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Original Article

Neutrophil extracellular traps promoting fibroblast activation and aggravating limb ischemia through Wnt5a pathway

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Abstract: Although the formation of NETs contributes to cancer cell invasion and distant metastasis, its role in the pathological progression of limb ischemia remains unknown. This study investigated the functional significance of NETs in cell-cell crosstalk during limb ischemia. The changes of cell subsets in lower limb ischemia samples were detected by single-cell RNA sequencing. The expression of neutrophil extracellular traps (NETs) related markers in lower limb ischemia samples was detected by immunohistochemistry and Western blotting. The signaling pathway of NETs activation in fibroblasts was verified by immunofluorescence, PCR and Western blotting. Through single-cell RNA sequencing (scRNA-seq), we identified 9 distinct cell clusters, with significantly upregulated activation levels in fibroblasts and neutrophils and phenotypic transformation of smooth muscle cells (SMCs) into a proliferative state in ischemic tissue. At the same time, the interaction between fibroblasts and smooth muscle cells was significantly enhanced in ischemic tissue. NETs levels rise and fibroblast activation is induced in ischemic conditions. Mechanistically, activated fibroblasts promote smooth muscle cell proliferation through the Wnt5a pathway. In ischemic mice, inhibition of Wnt5a mitigated vascular remodeling and subsequent ischemia. These findings highlighting the role of cell-cell crosstalk in ischemia and vascular remodeling. We found that the NETs-initiated fibroblast-SMC interaction is a critical regulator of limb ischemia via Wnt5a pathway, a potential therapeutic target for the treatment.

Keywords: Single cell RNA sequencing, limb ischemia, neutrophil extracellular traps, fibroblasts, inflammation

Introduction

The incidence and mortality rates of peripheral vascular diseases, particularly chronic limb ischemia (CLI), are increasing annually [1, 2]. Impaired limb blood flow can lead to tissue damage, non-healing wounds, and even amputations. Additionally, limb ischemia patients often have a high prevalence of comorbidities such as diabetes, hypertension, dyslipidemia, obesity, and renal impairment [3, 4]. These comorbid conditions exacerbate the already compromised vascular function and impede tissue healing processes.

In recent years, the role of neutrophils in cardiovascular diseases has attracted attention. Under physiological conditions, neutrophils not only participate in microbial defense, but also regulate various physiological processes such as angiogenesis [5, 6]. When stimulated, neutrophils are activated and release neutrophil extracellular traps (NETs). NETs are composed of nuclear DNA, histones, and various intracellular proteins [7]. Many studies have found that NETs play crucial roles in tumors, including cancer cell invasion, cancer immune editing, and interactions between cancer cells and the immune system [8]. Albrengues et al. discovered that NETs have the ability to awaken dormant cancer cells. NETs released during chronic pulmonary inflammation can awaken dormant breast cancer cells and promote their metastasis and dissemination [9]. Systemic

dissemination and distant organ metastasis are the main causes of cancer-related deaths. The study has confirmed a significant correlation between NETs and liver metastasis in breast cancer and colorectal cancer patients. In the tumor microenvironment, the binding activity of CCDC25 with NETs DNA increases, thereby activating the ILK-β-parvin pathway to attract cancer cells [10]. Furthermore, NETs facilitate cancer cell migration by releasing MMP and NE to degrade the extracellular matrix [9]. The occupant lesion formed by the distant metastasis and settlement of cancer cells may cause vascular stenosis, leading to limb ischemia. However, the impact of NETs on limb ischemia caused by vascular stenosis remains unknown.

Vascular fibrosis is a crucial marker of limb ischemia, involving different types of fibroblasts [11]. Previous studies have indicated that fibroblasts can be activated by cytokines released by macrophages [12, 13]. There is also evidence suggesting that NETs can stimulate fibroblast proliferation [14]. However, little is known about the interactions among neutrophils, fibroblasts, and smooth muscle cells in peripheral arterial diseases. In our study, through panoramic analysis of non-skeletal muscle cells from limb ischemia patients, we identified and described specific intercellular communication patterns between these cells. Furthermore, we demonstrated that NETs can activate the non-canonical WNT signaling pathway in the communication process between fibroblasts and smooth muscle cells. These findings contribute to a better understanding of smooth muscle cell proliferation and the mechanisms underlying peripheral vascular diseases.

Methods

Human amputation specimens

Lower limb ischemic tissue samples were obtained from patients with arteriosclerosis obliterans (ASO) who underwent amputation. Healthy lower limb tissue samples were collected from traumatic patients who also underwent amputation. The baseline characteristics of the patients are summarized in Table S1. All human lower limb amputation specimens were obtained from Department of Vascular Surgery at the First Affiliated Hospital of Zhejiang University School of Medicine in accordance with the Institutional Review Board-approved protocol for use of discarded human tissues (institutional review board approval No. 2020/98). All experiments were conducted according to the principles expressed in the Declaration of Helsinki. Approval was granted by the Research Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (approval No. 2020/98).

Mouse model of hindlimb ischemia

The animal experiments conducted in this study have received ethical approval from the Institutional Animal Research Ethics Committee of the School of Medicine, Zhejiang University, and adhere to the national guidelines established by the Ministry of Science and Technology of China. Adult male C57BL/6 mice weighing approximately 20-25 g were obtained from the Experimental Animal Limited Company of Zhejiang University (Hangzhou, China) and housed at the Animal Experimental Center of the First Affiliated Hospital, School of Medicine, Zhejiang University. Throughout the experiment, the mice were maintained on a 12-hour light/dark cycle and housed in well-ventilated and clean cages at a constant temperature of 22±1°C. They had ad libitum access to standard experimental feed and water. Place the mice in an anesthesia induction box connected to a vaporizer, with the vaporizer set to 3.0 Vol% of isoflurane and a 100% oxygen flow of 1 L/min, to induce global anesthesia. In the surgical group, the femoral artery of the left lower limb was ligated. In the sham surgery group, the femoral artery was not ligated. At the end of the experiment, euthanasia was performed through intraperitoneal injection of an overdose of sodium pentobarbital. Subsequently, limb tissues were harvested for further subsequent experiments.

Neutrophil extracellular traps extraction

Add 5 ml of neutrophil separation medium (LSM) agent A (Solarbio, P9040) into a new tube at RT and move blood sample (about 5 ml) carefully above the LSM layer, creating a sharp LSM-blood interface. Centrifuge at 800 g for 30 min at RT without break, after which will present two milky layers. The monocyte is on the top layer while neutrophil layer is on the bottom. Move the neutrophil layer into a clean new

tube and wash with 10 ml PBS. Centrifuge at 500 g for 10 min at RT and abandon supernatant twice. Then resuspended neutrophil in RPMI 1640 (Invitrogen™, 11875093) for further experiment. And 1.8×10^6 cells were seeded per well in six-well plates. Following stimulation with 50 nM PMA for 4 h, medium was removed and wells were washed with RPMI. PMA, at this concentration, does not promote apoptosis or necrosis; rather, it induces typical features of NET formation. To collect NETs, 2 ml RPMI per well was added and NET was collected in 15-ml tubes by vigorous agitation. After centrifugation at 20 g for 5 min, NET was collected in the supernatant and subjected to different treatments. In selective experiments, NETs were isolated and the concentration of NET-DNA was determined with Quant-iT™ PicoGreen™ dsDNA Assay Kits and dsDNA Reagents (Invitrogen, P11496). Isolated NETs were either used immediately or flash-frozen in LN for storage at -80°C.

Western blotting

Cells and muscle tissue were lysed on ice using RIPA lysis buffers. The protein concentration was determined using the BCA protein assay kit (Beyotime). The proteins were resolved by SDS-PAGE and transferred onto a polydiethylene fluoride membrane (PVDF) (Millipore, 30100-40001). The membrane was then incubated with the primary antibody and the secondary antibody. Western blot images were captured using the BIO-RAD ChemiDoc MP multi-functional chemiluminescence imager. Band intensity was quantified using Image J software (NIH, USA). Details of the primary antibodies used in this study can be found in Table S2.

RT-qPCR

RNA was extracted using TRIzol reagent (Takara, 9109). PrimeScript RT reagent Kit (Takara, RR037Q) and specific reverse transcription primers were used for RNA reverse transcription. GAPDH was used as an internal control for mRNA-related experiments. Quantitative real-time polymerase chain reaction (RT-qPCR) was carried out using SYBR Green PCR Master Mix (TaKaRa Biotechnology). The PCR primers used in this study are listed in Table S3.

SiRNA transfection

Fibroblasts were seeded onto 6-well plates. When the cell density reached 70%, siRNA transfection was performed using Opti-MEM™ I reduced serum medium (Gibco, 31985070) and Lipofectamine RNAiMAX transfection reagent (Invitrogen, 13778150). The cells were transfected with control siRNA and wnt5a siRNA. After 6 hours of transfection, the complete culture medium was replaced. The cells were then further incubated at 37°C with 5% CO_2 for 48 hours before subsequent analysis.

In vivo, the concentration of wnt5a siRNA was 10 ug/g. Starting on day 0, once every 3 days for 2 weeks, intramuscular injection.

Edu in proliferation essay

After the cells reached stable growth, they were incubated with EdU solution. Subsequently, fixation was performed using 4% paraformaldehyde. Following fixation, according to the manual, the corresponding staining solutions and Hoechst solution were added sequentially for staining. All imaging was acquired using a Nikon SMZ18 fluorescence stereomicroscope.

Single-cell RNA sequencing and data analyses

Both control and ischemia tissues were fully digested. The cell pellet was suspended in PBS and were subjected to single cell RNA sequencing as described previously [15, 16]. A total of 63,645 cells remained post-filtering and data were normalized by "LogNormalize". The top 2,000 highly variable genes were selected and scaled by using "ScaleData". The cell distribution was visualized with t-distributed stochastic neighbor embedding (t-SNE) plot. "FindAllMarkers" was used to identify marker genes upregulated in each cluster (min.pct = 0.25, logfc.threshold = 0.25). Integrative analysis of the femoral artery data at different time point was also analyzed with Seurat v4.0.3 using Canonical Correlation Analysis to remove batch effects. R package ggplot2 and Seurat were applied to draw violin plots, heastmap, bar plots and dot plots. Gene enrichment analyses were performed using R package clusterProfiler v3.18.1 [17].

Statistical analyses

Data are expressed as mean ± standard deviation. D'Agostino & Pearson test was used to

test the normality of the data, followed by an unpaired two-tailed t test. If no standard deviation is assumed to be equal by F test, Welch's correction is used. For comparisons of more than two groups, similar variances were assessed using Brown-Forsythe test, followed by ordinary analysis of variance or Welch ANOVA test for hypothesized or unhypothesized similar variances, respectively. Mann-Whitney U test was used for non-normal distribution data, and Kruskal-Wallis test and Dunn's multiple comparison test were used for more than two groups. The number of repeats of biological experiments in each group is shown in the figure, and the exact *P*-value of the result is shown in the figure. P<0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0 software.

Results

Single-cell RNA-seq reveals the cellular diversity and heterogeneity of ischemic tissue

Immunohistochemistry revealed significant inflammatory cell infiltration in the ischemic tissue and significantly elevated fibrosis levels (**Figure 1A**, **1B**). We then isolated mononuclear cells from normal and ischemic tissue to describe their precise properties and cell heterogeneity (**Figure 1C**). A total of 63,645 cells passed quality control indicators and were analyzed (Figure S1). A total of nine cell populations were identified, including smooth muscle cells (SMC), endothelial cells (EC), fibroblasts, muscle cells, macrophages, pericytes, T/NK cells, and neutrophils (**Figure 1D**). Based on the differential expression of genes with different characteristics, these clusters are further attributed to assumed biological characteristics (**Figure 1F**). Fibroblasts accounted for the largest proportion of all cell clusters. In addition, the proportions of fibroblasts, neutrophils, and T/NK cells are significantly upregulated during ischemia (**Figure 1E**). Further analysis of differential gene expression between the control group and the ischemia group showed that inflammation and extracellular strom-related genes were significantly upregulated in the ischemia group (**Figure 1G**). Functional enrichment analysis showed that the role of cells in ischemic tissue was mainly involved in the regulation of extracellular matrix remodeling and inflammatory cell chemotaxis (**Figure 1H**). These results suggest that fibroblasts and inflammatory cells may be key cellular components in the pathological process of lower limb ischemia. In addition, based on sequencing analysis, we found that cell interactions were significantly enhanced in the ischemic environment, particularly between fibroblasts and smooth muscle cells (**Figure 1I**). These cell clusters have obvious molecular characteristics, reflecting the diversity and heterogeneity of cells in lower limb ischemic tissue.

Single-cell transcriptomes of fibroblasts in ischemic tissues

In order to further explore the functional changes of fibroblasts during ischemia, we conducted subpopulation analysis of fibroblasts based on differential expression of distinct feature genes. We classified fibroblasts into 5 subgroups, namely Col15A1_hi Fb, CTHRC1_hi Fb, FBN1_hi Fb, Myo Fb, and SFRP1_hi Fb, according to characteristic gene expression and function (**Figures 2A** and S2A). Further analysis of the changes in characteristic gene expression of each subgroup before and after ischemia revealed significant upregulation of COL15A1, ACTA1, and SFRP1 in the ischemic environment, with similar levels of specific gene expression observed in Col15A1_hi Fb and SFRP1_hi Fb subgroups. Moreover, spearman correlation analysis indicated a high correlation in the expression levels of top characteristic genes between Col15A1_hi Fb and SFRP1_hi Fb subgroups (**Figure 2D**). Additionally, the expression levels of transcription factors in these two subgroups were significantly upregulated in the ischemic conditon (**Figure 2F**). Therefore, we defined Col15A1_hi Fb and SFRP1_hi Fb as activated fibroblast subgroups (**Figure 2B** and **2E**). Interestingly, the proportion of Col15A1_hi Fb and SFRP1_hi Fb subgroups was significantly increased in the ischemic tissue, suggesting activation of fibroblasts during ischemia (**Figure 2C** and **2G**). Subsequently, we analyzed the functions and signaling pathways of Col15A1_hi Fb and SFRP1_hi Fb subgroups using the GOBP and REACTOME databases. The analysis revealed enrichment of multiple biological processes in the Col15A1_hi Fb and SFRP1_hi Fb subgroups in the ischemic environment, such as regulation of cell development, regulation of ossification, collagen fibril organization, and

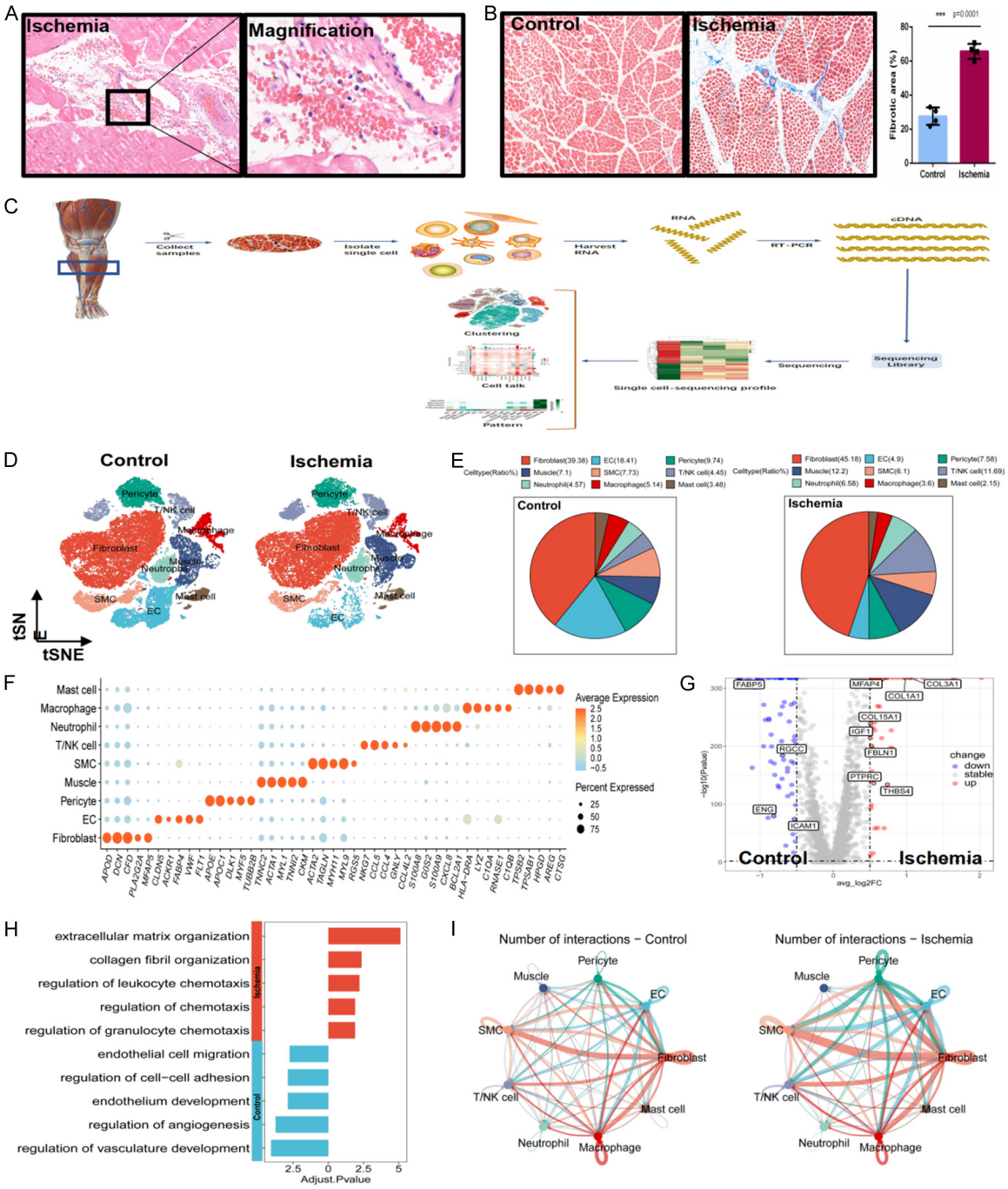

Figure 1. Single-cell RNA-seq reveals the cellular diversity and heterogeneity of ischemic tissue. A. Representative HE staining of inflammation response in ischemic tissues of human. Scale bars, 50 μm and 10 μm. B. Representative masson staining of control and ischemic tissues of human. Scale bars, 200 μm. C. Sketch of the experimental design for scRNA-sequencing of human tissue. D. Visualization of unsupervised clustering in a t-distributed stochastic neighbor embedding (t-SNE) plot of cells isolated from human limb tissues. E. Pie chart of percentage shifts of major cell types in each group. F. Dot plot showing average scaled expression levels (color-scaled, column-wise Z scores) of top 5 differentially expressed genes (DEGs; columns) across the total cell populations (rows), cells with a value >0 represent cells with expression above the population mean. Dot size reflects the percentage of cells expressing the selected gene in each population. G. Volcano plot showing the change of gene expression in two groups (red for ischemia, blue for control). H. GO biological processes analysis of DEGs enriched in control and ischemia groups. I. Circle plot displaying the number of the interaction between each population respectively.

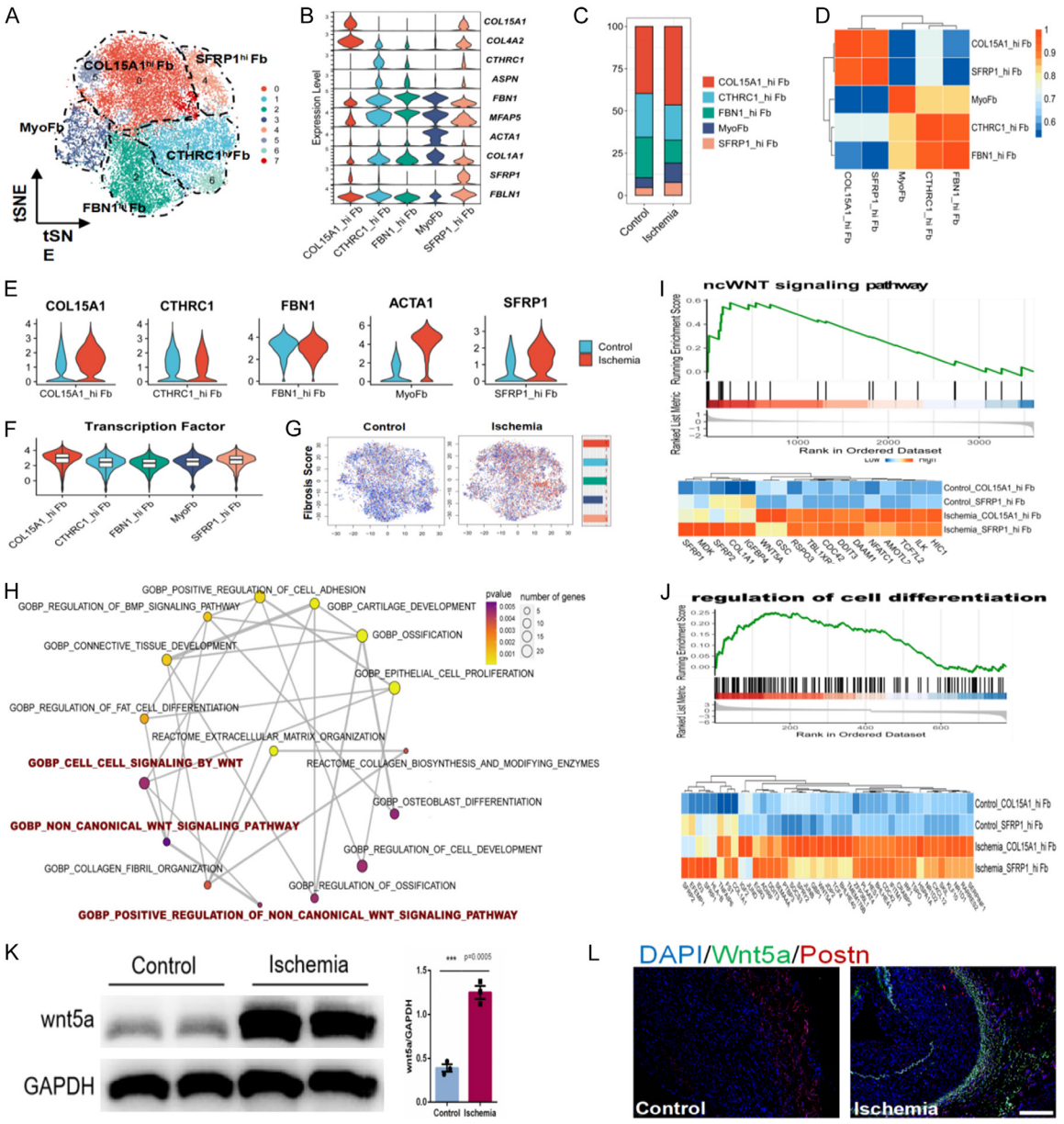

Figure 2. Single-cell transcriptomes of fibroblasts in ischemic tissues. A. Visualization of unsupervised clustering in a t-SNE plot of fibroblasts and dashed lines circles the subclusters via specific genes and biological function. B. Violin plot showing expression of specific genes in subclusters of fibroblasts. C. Bar charts showing the percentage of fibroblasts clusters in control and ischemia groups. D. Heatmap showing Spearman correlation analysis between fibroblast subcluster, calculated using the subset mean value of gene matrix. E. Violin plot showing the changes of the specific gene in the subcluster in control and ischemia groups. F. Violin combined with box plot showing the average expression of transcription factor in subclusters. G. The distribution of fibrosis score in t-SNE plot respectively in control and ischemia groups. Red means high fibrosis score while blue means low. The bar plot shows odds ratio of fibrosis score in each subcluster, the red dash line mead the odds ratio = 1. H. Net plot showing the relation of the biological process enriched in COL15Ahi and SFRP1hi subclusters compared ischemia versus control, the length of grey line means the number of reduplicate genes in adjacent function. I, J. Enrichment plots (upper panel) and leading-edge gene expression heatmaps (lower panel) for representative signaling pathways upregulated in COL15Ahi and SFRP1hi subclusters compared ischemia versus control. K. Representative western blot analyses and quantification of wnt5a in human ischemic tissues. n = 3 per group. L. Immunostaining for wnt5a and postn in blood vessels of control and ischemic human. Scale bars, 50 μm.

positive regulation of non-canonical Wnt signaling pathway (**Figure 2H**). GSEA analysis indicated significant enrichment of the ncWNT pathway in activated fibroblasts in the ischemic

environment, with markedly upregulated expression of Wnt5a in the non-canonical WNT pathway, while no significant difference was observed in the expression of genes related to the canonical WNT pathway (**Figures 2I** and S2B, S2C). To further verify the sequencing results, we confirmed that the expression of Wnt5a was significantly increased in ischemic tissues by Western blotting (**Figure 2K**). Immunofluorescence analysis revealed that Wnt5a was primarily expressed in the vascular adventitia (**Figure 2L**). Furthermore, analysis of the functions of ncWNT pathway-related genes demonstrated their involvement in regulating cell differentiation (**Figure 2J**). These results suggest that fibroblasts are activated in the ischemic environment and involved in regulation of cell differentiation by the WNT pathway.

Cellular heterogeneity and regulatory changes of smooth muscle cells in ischemic tissues

During the process of lower limb ischemia, the excessive proliferation of smooth muscle cells plays a crucial role in vascular remodeling. Therefore, we conducted an analysis of the functional changes of smooth muscle cells. Based on distinct feature genes and functions, we classified SMCs into 5 subgroups, namely developmental SMC, contractile SMC, inner SMC, immune SMC, and secretory SMC (**Figure 3A** and **3C**). Through functional enrichment analysis, we discovered that the developmental SMC subgroup is primarily involved in regulating cell proliferation and differentiation, the contractile SMC subgroup's function is mainly focused on muscle contraction, while the secretory SMC subgroup is mainly involved in extracellular matrix remodeling. Therefore, we consider the developmental SMC subgroup to be involved in vascular remodeling (**Figure 3D**). Furthermore, following ischemia, there was a significant increase in the proportion of the developmental SMC subgroup (**Figure 3B**). To further validate the sequencing results, we confirmed a significant enhancement of vascular smooth muscle cells proliferation in ischemic patients through immunofluorescence (**Figure 3E**). Moreover, H&E staining showed a significant increase in vessel wall thickness in ischemic tissue compared to control (**Figure 3F**). These results indicate that during the process of lower limb ischemia, SMCs exhibit a highly proliferative phenotype and are involved in vascular remodeling.

It has been shown that cell-cell interaction plays a critical role in vascular diseases. As shown in **Figure 1I**, the interaction between fibroblasts and smooth muscle cells is significantly enhanced in an ischemic environment. Further analysis of the interaction strength between the cell subgroups showed that the interaction between COL15A1_hi Fb and developmental SMC was significantly enhanced during ischemia (**Figure 3G**). This suggests that activated fibroblasts may mediate vascular remodeling by regulating the proliferation of smooth muscle cells during ischemia. To explore the underlying mechanism of phenotypic transformation of lower limb ischemic cells, we analyzed the signaling patterns of the two groups. The results showed that ncWNT pathway, BMP pathway, SEMA4 pathway and MK pathway were highly expressed in ischemic tissue (**Figure 3H**). In our earlier work, we found that the ncWNT pathway, especially the Wnt5a pathway, is highly enriched in the COL15A1_hi Fb subgroup (**Figure 2I**). We then investigated whether activated fibroblasts modulated smooth muscle cell proliferation via the ncWNT pathway. As shown in **Figure 3I**, the expression of WNT5a-MCAM ligand is the most obvious in the interaction between COL15A1_hi Fb and developmental SMC. This suggests that activated fibroblasts may regulate the proliferation of smooth muscle cells by secreting Wnt5a on MCAM receptors of smooth muscle cells.

Expression of NETs in lower limb ischemia

As is well known, inflammation is a key promoting factor for vascular remodeling during lower limb ischemia [18, 19]. As shown in **Figure 1A**, the ischemic tissue had significant inflammatory cell infiltration compared with the control group. At the same time, the ratio of neutrophils was found to increase in the ischemic tissue by RNA-Seq (**Figure 1E**). Neutrophils play an important role in the early inflammatory response of ischemic diseases. Further analysis of neutrophils through scRNA-seq revealed that subgroup 2 expressed high levels of CXCR2 and NCF1, indicating their activation state (**Figure 4A**, **4B**). Meanwhile, in the ischemic group, the proportion of activated neutrophils increased (**Figure 4C**). NETs are characteristic products of activated neutrophils. We found that the levels of NETs were significantly increased in the serum and ischemic tissues of ischemic patients (**Figures 4D**, **4E** and S3).

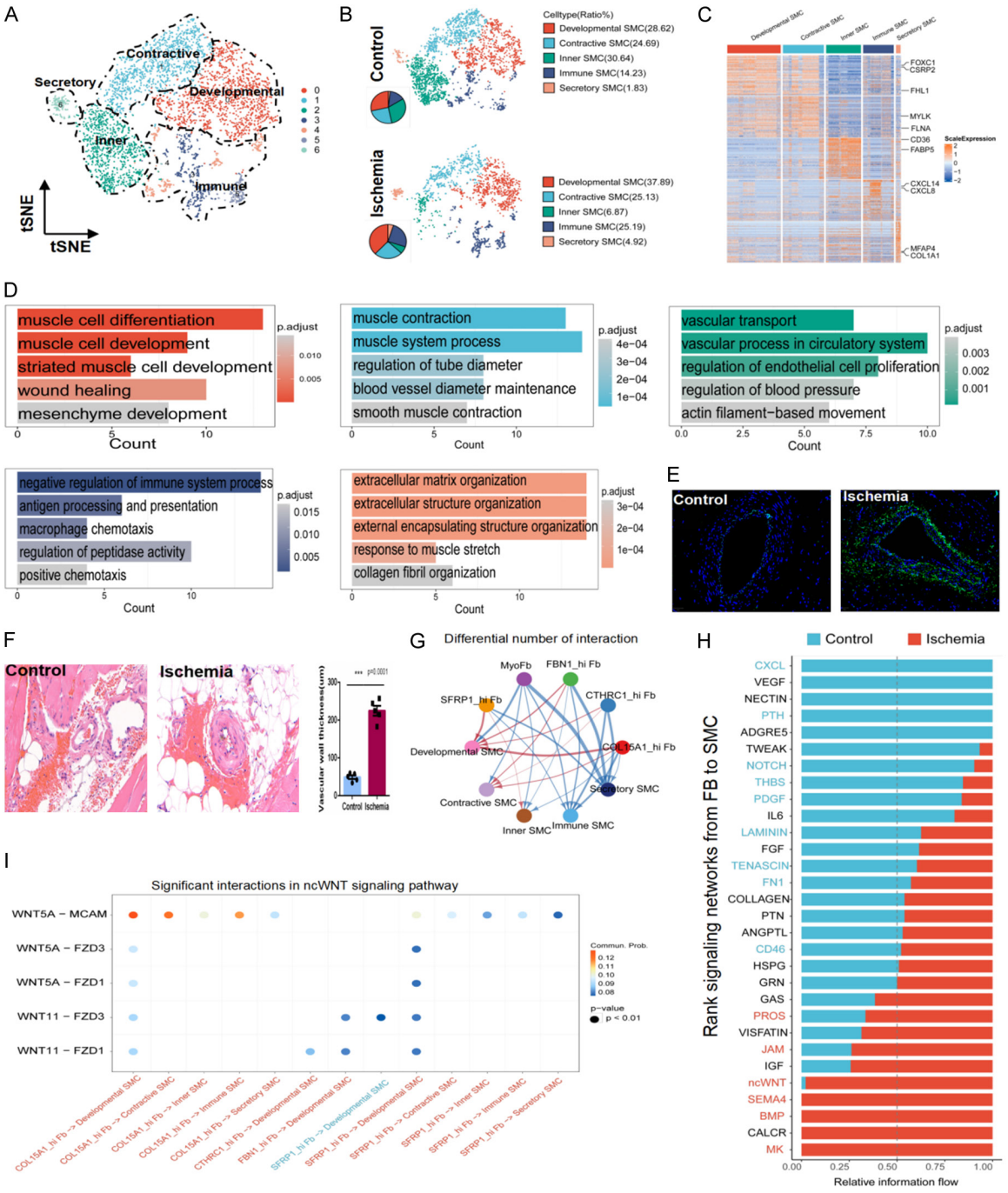

Figure 3. Cellular heterogeneity and regulatory changes of smooth muscle cells in ischemic tissues. A. T-SNE plot visualize the clusters of smooth muscle cells and dashed lines circles the subclusters via specific genes and biological function. B. T-SNE plots split by control and ischemia group and the pie charts showing the proportion of subclusters respectively. C. Heatmap showing expression of the top different genes in subclusters of smooth muscle cells and the specific genes are labeled. D. Top GO biological process in each subcluster. E. Immunostaining for Ki67 in control and ischemic tissues of human. Scale bars, 50 μm. F. Representative HE staining of vascular wall thickness of control and ischemic tissues of human. Scale bars, 20 μm. G. Circle plot showing the change of the number of interaction from fibroblast subclusters to smooth muscle cell subclusters. Red arrow means increase in ischemia group while blue means increase in control. H. Stack-bar plot showing the difference of signaling networks from fibroblast subclusters to smooth muscle cell subclusters. I. Dot plot showing the change of ncWNT related ligand-receptor interaction pairs between fibroblast and smooth muscle cell subsets, based on the communication probability. Each dot indicates the change of interaction between 2 specific cell types. Red means increase in ischemia group while blue means increase in control.

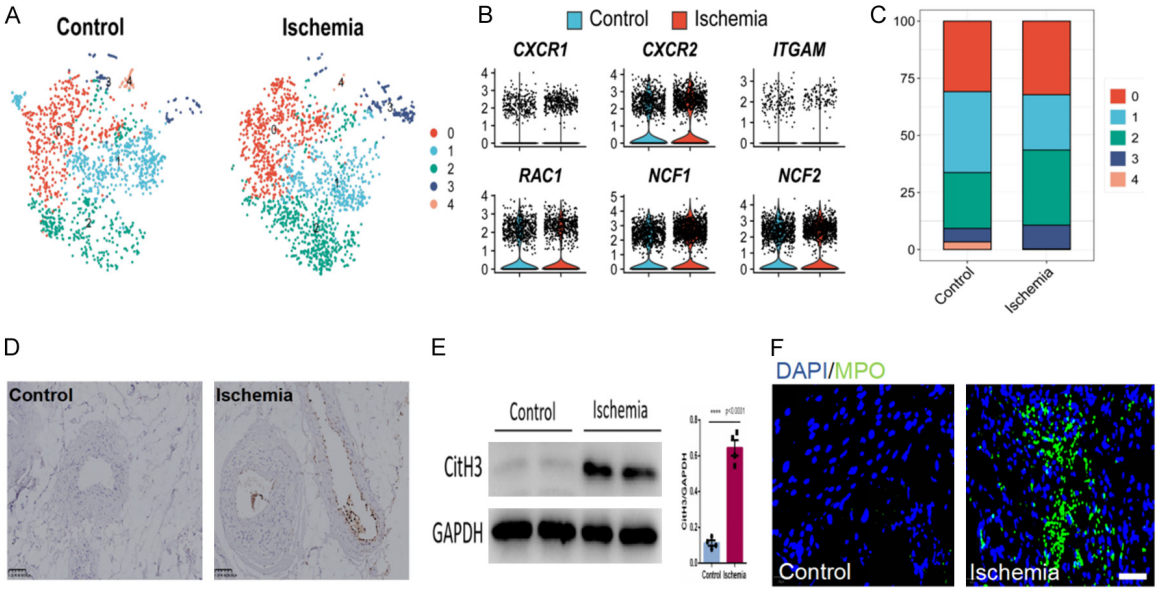

Figure 4. Expression of NETs in lower limb ischemia. A. Visualization of unsupervised clustering in a t-distributed stochastic neighbor embedding (t-SNE) plot of neutrophil isolated from control and ischemia. B. Violin plots comparing the expression of neutrophil-activate genes between control and ischemia. C. Bar chart showing the proportion of each neutrophil cluster across control and ischemia samples. D. Representative IHC staining of human ischemic tissues. Scale bars, 100 μm. E. Representative western blot analyses and quantification of CitH3 in human ischemic tissues. n = 3 per group. F. Immunostaining for MPO in mice ischemic tissues. Scale bars, 20 μm.

Additionally, compared to the control group, a significant increase in NETs levels was also observed in a mouse model of lower limb ischemia (**Figure 4F**). These results suggest that during the process of lower limb ischemia, NETs may regulate the proliferation of smooth muscle cells by activating fibroblasts.

NETs triggger fibroblast activation by Wnt5a pathway

To further elucidate the role of NETs, we found that fibroblasts were significantly activated in response to NETs stimulation (**Figure 5A**). Western blotting and PCR results showed that NETs promoted the expression of Wnt5a in fibroblasts compared with the control group (**Figure 5B**, **5C**). In addition, we co-cultured the supernatant of activated fibroblasts with smooth muscle cells, and the results showed that activated fibroblasts promoted the proliferation of smooth muscle cells (**Figure 5D**, **5E**). To further confirm the role of Wnt5a in the interaction between fibroblasts and smooth muscle cells, we transfected fibroblasts with Wnt5a-siRNA to specifically knock down the expression of Wnt5a in fibroblasts. Western blotting confirmed the knockdown efficiency of siRNA transfection with Wnt5a (Figure S4A). The results of CCK-8 showed that down-regulation of Wnt5a expression inhibited the promoting effect of fibroblasts on SMCs proliferation (**Figure 5F**).

To further explore the potential therapeutic effect of inhibition of Wnt5a on lower limb ischemia, we treated a mouse model of lower limb ischemia with Wnt5A-siRNA (Figure S4B). Experimental results showed that inhibition of Wnt5a could reverse tissue fibrosis, tissue ischemia and vascular remodeling, thereby restoring blood perfusion in the lower extremities (**Figures 5G-I** and S4C). These results suggest that inhibition of Wnt5a can alleviate vascular remodeling under ischemic conditions. Based on the above results, we believe that NETs can induce fibroblast activation and promote smooth muscle cell proliferation via the Wnt5a pathway (**Figure 5J**).

Discussion

According to estimates, there are currently 230 million patients worldwide suffering from peripheral arterial disease [20]. Despite significant progress in related research on lower limb

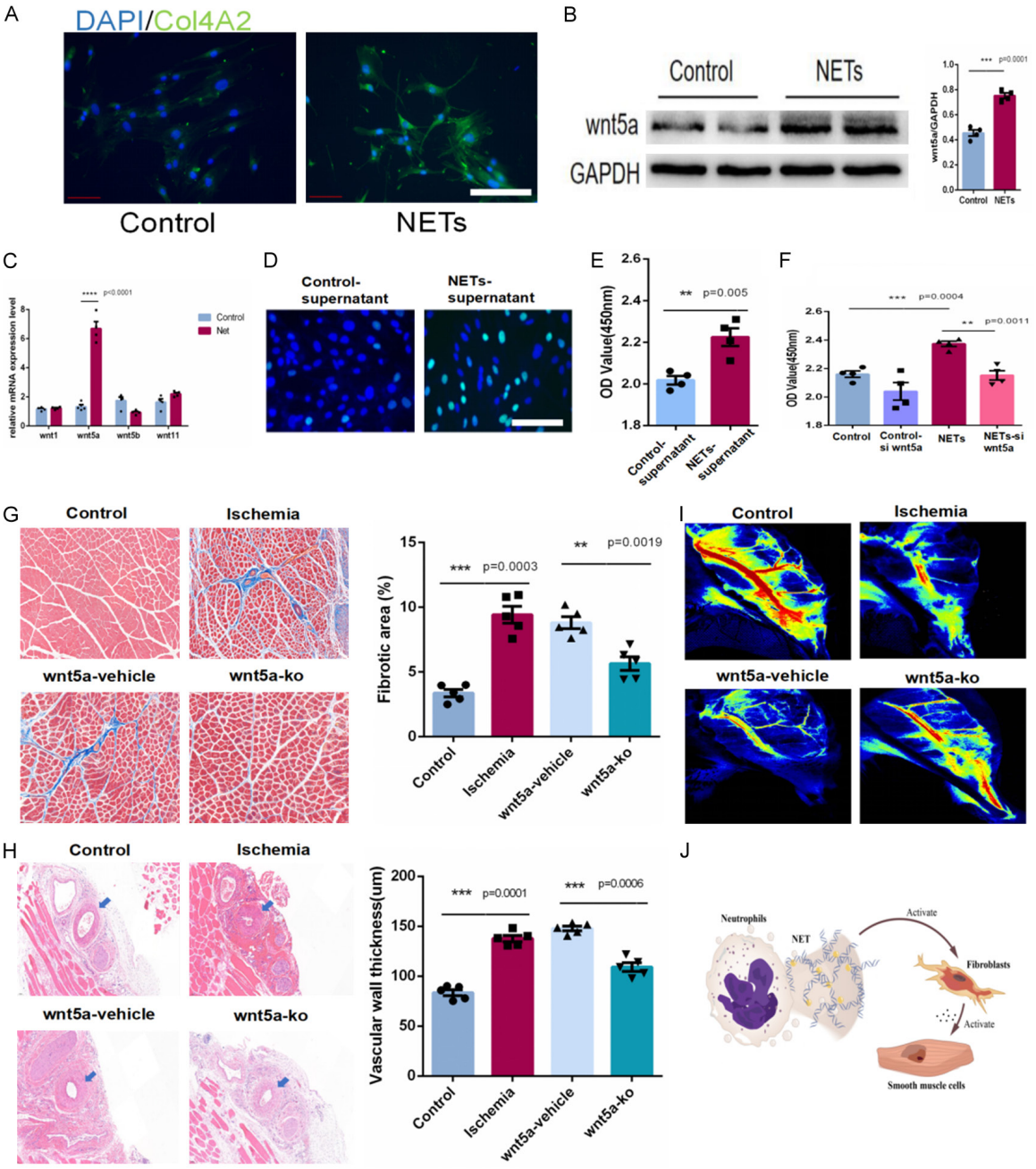

Figure 5. NETs triggger fibroblast activation by WNT5a pathway. A. Immunostaining for Col4A2 in fibroblasts. B. Representative western blot analyses and quantification of wnt5a in Fibroblasts. n = 3 per group. C. Quantitative PCR analyses for mRNA expression of wnt subtypes in fibroblasts. D. Proliferation of SMC treated with superantant of fibroblast by EdU assay. E. Proliferation of SMC treated with superantant of fibroblast by CCK-8 assay. F. Proliferation of SMC treated with superantant of fibroblast transfected with control or wnt5a siRNA by CCK-8 assay. G. Representative Masson staining of ischemic tissues from different treated mice. Scale bars, 100 μm. H. Representative HE staining of vascular wall thickness in ischemic tissues from different treated mice. Scale bars, 100 μm. I. Representative images of blood flow of the lower limbs from different treated mice. J. Schematic showing Fibroblast-SMC interaction induced by NETs.

ischemia, its pathogenesis remains unclear. In this study, we aimed to explore the potential mechanisms of lower limb ischemia and provide fundamental information for clinical treatment. We first described the cellular diversity and heterogeneity of normal and ischemic

lower limb tissues. Subsequently, we depicted the single-cell transcriptomes of fibroblasts and smooth muscle cells, elucidating intercellular communication. Importantly, signaling patterns were revealed during lower limb ischemia. We confirmed the critical role of NETs in the inflammatory response of lower limb ischemia, promoting interactions between fibroblasts and smooth muscle cells. Finally, we elucidated the potential mechanisms by which NETs induce functional changes in fibroblasts and smooth muscle cells. Therefore, our findings not only provide a gene expression profile at the single-cell level, but also suggest potential targets for the treatment of peripheral arterial disease.

Tissue ischemia is known to trigger an inflammatory response, initiating intercellular interactions that lead to vascular remodeling in peripheral arteries [21, 22]. This includes the expression of pro-inflammatory genes in endothelial cells, which is crucial for recruiting macrophages and T cells to the arterial wall [23, 24]. Neutrophils are among the shortest-lived immune cells and are continuously replenished from the bone marrow into circulation [25, 26]. In recent years, the formation of neutrophil extrinsic traps (NETs) has received much attention as a state of neutrophil activation. Tumor biological evidence suggests that NETs can be used as metastasis mediators to trap circulating tumor cells and promote their accumulation in peripheral tissues or assist in the colonization of malignant cells in peripheral tissues. For example, the CTSC-PR3-IL-1β axis induces the production of reactive oxygen species and the formation of NETs in neutrophils, thereby supporting metastatic colonization of lung cancer [27]. In recent years, researchers have increasingly focused on the role of NETs in cardiovascular disease. Tian et al. demonstrated that activated neutrophils participate in the development of abdominal aortic aneurysms by releasing NETs [28], and NET-mediated endothelial cell damage has been associated with glomerulonephritis, leading to histone-induced renal thrombotic microangiopathy and glomerular necrosis [29]. In peripheral arterial disease, atherosclerosis is the main cause of vascular remodeling. NETs act as a bridge in the communication between neutrophils and macrophages, stimulating macrophages to infiltrate into atherosclerotic plaques and regulate inflammatory responses [30]. Furthermore, NETs can activate plasmacytoid dendritic cells in the vessel wall, leading to a strong type I interferon response, thereby driving atherosclerosis [31]. In our study, we found elevated levels of NETs in the peripheral blood of patients with peripheral arterial disease. Single-cell RNA sequencing analysis of neutrophils revealed a significant increase in activated neutrophils in ischemic tissues. These results suggest that ischemia may activate neutrophils, leading to increased production of NETs and promoting the development of lower limb ischemia.

Vascular remodeling is the key event of lower limb ischemia, so we will pay more attention to the remodeling process during ischemia. Recent studies have indicated that vascular fibrosis is a key event leading to arterial occlusion [32]. Investigating the nature and function of various cell types in the ischemic microenvironment would be intriguing, and single-cell RNA sequencing (scRNA-seq) provides a powerful tool for this purpose [33]. In this study, 9 major cell types were identified, among which fibroblasts (45.18%) expressed higher levels of ECM tissue-related genes in patients. We found that in ischemic tissues, more activated fibroblasts aggregated around the intramuscular arteries. Regarding the activation of fibroblasts, reports have shown that various cytokines play a role in this process [34]. Recent studies also suggest that NETs may be involved in the activation of fibroblasts [35, 36], but the potential mechanisms underlying this activation are not yet clear. Single-cell RNA sequencing analysis results indicate a high enrichment of the non-canonical WNT signaling pathway in activated fibroblasts. Wan et al. discovered that FOSL2 transcriptionnally activates Wnt5a in breast cancer-associated fibroblasts, specifically activating FZD5/NF-κB/ERK signaling in HUVECs, promoting VEGF-independent angiogenesis [37]. Therefore, we speculate that NETs may activate fibroblasts during ischemia and promote the up-regulation of the ncWNT pathway in fibroblasts. To confirm this hypothesis, we stimulated fibroblasts with isolated and purified NETs and found that NETs not only activated fibroblasts but also upregulated Wnt5a in fibroblasts, thereby detecting active non-canonical WNT signaling.

As is well known, smooth muscle cells have two classic phenotypes - contractile and synthetic, with the contractile phenotype being predomi-

nant under physiological conditions [38, 39]. The progression of ischemia may trigger phenotype conversion, causing smooth muscle cells to transition to a synthetic phenotype. What are the potential mechanisms of smooth muscle cell phenotype conversion during ischemia? Analysis of cell-cell interactions through single-cell RNA sequencing data revealed a significant enhancement of interactions between activated fibroblasts and a highly proliferative subpopulation of smooth muscle cells during ischemia, and up-regulation of WNT5a-MCAM ligand. Therefore, we speculate that activated fibroblasts play a key role in promoting smooth muscle cell proliferation through the Wnt5a pathway. Co-culturing fibroblasts treated with NETs with smooth muscle cells led to a significant increase in smooth muscle cell proliferation. NETs can activate fibroblasts, promote the secretion of Wnt5a, and stimulate smooth muscle cell proliferation. It is worth noting that high levels of Wnt5a were detected in ischemic tissue, which is associated with activated fibroblasts. Based on these results, we suggest that ischemia may trigger the formation of NETs, thereby activating fibroblasts and promoting smooth muscle cell proliferation via the Wnt5a pathway.

Based on our discovery of the Wnt5a signaling pathway, we attempted to explore a new approach to alleviate peripheral artery disease-related vascular stenosis. After successfully establishing a mouse hind limb ischemia model and conducting siRNA transductions, we evaluated the blood supply situation. The results showed that silencing Wnt5a improved fibrosis and vascular remodeling in ischemic tissue. Evidence indicates that local inhibition of non-canonical WNT signaling can alleviate ischemia and improve blood supply. Our findings suggest that non-canonical WNT signaling, as a novel pattern of fibroblast-smooth muscle cell interaction, regulates the development of lower limb ischemia and has potential clinical applications. Despite achieving significant results, we are also aware of the limitations of this experiment, including the unclear detailed mechanism of improving blood supply to ischemic limbs after in vivo application of ncWNT-siRNA.

In conclusion, we have mapped the cellular landscape of human lower limb ischemic tissue and identified the critical role of intercellular communication in patients. During lower limb ischemia, neutrophils produce a large amount of NETs, which stimulates smooth muscle cell proliferation and enhances vascular remodeling by activating fibroblasts through the Wnt5a pathway. Wnt5a is expected to be a potential target for improving blood supply in ischemic lower limbs, providing a new avenue for the development of effective potential therapies for peripheral artery disease.

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Disclosure of conflict of interest

None.

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Table S1. Baseline characteristics of patients

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Basic Information						
Gender	Male	Male	Male	Male	Male	Male
Age	83	68	73	78	70	67
Primary Diagnose	ASO	ASO	ASO	Trauma	Trauma	Trauma
Operation type	Amputation (R-Thigh)	Amputation (R-Leg)	Amputation (R-Leg)	Amputation (R-Leg)	Amputation (L-Thigh)	Amputation (R-Thigh)
Pneumonia	Y	Y	N	N	N	N
T2DM	N	Y	N	N	N	N
Hypertension	N	N	Y	N	N	N
Hyperlipemia	N	N	N	N	N	N
Hyperuricemia	N	N	N	N	N	N
Gangrene or Ulcer	N	N	N	N	N	N
Vascular Surgery History	Y	Y	N	N	N	N
Stent Graft Implantation History	Y	Y	N	N	N	N
In-stent Restenosisor Occlusion	Y	Y	N	N	N	N
SampleHarvest Date	20210219	20210225	20210813	20201225	20210224	20201210
Cardial Vascular Inspection Results						
ECG	Normal	Normal	Normal	Normal	Normal	Normal
Carotid artery	AS Plaque	AS Plaque	AS Plaque	Normal	Normal	Normal
Illiac artery	AS Plaque & Right Illiac Artery Dissection	AS Plaque & Stenosis & Tortuous	AS Plaque	Normal	Normal	Normal
Femoral Artery	Occlusion	Occlusion	Occlusion	Normal	Injured	Injured
Superficial Femoral Artery	Occlusion	Occlusion	Occlusion	Normal	Injured	Injured
Anterior Tibial Artery	Occlusion	Occlusion	Occlusion	Injured	Normal	Normal
Posterior Tibial Artery	Stenosis	Occlusion	Occlusion	Injured	Normal	Normal
Popliteal Artery	Stenosis	Occlusion	Occlusion	Injured	Normal	Normal

Table S2. Antibody list

Name	Manufacturer	Code	Application
Anti-Histone H3 antibody	Abcam	ab5103	WB
Anti-Myeloperoxidase	Abcam	ab208670	IF, IHC
Anti-WNT5a	Abcam	ab235966	IF, WB
Anti-Ki67	Proteintech	28074-1-AP	IF
Anti-Collagen 4A2	Proteintech	55131-1-AP	IF
Anti-GAPDH	GNI	GNI4310-GH	WB

Table S3. List of PCR primers

Name	Sequence
GAPDH-F	GTCTCCTCTGACTTCAACAGCG
GAPDH-R	ACCACCCTGTTGCTGTAGCCAA
WNT1-F	CTCTTCGGCAAGATCGTCAACC
WNT1-R	CGATGGAACCTTCTGAGCAGGA
WNT5a-F	TACGAGAGTGCTCGCATCCTCA
WNT5a-R	TGTCTTCAGGCTACATGAGCCG
WNT5b-F	CAAGGAATGCCAGCACCAGTTC
WNT5b-R	CGGCTGATGGCGTTGACCACG
WNT11-F	CTGTGAAGGACTCGGAACTCGT
WNT11-R	AGCTGTCGCTTCCGTTGGATGT

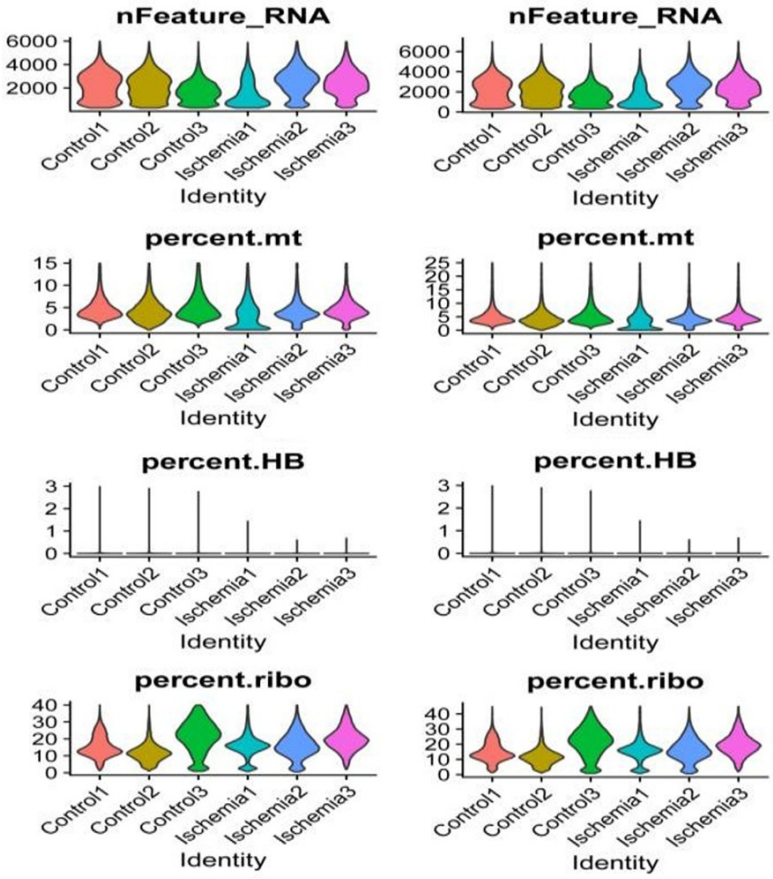

Figure S1. Violin plots showing the indicators of before andafter quality control, including the number of feature RNA, and percent of mitochondria, hemoglobin and ribosome genes.

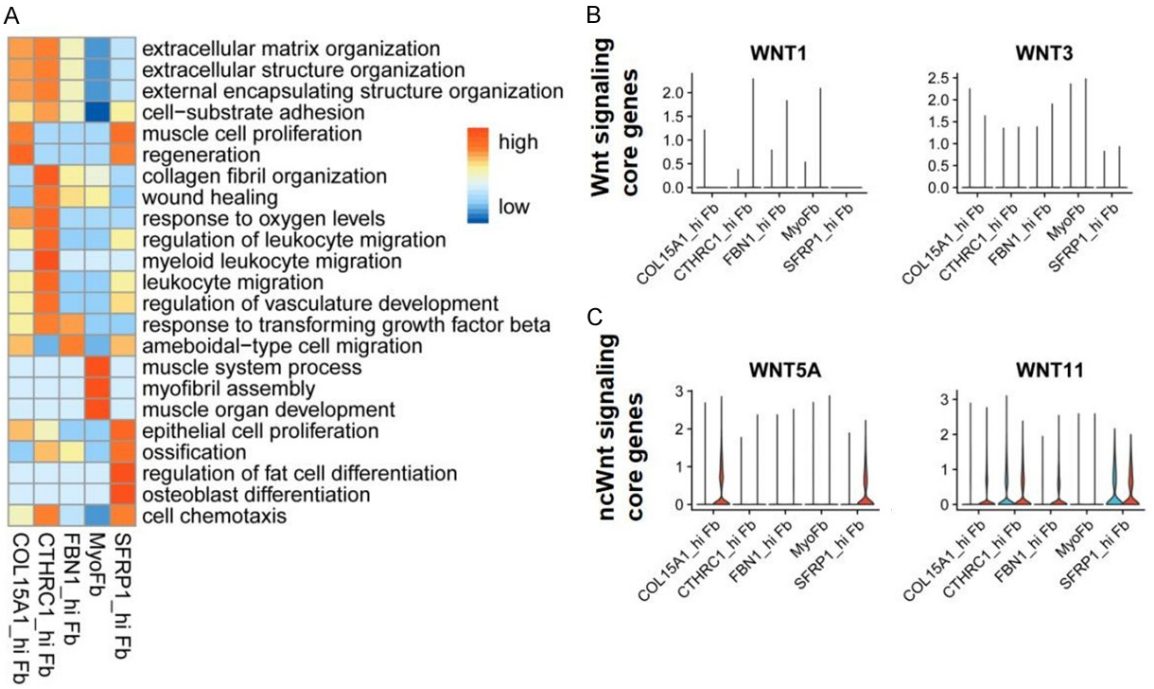

Figure S2. A. Heatmap showing the most significant biological process in each subcluster of fibroblast. B, C. Violin plots showing the expression of core genes of WNT and ncWnt pathways. Left (blue) means control and right (red) means ischemia.

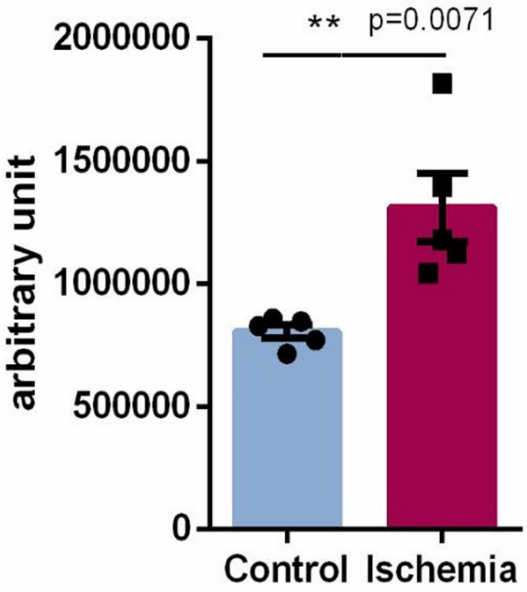

Figure S3. Bar plot showing the level of NETs in serum of human.

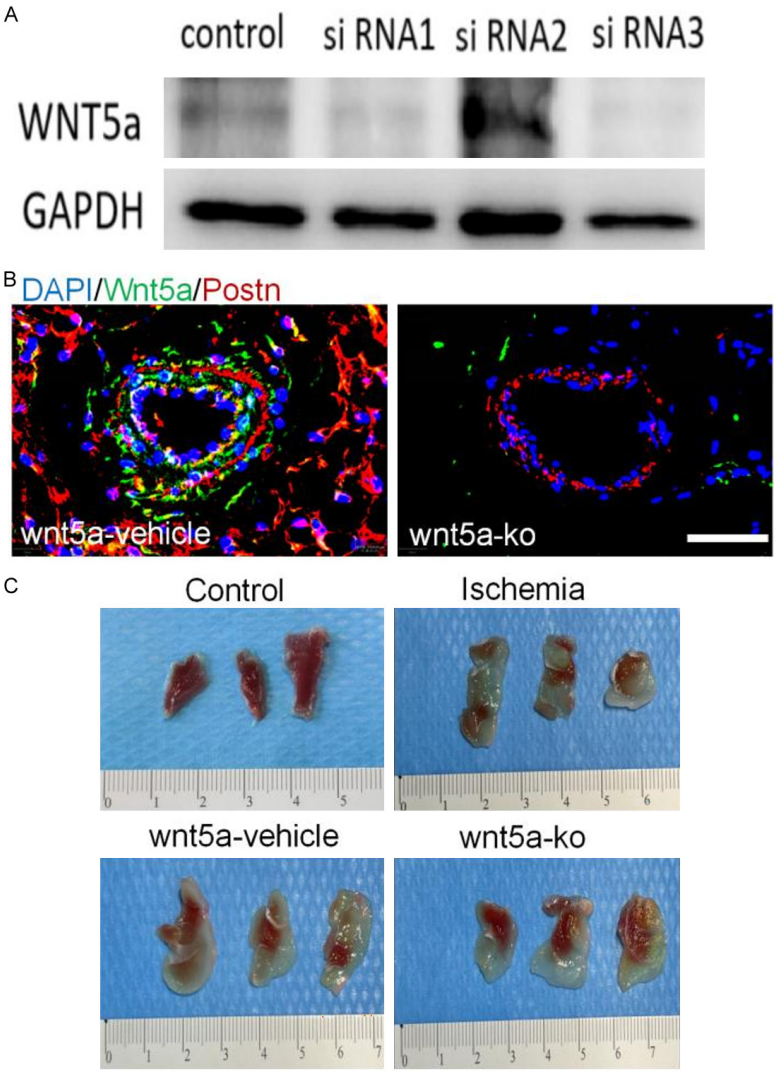

Figure S4. A. Western blot analyses of knockdown efficiency of siRNA transfection for WNT5A. B. Immunostaining for Wnt5a and Postn in mice ischemic tissues transfected with control-siRNA and Wnt5a-siRNA. Scale bars, 20 μm. C. Representative TTC staining of ischemic tissues from different treated mice. Scale bars, 100 μm.