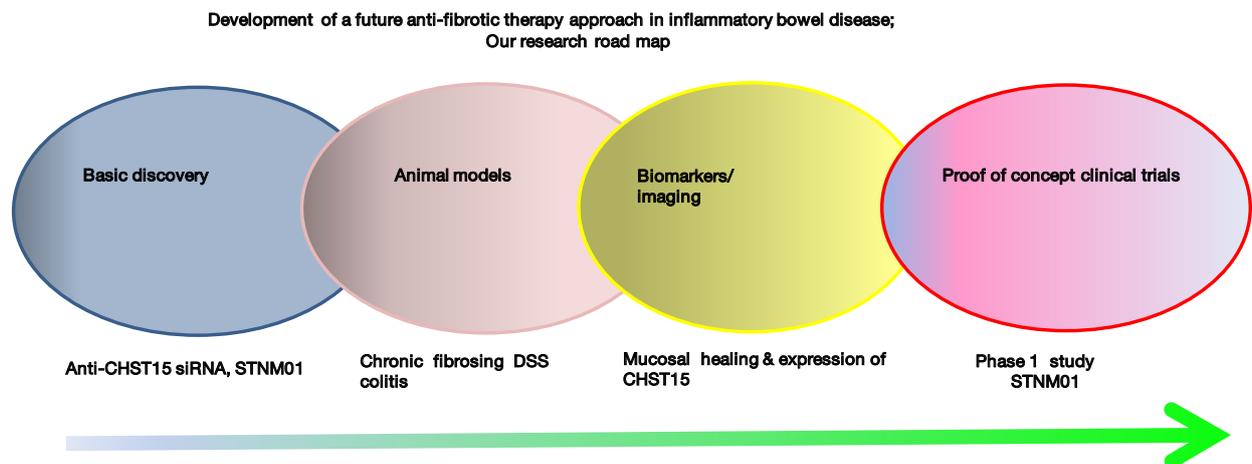


Figure 7 Development of a future anti-fibrotic therapy approach in inflammatory bowel disease; our research road map.

pancolonic delivery. We also established animal model that can bring insights into the pathogenesis of intestinal fibrosis (30) and demonstrated the therapeutic effect of STNM01 in view of anti-fibrosis as well as MH (25). As the balance between MH and fibrosis would contribute to disease outcome in tissue remodeling in IBD, we have investigated both endoscopic MH and histological fibrosis in animal and patients and shown endoscopy would be a feasible tool to predict the anti-fibrotic efficacy. We thus consider both endoscopic finding of MH and expression of CHST15 in the local site of intestine as a golden marker which predict fibrosis and response to therapy.

Based on the repression of pre-established fibrosis in patients with CD by the treatment with STNM01, CHST15 is demonstrated as a good therapeutic target for intestinal fibrosis in human. Considering anti-fibrotic actions of CHST inhibition in other organs like heart and lung (27,28), further modification to systemically applicable formulation including oral or systemic siRNA or screening of small molecule inhibitors is of great interest in the future.

Conclusions

Our studies document the translational practice from nonclinical studies into first-in-patient study by establishing the specific therapeutic target that could modulate multiple factors in tissue remodeling, clinically feasible application for siRNA and tissue-specific endpoints. The patient results also demonstrate an opportunity for new endoscopic anti-fibrosis therapy in IBD.

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Footnote

Conflicts of Interest: K Suzuki is an inventor of a patent related to submucosal injection. H Yoneyama founded Stelic Institute & Co., Inc. in 2004. Stelic filed and issued patents related to the treatment of intestinal fibrosis/inflammatory bowel disease with oligonucleotide-based medicine targeting CHST15.

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