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Neutrophil extracellular traps regulate LDHA expression to promote colorectal cancer liver metastasis

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Abstract

Metastasis is the most common cause of colorectal cancer (CRC)-related death. Neutrophil extracellular traps (NETs) promote tumor progression and distant metastasis. This study aimed to explore the role of NETs in CRC liver metastasis. Through analysis of publicly available single-cell transcriptome sequencing databases, in vitro experiments and nude mouse liver xenograft model experiments, we revealed that NETs promote CRC metastatic progression. Using scRNA-Seq technology, we showed that NETs marker expression was higher in metastatic lesions than in primary tumors. NET marker expression was high in colorectal cancer tissues and correlated with advanced tumor pathological grade. In addition, treatment with NETs enhanced the proliferation, migration and invasion of CRC cells in vitro by inducing EMT, as indicated by downregulation of E-cadherin and upregulation of N-cadherin and Vimentin. Cell-cell communication analysis revealed that NETs are related to the PI3K/AKT pathway and regulate the expression of LDHA, a key enzyme in glucose metabolism. In vitro, treatment with NETs promoted LDHA production and cell invasion and migration in CRC, while knockdown of LDHA suppressed EMT. Further, inhibition of LDHA expression or NET formation effectively inhibited NET-induced liver metastasis. In summary, this study elucidates the mechanism by which NETs regulate LDHA expression to promote CRC liver metastasis.

Keywords Colorectal cancer, Neutrophil extracellular trap, scRNA-Seq, Lactate, Metastasis

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Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal tract cancers. Globally, CRC accounts for approximately 10% of newly diagnosed cancers; among cancers, it ranks third in incidence and second in mortality [1]. The liver is the most common site of CRC metastasis. The 5-year survival rate of patients with colorectal cancer liver metastasis (CRLM) is significantly lower than that of CRC patients without metastasis [2]. Despite advances in precision medicine and improvements in the understanding of CRLM, metastasis to distant sites, including the liver, remains the most common cause of CRC-related death [3, 4]. The mechanism of CRLM is complex, and the effectiveness of existing treatments, including hepatic metastasectomy in select patients, is limited, leading to recurrence and ultimately death [5]. Therefore, investigation of the mechanisms driving CRLM and identification of molecular therapeutic targets have important scientific value and clinical implications.

Neutrophils are the most abundant granulocytes in the human body. These cells are an essential component of the host's response to pathogens [6]. During infection, neutrophils migrate from the peripheral blood into tissues [7–9], eradicate pathogens after phagocytosis, and protect the host cells by releasing antibacterial factors [6–12], which include granzymes, proteins, oxidants, as well as neutrophil extracellular traps (NETs) [13, 14]. NETs are network structures composed of DNA, histones, and antibacterial proteins. In more detail, NETs are DNA-based structures that contain histones (mainly citrullinated histone 3, CitH3), neutrophil elastase (NE), myeloperoxidase (MPO), and neutrophil elastase (NE). NETs contribute to tumor progression and are key mediators of immune response regulation [15, 16]. In recent years, a large number of studies have shown that NETs play multiple roles in the development of cancer. First, NETs directly or indirectly promote tumor growth, progression and metastasis to distant sites [15, 17]. NETs can be detected in the blood of patients after surgical tumor resection—high abundance of NETs is associated with an increased risk of cancer recurrence [18]. Watany et al. [19] emphasized the importance of exploring novel biomarkers in hepatocellular carcinoma (HCC), noting the limitations of traditional biomarkers like α -fetoprotein (AFP). Our findings, which reveal that NETs play a crucial role in promoting the proliferation, invasion, and migration of CRC cells during liver metastasis, align with Watany et al.'s call for identifying new biomarkers and mechanisms to enhance HCC diagnosis and treatment. Here, we report that NETs are abnormally enriched in CRC. NETs can upregulate lactate dehydrogenase (LDHA) expression, thereby promoting CRC invasion and metastasis.

Metabolic reprogramming is a hallmark of tumors [20–22]. Despite the presence of sufficient oxygen, aerobic glycolysis in mitochondria occurs in more than 85% of cancer cells, a phenomenon known as the Warburg effect. In response to severe metabolic stress, CRC cells exhibit high rates of glycolysis, which leads to the production of massive amounts of lactate [23, 24]. Glycolysis contributes to cell proliferation, angiogenesis, cell adhesion, and epithelial–mesenchymal transition (EMT), which contribute to the metastatic phenotype of cancer cells [25–27]. In 2019, a study by Zhao et al. reported that histone lactylation, a posttranslational modification, is a metabolic regulation mechanism that plays a key role in gene transcription regulation [28]. This recent discovery has prompted medical biologists to re-examine the relationship between lactate and the Warburg effect in cancer.

LDHA plays a key role in glucose metabolism and is the rate-limiting enzyme that converts glucose-derived pyruvate into lactate in glycolysis. Studies have shown that the expression of LDHA is regulated by a series of complex transcriptional, posttranscriptional and posttranslational mechanisms. At the transcriptional level, LDHA is regulated by genes such as c-MAP, HIF and c-MYC [29, 30]; at the posttranscriptional level, several microRNAs (miR-33b, miR-33b/c, etc.) are closely related to the expression of LDHA; at the posttranslational level, the activity of LDHA can be modulated via the acetylation or phosphorylation of various amino acid residues [31]. Therefore, in various tumors, aberrant LDHA expression is an important factor leading to the occurrence and development of tumors.

Lactate Promotes Tumor Metastasis through Multiple Regulatory Mechanisms [32]. Elevated lactate levels have been associated with metastatic niche formation [33], and its immunosuppressive role within the tumor microenvironment (TME) is increasingly recognized. Lactate facilitates tumor progression by inducing and recruiting immunosuppressive cells and molecules, thereby altering tumor cell migratory and invasive capacities. Brand et al. demonstrated that tumor cells promote lactate production via LDHA, and increased lactate disrupts the production of cytokines such as IFN- γ in tumor-infiltrating T cells and NK cells, consequently fostering tumor growth [34]. In colorectal cancer (CRC) tissues, lactate promotes the secretion of specific chemokines and the expression of E-cadherin by activating the PI3K-AKT signaling pathway. This recruits CD4⁺ T cells into the TME, facilitating the formation of a pre-metastatic niche for CRC bone metastasis [35]. Furthermore, in both cancer and endothelial cells, lactate is oxidized to pyruvate by lactate dehydrogenase A (LDHA), fueling angiogenesis and signaling [36, 37].

Neutrophils rely on glycolysis for the energy required for functions such as chemotaxis and phagocytic activity [38–40]. Glucose metabolism and oxygen are crucial for mediating neutrophil recruitment post-activation, neutrophil extracellular trap (NET) formation, and their overall function. Nunn and colleagues reported decreased lactate, the end product of non-oxidative glycolysis, in apoptotic neutrophils [41]. Recent studies have observed a significant increase in lactate accumulation during both NOX-dependent and NOX-independent NETosis, associated with glycolytic activation. Although the precise role of neutrophils within the TME remains incompletely defined, existing evidence suggests lactate promotes their pro-tumorigenic functions [42]. Chen et al. demonstrated that in vitro pre-treatment of neutrophils with lactate suppressed the expression of surface CXCR4, maturation/activation markers (CD10, CD101), degranulation marker (CD63), and glycolytic proteins (HIF1A, HK2, GLUT1, LDHA), while also reducing NET formation [43]. An intricate link between lactate and NETs is evident. However, the specific mechanistic roles of lactate and NETs in CRC metastasis remain unelucidated.

Therefore, we hypothesize that NETs promote CRC metastasis by activating LDHA expression. This study aims to elucidate the mechanism of NETs in CRC metastasis using clinical samples, cell line models, and animal models, providing potential novel targets for the early diagnosis and treatment of colorectal cancer. In this study we applied single-cell RNA sequencing (scRNA-seq) technology to construct a single-cell transcriptome map of CRC samples and identified 14 cell types including neutrophils, myeloid cells, epithelial cells, and fibroblasts. Subsequent analyses were conducted using methods such as pseudotime analysis and NicheNet, with a focus on neutrophils and CRC cells. Our study aimed to determine whether NETs regulate epithelial-mesenchymal transition (EMT) and distant metastasis in CRC. In addition, we aimed to study whether NETs promote glycolysis by increasing the expression of LDHA, thereby promoting CRC metastasis.

Materials and methods

Data download and preprocessing

In this study, a total of 17 patient tumor tissues were selected as the test samples [44]. CRC scRNA-seq data were downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). The data came from the GSE164522 data set. Finally, we used the R package Seurat to perform screening, which yielded 199,179 cells for subsequent analysis.

Single-cell data integration analysis and cell type annotation

In order to avoid the influence of sample batch effects, we use the canonical correlation analysis (CCA) algorithm to unbiasedly integrate multiple samples. High-dimensional data analysis is challenging considering that each gene in the sample is considered a dimension. Therefore, we used dimensionality reduction techniques to represent the true structure of the data using reduced dimensions. We used the “RunPCA” function in the Seurat software package to perform dimensionality reduction on the obtained set of highly variable genes. Subsequently, we used the “FindNeighbors” and “FindClusters” functions in the Seurat package to cluster the data with reduced dimensionality. To enhance the visualization of clustering results, we adopt the t-distributed stochastic neighbor embedding (t-SNE) method, which considers both the t-distribution and stochastic neighbors. By using the “RunTSNE” function in the Seurat package with parameters set to dim=1:15 and resolution=1.0, we achieved efficient visualization of clusters.

Single-cell status scoring

We used single-cell scoring algorithms to evaluate the enrichment level of genes in specific functional gene sets or pathways in single cells. We used the AddModuleScore function in Seurat software to complete the single-cell scoring process.

Subgroups of cell types

To subclassify a certain annotated cell type, we first extracted all the cells of that cell type through the subset function and then performed single-cell data integration analysis and cell type annotation.

Single-cell data visualization

Single-cell data result visualization was performed using the following functions or R packages: The DimPlot function was used for the visualization of t-SNE two-dimensional spatial data; each point represents a cell, and the colors correspond to different cell types. The FeaturePlot function was used to visualize the expression levels of specific genes on the t-SNE dimensionality reduction clustering diagram; each point represents a cell, and the color corresponds to the expression level—the higher the expression was, the darker the color. The DotPlot function was used to generate bubble charts to display the expression levels and expression rates of genes in different cell types; the size of the dots represents the percentage of cells with positive expression, and the color of the dots represents the expression level.

Single-cell data pathway and functional enrichment analyses

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to explore potential biological functions and associated signaling pathways of each cell type. GO and KEGG analyses were performed using the compareCluster function of R package “clusterProfiler”(4.6.2). For functional enrichment analysis of differential genes between cell types, we used the R packages ‘ClusterGVis’ and ‘ComplexHeatmap’ for visualization. In analysis, pathways or functions with an p value < 0.05 were considered as significantly enriched items.

Pseudotime differentiation trajectory inference and analysis

Pseudotime analysis was performed using “Mococle2”. We first extracted all neutrophil and CRC cell clusters and divided them into different subpopulations based on different cell marker genes. Subsequently, we used the “DDRTree” method to reduce dimensionality and the “orderCell” method to determine the cell differentiation status. At the same time, we visualized the expression levels of differentially expressed metabolism-related genes (DE-MRGs) at different differentiation stages.

Cell – cell communication

Cell – cell communication mediated by cell surface ligand and receptor interactions plays a crucial role in a variety of biological processes. To study the interactions between different cell types, we constructed a cellular communication network using the “NicheNet” package. NicheNet utilizes the gene expression profiles of ligands and receptors of NETs and CRC cells to infer interactions between them and infers the difference in interaction strength between receptors and ligands between the two cell types.

Patient information and organization of the clinical study

This study included 60 primary CRC patients newly diagnosed via pathological examination at the Second Affiliated Hospital of Harbin Medical University. We based the tumor – node – metastasis classification of the CRC samples on the 8th edition of the American Joint Commission on Cancer staging manual. A total of 20 CRC tissue samples were collected and stratified by tumor stage as stage I, stage II, or stage III. The inclusion criteria were as follows: age of 18–70 years old; no comorbidities of endocrine, cardiovascular, vascular, blood or infectious diseases. The exclusion criteria were as follows: age younger than 18 years old or older than 70 years old; no systemic or radiation treatment before surgery. This study was approved by the Scientific Ethics Committee of the Second Affiliated Hospital of Harbin Medical University,

China. The study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from each participant before the study began (approval number: KY2023-012).

Isolation of neutrophils and extraction of NETs

In this investigation, neutrophils were separated from preoperative venous blood obtained from patients with CRC using a neutrophil isolation kit (TBD Science, Tianjin, China). Isolation of bone marrow (BM) neutrophils was performed using Histopaque gradient centrifugation. After obtaining the purified neutrophils, they were seeded in a 6-well plate, stimulated with 50 $\mu\text{mol/ml}$ PMA (Solarbio), and cultured at 37 °C and 5% CO₂ for 2 h. The culture medium was then gently removed, and NETs and neutrophils were eluted with pre-chilled PBS. The liquid samples were collected and centrifuged at 450 $\times g$ for 10 min at 4 °C, and then the supernatants were collected and centrifuged at 15,000 $\times g$ for 15 min at 4 °C; the NET concentration was measured with a spectrophotometer (BioSpec-nano, Japan), and the samples were store at – 20 °C.

Cell culture

The human CRC cell lines HCT116, HT29, SW480, SW620, and Lovo (purchased from Procell, Wuhan, China) were maintained in DMEM-F12 medium (Gibco) supplemented with 10% fetal calf serum (Gibco) and cultured in an incubator at 37 °C with 5% CO₂.

Colony formation experiment

Complete culture medium containing 1,000 cells was added into a 6-well plate and cultured for 7 to 14 days. After colony formation, the cells were fixed with 4% paraformaldehyde and stained with crystal violet (Beyo-time). Quantification was performed via ImageJ (National Institute of Health, Bethesda, USA, MD, Bethesda, USA). The colony formation rate was calculated as follows: Colony formation rate = number of colonies/total number of cells inoculated and cultured $\times 100\%$.

5-Ethyl-2'-deoxyuridine (EdU) incorporation assay

For this experiment, an EdU cell proliferation assay kit (Cell-Light EdU Apollo 567 kit; RiboBio, China) was used as described previously.

Transwell assays

Transwell assays were performed using 24-well plates and polycarbonate filters (8 μm pores, Corning). To assess the cell migration ability, Transwell chambers without Matrigel were used, and to assess the cell invasion ability, Transwell chambers containing Matrigel were used. A total of 2×10^4 cells were suspended in serum-free medium and placed in the upper chamber, and 600 μL of complete

medium was added to the lower chamber. After 24 h of incubation, cells on the Transwell membrane were fixed with paraformaldehyde and stained with 0.25% crystal violet. Cells that migrated to the lower chamber were imaged and counted.

Wound healing

We prepared cell suspensions with a concentration of 5×10^5 cells per well and then inoculated them onto a six-well plate. We used an optical microscope to take pictures in three random fields as a 0-h control and to take pictures again 24 h and 48 h later. Before each photo was taken, the detached cells were rinsed away with PBS, and fresh culture medium containing 1% fetal bovine serum was added.

Immunohistochemistry

Tissue specimens were fixed, dehydrated, and embedded in paraffin to prepare pathological sections. The cells were incubated with specific primary antibodies used to identify neutrophils [anti-LDHA antibody (Abcam) and anti-CD66B antibody (Affinity)] and NETs [anti-cit-H3 antibody (Affinity)] overnight at 4 °C. Images were acquired and analyzed after incubation with secondary antibodies.

Immunofluorescence assay

Slides and cells were added to a 24-well plate. The slides were removed when the cells grew to approximately 90% confluence. The cells were fixed with 4% paraformaldehyde for 20 min and washed 3 times with PBS. Then, 0.5% Triton X-100 was added at room temperature to permeabilize the cells, and the samples were blocked with 5% BSA for 2 h at room temperature. Primary antibodies against LDHA (ab52488; Abcam), N-cadherin (ab18203; Abcam), Vimentin (60,330-1-Ig; Proteintech), and E-cadherin (ab135384; Abcam) were added to the 24-well plate and incubated overnight at 4 °C. The next day, the secondary antibody (AF-488 or AF-594; 1:200; Abcam, USA) was diluted and added to the plate, which was incubated in a dark box at 37 °C for 1 h. DAPI was added to the plate, which was incubated in a dark box at room temperature for 20 min. The slide was removed, sealed with anti-fluorescence quenching agent mounting solution, and attached to a polylysine slide; pictures of random fields were taken under a confocal microscope (LSM800; Zeiss, Germany).

Western blotting

HCT-116, Lovo, SW480, SW620 and HT-29 cells were harvested when their confluence reached approximately 80%. Prepared protein samples were stored at -20 °C for long-term storage. Protein samples were separated by electrophoresis (SDS-PAGE) and transferred to

polyvinylidene fluoride membranes (Millipore Corporation, Billerica, MA, USA). After blocking for 2 h, primary antibodies against LDHA (ab52488; Abcam), N-cadherin (ab18203; Abcam), Vimentin (60,330-1-Ig; Proteintech), E-cadherin (ab135384; Abcam), AKT (ab38449; Abcam), p-AKT (ab8805; Abcam), PI3K (ab302958, Abcam), p-PI3K (AF4369; Affinity), CDCC25 (21,209-1-AP; Proteintech), and L-lactyl lysine (PTM-1401RM; PTM BIO) overnight at 4 °C in the refrigerator. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse or goat anti-rabbit antibody (1:5000, Affinity) for 1 h at room temperature. The target protein levels were normalized to those of the control.

Lentivirus transfection

The sequences of siRNAs used in this study were as follows: LDHA siRNA#1, 5'-CGAAGACAAATTGAAGGGAGA-3'; LDHA siRNA#2, 5'-GACTGATAAAGATAAGGAACA-3'; LDHA siRNA#3, 5'-ACCTACGTGGCTTGAAGATA-3'. Scrambled siRNA was used as a negative control. All siRNAs were purchased from GENECHM. Briefly, 1.5×10^5 tumor cells were seeded in a 24-well plate, placed in 500 µl culture medium, and incubated at 37 °C overnight. When the cell density reached 30–50%, polybrene was used to facilitate lentiviral transduction infection. After 24 h, the medium was replaced with 500 µl of fresh culture medium, and culture was continued. After 48 to 72 h, the infection efficiency was assessed with a fluorescence microscope. Stably transduced cells were selected with puromycin (Solarbio, Beijing, China) for 3–4 weeks, and LDHA expression was confirmed via Western blotting for subsequent experiments.

Sphere formation assay

CRC cells (5×10^3 cells/well) were seeded in Corning ultra-low attachment 6-well plates (Cat. No. 3471; Corning, USA) with 2 ml culture medium (DMEM-F12 culture medium containing penicillin/streptomycin, L-glutamine, insulin, bovine serum albumin, glucose, bFGF and recombinant human epidermal growth factor). The cells were cultured for 5 to 7 days, and the morphology of tumor spheres was observed and photographed under a microscope.

Xenograft mouse model

BALB/c nude mice (male, 6 weeks old, 18–20 g) were purchased from Vitong Lever Laboratory Animal Center, and all animal experiments were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, China (SYDW2023-012). All animals were housed in individually ventilated cages under specific-pathogen-free (SPF) conditions and had free access to standard SPF mouse food and water. A

suspension of Lovo cancer cells expressing the luciferase gene was prepared for group experiments and divided into the Ctrl group, shLDHA group and DNase-1 inhibitor group. Each group consisted of at least 5 mice. Nude mice aged 6 to 8 weeks were anesthetized in accordance with laboratory animal rights protocols, and an 8-mm incision was made in the skin just above the spleen. Within 30 to 60 s, 100 μ l of cells were injected into the exposed spleen tip, and 5 min after injection, the peritoneal cavity was closed. Tumor metastasis was monitored weekly via bioluminescence imaging of luciferase signals. Mice were sacrificed after 6 weeks. Hematoxylin – eosin staining was performed for histological analysis.

Results

ScRNA-seq reveals the cell type composition in primary and metastatic CRC

To study the characteristics of different cell populations in primary and metastatic CRC, we collected data for 17

CRC tissues from the GEO database. Each sample was screened to remove low-quality data, and 199,179 cells were retained for scRNA-seq analysis. We performed dimensionality reduction clustering and annotated 14 cell types, including 8 CD8+ T subtypes, 3 CD4+ T subtypes, B cells, neutrophils, myeloid cells, epithelial cells, and fibroblasts. Cells are colored by cell type and cell tissue origin (Fig. 1A, B).

Next, we assessed the proportions of different cell types in different tissues and found that the proportions of cell types greatly varied by organ type. Neutrophils accounted for a smaller proportion of cells in primary tumors and normal lymph nodes, and a larger proportion of cells in metastatic tumors and metastatic lymph nodes (Fig. 1C, D). Notably, different cell types play different roles in the immune microenvironment. T cell subsets are related to immune responses and immune cell activation and differentiation. B cells are related to the response to endoplasmic reticulum stress. Epithelial cells are related to



Fig. 1 Characteristics of different cell populations in CRC. **A** Cell types of CRC cells. **B** Tissue origin of CRC cells. **C** and **D** The proportion of different cell types in different tissues. **E** Functions of different cell types

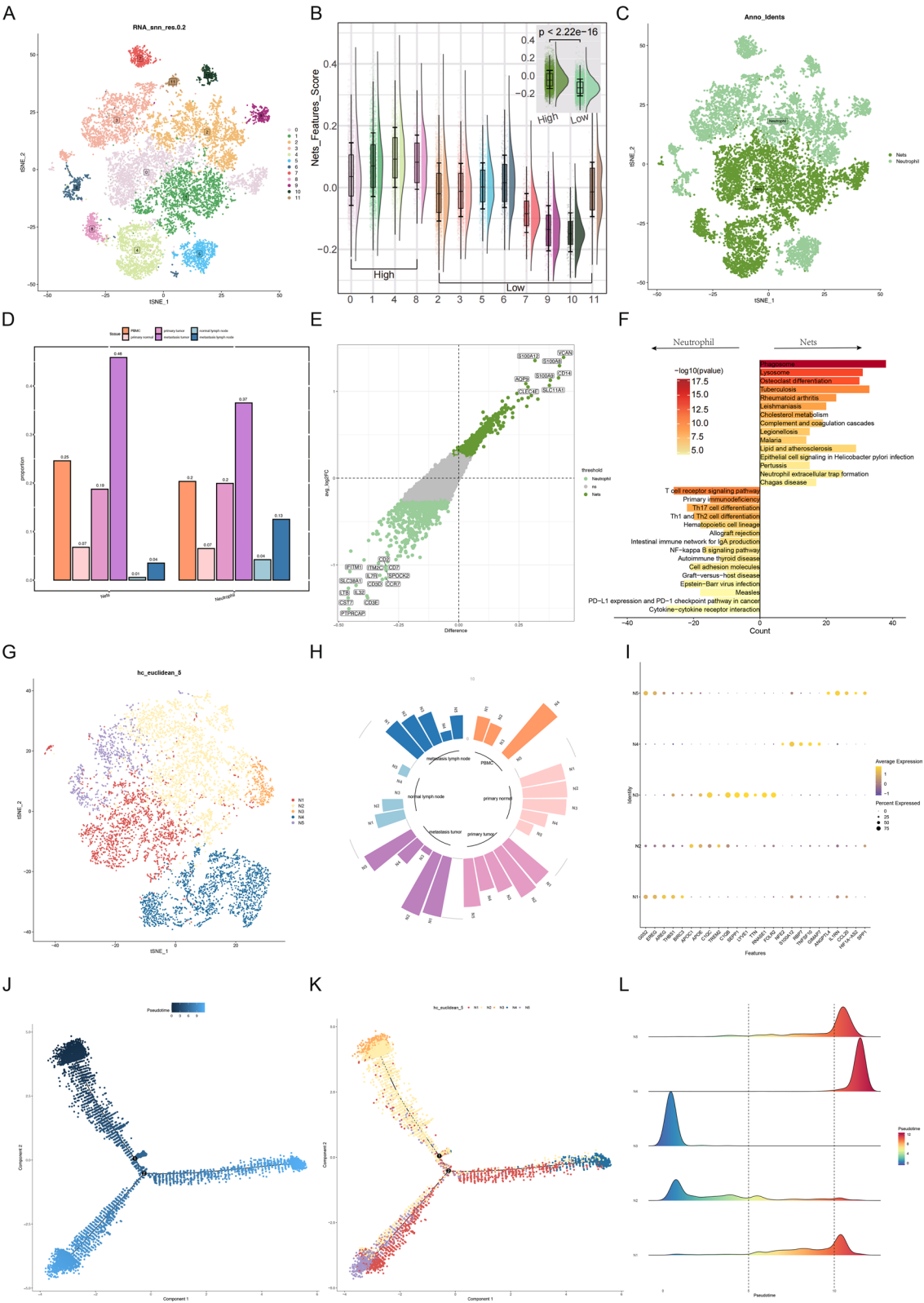


Fig. 2 (See legend on next page.)

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Fig. 2 Subtype and pseudotime analysis of NETs cells in scRNA-seq. **A** Cell types of neutrophils. **B** NETs-related gene scoring. **C** t-sne diagram, identifying two cell subtypes of neutrophils based on the corresponding marker genes. **D** Proportions of different cell subtypes in different tissues. **E** Differential gene analysis of two subtypes. **F** Cell functional enrichment of NETs. **G** NETs cell subtypes. **H** Cell numbers of NETs subtypes in different tissue sources. **I** Bubble plot of marker genes expressed in NETs. Dot color reflects expression level, and dot size represents the percentage of cells expressing the marker gene in different cell types. **J** Trajectory diagram of NETs cell development time. **K** Trajectory diagram of NETs cell subtypes. **L** Density changes of different cell subclusters over time

cell–cell interactions and connective tissue, and fibroblasts are mainly related to cell matrix adhesion; neutrophils—our focus—mainly function by producing NETs in response to external stimuli (Fig. 1E). Studies have shown that NETs can promote the migration and invasion of cancer cells through multiple pathways [15]. Therefore, we hypothesized that NETs play an important role in the metastasis of CRC.

Pseudotime analysis revealed the transformation of NETs in CRC progression.

To test our hypothesis, we perform dimensionality reduction and clustering on neutrophils and identified 12 clusters (Fig. 2A). We scored these 12 clusters using NET-related marker genes collected from the literature and the AddModuleScore function in the Seurat package [45]. We defined the clusters with high scores (C0, C1, C4, C8) as NET-containing cells, and the clusters with low scores (C2, C3, C5, C6, C7, C9, C10, C11) as neutrophils (Fig. 2B). We used this method to divide neutrophils into NET-containing cells and normal neutrophils for subsequent experiments (Fig. 2C).

The proportions of these two subtypes in peripheral blood, normal colorectal tissue and lymph nodes, primary CRC tissue, and metastatic CRC tissue and lymph nodes were examined. The proportion of NET-containing cells in liver metastatic tissues was higher than that in normal tissues and primary tissues, and the proportion of NET-containing cells in metastatic lymph nodes was also higher than that in normal lymph nodes (Fig. 2D). Next, we performed differential expression analysis on the two cell subtypes (Fig. 2E) and performed functional enrichment analysis of the upregulated genes in NET-containing cells and neutrophils. We found that the upregulated genes in NET-containing cells were mainly enriched in gene sets related to NET formation, cholesterol metabolism, phagosomes, neutrophil interaction with T cell signaling receptors, T cell differentiation, and cytokine–cytokine receptor interactions (Fig. 2F).

We conducted further analysis of NETs. Cell reclustering was performed on NET-containing cells, and 5 clusters were identified (Fig. 2G). In the analysis of the number of cells in each cluster in different tissue sources, we found that the proportions of different neutrophil clusters differed across tissues. N3 cells were mainly derived from primary tumor tissue; N1, N2, and N5 cells were mainly derived from metastatic tissue; and N4 cells

were mainly derived from peripheral blood (Fig. 2H). We also looked at the highly expressed genes specific to these five clusters (Fig. 2I). N1 cells specifically expressed AREG, an exocytic protein that is closely related to cancer malignant growth, acquired drug resistance, and distant metastasis. There is a clear pathological relationship. APOE was significantly upregulated in N2 vs. the other clusters. APOE plays roles in many immune processes (including inhibition of T cell proliferation, regulation of macrophage function, promotion of lipid antigen presentation to natural killer T cells, regulation of inflammation and oxidation). LYVE-1 was highly expressed in the N3 cluster, and studies have shown that LYVE-1 can be used as a marker of lymphoid tissue and/or lymphangiogenesis. S100A12, a highly expressed gene in N4, is mainly expressed by neutrophils and monocytes and mainly plays a pro-inflammatory role in diseases. N5 cells highly expressed SPP1, and some studies have confirmed that SPP1 + tumor-associated macrophages (TAMs) mainly promote angiogenesis and tumor metastasis.

Pseudotime analysis, also known as cell trajectory analysis, simulates the developmental trajectories of different cells based on the expression patterns of time genes in single-cell samples. We extracted NET-containing cells to demonstrate their developmental trajectories, revealing two branches of NET-containing cell subtypes, describing the temporal sequence of cell subtype differentiation, with darker cells gradually transitioning to lighter cells (Fig. 2J). It shows that NET-containing cells exhibit 5 differentiation states during development. The cell subtypes showed a trend of N3 and N2 cell differentiation into N4 and N5 subtypes, respectively (Fig. 2K). The density changes of different cell subclusters over time were also examined in this analysis (Fig. 2L). The results showed that in the course of disease progression, metastasis-related NET-containing cells (N5) appear in the late stage of cell differentiation, indicating that NETs play a role in CRC metastasis.

Identification of tumor cells and metastatic cell clusters in colorectal epithelial cells

To further validate the mechanism of CRC metastasis, we utilized Inferno to identify malignant epithelial cells among colorectal epithelial cells (Supplementary Fig. 1A). Copy number variation (CNV) analysis has been widely used in scRNA-seq to investigate the evolution and development mechanisms of disease [46, 47].

To distinguish malignant from non-malignant clusters, we assessed CNV levels across all epithelial cells and per epithelial cell cluster (Supplementary Fig. 1B). Subcluster analysis was performed on the identified cancer cells, and 5 clusters were identified (Supplementary Fig. 1C). We assessed the distribution of these clusters in different

tissues (Fig. 3A); C2 and C5 clusters were considered primary tumor cells, and C4 cells were considered meta-static tumor cells.

We then performed differential expression analysis of these two cell clusters to identify key genes that play a role in tumor progression. KEGG enrichment analysis

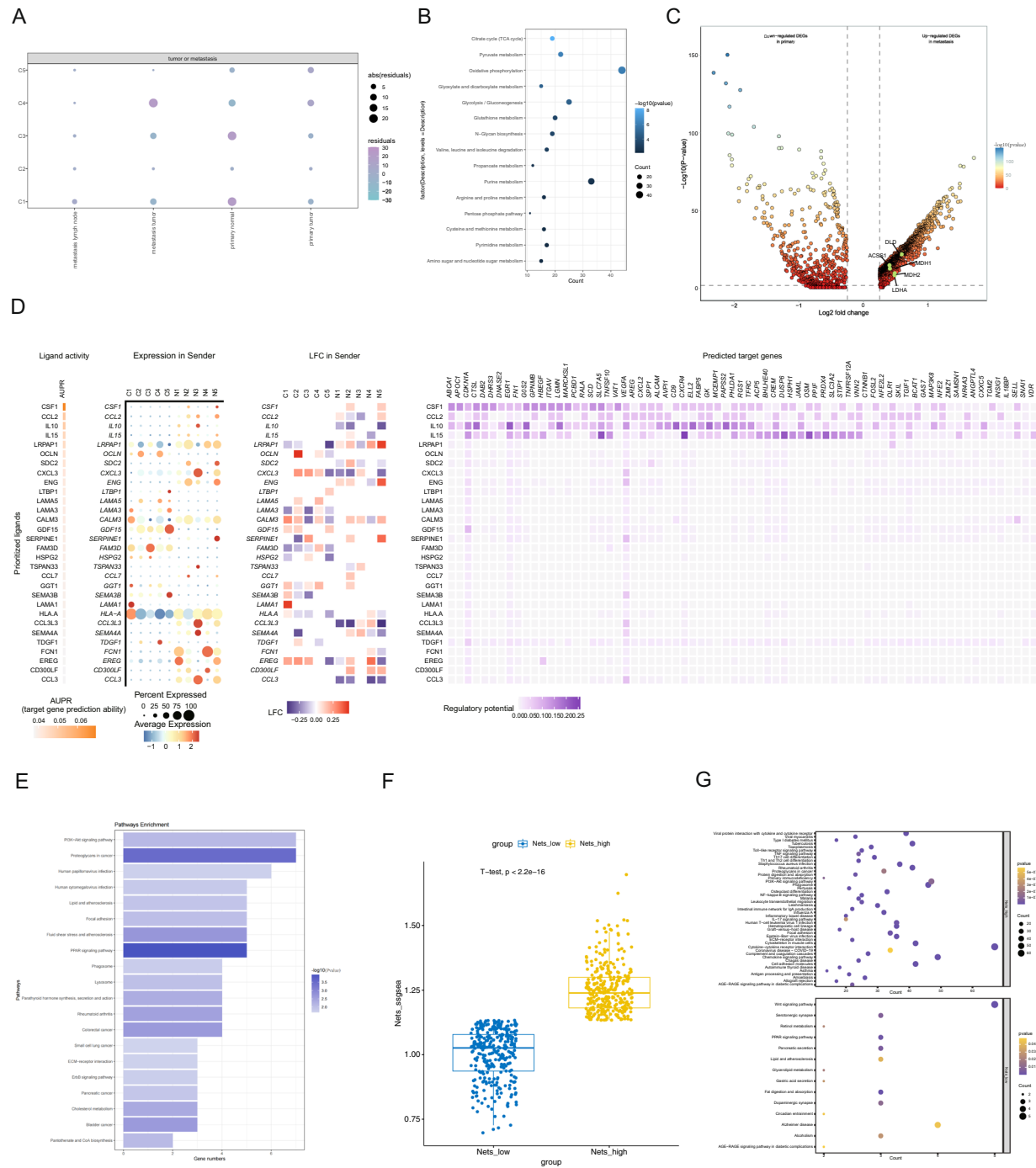


Fig. 3 Dynamics of cell–cell interaction networks in the tumor microenvironment of CRC tissues. **A** Distribution of different clusters of colorectal cancer cells in tissues. **B** KEGG enrichment analysis of colorectal cancer cell clusters. **C** Pathway significantly expressed genes. **D** Cell-to-cell communication between CRC and NETs. **E** KEGG enrichment analysis of C-ligand receptor target genes. **F** NETs marker gene differential analysis. **G** KEGG enrichment analysis

was performed on the genes with significant differences in expression between the primary tumor and metastatic tumor cell clusters (Fig. 3B), and the enriched pathways were intersected with metabolism-related pathways downloaded from the KEGG database to identify 15 enriched metabolism-related pathways such as glycolysis, pyruvate metabolism, propionate metabolism, and cysteine and methionine metabolism. Five of the highly expressed genes, LDHA, MDH1, MDH2, ASSC1, and DLD, were frequently involved in these pathways (Fig. 3C).

Validation of the role of NETs in CRC metastasis and the underlying mechanisms

The NicheNet method was used to predict the ligand–receptor pairs for cell–cell interaction analysis between CRC cells and NETs. The activity of the ligands was first assessed; we also assessed the expression of the ligands in the five cancer cell clusters (C1-5) and NET-containing cell clusters (N1-5) and the differential expression of these ligands in the primary and metastatic tumor cells. In addition, we predicted the targets of action of these ligand-receptor pairs (Fig. 3D). Furthermore, the strength of the interactions between these ligands and the predicted receptors is shown in the heat map (Supplementary Fig. 1D). Then, KEGG enrichment analysis of the predicted target genes of these ligands showed that these target genes were mainly enriched in the P13k-akt signaling pathway, PPAR signaling pathway, ECM-receptor interaction, and cholesterol metabolism (Fig. 3E).

Finally, we used the NET marker gene set to score TCGA samples (ssGSEA), divided the samples ($n=606$) into high and low score groups according to the median NET score (Fig. 3F), performed differential expression analysis on the high and low score groups, and performed KEGG enrichment analysis based on the significantly differentially expressed genes. The genes upregulated in the NET score group were significantly related to the P13k-akt signaling pathway, intestinal immune network for IgA production, and inflammatory bowel disease. The upregulated genes in the NET score group were mainly enriched in the Wnt signaling pathway, estrogen signaling pathway, and Hippo signaling pathway (Fig. 3G). Among these pathways, the PI3K signaling pathway activates LDHA and promotes glycolysis [48]. The occurrence of glycolysis in turn promotes the activation of PI3K pathway, which further promotes cell differentiation and proliferation [49]. PI3K/AKT signal transduction pathway is particularly important in the EMT process of tumor cells [50]. The PI3K/AKT pathway plays an important role in the regulation of E-cadherin (E-Cad) expression and in the process of EMT induction in coordination with other signaling pathways [51–53].

NETs and LDHA are highly expressed in CRC

In order to verify the expression of NETs markers and LDHA in CRC, we first used clinical sample tissue sections from CRC patients to detect the expression of NETs markers and LDHA in different stages of CRC tumors. The results showed that as CRC malignancy increases in terms of stage, the expression of markers of NETs and LDHA increase. The expression of cit-H3, CD66b and LDHA gradually increased, and their changing trends were consistent (Fig. 4A). Additionally, we conducted measurements on the levels of NETs and LDHA in the bloodstream of colorectal cancer patients at various stages. We discovered the expression of NETs and LDHA were significantly correlated with the advanced pathological grade of colorectal cancer. As the malignancy increased, so did the levels of these biomarkers (Fig. 4B,C). Furthermore, no significant associations were observed between LDHA and patient age, gender, N stage, or TNM stage (Table 1). In order to further verify the colocalization relationship between NETs and LDHA, at the same time once again to verify their positive correlation with tumor stage, we performed immunofluorescence analysis of tissue frozen sections and found that the expression of NET markers was upregulated in CRC tissues and correlated with stage (Fig. 4D). Previous studies have shown that CCDC25 is a transmembrane protein that can serve as a receptor for NETs, specifically bind to NET-DNA, trigger intracellular signals, enhance cell motility, and promote cancer metastasis [15, 54]. We found through Western blotting experiments that CCDC25 is expressed in HCT-116, HT-29, SW480, SW620 and Lovo cells (Fig. 4E). Moreover, the expression level was higher in HCT-116 and Lovo cell lines than in the other cell lines. We selected these two cell lines for subsequent experiments.

The proliferation, invasion, and migration abilities of CRC cells are enhanced after treatment with NETs

The proliferation and invasion of tumor cells are extremely complex processes that are regulated by various mechanisms and factors. Cytological tests were conducted in order to assess the impact of NETs on the phenotypic regulation of CRC cells in vitro. The experiment included three groups: the CTRL group, the NETs group, and the DNase-1 group. The follow-up experiment was carried out 24 h after the addition of 0.5 $\mu\text{g}/\text{ml}$ of NETs isolated from human peripheral blood to HCT-116 and Lovo cells in the NETs group. In a similar manner, NETs and DNase-1 were simultaneously used to treat the DNase-1 group for a full day. The proliferation capacity of HCT-116 and Lovo cells was increased following NETs activation, but it was partially decreased following DNase-1 treatment, according to the results of our EdU and plate colony formation assays (Fig. 5A,

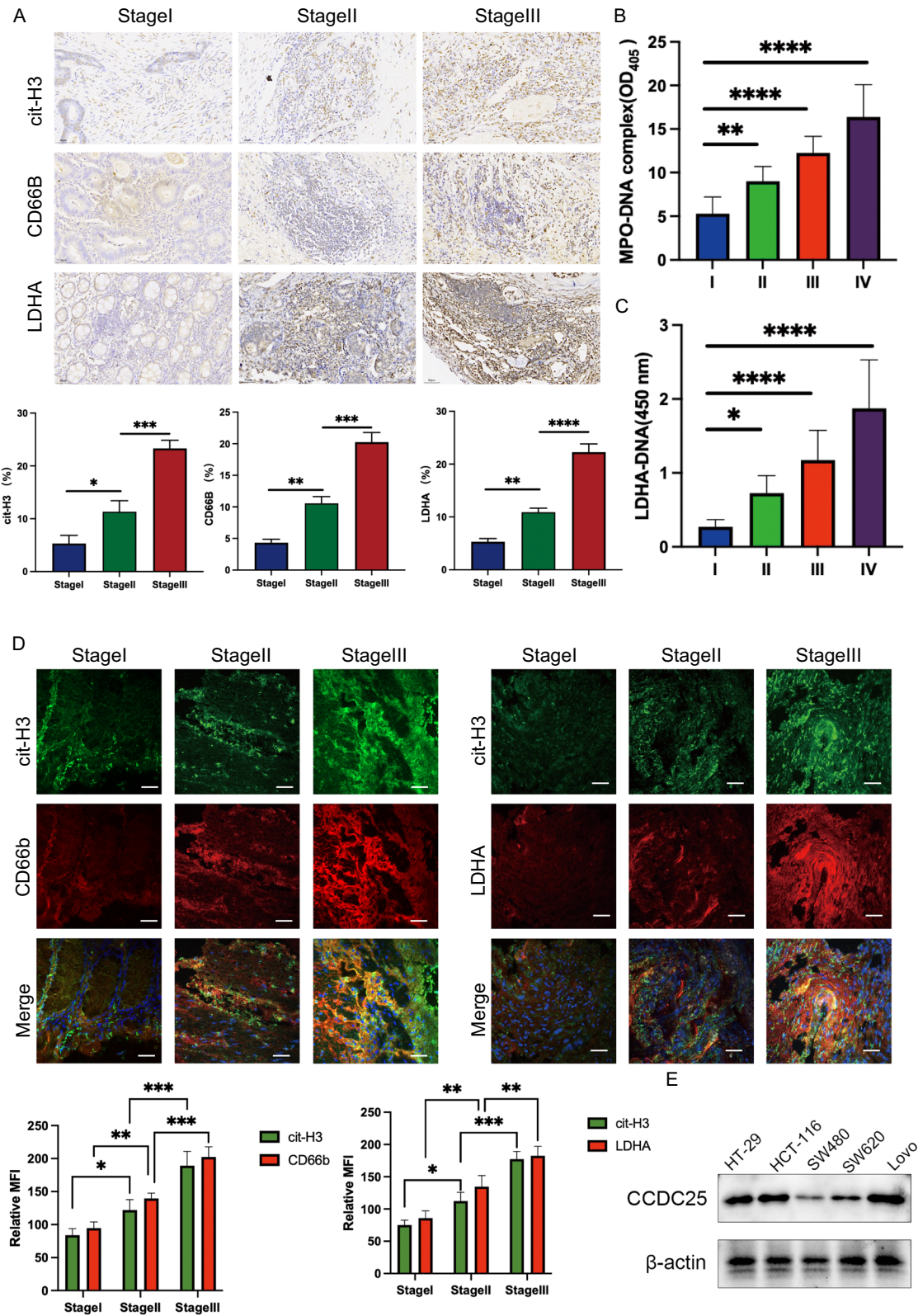


Fig. 4 (See legend on next page.)

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Fig. 4 Expression of nets in colorectal cancer. **A** Representative IHC staining images (40X, Scale bars, 50 μ m) of tissue specimens showing NENs expression at different stages of CRC tumorigenesis (n=20). **B** Determination of plasma NETs in patients with colorectal cancer of different stages using MPO-DNA ELISA. **C** Determination of plasma LDHA content in patients with colorectal cancer of different stages using ELISA method. **D** Confocal microscopy was used to detect the expression and spatial distribution of neutrophils and NETs in CRC tissues, and Image J software was used to analyze the expression levels and express them as mean fluorescence intensity (MFI). **E** Expression of CCDC25 in colorectal cancer cell lines. All values are mean $\pm$ SD. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

B). These findings imply that NETs have the potential to greatly accelerate the growth of CRC cells.

Invasion and migration play an important role in tumor development and metastasis. The role of NETs in the progression of CRC was explored through wound healing and in vitro transwell assay. The wound healing assay results showed that stimulation with NETs significantly promoted the migration of HCT-116 and Lovo cells (Fig. 5C). Our Matrigel-coated (for invasion) and Matrigel-uncoated (for migration) transwell experiments also showed that stimulation with NETs significantly promoted the invasion and migration of both cell lines (Fig. 5D).

Cancer stem cells are a group of cells that have self-renewal ability and strong invasion and migration capacities. They play an important role in tumor survival, proliferation, metastasis and recurrence [43]. The tumor sphere formation assay, which is used to assess spheroid size and number, is the gold standard for measuring the stemness of tumor cells. In order to study the effect of NETs on tumor cell stemness, we conducted a spheroid formation experiment. We found that stimulation with NETs significantly promoted the formation of tumor spheroids of CRC cells, while DNase-1 treatment reduced the number and size of the spheroids (Fig. 5E).

NETs can promote the invasion and migration of CRC cells by regulating LDHA

Next, we wanted to elucidate the specific molecular mechanisms by which NETs regulate CRC progression. LDHA is known to be a key enzyme in the glycolytic pathway. It plays an important role in the development of tumors. Here, we verified the regulation of LDHA by NETs. Once more, the experiment was split up into three groups: the Ctrl group, NETs group, and DNase-1 group. NETs and DNase-1 were used to activate HCT-116 and Lovo cells, and proteins were extracted for WB studies. The outcomes demonstrated that following NETs treatment, LDHA expression was likewise upregulated. Between the DNase-1 group and the Ctrl group, there was no discernible difference in the expression of LDHA (Fig. 6A).

In order to further verify the relationship between NETs and LDHA and the mechanism of action in CRC, cells were stably transduced with lentiviral particles carrying LDHA shRNA to generate stable LDHA knockdown cell lines. Western blotting verified the stable

knockdown of LDHA protein expression in cell lines (Supplementary Fig. 2A). We next verified the relationship between NETs and LDHA using three groups of cells: the NC group, NETs group, NETs + shLDHA group and shLDHA group. We performed EdU (Fig. 6B), plate colony formation (Supplementary Fig. 2B), wound healing (Fig. 6C) and transwell experiments (Fig. 6D) to verify our conjectures. The results showed that in the shLDHA group, the proliferation, invasion and migration abilities of HCT-116 and Lovo cells were reduced, and these abilities were partially restored in NETs + shLDHA group. All the above results indicate that the effect of NETs on the proliferation, invasion and metastasis of CRC is regulated by LDHA.

NETs regulate EMT protein levels in CRC through LDHA

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway is one of the most commonly activated pathways in human cancer, and it promotes tumor cell survival, proliferation, metabolism, invasion, and angiogenesis. Therefore, in the present study, we evaluated changes in the expression of proteins in this signaling pathway. Western blotting results for representative proteins showed that compared with control treatment, stimulation with NETs induced the expression of p-PI3K and p-AKT proteins in HCT116 and Lovo cells but had no effect on the total PI3K and AKT levels in HCT116 and Lovo cells. Application of DNase-1 to inhibit NET formation reduced the expression levels of p-PI3K and p-AKT proteins (Fig. 7A). More importantly, according to research, the PI3K/AKT signaling pathway can activate LDHA and promote glycolysis [48]. The ATP produced by glycolysis supplies energy to NETs and the PI3K/AKT signaling pathway, and glycolysis and its metabolite LDHA can in turn promote the production of NETs [44], thereby forming a positive feedback loop that promotes CRC progression and metastasis.

To better understand the effect of stimulation with NETs in CRC cells, we analyzed the levels of proteins related to the EMT process. Importantly, EMT mediates the progression and metastasis of CRC [57]. Therefore, we performed immunofluorescence and Western blotting experiments (Fig. 7B,C). We detected the changes in the expression of these proteins in CRC cells. stimulation with NETs inhibited the expression of E-cadherin protein in HCT-116 and Lovo cells, but the expression of

Table 1 Association of LDHA expression with clinicopathological parameters from colon cancer patients

Variables		LDHA expression		n	χ^2 value	p value
		Low	High			
Age(years)					0.44	0.833
	<55	19	12	31		
	>55	17	12	29		
Sex					0.423	0.515
	Female	21	16	37		
	Male	15	8	23		
Grade					5.338	0.013*
	I/n	34	2	38		
	III	6	20	22		
T stage					0.084	0.76
	1/2	11	13	24		
	3/4	18	18	36		
N stage					0.36	0.635
	NO	21	17	38		
	N1/N2	9	13	22		

Note: **p* < 0.05

N-cadherin and Vimentin was upregulated. These data indicate that NETs affect the EMT of CRC cells, thereby affecting the migration and invasion capabilities of tumor cells. In order to clarify whether the effect of NETs on the EMT process is through LDHA, we also performed the LDHA knockdown experiments (Fig. 7D,E), The results showed that the expression of the EMT-related proteins N-cadherin and Vimentin decreased, while the expression of E-cadherin protein increased in LDHA knockdown cells. LDHA knockdown partially attenuated the effects induced by NET stimulation. This suggests that LDHA knockdown suppresses the proliferation, invasion and migration of CRC cells and that NETs can regulate the effects of LDHA on CRC. Therefore, we verified the role of the NETs/LDHA/EMT signaling axis in CRC metastasis.

Inhibitory effects of inhibition of NET formation and downregulation of LDHA expression on CRC cell metastasis in vivo

We further explored the effect of the NETs/LDHA/EMT signaling axis on CRC progression in vivo. First, We found that knock LDHA and use the DNase—1 can reduce the growth rate of Lovo cells in vivo(Fig. 8A). Immunofluorescence assay showed that the expression of LDHA in the Control group was significantly higher than that in the DNase-1 and shLDHA groups(Fig. 8B). We used a metastasis-derived CRC cell line (Lovo) to establish a mouse liver metastasis model by injecting the tumor cells into the spleen of nude mice. Six weeks later, the mice were sacrificed for analysis of the liver metastasis of tumor cells, and H&E staining showed that LDHA knockdown together with NETs inhibitor treatment significantly suppressed tumor growth. The number of

liver metastases was significantly reduced in the LDHA knockdown group and NETs inhibitor group compared with NC group (Fig. 8C,D). Moreover, the liver metastases of nude mice injected with NETs inhibitor were also significantly smaller than those in the NC group (Fig. 8E). In conclusion, inhibition of NET formation and downregulation of LDHA expression attenuated the liver metastasis of CRC cells in vivo.

Discussion

NETs are extracellular structures composed of chromatin and decorated with granule proteins, and neutrophils deploy these structures to ensnare and neutralize microorganisms [58, 59].In this study, we show the NETs play in important role in the development of CRC liver metastasis.

ScRNA-seq revealed that NETs account for a smaller proportion of cells in primary tumors than in metastases. We showed that NETs are transformed in the progression of CRC and that NET-containing cells interact with CRC cells. Further, the ability of CRC cells to proliferate, invade and migrate is enhanced in the presence of NETs. NETs can promote the invasion and migration of CRC cells by regulating LDHA and can regulate EMT protein levels via LDHA. Finally, we showed that NETs regulate CRC progression through PI3K/AKT signaling pathway and lactylation modification (Fig. 9).

While the mechanisms of action of NETs in cancer cells are unclear, recent evidence indicates that NETs may promote cancer progression. Park et al. demonstrated that lung metastasis can ensue following injection of 4T1 metastatic breast cancer cells into the tail veins of LysM-EGFP mice, accompanied by pronounced formation of NETs within lung metastatic foci. Immunofluorescence

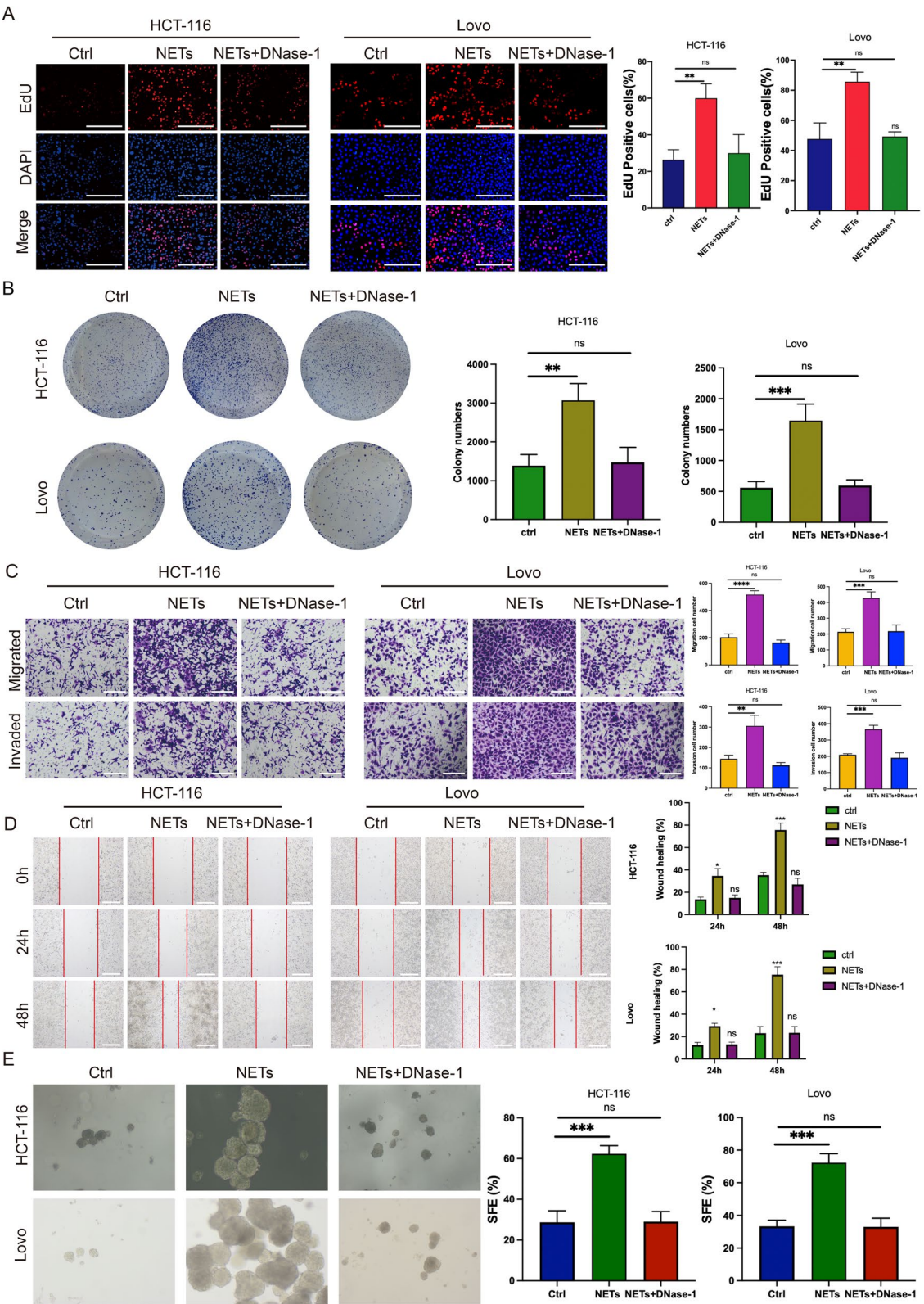


Fig. 5 NETs promote colorectal cancer cell proliferation, migration and invasion. **A** EdU experiment and **B** Plate cloning experiment to detect the proliferation ability of CRC cells HCT-116 and Lovo after NETs and DNase-1 treatment. **C** Wound healing detection after HCT-116 and Lovo cell NETs stimulation or DNase-1 treatment. **D** HCT-116 and Lovo cell migration and invasion assay after NETs stimulation or DNase-1 treatment. **E** Sphering test reflects the stemness of colorectal cancer cells after NETs stimulation. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

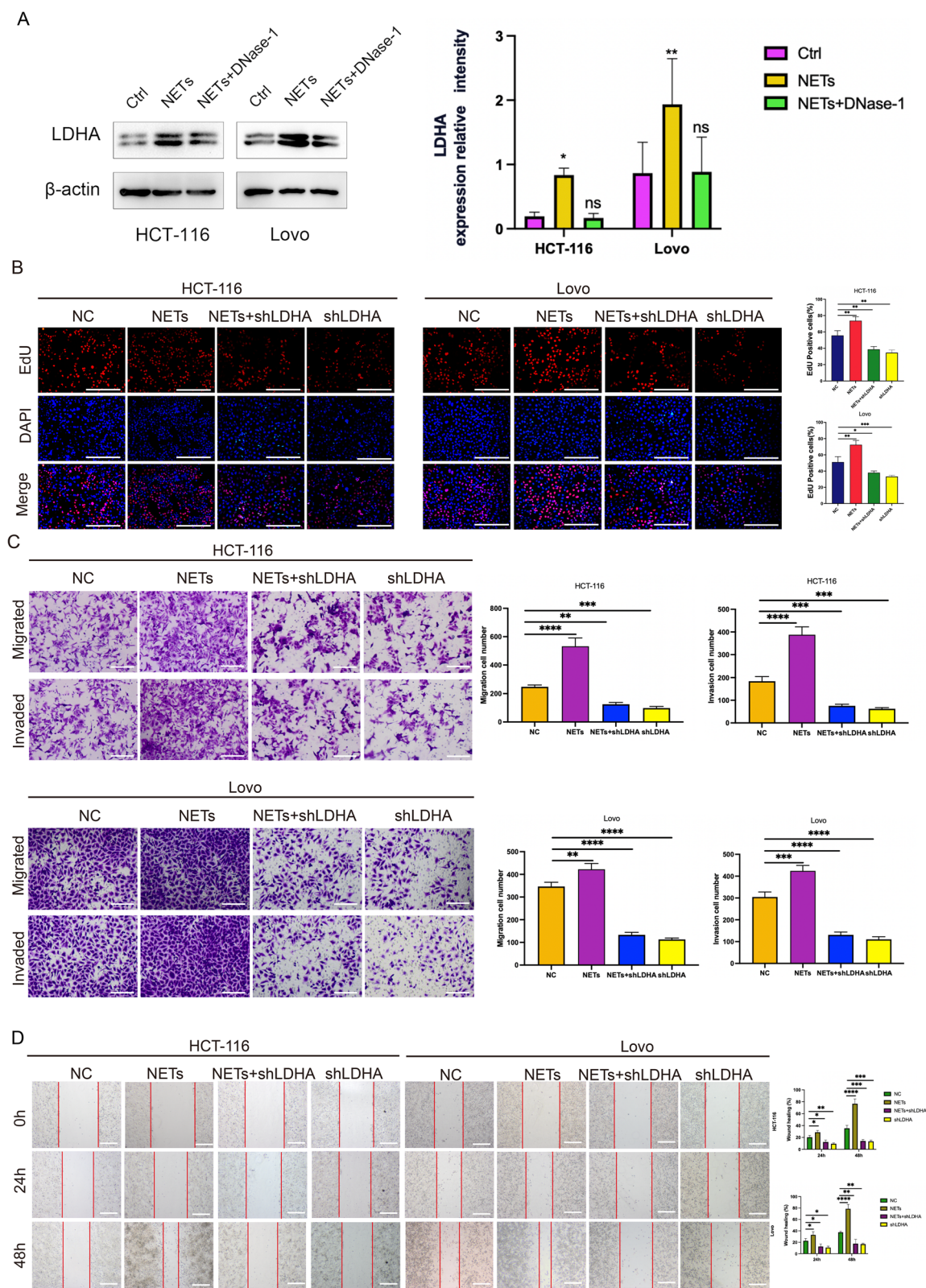


Fig. 6 The proliferation, migration and invasion abilities of colorectal cancer cells are reduced after the expression of LDHA is reduced. **A** The expression changes of LDHA after NETs stimulation. **B** Changes in the proliferation ability of HCT-116 and Lovo cells stimulated by NETs after shLDHA. **C** Wound healing ability of HCT-116 and Lovo cells in NC group and shLDHA group after NETs stimulation or DNase-1 treatment. **D** Detection of migration and invasion abilities of HCT-116 and Lovo cells after stimulation with shLDHA and NETs. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

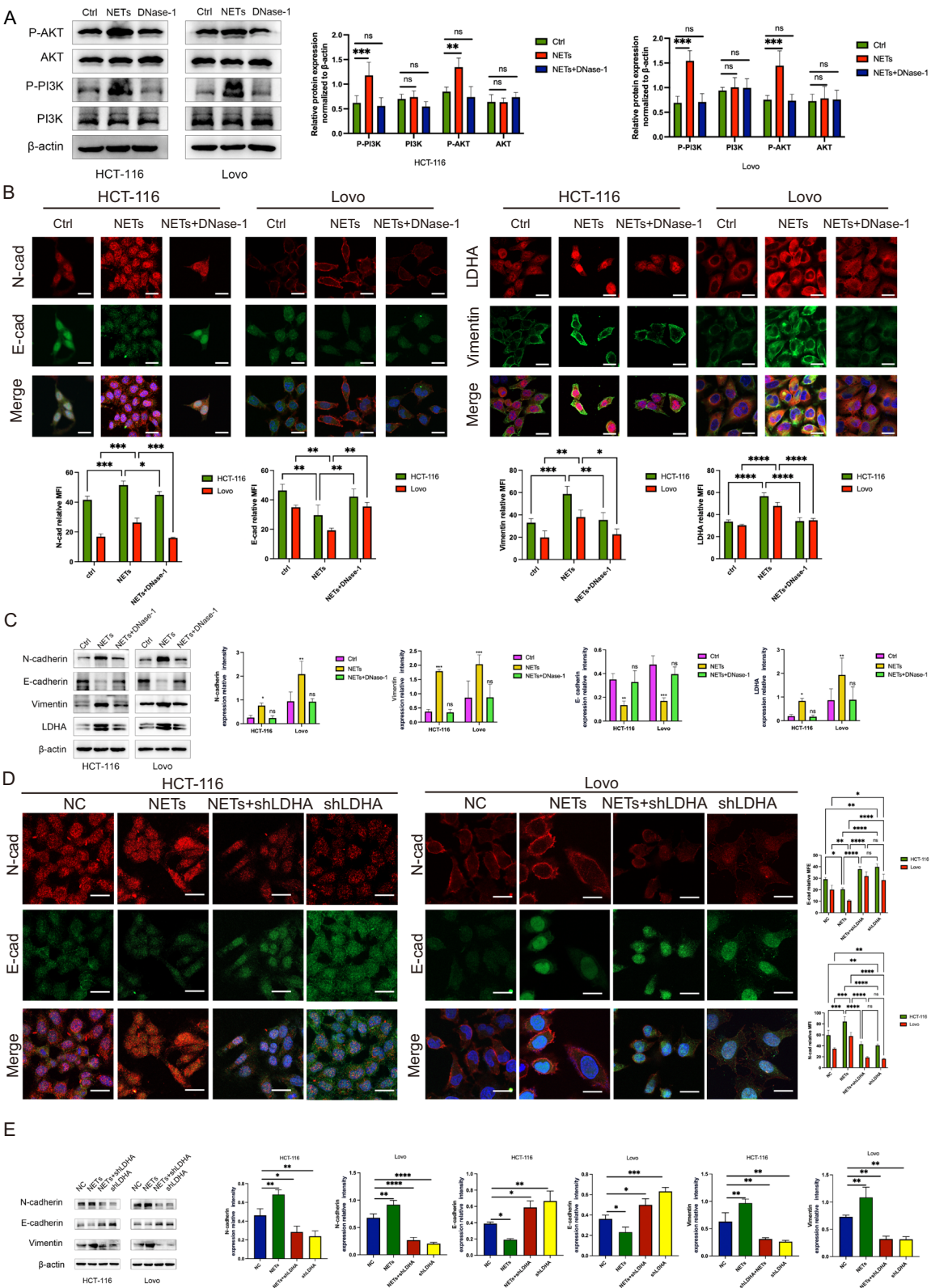


Fig. 7 NETs regulate EMT levels in colorectal cancer through LDHA. **A** Western blot analysis of the expression of p-PI3K, PI3K, p-AKT and AKT after NETs stimulation. **B,C** Immunofluorescence H and Western blot diagrams of the expression of emt-related proteins and LDHA in HCT-116 and Lovo cells after NETs stimulation. **D,E** Immunofluorescence and Western blot images of emt-related protein expression in HCT-116 and Lovo cells after NETs treatment after LDHA knockdown. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Scale bar: 50 μ m.

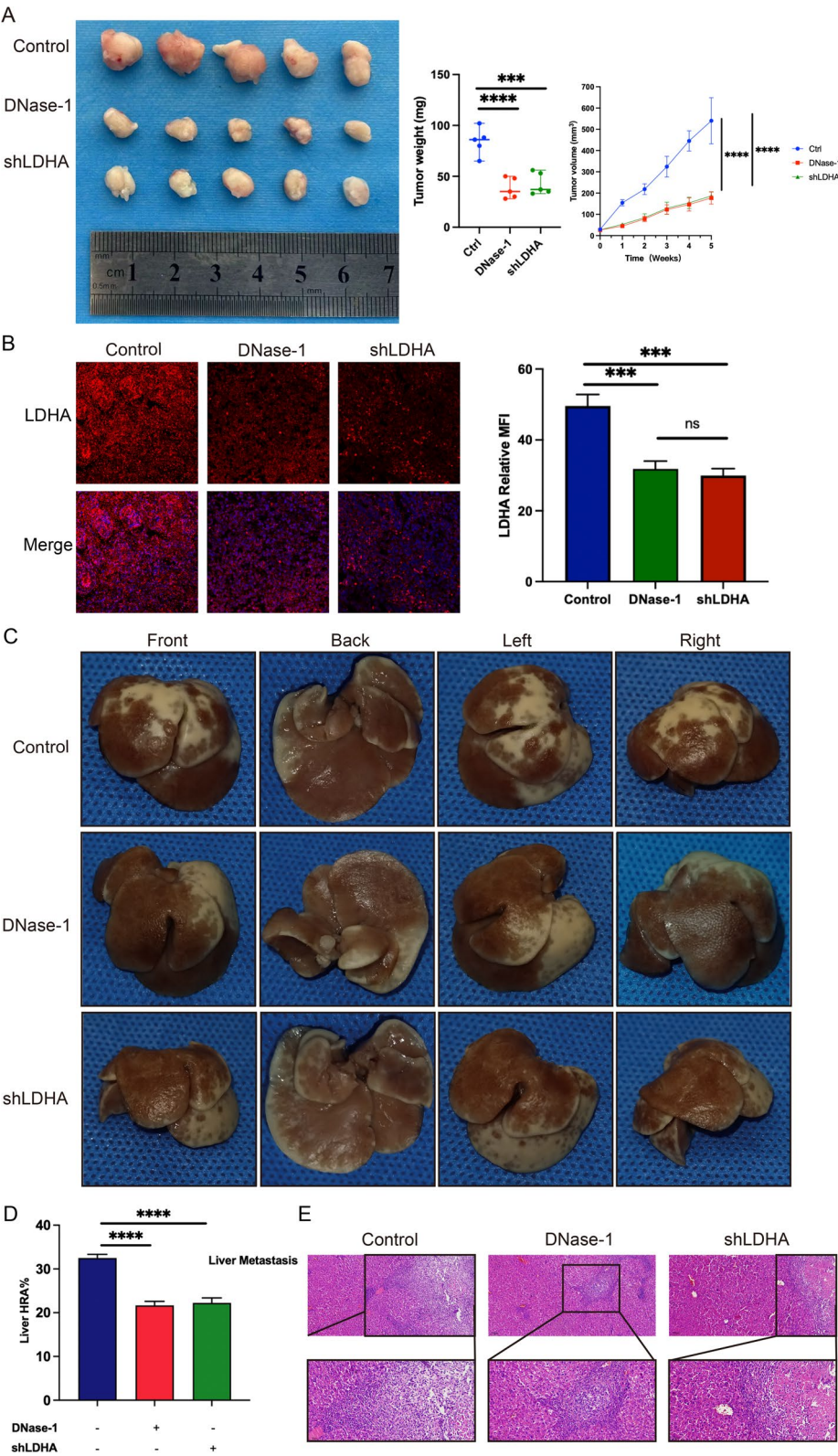


Fig. 8 Inhibitory effect of down-regulating NETs and LDHA expression on colorectal cancer cell metastasis in vivo. **A** LDHA knockdown inhibits the growth of tumours in vivo, and tumour growth curves as well as tumour weight show the inhibitory effect of LDHA. **B** LDHA expression in different groups. **C** Liver metastatic tumor samples from nude mice control group, DNase-1 group and shLDHA group (n=5). **D** The proportion of liver tissue replaced by hepatic metastatic tumor was counted as the liver replacement area (HRA%). (*****P* < 0.0001, n = 5) **E** HE staining was used to detect the tissues in the control group, DNase-1 group and shLDHA group. All results are presented as mean ± SD.

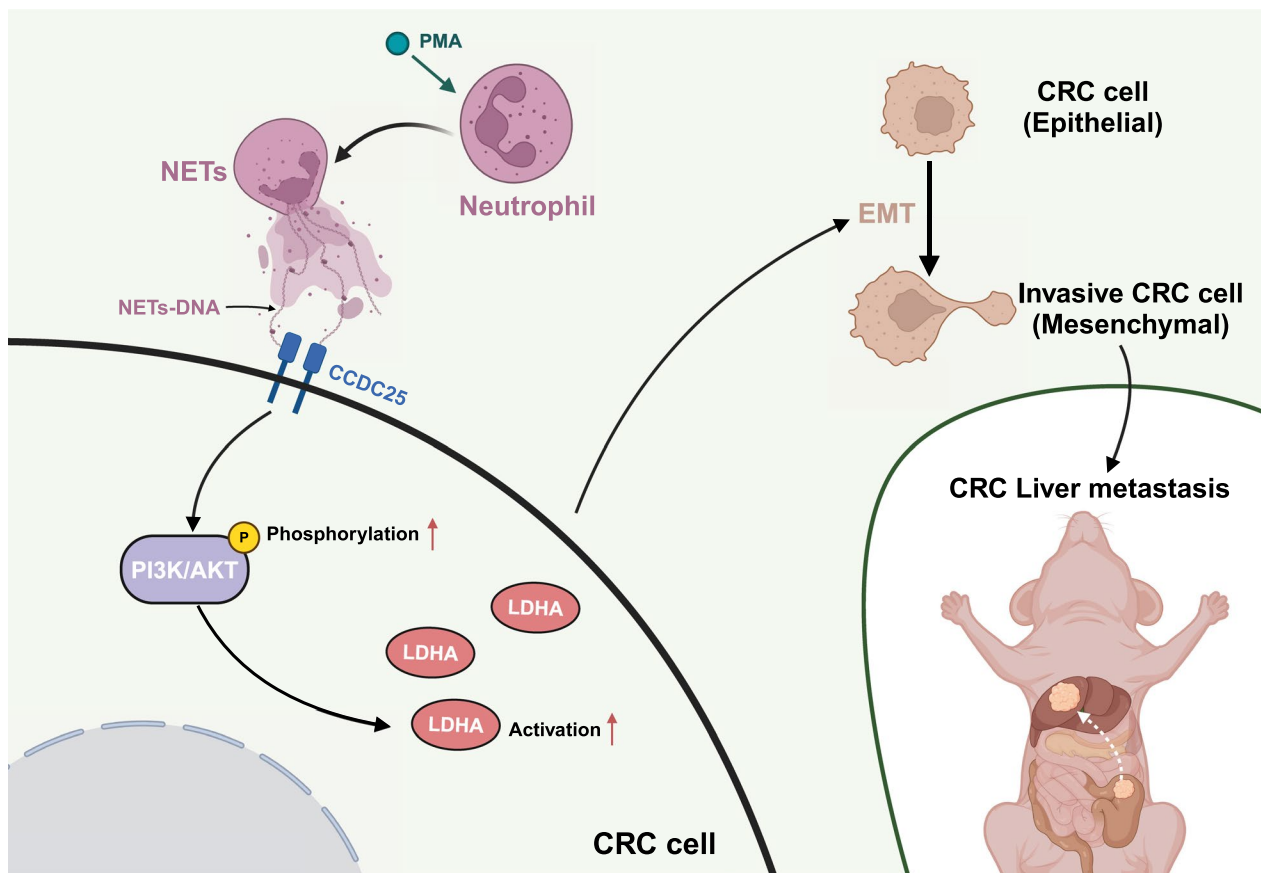


Fig. 9 Schematics highlighting the major findings of this study. Overview of the mechanism by neutrophil extracellular traps (NETs) promote colorectal cancer liver metastasis. Elevated expression of NETs interacts with the transmembrane protein CCDC25 via NETs-DNA, leading to activation of the PI3K/AKT pathway and subsequent upregulation of LDHA, after that inducing epithelial-to-mesenchymal transition (EMT) in colorectal cancer cells and promoting liver metastasis.

assays conducted on lung tissue sections revealed a significant increase in the number of neutrophils undergoing NETosis four days post tumor cell injection, and administration of DNase-I decreased the number of lung tumor metastases formed in mice [60]. Our previous study showed that NETs promote angiogenesis in gastric cancer. However, the effect of NETs in CRLM is unclear. In the present study, we explored the biological and clinical significance and underlying mode of action of NETs in CRC liver metastasis.

Within the tumor microenvironment, tumor cells can directly induce the formation of neutrophil extracellular traps (NETs). Tumor cells commonly express granulocyte colony-stimulating factor (G-CSF) and CXCL1. Elevated G-CSF levels induce the linear expansion of myeloid progenitor populations, including megakaryocyte-erythroid progenitors (MPPs), granulocyte-monocyte progenitors (GMPs), and mature neutrophils. Other tumor-derived cytokines, such as IL-1, IL-6, interferon-gamma (IFN- γ), and tumor necrosis factor-alpha (TNF- α), further promote granulopoiesis in the bone marrow. This collectively leads to an expansion of mature, naïve neutrophils in the

circulation, thereby increasing the NET-forming capacity of the neutrophil population [61–64]. In pancreatic ductal adenocarcinoma (PDAC), IL-17 recruits neutrophils into the pancreatic tumor microenvironment. Higher expression of both IL-17 and peptidylarginine deiminase 4 (PADI4) in human PDAC correlates with poorer prognosis, and serum from PDAC patients exhibits enhanced NETotic potential [14]. Furthermore, studies in various murine cancer models demonstrate that neutrophils can form NETs within tumors. These NETs contribute to the maintenance of tumor blood supply and nutrient support by promoting angiogenesis, primarily through their associated matrix metalloproteinase 9 (MMP9) [65–67].

Large deposits of NETs are found in the blood and tumor tissue patients with many types of solid tumors. Lin et al. found that more NETs were present in triple-negative breast cancer (TNBC) than in non-TNBC tissues, and the abundance of NETs was significantly related to tumor size, Ki67 levels and lymph node metastasis in TNBC patients [68]. Moreover, the study also found that the higher the number of NETs, the greater the risk of tumor recurrence in patients [15]. Elwan et al.^[71] found

significantly increased numbers of myeloid-derived suppressor cells (MDSCs) in the peripheral blood and ascitic fluid of cirrhotic and HCC patients, which suppress immune responses and promote tumor development. Analogously, our study reveals that NETs also play a role in CRC liver metastasis, suggesting that multiple cellular components in the tumor microenvironment may synergistically promote tumor invasion and metastasis. Our results reveal that NET markers are highly expressed in CRC. In order to further verify the relationship between NET marker expression and tumor stage, we performed immunofluorescence analysis of tissue frozen sections and found that the expression of NET markers was upregulated in CRC tissues vs. normal controls and was correlated with stage. Therefore, NETs are candidate markers for CRC risk and treatment response prediction.

Reminiscent of the classification of LDHA is known to be a key enzyme in the glycolytic pathway. It plays an important role in the development of tumors. Lactate serves not only as an energy substrate for tumor cells but also influences tumor proliferation, invasion, and distant metastasis by mediating processes such as immune evasion and angiogenesis within the tumor microenvironment [70]. Furthermore, intracellular lactate can directly bind to N-myc downstream regulated gene 3 (NDRG3). This binding prevents NDRG3 from interacting with prolyl hydroxylase 2 (PHD2), thereby blocking its PHD2-mediated degradation. Consequently, intracellular NDRG3 accumulates and stimulates tumor neovascularization through activation of the Raf-ERK signaling pathway [37].

Relevant studies have also proven that the process by which NETs promote metastasis is related to glycolysis energy supply and the accumulation of lactic acid [71], and lactic acid plays an indispensable role in tumor progression and metastasis [72]. The high predictive accuracy of serum markers such as sPD-L1 for HCC metastasis [73], which provides a reference paradigm for the clinical application of netS-derived markers in colorectal cancer liver metastasis. For instance, treatment with the tyrosine kinase receptor inhibitor lenvatinib recruits neutrophils to hepatocellular carcinoma (HCC) tissues. These recruited neutrophils predominantly polarize towards an N2 phenotype and exhibit high expression of programmed death-ligand 1 (PD-L1), which significantly compromises the therapeutic efficacy of lenvatinib. Tumor cell-derived lactate plays a critical role in shaping this immunosuppressive phenotype. Mechanistically, lactate synergizes with interferon-gamma (IFN γ) derived from immune cells to upregulate PD-L1 expression on neutrophils by activating the nuclear factor kappa B (NF- κ B) signaling pathway and cyclooxygenase (COX) activity. Consequently, co-administration of the COX inhibitor celecoxib with lenvatinib significantly enhances

therapeutic efficacy [74]. Therefore, we actively explored the specific action mechanisms of NETs and LDHA in CRC. Our scRNA sequencing result showed significant differences in expression between the primary tumor and metastatic tumor cell clusters, and LDHA-related pathways were commonly enriched in the KEGG analysis. Overall, this study explored the mechanism by which NETs affect CRC progression through LDHA and provides a theoretical basis for clinical treatment of CRC.

The PI3K/AKT signaling pathway is involved in metastasis and is activated in various cancers including CRC [38, 75]. Our results showed that AKT signaling pathway activation and its downstream effects were required for CRC liver metastasis. We found that NETs increased P-PI3K/P-AKT activity but had minimal impact on PI3K and AKT expression. Recovery experiments confirmed that PI3K/AKT signaling pathway activation contributes to the oncogenic effects of NETs in CRC cells. We observed a positive correlation between NETs and p-AKT in CRC cells. Therefore, the combination of NETs and P-AKT had a greater prognostic value than either factor alone. We concluded that NETs promotes CRC liver metastasis, at least in part, by activating the PI3K/AKT signaling pathway.

Further confirmation of the NETs promote metastatic potential in CRC cells was carried out by the investigation of the expression of EMT markers such as E-cadherin, N-cadherin, and vimentin. Opposite effects on the expression of E-cadherin (downregulated) and N-cadherin (upregulated) were observed, and high levels of vimentin were detected mainly in the Lovo cell line upon treatment with NETs. This indicates that high LDHA expression in CRC cells leads to a more aggressive phenotype and increased susceptibility to cancerous and metastatic ability, similar to the effects of NETs treatment. Of note, in order to further verify the relationship between LDHA and EMT, we knocked down LDHA, which suppressed the proliferation, invasion and migration of CRC cells; these effects were partially attenuated in NETs group. All the above results indicate that NETs promote CRC cell EMT by regulating LDHA expression.

Critically, these findings establish the NETs-LDHA axis as a novel and druggable therapeutic vulnerability in CRC metastasis. Our work is the first to identify NETs—extracellular components of the tumor microenvironment—as direct upstream regulators of LDHA-driven metabolic reprogramming and EMT in cancer cells, revealing a previously unrecognized immune-metabolic crosstalk. Mechanistically, this defines LDHA not merely as a glycolytic enzyme but as an essential mediator of NETs-induced phenotypic plasticity. Therapeutically, this axis provides a strategic window for dual-targeting interventions: disrupting NETs formation (e.g., via DNase I or PAD4 inhibitors) concurrently with inhibiting LDHA

activity (e.g., using FX11 or GSK2837808A) to synergistically block metastatic progression at its microenvironmental trigger (NETs) and intracellular effector (LDHA). This approach overcomes limitations of single-target therapies and offers a clinically actionable strategy to mitigate metastasis in high-risk patients exhibiting elevated NETs and LDHA.

This study also has several limitations. First, the single-cell data were derived solely from public datasets, and our clinical sample size was relatively small. Consequently, the findings require further validation through analysis of larger-scale clinical cohorts. We plan to address this by conducting more extensive validation in subsequent research. Secondly, the investigation into the mechanism of LDHA modification mediated by NETs lacks comprehensive mechanistic insights. Therefore, in future work, we aim to perform further experiments to validate these findings and elucidate the underlying molecular pathways in greater detail.

Conclusions

The present study demonstrated that LDHA is upregulated in the presence of abundant NETs in CRC cells. It also showed that when they are present in high levels, NETs promote CRC liver metastasis both in vitro and in vivo by regulating EMT and activating the PI3K/AKT signaling pathway. These results indicate a central role of NETs in CRC progression and suggest that NETs have potential therapeutic value in the treatment of CRC. It could therefore be useful to design inhibitors targeting NET markers or to use NET inhibitors to control CRC liver metastasis. This hypothesis should be tested in a prospective study in patients with CRC liver metastasis following surgery. Therapeutically, dual targeting of NETs formation and LDHA activity synergistically blocks liver metastasis in colorectal cancer through intracellular effector mechanisms, providing a clinically actionable strategy for CRC patients. Further investigation into the role of neutrophil extracellular traps (NETs) in colorectal cancer metastasis will establish a robust theoretical foundation for discovering novel therapeutic targets, paving the way for precision medicine and personalized treatment.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-025-07174-y>.

Supplementary material1

Supplementary material2

Supplementary material3. **A** Identification map of Infercnv in epithelial cells. **B** Violin plot of CNV levels in clusters of epithelial cells. **C** Clustering of cancer cells. **D** Heat map of cellular ligand and receptor interactions in CRC and NETs

Supplementary material4. **A** Knockdown efficiency of LDHA. **B** Plate clon-

ing assay was used to verify the effect of shLDHA on the proliferation of HCT 116 and Lovo cells stimulated by NETs

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Not applicable.

Author contributions

NL: bioinformatics analysis, manuscript writing, vitro assays. TJ and HY: prepare tissue and conduct immunohistochemistry, animal experiment. XL: Conduct immunohistochemistry, manuscript editing. SK and XZ: experimental design, manuscript editing. CX: performed initial bioinformatics analysis. QH and JZ: bioinformatics analysis supervision. SY: bioinformatics analysis, graphical visualization. BS: conceptual design, manuscript revision, providing fund.

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Data availability

All the data and material could be traced from the paper or can be requested from the corresponding author.

Declarations

Ethics approval and consent to participate

Our study was approved by the Ethics Committee of the second affiliated hospital of Harbin Medical University (KY2023-012).

Consent for publications

All the listed authors have participated in the study, and have seen and approved the submitted manuscript.

Competing interests

The authors declare no conflict of interest.

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