

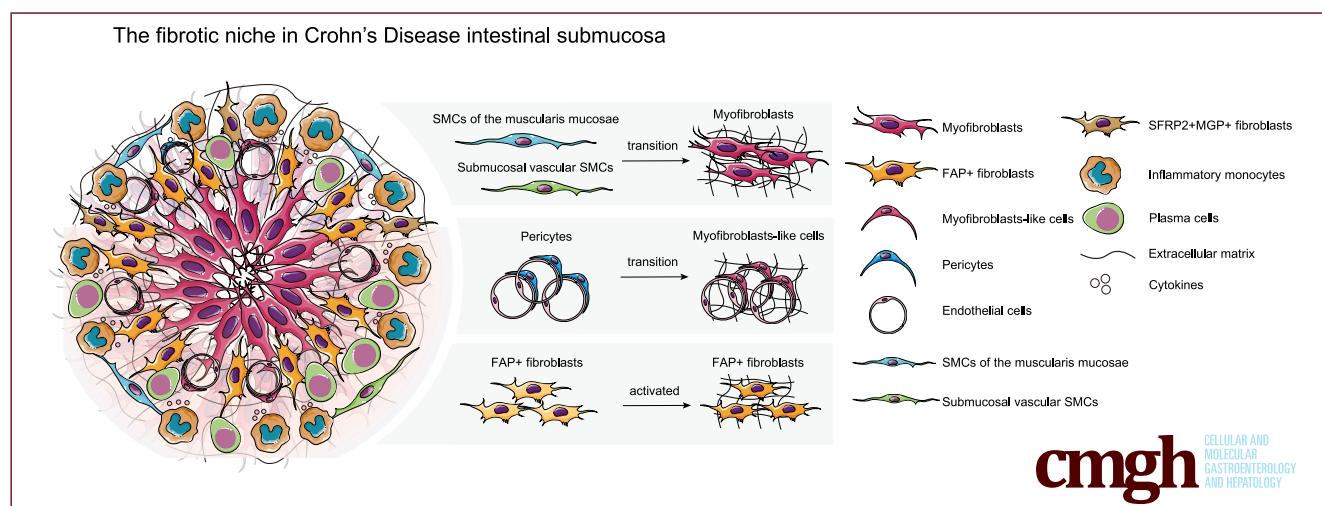
ORIGINAL RESEARCH

Elucidating a Myofibroblast-dominated Fibrotic Niche in Crohn's Disease-associated Fibrostenosis Through High-resolution Spatial Transcriptomics



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SUMMARY

High-resolution spatial transcriptomics defines a distinct fibrotic niche and reveals immune–stromal interactions, highlighting high extracellular matrix-producing myofibroblasts and pericytes as key stromal contributors to fibrotic remodeling in Crohn's disease.

WHAT YOU NEED TO KNOW

Background: Fibrostenosis in Crohn's disease features intestinal extracellular matrix remodeling and muscularization; the cellular identities and origins of the markedly expanded submucosal myoid cells remain poorly defined in human intestine.

Impact: Our study defines a distinct fibrotic niche in stricturing Crohn's disease; in situ identifies the expanded submucosal myoid cells as ECM-high myofibroblasts; and highlights these cells and pericytes as key antifibrotic therapeutic targets.

Future Directions: Further research should validate the fibrotic niche's generalizability in larger, clinically stratified cohorts; and dissect mechanisms driving submucosal myoid-cell and pericyte transitions toward fibrosis to nominate and develop rational antifibrotic targets.

BACKGROUND & AIMS: Fibrostenosis is a major complication of Crohn's disease (CD) characterized by intestinal remodeling and excessive extracellular matrix (ECM) deposition. A prominent feature is bowel wall muscularization, involving

expansion of submucosal myoid cells and muscularis propria smooth muscle cell (SMC) hyperplasia. However, the cellular identity and molecular mechanisms underlying submucosal myoid cell hyperplasia remain poorly characterized.

METHODS: Preoperative intestinal ultrasound from 117 patients with CD was retrospectively reviewed, and ileal tissues from 25 normal, 35 nonstenotic CD, and 44 stenotic CD cases were analyzed histologically. High-resolution spatial transcriptomics was applied to 1 nonfibrotic and 1 fibrostenotic ileal specimen with marked submucosal myoid cell expansion. Findings were validated using public single-cell RNA sequencing datasets (N = 158), immunofluorescence, primary pericyte cultures, and quantitative polymerase chain reaction.

RESULTS: Submucosal myoid cells were predominantly identified as high ECM-producing myofibroblasts, possibly representing the dominant stromal population expanded in fibrotic submucosa. Spatial and pseudotemporal analyses demonstrated their origin from muscularis mucosae and submucosal vascular SMCs. Additionally, pericytes underwent significant expansion and transcriptional reprogramming toward a myofibroblast-like phenotype. Fibroblast sub-clustering revealed spatial heterogeneity, with FAP⁺ fibroblasts enriched specifically in fibrotic regions. Inflammatory monocytes colocalized with stromal cells, exhibiting robust predicted ligand-receptor interactions indicative of immune-stromal crosstalk.

CONCLUSIONS: This case-level, high-resolution spatial analysis delineates a spatially organized fibrotic niche within a CD stricture, composed of distinct stromal and immune populations. We define the identity and origins of profibrotic myofibroblasts and characterize pericyte-to-myofibroblast reprogramming, thereby highlighting specific cell subtypes as prime therapeutic targets for antifibrotic strategies. (*Cell Mol Gastroenterol Hepatol* 2026;20:101701; <https://doi.org/10.1016/j.jcmgh.2025.101701>)

Keywords: Crohn's disease; Fibrostenosis; Fibrotic Niche; Myofibroblasts; Pericytes; Spatial Transcriptomics.

Intestinal fibrosis remains a major challenge in managing Crohn's disease (CD).^{1,2} Nearly all patients with CD experience some degree of fibrotic progression over the disease course, even without clinically apparent strictures.³ Approximately 50% of patients eventually develop clinically significant strictures from fibrotic remodeling, a condition known as fibrostenosis.^{4,5} With no effective antifibrotic therapies, surgical resection remains the primary treatment for CD-associated fibrostenosis. However, surgery does not prevent disease recurrence or progressive fibrogenesis in CD.^{1,5}

Intestinal wall muscularization—including smooth muscle hypertrophy in the muscularis propria (MP) and myoid cell hyperplasia in the submucosa—is increasingly recognized as a key contributor to luminal narrowing in CD.^{6–8} Previous studies have shown that creeping fat (CF) adjacent to the MP releases long-chain fatty acids that selectively stimulate MP smooth muscle cell (HIMC) proliferation, promoting MP thickening and intestinal stricture formation.^{9,10} In contrast, the submucosa exhibits marked α -smooth muscle actin (α -SMA)⁺ and desmin (DES)⁺ myoid cell hyperplasia, often accompanied by extensive extracellular matrix (ECM) deposition.¹¹ Stromal cells are the

principal source of ECM in intestinal tissue,¹¹ and their pathological activation and proliferation drive fibrogenesis in CD.^{12–14} However, although submucosal myoid cells are an important component of the intestinal stromal compartment, it remains unknown whether their hyperplasia directly contributes to fibrotic ECM deposition.

Recent advances in single-cell RNA sequencing (scRNA-seq) have enabled detailed characterization of fibroblast heterogeneity in full-thickness fibrostenotic intestinal specimens.^{12–14} Among the identified subsets, CXCL14⁺ and MMP⁺/WNT5A⁺ fibroblasts engage in pro-fibrotic signaling within CD strictures and co-express CDH11, a cadherin linked to ECM production and fibrotic remodeling.¹² Independent studies have also identified FAP⁺ fibroblasts in the submucosa that express core fibrotic genes and are implicated in intestinal fibrosis.^{13,14} However, despite these insights, scRNA-seq analyses of full-thickness intestinal tissue report that smooth muscle cells (SMCs) and myofibroblasts constitute only a small fraction of the stromal compartment—typically <10%—a finding that contrasts with histological observations in fibrostenotic tissue.^{13,14} These discrepancies suggest that current scRNA-seq approaches may not fully capture the cellular composition of the stromal compartment in fibrotic CD lesions.

The Visium HD high-resolution spatial transcriptomics platform addresses key limitations of scRNA-seq by enabling spatially resolved gene expression profiling in intact tissue at single-cell scale resolution.¹⁵ This approach preserves native spatial context and minimizes dissociation-induced artifacts (eg, cellular stress, apoptosis, and selective cell loss).^{16,17} Importantly, unlike earlier spatial methods that relied on scRNA-seq reference datasets, Visium HD allows direct, unsupervised in situ identification and clustering of transcriptionally distinct cell populations.¹⁵ In this study, we applied this platform to analyze 1 nonfibrotic and 1 fibrotic formalin-fixed, paraffin-embedded (FFPE) CD specimen, the

Abbreviations used in this paper: α -SMA, α -smooth muscle actin; ANOVA, analysis of variance; BEAM, branch expression analysis modeling; BSA, bovine serum albumin; BWS, bowel wall stratification; BWT, bowel wall thickness; CD, Crohn's disease; CDns, non-stenotic Crohn's disease; CDs, stenotic Crohn's disease; CF, creeping fat; DAPI, 4',6-diamidino-2-phenylindole; DEG, differentially expressed gene; DES, desmin; DMEM, Dulbecco's Modified Eagle Medium; EC, endothelial cell; ECM, extracellular matrix; FBS, fetal bovine serum; FC, fold change; FDR, false discovery rate; FFPE, formalin-fixed, paraffin-embedded; GEO, Gene Expression Omnibus; GO, Gene Ontology; GSEA, gene set enrichment analysis; H&E, hematoxylin and eosin; HBSS, Hanks' Balanced Salt Solution; HIMC, human intestinal muscularis propria (smooth) muscle cell; IL, interleukin; IUS, intestinal ultrasound; LEC, lymphatic endothelial cell; MACS, magnetic-activated cell sorting; MP, muscularis propria; NL, normal control; PBS, phosphate-buffered saline; PCA, principal component analysis; qRT-PCR, quantitative reverse transcription polymerase chain reaction; scRNA-seq, single-cell RNA sequencing; SD, standard deviation; SMC, smooth muscle cell; t-SNE, t-distributed stochastic neighbor embedding; TGF- β 1, transforming growth factor-beta 1; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier; VSMC, vascular smooth muscle cell.



Most current article

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latter featuring extensive submucosal myoid cell hyperplasia. By integrating these spatial data with 3 public scRNA-seq datasets (for cross-validation) and complementary in vitro experiments, we aimed to map the spatial cellular landscape of fibrostenotic CD tissue and elucidate the phenotypic identity and molecular underpinnings of submucosal myoid cell expansion.

Results

Hyperplasia of Submucosal Myoid Cells Contributes to Bowel Wall Thickening and Stenosis in Crohn's Disease

Intestinal ultrasound (IUS) is highly sensitive and specific for detecting bowel wall thickness (BWT) and stratification (BWS) in CD.^{18,19} It allows precise measurement of total BWT as well as individual layer thickness (mucosa, submucosa, and MP). In normal intestine, these three layers show no significant thickness differences.²⁰ To evaluate layer-specific thickening in stenotic segments, we analyzed preoperative IUS reports from 117 patients with CD who underwent surgery for intestinal stenosis (Supplementary Table 1). All stenotic segments had total BWT exceeding the 3-mm threshold for pathological thickening.²¹ Of the 3 layers, the submucosa was thickest (Figure 1A and B), suggesting that submucosal expansion is a major contributor to stenosis.

We next performed histological measurements on intestinal tissue from 25 normal (NL), 35 non-stenotic CD (CDns), and 44 stenotic CD (CDs) cases to investigate submucosal thickening (Supplementary Table 2). The submucosa was significantly thicker in CDs than in NL or CDns (Figure 1C–E). Histopathology revealed 3 prominent submucosal features in CDs: extensive ECM deposition (44/44 cases), inflammatory infiltration (44/44), and marked submucosal myoid cell hyperplasia (37/44) (Figure 1F). In affected cases, hyperplastic myoid cells spanned roughly one-third to two-thirds of the submucosal thickness and were consistently surrounded by dense ECM deposits (Figure 1G). Such extensive submucosal myoid cell hyperplasia was absent in NL and CDns. This spatial correlation suggests 2 mechanisms for submucosal thickening in CDs: direct expansion from myoid cell hyperplasia and indirect expansion via ECM production by these cells. Further studies are needed to validate this proposed dual mechanism.

Identification of a Distinct Submucosal Fibrotic Niche in Fibrostenotic CD

To characterize the cellular and molecular phenotypes underlying submucosal myoid cell hyperplasia, we performed high-resolution spatial transcriptomic profiling on 1 nonfibrotic and 2 fibrotic ileal specimen from 2 patients with CD (Supplementary Table 3; Figure 2A; Supplementary Figure 1A–D). The nonfibrotic region had well-preserved histology with normal mucosal, submucosal, MP, and serosal thickness. By contrast, the fibrotic region showed marked mucosal and submucosal thickening. Within the

fibrotic submucosa, ulcerated areas lacking epithelium exhibited extensive myoid cell hyperplasia accompanied by dense ECM deposition (Supplementary Figure 2A and B).

The Visium HD platform provides single-cell scale spatial resolution by capturing transcripts on contiguous $2 \times 2 \mu\text{m}$ barcoded spots. We binned spots into $8 \times 8 \mu\text{m}$ to approximate individual cell areas. The nonfibrotic and fibrotic tissues yielded $\sim 469,000$ and $\sim 520,000$ spots, respectively, each treated as an independent transcriptional unit. Unsupervised clustering identified 16 clusters in the nonfibrotic tissue and 22 in the fibrotic tissue (Figure 2B; Supplementary Figure 3A–D). Mixed-cell and unidentifiable clusters (likely due to multi-cell capture per spot) were excluded. Remaining clusters showed distinct marker gene profiles (Supplementary Figure 3E and F; Supplementary Tables 4 and 5) and spatial distributions mirroring the histological architecture (Figure 2B).

We mapped canonical cell markers to their spatial locations: FCGBP (enterocytes/goblet cells), TRAC (T cells), MS4A1 (B cells), JCHAIN (plasma cells), LYZ (monocytes/macrophages and Paneth cells), COL1A1 (stromal cells), MYH11 (myoid cells), and VWF (endothelial cells). Each marker displayed a specific spatial pattern consistent with expected anatomy (Figure 2C). In the fibrotic sample, we observed chronic inflammatory features, including localized T- and B-cell aggregates and monocyte/macrophage clusters, along with an increase in plasma cells (Figure 2C). Concurrently, classic fibrostenotic features were evident: extensive stromal expansion and localized myoid cell proliferation in the submucosal layer (Figure 2C).

We next quantified fibrosis by computing ECM signature scores. Using curated gene sets for collagens, glycoproteins, and proteoglycans^{22,23} (Supplementary Table 6) and the Gene Ontology (GO) term “extracellular matrix organization” (GO:0030198), we calculated average expression per spot to assign an ECM score. Higher scores indicate greater fibrosis. The fibrotic tissue contained submucosal regions of high ECM production with significantly elevated ECM scores compared with all other areas of the fibrotic tissue and to the nonfibrotic tissue (Figure 3A and B; Supplementary Figure 4A).

Spatial analysis delineated 3 submucosal regions based on ECM scores: a high-ECM region in the fibrotic tissue, an ECM-low region in the fibrotic tissue, and a corresponding ECM-low region in the nonfibrotic tissue (Figure 3C and D; Supplementary Figure 4B and C). These regions had distinct cellular compositions (Figure 3E and F). The high-ECM region was enriched in stromal and immune cells, with a pronounced expansion of myofibroblasts and increased numbers of high-ECM fibroblasts and pericytes, alongside more plasma cells and monocytes (Figure 3E–G). In contrast, 2 ECM-low regions consisted predominantly of fibroblasts distributed uniformly (Figure 3E–G). The diffuse fibroblast distribution in normal regions underscores their role in maintaining intestinal structural integrity. Meanwhile, the localized accumulation of ECM-overproducing stromal and immune cells in the fibrotic tissue defines a distinct fibrotic niche.^{24–27} The unique cellular composition and dynamic changes within this niche likely drive submucosal fibrotic progression in CD.

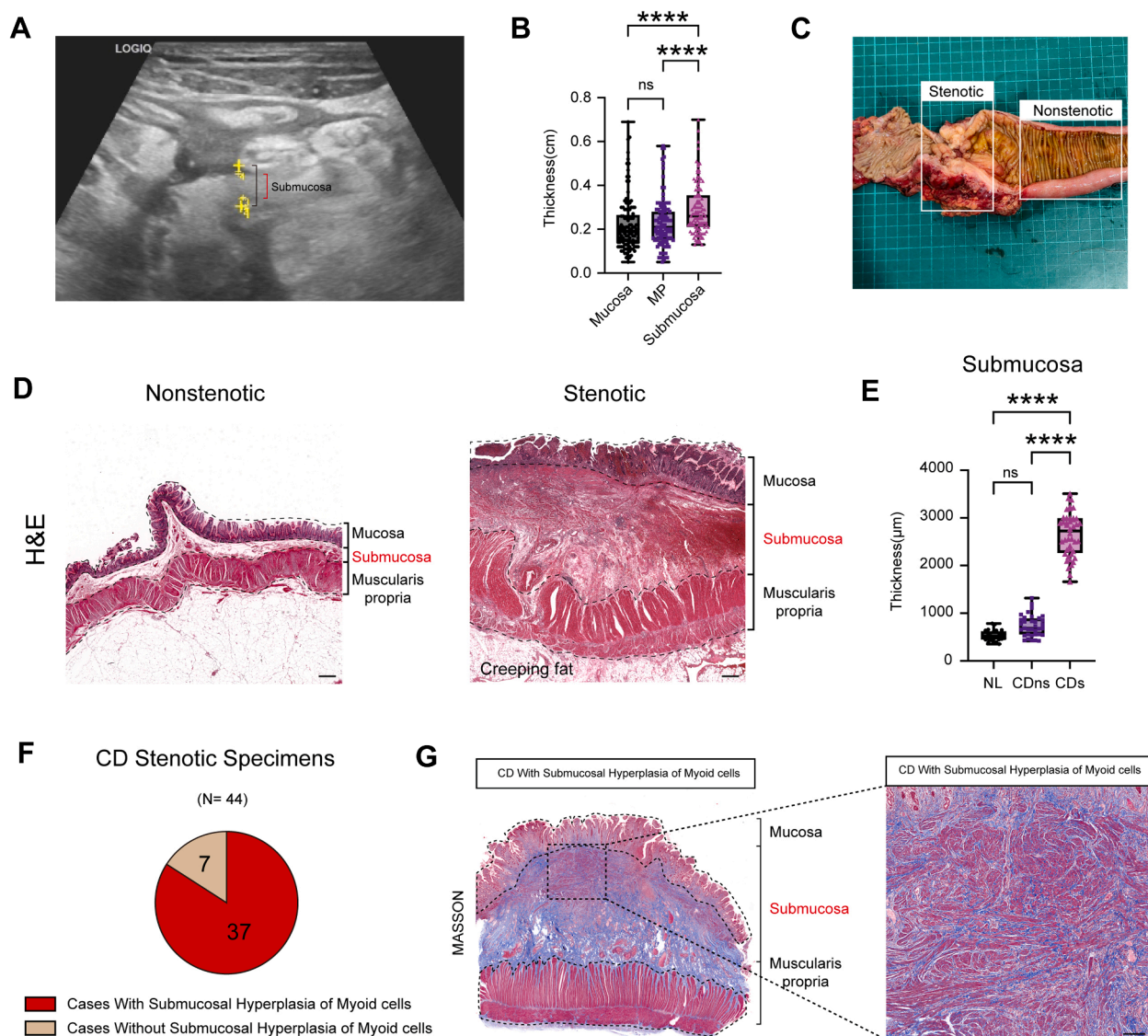


Figure 1. Hyperplasia of myoid cells in the submucosa of stenotic intestinal segments in CD. (A) Representative IUS image demonstrating marked thickening of both total bowel wall and submucosal layer in a patient with CD. (B) Quantification of individual layer thicknesses, showing significantly greater thickening of the submucosal layer compared with the mucosal layer and muscularis propria in stenotic CD segments. Statistical analysis by Friedman test with Bonferroni-adjusted Wilcoxon post hoc tests. **** $P < .0001$. (C) Gross pathology images of stenotic and nonstenotic intestinal regions from a surgical specimen of a patient with CD. (D) Representative H&E staining of stenotic and nonstenotic intestinal tissues from patients with CD. Scale bars, 500 μ m. (E) Quantification of submucosal thickness in non-lesional CD (CDns), stenotic CD (CDs), and normal (NL) tissues. Data are shown as individual values with box-and-whisker plots indicating median and interquartile range. **** $P < .0001$ by 1-way ANOVA with Tukey's post hoc test. (F) Histological analysis of CD surgical specimens showing submucosal hyperplasia of myoid cells in 37 of 44 cases. (G) Masson staining of stenotic CD tissue revealing extensive ECM deposition surrounding hyperplastic myoid cells in the submucosa. Scale bars: 1 mm (left), 200 μ m (right).

Submucosal Myoid Cells Are Identified as a Pathogenic ECM-producing Myofibroblast Subpopulation

Spatial transcriptomic analysis of the fibrotic submucosa identified 2 stromal subpopulations associated with myoid cell hyperplasia: myofibroblasts and SMCs. In the nonfibrotic tissue, no myofibroblasts were detected, and SMCs were confined to their usual anatomical locations (muscularis mucosae, MP, and perivascular regions),

validating our cell annotations (Figure 4A). In the fibrotic tissue, most SMCs remained in native compartments, whereas a distinct myofibroblast population emerged in the submucosa and underwent proliferative expansion, occupying about one-third of the submucosal thickness and directly contributing to wall thickening (Figure 4A). These myofibroblasts exhibited a hybrid phenotype, co-expressing SMC and fibroblast markers. Both SMCs and myofibroblasts expressed canonical smooth muscle genes (eg, ACTA2, TAGLN, DES, ACTG2, MYH11, MYL9, CNN1,

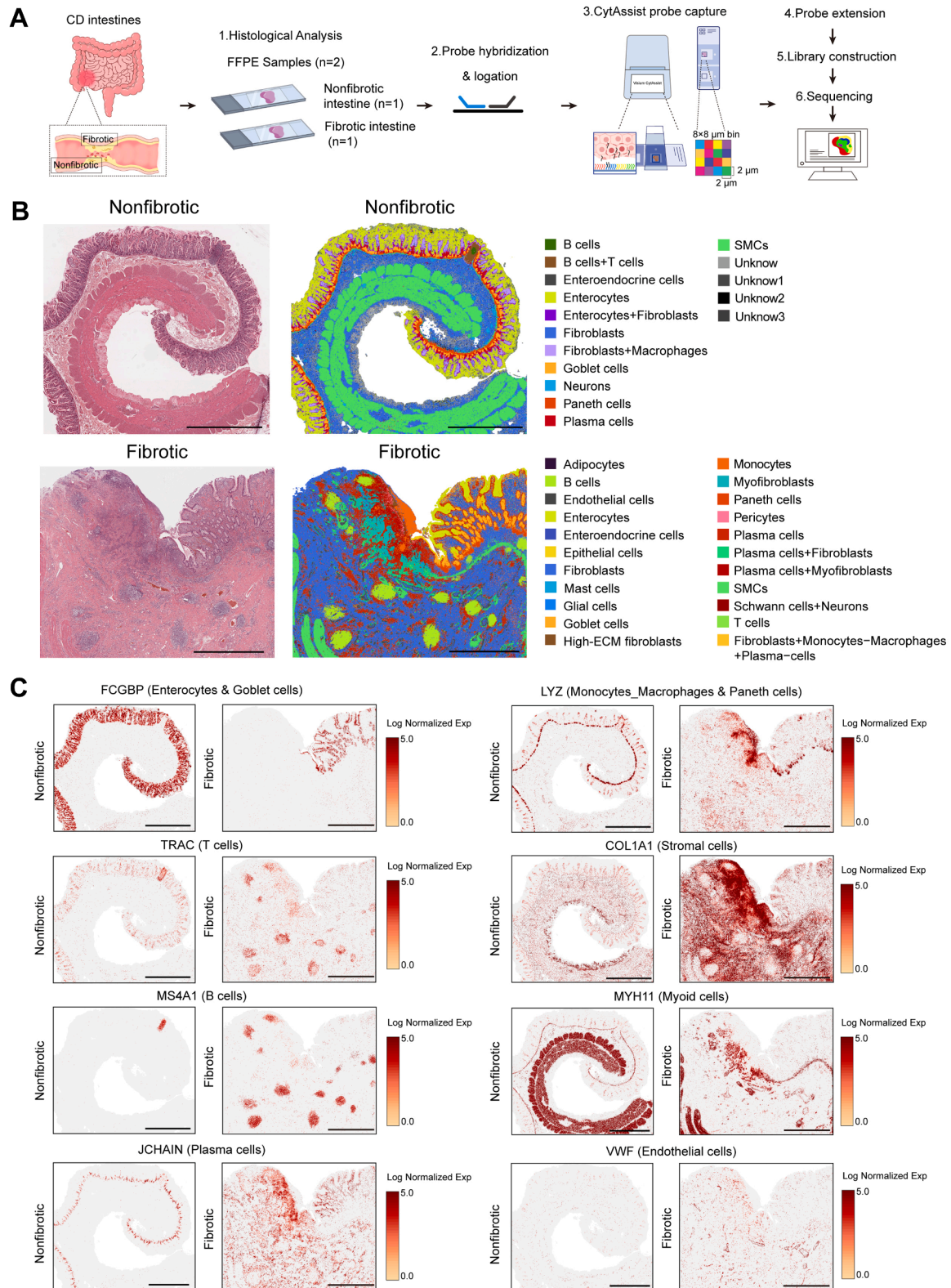


Figure 2. Spatial mapping of nonfibrotic and fibrotic intestinal tissues using Visium HD reveals high-resolution transcript localization. (A) Experimental workflow for spatial transcriptomic analysis of CD intestinal specimens using the 10x Genomics Visium HD platform. (B) H&E-stained tissue sections with corresponding spatial clustering maps of nonfibrotic and fibrotic specimens, displaying 8 μm bins color-coded by unsupervised clustering and manual annotation. Scale bars: 2.5 mm. (C) Spatially resolved expression patterns of selected cell-type markers in nonfibrotic vs fibrotic tissues, with expression levels indicated by color intensity. Scale bars: 2.5 mm.

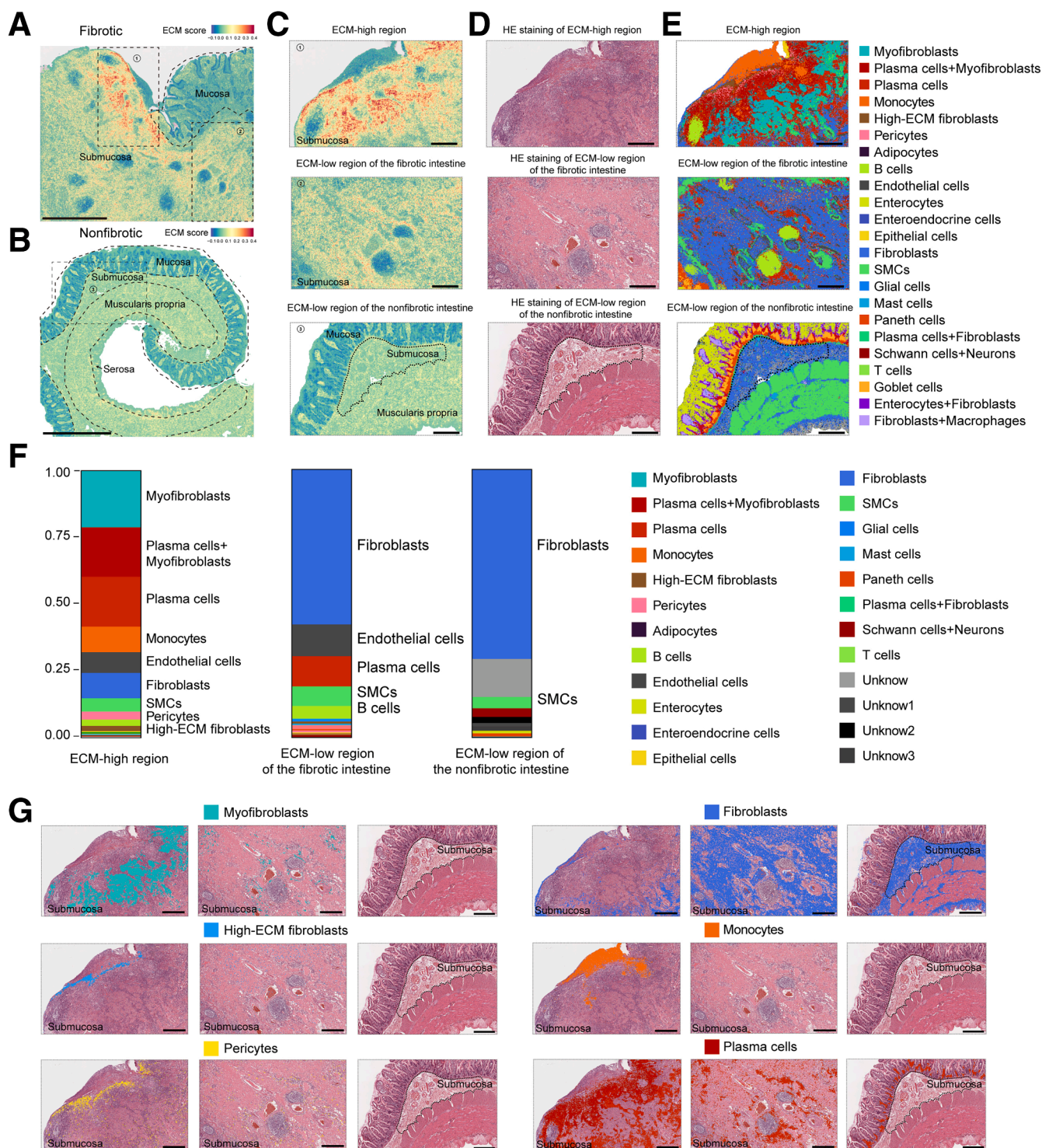
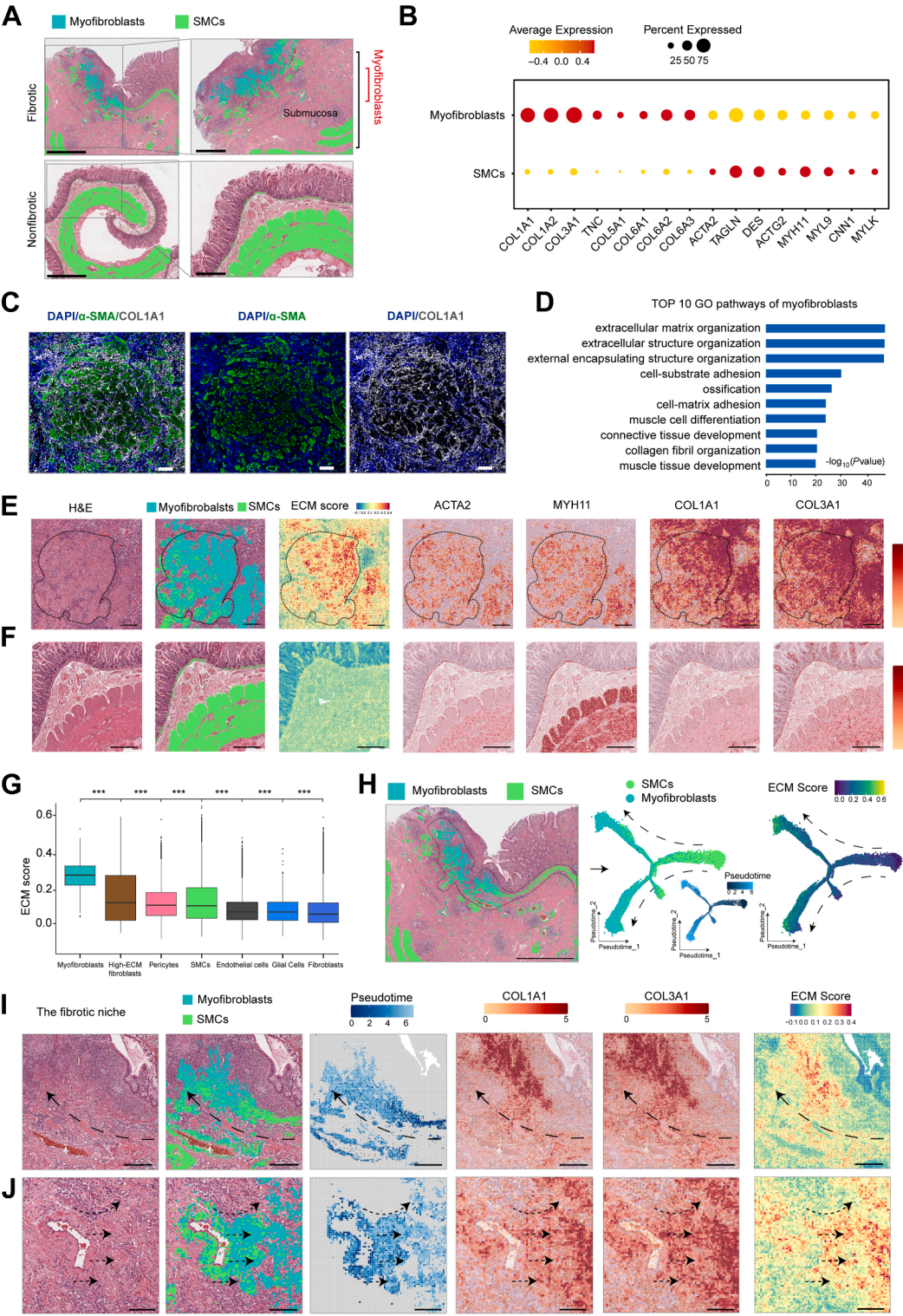


Figure 3. Characterization of the fibrotic niche and its cellular composition in fibrostenotic Crohn's disease intestine. (A–B) ECM signature scores in (A) fibrotic and (B) nonfibrotic sections, calculated based on the expression of collagen, proteoglycan, and glycoprotein genes. Red regions indicate higher ECM expression levels. Scale bars, 2.5 mm. (C–D) Comparative visualization of (C) ECM scores and (D) H&E staining across three spatially defined submucosal regions: ECM-high region from fibrotic submucosa (top), ECM-low region from fibrotic submucosa (middle), and ECM-low region from nonfibrotic submucosa (bottom). Scale bars, 500 μ m. (E) Spatial distribution of cell clusters within the same 3 regions. Scale bars, 500 μ m. (F) Quantitative comparison of cellular composition across the ECM-high region (left), ECM-low region from fibrotic submucosa (middle), and ECM-low region from nonfibrotic submucosa (right). Colored bars indicate the relative proportions of identified cell types. (G) Spatial localization of 6 key cell types—myofibroblasts, high-ECM fibroblasts, pericytes, fibroblasts (other), monocytes, and plasma cells—across the 3 regions. Scale bars, 500 μ m.

MYLK), but myofibroblasts showed significantly higher expression of ECM-related genes (eg, COL1A1, COL1A2, COL3A1, COL5A1, COL6A1–3, TNC) (Figure 4B). Immunofluorescence co-staining confirmed strong α -SMA and

COL1A1 protein expression in submucosal myofibroblasts (Figure 4C).

GO analysis highlighted functional differences: SMCs were enriched in muscle contraction and differentiation pathways



(Supplementary Figure 4D), whereas myofibroblasts were enriched in ECM organization, cell adhesion, and muscle differentiation pathways (Figure 4D). This supports classifying myofibroblasts as key ECM-producing cells retaining smooth muscle features. Spatially, abundant ACTA2⁺MYH11⁺ myofibroblasts in the fibrotic submucosa showed markedly elevated ECM scores and upregulation of fibrogenic genes (COL1A1 and COL3A1) compared with nonfibrotic tissue (Figure 4E and F). Quantitatively, myofibroblasts had the highest ECM scores and fibrogenic gene expression (COL1A1, COL1A2, COL3A1) among all stromal subsets (Figure 4G; Supplementary Figure 4E–H). Although high-ECM fibroblasts and pericytes also displayed increased ECM production and fibrogenic gene expression, their levels remained lower than those of myofibroblasts (Figure 4G; Supplementary Figure 4E–H).

To determine if the spatially identified ECM-producing myofibroblast subset was captured in previous scRNA-seq studies, we reanalyzed 3 public CD intestinal datasets (TI, CO, TI-1; total n = 158) (Supplementary Figure 5A).^{28,29} After quality control, we retained 60,316 stromal cells from TI, 33,877 from CO, and 3,188 from TI-1. Unbiased clustering identified 6 conserved stromal populations: fibroblasts, endothelial cells (ECs), glial cells, lymphatic endothelial cells (LECs), mural cells, and myoid cells (Supplementary Figure 5B and C). Myoid cells consistently expressed canonical smooth muscle markers (ACTA2, DES, MYH11) (Supplementary Figure 5D–F), accounted for <15% of stromal cells, and did not significantly differ between conditions (Supplementary Figure 5G and H).

GO analyses across all datasets indicated myoid cell enrichment in energy metabolism, mitochondrial function, and muscle contraction pathways (Supplementary Figure 6A). ECM scoring and differential gene expression analyses revealed myoid cells did not exhibit elevated ECM scores or upregulated fibrotic markers (COL1A1, COL1A2, COL3A1) compared with other stromal subsets (Supplementary Figure 6B–E). Thus, the high ECM-producing myofibroblast population identified by spatial transcriptomics was not captured in existing scRNA-seq datasets. Our spatial analysis therefore uncovered a previously unrecognized, numerically expanded pathogenic myofibroblast subset with robust ECM-producing capacity, suggesting a central role in CD-associated intestinal fibrosis and potential as a therapeutic target.

Myofibroblasts Arise From SMCs of the Muscularis Mucosae and Vascular SMCs in the Submucosa

Hematoxylin and eosin (H&E) staining combined with spatial transcriptomics suggested that submucosal myofibroblasts originate from 2 sources: SMCs in the muscularis mucosae and vascular smooth muscle cells (VSMCs) in the submucosa. To investigate this, we performed pseudotime trajectory analysis on SMCs and myofibroblasts in a representative fibrotic region (Figure 4H). The pseudotime ordering revealed a stepwise transition from SMCs to myofibroblasts accompanied by progressively increasing ECM scores along the differentiation trajectory (Figure 4H).

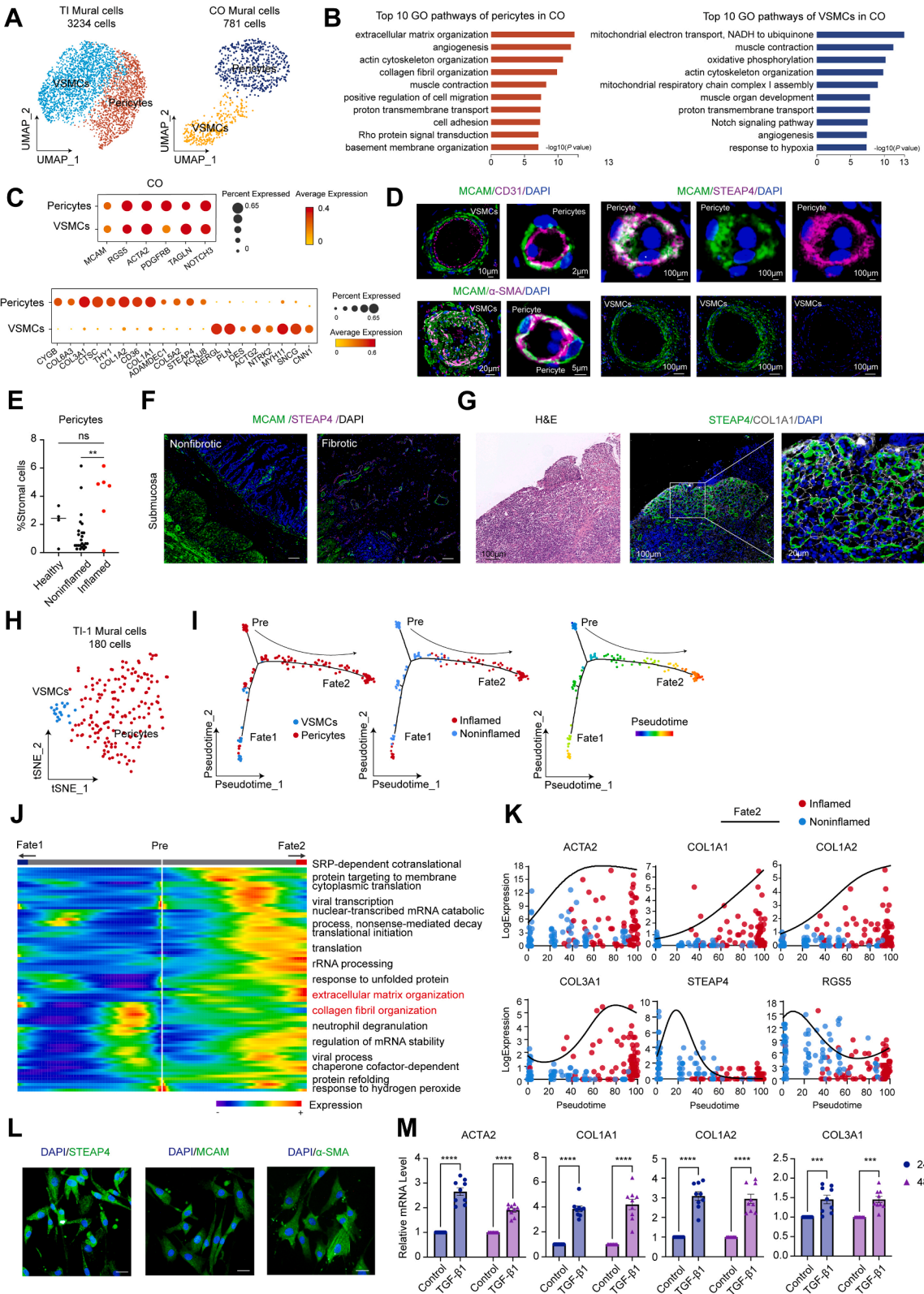
We further examined 2 distinct zones of the fibrotic niche: one enriched in muscularis mucosae-derived SMCs and myofibroblasts, and another containing submucosal VSMCs and myofibroblasts. Pseudotime analysis showed that in both zones, SMCs undergo proliferative expansion and differentiate into myofibroblasts. Cells at the end of the trajectories exhibited markedly elevated ECM scores and upregulated fibrogenic genes (COL1A1, COL3A1) compared with early-stage cells (Figure 4I and J). These findings indicate that SMC-to-myofibroblast transitions are associated with acquisition of a fibrogenic phenotype. Notably, in some fibrotic areas, VSMCs attached to vessel walls had begun transitioning toward a myofibroblast phenotype, evidenced by high ECM scores and COL1A1/COL3A1 expression (Supplementary Figure 7A). By contrast, ECM-low regions showed little proliferative or differentiative activity among muscularis mucosae SMCs or submucosal VSMCs (Supplementary Figure 7B). Collectively, these results support that pathogenic myofibroblasts in CD arise from both muscularis mucosae SMCs and submucosal VSMCs.

Pericyte Accumulation in the Fibrotic Niche and Their Transition to a Myofibroblast-like Phenotype

Mural cells, including VSMCs and pericytes, are located on blood vessels in close proximity to ECs.^{30,31} Although their roles in vascular biology are well-established, their contribution to intestinal fibrosis in CD remains unclear.^{32–34} To address this, we reanalyzed the public scRNA-seq datasets, identifying 2 distinct mural

Figure 4. (See previous page). Dominant ECM-producing phenotypes and cellular origins of myofibroblasts. (A) Spatial mapping of myofibroblasts (blue) and SMCs (green) in fibrotic (top) and nonfibrotic (bottom) sections. Scale bars: left, 2.5 mm; right, 500 μ m. (B) Dot plot of differentially expressed marker genes distinguishing myofibroblasts and SMCs. Dot color indicates average expression level; dot size indicates the percentage of cells expressing each gene. (C) Immunofluorescence staining confirming myofibroblast identity in the fibrotic submucosa. α -SMA (green), COL1A1 (gray), and DAPI (blue). Scale bars: 100 μ m. (D) Top 10 enriched GO pathways for myofibroblast-specific marker genes (hypergeometric test with FDR-adjusted *P* values). (E–F) Spatial localization of SMCs and myofibroblasts, ECM signature scores, and expression of smooth muscle-associated and fibrosis-related genes in fibrotic (top) and nonfibrotic (bottom) regions. Scale bars: 100 μ m. Red coloring indicates higher scores or expression levels. (G) ECM scores of stromal subclusters in the fibrotic specimen, calculated based on expression of collagen-, glycoprotein-, and proteoglycan-related genes. One-way ANOVA with Bonferroni correction. ****P* < .001. (H) Pseudotime trajectory (Monocle 2) showing a smooth progression from SMCs to myofibroblasts, accompanied by gradual elevation in ECM scores. Each point represents a single cell; transitional regions are outlined with dashed lines on the fibrotic section. Scale bar: 2.5 mm. (I–J) Representative regions of lineage transition from muscularis mucosae SMCs to myofibroblasts (I) and from VSMCs to myofibroblasts (J). Scale bars: top, 200 μ m; bottom, 100 μ m.

subsets—VSMCs and pericytes—in TI and CO (Figure 5A). GO analyses consistently indicated pericyte enrichment in ECM organization and angiogenesis, whereas VSMCs were associated with mitochondrial function, muscle contraction, and oxidative phosphorylation pathways (Figure 5B; Supplementary Figure 8A and B). Differential gene



expression validated these distinctions: pericytes highly expressed subtype-specific markers (STEAP4, KCNJ8) and ECM genes (COL1A1, COL1A2, COL3A1), whereas VSMCs exhibited high expression of RERGL, PLN, NTRK2, and canonical smooth muscle markers (MYH11, CNN1, ACTG2) (Figure 5C; Supplementary Figure 8C). Several markers (MCAM/CD146, RGS5, PDGFRB, ACTA2, NOTCH3) were shared by both subsets. Immunofluorescence confirmed α -SMA and MCAM expression in both cell types, validating STEAP4 as a pericyte-specific marker (Figure 5D).

Further analysis showed that pericyte numbers increased in inflamed tissues compared with healthy or noninflamed tissues in both the TI and CO cohorts (Supplementary Figure 8D and E). In the CO cohort, STEAP4⁺ pericytes were significantly more abundant in inflamed samples than in noninflamed controls (Figure 5E). Consistently, immunofluorescence confirmed prominent accumulation of STEAP4⁺ pericytes and ECs in the submucosa of fibrotic intestinal tissues from patients with CD (Figure 5F; Supplementary Figure 8F). Spatial transcriptomics demonstrated a preferential accumulation of pericytes within the fibrotic niche (Figure 3G). These cells had elevated ECM scores and increased expression of core fibrotic genes, including COL1A1, COL1A2, and COL3A1 (Figure 4G; Supplementary Figure 4E–H). Furthermore, co-staining of sequential sections for STEAP4 and COL1A1 confirmed that pericytes enriched in the fibrotic niche were surrounded by abundant ECM deposits (Figure 5G).

These observations suggest that pericytes in nonfibrotic tissues may undergo a phenotypic transition into an ECM-producing state in inflamed tissues. To test this hypothesis, we examined fibrotic gene expression in pericytes across disease states. Progressive increases in COL1A1, COL1A2, and COL3A1 expression were observed in pericytes from healthy, noninflamed, and inflamed samples (CO, TI-1) (Supplementary Figure 9A and B). Pseudotime trajectory analysis identified pericyte differentiation from a “pre-differentiation” state to an inflamed-enriched “Fate2” state, associated with fibrosis-related pathways (ECM organization, collagen fibril formation) and ECM-related gene

upregulation (COL1A1, COL1A2, COL3A1, COL4A1/2, COL6A1–3, POSTN), alongside ACTA2 induction and reduced STEAP4/RGS5 expression (CO, TI-1) (Figure 5H–K; Supplementary Figure 9D–F). To validate these findings, we isolated MCAM⁺CD36⁺ cells from nonfibrotic ileal CD tissues (identified as pericytes; Supplementary Figure 9G and Supplementary Table 7). These cells expressed STEAP4, MCAM, and α -SMA (Figure 5L). Upon stimulation with TGF- β 1 for 24 or 48 hours, they displayed robust upregulation of ACTA2, COL1A1, COL1A2, and COL3A1 (Figure 5M), confirming pericyte-to-myofibroblast transition potential. Collectively, these findings indicate that pericytes in fibrotic CD undergo significant expansion and phenotypic transformation toward a myofibroblast-like state, contributing substantially to intestinal fibrosis.

Increased Abundance of FAP⁺ Fibroblasts in the Fibrotic Niche

In this study, we performed fibroblast sub-clustering across 3 scRNA-seq datasets to categorize intestinal fibroblast subpopulations in CD. Although various single-cell clustering strategies have been reported, the resulting fibroblast subtypes remain largely uncharacterized and unvalidated.^{12–14} We performed fibroblast sub-clustering across 3 scRNA-seq datasets to define intestinal fibroblast subpopulations in CD. Consistently, we identified 3 main fibroblast subtypes in both the TI and TI-1 cohorts: CXCL14⁺ fibroblasts, CXCL14⁺ADAMDEC1⁺ fibroblasts, and SFRP2⁺MGP⁺ fibroblasts (Supplementary Figure 10A–C). In the CO cohort, we observed the same 3 subtypes plus an additional MMP3⁺MMP1⁺ subset (Supplementary Figure 10A and D). Spatial transcriptomics showed that CXCL14⁺ and CXCL14⁺ADAMDEC1⁺ fibroblasts were predominantly localized to the lamina propria in both specimens and had elevated ECM scores relative to surrounding epithelium (Figure 6A). The SFRP2⁺MGP⁺ fibroblasts spanned the submucosa, MP, and serosa but were nearly absent from the mucosa (Figure 6B). Based on their locations and ECM expression, these 3 subtypes likely maintain normal tissue architecture.

Figure 5. (See previous page). Identification, expansion, and myofibroblast-like transition of pericytes in fibrotic CD intestines. (A) UMAP plots showing 2 distinct mural cell subpopulations (VSMCs and pericytes) in TI (left) and CO (right) datasets. (B) Top 10 GO enrichment pathways for marker genes expressed by pericytes and VSMCs in the CO dataset (hypergeometric test with FDR-adjusted *P* values). (C) Dot plot of selected marker genes for VSMCs and pericytes in the CO dataset. Dot color indicates expression level in positive cells; dot size indicates the fraction of cells expressing each gene. (D) Immunofluorescence images showing MCAM (green), CD31 (magenta), α -SMA (magenta), STEAP4 (magenta), and DAPI (blue) staining in fibrotic CD intestinal tissues. (E) Comparison of pericyte abundance among stromal cells in healthy, non-inflamed, and inflamed CO samples. Kruskal-Wallis test with FDR-adjusted *P* values. (F) Immunofluorescence images showing MCAM (green), STEAP4 (magenta), and DAPI (blue) staining in the submucosa of nonfibrotic vs fibrotic intestines. Scale bars, 100 μ m. (G) Immunofluorescence confirming the localization of pericytes within the fibrotic niche (MCAM in green; STEAP4 in magenta). (H) t-SNE plots showing 2 mural cell subpopulations in the TI-1 dataset. (I) Monocle pseudotime trajectory of mural cell subpopulations in TI-1, colored by tissue source (inflamed vs noninflamed). Each point represents a single cell. (J) Heatmap showing genes differentially expressed along pseudotime trajectories from “Pre” to “Fate1” (left) and “Pre” to “Fate2” (right), with GO enrichment terms for genes upregulated on the “Pre”→“Fate2” trajectory. (K) Pseudotemporal expression dynamics of representative pericyte and myofibroblast marker genes along the “Pre” to “Fate2” trajectory. Each point represents a single cell, colored by tissue source (noninflamed or inflamed). (L) Immunofluorescence images showing STEAP4 (green), MCAM (green), α -SMA (green), and DAPI (blue) staining in cultured MCAM⁺CD36⁺ cells. Scale bar, 20 μ m. (M) Relative mRNA expression of myofibroblast marker genes (ACTA2, COL1A1, COL1A2, and COL3A1) in MCAM⁺CD36⁺ cells after 24 or 48 hours of TGF- β 1 stimulation. Each dot represents an individual sample. Statistical comparisons were performed using the Mann-Whitney *U* test with FDR-adjusted *P* values. ****P* < .001; *****P* < .0001.

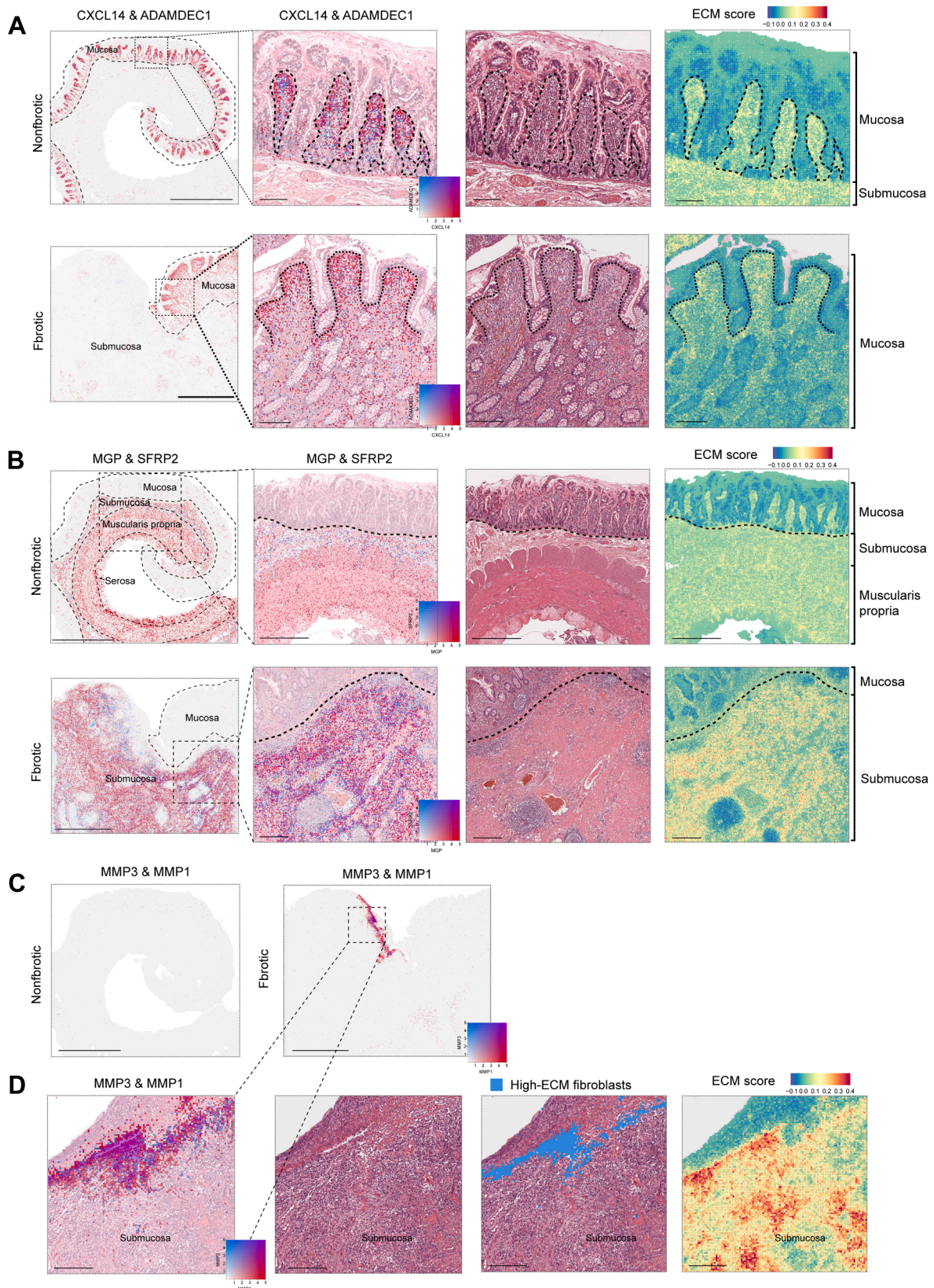


Figure 6. Spatial distribution of fibroblast subpopulations. (A) Spatial localization and ECM scores of CXCL14⁺ fibroblasts and CXCL14⁺ADAMDEC1⁺ fibroblasts in nonfibrotic and fibrotic sections. Darker colors indicate higher expression levels and ECM scores. Scale bars: *left*, 2.5 mm; *right*, 200 μ m. (B) Spatial localization and ECM scores of SFRP2⁺MGP⁺ fibroblasts in nonfibrotic and fibrotic sections. Darker colors indicate higher expression levels and ECM scores. Scale bars: *left*, 2.5 mm; *right*, 500 μ m. (C–D) Spatial localization and ECM scores of MMP3⁺MMP1⁺ fibroblasts in nonfibrotic (C) and fibrotic (D) sections. Darker colors indicate higher expression levels and ECM scores. Scale bars: (C) 2.5 mm; (D) 100 μ m.

We then focused on the $\text{MMP3}^+\text{MMP1}^+$ fibroblast population, which was significantly expanded in inflamed tissues (Supplementary Figure 11A). This subset expressed much higher levels of core fibrotic genes (COL1A1 , COL1A2 , COL3A1) than the other fibroblast subtypes (Supplementary Figure 11B). Notably, it also exhibited elevated FAP and TWIST1 expression—key markers of the FAP^+ fibroblast subset implicated in intestinal fibrosis.^{13,14,35,36} These findings suggest that the $\text{MMP3}^+\text{MMP1}^+$ subset corresponds to the FAP^+ fibroblast population described in previous studies. GO analysis supported their fibrogenic role, showing enrichment in collagen fibril and ECM organization pathways (Supplementary Figure 11C). Moreover, expression of COL1A1 , COL1A2 , COL3A1 , FAP, TWIST1, MMP1, and MMP3 was significantly upregulated in inflamed tissue compared with healthy or noninflamed controls (Supplementary Figure 11D).

Spatial co-expression mapping of MMP3 and MMP1 confirmed that $\text{MMP3}^+\text{MMP1}^+$ (FAP^+) fibroblasts were exclusively localized within the fibrotic niche of the fibrotic specimen (Figure 5C), overlapping with the high-ECM fibroblast regions identified by spatial analysis (Figure 5D). These fibroblasts were uniformly distributed within submucosal ulcerated regions lacking epithelium (Figure 5D). GO analysis of this subset revealed strong enrichment in ECM-related and wound-healing pathways (Supplementary Figure 11E). Furthermore, this population had significantly higher ECM scores than any other fibroblast subtype (Figure 5A, B and D). Collectively, these results indicate that the high-ECM fibroblasts identified via spatial transcriptomics correspond to the FAP^+ fibroblasts reported in previous studies.^{13,14} These cells are specifically localized within the fibrotic submucosa and are closely associated with ECM accumulation and tissue remodeling in fibrotic CD.

Spatial Colocalization and Interaction Between Inflammatory Monocytes and ECM-producing Stromal Cells Within the Fibrotic Niche

Our spatial transcriptomic analysis revealed a marked enrichment of inflammatory monocytes in the fibrotic niche, identified by expression of canonical monocyte markers LYZ and CD14 and the inflammatory proteins S100A8/S100A9 (calprotectin) (Figure 7A).^{37–39} This monocyte population also expressed SPP1 , IL1B , CXCL9 , and MMP9 (Figure 7B). Notably, SPP1 and IL1B are markers of CD150^+ inflammatory monocytes,¹⁴ whereas CXCL9 and MMP9 characterize CXCL9^+ macrophages.¹³ Both of these myeloid subsets have been implicated in activating intestinal FAP^+ fibroblasts. In line with this, GO analysis showed that the monocyte population was significantly enriched in cytokine-mediated signaling pathways (Figure 7C). Cell-cell interaction analysis further demonstrated that these inflammatory monocytes had the highest number and strength of predicted interactions with myofibroblasts, FAP^+ fibroblasts, and pericytes among all immune cell types (Figure 7D and E). Spatial mapping confirmed close colocalization of inflammatory monocytes with these key ECM-producing stromal cells in the fibrotic

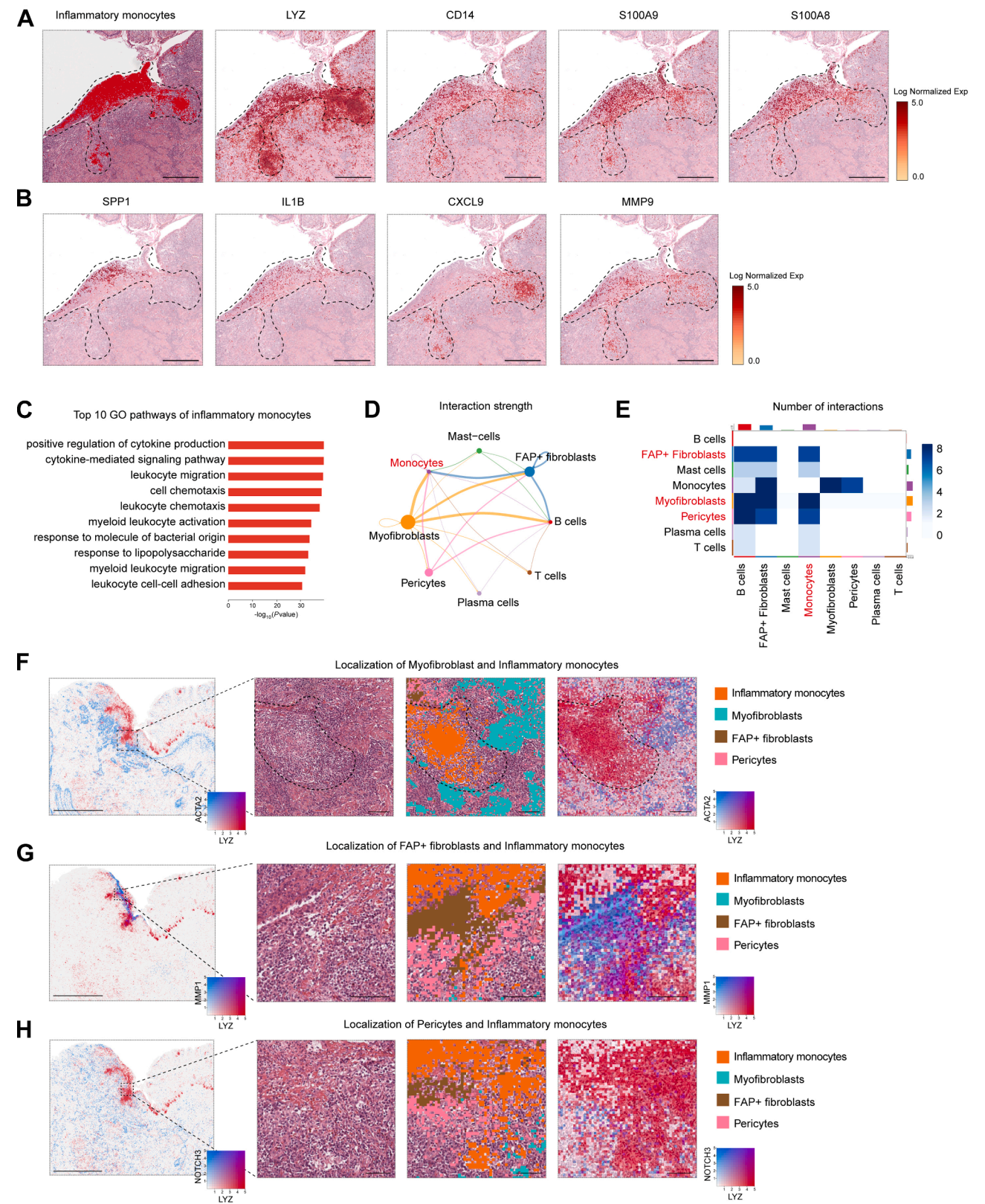
niche (Figure 7F–H). Transcriptomically, this colocalization was supported by co-expression of marker genes in the same regions: LYZ (monocytes), ACTA2 (myofibroblasts), MMP1 (FAP^+ fibroblasts), and NOTCH3 (pericytes) (Figure 7F–H). (Submucosal VSMCs were ruled out in these regions by corresponding H&E staining.) Collectively, these findings suggest that the enriched inflammatory monocytes may serve as key upstream activators of ECM-producing stromal cells in the fibrotic niche of CD.

Discussion

In this study, we employed high-resolution, whole-tissue spatial transcriptomic profiling to investigate the cellular phenotypes underlying extensive myoid cell hyperplasia in the thickened submucosa of fibrostenotic CD intestines. Our analysis revealed that the majority of these hyperplastic myoid cells are myofibroblasts—a distinct stromal population expressing markers of both SMCs and fibroblasts. Notably, this work provides the first in situ transcriptional evidence confirming the presence of myofibroblasts with high ECM-producing capacity in the fibrotic submucosal compartment of CD intestines. These myofibroblasts contribute to intestinal fibrostenosis through 2 principal mechanisms: (1) substantial cellular expansion within the submucosa, directly increasing BWT; and (2) robust ECM synthesis, leading to excessive ECM deposition and progressive luminal narrowing. Spatial mapping and pseudotemporal analysis further indicated that these myofibroblasts differentiate from both muscularis mucosae SMCs and submucosal VSMCs. In addition, integrated analysis of multiple scRNA-seq datasets, complemented by in vitro validation, demonstrated a significant expansion of pericytes within the submucosa of fibrostenotic CD tissues. These pericytes exhibited the potential to transdifferentiate into myofibroblast-like cells, thereby promoting ECM accumulation and contributing to fibrogenesis. We also observed pronounced spatial heterogeneity among fibroblast subpopulations: fibrosis-associated FAP^+ fibroblasts were preferentially enriched within the fibrotic submucosa. Moreover, we identified a population of inflammatory monocytes locally enriched in the submucosa of fibrostenotic CD intestines. These cells showed close spatial proximity and strong predicted ligand-receptor interactions with myofibroblasts, pericytes, and FAP^+ fibroblasts, suggesting they may constitute a critical immune subset driving stromal cell activation. Although not presented as universal, the presence, cellular composition, and putative dynamics of this niche observed in our specimen suggest a locally profibrotic architecture potentially contributing to CD intestinal fibrostenosis, thereby identifying myofibroblasts and pericytes as prime candidates for further mechanistic and therapeutic investigation.

Re-analysis of 3 public scRNA-seq datasets (TI, CO, and TI-1) identified a myoid cell population within the stromal compartment across all datasets. These cells expressed canonical smooth muscle markers (eg, ACTA2 , DES , MYH11) but constituted <15% of the total stromal population in every cohort and showed no significant

upregulation of core fibrotic genes (COL1A1, COL1A2, COL3A1) or enrichment of fibrosis-related pathways. Based on these characteristics, we infer that this population likely represents normal SMCs rather than the pathological ECM-producing myofibroblasts identified by our spatial transcriptomic analysis. In line with our findings, several recent



single-cell studies of fibrostenotic CD have also failed to identify this distinct myofibroblast population. For example, Mukherjee et al reported 3 myoid cell clusters (SMC_ACTA2, SMC_HHIP, SMC_DES_ACTG2), none of which exhibited elevated COL1A1, COL1A2, or COL3A1 expression or increased abundance in inflamed or stenotic tissues.¹² Likewise, Zhang et al described a stromal cluster termed “Myocytes,” characterized by smooth muscle markers (DES, ACTG2, MYH11, CNN1, HHIP) but low COL1A1/COL3A1 levels.¹³ Similarly, Ke et al identified an SMC cluster (ACTG2, HHIP, MYOCD) and a myofibroblast cluster (ACTA2, F3, SOX6), neither of which increased in inflamed or stenotic tissues or displayed high ECM scores.¹⁴ Collectively, these observations indicate that the failure of scRNA-seq to capture ECM-producing myofibroblasts is likely due to methodological constraints—specifically, the reliance on collagenase-based tissue dissociation, which may not adequately release cells embedded in dense ECM.⁴⁰ Consequently, ECM-rich populations such as myofibroblasts may be systematically underrepresented in single-cell profiles. This interpretation aligns with findings in idiopathic pulmonary fibrosis, where spatial transcriptomic methods have demonstrated superior capability relative to scRNA-seq in detecting ECM-embedded myofibroblast populations.⁴¹

We propose that the high ECM-producing myofibroblasts identified by spatial analysis originate from SMCs in the muscularis mucosae and from VSMCs in the submucosa. This conclusion is supported by convergent histological, spatial, and pseudotemporal evidence from the fibrotic specimen. Histologically, the muscularis mucosae in non-ulcerated regions was clearly delineated and unexpanded, whereas in ulcerated regions, it became markedly disorganized with indistinct boundaries and showed extensive myoid cell infiltration into the adjacent submucosa. This pattern closely aligns with prior histological reports of fibrostenotic CD,⁷ which described irregularly arranged smooth muscle bundles interwoven with fibrotic ECM, some extending outward from the muscularis mucosae. Our histological observations, combined with these earlier reports, indicate a structural continuum originating from the muscularis mucosae into the submucosa, providing anatomical evidence for an SMC-to-myofibroblast transition. Spatial transcriptomic analyses further substantiate this interpretation. SMCs in nonulcerated areas maintained expression of canonical smooth muscle genes without activation of fibrotic pathways, whereas cells in ulcerated regions displayed significantly elevated ECM scores and

upregulated fibrotic gene expression, warranting their designation as myofibroblasts. Pseudotime trajectory analysis provided additional support for this lineage transition, placing SMCs from nonulcerated regions at early pseudotime states and myofibroblasts from ulcerated regions at terminal states. A similar pseudotime pattern was observed for submucosal VSMCs: cells near the vascular wall corresponded to early pseudotime positions, whereas outward-migrating cells were identified as myofibroblasts at terminal pseudotime states. Collectively, these findings strongly indicate a fibrogenic differentiation pathway whereby both muscularis mucosae-derived SMCs and submucosal VSMCs give rise to myofibroblasts in fibrostenotic CD tissue. Further *in vitro* validation and mechanistic studies focusing on this phenotypic transition are warranted to fully elucidate its contribution to CD-associated intestinal fibrosis.

Pericytes play essential roles in vascular development, maturation, regulation of permeability, and tissue homeostasis. However, their heterogeneity, differentiation trajectories, and pathological contributions in CD remain insufficiently characterized, primarily due to difficulties in distinguishing pericytes from other stromal cells with overlapping markers.^{32,42} By integrating 3 public scRNA-seq datasets (TI, CO, and TI-1), we identified 2 transcriptionally distinct mural cell subsets: one expressing high levels of smooth muscle markers (annotated as VSMCs) and another defined by STEAP4 expression with significant enrichment of ECM-related genes (annotated as pericytes). These annotations were confirmed by immunofluorescence staining of CD intestinal tissues. Pericytes are known for their multipotency and lineage plasticity, and their involvement in fibrotic remodeling has been documented in multiple organs (for instance, kidney pericytes serve as precursors to scar-forming myofibroblasts,⁴³ and cardiac pericytes in ischemic myocardium upregulate fibrogenic programs contributing to ECM deposition).⁴⁴ In the context of CD, our spatial transcriptomic analysis demonstrated selective enrichment of pericytes within the submucosal fibrotic niche, accompanied by elevated ECM scores. Moreover, pseudotime analysis using integrated scRNA-seq data revealed that intestinal pericytes in CD undergo transcriptional reprogramming toward a myofibroblast-like phenotype, characterized by downregulation of classical pericyte markers and concurrent upregulation of key fibrogenic genes. This phenotypic transition was further validated *in vitro*, as pericytes isolated from nonfibrotic CD tissue adopted myofibroblastic characteristics upon

Figure 7. (See previous page). Enrichment of inflammatory monocytes in the fibrotic niche and their spatial proximity to ECM-producing stromal cells. (A–B) Spatial localization of inflammatory monocyte marker genes within the fibrotic niche. Darker colors indicate higher expression levels. Scale bars, 250 μ m. **(C)** Top 10 GO enrichment pathways for genes expressed by inflammatory monocytes (hypergeometric test with FDR-adjusted *P* values). **(D)** Bubble plot summarizing the interaction strengths between inflammatory monocytes and myofibroblasts, FAP⁺ fibroblasts, and pericytes among all immune cell populations in the CD fibrotic specimen. **(E)** Heatmap showing the number of predicted ligand-receptor interaction pairs between inflammatory monocytes and myofibroblasts, FAP⁺ fibroblasts, and pericytes across all immune cell types in the CD fibrotic specimen. **(F–H)** Spatial colocalization of cell clusters and representative marker gene expression for inflammatory monocytes and stromal subtypes (myofibroblasts, FAP⁺ fibroblasts, and pericytes) within the fibrotic niche. Scale bars: **(F)** 200 μ m; **(G)** 100 μ m; **(H)** 50 μ m.

exposure to pro-fibrotic stimuli. Collectively, these findings identify intestinal pericytes as previously unrecognized contributors to fibrotic remodeling in CD and underscore their potential as novel targets for antifibrotic interventions.

By performing fibroblast sub-clustering across 3 independent scRNA-seq datasets, we developed a novel classification framework for intestinal fibroblast subpopulations in CD. Although this classification may not precisely reflect the true biological composition of intestinal fibroblasts, it was reproducible across all cohorts. Importantly, fibroblast subsets defined by this strategy exhibited pronounced spatial heterogeneity in both fibrotic and nonfibrotic intestinal tissues. Based on their distinct transcriptional signatures and anatomical distribution patterns, we inferred partial functional roles for these subsets. CXCL14 and ADAMDEC1 are well-characterized fibroblast markers previously implicated in ulcerative colitis and various fibrotic disorders,⁴⁵ with ADAMDEC1⁺ fibroblasts reported in healthy CD mucosa.^{14,46} In our study, fibroblast populations characterized by expression of either CXCL14 alone or co-expression of CXCL14 and ADAMDEC1 were predominantly localized within the lamina propria of both fibrotic and nonfibrotic specimens. These subsets displayed elevated ECM scores compared with adjacent epithelial compartments, suggesting a role in maintaining mucosal structural integrity. Additionally, given the immunomodulatory function of CXCL14 as a chemokine that regulates leukocyte trafficking,^{47,48} CXCL14⁺ fibroblasts may also participate in modulating immune cell dynamics in the lamina propria. In contrast, the fibroblast subset characterized by SFRP2 and MGP exhibited a broader anatomical distribution, spanning the submucosa, MP, and serosa in both fibrotic and nonfibrotic tissues. However, their abundance was markedly reduced within the fibrotic niche, indicating that these cells likely represent resident fibroblasts essential for maintaining normal intestinal architecture. Notably, within the fibrotic niche, FAP⁺ fibroblasts were highly abundant, displayed elevated ECM scores, and showed increased expression of core fibrotic genes—second only to myofibroblasts. Although these findings reinforce the central role of myofibroblasts in intestinal fibrostenosis, our spatial transcriptomic data also highlighted a significant enrichment of FAP⁺ fibroblasts along ulcerated surfaces in the fibrotic submucosa. In these ulcerated regions, FAP⁺ fibroblasts exhibited significantly elevated expression of the matrix metalloproteinases MMP1 and MMP3, strongly indicating their involvement in ECM remodeling and tissue repair processes.⁴⁹ Consistent with previous studies implicating FAP⁺ fibroblasts in intestinal fibrosis, our findings further confirm their substantial contribution to fibrotic progression in CD-associated fibrostenosis.

Our spatial transcriptomic analysis identified a specific population of inflammatory monocytes enriched within the fibrotic niche, characterized by high expression of S100A8 and S100A9, which encode the calcium-binding proteins comprising calprotectin. Notably, fecal calprotectin is widely used as a clinical biomarker for monitoring intestinal inflammation in inflammatory bowel disease.³⁹ In

addition to S100A8/A9, this monocyte population expressed IL1B, MMP9, and CXCL9, markers previously associated with distinct pro-inflammatory monocyte and macrophage subsets. Specifically, interleukin (IL)1B has been recognized as a marker for CD150⁺ inflammatory monocytes,¹⁴ whereas MMP9 and CXCL9 are characteristic of CXCL9⁺ macrophages—2 subsets known to activate FAP⁺ fibroblasts in CD.¹³ Given this transcriptional overlap, the inflammatory monocytes identified here may correspond in part to these previously described subsets. Spatial mapping demonstrated that these monocytes colocalize with myofibroblasts, FAP⁺ fibroblasts, and pericytes within the fibrotic niche, and gene set enrichment analysis (GSEA) revealed significant activation of cytokine signaling pathways in this monocyte population. Furthermore, cell-cell interaction analysis showed that inflammatory monocytes had the greatest number and intensity of predicted interactions with ECM-producing stromal populations among all immune subsets. Based on our findings and prior evidence, we hypothesize that these inflammatory monocytes function as key immune effectors that activate myofibroblasts, FAP⁺ fibroblasts, and pericytes through cytokine-mediated signaling, thereby promoting stromal proliferation and excessive ECM production. This immune-stromal interplay may represent a pivotal mechanism underlying fibrotic remodeling and luminal narrowing in CD. Although further functional validation is necessary, our spatial and transcriptomic data provide robust evidence of coordinated crosstalk between myeloid cells and pro-fibrotic stromal cells within the CD fibrotic niche.

This study has certain limitations. Primarily, inherent heterogeneity of CD, coupled with our focus on high-resolution spatial transcriptomic analysis of a single fibrostenotic specimen matched to one control, suggests that the fibrotic niche we characterized likely represents a sample-specific profile. As such, its features may not extend to all fibrostenotic CD tissues. Validating the reproducibility of this spatial pattern and establishing its broader applicability will necessitate future studies utilizing larger, clinically stratified cohorts subjected to high-resolution spatial transcriptomics. Furthermore, advancing towards platforms capable of wider capture areas may be required to comprehensively assess spatial interactions across the entire bowel wall. Second, although the Visium HD platform provides enhanced spatial resolution compared to earlier technologies, its fixed 8 × 8 μm spot size inherently captures transcripts from multiple adjacent cells within each spatial unit. Consequently, signals from heterogeneous cellular populations may become mixed, potentially obscuring distinct cell identities. To mitigate these confounding effects, we excluded ambiguous spatial barcodes from downstream analyses. Thirdly, cross-validation utilized the most comprehensive accessible scRNA-seq datasets; however, these are predominantly derived from inflammatory CD contexts. The absence of well-annotated, cohort-scale fibrostenotic single-cell data currently limits robust external validation for fibrosis-specific mechanisms, highlighting a critical need for such resources. Lastly, although our study revealed critical stromal cell subpopulations and highlighted spatially coordinated immune-stromal interactions within

the fibrotic niche, the precise molecular underpinnings governing these crosstalk networks remain to be comprehensively defined. Future mechanistic work—integrating complementary *in vivo* models with *in vitro* systems—will be essential to delineate the signaling cascades and regulatory modules underlying fibrostenosis in CD, ultimately advancing targeted therapeutic strategies.

Taken together, our findings define a previously uncharacterized fibrotic niche in the CD intestine, defined by molecularly specialized stromal and immune subpopulations that spatially coordinate to drive fibrostenosis. Building on this framework, future studies should investigate the upstream regulatory mechanisms governing the differentiation of myofibroblasts from SMCs and VSMCs, as well as the transdifferentiation of pericytes into myofibroblast-like cells. In parallel, the immunological signals—particularly those derived from inflammatory monocytes—that modulate stromal activation and ECM production warrant further exploration. Ultimately, elucidating the dynamic interactions within this cellular ecosystem is essential to develop targeted antifibrotic strategies designed to counteract the pathology of CD-associated fibrostenosis.

Materials and Methods

Clinical Ultrasound Assessment

Preoperative IUS data were retrospectively collected from 117 patients with CD (sex was not taken into account) who underwent surgical resection for intestinal stenosis at Nanfang Hospital (Guangzhou, China) between 2020 and 2024. All examinations were performed by experienced sonographers using standardized imaging protocols. Total BWT and BWS were evaluated in the stenotic segments, and the thicknesses of individual layers—including the mucosa, submucosa, and MP—were measured when stratification was preserved. Segments with a total BWT >3 mm were considered pathologically thickened and included in the analysis. Ultrasound reports were independently reviewed by 2 investigators blinded to the histological findings.

Histological Analysis of Surgical Specimens

Full-thickness intestinal tissues were obtained from 25 NL, 35 CDns, and 44 CDs, all collected at Nanfang Hospital (Guangzhou, China). “Normal” samples were defined as histologically normal, full-thickness ileal segments taken from the healthy resection margins of patients undergoing right hemicolectomy for colorectal cancer. CD specimens were classified based on gross anatomical appearance into strictured (CDs) and nonstrictured (CDns) groups at the time of surgical resection. All specimens were FFPE, and sectioned at 4- μ m thickness. H&E staining was performed to assess overall tissue architecture, inflammatory cell infiltration, and myoid cell hyperplasia. Masson’s trichrome staining was used to evaluate ECM deposition, particularly collagen accumulation. Submucosal thickness was quantitatively measured in 3 randomly selected, well-oriented regions per specimen using ImageJ software (version 1.53, NIH). Histopathological evaluation was independently conducted by 2 blinded gastrointestinal pathologists. Myoid

cell hyperplasia was defined as a dense proliferation of spindle-shaped, smooth muscle-like cells occupying at least one-third of the total submucosal thickness.

All patients were enrolled in accordance with the ethical principles outlined in the Declaration of Helsinki. The study was approved by the Medical Ethics committee of Nanfang Hospital of Southern Medical University (approval number: NFEC-2024-495). Written informed consent was obtained from all participants prior to the collection of clinical data and surgical specimens. Both oral and written explanations of the study were provided, and each participant authorized the use of anonymized clinical information for publication.

Spatial Transcriptomics Analysis Using Visium HD

Sample collection and FFPE preparation. Fresh full-thickness ileal tissues were obtained from 2 patients with CD during surgical resection, including one fibrotic and stenotic sample and one nonfibrotic control sample (Supplementary Table 3). Tissue specimens were immediately fixed in 4% paraformaldehyde for 24 hours, followed by standard dehydration, clearing, and paraffin embedding procedures. FFPE tissue blocks were used for subsequent spatial transcriptomic analysis.

Visium HD library preparation and sequencing. Five-micrometer sections were cut from FFPE tissue blocks using a microtome and processed following the Visium CytAssist Spatial Gene Expression for FFPE – Tissue Preparation Guide (CG000518, Rev C). Sections were placed on plain glass slides for deparaffinization, H&E staining, and imaging according to the Visium HD FFPE Tissue Preparation Handbook (CG000684). Library construction steps—including probe hybridization, ligation, slide preparation, probe release, extension, and amplification—were carried out according to the Visium HD Spatial Gene Expression Reagent Kits User Guide (CG000685). Sequencing was performed on an Illumina NovaSeq 6000 platform with paired-end reads (Read 1: 43 cycles; i7: 10 cycles; i5: 10 cycles; Read 2: 50 cycles).

Data processing and clustering analysis. Raw sequencing data were processed using Space Ranger (v3.0), which aligned FASTQ files to the human reference genome (GRCh38), detected the tissue area on the slide, and generated spatially resolved gene-barcode matrices. Downstream analyses were performed using Seurat (v4.3.2) in R. Data were normalized, clustered, and annotated using default parameters.

Differential gene expression analysis. Differential expression analysis for each cluster was performed using Seurat’s FindMarkers function (v4.3.2). Significantly differentially expressed genes (DEGs) were defined by the following criteria: (1) detected in >10% of cells in either group; (2) $|\log_2 \text{fold-change}| > .25$; and (3) Bonferroni-adjusted $P < .05$ (Wilcoxon rank-sum test). A list of top 30 marker genes is provided in Supplementary Tables 4 and 5.

GO enrichment analysis. GO enrichment was performed using the clusterProfiler R package (v3.14.3) with a

hypergeometric test (significance threshold: $P < .05$). False discovery rate (FDR) control was applied using the Benjamini-Hochberg method. The background gene set was derived from the org.Hs.eg.db database (for human genes). Results were visualized using the barplot function in R.

Pathway signature scoring. Pathway module scores were computed using the AddModuleScore function in Seurat. ECM-related pathways were selected from MatrisomeDB 2.0 (collagen, glycoprotein, and proteoglycan categories; <https://matrisomedb.org/>) and from the GO biological process collection in MSigDB (category C5). Data visualizations were generated using the ggplot2 R package.

Trajectory inference analysis. To infer differentiation trajectories in myofibroblasts and SMCs, we used Monocle 2 (v2.14.0). Cells were filtered and normalized using the estimateSizeFactors and estimateDispersions functions. Pseudotime ordering was based on DEGs identified by Seurat's FindAllMarkers. Dimensionality reduction and cell ordering were performed using the DDRTree method and the orderCells function. DEG dynamics along pseudotime were visualized using the plot_cell_trajectory and plot_pseudotime_heatmap functions. Branch-dependent expression patterns were identified using the branch expression analysis modeling (BEAM) test and visualized with plot_genes_branched_heatmap.

Cell-cell communication analysis. Ligand-receptor interactions were inferred using CellChat (v1.5.0). Cell-cell communication networks were reconstructed with CellChat and the Connectome R toolkit (v0.2.2). Interaction counts and strengths were computed using the compareInteractions function. Filtered interaction edges were visualized using the CircosDiff and netVisual_chord_gene functions from the CellChat package.

Public scRNA-seq Dataset Acquisition and Analysis

Acquisition of public scRNA-seq datasets. Three publicly available scRNA-seq datasets were utilized. The TI and CO dataset was obtained from the Human Cell Atlas project via the Single Cell Portal and includes both relatively healthy and CD-associated intestinal tissue samples. Healthy samples were from terminal ileum (TI, $n = 13$) and colon (CO, $n = 32$), whereas CD samples included inflamed (TI, $n = 16$; CO, $n = 6$) and noninflamed segments (TI, $n = 49$; CO, $n = 20$), collected through surgical resection or endoscopic biopsy. The TI-1 dataset was retrieved from the Gene Expression Omnibus (GEO accession GSE134809) and includes paired inflamed (TI, $n = 11$) and noninflamed (TI, $n = 11$) ileal samples from patients with CD who underwent surgical resection for strictures.

Preprocessing and quality control. Initial processing of raw scRNA-seq data was conducted on the NovelBrain Cloud Analysis Platform. Reads were aligned and quantified using STARsolo (v2.7.10a) against the GRCh38 human reference genome. Cells expressing fewer than 200 genes or with $>10\%$ mitochondrial unique molecular identifier (UMI) content were excluded. Mitochondrial gene reads were removed from downstream expression matrices. Data

were normalized and variance-stabilized using Seurat (v4.1.1), regressing out total UMI counts and mitochondrial content. Principal component analysis (PCA) was performed on the top 2000 highly variable genes, and the top 10 principal components were used for t-distributed stochastic neighbor embedding (t-SNE) and uniform manifold approximation and projection (UMAP) for dimensionality reduction.

Clustering and cell type identification. Unsupervised clustering was performed using Seurat's graph-based algorithm on the top 10 principal components. Marker genes for each cluster were identified with the FindAllMarkers function (Wilcoxon rank-sum test) using thresholds of log2 fold change (FC) > 0.25 , $P < .05$, and expression in at least 10% of cells (min.pct > 0.1). For refined cell type annotation, clusters belonging to the same major lineage were subsetted and reanalyzed with additional rounds of t-SNE, clustering, and differential expression analysis.

DEG analysis. Differential expression between sample groups was assessed using the FindMarkers function in Seurat (Wilcoxon rank-sum test) under the following criteria: log2FC > 0.25 , $P < .05$, and min.pct > 0.1 . This approach enabled identification of functionally relevant gene signatures across disease states.

GO enrichment analysis. To determine the biological relevance of DEGs and cluster-specific markers, GO enrichment analysis was performed. GO annotations were obtained from the Gene Ontology Consortium (<http://geneontology.org/>). Fisher's exact test was used to identify enriched GO terms, and P values were adjusted using FDR correction.

Pseudotime trajectory analysis. Trajectory inference was performed using Monocle 2 (v2.14.0) to reconstruct dynamic transcriptional transitions. Prior to analysis, marker genes from Seurat clustering and raw count matrices of quality-controlled cells were imported into Monocle. Dimensionality reduction and pseudotime ordering were carried out using the DDRTree algorithm and orderCells. Branch-specific gene expression dynamics were examined with the BEAM function, and key bifurcation genes were visualized via branched heatmaps.

Immunofluorescence Staining

Tissue immunofluorescence. Paraffin-embedded full-thickness intestinal tissue sections were cut at 4- μ m thickness, deparaffinized in xylene, and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating sections to 100°C in 10 mM citrate buffer (pH 6.0). To quench endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide. After blocking with normal serum, sections were incubated for 16 hours at 4°C with the following primary antibodies: CD31 (rabbit monoclonal, 1:200; Servicebio, Cat. GB11063-2), CD146 (MCAM; rabbit monoclonal, 1:1000; Abcam, Cat. ab75769), α -SMA (rabbit monoclonal, 1:200; Cell Signaling Technology, Cat. 19245), COL1A1 (rabbit monoclonal, 1:1000; Cell Signaling Technology, Cat. 72026), and STEAP4 (rabbit monoclonal, 1:100; Proteintech, Cat. 11944-1-AP). After

washing, sections were incubated with secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG [H+L], 1:400, Abcam, Cat. ab150073; and Alexa Fluor 594 donkey anti-rabbit IgG [H+L], 1:400, Abcam, Cat. ab150076) for 1 hour at 22°C to 25°C. Immunofluorescence was visualized using an Olympus BX-53 fluorescence microscope (Olympus).

Cellular immunofluorescence. Human primary intestinal pericytes were seeded on sterile coverslips in 24-well plates and cultured to ~50% confluency. Cells were fixed with 4% paraformaldehyde for 15 minutes at 22°C to 25°C, permeabilized with 0.2% Triton X-100 for 10 minutes at 22°C to 25°C, and blocked with 5% bovine serum albumin (BSA) for 30 minutes at 22°C to 25°C. Cells were then incubated for 16 hours at 4°C with primary antibodies against STEAP4 (rabbit monoclonal, 1:100; Proteintech, Cat. 11944-1-AP), CD146 (rabbit monoclonal, 1:1000; Abcam, Cat. ab75769), and α -SMA (rabbit monoclonal, 1:200; Cell Signaling Technology, Cat. 19245). After washing, cells were incubated with Alexa Fluor 488 donkey anti-rabbit IgG (1:400; Abcam, Cat. ab150073) for 1 hour at 22°C to 25°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI), and images were captured using the Olympus BX-53 fluorescence microscope.

Isolation and Culture of Primary Stromal Cells From Human Intestine

Intestinal stromal cells were isolated from fresh, non-fibrotic ileal CD specimens ([Supplementary Table 7](#)). Within 5 minutes after resection, tissue samples were placed in prechilled Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS; Procell, Cat. 164210), 0.4 mg/mL gentamicin sulfate (LEAGENE, Cat. CA0055), 0.04 mg/mL ciprofloxacin (LEAGENE, Cat. CA0100), and 5% antibiotic-antimycotic solution (ECOTOP Scientific, Cat. ES-8440-100ml). After povidone-iodine disinfection and washing with calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) (Biosharp, Cat. BL559A), tissues were finely minced and processed in a GentleMACS dissociator using sequential programs (h_tumor_01, h_tumor_02, h_tumor_03). Enzymatic digestion was performed at 37°C in HBSS containing 0.2 mg/mL each of collagenase types I, II, and IV (Sigma-Aldrich) and 10 μ g/mL DNase I (Sigma-Aldrich), with gentle agitation every 10 minutes. The resulting cell suspension was passed through a 100- μ m cell strainer (Falcon), centrifuged at $250 \times g$ for 10 minutes at 20°C, and washed twice with phosphate-buffered saline (PBS). The cell pellet was resuspended and cultured in pericyte-conditioned medium (ScienCell, Cat. 1201), a low-glucose medium supplemented with 2% FBS, 1% penicillin-streptomycin, and 1% pericyte growth supplement. All primary stromal cells used for experiments were at passage 5 or earlier.

Purification of Pericytes by Magnetic-activated Cell Sorting

Subconfluent primary stromal cells with optimal growth were harvested for magnetic-activated cell sorting (MACS)

using the EasySep Human Cell Separation system (STEM-CELL Technologies), a column-free magnetic separation platform. Cells were detached with 0.25% trypsin, pelleted, and adjusted to 1×10^7 cells/mL in 100 μ L of sorting buffer (PBS + 2% FBS + 1 mM EDTA). To the cell suspension, we added 1 μ L of EasySep Human FC Blocker and 3 μ L of anti-human CD146 (MCAM) monoclonal antibody (BioLegend, Cat. 361006), mixing gently. After incubation with this antibody cocktail and magnetic RapidSpheres for 10 minutes at 22°C to 25°C, the volume was brought to 2.5 mL with sorting buffer, and the tube was placed in an EasySep magnet for exactly 5 minutes. The supernatant was discarded, and the CD146⁺ cells retained on the tube wall were collected and promptly transferred to culture medium for expansion. To enrich for pericytes, we employed a 2-step magnetic selection targeting CD146 and CD36, which together define the pericyte phenotype in human intestinal tissue. After initial expansion of the CD146⁺ fraction, cells were subjected to a second round of MACS using an anti-human CD36 monoclonal antibody (BioLegend, Cat. 336203) following the same protocol. The final CD146⁺CD36⁺ cell population was used for downstream experiments.

Transforming Growth Factor-beta 1 Stimulation Assay

Purified pericytes were cultured at a density of 80,000 cells per well in 6-well plates using pericyte-conditioned medium for 2 to 3 days until reaching optimal attachment and morphology. Cells were then serum-starved for 8 hours in pericyte-conditioned medium containing 0.2% FBS prior to stimulation with recombinant human transforming growth factor-beta 1 (TGF- β 1) (10 ng/mL; Novoprotein, Cat. CA59). Total RNA was harvested at 24 and 48 hours post-stimulation, and changes in gene expression were assessed by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Gene Expression Analysis by qRT-PCR

Total RNA was extracted from cultured cells using TRIzol Reagent (Takara) according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript RT Master Mix (Takara). Quantitative PCR was performed using a SYBR Green Master Mix (Takara) on a LightCycler 480 System (Roche). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping gene cyclophilin A. Primer sequences for target genes are provided in [Supplementary Table 8](#).

Statistical Analysis

All statistical analyses were performed using R (v4.3) and GraphPad Prism 8.0. Data are presented as mean $\pm$ standard deviation (SD) unless otherwise specified. Statistical significance was defined as $P < .05$ (or adjusted $P < .05$ for multiple comparisons). The specific statistical tests used are indicated in the corresponding figure legends.

Differential gene expression and enrichment analyses were corrected for multiple testing using FDR or Bonferroni adjustments as appropriate. Spatial gene expression patterns were visualized using Loupe Browser 8 (10x Genomics). No statistical method was used to predetermine sample size. All data were included for analysis, and investigators were not blinded during experiments or outcome assessment.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the full text version at <https://doi.org/10.1016/j.jcmgh.2025.101701>.

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Conflicts of interest

The authors disclose no conflicts.

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Data Availability

The spatial transcriptomics dataset supporting the conclusions of this study have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE302569. Additional datasets and materials are available from the corresponding author upon reasonable request.