

Multi-Omics Reveals MTOR1-HIF1 α axis educates ADAM28+ Fibroblasts and COL1A2+ Fibroblasts Across Distinct Colorectal cancer Microsatellite States

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14 **Highlights**

15 ● **Multi-Omics for Decoding colorectal cancer Stromal environment:**

16 For the first time, by combination deep learning WSI morphomics,
17 single-cell RNA-seq, we leveraged artificial intelligence-processed
18 pathological slices integrated with multi-omics data to investigate the
19 stromal microenvironment of colorectal cancer with different
20 microsatellite statuses

21 ● **COL1A2⁺ fibroblasts and ADAM28⁺ fibroblasts drive**

22 **Immuno-vascular Dynamics:** We identified COL1A2⁺ fibroblasts and
23 ADAM28⁺ fibroblasts are vital clusters in MSS versus MSI-H colon
24 adenocarcinoma. Two subsets of stromal cells drive differences in
25 vascular and immune microenvironments and serve as key regulators
26 shaping the tumor microenvironment. We also identified distinct spatial

niches governing tumor immunity and neurovascular dynamics in the "immunologically cold" MSS phenotype.

● **COL1A2+ fibroblasts and ADAM28+fibroblasts**Evolutionary points:

Evolutionary analysis indicates that the MTOR1-HIF1alpha singal axis represents the central focus for the differentiation of COL1A2+ fibroblasts and ADAM28+fibroblasts

● **Therapeutic Stroma Remodeling COL1A2+ fibroblasts:** We further integrated drug databases to perform screening at the single-cell level and identified candidate potential compounds,and validated their efficacy in vitro to reverse stromal resistance and reprogram the cold tumor

Abstract

Background

Microsatellite status serves as a pivotal biomarker dictating prognosis and immunotherapy responsiveness in colon adenocarcinoma (COAD). In contrast to microsatellite instability-high (MSI-H) tumors, which exhibit pronounced immunotherapeutic benefits and superior overall survival, the vast majority of patients with microsatellite stable (MSS) disease present with an "immunologically cold" phenotype characterized by low tumor mutational burden and profound therapeutic resistance. While previous investigations have largely focused on tumor cell-intrinsic genomic alterations and neoantigen loads, the systemic evolutionary trajectories and spatial topological mechanisms by which the stromal microenvironment governs tumor immunity and neurovascular dynamics—thereby driving distinct microsatellite phenotypes and therapy outcomes—remain poorly understood.

Results

We firstly demonstrated that the difference of cells phenotypic and spatial heterogeneity between the stromal microenvironment represent core determinants distinguishing MSS from MSI-H colon cancer. We identified two key fibroblast dominated-programs driving this stromal dimorphism: ADAM28⁺ fibroblasts (ADAM28⁺ Fibro) are significantly enriched in MSI-H tumors, where they stimulate pro-chemotactic factor secretion by ACKR1⁺ endothelial cells to facilitate T lymphocyte transendothelial migration and immune recruitment. Conversely, COL1A2⁺ fibroblasts (COL1A2⁺ Fibro) predominate in the MSS stroma, simultaneously promoting pathological angiogenesis and depositing a dense physical barrier while accelerating tumor vascularization. We uncovered a novel role for ADAM28⁺ Fibro and COL1A2⁺ Fibro in shaping type 1 conventional dendritic cell (cDC1) phenotypes, thereby modulating their antigen-presentation capabilities, which may lead to distinct immunotherapy response. Mechanistically, the transcription factor HIF1 α was pinpointed as the key molecular node controlling the lineage commitment of fibroblasts toward these distinct subpopulations. We further revealed via deep learning-based drug virtual screening tools that AZD2014 effectively targets the HIF1 α -mediated transcriptional network, reversing fibroblast commitment to the COL1A2⁺ Fibro fate, partially recapitulating an "MSI-H-like" stromal state, and attenuating the dismal prognosis associated with fibrotic MSS tumors.

Conclusions

This study systematically deconstructs the topological architecture and functional divergence of the stromal microenvironment in MSS and MSI-H colon adenocarcinoma, and further found out the upstream HIF1 α -driven mechanism dictating fibroblast fate decisions, and identifies a promising therapeutic candidate for stromal remodeling. These findings establish a

novel spatial theoretical framework and precise therapeutic targets to breach the immune tolerance barrier in MSS colon cancer.

Introduction

Colorectal adenocarcinoma (COAD) remains one of the leading causes of cancer-related incidence and mortality worldwide^[1]. As a cornerstone biomarker for prognostic stratification and precision oncology, microsatellite status directly governs the efficacy of immune checkpoint blockade (ICB) and combination regimens. For patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) tumors, anti-PD-1/PD-L1 therapies have achieved remarkable clinical breakthroughs^[2-4], radically transforming the therapeutic landscape of advanced COAD. Conversely, patients with microsatellite stable (MSS) disease—who represent over 85% of the total patient population—predominantly display an "immunologically cold" phenotype that confers widespread primary resistance to conventional immunotherapies^[5]. Prior investigations have extensively focused on the contributions of tumor cell-intrinsic genomic heterogeneity and tumor mutational burden (TMB) to differential treatment responses^[6]; however, altering tumor-intrinsic mutations or restoring neoantigen presentation in clinical settings presents formidable challenges. Consequently, deciphering the mechanisms underlying the immune tolerance bottleneck in MSS disease solely through the lens of cell-intrinsic genomic alterations remains exceptionally difficult, underscoring an urgent imperative to identify novel avenues capable of rewiring microenvironmental responsiveness.

A burgeoning body of evidence indicates that the tumor stromal microenvironment (TSME)—composed of non-malignant cell populations, and particularly the tumor matrix—functions as a pivotal barrier dictating immune cell infiltration, tumor progression, and therapeutic resistance^[7, 8]. The tumor stroma encompasses a highly complex and heterogeneous

110 assemblage of extracellular matrix (ECM) components, cancer-associated
111 fibroblasts (CAFs), vascular endothelial cells, and nerve endings^[9]. Although
112 the CMS4 subtype within the Consensus Molecular Subtypes (CMS)
113 classification is predominantly observed in microsatellite-stable (MSS)
114 colorectal cancer and is characterized by marked enrichment of
115 cancer-associated fibroblasts (CAFs), which has been strongly associated
116 with poor clinical outcomes and enhanced angiogenesis, substantial
117 knowledge gaps remain regarding the dynamic evolution of the tumor stroma,
118 stromal phenotypic diversification^[10], and the complex intercellular
119 communication networks within the stromal microenvironment across
120 distinct microsatellite states (MSS versus microsatellite instability-high^[11]
121 (MSS versus MSI-H). In particular, due to a lack of comparative analyses
122 contrasting stromal components in MSS and MSI-H tissues, the precise
123 spatial topology through which core stromal lineages such as CAFs
124 physically sequester immune cells or orchestrate an immunosuppressive
125 niche remains to be systematically elucidated^[12].

126 The explosive development of single-cell RNA sequencing (scRNA-seq) and
127 *in situ* Spatial Transcriptomics (ST) has provided revolutionary tools for
128 decoding the stromal barrier at unprecedented resolution. By overcoming the
129 fundamental limitation of traditional bulk RNA sequencing (Bulk
130 RNA-seq)—which obliterates spatial context—spatial omics platforms
131 enable the *in situ* mapping of gene expression profiles while preserving
132 tissue-level spatial topology, thereby uncovering localized cellular
133 neighborhoods and signaling pathway networks^[13, 14]. For instance, recent
134 spatial studies in breast^[15, 16] and pancreatic^[17-19] cancers have highlighted
135 the decisive role of the tumor-immune interface in shaping
136 immunotherapeutic responsiveness, as well as the compartmentalized
137 functions of specialized CAF subsets^[20, 21]. Nevertheless, in colorectal cancer,
138 a systematic, high-resolution comparative map detailing the spatial

architecture, microdomain distribution, and localized cell-cell communication networks through which stromal cells modulate immune and endothelial states in MSS versus MSI-H tumors remains elusive.

In recent years, the convergence of deep learning algorithms in artificial intelligence (AI) with computational pathology has opened a transformative dimension for extracting covert microenvironmental phenotypes embedded within pathological whole-slide images (WSIs)^[22]. Utilizing image segmentation, Multiple Instance Learning (MIL)^[23], and high-dimensional feature extraction, computational frameworks can automatically decipher sub-visual tissue architectures, stromal remodeling patterns, and histopathological features at the invasive front that evade conventional visual inspection^[24, 25]. A recent study published in further demonstrated that mining Tertiary Lymphoid Structure^[26] and morpho-spatial phenotypes from WSIs effectively stratifies patient prognosis and immune status^[27]. Integrating AI-driven high-throughput image feature extraction with single-cell and spatial multi-omics datasets has thus emerged as an indispensable paradigm to systematically dismantle the stromal barrier separating MSS and MSI-H tumors and discover clinically actionable, interpretive biomarkers.

To address these critical knowledge gaps, this study integrates multi-center pathological WSI cohorts, scRNA-seq, and spatial transcriptomics datasets, complemented by *in vitro* functional validation, to systematically deconstruct the core cellular subsets and molecular mechanisms governing stromal divergent behaviors in MSS and MSI-H colorectal cancers. Here, we establish for the first time that the cellular phenotypic heterogeneity and spatial topological remodeling of the stroma serve as core determinants defining the distinct biological behaviors of MSS and MSI-H COAD. Through comprehensive multi-omics profiling, we identify and define two

master fibroblast meta-programs—ADAM28⁺Fibro and COL1A2⁺ Fibro—that drive stromal dimorphism, mapping their *in situ* spatial neighborhoods and intercellular communication networks while uncovering their upstream regulatory roles in ECM remodeling, immunosuppression, and pathological angiogenesis. Crucially, leveraging Transformer-based gene language models (scTenifoldKnock) alongside computational pharmacology predictions, we identify the transcription factor HIF1 α as the master molecular watershed dictating lineage commitment between these two fibroblast states, leading to the computational identification and validation of a promising small-molecule candidate for targeted stromal intervention. Collectively, our work provides a novel spatial topological framework and molecular paradigm for understanding the stromal microenvironmental barriers underlying microsatellite disparities, offering compelling scientific rationale to breach the immune tolerance barrier in MSS COAD and advance stroma-targeted precision oncology.

Results

1. AI-driven profiling deciphered distinct stromal microenvironment architectures according to microsatellite status in COAD

As an integral component of the tumor microenvironment (TME), the tumor stroma plays a critical role in modulating the immune landscape^[28-34], vascular niches, cytokine gradients, and intercellular signaling cascades^[35]. Although microsatellite status serves as a decisive clinical biomarker for prognostic stratification and therapeutic decision-making in colorectal cancer (COAD), the distinct stromal microenvironmental features separating microsatellite stable (MSS) and microsatellite instability-high (MSI-H) tumors—and their subsequent impact on treatment efficacy—have not been systematically compared.

To delineate the key characteristics of the stromal microenvironment across different microsatellite states, we first analyzed whole-slide images (WSIs) from The Cancer Genome Atlas (TCGA) cohort according to the overall workflow (Fig. 1A). Leveraging a deep learning model inspired by DeepPATHO^[36], model performance was thoroughly evaluated (Fig. 1B). Five distinct histopathological patterns were identified and quantified according to TCGA microsatellite status (Fig. 1C–D). Notably, fibrotic regions occupied a significantly higher proportion in MSS tumors, potentially underlying ECM stiffening and the "immunologically cold" phenotype characteristic of MSS disease, which is corresponding to previous report^[7]. Conversely, inflammatory microenvironments were predominantly enriched in MSI-H tumors. Furthermore, we uncovered for the first time that MSI-H tumors exhibit a significantly higher density of tumor vascular regions—a finding aligned with recent clinical observations. However, the precise mechanistic pathways driving differential angiogenesis between MSI-H and MSS tumors warrant further investigation.

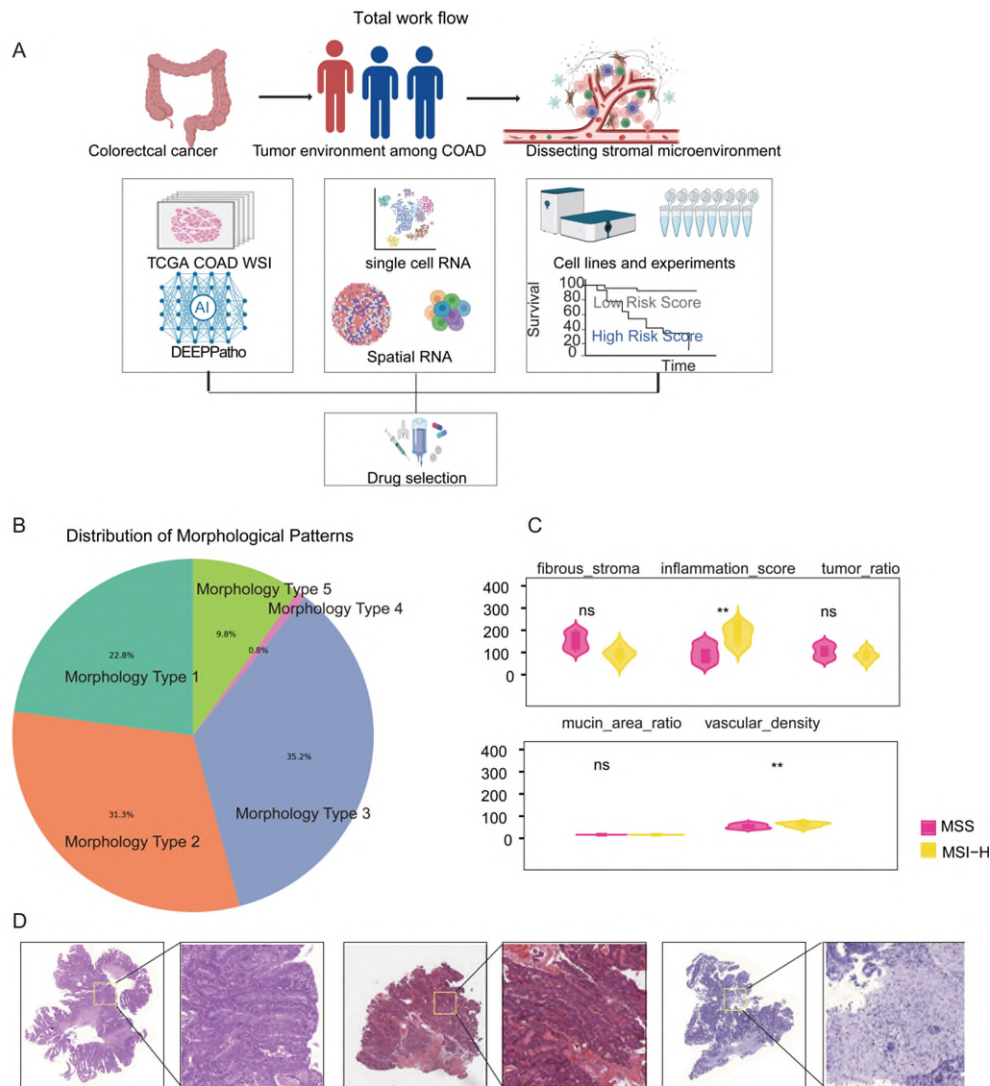


Figure 1 Modified AI model to discover COAD morpho-spatial structures

A. The total workflow for whole study B. 5 morpho-spatial structures were discovered via the modified AI model; C. Difference between 5 morpho-spatial structures among pMMR/MSS and dMMR/MSI-H Samples from TCGA; D. Typical features in WSI slices

2. Single-cell transcriptomic atlas resolved stromal microenvironment heterogeneity in COAD across microsatellite states

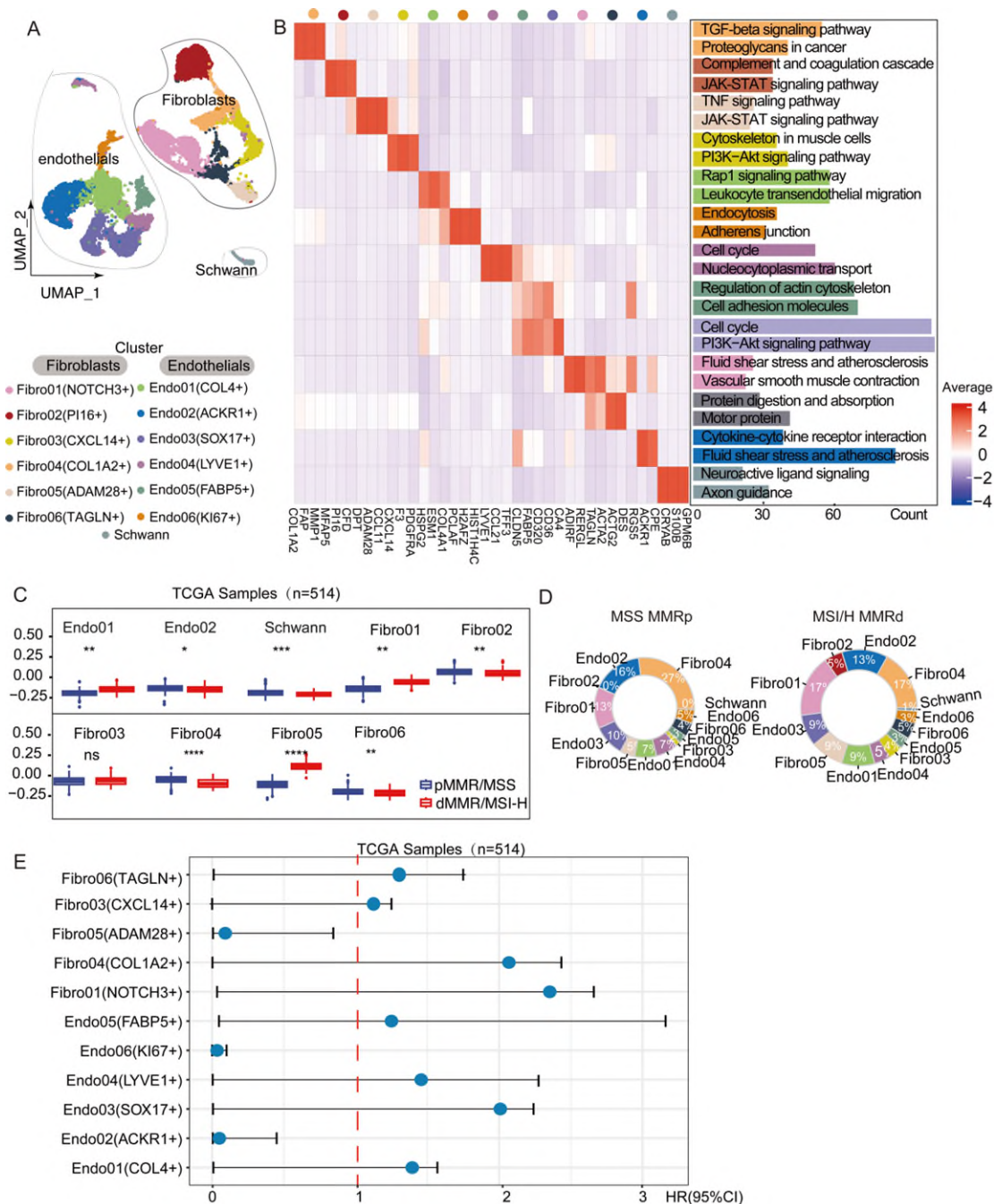
As AI-based morphological profiling of WSIs failed to work on cell-type-specific genomic resolution, we sought to systematically dissect the molecular and cellular heterogeneity of the COAD stromal landscape. We integrated single-cell RNA sequencing (scRNA-seq) datasets from eight

independent cohorts, comprising a total of 858,399 cells derived from 152 CRC patients (see Methods) from 8 published datasets. Batch effects across cohorts were integrated and corrected using the Harmony algorithm prior to downstream analysis. Based on established lineage marker genes, the total single-cell dataset was categorized into seven major lineages (Supplementary Fig. 1A) Epithelial cells (EPCAM and KRT8), Endothelial cells (VWF), Fibroblasts (COL1A1, ACTA2, and SOX9), Myeloid cells (S100A9 and LYZ), T cells (CD3D, CD3E, and CD8A), B cells (CD79A and MS4A1), Plasma cells (CD79A and MZB1), Schwann cells (GFAP, S100B) (Supplementary Fig. 1A–B, Supplementary table 1). To achieve finer granular resolution, stromal lineages (encompassing endothelial cells, fibroblasts, and Schwann cells) were selected for re-clustering and after dimensionally reduced, yielding six cancer-associated fibroblast (CAF) subclusters, six endothelial subclusters, and one Schwann cell cluster. Utilizing t-SNE visualization alongside tissue-of-origin annotations, we identified cluster-specific marker genes and performed gene-set KEGG enrichment analyses, revealing pronounced functional heterogeneity among stromal subclusters (Fig. 2A–B). Single-sample Gene Set Enrichment Analysis method (ssGSEA) was applied to project these stromal signatures onto the TCGA bulk RNA-seq cohort to evaluate their prognostic relevance. Though stromal cells were regarded as pro-tumor functions, we still found that several clusters, like ADAM28+ fibroblasts, ACKR1⁺ endothelial cells act anti-tumor function (Supplementary Fig. 2C).

To contrast the abundance and distribution of these functionally distinct stromal subsets between MSS and MSI-H tumors, we performed transcriptomic deconvolution on TCGA bulk data and validated the results in our integrated 8-cohort single-cell dataset. The analysis revealed that: Fibro04, which were characterized by prominent COL1A2 expression) was markedly enriched in MSS tumors. Fibro2 (PI16), generally regarded as

the start point of fibroblasts differentiation。Fibro5 (characterized by elevated ADAM28 expression—a marker recently implicated in sustaining T-cell interferon signaling and promoting immunotherapeutic responsiveness) was significantly enriched in MSI-H tumors as well.(Fig. 2C–D).

To further investigated the stromal clusters, multivariate Cox proportional hazards regression was indicated ,which revealed ADAM28+ fibroblasts, KI67⁺ endothelial cells, and ACKR1⁺ endothelial cells represent favorable prognostic factors for overall survival, whereas COL1A2⁺ fibroblasts strongly correlate with adverse patient outcomes (Fig. 2E). Taken together, these findings demonstrate that fibroblasts and endothelial cells exhibit profound numerical disparities and functional divergence between MSS and MSI-H tumors, pointing to distinct stromal-driven mechanisms in colorectal cancer biology.



264

265 Figure 2. Single-cell transcriptomic profiling resolves stromal heterogeneity and
 266 prognostic value across microsatellite states in COAD. (A) Single-cell dimensionality
 267 reduction and clustering identifying distinct cancer-associated fibroblast (CAF) and
 268 endothelial cell subclusters. (B) Lineage-specific marker gene expression profiles and
 269 representative KEGG pathway enrichment across stromal subclusters. (C)
 270 Transcriptomic deconvolution of the TCGA-COAD bulk cohort via ssGSEA
 271 demonstrating proportional disparities of Fibro05 (ADAM28⁺ Fibro) and Fibro04
 272 (COL1A2⁺ Fibro) between MSS and MSI-H tumors. (D) Proportional comparison of
 273 Fibro05 (ADAM28⁺ Fibro) and Fibro04 (COL1A2⁺ Fibro) subclusters in MSS versus
 274 MSI-H tumors within the integrated single-cell dataset. (E) Multivariate Cox

proportional hazards regression analysis illustrating the prognostic significance of individual stromal subclusters on overall survival.

3.ADAM28⁺ CAFs and COL1A2⁺ CAFs orchestrated divergent stromal microenvironments according to microsatellite status in COAD

The tumor stroma is shaped not only by malignant epithelial cells^[37], but also through frequent and intricate cross-talk among CAFs^[38], vascular structures^[39, 40], and peripheral nerves^[41, 42], which plays a pivotal role in extracellular matrix (ECM) remodeling and tumor angiogenesis. To decipher the cellular regulatory architecture and signaling networks within the colorectal cancer stroma, we performed cell-cell communication analyses and reconstructed intercellular networks utilizing Mathematica-based algorithms (see Methods). Network reconstruction confirmed that cancer-associated fibroblasts serve as master upstream regulators of the stromal microenvironment^[43, 44], directly modulating endothelial and immune cell states (Fig. 3A–B, Supplementary Fig. 2A–B). To further dissect how CAF heterogeneity manifests across distinct microsatellite states, we applied the Scissor algorithm^[45]—a transcriptomic-phenotype association pipeline for single-cell data—which revealed that ADAM28⁺Fibro and COL1A2⁺Fibro represent the primary Scissor-negative (associated with microsatellite instability) and Scissor-positive (associated with microsatellite stability) lineages, respectively (Fig. 3C). Functional GSEA enrichment analysis demonstrated that COL1A2⁺Fibros were strongly linked to collagen fibril assembly, matrix metalloproteinases (MMPs)-driven ECM degradation^[46], and matrix reorganization. Conversely, ADAM28⁺Fibro were associated with localized collagen catabolism and leukocyte migration, highlighting their opposing functional roles in ECM remodeling and local niche conditioning (Fig. 3D).

The stromal regulatory capacity of fibroblasts is intrinsically dependent on spatial proximity and localized intercellular cross-talk. AUCell-based

signature scoring showed that COL1A2⁺ Fibro localized to FAP⁺ACTA2⁺ stromal regions, whereas ADAM28⁺ Fibro resided near tumor margins and co-expressed key immune markers (Fig. 3E, Supplementary Fig. 2A–B). To map spatial niches—defined as discrete tissue compartments sharing similar cellular composition^[47]—we transformed estimated cell-type proportions from spatial transcriptomics spots into isometric log-ratio (ILR) coordinates and clustered the spots into distinct topological domains^[48] (Fig. 3F). Given that fibroblast functionality relies heavily on localized ecotypes governed by cell-cell units, we performed ISCHIA-based cellular neighborhood identification anchored on our integrated single-cell annotations (Fig. 3G–H, Supplementary Fig. 2C–D). To evaluate the clinical impact of ADAM28⁺ CAFs and COL1A2⁺ Fibro across diverse therapeutic modalities (including immunotherapy, anti-angiogenic therapy, and chemotherapy), we performed pseudo-bulk forest plot analyses and validated findings in pan-cancer treatment cohorts. ADAM28⁺ Fibro were significantly enriched in partial sensitive sample groups, whereas COL1A2⁺ Fibroblasts were enriched in those resistant individuals, confirming their opposite predictive value for therapeutic efficacy (Supplementary Fig. 2E). Mechanistically, COL1A2⁺ Fibro and ADAM28⁺ Fibro occupy upstream positions in stromal regulation, exerting distinct signaling cross-talk onto immune and endothelial compartments. CellChat communication modeling demonstrated that the impact of COL1A2⁺ Fibro and ADAM28⁺ Fibro was most pronounced on endothelial and immune subsets, whereas their differential influence on peripheral nerve innervation was modest—a observation potentially attributable to the low abundance of Schwann cells that warrants further investigation (Fig. 3E–G, Supplementary Fig. 3A–B).

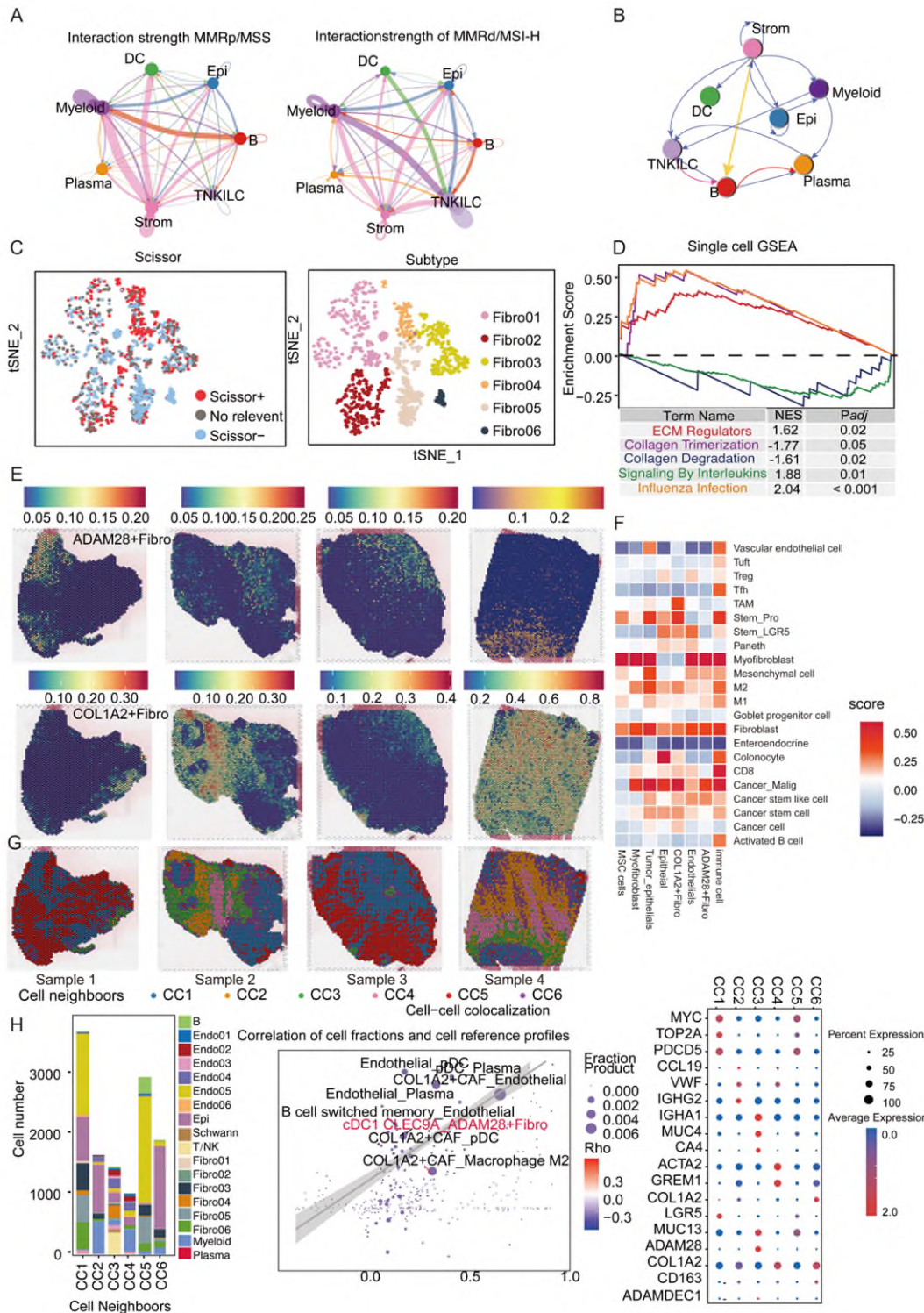


Figure 3. ADAM28⁺ CAFs and COL1A2⁺ CAFs orchestrate distinct stromal microenvironments according to microsatellite status in COAD. (A) CellChat analysis showing cell-cell communication networks across MSS and MSI-H single-cell transcriptomic cohorts. (B) Mathematical network reconstruction demonstrating that stromal cells occupy upstream regulatory nodes within intercellular signalling hierarchies. (C) Scissor algorithm pipeline and subtype UMAP projections establishing

the precise correspondence between Fibro4 or Fibro5 subsets and MSS or MSI-H clinical phenotypes. (D) GSEA functional enrichment illustrating ADAM28+ Fibro involvement in leukocyte migration and inflammatory responses, alongside COL1A2+ Fibro enrichment in matrix remodeling and collagen degradation pathways. (E–F) Spatial transcriptomic AUCell signature mapping defining the topological localization and microdomain co-localization of ADAM28+ Fibro and COL1A2+ Fibro. (G) ISCHIA spatial ecotype analysis identifying six discrete cellular niches along with their lineage-specific marker gene profiles. (H) Spatial co-localization and correlation analyses highlighting the strong positive association between cDC1 cells and ADAM28+ Fibro.

4. ADAM28+ CAFs and COL1A2+ CAFs differentially regulated the tumor vascular microenvironment

Differential cell-cell communication analysis revealed that ADAM28⁺ and COL1A2⁺ fibroblasts differentially regulated endothelial and immune compartments, thereby orchestrating distinct microenvironmental remodeling in colorectal cancer (CRC) tissues according to microsatellite status—a biological phenomenon that has not been previously reported. To elucidate the mechanisms through which ADAM28⁺ and COL1A2⁺ fibroblasts dictate endothelial vascular states, we extracted endothelial cells and performed subclustering analysis. Dimension reduction via t-SNE and UMAP stratified endothelial cells into six distinct subclusters defined by unique marker gene expression profiles (Fig. 4A–B). Proportional comparison stratified by microsatellite status revealed that ACKR1⁺ endothelial cells were markedly enriched in MSI-H tumors, whereas FABP5⁺ endothelial cells predominated in MSS tissues (Fig. 4C–D). To comprehensively capture how CAFs shape endothelial cells to regulate angiogenesis, we utilized the LIANA^[49] pipeline—an integrated benchmark framework incorporating multiple ligand-receptor databases, including secreted signaling networks and metabolic interaction pathways. LIANA modeling demonstrated that CAFs predominantly govern endothelial functionality via paracrine cytokine signaling: COL1A2⁺ fibroblasts engaged FABP5⁺ endothelial cells via COL1A2-CD36 signaling axes^[50, 51] (Fig. 4E–F), supporting the

pro-angiogenic potential of COL1A2⁺ fibroblasts. Conversely, ACKR1⁺ endothelial cells preferentially interacted with ADAM28⁺ CAFs. Prior studies have highlighted the favorable prognostic relevance of ACKR1⁺ endothelial cells in colorectal cancer, with recent evidence identifying ACKR1⁺ endothelial cells as specialized vascular gates that permit T-lymphocyte transendothelial migration and support immunologically active niches^[52, 53]. ADAM28⁺ CAFs engaged ACKR1⁺ endothelial cells to sustain T-cell abundance and function through immune recruitment and inflammatory niche preservation; transcriptomic signaling analysis and spatial co-localization validated these interactions (Fig. 4G). To functionally test this cross-talk *in vitro*, we collected condition medium from ADAM28-overexpressing or control CAFs to stimulate mice endothelial cell lines C166 and performed quantitative real-time PCR (rt-qPCR). Exposure to ADAM28⁺ CAF-conditioned medium significantly upregulated pro-chemotactic and tumor-killing cytokines, including CXCL8 and CCL12, without altering intrinsic ACKR1 expression levels, indicating that ADAM28⁺ Fibroblasts do not drive ACKR1 lineage commitment but rather enhance the immunostimulatory secretory profile of ACKR1⁺ endothelial cells. Together, these results underscore the essential role of CAF-endothelial cross-talk in sculpting vascular niches and shaping tumor-immune dynamics.

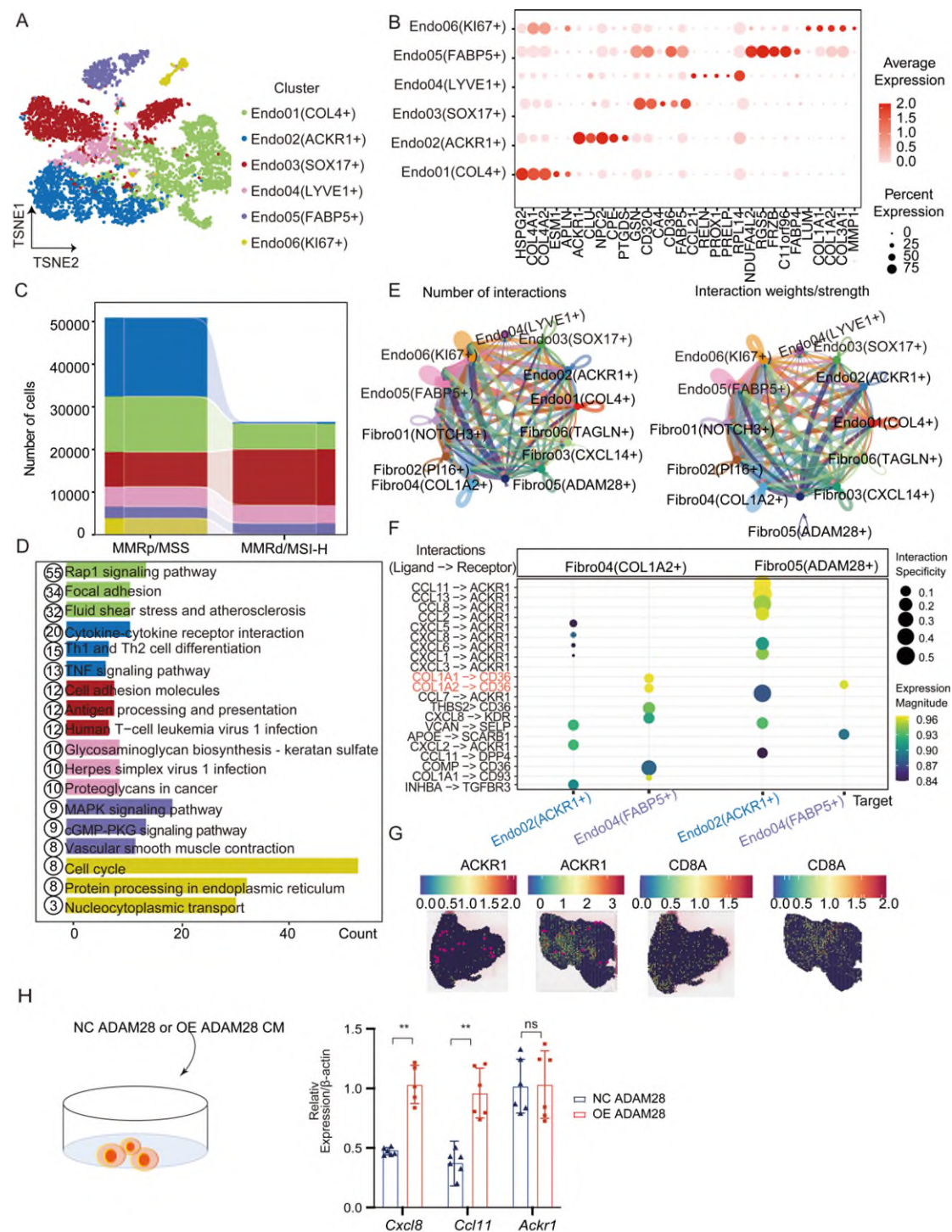


Figure 4. ADAM28⁺ Fibro and COL1A2⁺ Fibro shape distinct tumor endothelial cell states. (A) Dimensionality reduction and clustering of endothelial cells resolving six discrete subclusters. (B) Expression profiles of key marker genes across endothelial cell subclusters. (C) Proportional distributions of endothelial subclusters in MSS versus MSI-H COAD tissues. (D) Functional heterogeneity and pathway enrichment across endothelial

subclusters. (E) Cellular communication networks mediating cross-talk between ADAM28⁺ Fibro or COL1A2⁺ Fibro and endothelial subsets. (F) Inter cellular cross-talk showing preferential interactions of ADAM28⁺ Fibro and COL1A2⁺ Fibro with ACKR1⁺ and FABP5⁺ endothelial subclusters, respectively. (G) Spatial transcriptomic and histological co-localization demonstrating CD8⁺ T cell enrichment surrounding ACKR1⁺ endothelial cells. (H) Quantitative real-time PCR evaluating T-cell recruitment cytokine secretion by endothelial cells following stimulation with conditioned medium from ADAM28-overexpressing NIH3T3 fibroblasts.

5. ADAM28⁺ CAFs and COL1A2⁺ CAFs exerted opposing effects on cDC1 antigen-presenting phenotypes and immune activation

A recent study demonstrated the beneficial role of ADAM28⁺ CAFs in modulating T cell responses to foster immunotherapeutic efficacy^[54]. We further investigated transcriptomic differences in immune cell infiltration between microsatellite states in colorectal cancer. Activated dendritic cells, alongside CD4⁺ and CD8⁺ memory T cells as well as activated DC cells^[55], predominated in MSI-H tumors, reflecting heightened antigen presentation and cytotoxic T cell activity. Conversely, MSS tumors exhibited a marked enrichment of M2 macrophages and exhausted CD8⁺ T cells, aligned with an immunologically cold microenvironment (Fig. 5A). Dendritic cells are classically categorized into three main subsets: conventional DC1s (cDC1s: CLEC9A, XCR1, and CADM1), conventional DC2s (cDC2s: CD1C, FCER1A, and CLEC10A), plasmacytoid DCs (pDCs: LILRA4, GZMB, and MZB1), and mature DCs (LAMP3⁺ DCs: LAMP3, CCR7, and FSCN1)^[56]. Single-cell expression scoring revealed strong correlations between ADAM28⁺ Fibro or COL1A2⁺ Fibro signatures and specific DC subset profiles (Fig. 5B–C). Spatial transcriptomic analysis confirmed localized co-localization of ADAM28⁺ Fibro and COL1A2⁺ Fibro with activated cDC1

signatures (Fig. 5D–E). Because cDC1s represent a specialized anti-tumor DC phenotype with potent antigen-presenting capabilities, these spatial patterns indicate that ADAM28⁺ Fibro promote activated DC states, whereas COL1A2⁺ Fibro exert inhibitory effects. To functionally test this hypothesis *in vitro*, we stimulated the dendritic cell line DC2.4 with conditioned medium derived from ADAM28⁺ Fibro or COL1A2⁺ Fibro and evaluated phenotypic marker expression via flow cytometry. Exposure to COL1A2⁺ Fibro-conditioned medium significantly reduced the expression of the cDC1 marker CLEC9A while upregulating the pro-tumorigenic DC marker CD14^[57]; conversely, ADAM28⁺ Fibro-conditioned medium preserved and enhanced cDC1 marker expression while suppressing CD14 (Fig. 5F). Together, these findings demonstrate that ADAM28⁺ Fibro and COL1A2⁺ Fibro exert opposing regulatory functions on DC antigen-presentation capacity, thereby indirectly tuning downstream T cell cytotoxicity—a novel stromal-dendritic cell crosstalk mechanism not previously described.

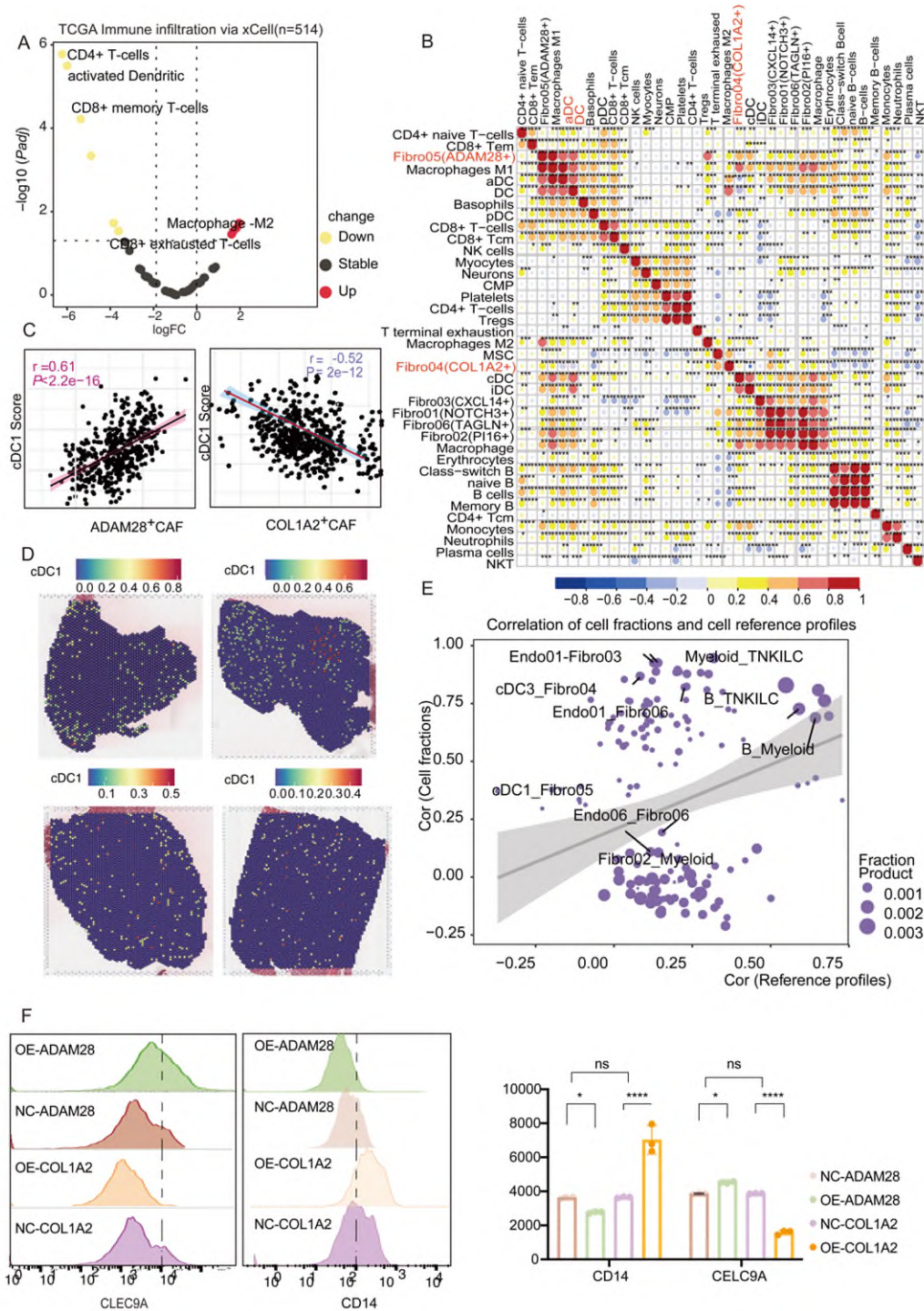


Figure 5. ADAM28⁺ CAFs and COL1A2⁺ CAFs exert opposing regulatory effects on anti-tumor dendritic cell functions. (A) Differential immune infiltration patterns between MSS and MSI-H COAD based on TCGA transcriptomic profiles, highlighting the predominant enrichment of activated dendritic cells, memory CD4⁺ T cells, and memory CD8⁺ T cells in MSI-H tumors, contrasted with M2 macrophages and exhausted CD8⁺ T cells in MSS tumors. (B) Correlation analysis of immune cell abundance across TCGA transcriptomic cohorts integrated with single-cell gene

signatures and immune infiltration profiles. (C) Correlation between signature scores of ADAM28⁺ Fibro or COL1A2⁺ Fibro and activated dendritic cell phenotypic scores. (D–E) Spatial localization of cDC1 cells alongside co-localization significance analysis of cDC1s with ADAM28⁺ Fibro and COL1A2⁺ Fibro across spatial transcriptomics tissue sections. (F) Flow cytometry analysis evaluating phenotypic differentiation changes in dendritic cells following stimulation with conditioned medium from control, (ADAM28-overexpressing, or COL1A2-overexpressing fibroblasts).

6. HIF1 α dictates lineage commitment between ADAM28⁺Fibro and COL1A2⁺ Fibro

We next sought to determine whether ADAM28⁺ fibroblasts directly interact with COL1A2⁺ Fibro to regulate the overall stromal microenvironment. We curated functional pathway gene sets related to fibroblast properties and evaluated their activity via ssGSEA transcriptomic scoring and correlation analyses. ADAM28⁺ Fibro displayed the strongest negative correlation with fibroblast migration signatures, suggesting an inhibitory effect on CAF trafficking toward tumor nests (Fig. 6A). To test this spatial interplay *in vitro*, we constructed overexpression plasmids for ADAM28 and COL1A2, transfected NIH3T3 cells, and established Transwell co-culture assays. Functional correlation analyses indicated a spatial antagonism between these two lineages, with COL1A2⁺ Fibro activation and migratory capacity being suppressed by ADAM28⁺ fibroblasts. Stable NIH3T3 fibroblast cell lines overexpressing murine ADAM28 or COL1A2 were established (see Methods), and conditioned media were collected to stimulate naive fibroblasts for 48 hours. Transwell assays confirmed that exposure to ADAM28⁺ fibroblast-conditioned medium significantly attenuated naive NIH3T3 cell migration (Fig. 6B).

To elucidate the upstream drivers governing ADAM28⁺ and COL1A2⁺ fibroblast lineage commitment, we performed single-cell pseudotime trajectory inference using PI16-high fibroblasts as the developmental root node, based on prior studies establishing PI16⁺ mesenchymal progenitor cells

as a major origin of CAFs^[58, 59]. Monocle2 trajectory analysis resolved three main lineage branches, with ADAM28⁺ Fibro and COL1A2⁺ Fibro committing to two divergent differentiation trajectories (Fig. 6C). Branched Expression Analysis Modeling (BEAM) identified key driver genes at the major fate-decision bifurcation node. Functional enrichment analysis of these fate-determining genes revealed that hypoxia-responsive regulatory networks constitute the predominant molecular program driving lineage divergence. Concurrent enrichment of mTOR and MAPK signaling pathways, together with other energy stress-related pathways, further suggested that metabolic reprogramming and cellular stress responses are critically involved in lineage commitment during CAF differentiation (Fig. 6D–E). In silico cell perturbation algorithms (scTenifoldKnock^[60] prophecies) predicted that virtual knockout of HIF1 α induced upregulation of ADAM28⁺ Fibro marker genes. Subsequent Western blot and quantitative real-time PCR (rt-qPCR) assays confirmed that HIF1 α knockdown or inhibition restored ADAM28 expression levels (Fig. 6F–G).

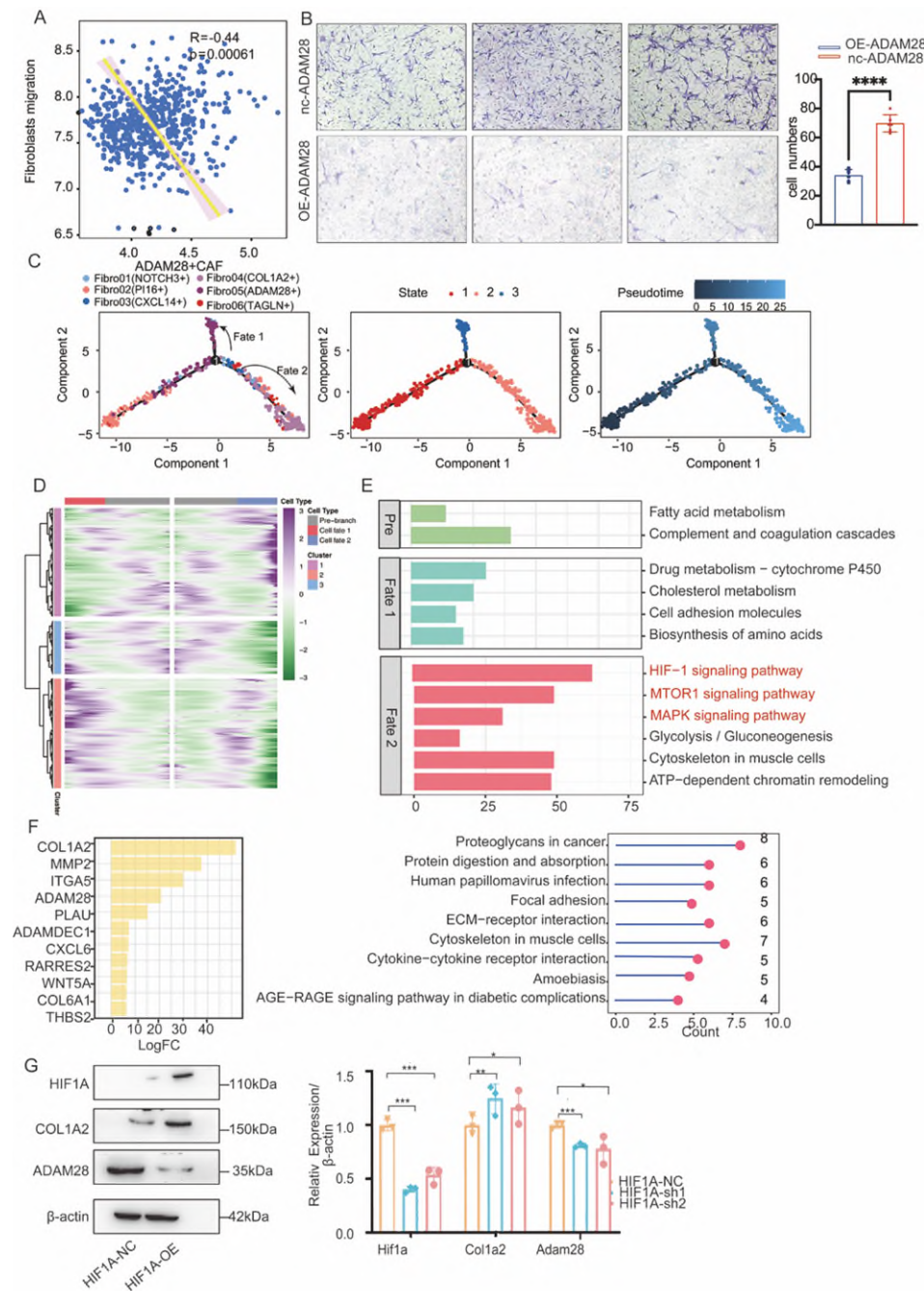


Figure 6. HIF1 α dictates lineage commitment between ADAM28⁺ CAFs and COL1A2⁺ CAFs. (A) Correlation analysis of cell-type scores demonstrating a significant negative association between the ADAM28⁺ Fibro signature and fibroblast migratory capacity. (B) Transwell cell migration assay evaluating the effect of ADAM28⁺ Fibro-conditioned medium on the transmembrane migratory capacity of fibroblasts. (C–E) Pseudotime trajectory analysis illustrating the divergent differentiation paths of ADAM28⁺ Fibro and COL1A2⁺ Fibro, identifying HIF1 α as the master driver gene at the key lineage fate-decision node. (F) In silico virtual knockout of HIF1 α predicting downstream transcriptomic alterations and functional pathway enrichment. (G) Quantitative

real-time PCR (rt-qPCR) and Western blot (WB) assays assessing expression changes of COL1A2 and ADAM28 in HIF1 α -knockout

7. Computational drug screening identifies candidate compounds AZD2014 to target the ADAM28⁺ CAF and COL1A2⁺ CAF lineage switch

To translate our biological findings into therapeutic applications, we utilized single-cell expression profiles and lineage annotations to perform computational drug screening, complemented by *in vitro* functional validation. Applying the scTherapy and Drug2Cell algorithms^[61, 62] to single-cell transcriptomic profiles, we systematically predicted potential candidate compounds capable of targeting COL1A2⁺ Fibro lineage-specific transcriptional programs. (Figure 7A-B). The drug AZD2014^[63, 64], a mTOR inhibitor, was both identified via two separate programs, which indicated its potential use in fibroblasts evolution. It has been demonstrated its preliminary *in vivo* therapeutic efficacy in colorectal cancer, we revealed for the first time, that ameliorating the stromal microenvironment restores the functional capacity of antigen-presenting cells^[64]. Real-time quantitative PCR (RT-qPCR) and Western blot assays both demonstrated that treatment with AZD2014 across a concentration gradient in NIH3T3 cell line induced a downward trend in key MTOR-HIF1 α pathway molecules (HIF1 α , MTOR1), as well as the two ECM components ADAM28 and COL1A2, with a particularly pronounced reduction at 200 μ g/mL AZD2014 (Figure 7C-D). In summary, the findings above revealed the differentiation trajectories of fibroblasts under distinct microsatellite stability statuses and their impact on the overall microenvironment, including vascular immunity and extracellular matrix states, providing a valuable reference for remodeling the cold tumor microenvironment of MSS colorectal cancer.(Figure 7 E).

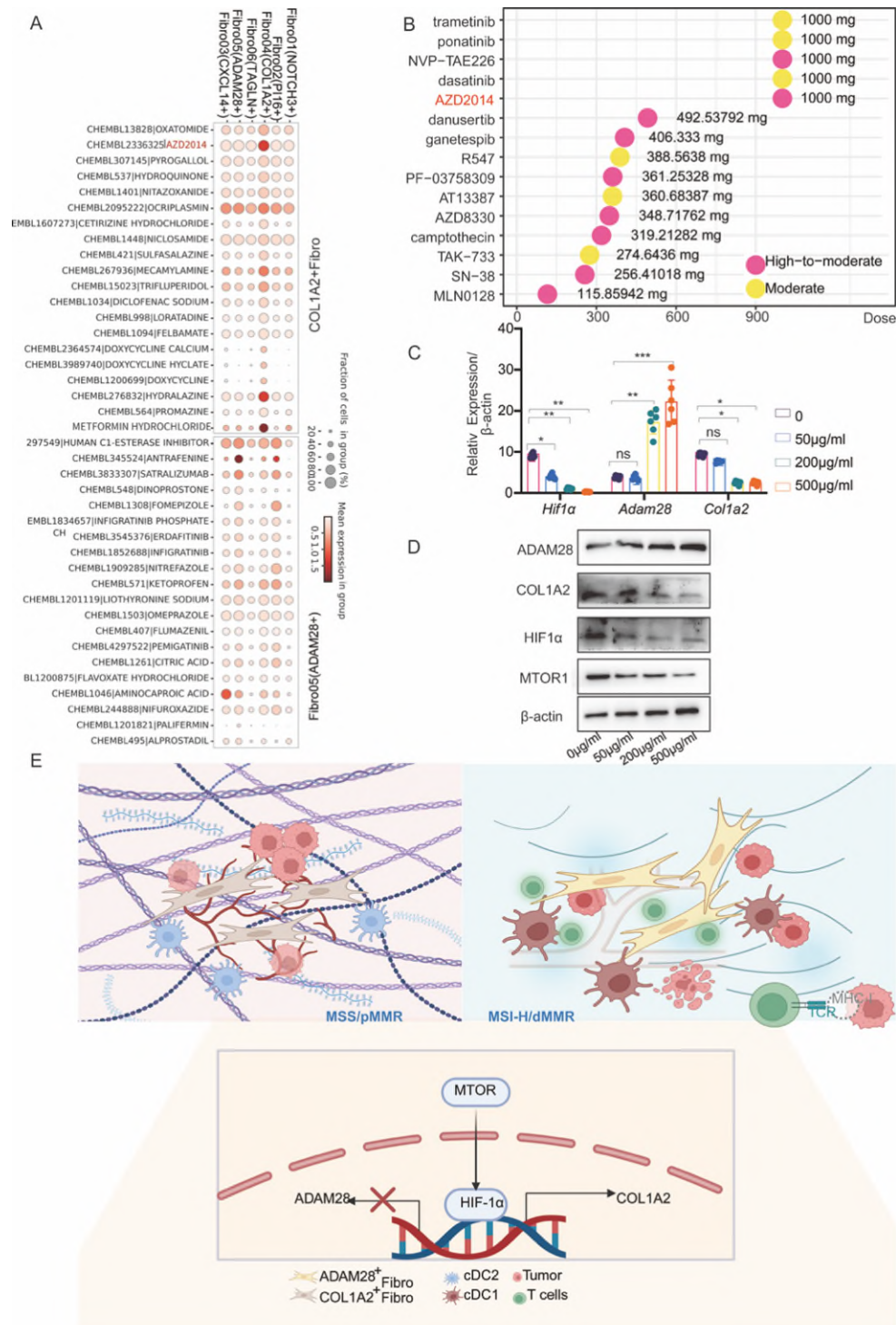


Figure 7. Drug screening identifies candidate compounds capable of modulating CAF differentiation and remodeling the stromal microenvironment.

A. Identification of candidate compounds predicted to regulate CAF differentiation using Drug2Cell based on computational drug screening. B. Identification of candidate compounds predicted to regulate CAF differentiation using scTherapy, providing an independent validation of the drug screening strategy. C. Quantitative real-time PCR (qRT-PCR) analysis of differentiation-associated gene expression

following treatment with increasing concentrations of the selected candidate compound AZD2014. D. Western blot analysis of protein expression of differentiation-associated markers in response to gradient concentrations of the candidate compound AZD2014. E. Schematic illustration summarizing the stromal microenvironmental remodeling and the molecular mechanism underlying CAF differentiation. The selected compound inhibits the transition toward the pathogenic CAF phenotype while promoting the differentiation of the immune-supportive CAF subtype, thereby reshaping the stromal ecosystem and enhancing antitumor immunity.

Discussion

The distinct immunotherapy response between microsatellite stable (MSS) and microsatellite instability-high (MSI-H) colon adenocarcinoma (COAD) has long been attributed primarily to tumor cell-intrinsic genomic mutational burden and neoantigen presentation defects.^[65] However, the systemic role of the tumor microenvironment factors especially stroma status in orchestrating these microsatellite-associated components has remained poorly understood. By integrating single-cell transcriptomics, spatial omics, and computational pathology, this study demonstrates for the first time that the cellular phenotypic heterogeneity and topological organization of the stroma serve as primary biological determinants distinguishing MSS from MSI-H tumors. We identified two dominant cancer-associated fibroblast (CAF) subclusters with distinct microsatellite distribution preferences: ADAM28⁺ Fibro were markedly enriched in the MSI-H stroma, whereas COL1A2⁺ Fibro predominated as the principal lineage in MSS disease. This discovery reframes the traditional view of CRC stromal fibroblasts as a homogeneous entity, providing a spatial and cellular framework to dismantle the immune tolerance barrier in MSS tumors.

To Decipher the lineage trajectories of these two CAF subsets for understanding stromal evolutionary dynamics, single-cell pseudotime modeling and Transformer-based gene language were used that we identified the transcription factor HIF1 α as the master fate-decision node dictating

lineage commitment toward either ADAM28⁺ Fibro or COL1A2⁺ Fibro. Within the MSS microenvironment, sustained and severe tissue hypoxia drives aberrant HIF1 α activation, inducing fibroblast trans differentiation toward a COL1A2⁺ Fibro phenotype characterized by excessive extracellular matrix (ECM) deposition and structural stiffening. Conversely, under the distinct metabolic and inflammatory conditions characteristic of MSI-H tumors, the ADAM28⁺ Fibro lineage trajectory remains favored. Pinpointing HIF1 α as the master molecular watershed provides mechanistic clarity regarding upstream drivers of stromal heterogeneity, demonstrating the feasibility of rewiring the microenvironment by targeting a single master regulator.

Within spatial tumor niches, complex intercellular cross-talk among CAFs, vascular endothelial cells, and immune populations dictates the physical and immunologic state of the microenvironment. Our findings show that ADAM28⁺ Fibro and COL1A2⁺ Fibro exert opposing functional effects on vascular niches and immune recruitment. ADAM28⁺ Fibro had created "immunologically hot" microenvironment of MSI-H tumor: This subpopulation secretes specific chemokines and cell adhesion molecules that stimulate T-lymphocyte transendothelial migration and enhance vascular immune recruitment signals with co-location with ACKR1+Endothelial cells. Thus, COL1A2⁺ Fibro act as pro-angiogenic and physical barrier effects conversely. COL1A2⁺ Fibro drive pathological angiogenesis and deposit a dense collagen matrix that functions as a physical barrier, sequestering immune cells at the tumor periphery and giving rise to the "immunologically cold" or "immune-excluded" phenotype typical of MSS disease. These contrasting roles suggest that therapeutic strategies designed to suppress COL1A2⁺ Fibro-mediated pathological vascularization while restoring ADAM28⁺ Fibro-like immune-recruiting vascular gates offer a promising approach to overcome immunotherapy resistance in MSS COAD.

Based on these mechanistic insights, selectively depleting or reprogramming COL1A2⁺ Fibro represents a promising strategy to breach the MSS stromal barrier. Therefore, we utilized deep learning frameworks and transcriptomic-based virtual screening, we identified AZD2014 as a candidate compound with potential stromal remodeling activity. Computational pharmacology predicted that AZD2014 targets the HIF1 α -driven transcriptional network, thereby reversing fibroblast commitment to the COL1A2⁺ Fibro fate and blunting the dense matrix barrier. These findings provide a concrete small-molecule candidate for targeting complex stromal phenotypes. Nevertheless, as these insights derive primarily from bioinformatic predictions and *in silico* modeling, the *in vivo* efficacy of AZD2014 in preventing COL1A2⁺ Fibro differentiation, normalizing vascular niches, and enhancing immunotherapy response requires preclinical validation in syngeneic animal models and patient-derived translational cohorts.

Certain limitations of this study warrant consideration. During our single-cell and spatial analyses, ADAM28⁺ Fibro and COL1A2⁺ Fibro displayed limited transcriptomic or spatial communication with adjacent peripheral nerves or Schwann cells. Extensive literature documents bidirectional neurotrophic and spatial guidance interactions among the ECM, Schwann cells, and tumor innervating fibers. The limited detection of these interactions in our dataset likely stems from technical sampling bias during tissue dissociation and single-cell preparation, as Schwann cells and neural components are low in abundance and highly susceptible to dissociation-induced apoptosis. Future studies incorporating high-density, spatially resolved multiplexed imaging platforms (such as CODEX or MIBI) alongside *in situ* neural tracing in intact tissue sections will be valuable to further explore these stromal-neural interactions.

630 Conclusion

631 In summary, this study deconstructs the cellular heterogeneity and spatial
632 architecture of the stromal microenvironment in MSS and MSI-H colorectal
633 adenocarcinoma. We firstly figure out that ADAM28⁺ Fibro and COL1A2⁺
634 Fibro serve as the dominant lineages driving MSI-H and MSS stromal states,
635 respectively, and identify HIF1 α as the master transcription factor governing
636 lineage commitment. These findings may provide a topological framework
637 for understanding the microenvironmental barriers across microsatellite
638 states, laying a scientific foundation for stroma-targeted combination
639 therapies designed to overcome immune tolerance in MSS cololorectal
640 cancer.

641 Methods

642 1. Bioinformatics and Computational Analysis

643 1.1. Single-cell RNA sequencing data acquisition and preprocessing

644 In this study, we collected and analyzed eight publicly available scRNA-seq
645 datasets: GSE178318(37,029 cells), GSE132465 (49,637 cells from 25 CRC
646 patients), GSE144735 (26,957 cells from 6 CRC patients), GSE166555
647 (67,254 cells from 12 CRC patients), GSE178341 (371,014 cells from 62
648 CRC patients), GSE188711 (27,435 cells from 6 CRC patients), GSE200997
649 (25,019 cells from 16 CRC patients), and GSE205506 (254,054 cells from 19
650 CRC patients) (Supplementary Table 1).

651 For each sample, raw count matrices were initially read and preprocessed
652 individually prior to merging per donor. Strict quality control metrics were
653 applied: cells with a library size of less than or equal to 2,000 UMIs or a
654 mitochondrial gene transcript ratio of greater than or equal to 5% were
655 excluded. Genes detected in fewer than 5 individual cells were defined as

undetected and removed. The optimal number of principal components (PCs) was determined by the JackStraw procedure utilizing the JackStraw and ScoreJackStraw functions in Seurat (v4.3.0). Graph-based cell clustering was performed using the FindNeighbors and FindClusters functions with the resolution parameter set to 0.6.

1.2. Batch effect correction

Batch effects across heterogeneous datasets and sequencing technology platforms were integrated and harmonized using the Harmony algorithm, preserving intrinsic cell-type lineage heterogeneity while projecting cells into a unified low-dimensional embedding space.

1.3. Spatial transcriptomics data processing

To investigate spatial organization and regional microenvironmental heterogeneity, spatial transcriptomics (ST) datasets from four tissue sections were downloaded from GEO (GSE225857). ST expression matrices were processed using the Seurat package in R. Quality control was conducted by retaining genes expressed in at least three cells and filtering out spatial spots containing fewer than 300 detected genes or fewer than 600 total UMIs. Each individual ST slide matrix was normalized and variance-stabilized via the SCTransform pipeline, with the number of top variable features fixed at 3,000.

1.4. Pseudotime trajectory inference

Single-cell pseudotime trajectory analysis was performed using Monocle 2 (v2.16.0) to reconstruct cellular lineage progression and state transitions. Highly variable genes were selected to construct the developmental trajectory, and dimensionality reduction was performed via the DDRTree algorithm. To identify dynamic transcriptional programs and switch-like

gene expression changes at developmental branch points, Branched Expression Analysis Modeling (BEAM) was executed across key bifurcation nodes.

1.5. Intercellular communication analysis across single-cell and spatial domains

Intercellular communication networks were modeled using CellChat (v1.6.0) anchored on the human CellChatDB signaling molecule interaction repository. Default parameters were applied to infer significant ligand-receptor interaction pairs. Network visualization and signaling pathway prioritization were conducted to characterize master communication axes across stromal, endothelial, and immune subclusters.

To contrast cell-cell communication dynamics across microsatellite states, distinct CellChat objects were constructed for the MSS and MSI-H groups and subsequently merged using the mergeCellChat function. Differential interaction numbers and strengths were quantified using compareInteractions, and differential signaling networks were visualized via netVisual_diffInteraction.

1.6. scTherapy-based malignant cell program and drug response prediction

Differentially expressed genes between malignant epithelial cells and normal hepatocytes were extracted and filtered via scTherapy, retaining biologically relevant expression signatures for drug sensitivity modeling. Refined transcriptomic signatures were submitted to a pre-trained scTherapy LightGBM model to predict single-agent therapeutic sensitivity, prioritizing candidate compounds with predicted high or moderate efficacy for downstream experimental testing.

1.7. LIANA-integrated ligand-receptor communication modeling

The LIANA framework was employed to integrate multiple ligand-receptor interaction resources and metabolic networks. Normalized Seurat objects containing stromal populations were processed through the `liana_wrap` function, and multi-resource rankings were combined via `liana_aggregate`. Significant ligand-receptor pairs were filtered using an aggregated rank of less than 0.05, and the top 30 interaction pairs were selected for comparative quantification.

1.8. In silico virtual gene knockout

In silico gene perturbation was conducted using the `scTenifoldKnk` computational framework. Stromal subclusters were designated as input target populations, and the top 2,000 highly variable genes were incorporated into network construction, including the candidate transcription factor HIF1 α . Virtual knockout of HIF1 α was executed with parameter settings: `qc_minLSize = 10`, `nc_nCells = 50`, `td_maxIter = 10`, and `td_maxError = 1e-02`. Genes exhibiting significant downstream network perturbations (p-value less than 0.05) were considered regulatory targets.

1.9. Spatial transcriptomic deconvolution and ecotype niche discovery

Spatial spot cell-type composition was estimated using the Robust Cell Type Decomposition (RCTD) algorithm, utilizing our annotated integrated single-cell dataset as the reference standard. Cell-type specific proportions were computed per spatial spot, yielding a proportion matrix across all tissue slides.

To identify spatial ecotypes, proportion matrices were analyzed using the ISCHIA framework in R. Spots were mapped into isometric log-ratio (ILR) transformed coordinates and clustered to extract six discrete microenvironmental neighborhoods. Cell type abundance per niche was

quantified using median cell proportions alongside top niche-specific marker gene expression profiles.

1.10. Transcriptomic COAD clinical cohorts

Bulk transcriptomic discovery cohorts were retrieved from TCGA-COAD and GEO (GSE39582). Immunotherapy and targeted therapy response cohorts were obtained from GEO datasets GSE28702, GSE62080, and GSE69657.

1.11. Cox proportional hazards regression and survival modeling

TCGA-COAD transcriptomic datasets were utilized for univariate and multivariate Cox proportional hazards regression analyses. Single-cell lineage signatures were quantified in bulk expression data using single-sample Gene Set Enrichment Analysis (ssGSEA) and CIBERSORTx deconvolution. Hazard ratios (HR) and survival metrics were computed using the coxph function in the R survival package, defining variables with a log-rank p-value of less than 0.05 as statistically significant prognostic factors. Results were visualized as forest plots using the forestplot package.

1.12. Network motif and topological hierarchy analysis

To decipher structural interaction patterns within the stroma, network motif analysis was conducted using Mathematica integrated with the IGLADFindSubisomorphisms function in the IgraphM package. Hierarchical sub-network architectures were systematically identified by searching for 2-node, 3-node, 4-node, and 5-node subgraphs across cell-cell communication topologies.

1.13. Drug2cell computational drug prioritization

Cell-type-specific drug targets were identified using the drug2cell Python package applied to single-cell data. Single-cell gene expression matrices paired with cell lineage annotations served as input to score and rank candidate small-molecule compounds targeting specific stromal populations.

2. In Vitro Experimental Validation

2.1. Cell lines and culture conditions

NIH3T3 murine fibroblasts, CT26 murine colon adenocarcinoma cells, and HCT116 human colorectal carcinoma cells were utilized in this study. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator at 37°C containing 5% CO₂. Cell line identities were authenticated via short tandem repeat (STR) profiling, and regular PCR screening confirmed the absence of mycoplasma contamination.

2.2. Plasmid construction and transient transfection

Overexpression plasmids encoding COL1A2 or ADAM28 alongside short hairpin RNA (shRNA) constructs targeting HIF1 α were synthesized by Obio Technology (Shanghai, China). Transfections were performed using LipoBooster 3000 reagent (Yeesen, China) according to the manufacturer's protocol. Overexpression and knockdown efficiencies were validated at 48 hours post-transfection by quantitative real-time PCR (rt-qPCR) and Western blot analyses (Supplementary Fig. 5). Target shRNA sequences are listed in Supplementary Table 2.

2.3. Western blot analysis

Cells were lysed in ice-cold RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails (NCM Biotech, China). Total protein concentrations were determined using a BCA Protein Assay Kit (FDbio, China). Equal amounts of protein lysates (20–30 µg) were resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBST for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies against COL1A2 (1:2,000, Proteintech Cat #14695-1-AP), ADAM28 (1:1,000, Proteintech Cat #22234-1-AP), HIF1 α (1:1,000, Proteintech Cat #20960-1-AP), and beta-ACTIN (1:1,000, Proteintech Cat #66009-1-Ig). Membranes were washed with TBST, incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature, and visualized using an enhanced chemiluminescence (ECL) detection system.

2.4. Flow cytometry analysis of human dendritic cells

Human monocyte-derived dendritic cells (MoDCs) or single-cell suspensions were washed twice with ice-cold FACS buffer (PBS containing 1% BSA and 2 mM EDTA). Cell viability was evaluated using the Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen, 1:1,000) for 15 minutes at room temperature in the dark. Fc receptors were blocked using Human TruStain FcX (BioLegend) at 4°C for 10 minutes.

Cells were subsequently stained with fluorophore-conjugated anti-human antibodies at 4°C for 30 minutes in the dark using the following antibody panel: FITC anti- CD14(Clone Bu15), APC anti- CLEC9A (Clone 2D10), PE anti-CD86 (Clone IT2.2), and PerCP/Cy5.5 anti-CD83 (Clone HB15e). Lineage exclusion was performed using an APC/Cy7-conjugated lineage cocktail (CD14, CLEC9A; BioLegend) to gate out non-DC cell lineages.

Cells were washed twice and resuspended in 200 µL FACS buffer. Flow cytometric acquisition was performed on a BD FACSCanto II flow cytometer (BD Biosciences).

2.5. Transwell cell migration assay

Fibroblast migratory capacity was evaluated in 24-well Transwell chambers equipped with 8.0 µm pore size polycarbonate membrane inserts (Corning). NIH3T3 cells transfected with ADAM28 overexpression or control plasmids (10,000 cells) were resuspended in 200 µL serum-free medium and seeded into the upper chamber. Target CAFs (10,000 cells) were seeded into the lower chamber containing 500 µL medium supplemented with 10% FBS. Chambers were incubated at 37°C for 36 hours. Following incubation, non-migrated cells on the upper surface were removed using a cotton swab. Migrated cells on the lower surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a light microscope across three randomly selected high-power fields.

2.6. Quantitative real-time PCR (rt-qPCR)

Total RNA was extracted from harvested cell pellets using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using 1 µg of total RNA with a PrimeScript RT Reagent Kit with gDNA Eraser (Takara). Quantitative real-time PCR was conducted on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) using TB Green Premix Ex Taq II (Takara). Target gene expression levels were normalized to GAPDH or beta-ACTIN as internal reference controls, and relative fold changes were calculated using the $2^{(-\Delta\Delta Ct)}$ method. Primer sequences are provided in Supplementary Table 2.

2.7. Statistical analysis

Statistical analyses were performed using R (v4.4.1), Python (v3.9), and GraphPad Prism (v9.0). Continuous variables were analyzed using two-tailed Student's t-test or Wilcoxon rank-sum test for two-group comparisons, and one-way ANOVA or Kruskal-Wallis test for multi-group comparisons. Correlation analyses were conducted using Pearson or Spearman correlation coefficients. Survival curves were generated using the Kaplan-Meier method and compared via log-rank tests. All experimental assays were performed with at least three biological replicates, and data are presented as mean $\pm$ standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

Supplement Materials

Data and Code Availability

Data Availability

All datasets analyzed in this study are publicly available. Detailed information regarding dataset accessions, sample counts, and original sources is provided in Supplementary Tables. No new raw sequencing data were generated during the current study.

Code Availability

This study utilized established, publicly available computational software, R packages, and Python toolkits as described in the Methods section. No custom computer code or novel algorithms were generated for this work.

Ethics declaration

The authors declare no competing interests. All patient-derived samples in this study were obtained from publicly available published databases. All ethical aspects of this research strictly adhered to the requirements and guidelines established by the Ethics Committee of Jinan University

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