Single-cell RNA-seq data reveals a critical role of pro-inflammatory macrophage and fibroblast cells in bone marrow environment after bone fracture

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Article

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Abstract

Single-cell RNA sequencing ("scRNA-Seq") examines the cell population at the single-cell level. The single cell changes in the osteoimmunological microenvironment in fresh and old fractures have not been studied. We used single cell transcriptomics in this study to uncover differences in the molecular composition and cellular signaling in bone tissue from fresh and old fractures.We first searched for and downloaded single-cell omics data from the GEO database, which included both fresh and old fracture samples from patients. After applying UMI detection, reducing the dimensions, and conducting principal component analysis, we visualized the data with tSNE and UMAP and identified the marker genes of the cell subsets. The differences of the differentially expressed genes and the signalling pathways of the cell-cell interaction between the two groups of samples were compared by means of Findmarkers and cellchat.The microenvironment in fracture tissue was analysed using a cell characterisation map, resulting in the identification of 18 distinct cell subsets, comprising of macrophages, fibroblasts, B cells, T cells, neutrophils and plasma cells. In comparison to fresh fractures, there was a significant increase in the number of macrophages in the old fracture samples. The number of fibroblasts was not significantly changed. The results of differential expression gene analysis showed that fibroblasts in old fractures were mainly enriched in immune, inflammatory and neutrophil degranulation reactions. TXNIP expression was significantly upregulated. Macrophages were mainly enriched in inflammatory response, immune response, antigen presentation response and cell migration signalling pathways. Among them, AREG was significantly upregulated in old fractures. In old fractures, the interaction between macrophages and other cells was significantly increased. Macrophages regulate other cells mainly through the ANXA1-FRP1 signalling pathway, thereby influencing the formation of callus and the healing of the fracture. Our findings uncovered that fibroblasts regulate inflammation and immune response via the TXNIP pathway. Macrophages influence fracture healing by changing their population and interacting with other cells via the ANXA1-FRP1 pathway.

Introduction

Bone fracture healing is a process of tissue regeneration. The whole process was mainly divided into hematoma stage, inflammation stage, fibrovascular stage, bone formation stage and remodeling stage. The process of fracture healing requires the synergistic interaction among immune cells, pluripotent stem cells, osteoblasts and osteoclasts [1]. Of significant concern is that different types of immune cells exert different function at different stages of fracture healing. For example, in the early stages of inflammation, neutrophils and macrophages are primarily activated, followed by mast cells and dendritic cells, where macrophages show phagocytosis that eliminates granulation and promotes osteogenic differentiation [2]. T-reg lymphocytes and macrophages had immunosuppressive effects in the late healing stage [3]. Inflammatory cells (macrophages, monocytes, lymphocytes, and osteoclasts) and fibroblasts migrate to the defect site to play their role through cell-cell communication [4]. At the stage of fracture repair, fibroblasts produce extracellular matrix and express VEGF to promote neovascularization. Fibroblasts and immune cells were detected in the synovium of patients with rheumatoid arthritis. The results

showed that the interaction between immune cells and fibroblasts resulted in the imbalance of immune cells and increased bone resorption of osteoclasts [5] .Recent work has also shown that fibroblasts play an important role in fracture healing. In the rat fracture healing model, specific fibroblasts actively deposited extracellular matrix to prevent callus bridging at the fracture site and hinder fracture healing [6]. Single-cell transcriptome sequencing has been used to detect cell heterogeneity during fracture healing in animal models, revealing the potential functions of different types of immune cells, such as B cells and T cells [7]. The above studies were mainly carried out in animal models, and the potential interaction mechanism and functions of immune cells and other cells in different stages of human fracture healing are not well understood.

Therefore, we propose the following hypothesis that during the process of fracture healing, immune cells may repair of bone tissue through affecting differentiation of stem cells, function of other cells.

In this project, single cell transcriptome data of bone and bone marrow from fresh and old fracture patients were downloaded from GSE142786, cell type identification was performed. Compared to the original paper, in addition to focusing on the heterogeneity of immune cells, we also identified fibroblasts and macrophages, which play an important role in fracture healing. This study focused on revealing the differential expression gene of fibroblasts and macrophages and their interactions with immune cells in different stages of fracture.

Results

scRNA-seq and cell constitution of human bone fracture

In order to probe the the constitution of the cell populations in human bone samples during fracture healing process, we downloaded the data sets of bone healing from GEO (GSE142786). These data contained two samples from the same patient. One sample was harvested at the time of the fracture and the other was 30 days after the fracture. After standard data processing and quality filtering (Method), we obtained single-cell transcriptomes from a total of 7762 single cells after data quality control screening.

18 clusters were identified by unbiased clustering of the cells based on uniform manifold approximation and projection (UMAP) analyses. According to the top principals each cluster was annotated, the marker genes were detected. There were as follows: (0) Neutrophils, (1) Granulocytes, (2) Neutrophils, (3) Neutrophils, (4) Neutrophils, (5) fibroblasts, (6) ISG expressing immune cells, (7) Erythroid-like and erythroid precursor cells, (8) CD8 + NKT, (9) Neutrophils, (10) Macrophages, (11) Effector CD4 + T cell, (12) CD8 + NKT, (13) Erythroid-like and erythroid precursor cells, (14) Myeloid dendritic cells, (15) Plasma B cells, (16) Pre B cells, (17) Plasmacytoid immune cells (Fig. 1A). Among them, 5 clusters were Neutrophil granulocyte, 2 clusters were red blood cell precursor. Differences in the expression of representative marker genes in cell populations were analyzed by statistical quantification to match biological annotations.

In order to determine differences in cellular proportion, each cell cluster of different sample data was compared. Compared to the cell types obtained in the original paper[8], the main increases were in the types of cluster 10 (macrophages) and cluster 5 (fibroblasts). The top3 characteristic genes of macrophages (cluster 10) are VCAN, HLA-DRA and KLF4, fibroblasts (cluster 5) are MMP9, PTGS2 and FPR1 (Fig. 1B-C). Compared to fresh fracture samples, the proportion of ISG expressing immune cells (cluster6), Effector CD4 + T cell (cluster11), CD8 + NKT (cluster12), Macrophage (cluster10) in old fracture increased significantly (Fig. 1D-E). The gene sets enriched for macrophage (cluster 10) were inflammation response, immune response and neutrophil degranulation related pathways (Fig S1). These results indicated that the number of immune cells was dominant in different stages of fracture repair, but the composition and function of immune cells changed with time.

Expression Of Differentiation Of Fibroblasts And Macrophage

The comparison of the differentially expressed genes per cell subpopulation in fresh and old fracture samples revealed that the amount of differentially expressed genes per cell subpopulation was more varied between the two sets. We focus on the cell types not detected in the original paper - macrophage (cluster10) and fibroblast (cluster5) (Fig. 2A). Of note, for fibroblasts, DEGs were enriched in immune response related pathway, such as neutrophil degranulation pathway, inflammatory response, innate immune response pathway, although proportion of cell numbers has little difference (Fig. 2, Fig S2). Previous studies have showed an prominent interaction between fibroblasts and immune cells, which further influence osteoclast bone resorption[5]. Our data suggested that fibroblasts also influence the immune response at the fracture site (Fig. 2B). TXNIP was significantly increased in fibroblasts of old fracture samples (Fig. 2D-E). The differential expressed genes detected by macrophage (cluster10) between the two types of fracture samples were not only enriched in the inflammation response and antigen presentation pathway, but also apoptosis and positive regulation of cell migration related pathways (Fig. 2C). Among them, Epidermal growth factor AREG was significantly increased in old fracture samples (Fig. 2D-E).

Interaction Between Marcophage And Fibroblast

In order to identify potential interactions between different cell subsets at the fracture site, we preformed Cellchat analysis on potential interactions among cell subsets. Our results showed that the strength of cell interactions increased significantly in fresh fracture samples (Fig. 3A). Comparing to old fracture, it was found that the intensity difference of interaction between macrophages with other cell subsets was the largest in the fresh fracture (Fig. 3B, FigS3B). Meanwhile, the inflammatory signaling pathways such as THBS, IL-1, and CXCL became significantly more active in old fractures(Fig. 3C). Therefore, we focused on cell-cell interaction between macrophage with fibroblast and other cells. The results showed that in old fracture samples, the interaction between ANXA1, a ligand on macropahge and FRP1, a receptor in Neutrophils, fibroblast, ISG expressing immune cells, and Myeloid dendritic cells was significantly increased (Fig. 3D). ANXA1, as a regulator of glucocorticoid-mediated inflammatory processes, plays an

important role in innate immune responses and has anti-inflammatory activity. This results indicated that macrophages were involved in bone remodeling by inhibiting inflammation in the late stage of bone repair.

Discussions

In this study, single cell transcription maps of bone and bone marrow tissue samples from patients with fresh and old fractures were systematically identified. Based on the gene expression characteristics of single cells, 18 different cell subgroups were identified, indicating the diversity of cell groups in bone tissue at different periods of fracture. In our analysis, 18 cell subsets were identified, and cluster10 (macrophages) and cluster5 (fibroblasts) were main newly discovered cell types compared with 13 cell subsets in the original article. By analyzing the changes of genes in different cell subpopulations in fresh and old fracture tissues, we found that in fibroblasts, the expression altered significantly by differential expressed genes identification.

Fibroblasts play an important role in fracture healing. Fibroblasts are regulated by inflammatory cytokines and secrete inflammatory cytokines that regulate immune processes. IL-1β and TNF-α promote fibroblast proliferation and also promote prostaglandin E2 expression, which in turn inhibits fibroblast proliferation [9]. Fibroblasts express a variety of TLR receptors and therefore secrete a large number of cytokines in response to pathogens and injuries [10]. Poly(I:C), LPS, bacterial lipoprotein, and flagellin stimulate fibroblasts to secrete IL-6 [11]. Cytokines secreted by fibroblasts regulate T cells, B cells and macrophages. These immune cells secrete a large amount of cytokines to promote the absorption of necrotic tissue and the formation of new blood vessels. At the same time, they can also regulate the balance of osteoblasts and osteoclasts [12, 13]. In addition, different cell subsets of fibroblasts have different functions, with some promoting inflammation and others inhibiting it [14].

In our study, the most prominent one is that the expression of TXNIP gene is significantly increased in fibroblasts of old fracture samples. This gene encodes a thioredoxin-binding protein that is a member of the alpha arrestin protein family. Thioredoxin is a thiol-oxidoreductase that is a major regulator of cellular redox signaling which protects cells from oxidative stress. This protein inhibits the antioxidative function of thioredoxin resulting in the accumulation of reactive oxygen species and cellular stress. This protein also functions as a regulator of cellular metabolism and of endoplasmic reticulum (ER) stress. Knocking down TXNIP in osteoblasts promoted osteogenic differentiation, and culture osteoclasts with TXNIP silenced osteoblast supernatant increased the activity of osteoclasts [15]. Recent study found that tanshinol activated TXNIP pathway to inhibit microcirculation disturbance, then improved bone quality [16]. We speculate that TXNIP elevation in old fracture samples inhibit osteoblast differentiation and promote osteoclast absorption. In addition, (cluster10) macrophages, as a versatile cell type, has also attracted attention in recent years for its role in the process of fracture repair.

Mcarophages take participate in inflammatory stage and play an important role in fracture healing [17]. In animal modal, depletion of macrophages reduced expression of oncostatin M, collagen type 1 and

decreased bone mineralization thorugh reducing the production of oncostatin M. Osteogenic differentiation of MSCs obviously reduced due to lack of macropahges induction, resulted in reduced callus formation and bone deposition [18, 19].

Macrophages produced a series of cytokines promote angiogenesis and formation of primary cartilaginous calluses [20], such as IL-1, IL-6, TNF-α, CCL2. Rencet study have shown that macrophages also play an important role in late fracture. Proinflammatory cytokines produced by macrophages affect osteoblast and osteoclast differentiation. Bone morphogenetic protein 2, bone morphogenetic protein 4, and TGF-b1 secreted by macrophages promote osteogenesis [19]. Recent study have shown that macrophages promote osteogenic differentiation of BMSCs through secreting macrophage scavenger receptor 1 to activate PI3K pathway [21].Recent studies have shown that M1, M2 and DC cells are recruited to the fracture site in the early stage of fracture repair, which may synergically regulate inflammation and participate in osteoblast recruitment in the later stage of fracture repair. The number of M1 cells at the fracture site was correlated with the expression of IL-1α, IL-1ß, IL-2, IL-17, Eotaxin, and MCP-1, and the number of DCs was correlated with the expression of IL-6, G-CSF, LIF, KC, and VEGF-A [22, 23]. Reducing the number of macrophages has no effect on the early stage of fracture healing, but will affect the formation of endochondral ossification in the later stage, leading to slower fracture healing. M2-type macrophages increased significantly in ossification stage [23].

Our results show that AREG expression is elevated in old fracture samples. AREG secreted by macrophages drived fibroblast proliferation [24]. AREG is related to epidermal growth factor EGF and TGF-α, which promote the growth of epithelial cells [25]. Expression of AREG influence bone development which was stimulated by Parathyroid hormone and bone growth promoting hormone in osteoblasts and bone tissue. Importantly, the tibial trabecular bones of AREG-deficient mice were significantly smaller than those of wild-type mice [26]. We speculated that the proliferation of macrophages in old bone tissue may influence the number of fibroblasts through the secretion of AREG, then hinder bone healing.

It was observed that macrophages and other cell subsets exhibited the most extensive and intensive connections when the interaction links between various cell groups were examined. Especially in the old fracture samples, Macrophages possess a broad array of ligands and engage in many cellular interactions. The most significant important of these is the ANXA1-FRP1 signaling pathway. Previous studies have shown that ANXA1-FRP1 signaling pathway plays an important role in inflammatory response, and their interaction enhance anti-inflammatory effects [27]. Old fracture samples are mainly at the stage of callus formation and bone remodeling. Macrophages at this stage mainly play an anti-inflammatory role, release tissue repair factors, and recruit stem cells to promote osteoblastic differentiation [28].

To sum up, we speculate that macrophages in fracture tissue affect the function of cluster0 (neutrophils), cluster5 (fibroblasts), ISG cells) and cluster14 (mDC cells) through ANXA1-FRP1 signal pathway. At present, the function of macrophages in fracture repair is unclear. This study further demonstrated that

macrophages influence the function of other immune cells in bone through ANXA-FRP1 signaling pathways in the late stage of fracture repair, thus affecting osteogenic differentiation.

Methods

Retrieval and process of public data

Unique molecular identifier (UMI) count matrix of single-cell RNA-seq data from 2 bone fracture tissues (bone and bone marrow), which extracting at different time (0 day,30 days), was downloaded from GSE142786. The UMI count matrix was converted into a Seurat object by the R package Seurat [29] (version 4.0.4). Cells with UMI numbers < 500 or with detected genes < 200 or with over 20% mitochondrial-derived UMI counts were considered low-quality cells and were removed. Genes detected in less than 5 cells were removed for downstream analyses.

Scrna-seq Data Preprocessing And Quality Control

After quality control, the UMI count matrix was log normalized. Then top 2000 variable genes were used to create potential Anchors with FindIntegrationAnchors function of Seurat. Subsequently, IntegrateData function was used to integrate data. To reduce the dimensionality of the scRNA-Seq dataset, principal component analysis (PCA) was performed on an integrated data matrix. With Elbowplot function of Seurat, top 50 PCs were used to perform the downstream analysis. The main cell clusters were identified with the FindClusters function offered by Seurat, with resolution set as default (res = 0.6). Finally, cells were clustered into 17 major cell types. And then they were visualized with tSNE or UMAP plots. To identify the cell type for each cluster, we detected gene markers for each cell clusters using the “FindMarkers” function in Seurat package (v4.0.4), then we annotated cell types using previously published marker genes [30, 31].

Differential Gene Expression Analysis

Differential expressed genes (DEGs) were determined with the FindMarkers / FindAllMarkers function from the Seurat package (one-tailed Wilcoxon rank sum test, pvalues adjusted for multiple testing using the Bonferroni correction). For computing DEGs, all genes were probed that the expression difference on a natural log scale was at least 0.5 and the difference of percent of detected cells was at least 0.15 and adjusted pvalue was less than 0.05.

Cell–cell Communication

Cell–cell interactions based on the expression of known ligand–receptor pairs in different cell types were inferred using CellChat [32] (v1.0.0). To identify potential cell–cell communication networks in bone fracture tissues, we followed the official workflow and loaded the normalized counts into CellChat and

applied the pre-processing functions identifyOverExpressedGenes, identifyOverExpressedInteractions and projectData with standard parameters set. As database, we selected the Secreted Signalling pathways and used the precompiled human Protein–protein-Interactions as a priori network information. For the main analyses the core functions computeCommunProb, computeCommunProbPathway and aggregateNet were applied using standard parameters and fixed randomization seeds. Finally, to determine the senders and receivers in the network, the function netAnalysis_signallingRole was applied on the netP data slot.

Other Statistical Analysis

The pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html) in R was used to perform the clustering based on Euclidean distance. Student's t-test was used for comparisons between two groups.

Declarations

CRediT authorship contribution statement

Min Zhou and Chao Jian: conceptualization, searching and analyzing data, writing original draft paper. Xin Xu and Hao zhang: Proofreading data and preparing data graph. Baiwen Qi: conceptualization and revise paper. All authors approved the final version of the manuscript.

Declaration of competing interest

All author declare that there is no competing interest.

Data availability

All data related to draw conclusions in the paper are present in the paper, and additional data related to this study might be requested from corresponding author. The data of bone healing was downloaded at GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE142786)

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Figures

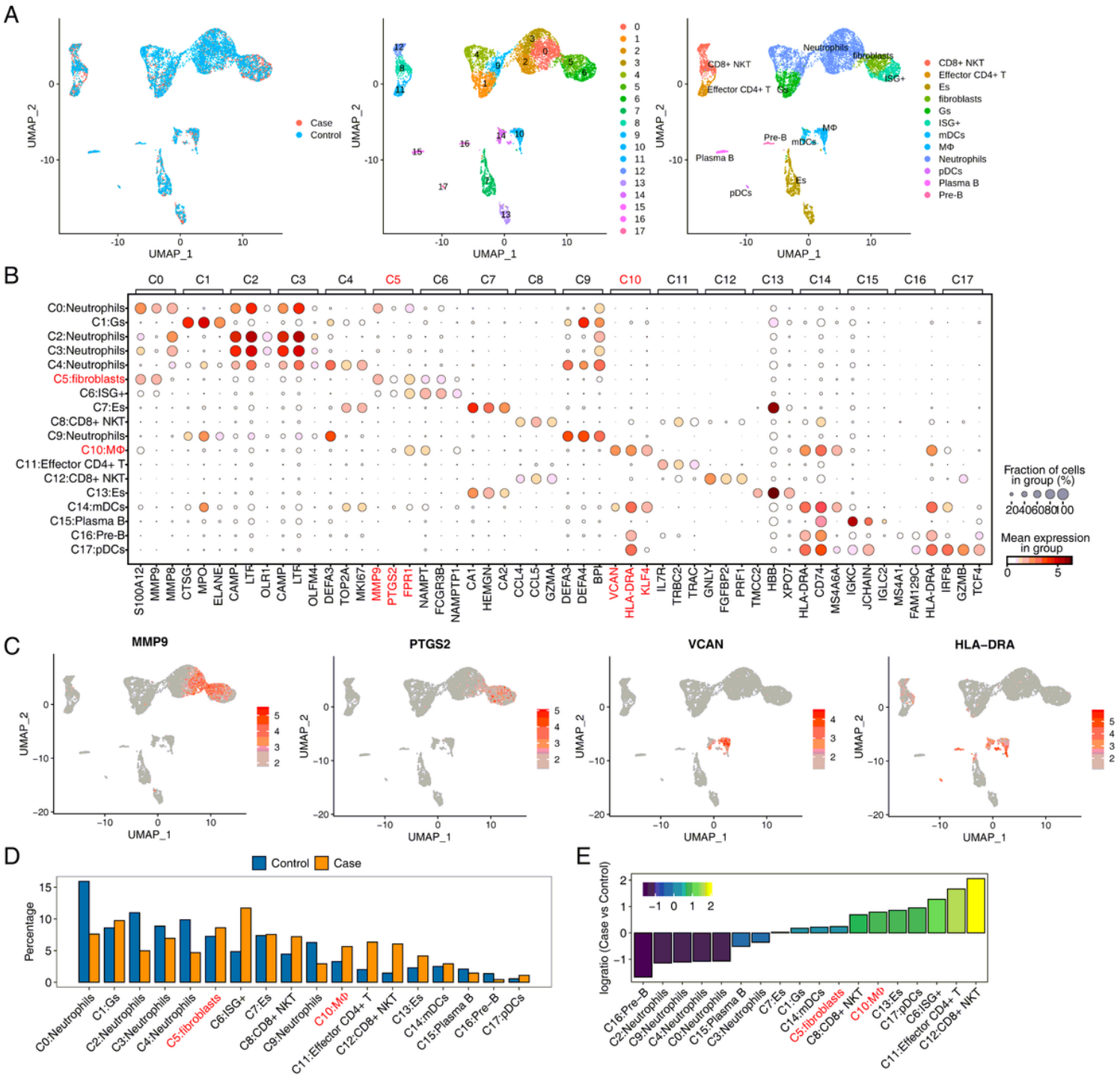

Figure 1

Global scRNA-seq analysis of bone and bone marrow from fresh and old fracture tissue identified distinct cells types.

(A) UMAP plot of composite single-cell transcriptomic profiles from all 2 samples from fresh fracture and old fracture patients. Colors indicate cell clusters along with annotations. Es: Erythroid-like and erythroid precursor cells; Gs: Granulocytes; ISG: ISG expressing immune cells; mDCs: Myeloid dendritic cells; pDCs: Plasmacytoid immune cells.

(B) Dot plot showing expression of representative top 3 genes in each cell type.

(C) Gene expression level of MMP9, PTGS2, VCAN, HLA-DRA in the UMAP plot split by different sample groups

(D) Bar plot comparing the proportions of cell populations of each cell type within each sample group.

(E) Rank order based on decreasing values of the relative frequency ratio between old fracture and fresh fracture sample.

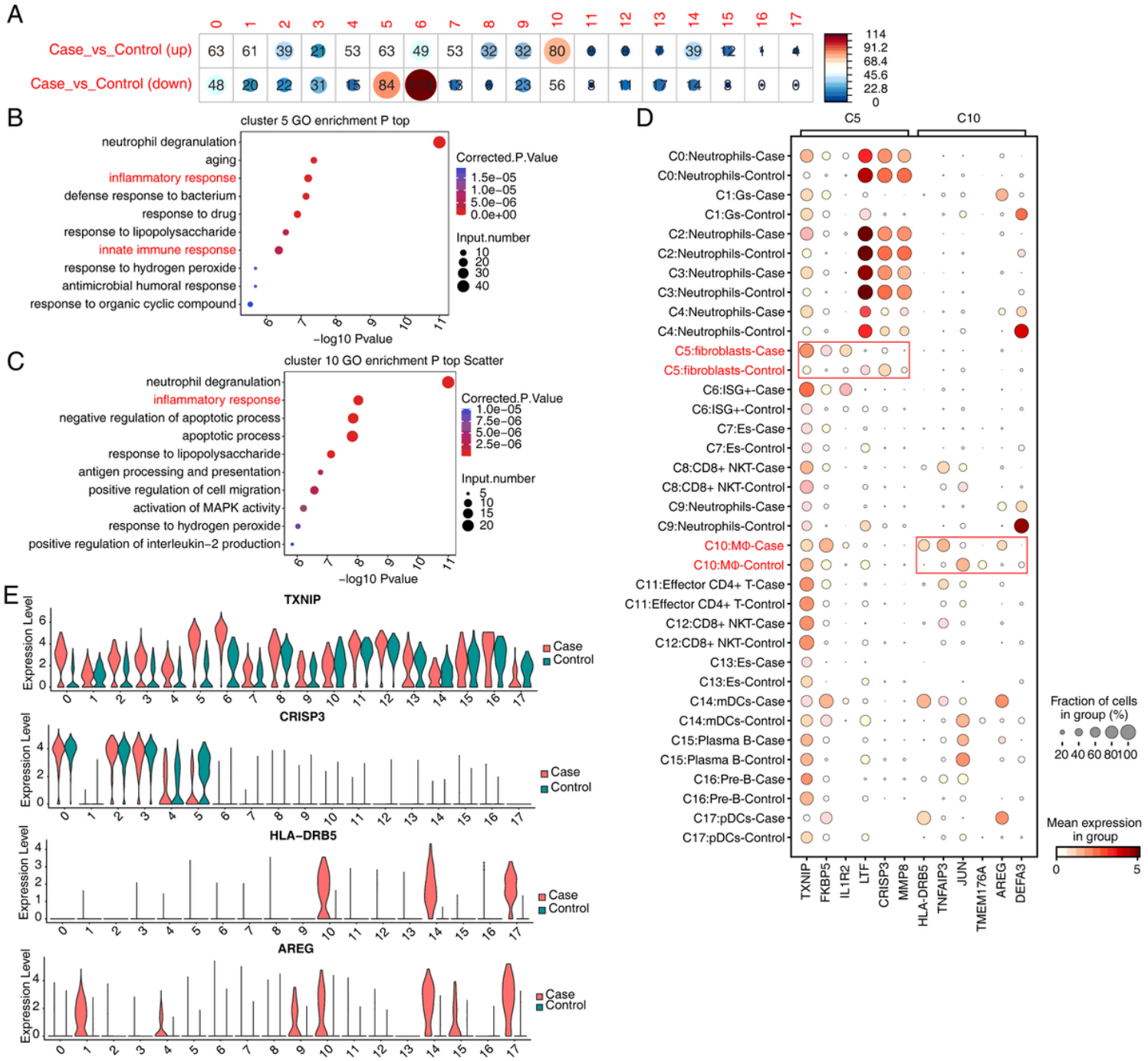

Figure 2

Single-cell analysis revealed different expression genes changes in old fracture and fresh fracture patients.

(A) Number of differentially expressed genes among old fracture and fresh fracture in each cell types.

(B) Gene Ontology biological process of all different genes in cluster5 in old fracture and fresh fracture tissue.

(C) Gene Ontology biological process of all different genes in cluster10 in old fracture and fresh fracture tissue.

(D) Dot plot showing expression of up and down top3 different expression genes in cluster5 and cluster10 cell types. Color of dots represents the mean expression in each cluster and dot size indicates percentage of cells in each cluster expressing the marker genes.

(E) Violin plot of TXNIP, CRISP3, HLA-DRB5, AREG in each cell type split by different sample groups.

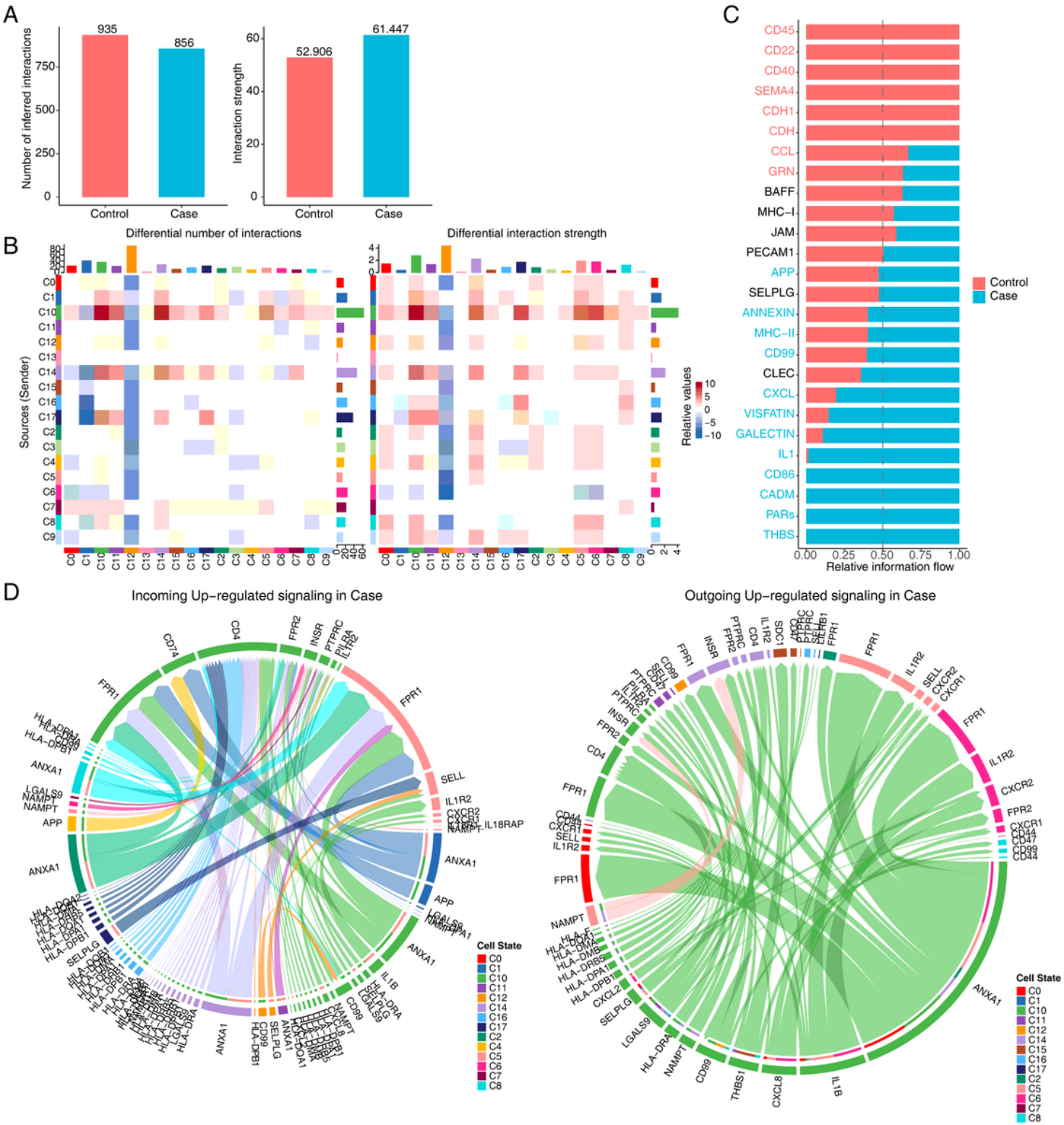

Figure 3

Intercellular signaling of macrophage and fibroblast cell with other cells showed dramatic changes

(A) Number (left) and strength (right) of CellChat-inferred interactions among the population in each sample groups.

(B) Heatmap showing the number of ligands and receptors estimated to have arisen at each paired cell populations for old fracture (Case) and fresh fracture (Control).

(C) Difference in interaction strength for key pathways in old fracture (Case) and fresh fracture (Control).

(D) Circular plot displaying up-regulated ligand-receptor pairs of cluster5 and cluster10 cell type. Lines originate at the ligand and connect to its receptor.

Supplementary Files

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- S1.png
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