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Supplemental information

Wound healing in aged skin exhibits systems-level alterations in cellular composition and cell-cell communication

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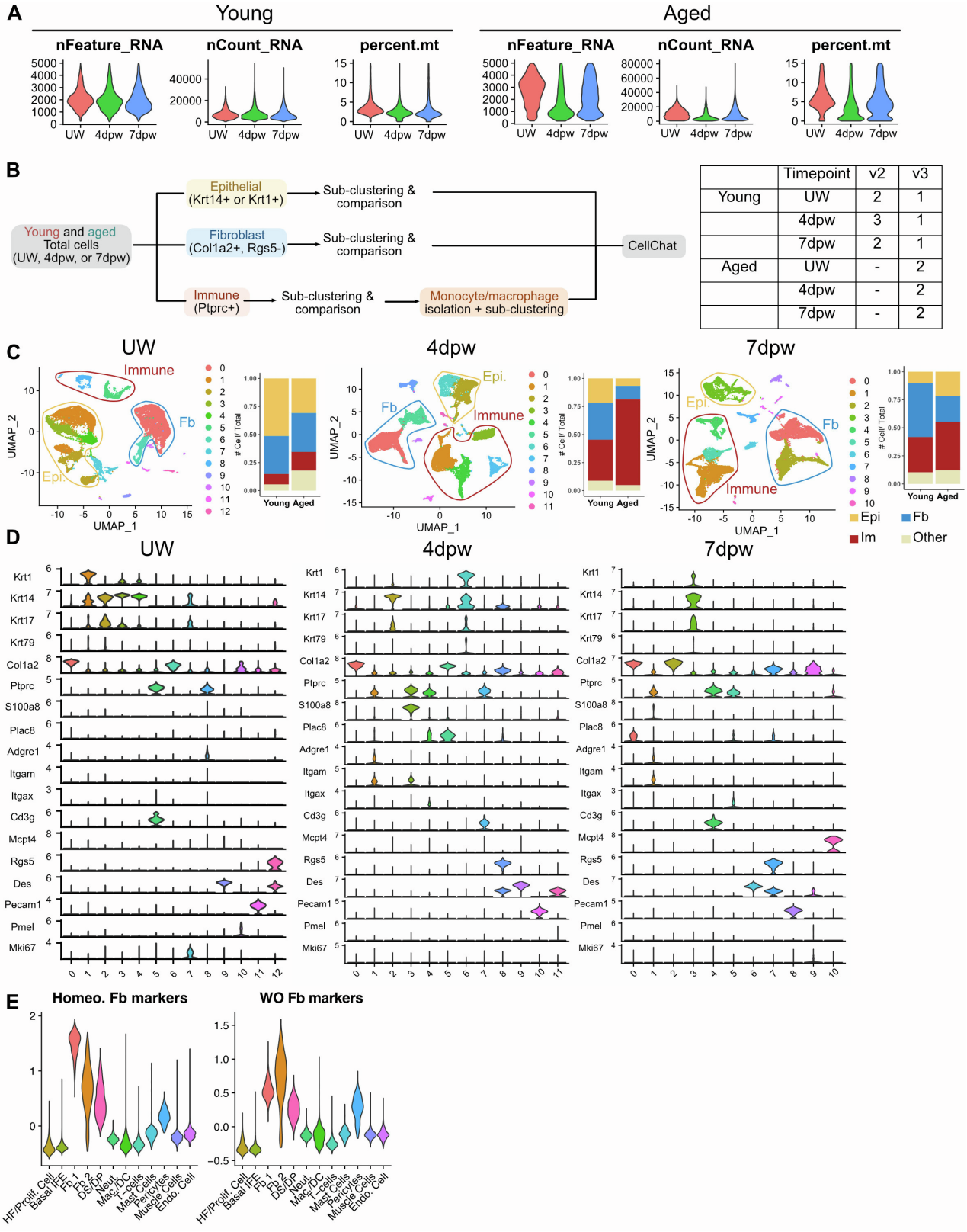
SUPPLEMENTARY FIGURES



	Timepoint	v2	v3
Young	UW	2	1
	4dpw	3	1
	7dpw	2	1
Aged	UW	-	2
	4dpw	-	2
	7dpw	-	2

Figure S1. Identification of major cell populations in UW, 4dpw and 7dpw wound samples using young+aged combined datasets. Related to Figures 1, 2, and 3.

A. Quality control metrices to eliminate dead and low-quality cells. Cells with nFeatures>5000 or <200 or percent mitochondrial genes >15% were eliminated from analysis.
B. Data analysis flowchart (left) and table showing the number of young and aged animals used for each wound timepoint and 10X kit chemistry (right).
C. UMAP and proportion of major cell populations present in the young+aged combined datasets and used for subsequent subclustered analysis.
D. Expression of marker genes identifying major cell populations in the young+aged combined datasets in C.
E. Gene scoring analysis of all cell types in aged UW, 4dpw and 7dpw samples for top 100 marker genes of homeostasis-associated and wound-induced fibroblast clusters in aggregated young samples.

Data presented in this figure include both v2 and v3 samples.

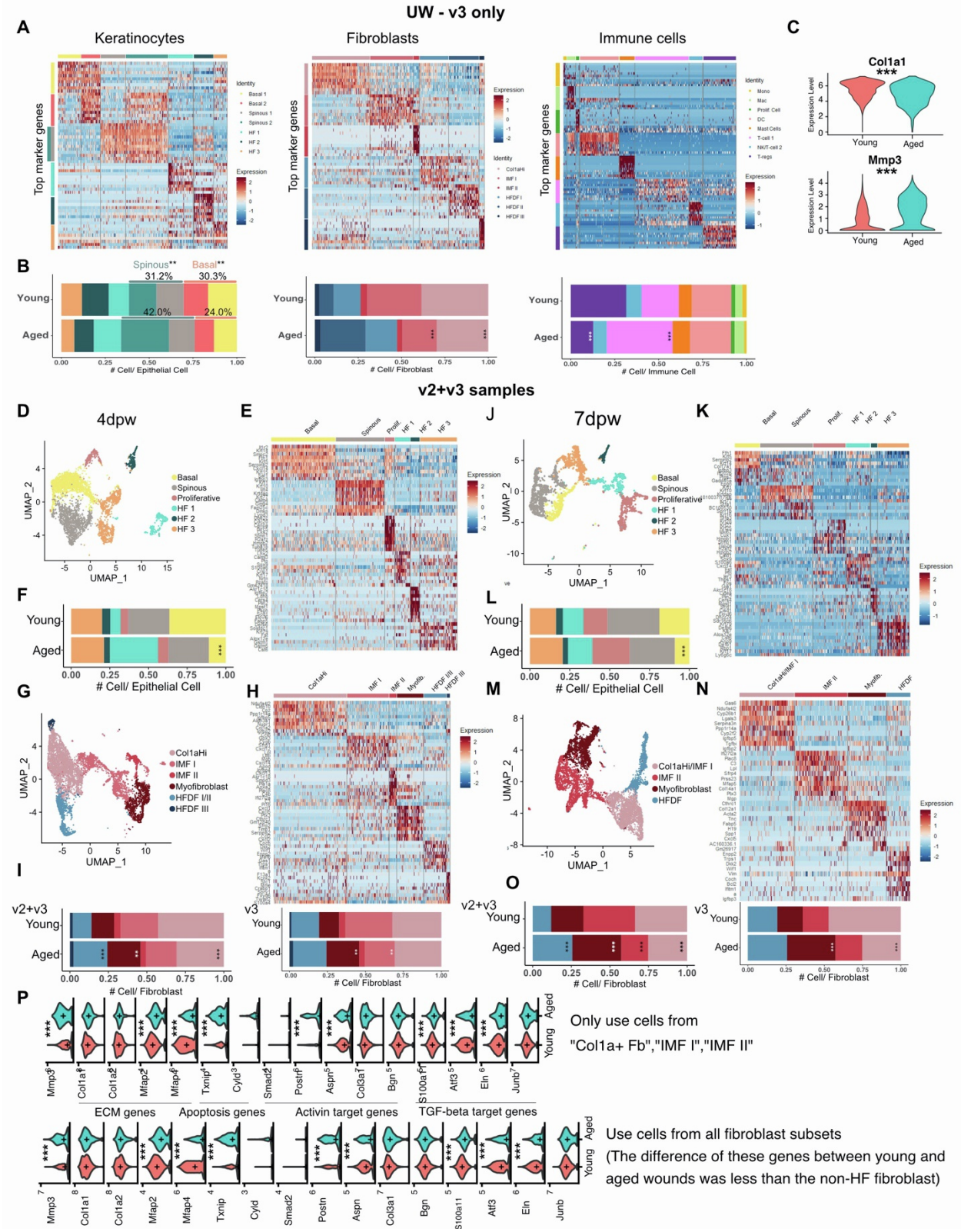

UW - v3 only
A
Keratinocytes
Fibroblasts
Immune cells
Top marker genes
C
Col1a1
***
Mmp3
***
Expression Level
Young
Aged
B
Spinous**
Basal**
31.2%
30.3%
42.0%
24.0%
Cell/ Epithelial Cell
Cell/ Fibroblast
Cell/ Immune Cell
v2+v3 samples
D
4dpw
UMAP_1
UMAP_2
Basal
Spinous
Proliferative
HF 1
HF 2
HF 3
E
J
7dpw
K
F
L
G
Col1aHi
IMF I
IMF II
Myofibroblast
HFDF I/II
HFDF III
H
M
Col1aHi/IMF I
HFDF
N
I
v2+v3
v3
O
P
Only use cells from
"Col1a+ Fb","IMF I","IMF II"
ECM genes
Apoptosis genes
Activin target genes
TGF-beta target genes
Use cells from all fibroblast subsets
(The difference of these genes between young and aged wounds was less than the non-HF fibroblast)

Figure S2. Heterogeneity of epidermal cells and fibroblasts in young and aged skin during homeostasis, and at 4dpw and 7dpw. Related to Figures 1, 6 and 7.

(A-C) v3-only young and aged samples. (D-O) v2+v3 young and aged samples.

A. Heatmap showing expression of top 10 marker genes found using v2+v3 data in each epithelial, fibroblast, and immune cell subpopulation in v3-only aggregated young+aged UW samples.
B. v3-only data corresponding to v2+v3 data in Figure 1D-E (epithelial cells), 1G-H (fibroblast), and 1L-M (immune cells).
C. v3-only data corresponding to v2+v3 data in Figure 1I.
D. UMAP of epithelial cells present in the aggregated young+aged combined datasets at 4dpw.
E. Heatmap of top 10 marker genes for each epithelial subpopulation in D.
F. Proportion of each epithelial subpopulation out of the total epithelial population in D.
G. UMAP of fibroblasts present in the aggregated young+aged combined datasets at 4dpw.
H. Heatmap of top 10 marker genes for each fibroblast subpopulation in G.
I. Proportion of each fibroblast subpopulation out of the total fibroblast population in G.
J. UMAP of epithelial cells present in the aggregated young+aged combined datasets at 7dpw.
K. Heatmap of top 10 marker genes for each epithelial subpopulation in J.
L. Proportion of each epithelial subpopulation out of the total epithelial population in J.
M. UMAP of fibroblasts present in the aggregated young+aged combined datasets at 7dpw.
N. Heatmap of top 10 marker genes for each fibroblast subpopulation in M.
O. Proportion of each fibroblast subpopulation out of the total fibroblast population in M.

P. Violin plots showing expression of the indicated genes in fibroblasts of young vs. aged skin.

p-values were calculated using the prop.test function in R (B, F, L, I, O) or Wilcoxon rank sum test (C, P). ***$p<0.001$, ** $p<0.01$.

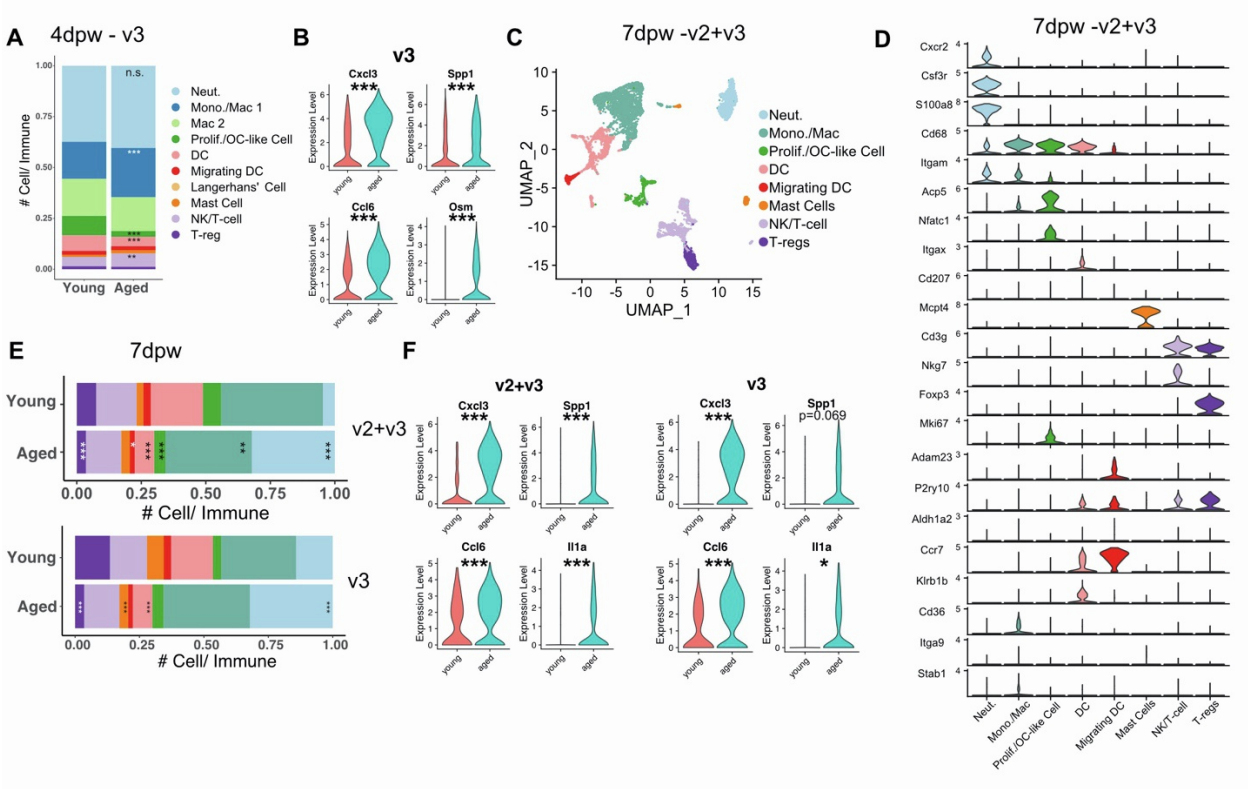

A
4dpw - v3
Cell/ Immune
Young
Aged
n.s.
Neut.
Mono./Mac 1
Mac 2
Prolif./OC-like Cell
DC
Migrating DC
Langerhans' Cell
Mast Cell
NK/T-cell
T-reg
B
v3
Cxcl3
Spp1
Ccl6
Osm
Expression Level
young
aged
C
7dpw -v2+v3
UMAP_1
UMAP_2
Neut.
Mono./Mac
Prolif./OC-like Cell
DC
Migrating DC
Mast Cells
NK/T-cell
T-regs
D
7dpw -v2+v3
Cxcr2
Csf3r
S100a8
Cd68
Itgam
Acp5
Nfatc1
Itgax
Cd207
Mcpt4
Cd3g
Nkg7
Foxp3
Mki67
Adam23
P2ry10
Aldh1a2
Ccr7
Klrb1b
Cd36
Itga9
Stab1
Neut.
Mono./Mac
Prolif./OC-like Cell
DC
Migrating DC
Mast Cells
NK/T-cell
T-regs
E
7dpw
Young
Aged
v2+v3
v3
Cell/ Immune
F
v2+v3
v3
Cxcl3
Spp1
Ccl6
Il1a
p=0.069
Expression Level
young
aged

Figure S3. Differential immune cell heterogeneity in young vs. aged skin wounds at 7dpw. Related to Figure 3.

A. v3-only data corresponding to v2+v3 data in Figure 3B.
B. v3-only data corresponding to v2+v3 data in Figure 3D. Full list of differentially expressed genes is included in Table S4.
C. UMAP of immune cell populations present in aggregated young+aged skin wound samples at 7dpw (v2+v3).
D. Marker genes used for identification of immune cell populations at 7dpw in C.
E. Population proportion of each immune cell type in C. Both v2+v3 (top) and v3-only (bottom) results are shown.
F. Expression of the indicated genes in neutrophils from young vs. aged 7dpw skin. Full list of differentially expressed genes is included in Table S4.

p-values were calculated using the prop.test function in R (A, E) or Wilcoxon rank sum test (B, F). ***$p<0.001$, ** $p<0.01$, * $p<0.05$.

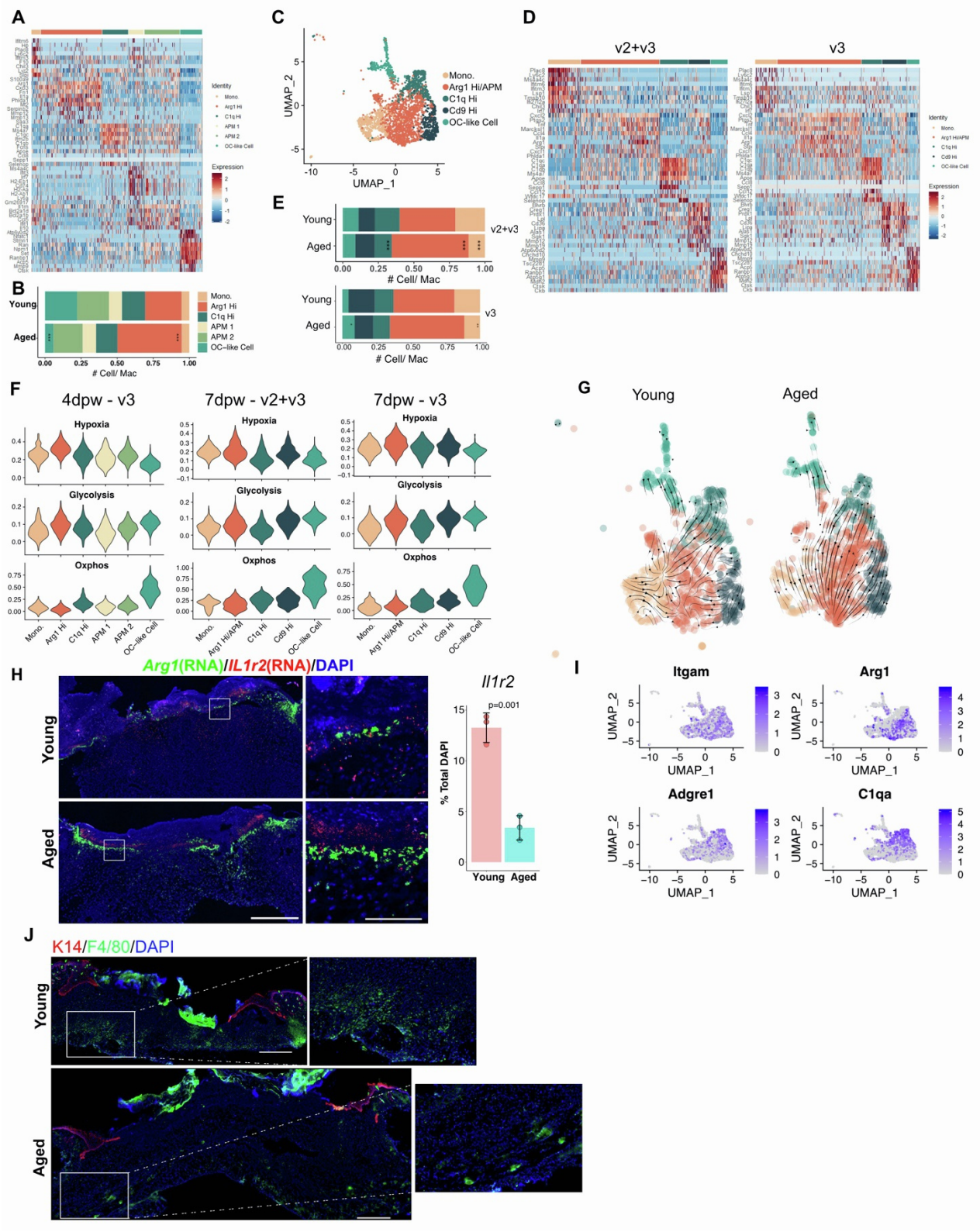

A
Identity
Mono.
Arg1 Hi
C1q Hi
APM 1
APM 2
OC-like Cell
Expression
B
Young
Aged
Mono.
Arg1 Hi
C1q Hi
APM 1
APM 2
OC-like Cell
Cell/ Mac
C
UMAP_2
UMAP_1
Mono.
Arg1 Hi/APM
C1q Hi
Cd9 Hi
OC-like Cell
D
v2+v3
v3
Identity
Expression
E
Young
Aged
v2+v3
Cell/ Mac
v3
F
4dpw - v3
7dpw - v2+v3
7dpw - v3
Hypoxia
Glycolysis
Oxphos
Mono.
Arg1 Hi
Arg1 Hi/APM
C1q Hi
Cd9 Hi
APM 1
APM 2
OC-like Cell
G
Young
Aged
H
Arg1(RNA)/Il1r2(RNA)/DAPI
Young
Aged
Il1r2
p=0.001
% Total DAPI
Young
Aged
I
Itgam
Arg1
Adgre1
C1qa
UMAP_2
UMAP_1
J
K14/F4/80/DAPI
Young
Aged

Figure S4. Differential macrophage heterogeneity in young vs. aged skin wounds at 4dpw and 7dpw. Related to Figure 4.

A. v3-only data corresponding to v2+v3 data in Figure 4B.

B. v3-only data corresponding to v2+v3 data in Figure 4C.

C. UMAP of macrophage subpopulations present in young+aged skin wounds at 7dpw (v2+v3).

D. Heatmap of top 10 differentially expressed marker genes in C. v3-only heatmap of the same 10 markers is shown on the right.

E. Population proportion of each immune cell type in C. Corresponding v3-only data is shown at the bottom.

F. Gene scoring analysis of 4dpw and 7dpw macrophage subpopulations for hypoxia-, glycolysis-, and oxidative phosphorylation-associated signatures using v2+v3 or v3-only young and aged samples.

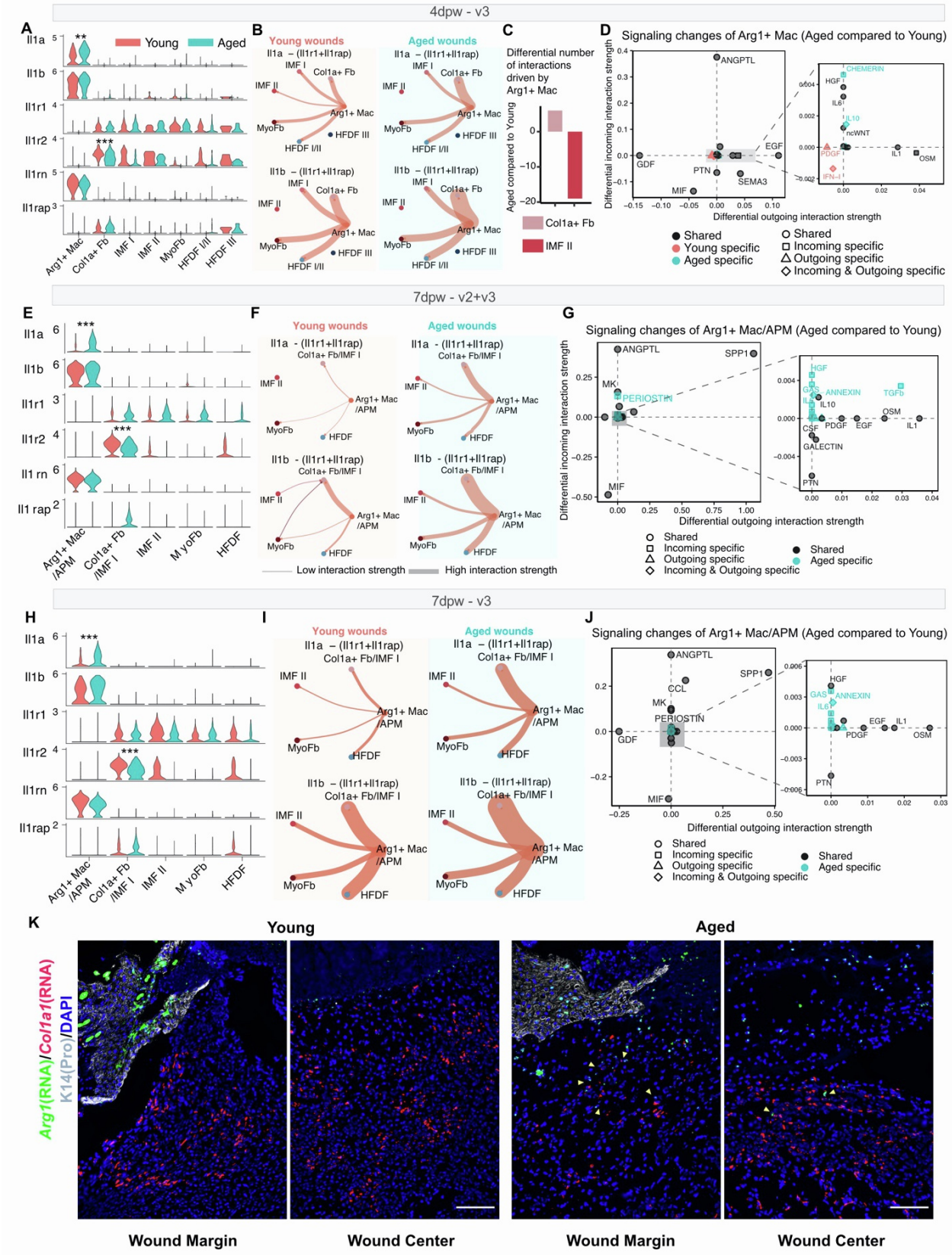
G. RNA velocity analysis of macrophages from young and aged skin wounds at 7dpw (v2+v3).

H. RNAScope data showing spatial distribution of *Arg1* and *Il1r2* transcripts in young and aged skin wounds at 4dpw. DAPI stains the nuclei. Scale bars: 500 μm in low-magnification image (left); 100 μm in high-magnification images (right). Quantification of *Il1r2*$^{+}$ cells per total DAPI-stained nuclei in young and aged skin wound beds is shown on the right (n=3 pairs). Bar graphs represent mean±SD.

I. Feature plots showing expression of *Itgam*, *Arg1*, *Adgre1*, and *C1qa*, in macrophage populations of young+aged skin wound samples at 7dpw (v2+v3).

J. Immunofluorescence of K14 and F4/80 proteins in young and aged skin wounds at 4dpw. DAPI stains the nuclei. Scale bars: 500 μm.

p-values were calculated using the prop.test function in R (B, E) or unpaired two-tailed Student's t-test (H). ***$p<0.001$, ** $p<0.01$, * $p<0.05$.


4dpw - v3
A
Young
Aged
Il1a
Il1b
Il1r1
Il1r2
Il1rn
Il1rap
Arg1+ Mac
Col1a+ Fb
IMF I
IMF II
MyoFb
HFDF I/II
HFDF III
B
Young wounds
Aged wounds
Il1a – (Il1r1+Il1rap)
Il1b – (Il1r1+Il1rap)
C
Differential number of interactions driven by Arg1+ Mac
Aged compared to Young
Col1a+ Fb
IMF II
D
Signaling changes of Arg1+ Mac (Aged compared to Young)
Differential incoming interaction strength
Differential outgoing interaction strength
ANGPTL
EGF
GDF
PTN
SEMA3
MIF
CHEMERIN
HGF
IL6
IL10
ncWNT
PDGF
IL1
OSM
IFN-I
Shared
Young specific
Aged specific
Incoming specific
Outgoing specific
Incoming & Outgoing specific
7dpw - v2+v3
E
F
Arg1+ Mac /APM
Col1a+ Fb /IMF I
M yoFb
HFDF
Low interaction strength
High interaction strength
G
Signaling changes of Arg1+ Mac/APM (Aged compared to Young)
SPP1
MK
PERIOSTIN
ANNEXIN
TGFb
GAS
CSF
GALECTIN
7dpw - v3
H
I
J
CCL

K
Young
Aged
Arg1(RNA)/Col1a1(RNA)
K14(Pro)/DAPI
Wound Margin
Wound Center
Wound Margin
Wound Center

Figure S5. Macrophage-mediated signaling in young and aged skin wounds. Related to Figure 5.

A-D: v3-only analysis using young and aged skin wound data at 4dpw.

A. v3-only data corresponding to v2+v3 data in Figure 5D.

B. v3-only data corresponding to v2+v3 data in Figure 5E.

C. v3-only data corresponding to v2+v3 data in Figure 5F.

D. v3-only data corresponding to v2+v3 data in Figure 5G.

E-G: v2+v3 analysis of *Arg1*^{Hi} macrophage/APM-mediated signaling in young and aged skin wounds at 7dpw. See Figure 5 legends for additional information.

H-J: v3-only data corresponding to v2+v3 data in (E-G) above.

K. RNAScope data showing spatial distribution of *Arg1* and *Col1a1* transcripts in young and aged skin wounds at 4dpw. Scale bars: 100 μm. Yellow arrowheads indicate instances of spatial proximity between *Arg1*$^{+}$ and *Col1a1*$^{+}$ cells.

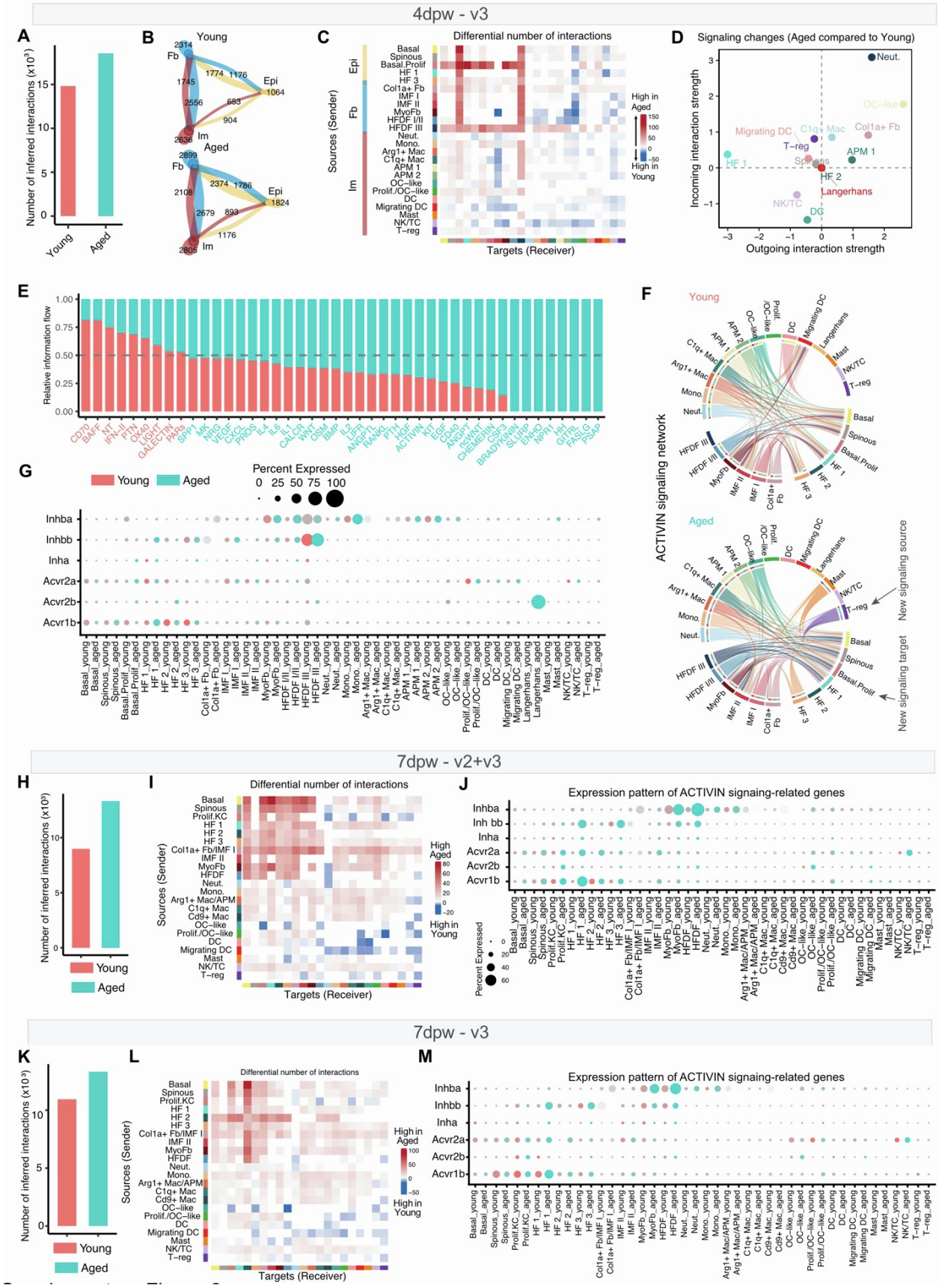

4dpw - v3
A
Number of inferred interactions (x10³)
Young
Aged
B
Young
Fb
Epi
Im
Aged
C
Differential number of interactions
Sources (Sender)
Targets (Receiver)
High in Aged
High in Young
D
Signaling changes (Aged compared to Young)
Incoming interaction strength
Outgoing interaction strength
Neut.
OC−like
Migrating DC
C1q+ Mac
Col1a+ Fb
T−reg
APM 1
HF 1
HF 2
Langerhans
NK/TC
DC
E
Relative information flow
F
Young
Aged
ACTIVIN signaling network
New signaling source
New signaling target
G
Percent Expressed
Young
Aged
Inhba
Inhbb
Inha
Acvr2a
Acvr2b
Acvr1b
7dpw - v2+v3
H
Number of inferred interactions (x10³)
Young
Aged
I
Differential number of interactions
Sources (Sender)
Targets (Receiver)
High in Aged
High in Young
J
Expression pattern of ACTIVIN signaing-related genes
Inhba
Inh bb
Inha
Acvr2a
Acvr2b
Acvr1b
Percent Expressed
7dpw - v3
K
Number of inferred interactions (x10³)
Young
Aged
L
Differential number of interactions
Sources (Sender)
Targets (Receiver)
High in Aged
High in Young
M
Expression pattern of ACTIVIN signaing-related genes
Inhba
Inhbb
Inha
Acvr2a
Acvr2b
Acvr1b

Figure S6. Aging induces substantial changes in both structure and strength of putative cell-cell communications. Related to Figure 6.

A-G: v3-only analysis using young and aged skin wound data at 4dpw.

A. v3-only data corresponding to v2+v3 data in Figure 6A.

B. v3-only data corresponding to v2+v3 data in Figure 6B.

C. v3-only data corresponding to v2+v3 data in Figure 6C.

D. v3-only data corresponding to v2+v3 data in Figure 6D.

E. v3-only data corresponding to v2+v3 data in Figure 6F.

F. v3-only data corresponding to v2+v3 data in Figure 6G.

G. v3-only data corresponding to v2+v3 data in Figure 6H.

H-J: v2+v3 analysis for young and aged skin wounds at 7dpw. See Figure 6 legends for additional information.

H. 7dpw data related to 4dpw data in Figure 6A.

I. 7dpw data related to 4dpw data in Figure 6C.

J. 7dpw data related to 4dpw data in Figure 6H.

K-M: v3-only data corresponding to v2+v3 data in (H-J) above.

.

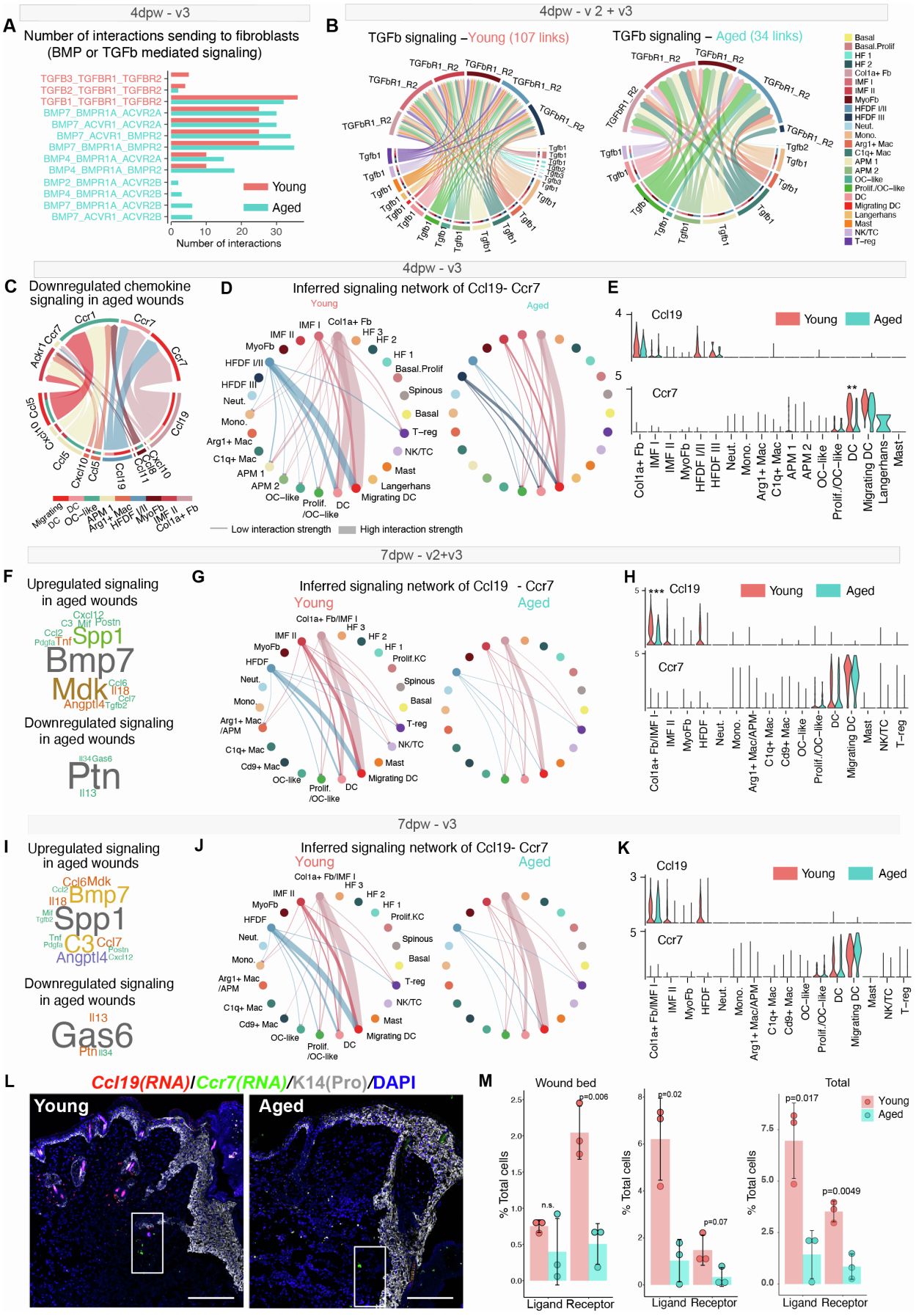

4dpw - v3
A
Number of interactions sending to fibroblasts
(BMP or TGFb mediated signaling)
TGFB3_TGFBR1_TGFBR2
TGFB2_TGFBR1_TGFBR2
TGFB1_TGFBR1_TGFBR2
BMP7_BMPR1A_ACVR2A
BMP7_ACVR1_ACVR2A
BMP7_ACVR1_BMPR2
BMP7_BMPR1A_BMPR2
BMP4_BMPR1A_ACVR2A
BMP4_BMPR1A_BMPR2
BMP2_BMPR1A_ACVR2B
BMP4_BMPR1A_ACVR2B
BMP7_BMPR1A_ACVR2B
BMP7_ACVR1_ACVR2B
Young
Aged
0
10
20
30
Number of interactions
4dpw - v 2 + v3
B
TGFb signaling –Young (107 links)
TGFb signaling – Aged (34 links)
TGFbR1_R2
Tgfb1
Tgfb2
Tgfb3
Basal
Basal.Prolif
HF 1
HF 2
Col1a+ Fb
IMF I
IMF II
MyoFb
HFDF I/II
HFDF III
Neut.
Mono.
Arg1+ Mac
C1q+ Mac
APM 1
APM 2
OC–like
Prolif./OC–like
DC
Migrating DC
Langerhans
Mast
NK/TC
T–reg
4dpw - v3
C
Downregulated chemokine
signaling in aged wounds
Ccr1
Ccr7
Ackr1
Cxcl10
Ccl5
Cxcl10
Ccl5
Ccl19
Ccl11
Ccl8
Cxcl10
Ccl19
Migrating DC
DC
OC–like
APM 1
Arg1+ Mac
HFDF I/II
MyoFb
IMF II
Col1a+ Fb
D
Inferred signaling network of Ccl19- Ccr7
Young
Aged
Low interaction strength
High interaction strength
E
Ccl19
Young
Aged
Ccr7
**
Col1a+ Fb
IMF I
IMF II
MyoFb
HFDF I/II
HFDF III
Neut.
Mono.
Arg1+ Mac
C1q+ Mac
APM 1
APM 2
OC–like
Prolif./OC–like
DC
Migrating DC
Langerhans
Mast
7dpw - v2+v3
F
Upregulated signaling
in aged wounds
Cxcl12
C3
Mif
Postn
Ccl2
Pdgfa
Tnf
Spp1
Bmp7
Mdk
Ccl6
Il18
Ccl7
Angptl4
Tgfb2
Downregulated signaling
in aged wounds
Il34
Gas6
Ptn
Il13
G
Inferred signaling network of Ccl19 - Ccr7
Young
Aged
H
***
Ccl19
Young
Aged
Ccr7
Col1a+ Fb/IMF I
IMF II
MyoFb
HFDF
Neut.
Mono.
Arg1+ Mac/APM
C1q+ Mac
Cd9+ Mac
OC–like
Prolif./OC–like
DC
Migrating DC
Mast
NK/TC
T–reg
7dpw - v3
I
Upregulated signaling
in aged wounds
Ccl6
Mdk
Ccl2
Bmp7
Il18
Mif
Tgfb2
Spp1
Tnf
Pdgfa
C3
Ccl7
Postn
Angptl4
Cxcl12
Downregulated signaling
in aged wounds
Il13
Gas6
Ptn
Il34
J
Inferred signaling network of Ccl19- Ccr7
Young
Aged
K
Ccl19
Young
Aged
Ccr7
L
Ccl19(RNA)/Ccr7(RNA)/K14(Pro)/DAPI
Young
Aged
M
Wound bed
Total
Young
Aged
% Total cells
p=0.006
n.s.
p=0.02
p=0.07
p=0.017
p=0.0049
Ligand Receptor

Figure S7. Dysregulated signaling in aged skin wounds compared to young skin wounds. Related to Figure 7.

A. v3-only data corresponding to v2+v3 data in Figure 7B.

B. Inferred TGF-β-mediated cell-cell communications from non-fibroblast subsets to fibroblast subsets in young (left) and aged (right) skin wounds at 4dpw using v2+v3 data. The number of putative interactions (i.e., links in the diagram) is indicated on the top.

C-E: v3-only analysis using young and aged skin wound data at 4dpw.

C. v3-only data corresponding to v2+v3 data in Figure 7D.

D. v3-only data corresponding to v2+v3 data in Figure 7E.

E. v3-only data corresponding to v2+v3 data in Figure 7F.

F-H: v2+v3 analysis for young and aged skin wounds at 7dpw. See Figure 7 legends for additional information.

F. 7dpw data related to 4dpw data in Figure 7A.

G. 7dpw data related to 4dpw data in Figure 7E.

H. 7dpw data related to 4dpw data in Figure 7F.

I-K: v3-only data corresponding to v2+v3 data in (F-H) above.

L. Low-magnification images of additional RNAScope data showing spatial distribution of *Ccl19* and *Ccr7* transcripts in young and aged skin wounds (see high-magnification images in Figure 7G). DAPI stains the nuclei. Scale bar: 100 μm.

M. Quantification of $Ccl19^+$ cells and $Ccr7^+$ cells per total DAPI-stained nuclei in young and aged skin wound beds (n=3 pairs). Bar graphs represent mean±SD.

p-values were calculated using Wilcoxon rank sum test (E, H) or unpaired two-tailed Student's t-test (M). ***$p<0.001$, ** $p<0.01$.