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Significant contribution of TRPC6 channel-mediated Ca²⁺ influx to the pathogenesis of Crohn's disease fibrotic stenosis

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Abstract

Intestinal fibrosis is an intractable complication of Crohn's disease (CD), and, when occurring excessively, causes severe intestinal obstruction that often necessitates surgical resection. The fibrosis is characterized by an imbalance in the turnover of extracellular matrix (ECM) components, where intestinal fibroblasts/ myofibroblasts play active roles in ECM production, fibrogenesis and tissue remodeling, which eventually leads to the formation of stenotic lesions. There is however a great paucity of knowledge about how intestinal fibrosis initiates and progresses, which hampers the development of effective pharmacotherapies against CD. Recently, we explored the potential implications of transient receptor potential (TRP) channels in the pathogenesis of intestinal fibrosis, since they are known to act as cellular stress sensors/transducers affecting intracellular Ca²⁺ homeostasis/dynamics, and are involved in a broad spectrum of cell pathophysiology including inflammation and tissue remodeling. In this review, we will place a particular emphasis on the intestinal fibroblast/myofibroblast TRPC6 channel to discuss its modulatory effects on fibrotic responses and therapeutic potential for anti-fibrotic treatment against CD-related stenosis.

Key words: Inflammatory Bowel disease, myofibroblast, TRP channel, fibrotic stenosis, Ca²⁺

Methods

The details of the procedures used for cell culture, [Ca²⁺]ᵢ measurement, immunostaining, and real-time RT-PCR are described elsewhere (1). Biopsy samples were obtained from CD patients according to their in-

formed consents. Statistical analysis was performed as described previously (1). The Fukuoka University Hospital Ethics Committee approved the protocol, and written informed consent was obtained from all patients.

Expression array analysis

Total RNA isolation for array: The total RNA was isolated from the cerebellum of each individual animal using TRIzol Reagent (nitrogen) and purified using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RNA samples were quantified by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was confirmed with an Experion System (Bio-Rad Laboratories, Hercules, CA).

Gene expression microarrays: The cRNA was amplified, labeled using GeneChip® WT Terminal Labeling and Control Kit, and hybridized to an Affymetrix Human Genome U133 Plus 2.0 array according to the manufacturer's instructions. All hybridized microarrays were scanned by an Affymetrix scanner. Relative hybridization intensities and background hybridization values were calculated using the Affymetrix *Expression Console™*.

Data analysis and filter criteria: Raw signal intensities for respective probes were calculated from hybridization intensities. Then the raw signal intensities of two samples were $\log_2$-transformed and normalized by RMA (Robust Multi-array Average) and quantile algorithm [P] with Affymetrix® Expression Console™ 1.1 software. To identify up- or down-regulated genes, we calculated Z-scores [Z] and ratios (non-log scaled fold-change) from the normalized signal intensities of respective probes for comparison between control and experiment sample. Finally, we established the criteria for regulated genes: (up-regulated genes) Z-score $\geq$ 2.0 and ratio $\geq$ 1.5-fold, (down-regulated genes) Z-score $\leq$ –2.0 and ratio $\leq$ 0.66.

—————— Intestinal Fibroblast/Myofibroblast and fibrosis ——————

Fibrosis is the common final pathway to organ failure in diseases of the heart, kidney, liver, lung, and intestine. It has been estimated that about 45% of human deaths are associated with fibroproliferative disorders including fibrosis (2). Intestinal fibrosis is a major complication of inflammatory bowel disease (IBD) and can occur in both ulcerative colitis (UC) and CD, but is much more prevalent in CD (3). Approximately 40% of CD patients with ileal disease develop clinically apparent strictures throughout their lifetime, which significantly influences the quality of life (4, 5). The excessive presence of fibrous tissue increases the thickness of bowel wall, reducing the elasticity and the function over the affected area. Even surgical removal performed to eliminate the fibrotic stenosis and obstructing strictures often fails to prevent a recurrence in the same patient (6). Anti-inflammatory therapies are not efficient in resolving the fibrosis, CD patients treated with biologics still develop strictures and associated complications (7). Currently, there is not much information known about pathogenic mechanisms associated with detrimental fibrosis, but a few clues have been hinted by experimental models (8–10). The critical role of intestinal fibroblasts/myofibroblasts in wound healing and development of fibrosis is well recognized (11–13). Persistent myofibroblastic activity can underlie hypertrophic scarring, loss of tissue compliance, and even rampant fibrosis that is the basis for fibrotic disorders of the heart, skin, lung, kidney, skeletal muscle, and liver (8, 14, 15). As shown in Fig. 1, the origin of fibroblasts and myofibroblasts is very controversial and potentially includes resident fibroblasts, bone marrow derived mesenchymal precursors (fibrocytes), and epithelial cells undergoing the epithelial-to-mesenchymal transition (EMT). Fibroblasts isolated from IBD mucosa proliferate faster than normal, and this increase occurs after exposure to growth factors and proinflammatory cytokines, and after direct cell-to-cell contact with inflammatory cells (10, 16).
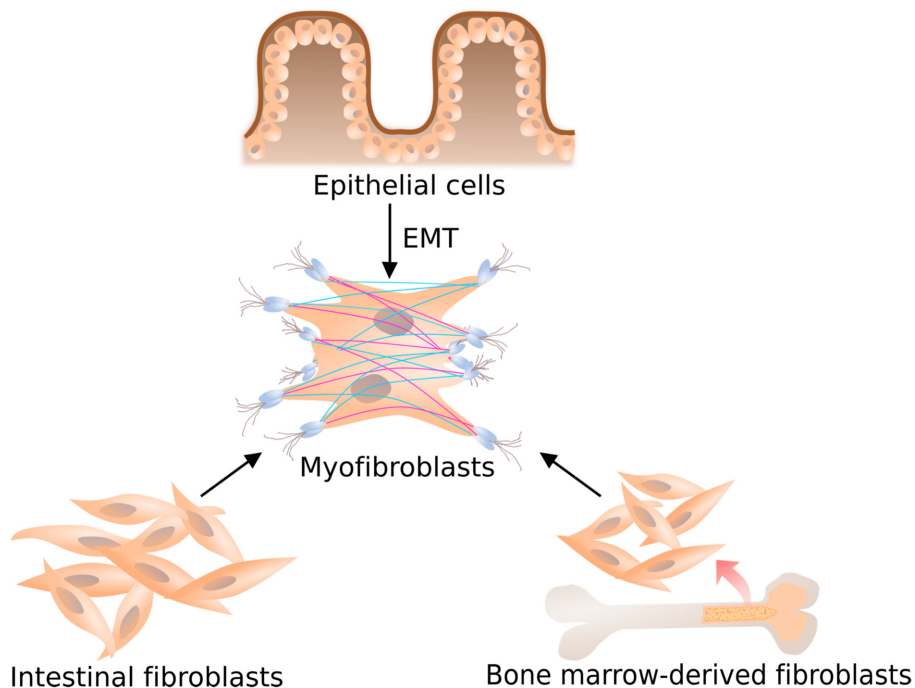
Fig. 1. Schematic illustration showing the evolution of the myofibroblast phenotype. Distinct cell types are involved in intestinal fibrosis, such as ECM-producing cells derived from epithelial, local or bone marrow-derived fibroblasts. Following an increase in mechanical tension, and fibrotic cytokine such as TGF-β1 further differentiation occurs to a contractile phenotype, termed a differentiated myofibroblast, characterized by the expression of alpha smooth muscle actin.

During the inflammatory process, to repair and regenerate homeostasis, these tissue-resident fibroblasts are activated and transformed into myofibroblasts, contractile cells expressing α-SMA and myosin bundles. Myofibroblasts secrete ECM and collagens, and are vital players in the fibrotic stenotic tissue, by aiding tissue contracture and healing. In the wound-healing program, a substantial portion of myofibroblasts could also arise from regenerating epithelial or endothelial cells or from epithelial stem cell progenitors via EMT. Circulating fibrocytes appear universally involved in organ fibrosis. A complex array of cytokines, chemokines and growth factors regulate fibrocyte biology, and these are associated with fibrogenesis in CD. The cytokines transforming growth factor β1 (TGF-β1), connective tissue growth factor and interleukin 13 (IL-13), overexpressed in the strictured Crohn's intestine, promote fibrocyte generation and/or differentiation (17, 18). Increased resident fibroblast/myofibroblast populations are pivotal to fibrosis development. During inflammatory process, profibrotic cytokines and chemokines (TGF-β, IL-13, IL-17), the peptide hormone Angiotensin II, growth factors (CTGF and PDGF) and matrix factors (hyaluronan fragments, mechanical stress and/or stiffness) are secreted from mesenchymal and inflammatory cells to induce or augment myofibroblast transformation (8, 19). These changes subsequently induce extracellular matrix deposition, metalloproteinase inhibition, and fibroblast activation.

TGF-β is central to the development of fibrotic stenosis in CD. In numerous cell types, TGF-β secretion augments myofibroblast transformation. There are three TGFβ isoforms in mammals, namely TGFβ1, TGFβ2 and TGFβ3 which are expressed in myofibroblasts, vascular smooth muscle cells, endothelial cells, and macrophages (20). The human TGFβ1 gene produces a 390 amino acid propeptide which is cleaved intracellularly into two identical 112 amino acid peptide subunits joined together by a disulphide bond (21). The canonical

TGF-β signaling pathway commences with binding of TGF-β to a TGF-β type 2 receptor, which subsequently heterodimerizes with a TGF-β type l receptor to form an active TGFβR1 complex. TGF-β and its receptors are up-regulated in CD strictures, and abnormal TGF-β signaling impairs the intestinal immune tolerance and tissue repair (22). Blockade of TGFβR1 signaling by an injectable inhibitor (SD-208) was evaluated in two experimental animal models of intestinal fibrosis: anaerobic bacteria- and trinitrobenzensulphonic acid-induced colitis (TNBS). SD-208 reduced fibroblast activation, phosphorylation of Smad2 and Smad3 proteins, and intestinal wall collagen deposition in both models (23). Although TGFβ1 and mechanical stress (generated by ECM stiffness) are recognized as major mediators of myofibroblast differentiation, the molecular signals in these soluble and mechanical signals are still elusive. Furthermore, intestinal stricture formation in CD is driven by local excessive production of TGF-β (13, 24). In addition to TGF-β1, emerging evidence has shown that IL-13 and IL-17 are involved in intestinal fibrosis. In TNBS-induced colitis, inhibition of IL-13 signaling by administration of small interfering RNA targeting the IL-13Rα2, reduces fibrosis and expression of TGFβ (25). IL-17A expression was found to be increased in the inflamed areas of patients with inflammatory bowel disease (26). But, blockade of IL-17A by administration of the anti-IL-17A antibody, secukinumab, failed to meet its primary endpoint, in a clinical trial of CD patients (27).

Myofibroblasts synthesize ECM components and generate high contractile forces for wound retraction or tissue remodeling in developmental processes. It is well known that fibrosis is associated with excessive accumulation of ECM components, such as collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) (2, 28, 29). This is mainly owing to increased synthesis and decreased degradation of ECM components. Notably, MMPs that degrade the ECM are upregulated, whereas TIMPs are down-regulated (30). In addition, other ECM proteins, such as fibronectins, elastin, and fibrillins, are upregulated during the development of fibrosis. In response to tissue injury and profibrotic mediators, including TGF-β, IL-13 and IL-17, fibroblasts differentiate into myofibroblasts, and the activation and/or recruitment of fibroblasts resistant to apoptosis result in fibrogenesis and subsequent fibrosis (31, 32). A defining feature of fibroblast to myofibroblast differentiation is the formation of αSMA stress fibers that provide a structural network for generating contractile forces (10, 16, 33). The α-SMA expression is suppressed by extracellular fibrotic collagen and by anti-fibrotic cytokines, such as IL-10 and IL-11 (34–36). Increased constitutive N-cadherin expression in fibroblasts has been shown to potentiate stricture formation in CD patients (37).

—— Fibroblast/Myofibroblast TRP channels and tissue remodeling ——

Interestingly, calcium signaling has recently gained much attention as a regulator of myofibroblast contractile activity but it is not known whether calcium signaling is required for the differentiation of fibroblasts to myofibroblasts (32, 38). Ca^{2+} is an essential signaling molecule implicated in various long-term cellular consequences, such as differentiation, gene expression, and cell proliferation, growth and death, and it plays a significant role in regulating fibroblast functions (39–41). In general, there are two distinct sources of Ca^{2+} for elevating intracellular Ca^{2+} levels: Ca^{2+} influx across the plasma membrane and Ca^{2+} release from the endoplasmic reticulum. Ca^{2+} influx can occur through three functionally distinct classes of Ca^{2+}-permeable channels, i.e. voltage-gated Ca^{2+} channels, receptor-operated Ca^{2+}–permeable channels (ROC) and store-operated Ca^{2+} channels (SOC). Through extensive survey of their molecular identification, the majority of ROCs and some of SOCs have been closely linked to the transient receptor potential (TRP) superfamily (42, 43). Several lines of evidence suggest that fibrosis-associated events in myofibroblasts are controlled by the cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$), which is mediated by some members of TRP superfamily (44–47). TRP channels are

cellular sensors for a variety of physical and chemical stimuli (48–50). Gastrointestinal TRP channels are involved in the sensation of smell, taste, touch, temperature, and pain (48, 51–53). TRP channels also play essential roles in cell signaling and responses to benign or harmful environmental changes (54–57). In addition to Ca^{2+}, TRP channels change the membrane potential, translocate important signaling ions across the cell membrane, change enzymatic activities, and initiate endocytosis or exocytosis (48, 58, 59). The TRPC family consists of seven distinct isoforms designated as TRPC1–TRPC7 (44, 58, 60, 61). TRPC family members can be transcriptionally induced and/or are directly activated by G-protein coupled-receptor (GPCR) signaling through diacylgylcerol, and are susceptible to the depletion of intracellular Ca^{2+} stores or to the stretch of the plasma membrane (55). TRPC channel-mediated Ca^{2+} influx can directly activate the Ca^{2+}-sensitive protein phosphatase calcineurin to induce diverse intracellular responses through its downstream transcriptional effector nuclear factor of activated T-cells (NFAT) (46). TRPC1-mediated Ca^{2+} influx is essential for intestinal homeostasis/inflammation and progesterone-induced endometrial decidualization (57, 62, 63). Low intensity irradiation with 635 ± 5 nm diode laser inhibited TGF-β1/Smad3-mediated fibroblast-myofibroblast transition and this effect involved the modulation of TRPC1 ion channels (64). Ca^{2+} signaling via the TRPM7 channel likely plays a key role in TGF-β1-elicited fibrogenesis in human atrial fibroblasts (47). Cell-cell contact formation is governed by Ca^{2+} signaling via TRPC4, which co-immunoprecipitates with the junction proteins β-catenin and cadherin in vascular endothelial cells (65). However, whether TRP channels play roles in intestinal fibrosis remains to be investigated.

——— Intestinal Fibrotic stenosis and Myofibroblast TRPC6 channels ———

We investigated whether TRP channels are involved in the expression of fibrosis-associated molecules and TGF signaling in InMyoFib cells. At first, we examined TGF-β1-induced morphological changes and TRP channel expression in InMyoFib (intestinal myofibroblast cell line: Fig. 2A). We found that TGF-β1 significantly upregulates TRPC6 mRNA (Fig. 2B) and protein expression. Upregulated TRPC6 expression is essential for the formation of α-SMA stress fibers and N-cadherin-mediated adherens junctions, which respectively enable myofibroblasts to gain contractility and reinforce mutual intercellular connections (6, 8, 66). The hallmarks of myofibroblast differentiation are stress fiber development and *de novo* α-SMA expression. Incorporation of α-SMA into stress fibers confers high contractility to myofibroblasts, promoting the formation of specialized contacts within extracellular matrix regions termed "supermature focal adhesions" *in vitro* and "fibronexus" *in vivo* (67). Fibroblasts from the strictured regions of CD patients show increased constitutive expression of N-cadherin and exhibit enhanced basal cell migration (37). TGF-β1 potently induces N-cadherin expression in intestinal fibroblasts and cell's migration ability (37, 68). Interestingly, adherens junctions appear in fibrotic tissue, but are absent in normal tissue in which fibroblasts do not develop stress fibers (67). Thus, direct links between subcellular stress fibers and cell surface cadherins may serve to maintain the tension created between adjacent cells. In the current review, it is well accepted that cellular adhesion molecules, integrins and cadherins, may contribute to the development of tissue fibrosis (69, 70).

Among the members of the TRP channel family, TRPC6 is a receptor-operated cation channel that can be activated by angiotensin II or endothelin I through stimulation of their corresponding receptors and secondary generation of diacylglycerol. Additionally, TRPC6 participates in the development and pathogenesis of fibrotic diseases, such as hepatic, renal, pulmonary, and cardiac fibrosis (45, 71, 72). TRPC6 and calcineurin are required to promote myofibroblast differentiation, suggesting the presence of a comprehensive pathway for the differentiation associated with TGFβ, p38 MAPK and serum response factor (33). We also tested the
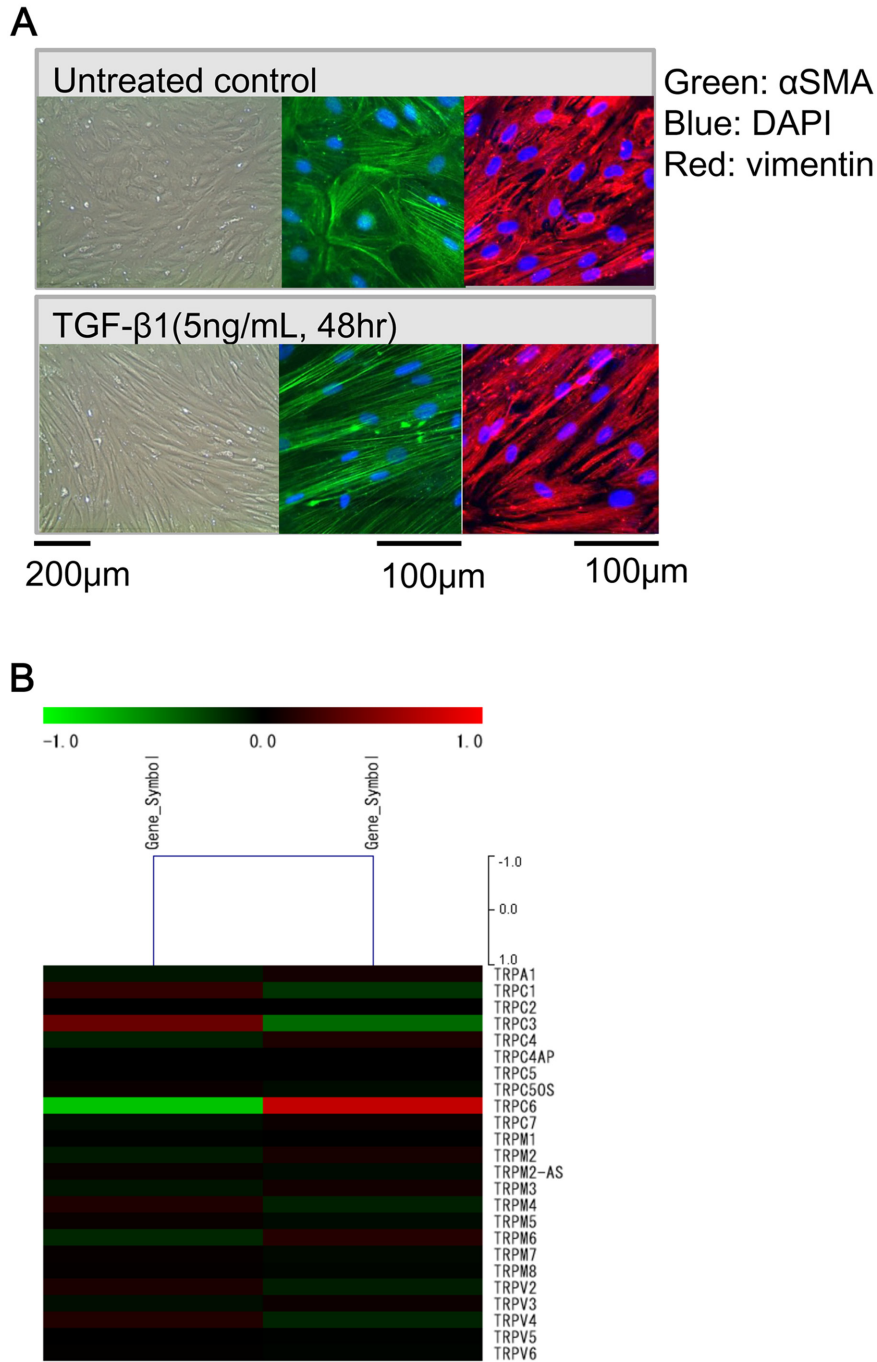
Fig. 2. TGF-β1-induced morphological changes and TRPC6 expression in InMyoFib cells.
A: Phase-contrast (left) or immunostaining images of InMyoFib cells stained with anti-α-SMA (green) and anti-vimentin (red) antibodies on the day of plating (untreated control), or 48-h post-treatment with TGF-β1 (5 ng/ml). Modified from (1)
B: Fold change of TRP isoform mRNAs in InMyoFibs by expression array analysis. Control vs TGF-β1 (5 ng/ml, 24 h) treated InMyoFib.

abilities of the muscarinic agonist carbachol (CCh) and a membrane-bulging agent TNP to induce Ca^{2+} influx and potentiation (73). The magnitude of the CCh-induced Ca^{2+} influx and its enhancement by TNP were nearly abolished by *TRPC6*-si treatment (Fig. 3). These results strongly suggested that TRPC6 makes a critical contribution to TGF-β1-mediated enhancement of both basal and biochemically/mechanically-induced Ca^{2+}
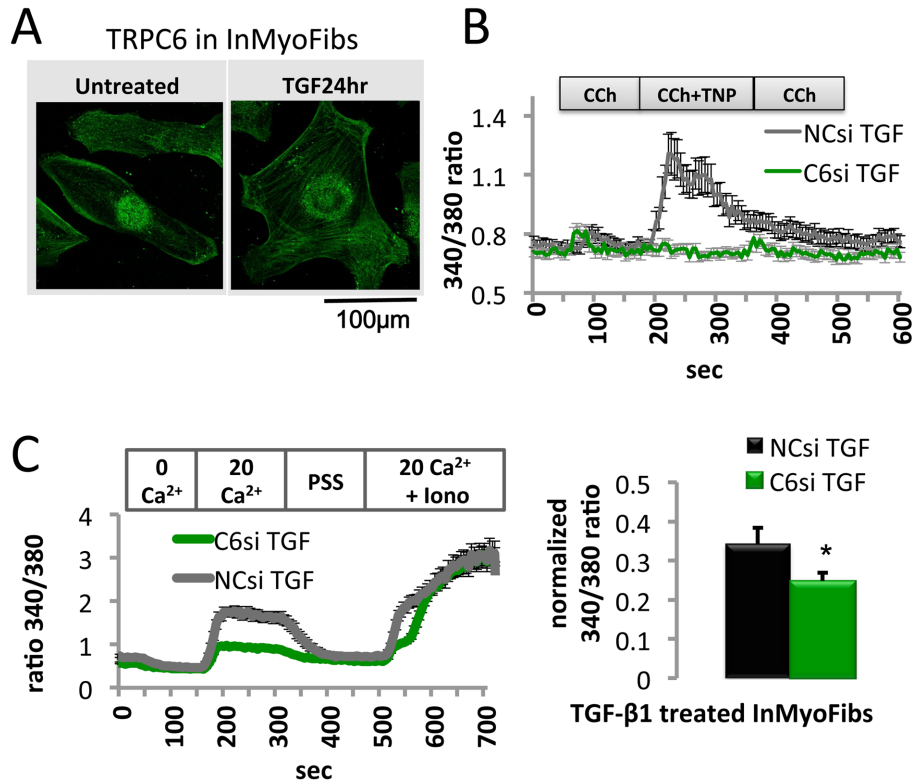
Fig. 3. TRPC6-associated Ca^{2+} influx by TGF-β1 treatment. A: InMyoFib plasma membrane expression of TRPC6. Immunofluorescence double staining of untreated or TGF-β1-treated (5 ng/ml) InMyoFib cells with TRPC6-Alexa488 (green) primary antibodies. Scale bar = 100 μm. B: TRPC6si pre-treatment diminished shear stress-induced Ca^{2+} entry. Representative [Ca^{2+}]$_i$ responses of TGF-β1-treated (5 ng/ml) cells. Cells were exposed to CCh (10 μM), CCh + TNP (500 μM), or CCh (10 μM). C: Representative [Ca^{2+}]$_i$ responses of TGF-β1 (5 ng/ml)-treated cells that were transfected with negative control siRNA (NCsi) or *TRPC6* siRNA (C6si) during a pre-treatment step. Data points represent mean ± S.E.M. from > 30 cells. Modified from (1).

influxes in InMyoFibs. Overexpression of TRPC6 siRMA, dominant-negative TRPC6 mutants (Δ130-TRPC6 and 3A-TRPC6) (46) or administration of SKF resulted in the enhanced phosphorylation of SMAD-2, ERK1/2, and p38-MAPK (1). Moreover, treatment with cyclosporin A or FK506 significantly enhanced TGF-β1-induced phosphorylation of SMAD-2, ERK1/2, and p38-MAPK (1). These lines of evidence suggest that Ca^{2+} influx through this channel negatively regulates TGF-β1-SMAD/p38-MAPK/ERK1/2 signaling via calcineurin activation.

As summarized in Fig. 4, while TGF-β1-mediated increase in TRPC6 activity promotes the expression of α-SMA and N-cadherin and strengthened their interactions with TRPC6 protein, it also negatively regulates collagen synthesis and the secretion of anti-inflammatory/anti-fibrotic factors. These pleiotropic effects appear to be mediated by distinct downstream pathways of TGF-β1 signaling, suggesting that TRPC6 may be involved in fibrosis in a very intricate way. Obviously, more in-depth investigation is necessary to decipher how stimulation of TGF receptor(s) leads to the activation of distinct TRPC6-mediated signaling cascades linked to both anti- and pro-fibrotic consequences during the transition from wound healing to fibrosis. This may open an avenue of discovering a new TRPC6-targeting therapy which would be more appropriate for CD patients with intestinal fibrosis (1).
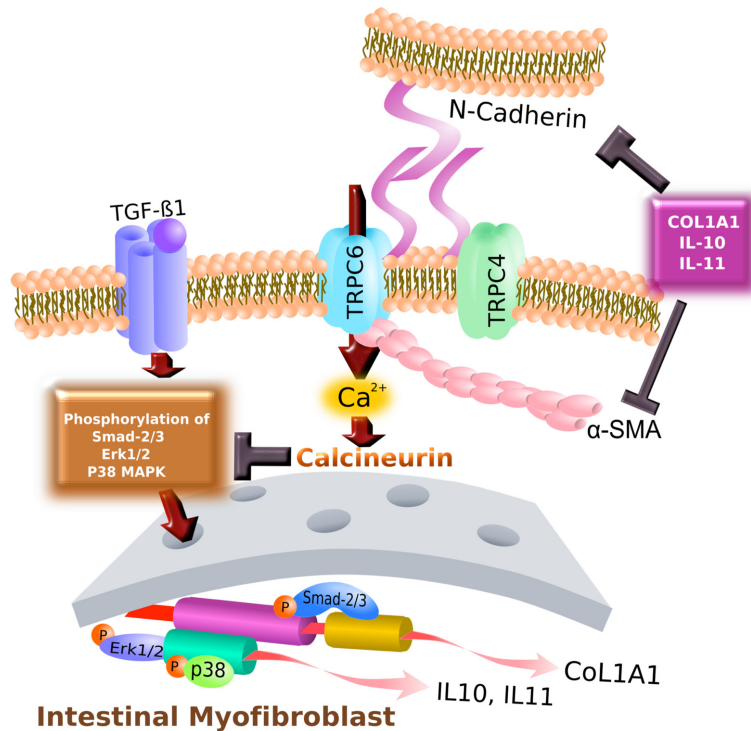
Fig. 4. Hypothetical TRPC4- / TRPC6-mediated signaling pathway downstream of TGF-β1 in InMyoFib cells. TRPC6 interacted directly with α-SMA, N-cadherin, activating its expression, and indirectly supported α-SMA, N-cadherin expression by downregulating the negative regulators COL1A1, IL-10, and IL-11. The TRPC6 channel negatively regulates COL1A1, IL-10, and IL-11 expression, as well as Smad-2, ERK, and p38-MAPK phosphorylation in intestinal myofibroblasts. AKT signal transduction may involve TRPC6 upregulation downstream of the TGF-β1 receptors.

TGF-β1-induced secretion of collagen, IL-10, and IL-11 appears to negatively regulate α-SMA and N-cadherin expression. This mechanism may serve as negative feedback regulation by anti-fibrotic factors. The observed negative regulation of TGF-β1-SMAD signaling via calcineurin, which is activated through increased TRPC activity, may be an important anti-fibrotic mechanism. Indeed, in cultured mesangial cells, the calcineurin inhibitors, cyclosporin A and FK506, were found to activate this signaling pathway, thereby initiating fibrogenic gene expression [25]. Calcineurin dephosphorylates a variety of kinase substrates (74). In fact, calcineurin dephosphorylates several phosphorylated proteins involved in TGF-β1 signaling (e.g., SMAD, ERK1/2, or p38-MAPK) independently of NFAT activation (75). This raises an intriguing possibility that targeting the TRPC6-calcineurin signaling axis may be a useful therapeutic strategy for reinforcing the anti-fibrotic potential in fibrotic diseases, such as CD. In fact, it is known that inhibition of calcineurin has healing effects on erosions/ulcers in ulcerative colitis (UC), and a calcineruin inhibitor FK506 (tacrolimus) has been used in clinical practice for treating the patients with UC (76). Moreover, clinical trials of tacrolimus treatment for fistulas in CD are already underway (77, 78). However, our results have indicated that this compound may also facilitate fibrogenic processes in the gut. These "double-edged sword" effects of tacrolimus tell us that, besides its primary therapeutic goal of wound healing, a simple strategy to inhibit calcineurin would also bring about the undesired adverse effect, fibrosis.

Additionally, we obtained 12 paired biopsy samples from stenotic and non-stenotic ileal regions of six
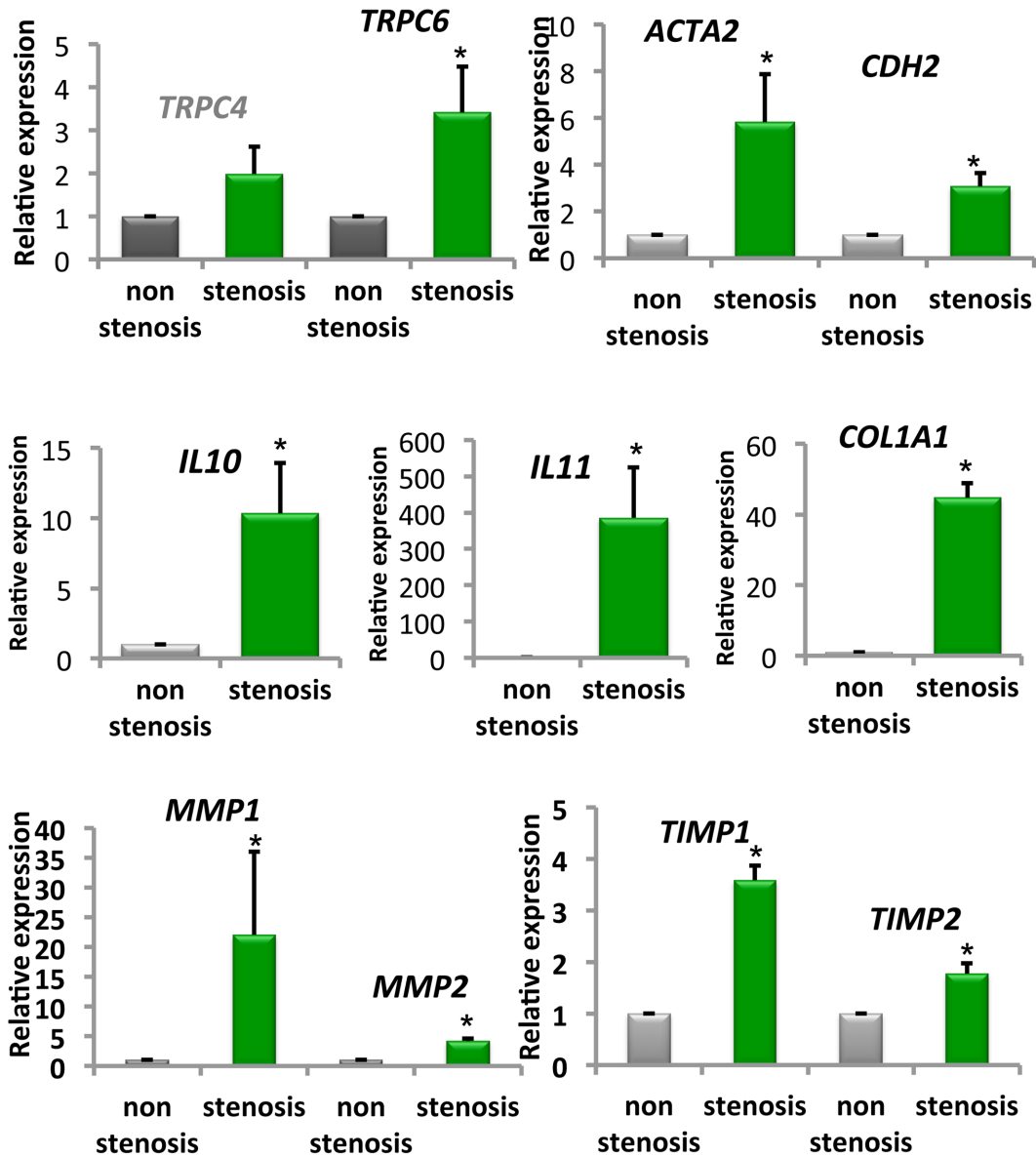
Fig. 5. Crohn's disease (CD) patient biopsies from non-stenotic or stenotic intestinal areas. mRNA levels of *TRPC4*, *TRPC6*, *ACTA2* (α-SMA), *CDH2* (N-cadherin), *IL10*, *IL11, COL1A1*, *MMP1*, *MMP2*, *TIMP1,* and *TIMP2* in biopsies were examined by real-time RT-PCR in non-stenotic or stenotic inflamed mucosal tissues of CD patients. *$P < 0.05$ vs. non-stenotic sample (12 paired biopsy samples obtained from 6 patients). Modified from (1).

CD patients, five of which received anti-TNF agents. We examined the expression levels of TRP channels and fibrosis-associated factors. Stenotic lesions can be inflammatory, fibrogenic, or neoplastic, or can possess all of these characteristics (1, 7). The mRNA levels of *TRPC6*, *ACTA2*, *CDH2*, *IL-10*, *IL-11*, and *COL1A1* were significantly higher in stenotic areas than in non-stenotic mucosal areas in CD patients (Fig. 5). This new finding indicates that TRPC6 vitally contributes to the progression of excessive fibrosis in both an experimental model and human tissues, which should help elucidate the mechanism underlying the fibrotic process. These mechanisms may be relevant not only to intestinal fibrosis, but also to other fibrotic lesions of the skin, lung, and liver, where these channels are expressed at significant levels.

──────────── **Summary** ────────────

We focused on TGF-β1-mediated signaling and regulation by TRPC6 channels, which modulate myofibroblast functions associated with wound repair, such as stress fiber formation, cell-cell adhesion, ECM synthesis, and cytokine secretion. Our results showed that TGF-β1-mediated signaling in intestinal myofibroblasts comprises several phosphorylation events, and forms an intricate network involving TRPC6-mediated signaling pathways, the result suggesting a new anti-fibrotic strategy for treating chronic intestinal inflammatory diseases. The final consequence of the activation of this network appears to be a complex balance between pro-fibrotic and anti-fibrotic activities. Further studies investigating the spatiotemporal heterogeneity of TGF-β1-mediated signaling may help to elucidate the pathways underlying progression of CD-associated fibrosis.

The above findings are consistent in part with a previous study that TRPC6-mediated Ca^{2+} influx was obligatory for myofibroblast differentiation in dermal and cardiac wound healing, but simultaneously suggest a greater complexity of TRPC6-mediated signaling in the intestinal fibrotic processes. Furthermore, the expression profile in CD patient samples indicated similarities between the strictured regions in CD patients and InMyoFibs in terms of pro- and anti-fibrogenic factors. Collectively, the present results suggest that actual signaling pathways activated during TGF-β1-induced fibrosis include many factors that interact via an interconnected network, as recapitulated in the scheme shown. Although some improvement has been made in elucidating the patho-mechanism for fibrosis, the knowledge is still devoid of effective anti-fibrotic agents (79, 80). In this regard, it is highly probable that TRP channels are attractive therapeutic candidates involved in a large spectrum of human intestinal health and diseases, including infectious, indefinite complaint, and inflammatory diseases, which will deserve continuous investigation in future.

──────────── **Conflict of interest** ────────────

The authors have no conflict of interest directly relevant to the content of this article.

──────────── **References** ────────────

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