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Research article

Single-cell transcriptomics reveals variable trajectories of CSPCs in the progression of osteoarthritis

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ABSTRACT

Osteoarthritis (OA) is characterised by cartilage destruction; however, there are no specific drugs available for its treatment. Cartilage-derived stem/progenitor cells (CSPCs) are multipotent cells that play an essential role in cartilage renewal and may provide critical insights into the medical needs for OA treatment. However, alterations in cell function and fate of CSPCs during OA progression have seldom been analysed, especially at the single-cell level. Additionally, it has been reported that CSPCs can migrate to the cartilage injury area, although the mechanism of migration remains elusive. Thus, understanding the changing patterns of CSPCs in the pathological process of OA is important in the effort to develop stem cell therapy for OA. Here, we downloaded single-cell transcriptomic data of patients with OA from the Gene Expression Omnibus (GEO) database and performed unbiased clustering of the cells based on gene expression patterns using the Seurat package. Using common stem cell markers and chondrogenic transcription factors, we traced CSPCs throughout all stages of OA. We further explored the dynamics of CSPCs in OA progression and validated the single-cell RNA sequencing data *in vitro* using qPCR, immunofluorescence, and western blotting. Specifically, we primarily explored the heterogeneity of CSPCs at the single-cell level and found that it was closely associated with OA progression. Our results indicate significantly reduced chondrogenic differentiation capacity in CSPCs during the late stage of OA, while their proliferation capacity tended to increase. We also found that genes implicated in fibrosis, cell motility, and extracellular matrix remodelling were upregulated in CSPCs during the progression of OA. Our study revealed the dynamics of stem cells in OA progression and may inform the development of stem cell therapy for OA.

1. Introduction

Osteoarthritis (OA), one of the most common musculoskeletal diseases among the elderly [1], is characterised by cartilage destruction, subchondral bone remodelling, synovitis, osteophyte formation, and increasing inflammation [2]. Chondrocytes are embedded in a framework of collagens, together with proteoglycans and glycoproteins, which act as link proteins to stabilise collagen [3]. Chondrocytes proliferate in the articular cartilage and secrete extracellular matrix-related proteins and cytokines to maintain cartilage homeostasis and resist compression [4, 5, 6]. OA can be induced by disturbing cell–matrix interactions; this involves the loss of the superficial zone and eventually produces deep surface fissures and collagen fibrillation [4, 7]. In addition, pro-inflammatory cytokines, such as interleukin (IL)-1β, tumour necrosis factor (TNF)-α, IL-6, etc. are involved in extracellular matrix catabolism [8, 9, 10]. Recently, stem cells or progenitor cells have been found in

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cartilage [11, 12, 13, 14]. Cartilage-derived stem/progenitor cells (CSPCs) express stem cell markers, including *CD44*, *CD51(ITGAV)*, *CD49e(ITGA5)*, *CD29(ITGB1)*, *CD54(ICAM1)*, *CD90(THY1)*, *CD105(ENG)*, *CD73(NT5E)*, and *CD106(VCAM1)*, and have multi-lineage differentiation potential, accompanied by high chondrogenic capacity and colony-forming ability [12, 15, 16, 17, 18, 19]. CSPCs contribute to tissue renewal and cartilage homeostasis. Evidence suggests that chondrogenic CSPCs play an important role in cartilage formation to attenuate OA development [14]. Moreover, migration abilities are important for CSPCs to arrive at injury sites to heal wounds and rapidly increase in the late stage of OA, but the underlying mechanism remains unclear [12, 15, 20, 21, 22]. In one study, different passages of CSPCs displayed heterogeneity, especially in surface stem cell markers and differentiation capacity [23, 24]. However, little is known about the heterogeneous dynamics of CSPCs during OA progression [25]. Therefore, it is meaningful to investigate the dynamics of CSPCs during OA progression to gain critical insights into the medical needs for OA treatment.

To explore the fate of CPSCs in OA, we analysed 1493 single cells from different stages of OA using a single-cell RNA-seq dataset (GSE104782) [26]. Based on canonical stem cell markers (CD44) and chondrogenic transcription factor (SOX9), we selected 532 CSPCs from all individuals. The characteristics of CSPCs change during OA progression, and increased expression levels of inflammation receptors and decreased differentiation was observed. Finally, we found that genes involved in fibrosis, migration, and extracellular matrix (ECM) remodelling were upregulated in CSPCs in the late stage of OA. Collectively, our results show that heterogeneity in CSPCs is largely associated with OA progression and reveal the dynamics of CPSCs in terms of cell behaviour and function during OA progression.

2. Materials and methods

2.1. Study design

The experiments were not randomised. No statistical methods were used to determine the sample size. The researchers were not blinded to the distribution during the experiments and outcome analyses.

2.2. Single-cell RNA sequencing (scRNA-seq) data retrieval and isolation of human articular chondrocytes

The single-cell sequencing data used in this study were downloaded from the Gene Expression Omnibus (GEO) data repository (GSE104782) [26]. Briefly, all articular cartilage samples were obtained from patients with knee OA who underwent knee arthroplasty. S0 (stage 0) is normal articular cartilage; S1 (stage 1) is softening of the articular cartilage; S2 (stage 2) is fibrillation or superficial fissures of the cartilage; S3 (stage 3) is deep fissuring of the cartilage without exposed subchondral bone; and S4 (stage 4) is exposed subchondral bone. The scRNA sequencing was performed on a total of 1464 chondrocytes from cartilage in 10 patients undergoing knee arthroplasty surgery using the 10X Genomic platform. To obtain articular cartilage, the femur and tibia were washed twice with sterile phosphate-buffered saline (PBS). As previously described [26], articular cartilage was obtained by digestion with 0.2% type II collagenase after being cut into 1 mm^3 pieces.

2.3. Single-cell transcriptomic data analysis

The main analyses in this study were performed using the Seurat R package (version 3.2.2) [27]. Individuals with fewer than 1000 detected genes and fewer than 10,000 detected transcripts were filtered. Raw counts were normalised by the "NormalizeData" function with the default parameters (normalisation method is LogNormalize and scale factor is 10,000). Using the "FindVariableGenes" function with the "vst" method and 4800 top variable features, 3800 highly variable genes were identified for principal component analysis (PCA). According to JackStrawPlot and ElbowPlot, 30 principal components were chosen to construct a shared nearest neighbour. The "FindClusters" function was used to cluster cells with a resolution of 0.7. The dimension reduction was based on t-SNE. "FindAllMarkers" with default parameters was used for differential expression analysis among different clusters. Cellular clusters were visualised using Dimplot software. Gene expression profiles were visualised using the VlnPlot function. A heat map was generated using the DoHeatmap function.

Pseudotemporal trajectory analysis was performed using the monocle2 R package (version 2.16.0) [27,28]. Normalised data and metadata were extracted from the Seurat object. The CellDataSet object for the monocle was created using the function "newCellDataSet". To mark genes of clustering in subsequent calls, setOrderingFilter was applied. Finally, "reduceDimension" was used to reduce dimension and identify trajectory of the input cells.

Gene ontology enrichment analysis was conducted using the clusterProfiler package (version 3.16.1) [29]. For precise visualisation of the data, biological pathway enrichment networks and transcriptional regulatory relationship (TRRUST) analyses were conducted using Metascape [30, 31]. Lineage scores were calculated according to a published article [32] by calculating the sum of the logarithm of counts per million across selected genes (see as Supplemental Table 1). Statistical analyses were performed using the ggsignif R package (version 0.6.0).

2.4. Ethics statement

All animal experiments in this study were approved by the Animal Care and Use Committee of Tongji University School of Medicine. Eight-week-old Sprague–Dawley (SD) rats (SLAC, Shanghai) were used for *in vivo* analysis. All rats were maintained on a 12-hour light cycle in the animal facility of the Animal Unit of Tongji University.

2.5. Isolation and culture of rat CSPCs

The CSPCs were isolated as previously described, with minor modifications [12, 33, 34, 35]. CSPCs were isolated from the tibial plateau of healthy SD rats (n = 5) using the migratory method. Briefly, articular cartilage tissues were manually dissected into 1 mm^3 pieces. The tissue pieces were then digested with 0.25% collagenase II (17101-015, Gibco) for 2 h. Then, digested cartilage chips were transferred into complete medium [DMEM/F12 (11320033, Gibco), 10% foetal bovine serum (10100147, Gibco), 1% penicillin/streptomycin (03-031-1B, BI) and 4.5 mM L-glutamine (03-020-1B, BI)] and incubated at 37 °C and 5% CO_2. Finally, the CSPCs outgrew from the cartilage chips within 10 days. When cultured to 80–90% confluence, the cells were digested with 0.05% trypsin (25300054, Gibco), harvested by centrifuging at 800 ×*g* for 5 min, and then cultured in 6-well plates with complete medium. CSPCs from passage 2–4 was used in all experiments.

2.6. Flow cytometry

CSPCs at passage 2 (~1,000,000 cells) were harvested by trypsin digestion and incubated for 30 min in the dark at 4 °C with conjugated antibodies against CD90-FITC (11-0900-81, Invitrogen), CD29-PE (12-0291-81, Invitrogen), and CD45-APC (17-0461-80, Invitrogen). After three washes with PBS (02-024-1A, BI), the labelled cells were resuspended in 400 μL PBS and collected through flow cytometry using the FACSCalibur system (BD Biosciences). Isotype controls were used to exclude nonspecific interactions between the antigens and antibodies. Flow cytometry data were analysed using the FlowJo software (version 10.0.7r2).

2.7. Colony forming assay

CSPCs at passage 2 were harvested and cultured in 6-well plates (500 cells/well). After 7 days of culturing, the cells were fixed in 4% paraformaldehyde for 20 min and then stained with 1% crystal violet dye (G1062, Solarbio) for 15 min. After washing with ddH_2O until clarified, the gross appearance of the colonies was imaged using Nikon digital photography system (ECLIPSE Ti, Nikon).

2.8. Multilineage differentiation and quantification

For chondrogenesis, 1×10^6 CSPCs at passage 2 were centrifuged in 15 mL polypropylene tubes to obtain cell pellets and cultured in MesenCult™-ACF chondrogenic differentiation medium (Catalogue #05455, Stem Cell) for 4 weeks. After differentiation, pellets were fixed and sectioned. Chondrogenesis was analysed by staining with toluidine blue (G3668, Solarbio). For osteogenesis, CSPCs at passage 2 were harvested and cultured in 24-well plates (20,000 cells/well). After 90% confluence, the osteogenic differentiation medium was replaced [low glucose DMEM (10567-014, Gibco) with 10% FBS, 10 mM β-glycerophosphate (50020, Sigma), 50 mM L-ascorbate-2-monophosphate (A7631, Sigma), and 1 μM dexamethasone (D1756, Sigma)] for 3 weeks. The osteogenic cells were fixed and stained with alizarin red (G1452, Solarbio). To induce adipogenesis, CSPCs at passage 2 were harvested and cultured in 24-well plates (20,000 cells/well). After 90% confluence, the osteogenic differentiation medium high glucose DMEM (11965-092, Gibco) was replaced with 10% FBS, 100 μM indomethacin (I7378, Sigma), 0.5 mM IBMX (I5879, Sigma), 10 μg/mL insulin (91077C, Sigma), and 1 μM dexamethasone (D1756, Sigma) for 2 weeks. Oil Red O (G1260, Solarbio) staining was performed to assess the adipogenicity potency.

2.9. In vitro model of inflammatory environment

CSPCs were resuspended in complete medium, seeded at 10^4 cells/well in 6-well plates containing different concentrations of IL-1β (0, 10, 30, 60 ng/mL), and cultured for 48 h. After the cells became confluent, a model was constructed, and the cells were suitable for subsequent experiments.

2.10. RNA extraction and quantitative reverse transcription PCR

Total RNA was extracted from CSPCs using the FastPure Cell/Tissue Total RNA Isolation Kit (RC401, Vazyme), following the manufacturer's instructions. Complementary DNA (cDNA) was synthesised with 1 μg of total RNA using a FastKing RT Kit (KR116, Tiangen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Q71102, Vazyme) with a Stepone Plus Realtime system (LightCycler 96, Roche). β-actin was used as an endogenous control, and the relative expression of mRNA was quantified using $2^{-\Delta\Delta}$CT. The primer sequences used in this study are listed in supplementary tables (see Supplemental Table 2).

2.11. Western blotting

Total protein was isolated from primed CSPCs using RIPA buffer (WB6001, Weiaolab) containing phenylmethanesulfonyl fluoride (PMSF) (WB2122-3, Weiaolab). Samples were loaded onto 10% SDS-PAGE (PG112, EpiZyme) and transferred to 0.45 PVDF membranes (IPVH00010, Merck Millipore). After blocking with 5% non-fat dry milk (Anchor, New Zealand) in TBST for 2 h at room temperature, membranes were incubated with primary antibodies, anti-PCNA (24036-1-AP, Proteintech), anti-SOX9 (ab185966, Abcam), and anti-β-actin (ab8226, Abcam) overnight at 4 °C, followed by secondary antibodies, anti-rabbit (SA00001-2, Proteintech) and anti-mouse (SA00001-1, Proteintech), at room temperature for 2 h. Finally, membranes were treated with an ECL chemical luminescence kit (P0018FM-2, Beyotime) and images were captured using the Odyssey imaging system.

2.12. Immunofluorescence

The CSPCs were fixed with 4% paraformaldehyde (P1110, Solarbio) for 15 min at room temperature and permeabilised using 0.25% Triton (A11094, Sangon Biotech) for 20 min. The cells were blocked with 3% donkey serum (017-000-121, Jakson) for 2 h and incubated overnight with anti-PCNA (24036-1-AP, Proteintech) and anti-SOX9 (ab185966; Abcam). After washing, the cells were incubated with Alexa Fluor 568-conjugated donkey anti-rabbit secondary antibody (A10042, Thermo Fisher Scientific) and DAPI (D9542, Sigma) for 1 h at room temperature. Images were captured using a Leica microscope system (TCS SP8, Leica).

2.13. Statistical analysis

The experimental data were tested using Dunn's multiple comparison test for non-normally distributed unpaired groups using GraphPad Prism (v.8.2.1). Bioinformatic data were statistically analysed using unpaired, two-tailed Mann–Whitney *U* tests with R. Data are represented as the mean ± S.D.

3. Results

3.1. Identification of CSPCs in human OA cartilage

The reported single-cell RNA-seq dataset, including 1493 chondrocytes from patients with OA, was used as the input data for the Seurat R package. After data quality control and normalisation, ten clusters of chondrocyte populations were identified using an unbiased clustering algorithm (Figure 1a, b). Primarily, all individuals were artificially divided into three stages, as described in a previous paper [26]: early stage (S0, S1), middle stage (S2), and late stage (S3, S4) (Figure 1c). To analyse the dynamics of chondrocyte progenitors, we chose cells co-expressing CD44 and SOX9 (expression level >1) for further analysis, following a previous paper [36] (Figure 1d). A total of 532 CSPCs expressing CD44 and SOX9 were identified, and the cells were projected onto a t-SNE plot for visualisation (Figure 1e). Additionally, the percentage of CSPCs among the different stages is shown in Figure 1f: 39.47% in the early stage, 22.37% in the middle stage, and 38.16% in the late stage (Figure 1f). To confirm the function of the selected CSPCs, we analysed differentially expressed genes (DEGs) between assumed and rested cells. It was noted that stem cell-related genes (*THY1*), cell fate-related genes (*KLF4*, *MCL1*, *BMP2*, *SFRP2,* and *HES1*), and stem cell maintenance genes such as *KLF4* and *HES1* were expressed increasingly in the assumed CSPCs, indicating that this standard worked well (Figure 1g–h). Together, this single-cell RNA-seq screen not only confirmed the previously shown result that CSPCs existed widely in cartilage chondrocytes, but also revealed previously unidentified differences. Next, we used these data to evaluate heterogeneity within CSPCs in relation to possible differentiation.

3.2. CSPC heterogeneity among different stages of OA

To further explore the characteristics of CSPCs, we re-clustered all CSPCs (Figure 2a). Intriguingly, we found that recalculated Seurat clusters matched well with the arrangement of OA stages, especially in the early and late stages (Figure 2b). Cells from the early stage were mainly distributed in area I and cells from the late stage were mainly distributed in area III, while cells in the middle stage were scattered evenly in all three areas (Figure 2c). These results suggest that discrepancies among CSPCs are largely associated with OA progression. We also calculated cell cycle scores among selected cells, and the results showed that cell cycle-related genes made a small contribution to clustering, further supporting that OA progression is a key factor resulting in the heterogeneity of

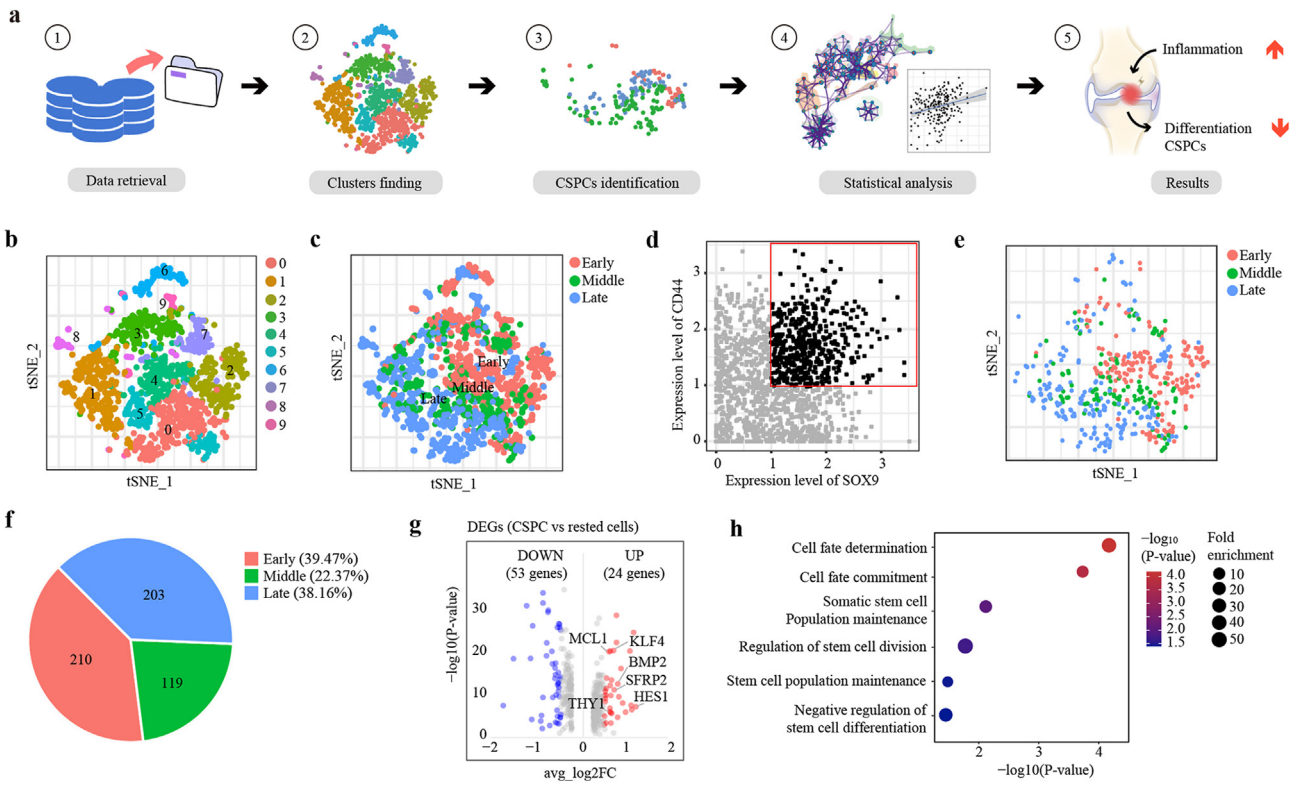

Figure 1. Identification of CSPCs in human OA cartilage chondrocyte. a) Schematics of the whole research. b) t-SNE plot for all ten Seurat clusters. c) t-SNE plot for all ten Seurat clusters coloured by different stages. d) Scatter plot showing the expression of pan stem-cell markers among all cells. e) t-SNE plots showing the distribution of assumed CSPCs among different stages. f) Pie plot showing the percentage of CSPCs at different stages. g) Volcano plot of the different expression genes between CSPCs and non-CSPCs. h) Dot plots for selected GO term of upregulated DEGs in CSPCs.

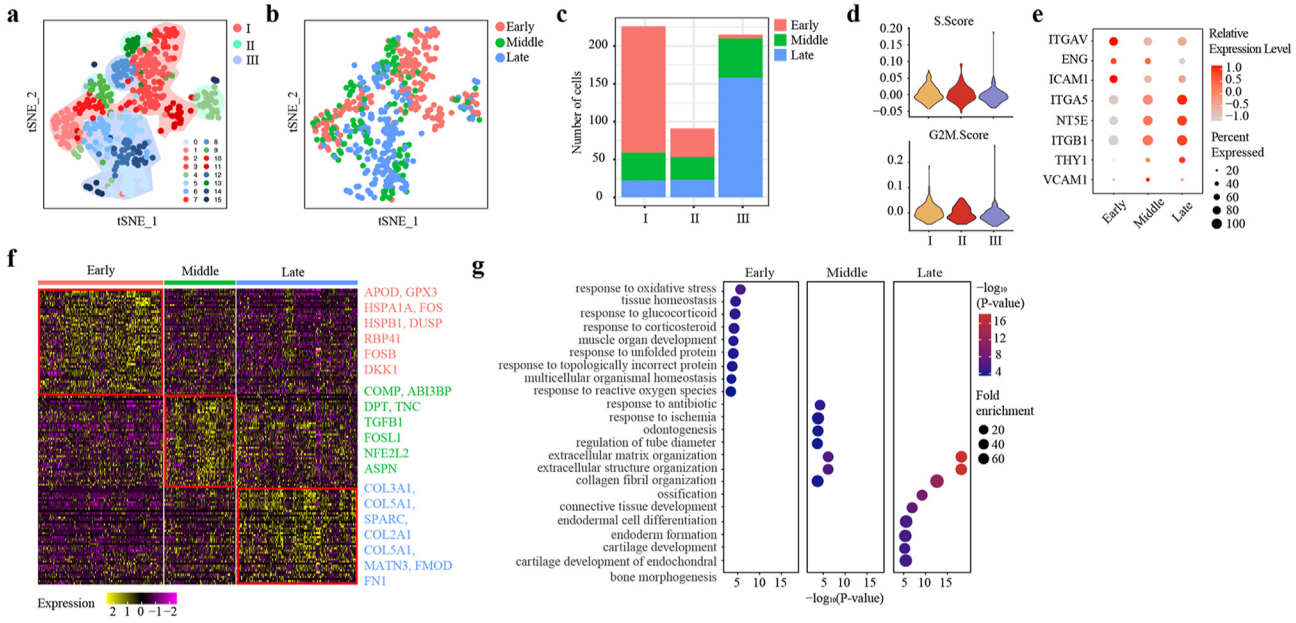

Figure 2. Heterogeneity of CSPCs among different stages of OA. a) t-SNE plot for CSPCs coloured by recalculated Seurat clusters and marked by manual boundaries. b) t-SNE plot for CSPCs coloured by stages. c) Histogram for the numbers of stem cells in recalculated Seurat clusters among different stages. d) Violin plot for S phase score and G2M phase score among different recalculated Seurat clusters. e) Dot plots showing the distribution of diverse stem cell marker among different stages. f) Heatmap for highly variable genes among different stages. g) Dot plot showing the GO terms of DEGs among different stages.

CSPCs (Figure 2d). Furthermore, we found that the expression of stem cell markers showed high variation among the different stages (Figure 2e). For example, the expression of some stem cell markers, such as *ITGAV*, *ITGA2,* and *ICAM1* decreased during the early to late stages, while the expression of other stem cell markers such as *ITGA5*, *NT5E,* and *ITGB1* gradually increased during OA progression (Figure 2e). To further delineate the heterogeneity of CSPCs among different stages, we calculated the highly variable genes among different stages from which we

selected the top 50 (Figure 2f). By subjecting these genes to GO analysis, we found that genes upregulated in the late stages were strongly associated with the extracellular matrix (ECM) and collagen fibril organisation, while genes upregulated in the early stages were mainly related to the response to the matrix microenvironment (Figure 2g). Simultaneously, genes upregulated in the middle stages were combined with those in the early and late stages, involving both extracellular matrix organisation and response to stimulation (Figure 2g). Together, these results indicate that the heterogeneity of CSPCs is strongly associated with the different stages of OA.

3.3. Variable behaviours of CSPCs in OA progression

To characterise the behaviours of CSPCs during OA progression, we first analysed these data on Monocle. Cells of late stages were mainly clustered at the onset of the trajectory, whereas cells from early stages appeared almost at the end of the trajectory (Figure 3a, Supplemental Figure 1). These results indicated that CSPCs in the late stage of OA tended to be at the beginning of the development trajectory. In addition, results of transcriptional regulatory relationship (TRRUST) analysis showed that DEGs in the late stage are regulated by most transcription factors (TFs), especially inflammation-related TFs such as JUN, RELA, and NFKB1, revealing that CSPCs are more sensitive to inflammatory stimulation during the development of OA (Figure 3b). Using a heatmap of the trajectory makes it evident that the expression of critical osteoarthritis mediators such as *TNFSF1A* increased with the progression of OA (Figure 3c, d). Furthermore, we found that the expression levels of chondrogenic-related genes, such as *SOX9*, decreased greatly in the late stage, whereas those of proliferation-related genes, such as *PCNA*, increased mildly during OA progression (Figure 3c, d).

Because differentiation and proliferation are essential parameters in evaluating the function of CSPCs, we used *in vitro* experiments to confirm the dynamics of differentiation and proliferation of CSPCs in OA progression. We isolated CSPCs from rats and cultured them in an inflammatory environment; they were identified by assay of multilineage differentiation and quantification, flow cytometry, and colony formation (Supplemental Figure 2). IL-1β is a major pro-inflammatory cytokine involved in the pathogenesis of OA and widely used to simulate the inflammatory environment *in vitro* [8, 9, 37, 38]. In this study, we used different doses of IL-1β to induce an inflammatory environment similar to the diverse stages of OA. Reassuringly, the results of real-time PCR analysis revealed that the expression pattern of either proliferation or differentiation genes was highly concordant with single-cell RNA-seq data. Notably, the expression levels of *Pcna* were relatively high in the late stage, while the expression levels of *Sox9* were significantly reduced in the late stage (Figure 3e). In parallel, immunofluorescence and western blotting for *PCNA* and *SOX9* in IL-1β-treated cells displayed consistent results with qPCR (Figure 3f, g). Taken together, we show that inflammation may drive dysfunction of CSPCs, leading to depression in differentiation.

3.4. Functional dynamics of CSPCs during OA progression

Because epithelial–mesenchymal transition (EMT) leads to morphological and functional alterations, we next assessed the expression of EMT-related genes across all stages. We noted that expression levels of

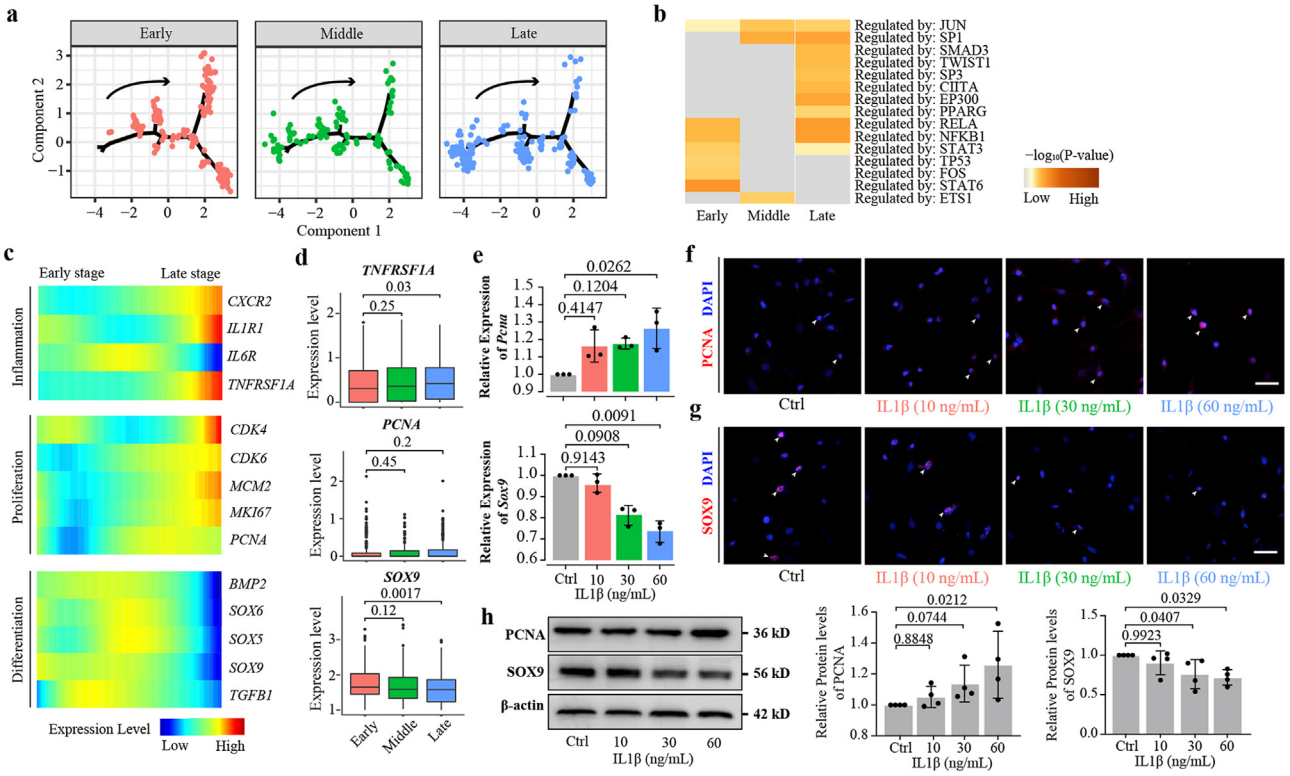

Figure 3. Behaviours of CSPCs in progressing of OA. a) Monocle pseudospace trajectory revealing the CSPC lineage progression coloured and separated by stages. b) Heatmap showing the results of TRRUST among different stages. c) Heatmap showing the expression changes of inflammation, proliferation and differentiation related genes. d) Box plot for specific representative genes showed in Figure 3c. e) qPCR analysis shows the expression of *Pcna* and *Sox9* among cells treated by different concentration of IL-1β (n = 3 biologically independent animals per group). Data are represented as mean ± S.D, adjusted *p* value was labelled on the top. f-g) Immunofluorescence analysis of PCNA and SOX9 among cells treated by different concentration of IL-1β (Representative images are shown from n = 3 biologically independent samples, Scale bar = 50 μm). h) Protein levels of PCNA and SOX9 among cells treated with different concentration of IL-1β by western blotting. Representative images are shown from n = 4 biologically independent samples, β-actin was used as a loading control. The blue fluorescence represents nuclear-staining DAPI confirming the presence of the cells. The uncropped images of (h) were referred in Supplemental Figure 3.

EMT-related translational factors, such as *SNAI1* and *SNAI2,* tended to be slightly upregulated in the late stage, whereas EMT-mediated fibrosis genes (*FN1*, *COL1A1*, and *COL3A1*) showed greatly increased expression in CSPCs in the late stage (Figure 4a). These findings indicate that CSPCs may become dysfunctional in the late stage. We further analysed the genes involved in cell adhesion and motility. Our data showed that cell adhesion-related genes (*DSP* and *PLEC*) and Rho-GTPase related genes (*RHOA*, *RHOC,* and *CDC42*) showed little change, but the expression of cell motility-related genes such as *ARPC1A*, *ARPC3*, *ARPC4*, *PTK2,* and *VCL* tended to increase during OA progression (Figure 4a). To explore the dynamics of cartilage resurfacing genes in CSPCs, we analysed the expression of key ECM remodelling genes (*COL2A1*, *COL12A1,* and *TIMP1*). Our results show that, in contrast to the aggravation of fibrosis, the expression of ECM remodelling genes such as *COL2A1* and *TIMP1* tends to increase in the late stage. To analyse this tendency comprehensively, we calculated the lineage scores of fibrosis, cellular adhesion, Rho-GTPase, and cell motility using the above genes. We found that the expression of EMT-and ECM remodelling-related genes increased considerably during OA progression (Figure 4b, c). Cell adhesion and Rho-GTPase-related genes showed no significant changes in expression among different stages, but cell motility genes were expressed increasingly in the late stages (Figure 4d–f). In summary, these data revealed that the function of CSPCs tends to decline in the late stage of OA, characterised by a surge in EMT and fibrosis.

4. Discussion

In this study, we investigated the dynamics of CSPCs in the progression of OA by analysing the single-cell sequencing dataset GSE104782. By analysing single-cell transcriptomic data, we found that CSPCs are involved in all three stages and heterogeneity of CSPCs has a strong relationship with OA progression. In addition, we found that genes involved in chondrocyte differentiation, such as *SOX9*, *BMP2*, *SOX5,* and *SOX6* were largely downregulated in the late stage, suggesting that the differentiation of CSPCs in the late stage was reduced; however, the expressions of genes involved in proliferation, such as *PCNA*, *MKI67* and *CDK6*, increased as OA progressed. Simultaneously, the capacity of CSPCs to repair damage, characterised by the sharp upregulation of genes involved in cellular motility and ECM repair, tended to increase in the late stage. Based on these findings, we hypothesised that although one of the most important functions of CSPCs, chondrogenic differentiation, shows a significant decrease, CSPCs tend to compensate for the deleted function by improving proliferation and damage repair abilities. Furthermore, we speculated that replenishing healthy stem cells in joints of patients with OA may bring about a therapeutic effect through the above mechanism.

Studies on progenitor cells in human OA are limited by materials and methodologies. Although previous studies have revealed changes in the microenvironment of osteoarthritic cartilage [26, 36], the dynamics of CSPCs during OA progression are still unclear. Instead of exploring other chondrocytes, we mainly focused on CSPCs. First, we investigated the distribution and heterogeneity of CSPCs at the different stages of OA. We also explored the dynamics of the main biological functions of CSPCs, such as differentiation, proliferation, and extracellular matrix remodelling. Compared with previous studies, we systematically analysed the dynamics of CSPCs in the process of OA, which is beneficial for understanding the principle of self-regulation in osteoarthritic articular cartilage and provides a theoretical basis for stem cell therapy.

As previously described, there are large differences among CSPCs and the underlying mechanisms are unclear [25]. In the current study, we found that the heterogeneity of CSPCs can be greatly influenced by OA progression, and markers such as *ITGAV*, *ITGA2,* and *ICAM1* are highly expressed in the early stage, while *ITGA5*, *NT5E,* and *ITGB1* are upregulated in the late stages. In addition to the above markers, the functions of CSPCs in different stages are diverse, responding to environmental stress in the early stage, initiating repair in the middle stage, and producing extensive ECM repair in the late stage. Previous studies have also confirmed that CSPCs with different markers have different functions [39, 40]. Based on these findings, we suggest that CSPCs display a great deal of heterogeneity in different periods of OA and this heterogeneity has a strong relationship with OA progression characterised by changes in stem cell markers.

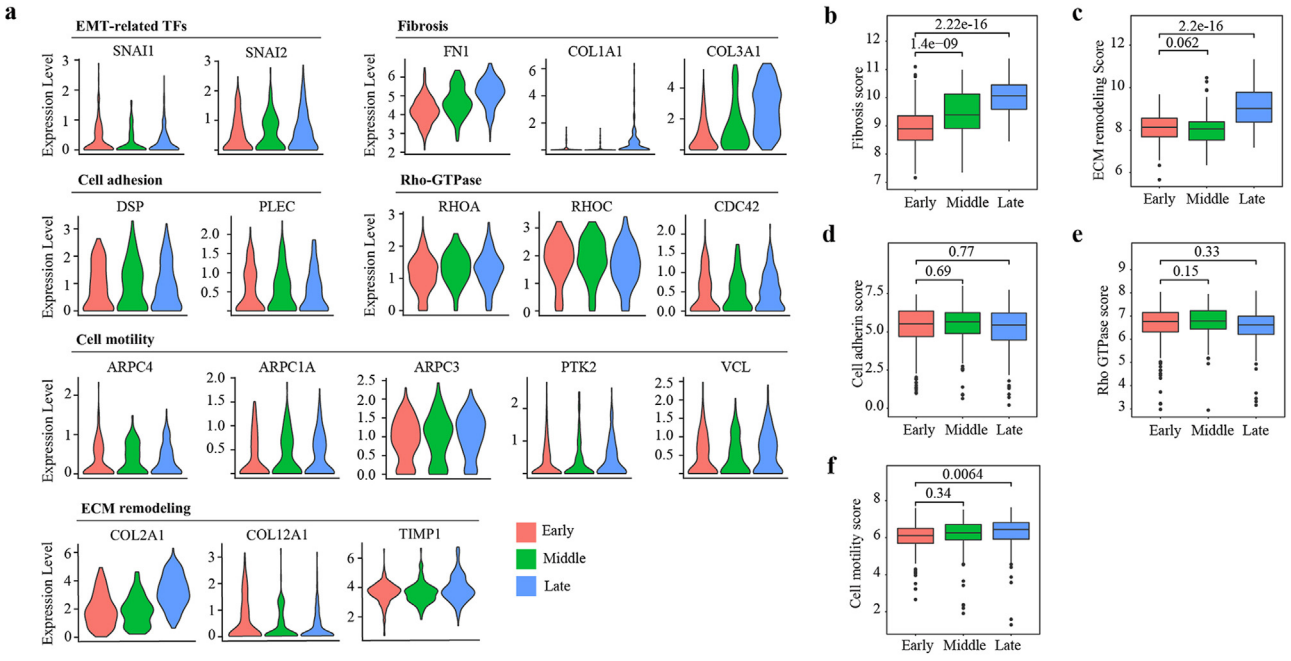

Figure 4. Function dynamics of CSPCs among different stages. a) Violin plot showing the expression level of EMT-related genes (*FN1*, *COL1A1*, *COL3A1*, *SNAI1*, *SNAI2*), cellular adhesion related genes (*DSP*, *PLEC*, *CDH1*), Rho GTPase related genes (*RHOA*, *RHOC*, *CDC42*), cell motility genes (*ARPC1A*, *ARPC3*, *PTK2*, *VCL*) and ECM remodelling related genes (*COL2A1*, *COL12A1*, *TIPM1*). b–e) Box plots showing values of EMT score (b), ECM remodelling score (c), cellular adhesion score (d), Rho GTPase score (e), and cell motility score (f).

Previous evidence has confirmed that differentiation potency is a critical parameter for evaluating the activity of CSPCs and maintaining normal cell function [35, 41, 42, 43, 44]. Once the capacity for chondrogenic differentiation decreases, renewal of articular cartilage is affected, and CSPCs can be regarded as dysfunctional to an extent. In addition, factors such as COL1A1 and COL3A1 have been identified as leading to fibrosis by disturbing normal ECM synthesis. In our study, we found that genes involved in chondrocyte differentiation were downregulated, while the expression levels of *COL1A1* and *COL3A1* increased greatly in the late stage of OA. These results suggest that a continuous progression of OA is likely to affect the function of chondrogenic differentiation in CSPCs, which may cause a defect in the self-renewal of chondrocytes. We also found that genes involved in processes of homeostasis, such as migration and ECM repair, tend to be highly expressed in the late stage of OA [12, 45]. These results suggest that during OA progression, the levels of self-renewal in cartilage decreased, but the capacity to restore damage improved, which likely resulted in self-regulation of the human body. Therefore, we believe that the future treatment of OA can be based on the above phenomenon and supplementing healthy mesenchymal stem cells in the cartilage of patients with OA, by moderating the lack of cartilage self-renewal ability and contributing to strengthening extracellular matrix repair.

Our study demonstrates the role of CSPCs during OA progression and defines some differences in function at the different stages. However, we did not further confirm our results *in vivo* due to a lack of specimens from humans and limitations in the double-staining immunofluorescence technique for cartilage chondrocytes. To further scrutinise the role of CSPCs during OA progression, it would be insightful to conduct *in vivo* lineage tracing from progenitor cells and examine the dynamics of dedifferentiation of these cells into new CSPCs. Furthermore, although we have observed that fibrosis, cell motility, and ECM remodelling genes were upregulated in CSPCs during OA progression, it remains to be elucidated whether these genes contribute to the observed effects jointly or independently.

Declarations

Author contribution statement

Lingbin Qi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jian Wang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xian Chen: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Bin Ling, Yanhui Ding: Contributed to reagents, materials, analysis tools or data.

Wenjun Wang: Analyzed and interpreted the data.

Jun Xu and Zhigang Xue: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at “Gene Expression Omnibus (GEO) data repository” under the accession number GSE104782.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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