

Single nuclei transcriptomics reveal the differentiation trajectories of periosteal skeletal/stem progenitor cells in bone regeneration

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

About eLife's process

Reviewed preprint version 1
January 5, 2024 (this version)

Posted to preprint server
September 27, 2023

Sent for peer review
September 25, 2023

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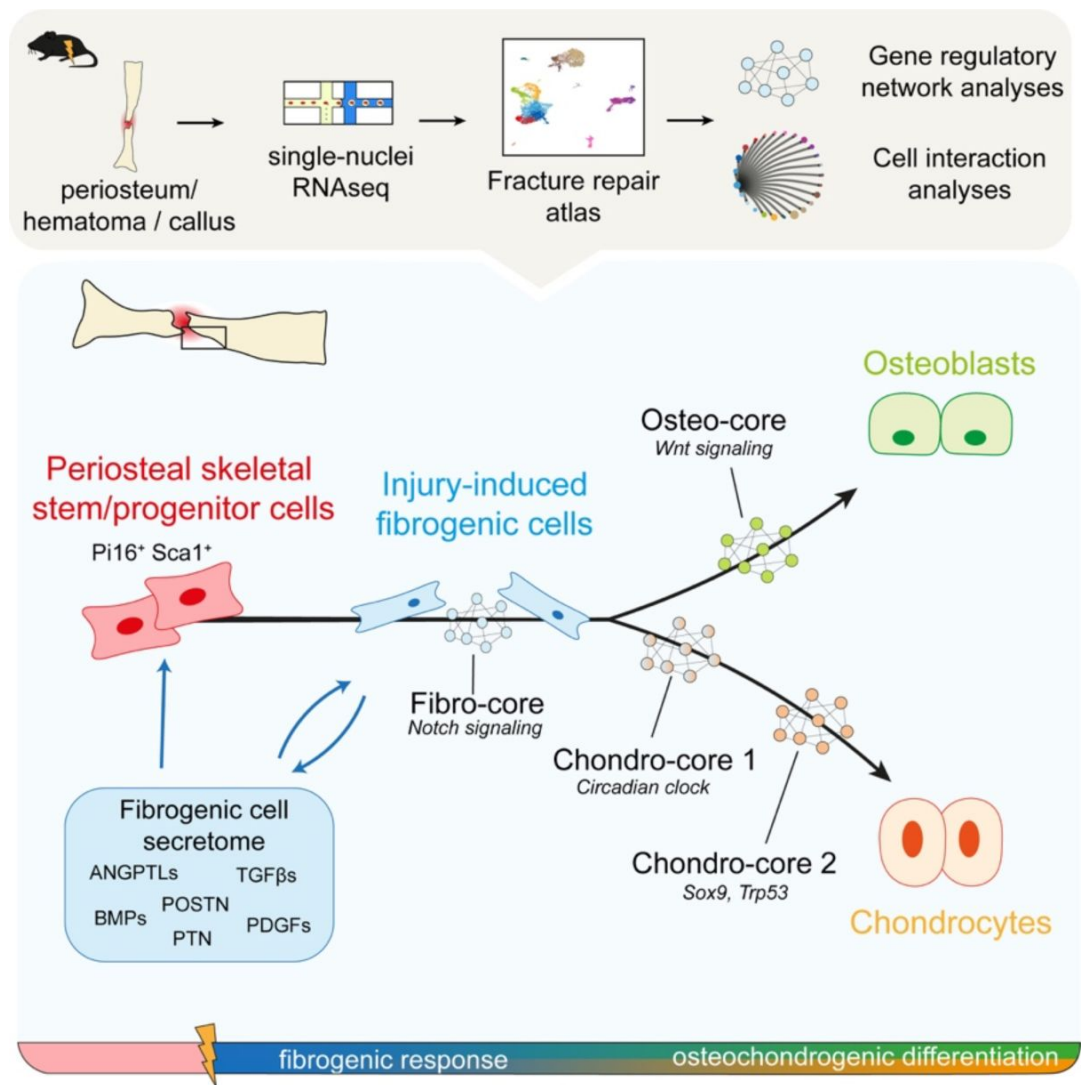
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Abstract

Bone regeneration is mediated by skeletal stem/progenitor cells (SSPCs) that are mainly recruited from the periosteum after bone injury. The composition of the periosteum and the steps of SSPCs activation and differentiation remain poorly understood. Here, we generated a single-nuclei atlas of the periosteum at steady-state and of the fracture site during early stages of bone repair. We identified periosteal SSPCs expressing stemness markers (Pi16 and Ly6a/Sca1) and responding to fracture by adopting an injury-induced fibrogenic cell (IIFC) fate, prior to undergoing osteogenesis or chondrogenesis. We identified distinct gene cores associated with IIFCs and their engagement into osteogenesis and chondrogenesis involving Notch, Wnt and the circadian clock signaling respectively. Finally, we show that IIFCs are the main source of paracrine signals in the fracture environment, revealing a crucial paracrine role of this transient IIFC population during fracture healing. Overall, our study provides a complete temporal topography of the fracture healing stages and the dynamic response of periosteal SSPCs to injury, redefining our knowledge of bone regeneration.

Graphical Abstract



eLife assessment

This **important** study characterizes various cell populations and describes a developmental trajectory using snRNAseq data, highlighting the cell state transitions including periosteal stem cells during bone repair. However, there was a general consensus that the evidence provided is currently **incomplete**, necessitating the additional data and a more thorough verification of the conclusions. Despite this, the work provides a helpful resource that will be of broad interest to the bone community.

Introduction

The skeleton provides structural support and protection for internal organs in the vertebrate body. Bones can fracture, but regenerate themselves efficiently without scarring. Bone regeneration is mediated by the action of resident skeletal stem/progenitor cells (SSPCs) from periosteum and bone marrow, and SSPCs from skeletal muscle adjacent to the fracture site^{1,2,3,4}. SSPCs are

activated during the inflammatory phase of healing and differentiate into osteoblasts and/or chondrocytes to repair bone via a combination of intramembranous and endochondral ossification. During intramembranous ossification SSPCs differentiate directly into osteoblasts, while during endochondral ossification SSPCs first differentiate into chondrocytes to form an intermediate cartilage template subsequently replaced by bone.

The periosteum, an heterogeneous tissue located on the outer surface of bones, is a crucial source of SSPCs during bone healing^{1,2,5,7}. Periosteal SSPCs exhibit a high regenerative potential. They also display a unique osteochondrogenic potential after injury compared to bone marrow SSPCs that are mostly osteogenic and skeletal muscle SSPCs that are mostly chondrogenic^{1-4,8}. Periosteal SSPCs (pSSPCs) are still poorly characterized and the origin of their bipotentiality and response to bone injury remain elusive. Recent advances in single cell/nuclei transcriptomic analyses provided new insights in stem cell population heterogeneity and regeneration processes in many organs⁹⁻¹¹. However, few studies have investigated bone fracture healing at the single cell level and these studies have focused on cultured cells or late stages of bone repair^{3,12}. Therefore, a complete dataset of periosteum and bone regeneration is lacking and is essential to decipher the mechanisms of pSSPC activation and regulation. Here, we created a single-nuclei atlas of the uninjured periosteum and its response to bone fracture. We generated single-nuclei RNAseq (snRNAseq) datasets from the uninjured periosteum and from the periosteum and hematoma/callus at days 3, 5 and 7 post-tibial fracture. Our analyses thoroughly describe the heterogeneity of the periosteum at steady state and the steps of pSSPC activation and differentiation after injury. We show that pSSPC represent a single population that can provide osteoblasts and chondrocytes for bone repair by first generating a common injury-induced fibrogenic cell (IIFC) population that can then engage into osteogenesis and chondrogenesis. We identified the gene networks regulating pSSPC fate after injury and IIFCs as the main population producing paracrine factors mediating the initiation of bone healing.

Results

Heterogeneity of the periosteum at steady state

To investigate the heterogeneity of the periosteum at steady-state, we performed snRNAseq of the periosteum of wild-type mice (**Fig. 1A-B**). After filtering, we obtained 1189 nuclei, corresponding to 8 cell populations: SSPC (expressing *Pi16*), fibroblasts (expressing *Pdgfra*), osteogenic cells (expressing *Runx2*), Schwann cells (expressing *Mpz*), pericytes/SMC (expressing *Tagln*), immune cells (expressing *Ptpcr*), adipocytes (expressing *Lpl*), and endothelial cells (ECs, expressing *Pecam1*) (**Fig. 1C-D**, Fig. S1). We performed in depth analyses of the SSPC, fibroblast and osteogenic cell populations. Subset analyses of clusters 0 to 5 identified 5 distinct SSPC/fibroblast populations expressing *Pdgfra* and *Prrx1*: *Pi16*⁺, *Csmd1*⁺, *Hsd11b1*⁺, *Cldn1*⁺, and *Luzp2*⁺ cells (**Fig. 1E-G**). *Pi16*⁺ cells (i.e., cluster 0) were the only cell cluster expressing stemness markers including *Ly6a* (Sca1), *Dpp4*, and *Cd34* (**Fig. 1H**). Cytotrace scoring identified *Pi16*⁺ cells as the population in the most undifferentiated state, suggesting that this population corresponds to periosteal SSPCs (**Fig. 1I**). We explored the expression of other known markers of periosteal SSPCs, including *Ctsk*, *Acta2* (αSMA), *Gli1*, and *Mx1*, but no marker was fully specific to one cell cluster^{1,2,5,7,13-17} (Fig. S2).

The fracture repair atlas

To investigate the periosteal response to bone fracture, we collected injured periosteum with hematoma or callus at days 3, 5 and 7 post-fracture, extracted the nuclei and processed them for snRNAseq (**Fig. 2A**). We combined the datasets with the uninjured periosteum from **Figure 1** and obtained a total of 6213 nuclei after filtering. The combined dataset was composed of 25 clusters corresponding to 11 cell populations: SSPCs (expressing *Pi16*), injury-induced fibrogenic

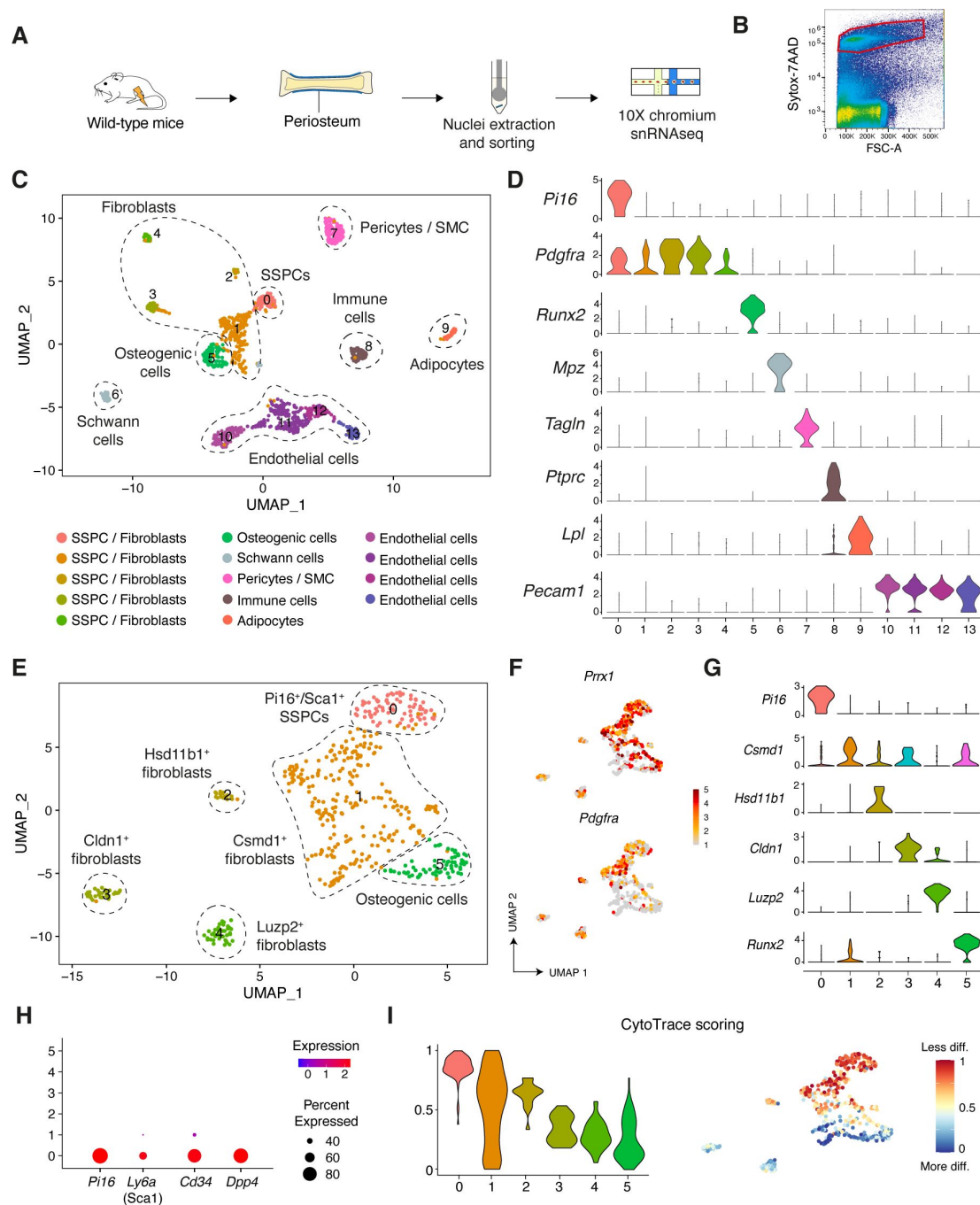


Figure 1

Heterogeneity of the periosteum at steady state

A. Experimental design. Nuclei were extracted from the periosteum of uninjured tibia and processed for single-nuclei RNAseq. **B.** Sorting strategy of nuclei stained with Sytox-7AAD for snRNAseq. Sorted nuclei are delimited by a red box. **C.** UMAP projection of color-coded clustering of the uninjured periosteum dataset. Six populations are identified and delimited by black dashed lines. **D.** Violin plots of key marker genes of the different cell populations. **E.** UMAP projection of color-coded clustering of the subset of SSPC/fibroblasts. **F.** Feature plots of *Prrx1* and *Pdgfra* in the subset of SSPC/fibroblasts. **G.** Feature plots of key marker genes of the different cell populations. **H.** Dot plot of the stemness markers *Pi16*, *Ly6a* (Sca1), *Cd34*, and *Dpp4*. **I.** Violin and feature plots of CytoTrace scoring in the subset of SSPC/fibroblasts, showing that Sca1⁺ SSPCs (cluster 0) are the less differentiated cells in the dataset.

cells (IIFC, expressing *Postn*), osteoblasts (expressing *Ibsp*), chondrocytes (expressing *Col2a1*), osteoclasts (expressing *Ctsk*), immune cells (expressing *Ptprc*), Schwann cells (expressing *Mpz*), endothelial cells (expressing *Pecam1*), pericytes (expressing *Rgs5*), SMCs (expressing *Tagln*) and adipocytes (expressing *Lpl*) (**Fig. 2B-C**, Fig. S3A). Next, we observed the dynamics of the cell populations in response to bone fracture (**Fig. 2D**, Fig. S3B). After injury, the percentage of SSPCs was strongly decreased and the percentage of IIFCs progressively increased (**Fig. 2D-E**). The percentage of chondrocytes and osteoblasts increased from day 3 post-fracture. Immune cells were drastically increased at day 3 after injury, before progressively decreasing at days 5 and 7 post-fracture (**Fig. 2E**).

Spatial organization of the fracture callus

To evaluate the spatial distribution of the main cell populations identified in the snRNAseq data, we performed in situ immunofluorescence on day 3, 5 and 7 post-fracture hematoma/callus tissues. At day 3 post-fracture, we observed periosteal thickening and the formation of a fibrous hematoma (**Fig. 3A**). We did not detect SSPCs at both sites, consistent with their absence in day 3 snRNAseq dataset compared to uninjured periosteum. POSTN⁺ IIFCs and immune cells were the main populations present in hematoma and activated periosteum (**Fig. 3B**). Few IIFCs in the activated periosteum expressed SOX9 and OSX and only the periosteum was vascularized. At days 5 and 7 post-fracture, the callus was formed mainly of fibrotic tissue, bone formed on the periosteal surface at the periphery of the callus and small cartilage islets were detected in the center of the callus near the periosteal surface (**Fig. 3C-F**). The fibrotic tissue contained mostly IIFCs, as well as immune and endothelial cells. We also observed SOX9⁺ and OSX⁺ cells in the fibrotic tissue, while the cartilage was solely composed of SOX9⁺ chondrocytes. OSX⁺ osteoblasts were the main cell population detected in the new bone at the periosteal surface. We also observed a progressive reduction of POSTN⁺ cells and immune cells from day 5 to 7 and increased vascularization in the newly formed bone (**Fig. 3C-F**).

Periosteal SSPCs differentiate via an injury-induced fibrogenic stage

To understand the differentiation and fate of pSSPCs after fracture, we analyzed the subset of SSPC, IIFC, chondrocyte and osteoblast clusters from the combined fracture dataset (**Fig. 4A**). We performed pseudotime analyses to determine the differentiation trajectories, defining the starting point in the pSSPC population, where the uninjured and undifferentiated cells cluster. We identified that pSSPC differentiate in 3 stages starting from the pSSPC population (expressing *Ly6a*, *Pi16* and *Cd34*), predominant in uninjured dataset (**Fig. 4B-C**). Periosteal SSPCs then transition through an injury-induced fibrogenic stage predominant at days 3 and 5 post-injury. In this intermediate fibrogenic stage, pSSPC-derived cells express high levels of extracellular matrix genes, such as *Postn*, *Aspn* and *Tnc*. Later, injury-induced fibrogenic cells (IIFCs) differentiate into chondrocytes (expressing *Acan*, *Col2a1* and *Sox9*) or osteoblasts (expressing *Sp7*, *Alpl* and *Ibsp*), both predominant at days 5 and 7 (**Fig. 4B-C**, Table S1). These results show that pSSPCs respond to fracture via an injury-induced fibrogenic stage common to chondrogenesis and osteogenesis and independent of their final fate (**Fig. 4D**). To visualize the transition of IIFCs towards chondrocytes and osteoblasts in the fracture callus, we performed co-immunofluorescence on day 5 post-fracture hematoma/callus. We observed a progressive increase in SOX9 and OSX signals in IIFCs at the fibrosis-to-cartilage and fibrosis-to-bone transition zones respectively (**Fig 5A**). To functionally validate the steps of pSSPC activation, we isolated SCA1⁺ GFP⁺ pSSPCs from *Prx1^{Cre}; R26^{mTmG}* mice and grafted them at the fracture site of wild type hosts. We observed that grafted GFP⁺ pSSPCs formed POSTN⁺ IIFCs at day 5 post-fracture (**Fig 5B**). Then, we isolated GFP⁺ IIFCs from the fracture callus of *Prx1^{Cre}; R26^{mTmG}* mice at day 3 post fracture and grafted the GFP⁺

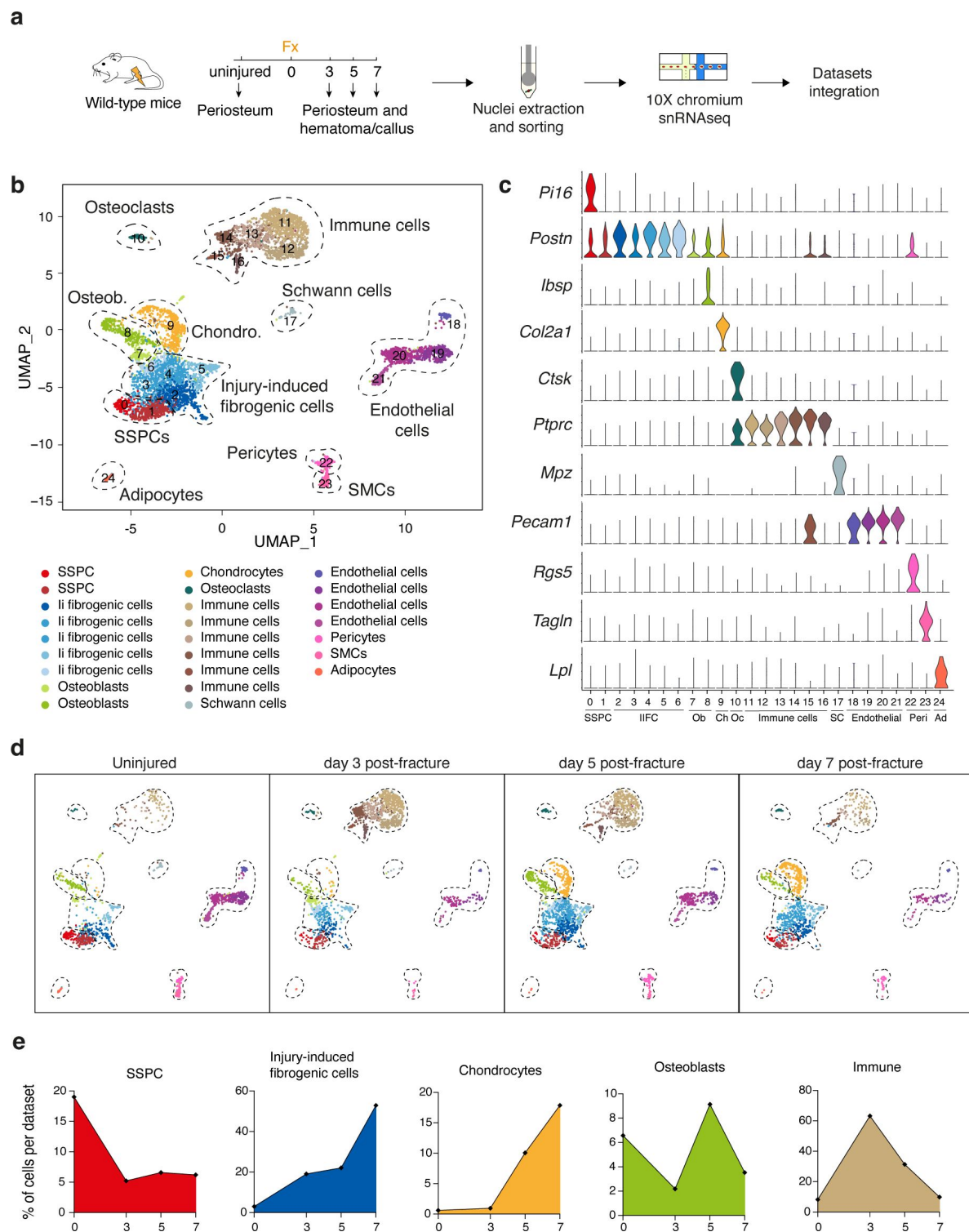


Figure 2

Periosteal response to fracture at single-nuclei resolution

A. Experimental design. Nuclei were extracted from the periosteum of uninjured tibia of wild-type mice and from the periosteum and hematoma at days 3, 5 and 7 post-tibial fracture and processed for single-nuclei RNAseq. **B.** UMAP projection of color-coded clustering of the integration of uninjured, day 3, day 5 and day 7 datasets. Eleven populations are identified and delimited by black dashed lines. **C.** Violin plots of key marker genes of the different cell populations. **D.** UMAP projection of the combined dataset separated by time-point. **E.** Percentage of cells in SSPC, injury-induced fibrogenic cell, osteoblast, chondrocyte, and immune cell clusters in uninjured, day 3, day 5 and day 7 datasets.

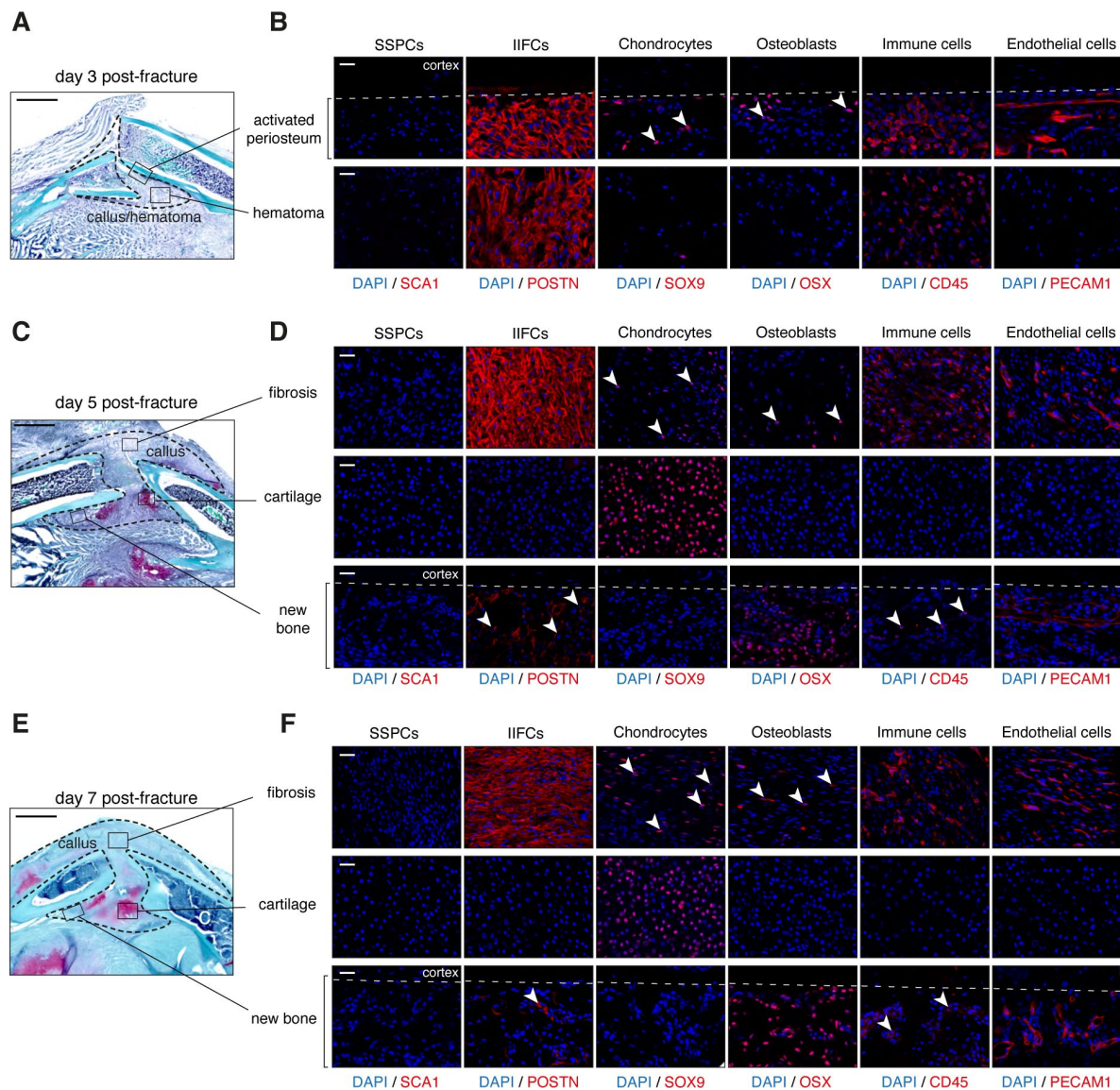


Figure 3

Cellular organization of the fracture callus

A. Safranin'O staining of longitudinal callus sections at day 3-post tibial fracture. **B.** Immunofluorescence on adjacent sections show absence of SSPCs (SCA1⁺), and presence of IIFCs (POSTN⁺) and immune cells (CD45⁺) in the activated periosteum and hematoma at day 3-post fracture. Chondrocytes (SOX9⁺, arrowhead), osteoblasts (OSX⁺, arrowhead) and endothelial cells (PECAM1⁺) are detected in activation periosteum (n=3 per group). **C.** Safranin'O staining of longitudinal callus sections at day 5-post tibial fracture. **D.** Immunofluorescence on adjacent sections show IIFCs (POSTN⁺), chondrocytes (SOX9⁺, arrowhead), osteoblasts (OSX⁺, arrowhead), immune cells (CD45⁺) and endothelial cells (PECAM1⁺) in the fibrosis, chondrocytes (SOX9⁺) in cartilage and osteoblasts (OSX⁺), immune cells (CD45⁺, arrowhead) and endothelial cells (PECAM1⁺) in new bone. (n=3 per group). **E.** Safranin'O staining of longitudinal callus sections at day 7-post tibial fracture. **F.** Immunofluorescence on adjacent sections show IIFCs (POSTN⁺), chondrocytes (SOX9⁺, arrowhead), osteoblasts (OSX⁺, arrowhead), immune cells (CD45⁺) and endothelial cells (PECAM1⁺) in the fibrosis, chondrocytes (SOX9⁺) in cartilage and osteoblasts (OSX⁺), immune cells (CD45⁺, arrowhead) and endothelial cells (PECAM1⁺) in new bone (n=3 per group). Scale bars: A-B-E: 1mm, B-D-F: 100µm.

IIFCs at the fracture site of wild type hosts. We showed that grafted cells formed bone and cartilage at day 14 post-fracture. These results confirmed that pSSPCs first become IIFCs that differentiate into osteoblasts and chondrocytes.

Characterization of injury-activated fibrogenic cells

We performed in depth analyses of the newly identified IIFC population. Gene ontology (GO) analyses of upregulated genes in IIFCs (clusters 2 to 6) showed enrichment in GOs related to tissue development, extracellular matrix (ECM), and ossification (**Fig. 6A** [↗](#)). We identified several ECM-related genes specifically upregulated in IIFCs, including collagens (*Col3a1*, *Col5a1*, *Col8a1*, *Col12a1*), *Postn*, and *Aspn* (**Fig. 6B-C** [↗](#)). We also identified GO terms related to cell signaling, migration, differentiation, and proliferation, indicating that this step corresponds to an injury-induced activation. To further understand the mechanisms regulating SSPC activation and fate after injury, we performed gene regulatory network (GRN) analyses on the subset of SSPCs, IIFCs, osteoblasts and chondrocytes using SCENIC package (Single Cell rEgulatory Network Inference and Clustering)¹⁸ [↗](#). We identified 280 activated regulons (transcription factor/TF and their target genes) in the subset dataset. We performed GRN-based tSNE clustering and identified SSPC, IIFC, chondrocyte and osteoblast populations (**Fig. 6D-E** [↗](#), Fig. S4A). Fibroblasts from uninjured periosteum (*Hsd11b1*⁺, *Cldn1*⁺ and *Luzp2*⁺ cells) clustered separately from the other population, indicating the absence of their contribution to bone healing. Analysis of the number of activated regulons per cell indicated that SSPCs are the most stable cell population (higher number of activated regulons), while IIFCs are the less stable population, confirming their transient state (**Fig. 6F** [↗](#)). We then investigated cell population specific regulons. SSPCs showed activated regulons linked to stemness, including *Hoxa10* (16g), *Klf4* (346g), *Pitx1* (10g), and *Mta3* (228g)¹⁹ [↗](#)–²¹ [↗](#), and immune response, including *Stat6* (30g), *Fiz1* (72g) and *Stat5b* (18g)²² [↗](#)–²⁴ [↗](#) (**Fig. 6G** [↗](#)). Osteoblasts and chondrocytes display cell specific activated regulons including *Sp7* (18g) and *Sox9* (77g) respectively. We identified 21 regulons that we named fibro-core and that were upregulated specifically in the IIFC population (**Fig. 6F-I** [↗](#), Table S2). Several fibro-core regulons, such as *Meis1* (1556g), *Pbx1* (11g), *Six1* (20g), and *Pbx3* (188g) are known to be involved in cell differentiation during tissue development and repair²⁵ [↗](#)–²⁸ [↗](#). Reactome pathway analysis showed that the most significant terms linked to IIFC-specific TFs are related to Notch signaling (**Fig. 6J** [↗](#), Table S3). We confirmed that Notch signaling is specifically increased in the IIFC stage (**Fig. 6K** [↗](#)), suggesting its involvement in the fibrogenic phase of bone repair.

Distinct gene cores regulate the engagement of IIFCs into chondrogenesis and osteogenesis

We sought to identify the drivers of the transition of IIFCs to chondrocytes or osteoblasts. We identified 2 cores of regulons involved in chondrogenic differentiation. Chondro-core 1 is composed of 9 regulons specific to the transition of IIFC to chondrocytes, including *Maf* (17g), *Arntl* (1198g), and *Nfatc2* (37g) and chondro-core 2, composed of 14 regulons specific to differentiated chondrocytes (**Fig. 7A** [↗](#), Fig. S4B). Chondro-core 1 regulons are known to be regulators of the circadian clock (*Npas2*, *Arntl*) or of the T/B-cell receptor cellular response (*Bach2*, *Nfatc2*, *Nfkb1*). STRING network analysis showed that TFs from the chondro-core 1 are interacting between each other and are at the center of the interactions between the chondro-core 2 TFs, including *Sox9*, *Trp53* and *Mef2c* (**Fig. 7B** [↗](#), Fig. S4C). We observed that chondro-core 1 is only transiently activated when IIFCs are engaging into chondrogenesis and precedes the activation of chondro-core 2 (**Fig. 7C-D** [↗](#)). Chondro-core 1 activity was high in early differentiated chondrocytes (low *Acan* expression) and progressively reduced as chondrocytes undergo differentiation, while chondro-core 2 activity was gradually increased as chondrocytes differentiate (**Fig. 7D** [↗](#)). This suggests that transient activation of the chondro-core 1 allows the transition of IIFCs into chondrocytes. Then, we investigated the osteogenic commitment of IIFCs. We identified 8 regulons forming the osteo-core and activated in IIFCs transitioning to osteoblasts,

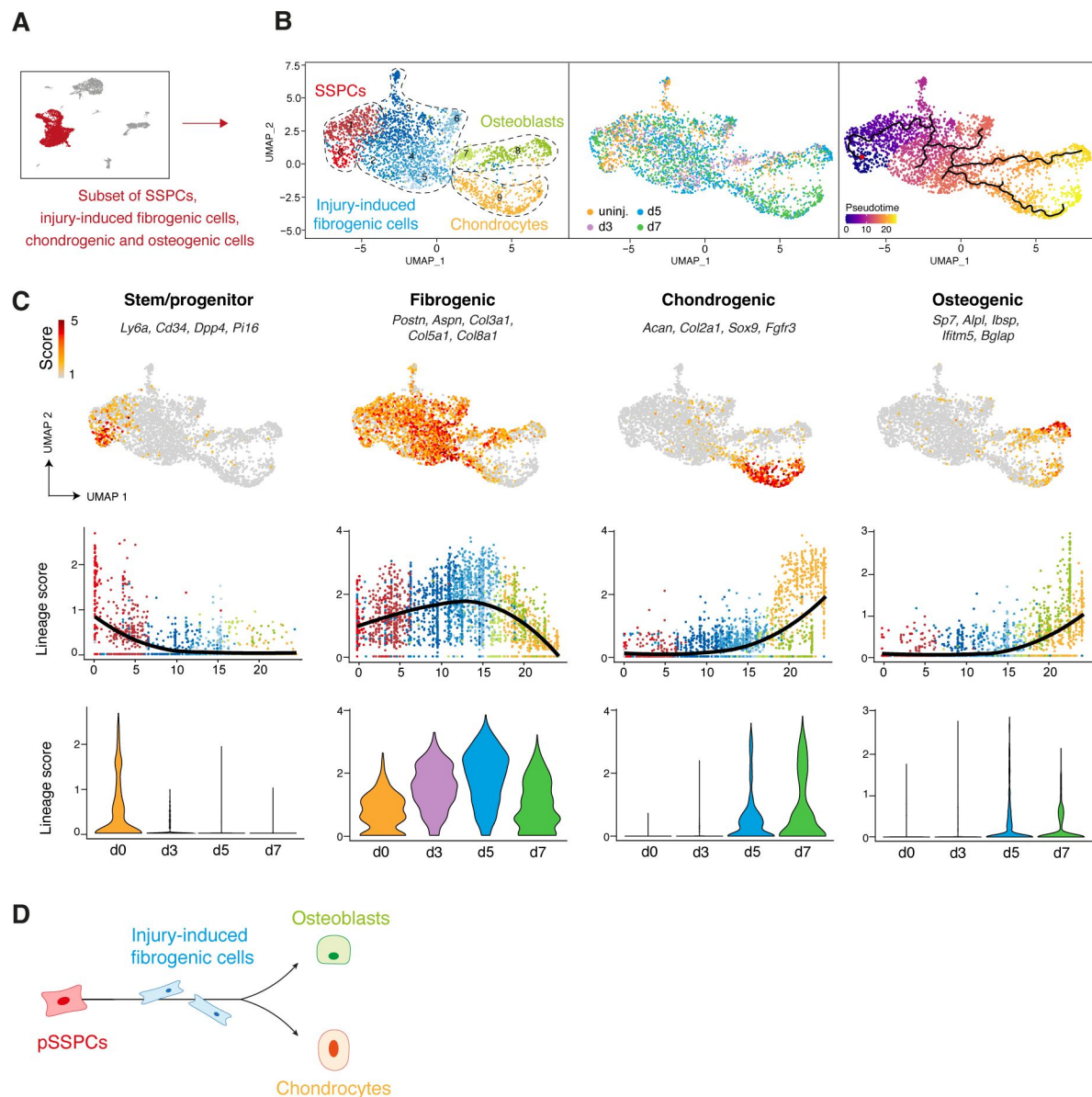


Figure 4

Periosteal SSPCs activate through a common fibrogenic state prior to undergoing osteogenesis or chondrogenesis

A. SSPCs, injury-induced fibrogenic cells (IIFCs), chondrocytes and osteoblasts from integrated uninjured, day 3, day 5 and day 7 post-fracture samples were extracted for a subset analysis. **B.** UMAP projection of color-coded clustering (left), color-coded sampling (middle) and monocle pseudotime trajectory (right) of the subset dataset. The four populations are delimited by black dashed lines. **C.** (top) Feature plots of the stem/progenitor, fibrogenic chondrogenic and osteogenic lineage scores (middle) Scatter plot of the lineage scores along pseudotime. (bottom) Violon plot of the lineage score per time point. **D.** Schematic representation of the activation trajectory of pSSPCs after fracture.

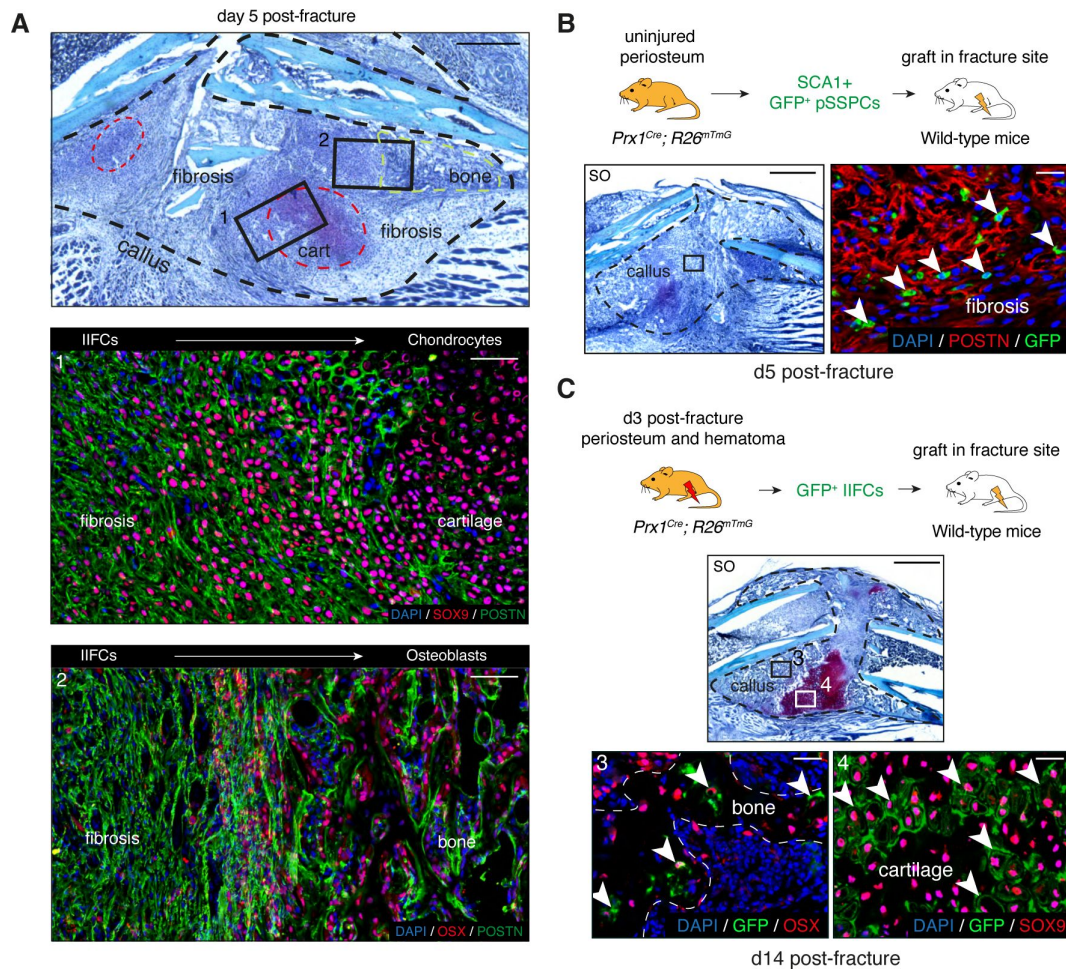


Figure 5

In vivo validation of pSSPC activation trajectory

A. (Top) Representative Safranin'O staining on longitudinal sections of the hematoma/callus at day 5 post-fracture. The callus is composed of fibrosis, cartilage (red dashed line) and bone (green dashed line). (Middle, box 1) Immunofluorescence on adjacent section shows decreased expression of POSTN (green) and increased expression of SOX9 (red) in the fibrosis-to-cartilage transition zone. (Bottom, box 2) Immunofluorescence on adjacent section shows decreased expression of POSTN (green) and increased expression of OSX (red) in the fibrosis-to-bone transition zone (n=3 per group). **B.** Experimental design: GFP+ Sca1+ SSPCs were isolated from uninjured tibia of *Prx1^{Cre}; R26^{mTmG}* mice and grafted at the fracture site of wild type mice. Safranin'O staining of callus sections at day 5 post-fracture and high magnification of POSTN immunofluorescence of adjacent section showing that GFP+ cells contribute to the callus and that grafted SSPCs differentiate into POSTN+ IIFCs (white arrowheads) (n=4 per group). **C.** Experimental design: GFP+ IIFCs from day 3 post-fracture tibia were isolated from *Prx1^{Cre}; R26^{mTmG}* mice and grafted at the fracture site of wild type mice. Safranin'O of callus sections at day 14 post-fracture and high magnification of OSX and SOX9 immunofluorescence of adjacent sections showing that GFP+ cells contribute to the callus and that grafted IIFCs differentiate into OSX+ osteoblasts (box 3, white arrowheads) and SOX9+ chondrocytes (box 4, white arrowheads) (n=4 per group). Scale bars: Low magnification: A: 500µm, B-C: 1mm. High magnification: 100µm.

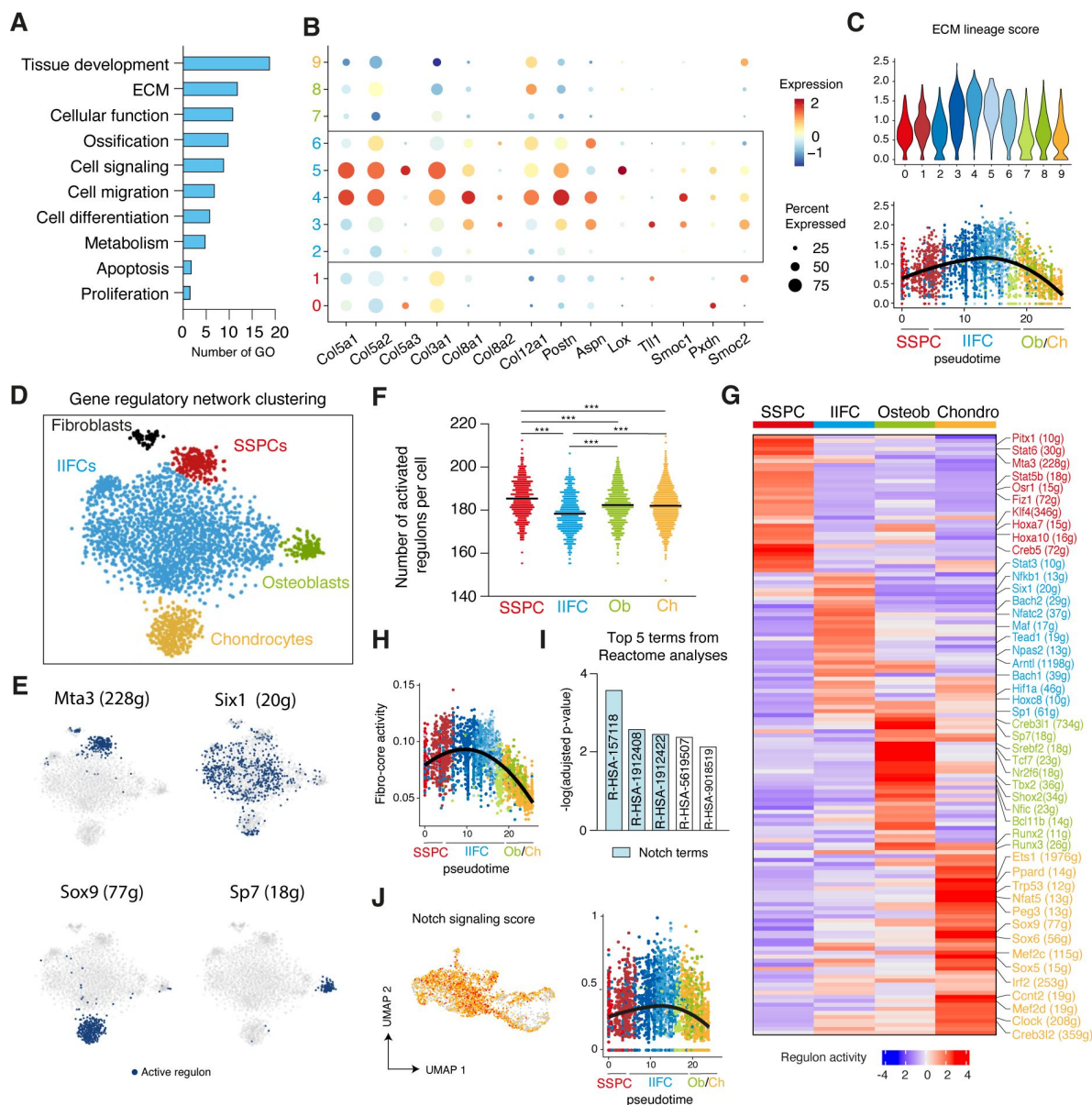


Figure 6

Characterization of injury-activated fibrogenic cells

A. Gene ontology analyses of upregulated genes in IIFCs (clusters 2 to 6 of UMAP clustering from Fig. 4). **B.** Dot plot of ECM genes in UMAP clustering from Fig. 4. **C.** Feature plot per cluster and scatter plot along pseudotime of the mean expression of ECM genes. **D.** Gene regulatory network (GRN)-based tSNE clustering of the subset of SSPCs, IIFCs, chondrocytes and osteoblasts. **E.** Activation of Mta3, Six1, Sox9 and Sp7 regulons in SSPCs, IIFCs, chondrocytes and osteoblasts. Blue dots mark cells with active regulon. **F.** Number of regulons activated per cell in the SSPC, IIFC, osteoblast (Ob) and chondrocyte (Ch) populations. Statistical differences were calculated using one-way ANOVA. ***: p-value < 0.001. **G.** Heatmap of activated regulons in SSPC, IIFC, osteoblast and chondrocyte populations. **H.** Scatter plot of the activity of the combined fibrogenic regulons along monoclone pseudotime from Fig. 4. **I.** Reactome pathway analyses of the fibrogenic regulons shows that the 3 most significant terms are related to Notch signaling (blue). **J.** Feature plot in Seurat clustering and scatter plot along monoclone pseudotime of the Notch signaling score.

such as *Tcf7* (23g), *Bcl11b* (14g), and *Tbx2* (36g) (**Fig. 7E**, Fig. S4D). STRING network analysis showed that the genes with the strongest interaction with the osteo-core TFs are mostly related to Wnt signaling (**Fig. 7F**). This reveals the role of Wnt signaling in this transition from early fibrogenic activation of pSSPCs to osteogenic differentiation during bone repair. We calculated the osteo-core activity, and observed that it is gradually increased and maintained in osteogenic cells, showing that the osteo-core is required for the transition and maturation of IIFC into osteoblasts (**Fig 7G-H**). Overall, we identified distinct cores of regulons with distinct dynamics driving the transition of IIFCs into chondrocytes and osteoblasts.

IIFCs mediate paracrine interactions during bone repair

Paracrine cell interactions are crucial drivers of tissue regeneration and stem cell activation. To identify key cell interactions during bone repair, we performed cell interaction analyses using CellChat package²⁹. We observed that IIFCs are the predominant source of outgoing signals during bone repair, and are also important receivers of signals, highlighting their central role in mediating cell interactions after fracture (**Fig. 8A-B**). Endothelial cells were mostly receiving signaling, while chondrocytes, osteoblasts and most immune cells exhibited reduced interactions with the other cell types in the fracture environment. IIFCs interact with all cell populations in the fracture environment, but the strongest interactions were with SSPCs and IIFCs (Fig. S5A). CellChat analyses of the subset of SSPCs, IIFCs, osteoblasts and chondrocytes confirmed that IIFCs are the major source and receiver population of paracrine signalings after fracture (Fig. S5B). We then analyzed the main secreted factors from IIFCs. IIFCs secreted periostin (*Postn*), BMPs (*Bmp5*), pleiotropin/PTN (*Ptn*), TGFβs (*Tgfb2*, *Tgfb3*), PDGFs (*Pdgfc*, *Pdgfd*) and angiopoietin-like/ANGPTLs (*Angplt2*, *Angplt4*) (**Fig. 8C-D**, Fig. S5C). We observed differences in the dynamics of these factors, as some of them peak at day 3 post-fracture such as TGFβ, while others peak at day 5 such as BMP, POSTN, PTN and ANGPLT (**Fig. 8E**). We assessed the dynamics of ligand and receptor expression and observed that ligand expression was specific of the IIFC phase and of the fracture response (**Fig. 8F**). Receptor expression was high in both SSPCs and IIFCs, and receptors were expressed from steady state, suggesting that SSPCs can receive signals from IIFCs. Analysis of SSPC incoming signals confirmed that IIFCs are the major source of paracrine factors for SSPCs (**Fig. 8G**), indicating that they contribute to SSPC recruitment after fracture.

Discussion

The periosteum is the main driver of bone regeneration. Yet the periosteum composition and its response to bone fracture are poorly described. Here, we used single-nuclei transcriptomics to understand the heterogeneity of the periosteum at steady state and the changes occurring within periosteum after bone fracture. We developed a protocol to extract nuclei from freshly dissected periosteum, allowing to capture their intact transcriptomic profile without enzymatic digestion-induced stress^{30–32}. In addition, we performed snRNAseq without sorting specific populations, allowing the identification of all cell types located in the periosteum and the fracture environment from the early stages of repair. Our study provides the first complete fracture repair atlas, a key tool to understand bone regeneration.

First, we described the heterogeneity of the periosteum at steady state. While previous studies performed scRNAseq on sorted periosteal cell populations, our dataset uncovers the diversity of cell populations within the periosteum. We describe fibroblast populations, as well as tissue resident immune cells, adipocytes, and blood vessel/nerve-related cells. We identified one SSPC population of undifferentiated cells expressing markers such as *Sca1*, *Dpp4* and *Pi16*, known to mark fibroblasts with stemness potential³³. No markers previously described such as *Ctsk*, or *Gli1* were fully specific of one cell cluster and of the undifferentiated SSPC population, suggesting that these markers may label heterogeneous cell populations^{2,5,6,15}.

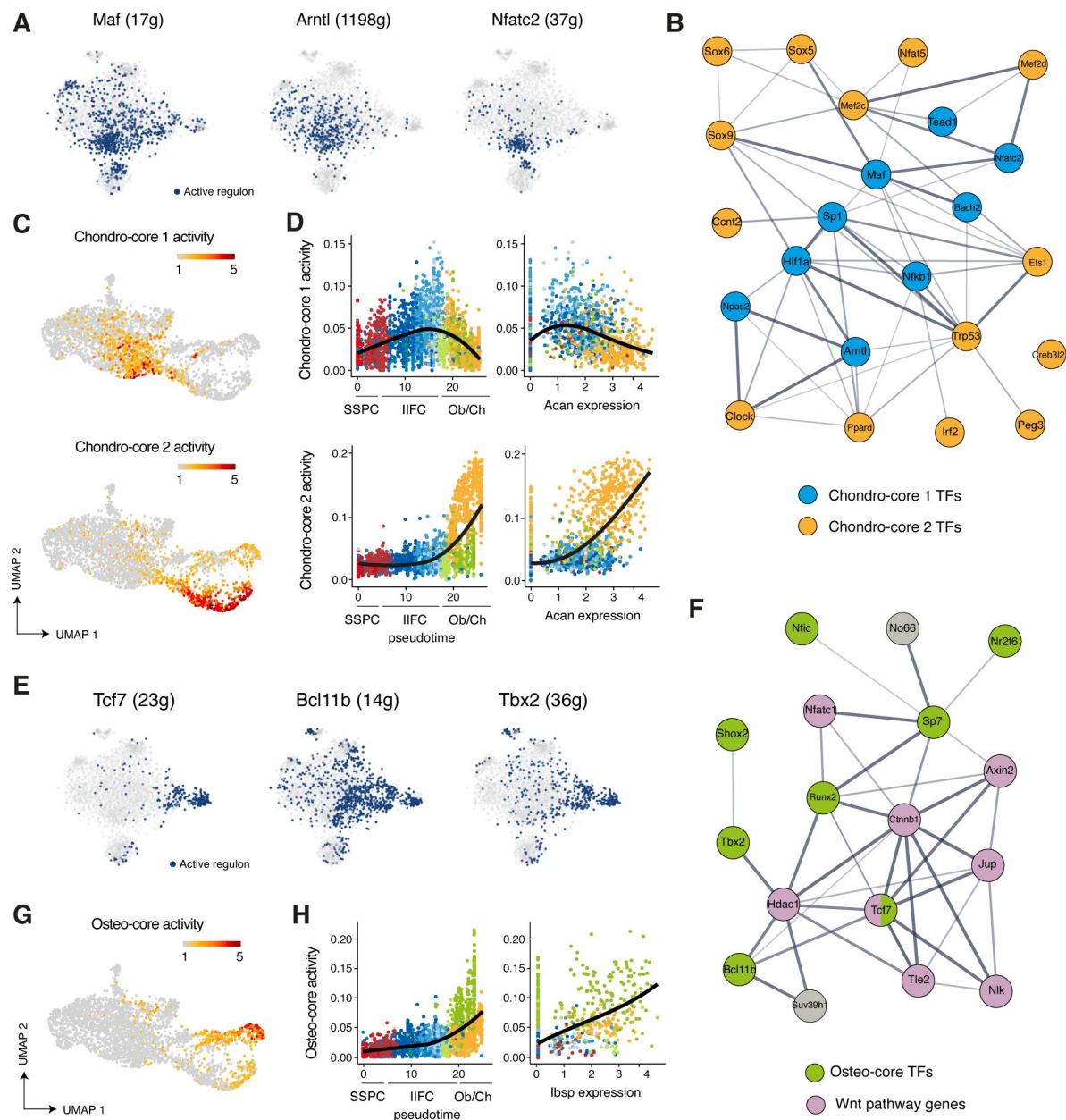


Figure 7

Gene regulatory network analyses identifies gene cores driving fibrogenic to chondrogenic and osteogenic transition.

A. Activation of Maf, Arntl, and Nfatc2 regulons in SSPCs, IIFCs, chondrocytes and osteoblasts. **B.** STRING interaction network of the chondro-core 1 and 2 transcription factors (blue and orange respectively). **C.** Feature plot of chondro-core 1 (top) and chondro-core 2 (bottom) activities in SSPCs, IIFCs, chondrocytes and osteoblasts in Seurat UMAP from [Fig. 4](#). **D.** Scatter plot of chondro-core 1 (top) and chondro-core 2 (bottom) activities along monocle pseudotime and *Acan* expressing. **E.** Activation of Tcf7, Bcl11b and Tbx2 regulons in SSPCs, IIFCs, chondrocytes and osteoblasts. **F.** STRING interaction network of the osteo-core transcription factors (green) and their related genes shows that most of osteo-core related genes are involved in Wnt pathway (purple). **G.** Feature plot of the osteo-core activity in SSPCs, IIFCs, chondrocytes and osteoblasts in Seurat UMAP from [Fig. 4](#). **H.** Scatter plot of osteo-core activity along monocle pseudotime and *Ibsp* expressing.

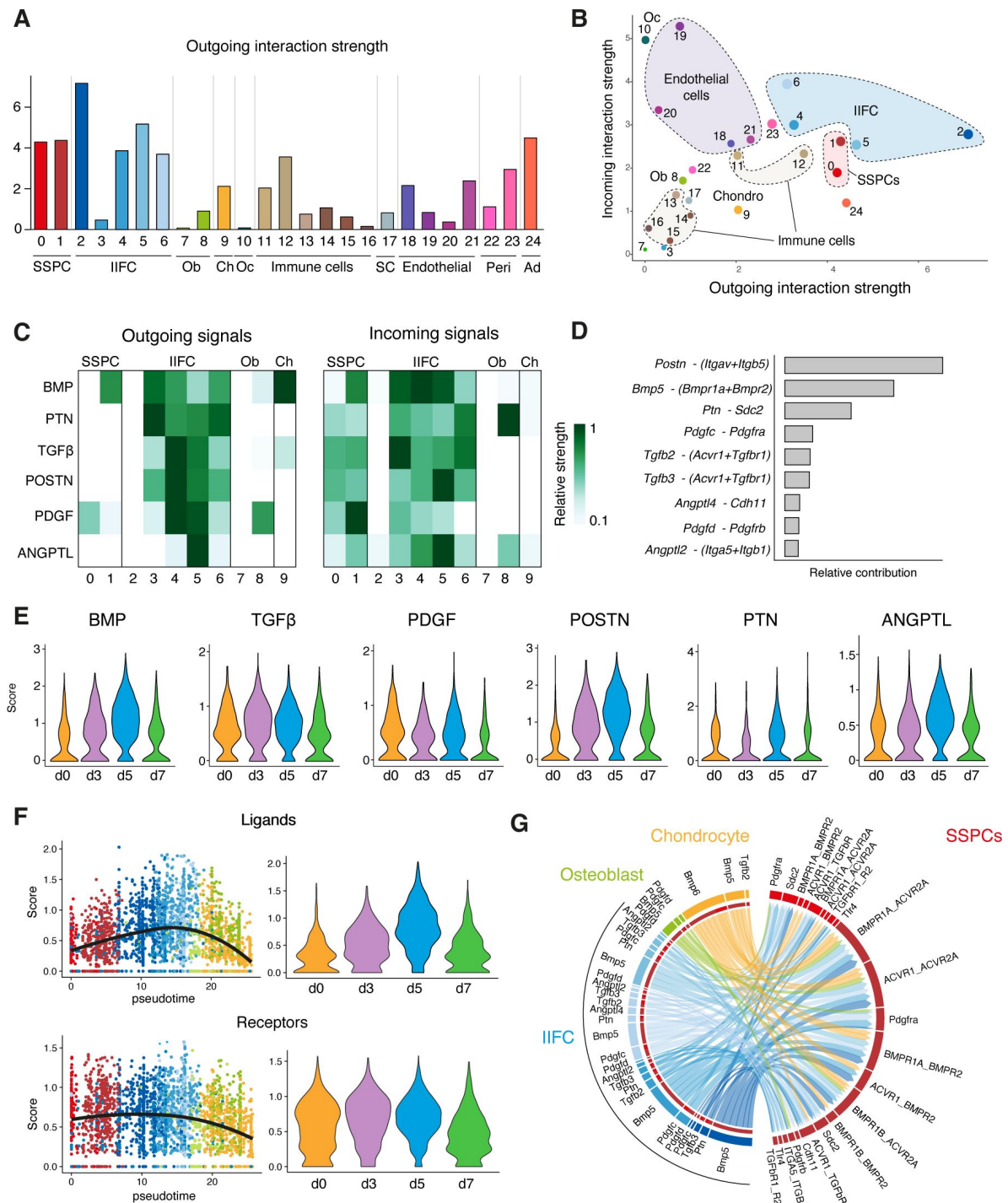


Figure 8

IIFCs are the main source of paracrine factors after fracture.

A. Outgoing interaction strengths of the different cell populations of the fracture environment determined using CellChat package. **B.** Comparison of incoming and outgoing interaction strengths across SSPC, IIFC, chondrogenic and osteogenic populations. **C.** Outgoing and incoming signaling from and to SSPCs, IIFCs, chondrocytes and osteoblasts. **D.** Cell-cell interactions identified between SSPCs, IIFCs, chondrocytes and osteoblasts. **E.** Feature plots of the score of BMP, TGFβ, PDGF, POSTN, PTN and ANGPTL signaling per timepoint. **F.** Scatter plot along pseudotime and feature plot per time point of the mean expression of the ligand and receptors involved in signaling from IIFCs. **G.** Circle plot of the interactions between SSPCs, IIFCs, chondrocytes and osteoblasts, showing that most signals received by SSPCs are coming for IIFCs.

After fracture, the composition of the periosteum changes drastically, with the appearance of IIFC and immune cells from day 3 post-fracture, and of chondrocytes and osteoblasts from day 5 post-fracture. Previous studies based on in vitro analyses and in vivo lineage tracing demonstrated that the periosteum displays the unique potential to form both bone and cartilage after fracture^{1,3,5}. Yet, it was still unknown if the periosteum contains a bipotent SSPC population or several SSPC populations with distinct potentials after injury. Here, we show that pSSPCs activate and form bone and cartilage via a single trajectory emerging from Sca1+ SSPCs. Periosteal Sca1+ SSPCs become IIFCs, a state marked by a decreased expression of stemness markers and a strong expression of extracellular matrix genes, such as *Postn* and *Aspn* and activation of Notch-related TFs. Thus, bone injury does not induce an expansion of Sca1+ SSPCs, rather a transition toward IIFCs that progressively expand and represent the main cell population within the fracture callus until day 7 post-fracture. Following this unique fibrogenic step, IIFCs undergo either osteogenesis or chondrogenesis. This newly identified transient IIFC state represents the crossroad of bone regeneration and SSPC differentiation.

GRN analyses identified TFs regulating SSPC response to fracture. We found that Notch signaling was specific to the injury-induced fibrogenic phase. Previous studies reported the role of Notch and Wnt in osteogenesis, and their temporal separation during intramembranous ossification^{8,34,35}. Our results show that Notch/Wnt activation after fracture correlates with the successive stages of pSSPC fibrogenic and osteogenic differentiation. However, we discover that Wnt is only upregulated in IIFCs engaging into osteogenesis, while Notch is activated early in IIFCs prior to their commitment to osteogenesis or chondrogenesis. In addition, we identified a chondro-core of 9 regulons transiently activated when IIFCs engage into chondrogenesis. Among these regulons, several are involved in the autonomous circadian clock, including *Npas2* and *Arntl* (*Bmal1*). *Bmal1* was previously shown to be a regulator of cartilage differentiation in bone development and homeostasis³⁶. *Bmal1* inactivation leads to chondrocyte apoptosis and disruption of signaling involved in cartilage formation, including TGFβ, *Ihh*, HIF1α and NFAT^{37–40}. This suggests a role of the circadian clock genes as key regulators of chondrogenesis during bone repair. IIFCs are also crucial regulators of the fracture paracrine environment. IIFCs secrete factors including BMPs, PDGFs, TGFβs, and POSTN, known to be required for successful bone healing^{1,3,14,41}. These signals allow IIFCs to interact with all cell types in the fracture environment, but primarily with SSPCs and others IIFCs. Thus, IIFCs are contributing to the recruitment of SSPCs and promoting their own maturation and differentiation.

Overall, our study provides a complete dataset of the early steps of bone regeneration. The newly identified SSPC activation pattern shows the importance of the temporal dynamic of cell phenotypes and signaling pathways after injury. IIFCs appear as a transient cell population that plays essential roles in the initial steps of bone repair. Deeper understanding of IIFC regulation will be crucial as they represent the ideal target to enhance bone healing and potentially treat bone repair defects.

Materials and Methods

Mice

C57BL/6ScNj were obtained from Janvier Labs (France). *Prx1^{Cre42}* and *Rosa26-mtdTomato-mEGFP (R26^{mTmG})*⁴³ were obtained from Jackson Laboratory (Bar Harbor, ME).). All SSPCs, including pSSPCs, are marked by GFP in *Prx1^{Cre}; R26^{mTmG}* mice. Mice were bred in animal facilities at IMRB, Creteil and kept in separated ventilated cages, in pathogen-controlled environment and ad libitum access to water and food. All procedures performed were approved by Paris Est Creteil University Ethical Committee. Twelve-week-old males and females were mixed in experimental groups.

Tibial fracture

Open non-stabilized tibial fractures were induced as previously described ⁴⁴. Mice were anesthetized with an intraperitoneal injection of Ketamine (50 mg/mL) and Medetomidine (1 mg/kg) and received a subcutaneous injection of Buprenorphine (0.1 mg/kg) for analgesia. Mice were kept on a 37°C heating pad during anesthesia. The right hindlimb was shaved and sanitized. The skin was incised to expose the tibia and osteotomy was performed in the mid-diaphysis by cutting the bone. The wound was sutured, the mice were revived with an intraperitoneal injection of atipamezole (1 mg/kg) and received two additional analgesic injections in the 24 hours following surgery. Mice were sacrificed 3, 5, 7 or 14 days post-fracture.

Nuclei extraction

Nuclei extraction protocol was adapted from ^{45,46}. We generated 4 datasets for this study: uninjured periosteum, periosteum and hematoma at days 3, 5 and 7 post-tibial fracture. The uninjured and day 3 post-fracture datasets were generated in duplicates. For uninjured periosteum, tibias from 4 mice were dissected free of muscle and surrounding tissues. The epiphyses were cut and the bone marrow flushed. The periosteum was scraped from the cortex using dissecting Chisel (10095-12, Fine Science Tools). For days 3, 5 and 7 post fracture, injured tibias from 4 to 9 mice were collected and the surrounded tissues were removed. The activated periosteum was scraped and collected with the hematoma. Collected tissues were minced and placed 5 min in ice-cold Nuclei Buffer (NUC101, Merck) before mechanical nuclei extraction using a glass douncer. Extraction was performed by 20 strokes of pestle A followed by 5-10 of pestle B. Nuclei suspension was filtered, centrifuged and resuspended in RNase-free PBS (AM9624, ThermoFischer Scientific) with 2% Bovine Serum Albumin (A2153, Merck) and 0.2 U/μL RNase inhibitor (3335399001, Roche). A second step of centrifugation was performed to reduce contamination by cytoplasmic RNA. Sytox™ AADvanced™ (S10349, ThermoFischer Scientific) was added (1/200) to label nuclei and Sytox-AAD+ nuclei were sorted using Sony SH800.

Single nuclei RNA sequencing

The snRNA-seq libraries were generated using Chromium Single Cell Next GEM 3' Library & Gel Bead Kit v3.1 (10x Genomics) according to the manufacturer's protocol. Briefly, 10 000 to 20 000 nuclei were loaded in the 10x Chromium Controller to generate single-nuclei gel-beads in emulsion. After reverse transcription, gel-beads in emulsion were disrupted. Barcoded complementary DNA was isolated and amplified by PCR. Following fragmentation, end repair and A-tailing, sample indexes were added during index PCR. The purified libraries were sequenced on a Novaseq (Illumina) with 28 cycles of read 1, 8 cycles of i7 index and 91 cycles of read 2. Sequencing data were processed using the Cell Ranger Count pipeline and reads were mapped on the mm10 reference mouse genome with intronic and exonic sequences.

Filtering and clustering using Seurat

Single-nuclei RNAseq analyses were performed using Seurat v4.1.0 ^{47,48} and Rstudio v1.4.1717. Aligned snRNAseq datasets were filtered to retain only nuclei expressing between 200 et 5000 genes and expressing less than 2% of mitochondrial genes and 1.5% of ribosomal genes. Contamination from myogenic cells were removed from the analyses. After filtering, we obtained 1378 nuclei from uninjured periosteum, 1634 from day 3 post-fracture, 2089 from d5 post-fracture and 1112 from day 7 post-fracture. The replicates of the uninjured dataset were integrated using Seurat. The integrated dataset was regressed on cell cycle, mitochondrial and ribosomal content and clustering was performed using the first 15 principal components and a resolution of 0.5. SSPC/fibroblast and osteogenic cells were isolated and reclustered using the first 10 principal components and a resolution of 0.2. Uninjured, d3, d5 and d7 datasets were integrated using Seurat. The integrated dataset was regressed on cell cycle, mitochondrial and ribosomal content. Clustering was performed using the first 20 principal components and a resolution of 1.3. SSPC,

IIFC, chondrogenic and osteogenic clusters from the integration were isolated to perform subset analysis. The subset was reclustered using the first 15 principal components and a resolution of 0.6.

Pseudotime analysis using monocle3

Monocle3 v1.0.0 was used for pseudotime analysis⁴⁹. Single-cell trajectories were determined using monocle3 default parameters. The starting point of the pseudotime trajectory was determined as the cells from the uninjured dataset with the highest expression of stem/progenitor marker genes (*Ly6a*, *Cd34*, *Dpp4*, *Pi16*). Pseudotime value were added in Seurat object as metadata and used with Seurat package.

Differentiation state analysis using CytoTRACE

To assess the level of differentiation of the cell clusters, we performed analyses using CytoTRACE with the default parameters. CytoTRACE scoring was plotted in violin plot and on the Seurat UMAP clustering.

Gene Ontology and Reactome and analyses

Reactome and GO analyses were performed using EnrichR⁵⁰. All significant GO terms from upregulated genes in clusters 2 to 6 of the subset of SSPCs, IIFCs, osteoblasts and chondrocytes were manually categorized. The 5 more significant terms of the Reactome analysis from the fibrogenic TFs of Fig. 6 are presented in Table S3.

Single cell regulatory network inference using SCENIC

Single cell regulatory network inference and clustering (SCENIC)¹⁸ was used to infer transcription factor (TF) networks active in SSPCs, IIFCs, osteoblasts and chondrocytes. Analysis was performed using recommended parameters using the packages SCENIC v1.3.1, AUCell v1.16.0, and RcisTarget v1.14 and the motif databases RcisTarget and GRNboost. SCENIC package was used to perform regulon based tSNE clustering and identified population specific regulons.

Cell-cell interaction using CellChat

Cell communication analysis was performed using the R package CellChat²⁹ with default parameters on the complete fracture combined dataset and on the subset of SSPCs, IIFCs, osteoblasts and chondrocytes.

STRING network analyses

To assess protein-protein interaction network, we used the STRING v11.5 database⁵¹. To assess interaction in the chondro-core, we performed the analysis on the chondro-core and chondro-specific TFs identified in our analysis. For osteo-core analyses, we performed the analysis with osteo-core genes and the most significant interactions.

Histology and immunofluorescence

Mouse samples were processed as previously described⁴⁴. Tibias were collected and fixed in 4% PFA (sc-281692, CliniSciences) for 4 hours at 4°C. Then, samples were decalcified in 19% EDTA for 10 days (EU00084, Euromedex), cryoprotected in 30% sucrose (200-301-B, Euromedex) for 24h and embedded in OCT. Samples were sectioned in 10µm thick sections. Cryosections were defrosted and rehydrated in PBS. For Safranin-O staining, sections were stained with Weigert's solution for 5 min, rinsed in running tap water for 3 min and stained with 0.02% Fast Green for 30 seconds (F7252, Merck), followed by 1% acetic acid for 30 seconds and Safranin'O solution for 45 min (S2255, Merck). For immunofluorescence, sections were incubated 1 hour at room temperature in 5% serum, 0.25% Triton PBS before incubation overnight at 4°C with the following antibodies: Rat

monoclonal to mouse Sca1 (740450, BD Biosciences), Rabbit monoclonal to mouse SOX9 (ab185230, Abcam), Rabbit polyclonal to mouse Osterix/Sp7 (ab22552, Abcam), Goat polyclonal to mouse Periostin (AF2955, R&D Systems), Goat polyclonal to mouse PECAM1 (AF3628, Biotechne), Rat monoclonal to mouse CD45 (552848, BD Bioscience). Secondary antibody incubation was performed at room temperature for 1 hour. Slides were mounted with Fluoromount-G mounting medium with DAPI (00-4959-52, Life Technologies).

Tissue dissociation and cell sorting

Periosteal SSPC isolation

To isolate periosteal cells, uninjured tibias from *Prx1^{Cre}; R26^{mTmG}* mice were collected and all surrounding soft tissues were carefully removed. Epiphyses were embedded in low melting agarose and tibias were placed for 30 min at 37°C in digestion medium composed of PBS with 3mg/ml of Collagenase B (C6885, Merck), 4mg/ml of Dispase (D4693, Merck) and 100U/mL of DNase I (WOLS02007, Serlabo, France). After digestion, tibias were removed and the suspension was filtered, centrifuged and resuspended.

Injury-induced fibrogenic cell isolation

The fracture hematoma and the activated periosteum were collected from *Prx1^{Cre}; R26^{mTmG}* mice 3 days post-fracture. Tissues were minced and digested at 37°C for 2 hours in DMEM (21063029, Life Technologies) with 1% Trypsin (15090046, Life Technologies) and 1% collagenase D (11088866001, Roche). Cells in suspension were removed every 20 min and digestion medium was replaced. After 2 hours, the cell suspension was filtered, centrifuged and resuspended.

Cell sorting and transplantation

Digested cells were incubated 30 minutes with anti-Sca1-BV650 (BD Biosciences, 740450) or anti-CD146-BV605 (BD Biosciences, 740636). Cells were sorted using Influx Cell Sorter. Prior sorting, Sytox Blue (S34857, Thermo Fisher Scientific) was added to stain dead cells. For pSSPCs, living single GFP⁺ Sca1⁺ cells were sorted. For IIFCs, living single GFP⁺ CD146⁺ cells were sorted. Cell transplantation was performed as described in [44](#). 30000 to 45000 sorted GFP⁺ cells were embedded in Tisseel Prima fibrin gel, composed of fibrinogen and thrombin (3400894252443, Baxter S.A.S, USA), according to manufacturer's instructions. Briefly, the cells were resuspended in 15 µL of fibrin (diluted at 1/4), before adding 15 µL of thrombin (diluted at 1/4) and mixing. The pellet was then placed on ice at least 15 min for polymerization. The cell pellet was transplanted at the fracture site of wild type mice at the time of fracture.

Statistical analyses

Statistical difference between the number of activated regulons per cell was determined using one way-ANOVA followed by post-hoc test. A p-value < 0.001 is reported as ***.

Resource availability

Single nuclei RNAseq datasets are deposited in the Gene Expression Omnibus (GSE234451). This paper does not report original code. Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Céline Colnot (celine.colnot@inserm.fr).

Acknowledgements

This work was supported by ANR-18-CE14-0033, ANR-21-CE18-007-01 to C.C., NIAMS R01 AR072707 to C. C. and Ted Miclau, R01 AR081671 to C.C. and Ralph Marcucio. S. Perrin was supported by a PhD fellowship from Paris Cité University. We thank O. Pellé from the Flow Cytometry platform at Imagine Institute and all the staff from the Imagine genomic core facility at Imagine Institute. We thank A. Julien, S. Protic, M. Ethel, C. Goachet and Y. Hachemi for technical assistance or advice.

Conflict of interests

Authors declare no competing interests.

Author contributions

Conceptualization: S.P. and C.C. Methodology: S.P. and C.C. Formal analysis: S.P. and C-A.W. Investigation: S.P., M.L., F.C. and C.M. Resources: F.C. and M.M. Writing – Original Draft: S.P. and C.C. Visualization: S.P. Supervision: C.C. Project administration: C.C. Funding Acquisition: C.C.

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Reviewer #1 (Public Review):

This study delineates an important set of uninjured and injured periosteal snRNAseq data that provides an overview of periosteal cell responses to fracture healing. The authors also took additional steps to validate some of the findings using immunohistochemistry and transplantation assays. This study will provide a valuable publicly accessible dataset to reexamine the expression of the reported periosteal stem and progenitor cell markers.

Strengths:

1. This is the first single-nuclei atlas of periosteal cells that are obtained without enzymatic cell dissociation or targeted cell purification by FACS. This integrated snRNAseq dataset will provide additional opportunities for the community to revisit the expression of many periosteal cell markers that have been reported to date.
2. The authors delved further into the dataset using cutting-edge algorithms, including CytoTrace, SCENIC, Monocle, STRING, and CellChat, to define the potential roles of identified cell populations in the context of fracture healing. These additional computation analyses generate many new hypotheses regarding periosteal cell reactions.
3. The authors also sought to validate some of the computational findings using immunohistochemistry and transplantation assays to support the conclusion.

Weaknesses:

1. The current snRNAseq datasets contain only a small number of nuclei (1,189 nuclei at day 0, 6,213 nuclei on day 0-7 combined). It is unclear if the number is sufficient to discern subtle biological processes such as stem cell differentiation.
2. The authors' designation of Sca1+CD34+ cells as SSPCs is not sufficiently supported by experimental evidence. It will be essential to demonstrate stem/progenitor properties of Sca1+CD34+ cells using independent biological approaches such as CFU-F assays. In addition, the putative lineage trajectory of SSPCs toward IIFCs, osteoblasts, and chondrocytes remains highly speculative without concrete supporting data.
3. The designation of POSTN+ clusters as injury-induced fibrogenic cells (IIFCs) is not fully supported by the presented data. The authors' snRNAseq datasets (Figure 1d) demonstrate that there are many POSTN+ cells prior to injury, indicating that POSTN+ cells are not specifically induced in response to injury. It has been widely recognized that POSTN is expressed in the periosteum without fracture. This raises a possibility that the main responder of fracture healing is POSTN+ cells, not SSPCs as they postulate. The authors cannot exclude the possibility that Sca1+CD34+ cells are mere bystanders and do not participate in fracture healing.
4. Detailed spatial organization of Sca1+CD34+ cells and POSTN+ cells in the uninjured periosteum with respect to the cambium layer and the fibrous layer is not demonstrated.

5. Interpretation of transplantation experiments in Figure 5 is not straightforward, as the authors did not demonstrate the purity of Prx1Cre-GFP+SCA1+ cells and Prx1Cre-GFP+CD146+ cells to pSSPCs and IIFCs, respectively. It is possible that these populations contain much broader cell types beyond SSPCs or IIFCs.

- <https://doi.org/10.7554/eLife.92519.1.sa2>

Reviewer #2 (Public Review):

Summary:

The authors described cell type mapping was conducted for both WT and fracture types. Through this, unique cell populations specific to fracture conditions were identified. To determine these, the most undifferentiated cells were initially targeted using stemness-related markers and CytoTrace scoring. This led to the identification of SSPC differentiating into fibroblasts. It was observed that the fibroblast cell type significantly increased under fracture conditions, followed by subsequent increases in chondrocytes and osteoblasts.

Strengths:

This study presented the injury-induced fibrogenic cell (IIFC) as a characteristic cell type appearing in the bone regeneration process and proposed that the IIFC is a progenitor undergoing osteochondrogenic differentiation.

Weaknesses:

This study endeavored to elucidate the role of IIFC through snRNAseq analysis and in vivo observation. However, such validation alone is insufficient to confirm that IIFC is an osteochondrogenic progenitor, and additional data presentation is required.

- <https://doi.org/10.7554/eLife.92519.1.sa1>

Reviewer #3 (Public Review):

In this manuscript, the authors explored the transcriptional heterogeneity of the periosteum with single nuclei RNA sequencing. Without prior enrichment of specific populations, this dataset serves as an unbiased representation of the cellular components potentially relevant to bone regeneration. By describing single-cell cluster profiles, the authors characterized over 10 different populations in combined steady state and post-fracture periosteum, including stem cells (SSPC), fibroblast, osteoblast, chondrocyte, immune cells, and so on. Specifically, a developmental trajectory was computationally inferred using the continuum of gene expression to connect SSPC, injury-induced fibrogenic cells (IIFC), chondrocyte, and osteoblast, showcasing the bipotentials of periosteal SSPCs during injury repair. Additional computational pipelines were performed to describe the possible gene regulatory network and the expected pathways involved in bone regeneration. Overall, the authors provided valuable insights into the cell state transitions during bone repair and proposed sets of genes with possible involvements in injury response.

While the highlights of the manuscript are the unbiased characterization of periosteal composition, and the trajectory of SSPC response in bone fracture response, many of the conclusions can be more strongly supported with additional clarifications or extensions of the analysis.

1. As described in the method section, both the steady-state data and full dataset underwent integration before dimensional reduction and clustering. It would be appreciated if the authors could compare the post-integration landscapes of uninjured cells between steady state and full dataset analysis. Specifically, fibroblasts were shown in Figure 1C and 1E, and

such annotations did not exist in Figure 2B. Will it be possible that the original 'fibroblasts' were part of the IIFC population?

2. According to Figure 2, immune cells were taking a significant abundance within the dataset, specifically during days 3 & 5 post-fracture. It will be interesting to see the potential roles that immune cells play during bone repair. For example, what are the biological annotations of the immune clusters (B, T, NK, myeloid cells)? Are there any inflammatory genes or related signals unregulated in these immune cells? Do they interact with SSPC or IIFC during the transition?

3. The conclusion of Notch and Wnt signaling in IIFC transition was not sufficiently supported by the analysis presented in the manuscript, which was based on computational inferences. It will be great to add in references supporting these claims or provide experimental validations examining selected members of these pathways.

- <https://doi.org/10.7554/eLife.92519.1.sa0>