SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Viability testing and quality control of scRNA-seq data. Related to Figure 1. (A) Flow cytometry analysis of synovial cells to assess major cell populations and viability. Small debris were first excluded (1) then single cells gated on (2,3). Dead cell exclusion (4, including mean percentage of live cells) was performed, and the resulting live single synovial cells analyzed for expression of CD31 (5, endothelial cells), CD45 (6, hematopoietic cells), or lack of expression of CD31 and CD45 (7, double negative cells including predominantly SFs). (B) Schematic illustrating the experimental design of the scRNA-seq experiment. (C) Quality control and filtering steps prior to analysis of scRNA-seq data. Corresponding plots are included for each step. (D) Cluster-by-cluster Spearman correlation analysis of biological replicates for each condition. Color bar represents the Spearman correlation co-efficient of the average expression for all genes from each replicate (ρ). FSC-A: forward scatter-area; SSC-A: side scatter-area; FSC-W: forward scatter-width; SSC-W: side scatter-width; FSC-H: forward scatter-height; SSC-H: side scatter-height.

Figure S2. Markers and annotation of all synovial cell types. Related to Figure 1. (A) Heatmap showing the top 5 genes per cluster as determined by FindAllMarkers in Seurat, corresponding to clusters in UMAP plots from Figure 1A, ranked by log2FC. (B) UMAP plot showing all synovial cells from all conditions integrated by canonical correlation analysis. Major cell groups have dashed outlines and cell type annotations are shown on the right. (C) Identification of synovial Schwann cells using the Mouse Brain Atlas (mousebrain.org)(1). The top 5 genes from *FindAllMarkers* for the putative Schwann cells cluster were entered to determine the identity of this cluster, and the following conditions set: all genes must be expressed, and trinarization score > 0.95. The entered genes were also highly expressed in oligodendrocytes (glial cells of the central nervous system, CNS), but Schwann cells (glial cells of the peripheral nervous system, PNS, which includes synovium) were the only cell type that fulfilled the search criteria.

Figure S3. Outgoing and incoming communication patterns for all synovial cells. Related to Figure 1. (A) Line plots for determining the number of outgoing communication patterns in CellChat analysis. (B) Heatmap of outgoing communication patterns for each cell type. (C) Heatmap showing the contribution of each signaling pathway to each outgoing communication pattern. (A-C) correspond to the river plot in Figure 1F. (D) Line plots for determining the number of incoming communication patterns. (E) Heatmap of incoming communication patterns for each cell type. (F) Heatmap showing the contribution of each signaling pathway to each incoming communication pattern. (G) River plot showing CellChat analysis of incoming signaling patterns to major synovial cell types and the pathways comprising each pattern, with contributions scores of each signaling pathway shown on the right. (D-F) correspond to the river plot in Figure S3G.

Figure S4. Markers and annotation of synovial fibroblast subsets. Related to Figure 2. (A) Heatmap showing the top 5 genes per cluster as determined by FindAllMarkers in Seurat, ranked by log2FC. Corresponding to clusters in UMAP plots from Figure 2A-B. (B) UMAP plot of SFs showing cluster borders with colored, dashed outlines. Duplicated elsewhere throughout figures. (C) Feature plots of additional SF subset marker genes for each cluster determined by FindAllMarkers analysis in Seurat. Color scales represent relative expression for each gene individually. OCP: osteochondral progenitor.

Figure S5. Functions and communication patterns of synovial fibroblast subsets. Related to Figure 2. (A) Pathway analysis using a GeneUniverse approach (with GO Biological Processes terms) to assess conserved functions across SF clusters. (B) Reactome pathway analysis showing unique functional terms for each cluster. (C) Line plots for determining the number of outgoing communication patterns. (D) Heatmap of outgoing communication patterns for each SF cluster. (E) Heatmap showing the contribution of each signaling pathway to each outgoing communication pattern in Figure 2I. (C-E) correspond to the river plot in Figure 2I. (F) Line plots for determining the number of incoming communication patterns.

(G) Heatmap of incoming communication patterns for each SF cluster. (H) Heatmap showing the contribution of each signaling pathway to each incoming communication pattern for each SF cluster. (I) River plot showing CellChat analysis of incoming signaling patterns to SF clusters and the pathways comprising each pattern, with contribution score shown on the right. (F-H) correspond to the river plot in Figure S5I. n_fg: number of foreground genes found in a GO term, FDR: false discovery rate.

Figure S6. Synovial fibroblast subsets in mouse and human arthritis. Related to Figure 2. Comparison of synovial fibroblast subsets identified in this study with human synovial fibroblasts from published scRNA-seq datasets. (A) Synovial fibroblasts and related cells in mouse PTOA (this study). Left: UMAP plot featuring dashed colored outlines that denote functionally defined clusters. Right: Feature plots showing expression patterns of genes used to designate each cluster. (B) Synovial cells in human rheumatoid arthritis (RA) from Zhang *et al*. Nat Immunol, 2019(2). Left: t-SNE plot featuring dashed colored outlines of synovial fibroblast clusters (SC-F1 to 4) and other major cell types are labeled. Right: Feature plots showing expression patterns of genes used to designate each cluster in mouse PTOA (this study). (C) CD45- synovial cells in human OA (top) and RA (bottom) from Wei *et al*. Nature, 2020(3). Left: UMAP plot featuring dashed colored outlines of sublining and lining fibroblasts and other major cell types are labeled. Right: Feature plots showing expression patterns of genes used to designate each cluster in mouse PTOA (this study). For (A-C), all feature plots have a corresponding relative expression scale bar below.

Figure S7. Wnt signaling is induced in synovium after joint injury. Related to Figure 3. (A) Relative contribution of Wnt pathway ligand-receptor pairs for the circle plot of all synovial cells in Figure 3A. (B) Relative contribution of Wnt pathway ligand-receptor pairs for the circle plot of SFs in Figure 3B. (C-D) Pseudobulk heatmaps of significantly differentially expressed genes (DEGs) from the GO term cell-cell signaling by Wnt (GO:0198738) in (C) all synovial cells or (D) SFs across conditions. Color scale (z-score) of expression for C and D is located above heatmaps.

Figure S8. Flow cytometric analysis of synovium from injured Wnt-GFP reporter mice. Related to Figure 3. (A) Strategy for gating live Wnt-GFP+ synovial cells by flow cytometry using a fluorescence-minus-one (FMO) control lacking endogenous Wnt-GFP (left) and a fully stained sample from a Wnt-GFP reporter mouse (right). TOPRO3 was used as a dead cell exclusion dye. (B) Wnt-GFP+ cells as a percentage of all synovial cells in contralateral (contra) and 7d ACLR synovium (n=4 mice). (C) Wnt-GFP+ CD31- CD45- cells as a percentage of all CD31- CD45- cells in contra and 7d ACLR synovium (n=4 mice). ns: not significant. Error bars are mean ± SEM.

Figure S9. R-spondin 2 is activated in synovium following joint injury. Related to Figure 4. (A) mRNA expression of *Rspo2* in 3d or 14d ACLR and contralateral synovium (n=6-8 mice per condition). *Rspo2* levels were normalized to *Atp5b* and the 3d contra condition set to 1. (B) Total number of CD31- CD45- cells expressing RSPO2 in contra, 7d or 28d ACLR synovium (n=3 mice per condition). (C) Relative expression of *Rspo* family member genes in SFs across conditions. (D) Feature plots of *Rspo1-4* in SFs with corresponding cluster outlines (right). Color scales are not equivalent between plots. (E) mRNA expression of *Rspo2* in cultured BMDM and knee-derived SFs, normalized to *Atp5b* and with BMDM set to 1 (n=3 biological replicates). (F) Immunofluorescent staining of RSPO2 in cultured knee-derived SFs. A secondary antibody (Ab) only control is shown (left) and nuclei were counterstained with Hoechst 33342. (G-J) Flow cytometry on hindpaw SFs from (G-H) passage 2 or (I-J) passage 4. Fibroblast markers Podoplanin, CD90.2 and CD55 were assessed against unstained SFs. The percentage of CD11b+ macrophages out of all live cells was also calculated (in red gate). For (A), unpaired two-tailed student's t-tests were used to compare expression in contra versus ACLR synovium at each time point, where $^{**}P<0.01$ compared to contralateral. For (B), two-way ANOVA with multiple comparisons and Tukey's post-hoc testing was used, where $^{*}P<0.05$, $^{***}P<0.001$ compared to contralateral. For (E), an unpaired two-tailed student's t-test was used where $^{*}P<0.05$. For A, B and E, error bars are mean ± SEM.

Figure S10. SOX5 regulates *Rspo2* expression and lining fibroblast emergence. Related to Figure 5. (A) Heatmap of co-regulated gene modules for the Dpp4+ to Prg4^{hi} lining trajectory in Figure 5A. (B) Proximal promoter sequence of mouse *Rspo2*, 300 bp upstream of the transcription start site (TSS) and 100 bp downstream (exon 1). Two SOX5 consensus binding motifs are shown in the *Rspo2* proximal promoter (JASPAR MA0087.1 and JASPAR MA0087.2). (C) Gene regulatory network for SOX5, generated by CytoScape. (D) Related to Figure 5G. Hindpaw SFs were transfected with a negative control siRNA, siRNA for *Sox5* (#1 and #2), or untransfected (Sham), then *Sox5* expression assessed after 48 h (n=3 biological replicates). *Atp5b* was used as the housekeeper gene, and the Sham transfection condition was set to 1. For D, one-way ANOVA with multiple comparisons and Tukey's post hoc testing was performed where **P*<0.05. Error bars are mean ± SEM.

Figure S11. PTOA severity subscores. Related to Figure 6. Graphs (left) and representative images of contralateral (vehicle-injected, n=4) or RSPO2-injected limbs (n=5) (right) are featured for each PTOA severity subscore. (A) Structural damage. (B) Proteoglycan loss. (C) Chondrocyte hypertrophy. (D) Subchondral bone thickening. Osteophyte size and maturity had scores of zero in both conditions and were not included here. F: femur; T: tibia; M: meniscus; S: synovium. Black arrows highlight each respective subscore criterion on representative images. Scale bars are 100 μm.

Figure S12. Synovitis subscores. Related to Figure 6. Graphs (left) and representative images of contralateral (vehicle-injected, n=5) or RSPO2-injected limbs (n=5) (right) are featured for each synovitis subscore. Images are of the intercondylar notch region. (A) Pannus. (B) Bone erosion. (C) Synovial lining hyperplasia. (D) Subsynovial inflammation. (E) Fibrosis. (F) Exudate. F: femur; T: tibia; M: meniscus; S: synovium. Black arrows highlight each respective subscore criterion on representative images. Scale bars are 100 μm.

Figure S13. R-spondin 2 orchestrates crosstalk between synovial fibroblasts and macrophages. Related to Figure 7. (A) Relative expression of *Lgr* family member genes in SFs across conditions, scaled for each gene individually, from scRNA-seq. (B) qPCR analysis of relative expression of *Lgr4-6* in hindpaw-derived SFs (left, n=7 biological replicates) and knee-derived SFs (right, n=10 biological replicates), with levels normalized to *Atp5b* and *Lgr4* set to 1. (C) Left: Representative images of hindpaw SFs treated for 4 h with veh, C59 (0.25 μM), RSPO2 (200 ng/mL) or CHIR99021 (10 μM) then stained for non-phosphorylated/active β-catenin. Scale bar is 100 μm. Right: Representative images from hindpaw SFs stained with secondary antibody only (top), isotype control and secondary antibody (middle), and anti-active β-catenin (non-phosphorylated) and secondary antibody (bottom). (D) Knee-derived SFs were treated for 24 h with vehicle, RSPO2 (200 ng/mL), Mianserin (20 μM) or RSPO2+Mianserin (n=3-10 biological replicates). Expression of *Axin2*, *Lef1* and *Rspo2* was measured by qPCR and normalized to *Atp5b* levels, with vehicle-treated samples set to 1. (E) Feature plots of *Lgr4-6* mapped onto all synovial cells showing distribution of expression. Major cell types are indicated. Color scales for expression of each gene are not equivalent. (F) Individual gene expression graphs for M1 (*Il1b, Il6, Nos2*) and M2 (*Mrc1/Cd206, Il10, Arg1*) genes from the 8 h treatment of BMDM with SF conditioned in Figure 7F-G (n=3 biological replicates). *Gapdh* was used as a housekeeping gene, and for each gene, the vehicle/vehicle condition was set to 1. For B and D, one-way ANOVA with multiple comparisons and Tukey's post hoc testing was performed, where **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars are mean ± SEM for B, D, and F.

Figure S14. R-spondin 2 orchestrates crosstalk between synovial fibroblasts and chondrocytes. Related to Figure 7. (A) Relative expression of *Lgr4-6* in ATDC5 cells following 21 d of chondrogenic differentiation (n=8), with levels normalized to *Gapdh* and *Lgr4* set to 1. (B) Conditioned media from SFs treated for 24 h with vehicle (veh) or RSPO2 (200 ng/mL) was given to differentiated ATDC5 cells for 24 h (n=3). Expression of osteogenic genes *Bsp* and *Ocn* was measured by qPCR and normalized to *Gapdh* levels, with veh-treated samples set to 1. (C) ATDC5 cells were differentiated for 21 d with veh or RSPO2 (200 ng/mL) (n=6). Expression of *Acan*, *Col2a1*, *Col10a1*, *Runx2*, *Ocn*, *Osx* and

Axin2 was measured by qPCR and normalized to *Gapdh* levels, with veh-treated samples set to 1. (D) ATDC5 cells were differentiated for 21 d then treated for 24 h with veh or RSPO2 (200 ng/mL) (n=8). Expression of *Acan*, *Col2a1*, *Col10a1*, *Ocn*, *Bsp*, *Axin2* and *Lef1* was measured by qPCR and normalized to *Gapdh* levels, with veh-treated samples set to 1. For (A), one-way ANOVA with multiple comparisons and Tukey's post hoc testing was performed, where **P*<0.05, ***P*<0.01, ****P*<0.001. For B-D, unpaired two-tailed student's t-tests were used, where **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars are mean ± SEM.

References

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