Original Research

Multiregion single cell analysis reveals a novel subtype of cancer-associated fibroblasts located in the hypoxic tumor microenvironment in colorectal cancer

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ABSTRACT

Background: The tumor microenvironment (TME) plays a critical role in shaping tumor progression and determining the outcome of the therapeutic response. In this study, we aimed to generate a comprehensive cellular landscape of the colorectal cancer (CRC) TME.

Methods: We generated a comprehensive single-cell atlas by collecting CRC cases that have been uploaded to the online database and conducting an in-depth secondary analysis. We then carried out spatial transcriptomic sequencing and multiple immunohistochemical analyses to verify the results of the single-cell analysis. Moreover, we applied our findings to the TCGA database and used tissue microarray (TMA) on CRC tissue specimens to validate clinical prognosis.

Findings: We re-analyzed the transcriptomes of 23,785 cells, revealing a pattern of cell heterogeneity in the tumor region, leading-edge region, and non-tumor region. A subtype of COL11A1+INHBA+ tumor-resident cancer-associated fibroblasts (CAFs) was identified, and marker genes, transcription factors, and tissue-specific expression differences were noted and suggested to have potential roles in promoting cancer. We further confirmed that COL11A1+INHBA+ tumor-resident CAFs are mainly located in the hypoxic TME and we propose that they interact with CD44+ CRC cells via INHBA. Elevation of INHBA in CRC is associated with a poor prognosis.

Interpretation: Our results demonstrated a single cell landscape of CRC in different regions and identified in hypoxic TME a special subtype of CAFs producing INHBA, which promotes CRC development and correlates with poor prognosis. This special subtype of CAFs is a candidate target for translational research.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the leading cause of global cancer morbidity and mortality [1]. Despite continuous improvements in surgery, chemotherapy, radiotherapy, and targeted therapy, a significant proportion of CRC patients develop drug resistance and their disease relapses over the course of treatment [2], which emphasizes the need for a better understanding of the mechanisms underlying CRC metastasis.

Over the past years, it has been proposed that the tumor microenvironment (TME) plays a pivotal role in CRC drug resistance and relapse [3]. The complex communication between the TME and cancer cells has far-reaching consequences for the progression and spread of CRC [4]. During the early stages of tumorigenesis, a finely tuned repertoire of cytokines and their receptors maintain the homeostatic balance between the TME and cancer cells [5]. This balance can be destroyed by selective pressures, leading to activation of carcinoma-associated fibroblasts (CAFs), which are the dominant component of the TME. CAFs secrete...
various stromal factors, contributing to the maintenance of colorectal cancer stem cells (CSCs), chemoresistance, and promoting metastasis [6]. Targeting bidirectional crosstalk may be a useful strategy for CRC therapy. However, CAFs in CRC are inhomogeneous and have different phenotypes and functions [7], and there is a lack of markers that accurately reflect their biological sources and functions [3,8,9]. Understanding the features that differentiate CAFs and the mechanisms by which CAFs regulate colorectal CSCs currently limits the pioneering efforts to develop drugs targeting CAFs.

Here, we performed a comprehensive single-cell analysis of publicly available data on CRC samples and identified a cancer-promoting heterogeneous cluster of CAFs. COL11A1 and INHBA are specifically expressed in these heterogeneous cluster CAFs, and elevation of these markers in CRC is associated with a poor prognosis. Heterogeneous clusters of CAFs are located in the hypoxic TME and may interact with CD44+ CRC cells. The interaction of INHBA secreted by heterogeneous clusters of CAFs and ACVR on CD44+ CRC cells might play an important role in maintaining colorectal CSCs, and thereby promote colorectal tumor development.

Results

Single-cell landscape of colorectal carcinoma in different regions

The colorectal carcinoma (CRC) microenvironment is a complex niche composed of parenchymal and mesenchymal cells, and in which the cellular composition remains highly heterogeneous. A comprehensive analysis of the distribution and subtypes of cells across disparate regions can help us to better understand the ontogeny and development of CRC and more efficiently identify therapeutic targets. For this reason, we used seven CRC scRNA-seq datasets that were uploaded to the online database to conduct an in-depth secondary analysis. The three CRC tissue sites in each case were T (tumor) region, L (leading-edge) region, and N (non-tumor) region. Overall, 23785 cells that passed the quality control inspection were eligible for subsequent analysis, yielding 22875 detectable genes. Spatial transcriptomic sequencing was also performed to verify our findings.

To better probe the quantitative changes of different cell clusters within CRC, unified manifold approximation and projection (UMAP) dimensionality reduction was implemented in the ‘Seurat’ package to visualize clustering of cells among different regions from all samples (Fig. 1a). The percentage of cell types in each sample is shown in Fig. 1d. All major cell types can be compared between different regions and samples, albeit at different proportions. Based on the expression of canonical genes, these clusters were annotated into eight main cell types: T/NK cells (CD3D, NKG7, and KLRD1), B/plasma cells (JCHAIN, MS4A1, and CD79A), myeloid cells (LYZ), epithelial cells (EPCAM), myofibroblasts (DCN and COL1A1), endothelial cells (VWF), enteric glial cells (SI100B), and stem-like cells (KIT) (Fig. 1a, b). The representative top 5 genes are shown in Fig. 1c. To further explore the heterogeneity of cells in different niches of CRC, a scatter plot of the differences in the proportion of cells across different sites was drawn for each cluster (Fig. 1e). Interestingly, compared with the L and N regions, we observed that the proportion of plasma cells (CD1) decreased dramatically in the T region. As for myofibroblast cells, C12 was enriched in the T and L regions, whereas C07 was substantially reduced, suggesting that tumor-associated fibroblasts (CAFΔs) and normal myofibroblasts (MFs) were replaced in different regions. In the T and L regions, T cells from C09 with high expression of TNFRSF4, TNFRSF18, BATF and IL13 were predominantly enriched. Additionally, enteric glial cells were absent in the tumor. Although the number remained low, the proportion of migratory dendritic cells (migDCs) expressing FSCN1 was significantly higher in the L region.

Identification of CRC parenchymal cells and hypoxia-induced JAK-STAT pathway activation in CD44+ stem cells

We sorted cells with an epithelial phenotype based on the expression level of EPCAM (Fig. 2a), and inferred copy number variation for each cell using the ‘inferCNV’ package. As shown in Fig. 2a, clusters of malignant cells were only from CRC01, CRC02, CRC03, and CRC04, just because no cancer cells were found in the original sequencing data of CRC05, CRC06, and CRC07. Correspondingly, cells were categorized as non-malignant (E06, E08, and E10) and malignant cells (C01, C02, C03, C04, C05, C07, and C09) (Fig. 2b), which was further determined by the distributional differences across regions (Fig. 2c). We found that non-malignant cells from different samples were discretely distributed among different dimensionality reduction clusters, whereas normal tissues adjacent to cancer were located in the same clusters. Compared to non-malignant cells, CRC malignant cells showed evident heterogeneity among individuals. Although there was no dense distribution of malignant cells, epithelial tissues adjacent to cancer cells had sporadic alternations in copy number, whereas there was no clonal proliferation or heterogeneity in the transcriptome (Fig. 2b).

Next, we calculated the cell cycle score (G1, G2M, and S) and differentiation (epithelial or mesenchymal) score for each cell and assigned each cell to a specific cycle and differentiation stage (Fig. 2d and e). When compared with para-cancer tissues, the inter-individual heterogeneity of tumors is also reflected in the divergence of the proliferation and differentiation states. It should be noted that the cell cycle or differentiation stage of cells in T and L regions in the same individual is not the same, and there is no identical underlying rule generalizable to all samples, which may be attributed to intra-tumor heterogeneity. Therefore, we explored intratumoral heterogeneity among these seven samples, from which sufficient malignant cells were collected from CRC01, CRC02, CRC03, and CRC04. Except for CRC04, which had only one cluster, the other three samples could be divided into two or three clusters in which differences in the transcriptome were observed. Among the three clusters of CRC01, C07 cells highly expressed CTSE, P13, SLPI, and IFI6, whereas C11 cells highly expressed LMO7, NABP2L2, HSP54, and NEAT1. When the two clusters from CRC02 were compared, C05 cells were characterized by high expression levels of RP11-462G2.1, UBE2C, CBX3, and CKS2. In the two CRC03 clusters, C09 cells expressed high levels of IGFBP3, PSCA, KRT16, and NEAT1 (Fig. 2f). Intriguingly, both C09 and C11 cells had upregulated expression of NEAT1, in agreement with our previous studies in liver cancer organoid models [10], and the elevated expression of NEAT1 was mainly co-expressed with CD44+ stem cells (Fig. 2g). Elevated expression of NEAT1 is associated with hypoxia [10]. Consistent with these findings, the upregulated genes of C09 and C11 were subjected to KEGG pathway enrichment, with the results indicating hypoxia-induced activation of the HIP1 signaling pathway (Fig. 2h). It was reported in our previous studies that upregulation of NEAT1 could also activate the JAK-STAT pathway downstream, thereby facilitating drug resistance and proliferation in stem cells [10]. We further found an enriched expression of genes related to the JAK-STAT signaling pathway in the C09 and C11 clusters, suggesting that the hypoxic environment might be related to stemness remodeling in primary CRC (Fig. 2i).

Negative immune regulation and lack of antigen presentation

Alterations in the immune microenvironment of CRC are critical in the ontogeny and development of malignancy. Notably, many studies have reported immunosuppressive states and a lack of antigen presentation in the core of tumors. This study specifically addressed changes in the cellular composition of immune cells in CRC, which is rarely observed in a stepwise manner. Thus, we identified and mapped clusters of T/natural killer (NK) cells, B/plasma cells, and myeloid cells in the T, L, and N regions of CRC (Fig. 3a).

We categorized T/NK cells into 15 clusters (T01-T15), including...
Fig. 1. Single cell landscape of colorectal carcinoma (CRC) in different regions.

a. Distribution of 20 cell clusters on UMAP plot including B/plasma cells (C01, C06, C18, C19), T/NK cells (C02, C04, C09), Myeloid cells (C05, C15, C20), Epithelium cells (C03, C08, C17), Myofibroblast cells (C07, C11, C12, C13), Endothelial cells (C10), Enteric glial cells (C14), and Stem-like cells (C16).

b. Violin plots displaying marker genes (B/plasma cells: JCHAIN, MS4A1, CD79A, T/NK cells: CD3D, NKG7, KLRD1, Myeloid cells: LYZ, Epithelium cells: EPCAM, Myofibroblast cells: DCN, COL1A1, Endothelial cells: VWF, Enteric glial cells: S100B, Stem-like cells: KIT).

c. Dot plot of top 5 genes in each cluster.

d. Scatter plot and statistical differences of 20 clusters (p < 0.05: * and p < 0.01: **).

e. Distribution and composition of clusters and different cell types in T (Tumor region), L (Leading-edge region) and N (Non-tumor region) among 7 samples.
naïve T cells (T01: IL7R+, CCR7+), regulatory T cells (Tregs) (T02, T09, T13: CD4+, IL2RA+, FOXP3+, CTLA4+), cytotoxic CD8+ T cells (T03: CD8+, GZMA+, GZMK+), MAITs (T04, T05, T06: KLRD1−, TRDC−, KLRR1+), γδT cells (T07: CD3D0−, TRDC+), exhausted CD4 T cells (T08: CD4−, PDCD1−), NKT cells (T10, T15: CD3D−, KLRD1−), NK cells (T11: CD3−, KLRD1−), memory CD8+ T cells (T12: CD8+, IL7R−, CCR7−) and exhausted CD8+ T cells (T14: CD8+, PDCD1+, LAG3+, HAVCR2+). Likewise, re-clustering analysis of B/plasma cells yielded 12 clusters (B01-B12), including plasma cells (B01, B03, B05, B06, B07, B10, B11, B12: MS4A1+), memory B cells (B02: CD38−, CD27+), naïve B cells (B04, B09: CD38+, CD27+), and plasmablasts (B08: JCHAIN+, MS4A1+). A total of 13 clusters emerged in myeloid cells (M01-M13), including: macrophages (M01: CD14+, CIQA+, CIQB+), double positive monocytes (M02, M03, M04, M05, M06, M09, M11: CD68+, CD14+, FCGR3A+), cDCs (M07: CD1C+, CD14+ monocyes (M08: CD68+, CD14+, FCGR3A+), neutrophils (M10: CD68−, S100A8+, S100A9+), migDCs (M12: CCR7+, LAMP3+), and pDCs (M13: LILRA4+, IRF4+) (Fig. 3b).

An in-depth study of the progression to the core region of the tumor showed that the proportion of Treg1 (T02) and naïve B2 (B04) increased significantly, whereas the ratio of cDC (M07) substantially diminished (Fig. 3c). In previous reports, the stronger regulatory capacity of Tregs with weaker effectiveness of antigen presentation and humoral immune response might be mediated by TGBF signaling, resulting in loss of adaptive immunity within tumors, as evidenced by the inactivation of T cells and their differentiation toward an exhausted phenotype. Accordingly, we probed the expression of functional molecules in the T lymphocytes (Fig. 3d). Cytotoxic CD8+ T cells accounted for only a small proportion of total T cells, which expressed intermediate levels of activation molecules, such as CD27. The activation of other functional T cell molecules involved in adaptive immunity is less pronounced. Furthermore, MAITs, a component of innate immune cells, were characterized by high expression of CD40LG, and T15 in NKT cells also showed upregulated TIGIT expression. Although γδT cells have an intermediate expression level of PRF1, a sudden drop in expression was minimal in the tumor tissues compared to the L and N sites. Only a fraction of NK cells expressed high levels of cytotoxic-related marker genes such as GNYL and PRF1. Severe malfunction of the adaptive immune response is deemed one of the pivotal pro-tumoral factors in the immune microenvironment of CRC.

**COL11A1+** tumor-specific CAF remodels the microenvironment

Fibroblasts play an essential role in tumor growth as they serve as an important matrix for oncogenesis. Due to the technical limitations of previous studies, it was difficult to effectively distinguish and analyze parenchymal and mesenchymal cells from tumors. Therefore, the characteristics and functions of some mesenchymal cells have not attracted sufficient attention. At single-cell resolution, we divided CAFs and MFs into 12 heterogeneous clusters (Fig. 4a). In light of the distribution characteristics (Fig. 4b), cells from F01, F02, and F05 were annotated as MFs in normal tissues, and F04 also had an identical expression pattern to F01 and F02 after taking into account the distributional relationship of the reduction plot. Notably, the cells from F03, F06, and F07 appeared to be predominantly enriched in the T and L regions. And F06 and F07 emerged in T and L regions in each sample of CRC01-CRC07 (Fig. S1). Since F07 cells specifically emerged in the T and L regions, it was suggested that cells from F07 represented a type of specialized fibroblast in tumors (Fig. 4b). The results of differential gene expression analysis are shown among disparate clusters in Fig. 4c, in which F07 had a unique expression profile and specifically expressed COL11A1, which can thus be invoked as one of its marker genes. Additionally, this cluster also displayed upregulation of POSTN, THBS2, and TNFRSF12A (Fig. 4d), all of which reportedly facilitates tumorigenesis and the development of CRC. Our study further clarifies the cellular sources of these molecules.

We next systematically investigated the expression levels of several functional molecules in fibroblasts, including marker molecules, matrix metalloproteinases, growth factors, tumor-promoting proteins, and chemokines (Fig. 4e). Of note with regard to possible marker genes, F07 highly expressed FN1 and SPARC. High expression of MMP11, MMP14, and TIMP3 may provide favorable conditions for tumor migration. More importantly, F07 widely expressed various growth factors, including MYDGF, PDGFC, TGFβ1, and VEGFB, with upregulated expression of IGFBP3 and IGFBP5, which have been demonstrated to prolong growth stimulation of insulin-like growth factors on tumors. However, they do not appear to play a direct role in chemotaxis. KEGG analysis revealed enrichment of genes associated with TGFβ, HIF-1, and stem cells regulating pluripotency signaling pathways in F07 (Fig. 4f), indicating its likely important roles in the TME and stem cell remodeling. It has been well established that the WNT signaling pathway is a key player in CRC (Fig. 4g). Although we observed no enrichment of genes in the WNT pathway, upregulated CTNNB1 and WNT5A in F07 also likely play a central role, as supported by the protein-protein interaction analysis. Next, we analyzed the evolutionary trajectory among all fibroblasts (Fig. 4h, i), in which pseudotime and highly expressed gene heat map clustering identified that F07 was enriched in the middle phase along pseudotime, consistent with roles in promoting tumor growth and shaping the TME.

**COL11A1+** INHBA+ tumor-specific CAF participates in intercellular communication

In addition to COL11A1, we also found that INHBA was significantly expressed in F07 (Fig. 4d); therefore, we hypothesized that COL11A1+INHBA+ tumor-specific CAFs may play an important role in intercellular communication. Considering the lack of spatial information on the RNA profile of the CRC tissue, we carried out spatial transcriptome sequencing from two primary colorectal cancer tissues to verify the existence of COL11A1+INHBA+ tumor-specific CAFs and to investigate the possibility of intercellular communication between COL11A1+INHBA+ tumor-specific CAFs and other cell types in primary CRC in situ (Fig. 5a). We found that the SF03 cluster in the UMAP plot had a phenotype highly similar to that of the F07 cluster (Fig. 5b, c). We further verified the existence of this cluster at the protein level by immunohistochemistry (Fig. 5d). Next, we studied the expression levels of ACTA2, COL11A1, INHBA, CD44, and HIF1A in the CRC tissues. The results suggested that COL11A1+INHBA+ tumor-specific CAFs were concentrated in the core region of tumors with a hypoxic TME, constituting a scaffold for tumor growth (Fig. 5e). Although CD44+ cells diffused among tumor tissues, the gather of CD44+ cells in the hypoxic...
Fig. 3. Antigen presentation decreased in CRC immune microenvironment and Treg negative regulation.

a. UMAP plot of 15 T/NK cell clusters, 12 B/plasma cell clusters and 13 myeloid cell clusters.
b. Violin plots showing marker genes.
c. Scatter plots of immune cell distribution differences in T, L and N ($p < 0.05$: *, $p < 0.01$: ** and $p < 0.001$: ***).
d. Dot plots of T cell functional genes.
TME with \textit{COL11A1+INHBA+} tumor-specific CAFs infiltration was observed (Fig. 5f).

\textit{COL11A1+INHBA+} tumor-specific CAFs expressed a variety of significant marker genes that correlated with cell interactions in the CRC microenvironment, including \textit{NRP2, TGFBI, WNT5A, INHBA, MDK, PLXNB2, VEGFB, MRC2, and TNRFSF12A} (Fig. 5g, h). Based on receptor-ligand interaction analysis, we identified evidence of intensive communication between \textit{NEAT1+CD44+ CSCs} (C09 and C11) and key immune cell subgroups (Tregs and cDCs) via these molecules. For example, \textit{VEGFA} can bind to \textit{NRP2} to enhance blood vessel permeability and promote the proliferation and migration of vascular endothelial cells. Tumor necrosis factor receptor superfamily member 12A (TNRFSF12A), also known as fibroblast growth factor-inducible immediate early response protein 14, is a weak inducer of apoptosis in certain cell types and is capable of promoting angiogenesis and endothelial cell proliferation. Although it is activated in the early stage of tumorigenesis, \textit{TGFBI} mainly exerts anti-cancer effects; with the downregulation of receptors on tumor cells, highly expressed \textit{TGFBI} can exert immunosuppressive functions, which inhibit activation and differentiation of T/B lymphocytes, resulting in immune dysregulation and immune escape. Based on the cell–cell communication analysis, cells from C09 and C11 were found to have completely divergent reactivity with respect to the \textit{TGFBI-TGFBR3} axis. In addition, the secretion of \textit{TGFBI} can regulate Tregs and cDCs via \textit{TGFBR1} and \textit{TGFBR2}, respectively.

Notably, the secretion of \textit{INHBA} by tumor-specific \textit{COL11A1+INHBA+} CAFs could directly act on stem phenotype cells through ACVR receptors, which heightens the risk of a high tumor burden through the activin pathway. Multiple immunohistochemical analyses showed that \textit{COL11A1+INHBA+} CAFs were spatially close to ACVR \textit{CD44+} cancer cells in CRC tissue, offering opportunities for direct intercellular communication (Fig. 5i).

We used the TCGA database to validate the association between \textit{COL11A1} and \textit{INHBA} with the prognosis of patients who developed malignant tumors, and found that patients with high expression of \textit{COL11A1} and \textit{INHBA} had a markedly worse clinical prognosis (Fig. 5j). Altogether, these results suggest that \textit{COL11A1+INHBA+} tumor-specific CAFs may play a crucial role in maintaining cancer cell stemness and promoting tumor progression, thereby leading to adverse outcomes.

\textit{High expression of INHBA protein in CRC tumors predicts poor survival}

To further evaluate the prognostic value of \textit{INHBA} among patients with CRC, we performed immunohistochemistry on our CRC tissue microarrays. INHBA protein expression was quantified based on artificial intelligence learning (Fig. 6a). The H-Score of 179 CRC patients ranged from 0.4136 to 66.1938, and we divided these into low-expression \textit{(n = 60)}, moderate-expression \textit{(n = 60)}, and high-expression \textit{(n = 59)} groups based on the H-Score (Fig. 6b). We analyzed the baseline data of 179 patients with CRC and found that age, sex, tumor location, preoperative CEA, preoperative CA199, pTNM stage, differentiation, and adjuvant therapy were not significantly different among the three groups (Table 1). Kaplan–Meier analysis showed that patients with high INHBA protein expression levels had shorter disease-free survival than those with moderate or low INHBA protein expression \textit{(all p < 0.05)} (Fig. 6c).

\textbf{Discussion}

With the rapid development of sequencing technology, scRNA-seq has become an important tool for analyzing tumor heterogeneity. scRNA-seq analysis enables comparisons between normal and tumor tissue, and has led to the emergence of a comprehensive interpretation of the distribution and subtype characteristics of cells across disparate regions. In this study, our results showed heterogeneous characteristics of parenchymal cells, immune cells, and CAFs in CRC.

Among the single-cell landscapes of different regions in CRC, we discovered a novel heterogeneous cluster of tumor-specific CAFs overexpressing \textit{COL11A1}. CAFs are key players in the TME and are known to secrete various factors that contribute to the maintenance of colorectal cancer stem cells (CSCs), chemoresistance, and metastasis \cite{6,11,12}. Although many studies have described the heterogeneity of CAFs in CRC, there is still a lack of markers that accurately reflect their different phenotypes and functions \cite{7}. Our results suggest that \textit{COL11A1} is a marker for a cluster of tumor-specific cancer-promoting CAFs in CRC. Collagen is the major extracellular matrix molecule that provides support for cell growth and is responsible for the mechanical resilience of connective tissues \cite{13}. Abnormally expressed collagen can enhance malignancy and the ability of tumor cells to escape the immune system by combining and storing bioactive molecules such as cytokines and growth factors that regulate the TME \cite{14}, and which are associated with the prognosis of various cancer types, including CRC \cite{15}. \textit{COL11A1} is the gene encoding \textit{COL11A1}, a fibrillar collagen which is mainly distributed in cartilaginous tissues \cite{16}. Its deficiency is associated with skeletal and chondroid disorders \cite{17,18}. Although high expression of \textit{COL11A1} has been reported to indicate poor prognosis in CRC \cite{19}, and the gene is overexpressed in CAFs compared to normal fibroblasts \cite{6}, few studies have focused on the mechanisms of \textit{COL11A1+} tumor-specific activation of CAFs and its possible functions in CRC.

In our study, we found that a cluster of CAFs specifically emerged in tumor regions, suggesting the existence of tumor-specific CAFs in CRC. Differential gene expression analysis showed that the cluster of CAFs had a unique expression profile and specifically expressed \textit{COL11A1}. Hence, \textit{COL11A1+} tumor-specific CAFs were used in our study. \textit{COL11A1+} tumor-specific CAFs upregulated a series of genes with known roles in facilitating tumorigenesis, providing favorable conditions for tumor migration, or prolonging growth stimulation of insulin-like growth factors. This suggests that these CAFs may have pivotal roles in promoting CRC development. Among the upregulated genes, \textit{INHBA} was specifically overexpressed in the \textit{COL11A1+} tumor-specific CAFs. \textit{INHBA} encodes inhibin subunit beta A (INHBA), which belongs to the transforming growth factor (TGF) superfamily \cite{20}. INHBA homodimers are known bioactive proteins that exist in various tissues \cite{21,24}, and are implicated in multiple biological processes, including neoplastic progression \cite{25,26}. \textit{INHBA} is predominantly expressed in stage IV CRC \cite{27}. However, few studies have focused on the mechanisms underlying INHBA secretion by \textit{COL11A1+} tumor-specific CAFs and the regulation...
Our results revealed that 1) \( \text{COL11A1} \) + tumor-specific CAFs were enriched in the middle phase along pseudotime; 2) TGFβ, HIF-1, and stem cells regulating pluripotency signaling pathways were activated, and 3) \( \text{HIF1A} \) played a central role in the protein-protein interaction network. Hence, we inferred that upon activation under a hypoxic TME, \( \text{COL11A1} \) + tumor-specific CAFs increase INHBA secretion through TGFβ signaling pathways, which promote tumor growth, shape the TME, and remodel cancer cell stemness. Hypoxia, a critical feature of the TME, promotes cancer stemness during tumor growth [28]. Hypoxia-inducible factor-1α (HIF-1α), a vital transcription factor for cell sensing and adaptation to hypoxia, plays an important role in normal metabolism [29]. For example, HIF-1α helps chondrocytes adapt to the hypoxic environment in cartilaginous tissues under normal physiological conditions [30]. Considering that \( \text{COL11A1} \) is expressed mainly in cartilaginous tissue [16], we can extrapolate that HIF-1α may have a potential effect in regulating \( \text{COL11A1} \) secretion. Previous studies have revealed that HIF-1α is upregulated in CRC patients and is associated with poor prognosis [31,32], suggesting a role of HIF-1α in CRC tumorigenesis, although how hypoxic TME regulates CAFs for

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**Fig. 5.** The distribution of \( \text{COL11A1} + \text{INHBA} + \text{F07} \) in situ and its Intercellular Communication.

a. Spatial transcriptome single-cell sequencing clusters distribution overlayed with tissue immunohistochemistry (IHC).
b. UMAP plots showing the co-localization of \( \text{COL11A1} \) and \( \text{INHBA} \).

c. Multiple immunohistochemistry verified co-localization of \( \text{COL11A1} \), \( \text{INHBA} \), and \( \text{ACTA2} \) at protein level.
d. The expression of \( \text{ACTA2} \), \( \text{COL11A1} \), \( \text{INHBA} \), \( \text{CD44} \), and \( \text{HIF1A} \) in spatial level.
e. The Venn diagram showing the overlap of spots with \( \text{ACTA2} \), \( \text{CD44} \), and \( \text{COL11A1} \) expression in two CRC tissue samples with spatial transcriptomics data analysis
f. Cell-to-cell communication heatmap among \( \text{F07} \), \( \text{CD44} \)+\( \text{NEAT1} \)+ cancer cells and immune cells.
g. Violin plots displaying communication molecules expression in CAFs and MFs.
h. Multiple immunohistochemistry verified co-localization of \( \text{COL11A1} \), \( \text{ACVR} \), and \( \text{CD44} \) at protein level.
i. The Kaplan-Meier (KM) curves presenting the prognosis disparity of \( \text{COL11A1} \) and \( \text{INHBA} \) expression in TCGA database.

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**Fig. 6.** Association between \( \text{INHBA} \) expression and patient survival in CRC.

a. Representative images for \( \text{INHBA} \) immunostaining in CRC tissues.
b. H-SCORE of immunohistochemistry distribution and group division in 179 CRC patients.
c. The Kaplan-Meier (KM) curves presenting the prognosis disparity of \( \text{INHBA} \) expression in 179 CRC patients.
maintenance of cancer cell stemness and chemoresistance is unclear. In this study, verifying the existence of COL11A1+ tumor-specific CAFs might partly answer these questions.

Our spatial transcriptome sequencing and data from multiple immunofluorescent staining experiments successfully verified the existence of COL11A1+INHBA+ tumor-specific CAFs in primary CRC in situ. These CAFs are located in the core region of tumors, potentially constituting a scaffold for tumor growth. The data further showed that COL11A1+INHBA+ tumor-specific CAFs were enriched in hypoxic regions in which HIF1A is upregulated and with neighboring CD44+ CRC cells. Analysis of potential receptor-ligand interactions suggested that COL11A1 tumor-specific CAFs communicate between CD44+ CRC cells in the hypoxic TME and key immune cell subgroups (Tregs and cDCs) via a series of molecules overexpressed in the COL11A1 tumor-specific CAFs. Among these molecules, INHBA strongly interacts with ACVR in CD44+ CRC cells. Previous studies have revealed that the INHBA homodimer-ACVR-SMAD2/3 signaling network in pancreatic cancer plays a pivotal role in regulating multiple mechanisms key to the development of chemoresistance, including enhancement of stem cell-like properties and tumorigenicity of pancreatic cancer cells, mediating cooperative interactions between pancreatic cancer cells and the surrounding stroma, and regulating the deposition of extracellular matrix proteins within the TME [20]. Here, we infer that under a hypoxic TME, COL11A1+ tumor-specific CAFs maintain the stemness of CRC cells through a ligand-receptor interaction between INHBA and ACVR.

Despite advances in the diagnostic and therapeutic tools for CRC, there is still a desperate need for better risk stratification tools. Here, we not only reconfirmed that CRC patients with high expression of COL11A1 or INHBA had a markedly worsening clinical prognosis using the TCGA dataset, but also explored the expression of INHBA in a well-characterized cohort of CRC and scored the protein expression using a digital pathological image analysis software. With the help of artificial intelligence learning tools, quantification of the expression of the INHBA protein by immunohistochemistry becomes feasible and reliable, which makes it a usable tool for prognostic analysis in CRC patients.

However, this study has some limitations. First, there is insufficient proof that cluster F07 identified in the single-cell sequencing data and cluster S03 found in the spatial transcriptome sequencing data had the exact same phenotype. Second, bulk RNA analysis from the TCGA database cannot detect the cell types that contribute to RNA expression levels. Third, we were not able to cleanly isolate CAFs and quantify the expression of INHBA in tissue microarrays. Finally, some clinicopathological parameters such as vascular invasion, perineural invasion, and microsatellite instability were not included in this analysis.

In conclusion, we propose that the hypoxic TME specifically activates COL11A1+ tumor-specific CAFs through HIF-1α. COL11A1+ tumor-specific CAFs act as cancer-promoting CAFs by secreting a series of molecules that interact with CRC cells and key immune cell subgroups. COL11A1+ tumor-specific CAFs markedly overexpress INHBA via the TGFβ signaling pathway. INHBA promotes CRC development by binding to ACVR in CRC cells and is a potential marker for predicting CRC prognosis.

Materials and methods

scRNA-seq datasets availability

Raw single-cell RNA data were deposited in the ArrayExpress database of EMBL-EBI (www.ebi.ac.uk/arrayexpress) with accession number E-MTAB-8107. Detailed clinicopathological information of the seven CRC patients is available in the Supplementary Materials of the article published in Cell Research (2020) 0:1–18 (https://doi.org/10.1038/s41422-020-0355-0).

Single-cell RNA-seq data analysis

Analyses were performed by using R software version 4.0.3 and the ‘Seurat’ package version 3.1.1, ‘Monocle2’ package version 2.18.0, ‘inferncv’ package version 1.6.0, ‘corrploit’ package version 0.92, and CellPhoneDB Python 3.8.5. Cells with less than 200 detectable genes and mitochondrial content greater than 10% were filtered out during the quality control process. After excluding low-quality cells, we then used the ‘NormalizeData’ function to normalize filtered UMI counts, for which the normalization method is set as ‘logNormalize’, with a scaling factor of 10000. The ‘FindVariableGenes’ function was conducted with default parameters in order to select highly variable genes, which were subsequently applied for linear dimensionality reduction. Next, principal component analysis (PCA) was performed using the top 2000 highly variable genes via the ‘RunPCA’ function. Afterwards, a number of PCs were selected corresponding to ElbowPlot, followed by performing the ‘RunUMAP’ function with a perplexity value of 30 to acquire bidimensional coordinates for single cells. Meanwhile, we used unsupervised cell clustering by ‘FindClusters’ function with a resolution of 0.6 based on identical PCs as for ‘RunUMAP’ function. The datasets were visualized using UMAP plots. Based on normalized data, function ‘FindAllMarkers’ or ‘FindMarkers’ were used to identify DEGs across different clusters. We applied the Bonferroni correction method to adjust the P value and excluded DEGs whose adjusted P value was higher than 0.05. The non-parametric Wilcoxon rank sum test from the ‘Seurat’ package was designated for differential expression analysis between each subpopulation.

Spatial transcriptomics data analysis

CRC tissues were collected from a CRC patient who underwent radical resection at the Department of Colorectal Surgery, Changhai Hospital, Naval Medical University, Shanghai, China. Two tissue samples from the primary tumor were collected. The CRC tissues were pretreated, snap-frozen with dry ice, cut into 10 μm thick cryosections, and mounted onto spatial transcriptomics arrays. Each spot had a diameter of 100 μm and spots were 200 μm from center to center, covering an area of 6.1 × 6.5 mm². The spots were printed with approximately 2 × 10⁶ oligonucleotides containing an 18-mer spatial barcode, randomized 7-mer (unique molecular identifier) UMI,
poly-20TVN transcript capture region.

After tissue permeabilization, cDNA synthesis, and probe release, the barcoded mRNA/cDNA material was cleaved from the arrays and sequencing libraries were prepared in solution. Second-strand cDNA was synthesized, followed by in vitro transcription and adapter ligation. Sequencing handles and indexes were added to the PCR mix, and the completed libraries were sequenced on the 10x Genomics platform (Illumina NextSeq, HiSeq4000, or NovaSeq6000) with paired-end sequencing.

**Cell cycle and differentiation analysis and pseudo-time transcriptional trajectory analysis**

Based on the expression of genes related to G2/M and S phases as well as differentiation (epithelial and mesenchymal) states, cells are categorized into particular cell cycle and differentiation stages. Briefly, we applied the "CellCycleScoring" function to calculate the cell cycle and differentiation scores for each cell, followed by matching to the metadata.

The "Monocle2" package was used for trajectory and pseudo-time analysis containing 400 marker genes from the "differentialGeneTest" function, which is designed to infer potential pedigree differentiation trajectories. RNA counts in all cells from clusters were selected as input to "Monocle2" for downstream analyses. A generalized additive model (GAM) was constructed to generate an average expression for each isotype. The lineage difference trajectory among CAFs and MFs clusters was performed using default parameters of "Monocle" after dimensionality reduction and cell ordering.

**Cell-cell interacting network analysis**

To gain insight into potential interactions between disparate cell types, we performed cell-cell network analysis to investigate potential ligand-receptor pairs using 'CellPhoneDB'. Predicted receptor-ligand interactions across different cell types were confirmed, corresponding to the expression level of receptors in one cell type and the expression level of homogenous ligands in another cell type. Bonferroni multiple test correction was used to adjust the P value; a ligand-receptor pair with an adjusted P value less than 0.01 was deemed a potential molecular partner in mediating cell-to-cell communication.

**Multiple immunofluorescence staining**

Co-staining of CRC tissues was performed using antibodies against COL1A1 (21841-1-AP, Proteintech, 1:100) and INHBA (10651-1-AP, Proteintech, 1:100).

ACVR1 (10227-T24, Sinobiological, 1:100), ACTA2 (19245, CST, 1:100), and CD44 (ab189524, Abcam, 1:100) were detected using a Four-color Fluorescence kit (Recordbio Biological Technology, Shanghai, China) based on the tyramide signal amplification (TSA) technology according to the manufacturer’s instruction. Briefly, paraffin sections of CRC tissues were treated with antigen retrieval buffers under optimal conditions, and then blocked successively with 3% H2O2 and 3% BSA-PBS. After incubation with the primary antibody overnight at 4°C and horseradish peroxidase (HRP) secondary antibody for 50 min at room temperature, 100 μl of TYR-520 (1:500), TYR-570 (1:300), TYR-690 (1:500), and DAPI were used successively for staining.

**Tissue microarray immunohistochemical assay**

Clinicopathological parameters were collected from 179 patients with CRC who underwent surgical resection at Changhai Hospital, Naval Medical University (Shanghai, China), between January 2001 and November 2011. Informed consent was obtained. Tissue microarrays were constructed from the donated CRC tissue specimens using a commercial company (OutDo Biotech, Shanghai, China). One core was used for each sample, with a core diameter of 1.2 mm. The TNM stage was reclassified according to the American Joint Committee on Cancer staging manual (seventh edition). This study was approved by the Institutional Review Board of Changhai Hospital.

INHBA staining on tissue microarrays was performed using an IHC kit (Servicebio Technology, Wuhan, China) according to the manufacturer’s instructions. Specimens were stained with antibodies. The sections were heated at 70°C for 1 h, dewaxed in xylene, and dehydrated through a gradient concentration of alcohol. After retrieving and blocking endogenous peroxidase and nonspecific staining with 3% H2O2 and normal bovine serum, the sections were incubated with primary antibody overnight at 4°C. The slides were then incubated with HRP-conjugated secondary antibody for 10 min at 37°C. Finally, the sections were visualized by dianminobenzidine (DAB) solution and counterstained with haematoxylin. The stained tissue microarray slides were analyzed at a resolution of ×20 (digital scanning via K-Viewer software; KBIO Co., Ningbo, Zhejiang, China). INHBA protein expression was quantified using Alipathwell software (Servicebio Technology, Wuhan, China), a digital pathological image analysis software based on artificial intelligence learning. The H-SCORE of each core was evaluated using the following formula: H-SCORE=∑(pi × i) = (percentage of weak intensity × 1)+ (percentage of moderate intensity × 2)+ (percentage of strong intensity × 3).

**Statistical analysis**

All statistical analyses were performed using the R software (version 4.0.3) and GraphPad Prism software version 8.0. All grouped data were summarized as mean ± standard error of the mean (SEM). Unpaired Student’s t-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of the comparison between two groups and more than three groups, respectively. Two-tailed P values less than 0.05 were considered to be statistically significant. Survival analysis was conducted using the Kaplan–Meier method.

**Credit authorship contribution statement**

W.Z. and F.C designed the study and drafted the manuscript. N.Z., R.W., L.Z., and Z.L. prepared the tables and figures and drafted the manuscript. The authors thank the TCGA Research Network: https://www.cancer.gov/tcga. The authors thank the TCGA Research Network: https://www.cancer.gov/tcga. The authors thank the TCGA Research Network. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Declaration of Competing Interest**

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101570.

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