Data Supplement

Single-cell analysis reveals the loss of FABP4-positive proliferating valvular endothelial cells relates to functional mitral regurgitation

Running title: single cell RNA sequencing and human mitral regurgitation

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Methods:

Echocardiographic

Patient medical histories and clinical characteristics, as recorded by physicians at our institution, were obtained without any modifications from electronic records. Vital signs were measured during the echocardiography procedure. Echocardiographic examinations were conducted by multiple trained sonographers and reviewed by cardiologists at Fuwai Hospital (Beijing, China) using commercially available machines, as part of routine clinical practice. The measurements were performed according to clinical recommendations for assessing regurgitation and cardiac cavities. Following the echocardiography, the cardiologists graded mitral regurgitation as none/trivial, mild, moderate, or severe by considering all signs and measures recommended by guidelines[13]. This included the quantification of effective regurgitant orifice area (EROA) and regurgitant volume, which were regarded as zero when no or trivial regurgitation was present. Echocardiographic data, both qualitative and quantitative, were obtained directly from the original reports via electronic transfer.

Gender/Age correlation analysis

We used AverageExpression function of Seurat package to count average expression level of top 500 HVGs (High Variable Features) within each gender (Male/Female) or age (Adult/Children) group. The HVGs were calculated earlier by FindVariableFeatures function. To perform the pearson correlation test between

gender group or age group, the cor.test function of stats (v 4.1.2) package was employed.

Software:

R (v4.1.2)	https://www.r-project.org/
Cell Ranger (v3.1.0)	https://support.10xgenomics.com/
Seurat (v4.1.1)	https://satijalab.org/seurat/
clusterProfiler (v4.2.2)	https://github.com/YuLab-SMU/clusterProfiler[14]
pySCENIC (v0.11.2)	https://github.com/aertslab/pySCENIC[15]
pheatmap (v1.0.12)	https://github.com/raivokolde/pheatmap
scran (v1.22.1)	https://github.com/MarioniLab/scran
ReactomePA (v 1.38.0)	https://github.com/YuLab-SMU/ReactomePA[17]
CellChat (v1.4.0)	https://github.com/sqjin/CellChat[18]
velocyto (v0.17.17)	https://github.com/velocyto-team/velocyto.py[19]
scvelo (v0.2.5)	https://github.com/theislab/scvelo[20]
slingshot (v2.5.2)	https://github.com/kstreet13/slingshot/[21]
tradeSeq (v1.8.0)	https://github.com/statOmics/tradeSeq [22]

ggplot2 (v3.3.6)	https://github.com/tidyverse/ggplot2
DESeq2 (v1.34.0)	https://github.com/mikelove/DESeq2

Immunohistochemistry (IHC) staining

IHC staining of mitral valve tissue was performed with FABP4 polyclonal antibody (1:300, ab92501, Abcam) according to standard protocols. The paraffin sections were placed in an oven at 55 °C for 12-16 hours, dewaxed and rehydrated with xylene and ethanol, and washed three times with phosphate-buffered saline (PBS) (pH = 7.4) for 2 min. The antigens were then repaired with AR6 buffer solution (PerkinElmer) and endogenous peroxidase was blocked with 3% hydrogen peroxide. The slides were blocked with 3% BSA at room temperature for 60 min and then incubated with FABP4 polyclonal antibody (1:300, ab92501, Abcam) at 4 °C overnight. Finally, the paraffin slices were subjected to secondary antibody blocking, DAB staining, nuclear restaining, and dehydration. The number of positive cells of IHC staining of FABP4 in valve tissue was scored by two independent pathologists.

Opal multicolor IHC staining and tissue image analysis

A total of 3 MR and 3 NC specimens were fixed in formalin, embedded in paraffin, and were sectioned (5 μm thick) sequentially. Antigen retrieval was carried out using AR6 buffer solution (PerkinElmer). The antigen block lasted for 10 min (PerkinElmer) and then incubated overnight with rabbit monoclonal anti-FABP4 antibody (1:200, ab92501, Abcam), mouse monoclonal anti-CD31 antibody (OTI8A9, Zsbio), rabbit

monoclonal anti-vWF antibody (1:200, ab6994, Abcam) and mouse monoclonal anti-ACTA2 antibody (1:300, ab7817, Abcam). The secondary antibody and staining were obtained from an Opal 7-color IHC detection kit (PerkinElmer), and 4′,6-diamidino-2-phenylindole (DAPI) was used to make the cell nuclei visible. The positive cell infiltration of the valve slides was scanned by a multi-spectrum imaging instrument for immunohistochemistry (PerkinElmer Vectra), and the images were analyzed using Phenochart software (PerkinElmer). Violin Plots were plotted using GraphPad Prism 9.0

Immunofluorescence staining

The cell immunofluorescence method involved fixing cells with 4% paraformaldehyde (P0099, Beyotime) when cell confluence reached 50-70%. Subsequently, cell permeabilization was achieved using 0.3% Triton X-100 (T8787, SIGMA). Nonspecific binding was blocked using a blocking solution (P0102, Beyotime,). Primary antibodies, including ki-67(1:200, 27309-1-AP, Proteintech,), CD34(1:100, 60180-1-Ig, Proteintech), and FABP4(1:200, ab92501, Abcam) were applied and incubated overnight at 4°C. The following day, cells were incubated with antibodies including Goat anti-Mouse Secondary Antibody(1:200, A32723, ThermoFisher) and Goat anti-Rabbit Secondary Antibody(1:200, A11037, ThermoFisher) . Finally, fluorescent mounting medium containing DAPI （Zsbio, ZLI-9556） was used for slide mounting. Imaging was conducted using a confocal

fluorescence microscope (Zeiss LSM800), and data analysis was performed using Image J (Version, 1.8.0) for quantification.

Cell scratch assay

First, cells are seeded into a 96-well plate. When the cell confluence reaches 70%, Cell Migration Kit (BA-04858, Incucyte) is used to create a scratch. Rinse each well twice with 100 μ l PBS. Then Serum-free DMEM was used for cell culture. Subsequently, cell migration is observed at 0, 12, and 24 hours, respectively. Images were captured using a Zeiss microscope (Axio Observer D1, Carl Zeiss, Jena, Germany). Data analysis was conducted using ImageJ software (version 1.49, National Institutes of Health, Bethesda, MD).

Edu assay

Cells were seeded at a density of 1,500 cells per well in 96-well plates. Following various treatments, VECs were cultured in a medium containing EdU for 2 hours at 37°C with 5% CO2. Afterward, the cells were fixed with 4% paraformaldehyde (P0099, Beyotime) for 15 minutes, washed several times with PBS, stained with Apollo staining solution for 30 minutes and then stained with DAPI. Finally, the cells were observed using a fluorescence microscope (Opera Phenix Plus High-Content Screening System, PerkinElmer). The EdU assay kit was procured from Ribobio Bio (Guangzhou, China).

Transient transfection

A siRNA (small interfering RNA) was used to silence FABP4 (si-FABP4-2, 5′-CAAGAGCACCAUAACCUUATT -3′, designed and synthetized by Hanbio; Shanghai, China) in VEC. Cells transfected with scramble siRNA (5′-UUCUCCGAACGUGUCACGUTT -3′, Hanbio) were used as controls. Cells were grown in 6-well dishes and transiently transfected with SmartPool siRNA (5 nM, Hanbio) using LipoFiter 3.0 (Hanbio). Experiments were performed 48h after transfection. Then, the cells and culture supernatant were collected for the subsequent experiments.

Western Blot

Protein samples are first separated by SDS-PAGE gel electrophoresis (NP0323BOX, Invitrogen). Subsequently, the separated proteins are transferred onto a PVDF membrane (IB401032, Invitrogen). The membrane is then blocked by blocking buffer (P0023B, Beyotime) and probed with primary antibody, including Vimentin (1:5000, ab247342, Abcam) and FABP4 (1:5000, Ab92501, Abcam) in 4 ℃ overnight. Following that, the Goat anti-Mouse Secondary Antibody (1:5000, A16072, Invitrogen) is used. Finally, Enhanced Chemiluminescence (32209, Invitrogen) are employed to visualize and quantify protein bands.

Table S1 in supplement. Information of patients enrolled in the study.

A. Information of patients for scRNA-seq

	NC1	NC2	NC3	MR1	MR2	MR3
Age, y	26	33	17	10	35	48
Diagnosis	RCM	HCM	ARVC	DCM	DCM	HCM
Hypertension	No	No	No	No	No	No
Diabetes mellitus	No	No	No	No	No	No
Hyperlipidemia	No	No	No	No	No	No
NYHA class	Ⅳ	Ⅲ	Ⅲ	Ⅳ	Ⅲ	Ⅲ
BMI, kg/m^2	24.9	21.2	25.6	21.1	24.6	20.8
Echocardiography						
LVEF, %	62	29	35	22	28	37
AV peak velocity, m/s	1.3	0.8	1.0	1.3	1	1.4
AV mean PG, mmHg	6.8	2.6	4.0	6.8	4	0.6
AV regurgitation	No	No	No	No	No	No
PV peak velocity, m/s	1.6	0.8	0.7	3	1	0.6
PV mean PG, mmHg	10.2	2.6	2.0	36	4	1.4
PV regurgitation	No	No	No	Severe	No	No
TV mean PG, mmHg	36	31.4	21.2	29.2	21.2	36
TV regurgitation	Mild	Mild	No	No	No	No
MV mean PG, mmHg	3.2	2.6	1.4	4	2.6	1
MV regurgitation	No	Trace	No	Severe	Severe	Moderate

B. Information of patients for staining

No	Age	Diagnosis	MV regurgitation
1	61	ARVC	Mild
2	46	HCM	None

3	62	ARVC	Trace
4	33	HCM	Mild
5	15	ARVC	Mild
6	10	DCM	Mild
7	50	DCM	Trace
8	54	DCM	Mild
9	10	DCM	Mild
10	26	RCM	Trace
11	49	HCM	Mild
12	33	HCM	Mild
13	49	DCM	Moderate
14	56	DCM	Moderate
15	63	DCM	Moderate
16	16	DCM	Moderate
17	66	DCM	Moderate
18	28	HCM	Moderate
19	53	DCM	Moderate
20	26	HCM	Moderate
21	55	DCM	Moderate
22	30	DCM	Moderate
23	7	DCM	Moderate
24	33	DCM	Severe

25	50	DCM	Severe
26	65	HCM	Severe
27	10	DCM	Severe
28	57	DCM	Severe
29	4	DCM	Severe
30	65	DCM	Severe
31	37	DCM	Severe
32	58	HCM	Severe
33	35	DCM	Severe

RCM, Restrictive cardiomyopathy; DCM, dilated cardiomyopathy; ARVC, arrhythmogenic right ventricular cardiomyopathy; HCM, hypertrophic cardiomyopathy; BMI, body mass index; LVEF, left ventricular ejection fraction; AV, aortic valve; PG, pressure gradient; PV, pulmonary valve; TV, tricuspid valve; MV, mitral valve;

Table S2 in supplement. Quality control results.

Sample	NC1	NC2	NC3	MR1	MR2	MR3
CellCount.Raw	8731	6418	8618	9053	6821	9807
HBBPositiveCellCount.Raw	493	31	124	550	573	6
CellCount.Filtered	7260	5858	7954	7571	5601	9137
MedianUMICount.Raw	4920	5712	6427	5249	3664	4630
MedianUMICount.Filtered	4462	5497	6263	5048	3022	4540
MeanUMICount.Raw	6609.0032	6568.7703	6958.2686	6479.6651	5850.4461	5584.3884
MeanUMICount.Filtered	4875.4534	5680.816	6389.6538	5322.4097	4315.9454	4934.5902
MedianGeneCount.Raw	2151	2431.5	2403	2135	1555	1905
MedianGeneCount.Filtered	2027	2370	2354	2080	1348	1884
MeanGeneCount.Raw	2318.4256	2481.5469	2388.8458	2240.7865	1895.5945	2038.8239
MeanGeneCount.Filtered	2018.1499	2321.0073	2283.1758	2053.6414	1620.6035	1923.5082
MedianPercentMT.Raw	1.9380775	1.2651698	1.1969881	2.4595613	1.2141816	3.0794406
MedianPercentMT.filtered	1.9850758	1.287779	1.2017659	2.4513473	1.1792453	3.0746706
MeanPercentMT.Raw	2.5106638	1.7529317	1.6471653	3.2568067	1.8147522	3.5989679
MeanPercentMT.filtered	2.2412468	1.6022727	1.408359	2.7945014	1.5444632	3.3241006

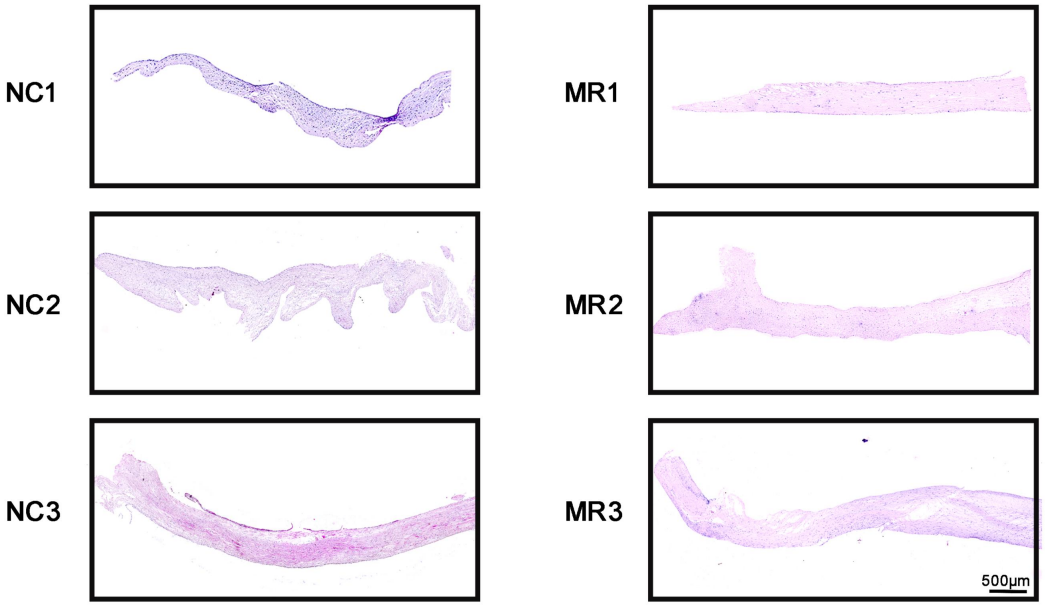

Figure S1. **Histological examination (HE staining) of mitral valve corresponding to each patient.** NC, non-diseased control; MR, mitral regurgitation.

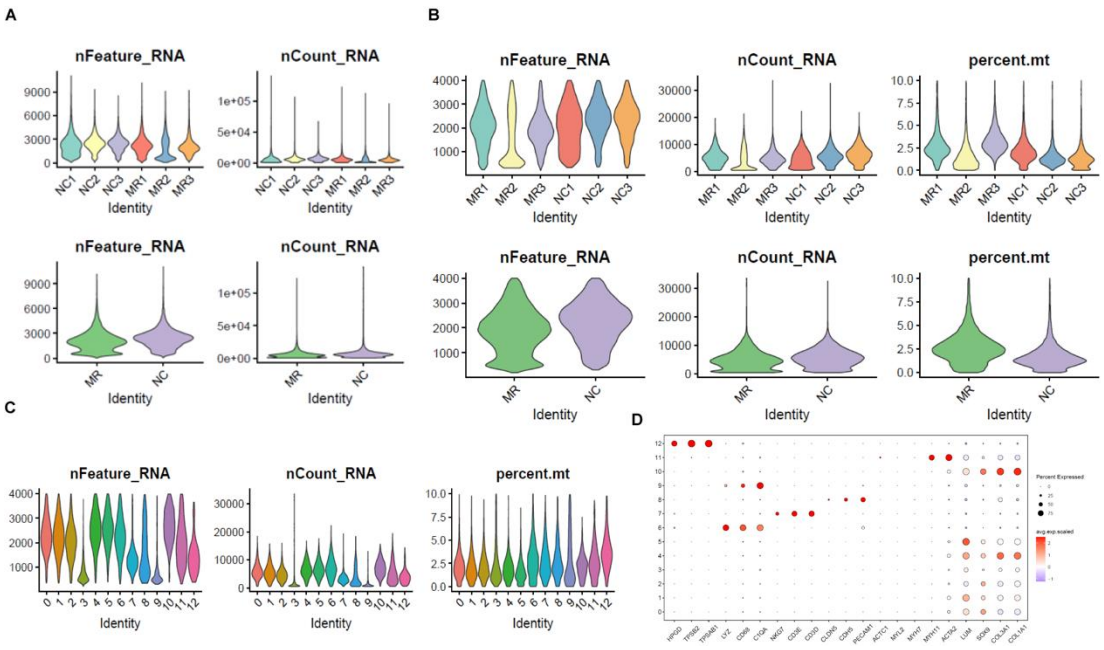

Figure S2. Quality control for scRNA-seq datasets. A-B. nFeature_RNA, nCount_RNA and Percent_mt of scRNA-seq datasets before (**A**) and after (**B**) filter. **C.** nFeature_RNA, nCount RNA and Percent of each cell cluster showed the low quality clusters. **D.** Canonical marker genes of different cell types defined cell clusters. NC, non-diseased control; MR, mitral regurgitation.

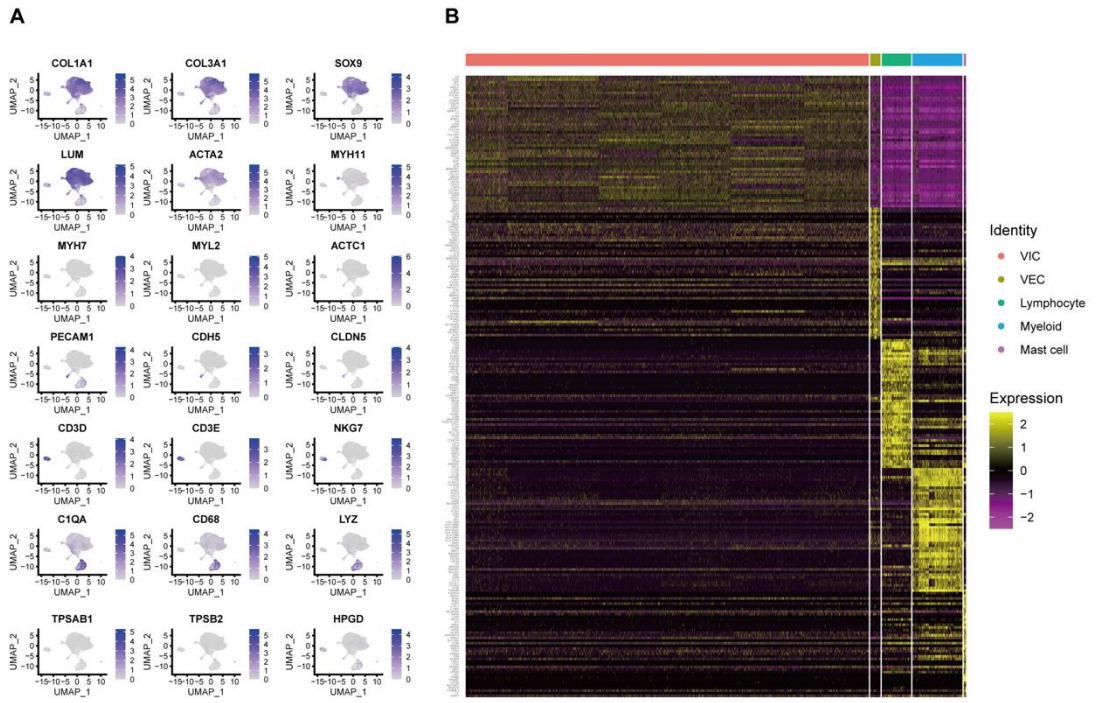

Figure S3. Identification of valve cells. A. UMAP of canonical marker genes in valve cell. UMAP plots showing post-QC data; cells are color-coded for canonical marker genes. Color scale: blue, high expression; gray, low expression. **B.** Gene-expression heatmap of the top 50 marker genes for each cell type. Color scale: yellow, high expression; purple, low expression. UMAP, uniform manifold approximation and projection; VIC, valvular interstitial cell; VEC, valvular endothelial cell.

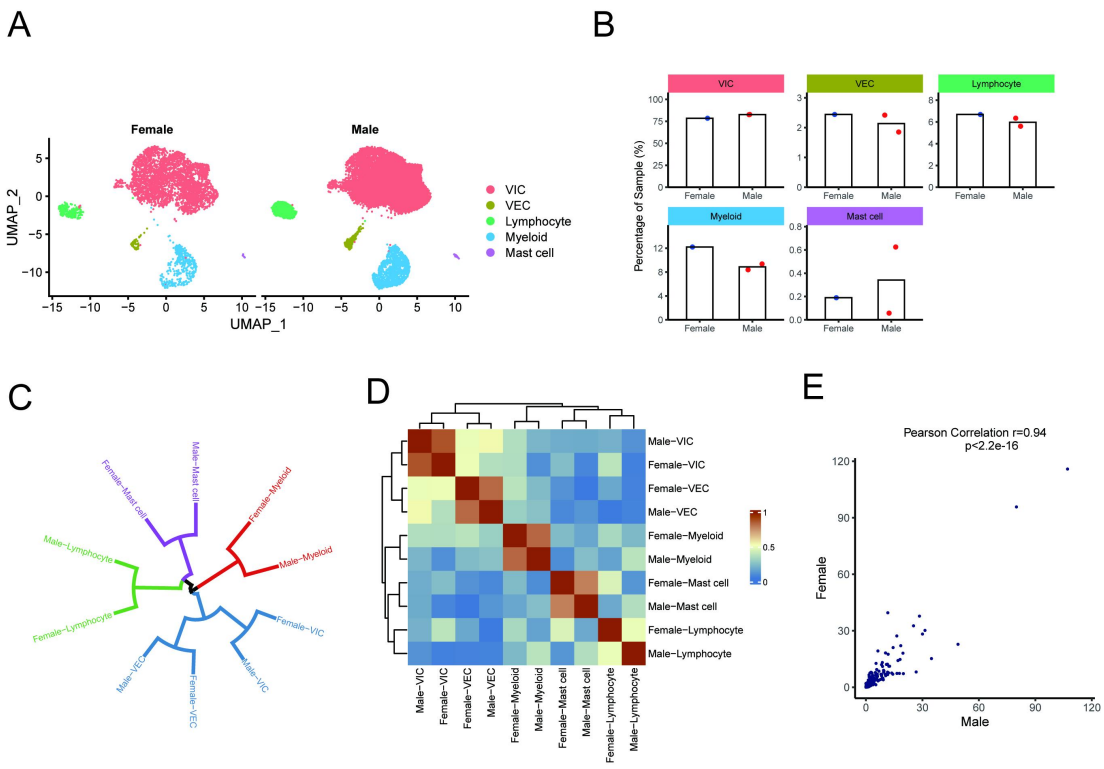

Figure S4. Potential effects of gender on the cellular landscape of mitral regurgitation valves. A. Separate UMAP plot for cells from two male and one female. **B.** The ratio of each cellular clusters in MR valves from male and female. Each dot represents one valve sample. The Student's t-test with Welch's correction was performed to compare the log transformed proportion of cell subpopulations. **C.** Hierarchical clustering of six cellular clusters among MR valves from male or female. **D.** Heatmap showing the Pearson correlation coefficient among six major cellular clusters among MR valves from male or female. **E.** Pearson correlation analysis of the all detected genes in valves from two male and one female.

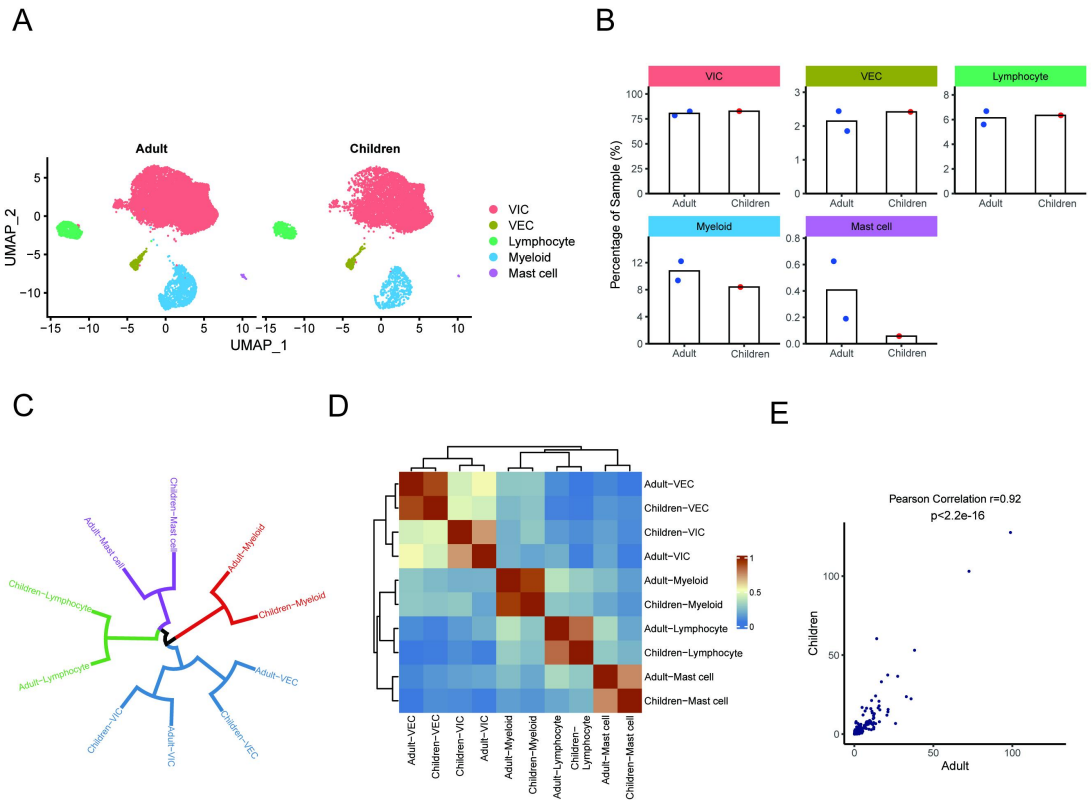

Figure S5. Potential effects of age on the cellular landscape of mitral regurgitation valves. A. Separate UMAP plot for cells from two adults and one child. **B.** The ratio of each cellular clusters in MR valves from adults and children. Each dot represents one valve sample. The Student's t-test with Welch's correction was performed to compare the log transformed proportion of cell subpopulations. **C.** Hierarchical clustering of six cellular clusters among MR valves from adult or children. **D.** Heatmap showing the Pearson correlation coefficient among six major cellular clusters among MR valves from adult or children. **E.** Pearson correlation analysis of the all detected genes in valves from two adults and one child.

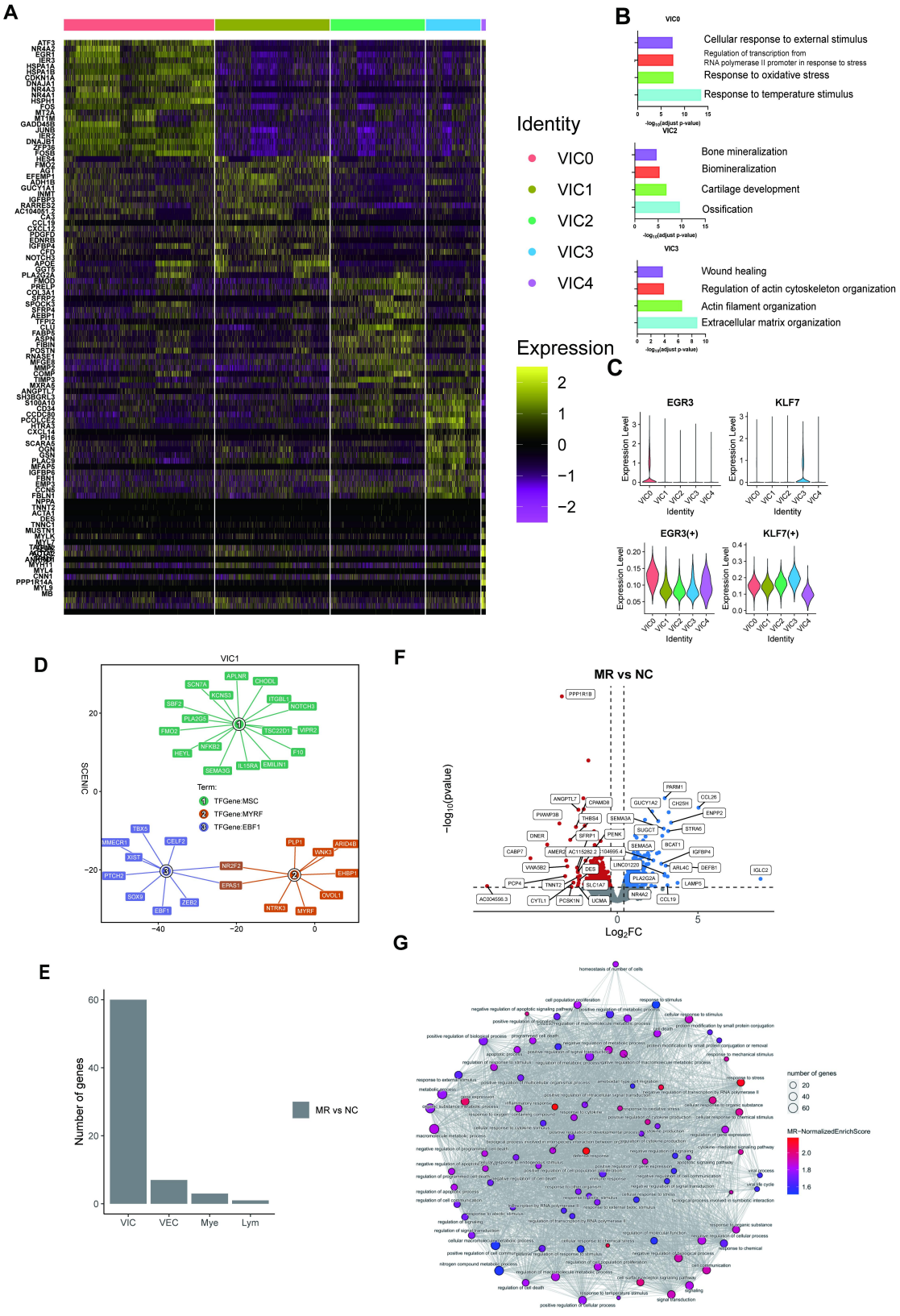

Figure S6. VIC subclusters helps the valve change its structure to accommodate hemodynamic changes. A. Gene-expression heatmap of the top 20 marker genes for

each VIC subcluster. Color scale: yellow, high expression; purple, low expression. **B.** Selected top GO enrichment terms related to corresponding differential expressed genes (DEGs). **C.** The expression level and regulon activity of TFs EGR3 and KLF7 in each VIC cluster. **D.** A network diagram of TFs and their downstream regulated genes. **E**. Bar chart depicting the number of differential genes among different subgroups. **F.** Differential gene expression of VIC2 in NC group and MR group are shown in a volcano plot. **G.** Network view of the differentially regulated REACTOME pathways in VIC in the TR. The size of the dot reflects the size of the gene set. The dots in red denote high-upregulated pathways, and the dots in blue represent low-upregulated pathways. The significance threshold of the GSEA test was set to a p value of 0.05. NC, non-diseased control; MR, mitral regurgitation.

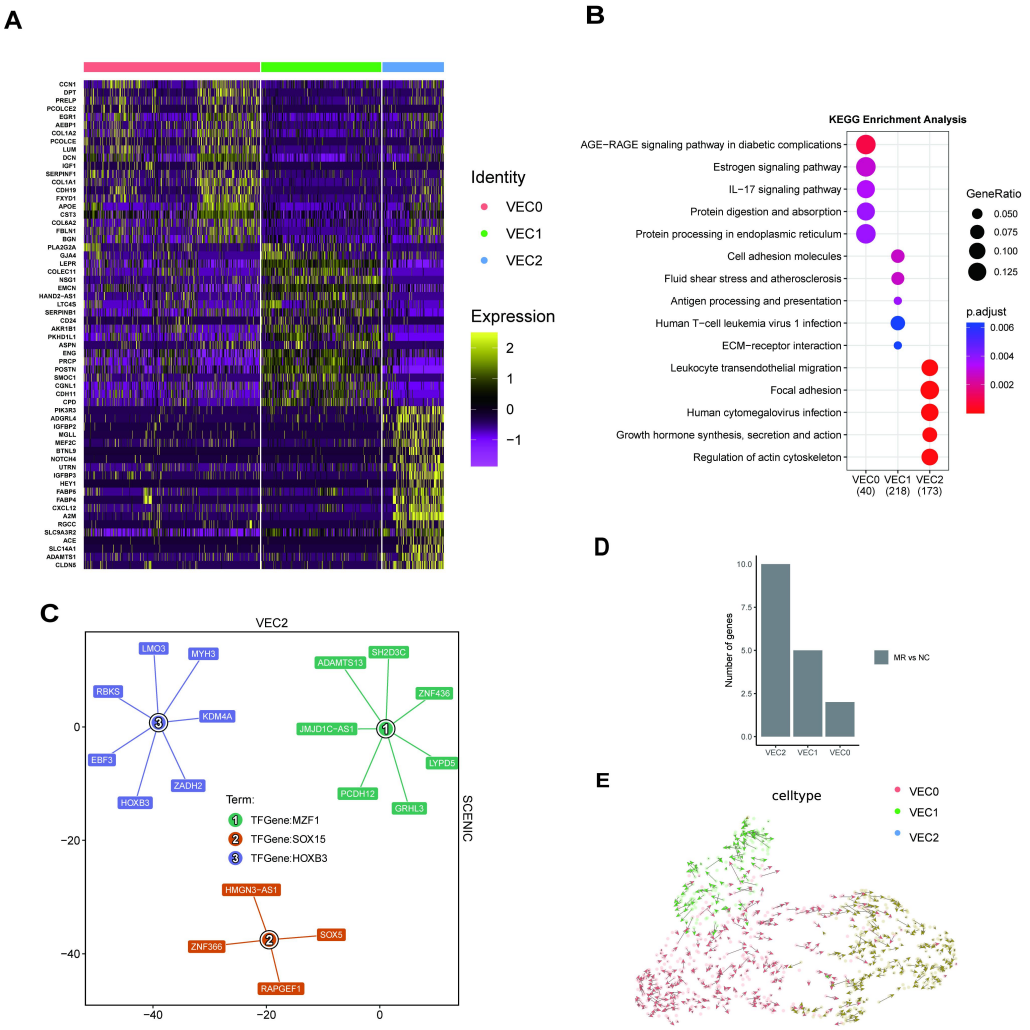

Figure S7. Supplementary figures on VEC analysis. A. Heatmap showing top 20 differentially expressed genes with a Log2 (Fold change) of >0.5 between one VEC cluster and other VEC clusters. **B.** The enriched KEGG terms of each VEC cluster. **C.** A network diagram of TFs and their downstream regulated genes. **D.** Bar chart depicting the number of differential genes among different subgroups. **E.** RNA velocity-based cell lineage of VEC. NC, non-diseased control; MR, mitral regurgitation.

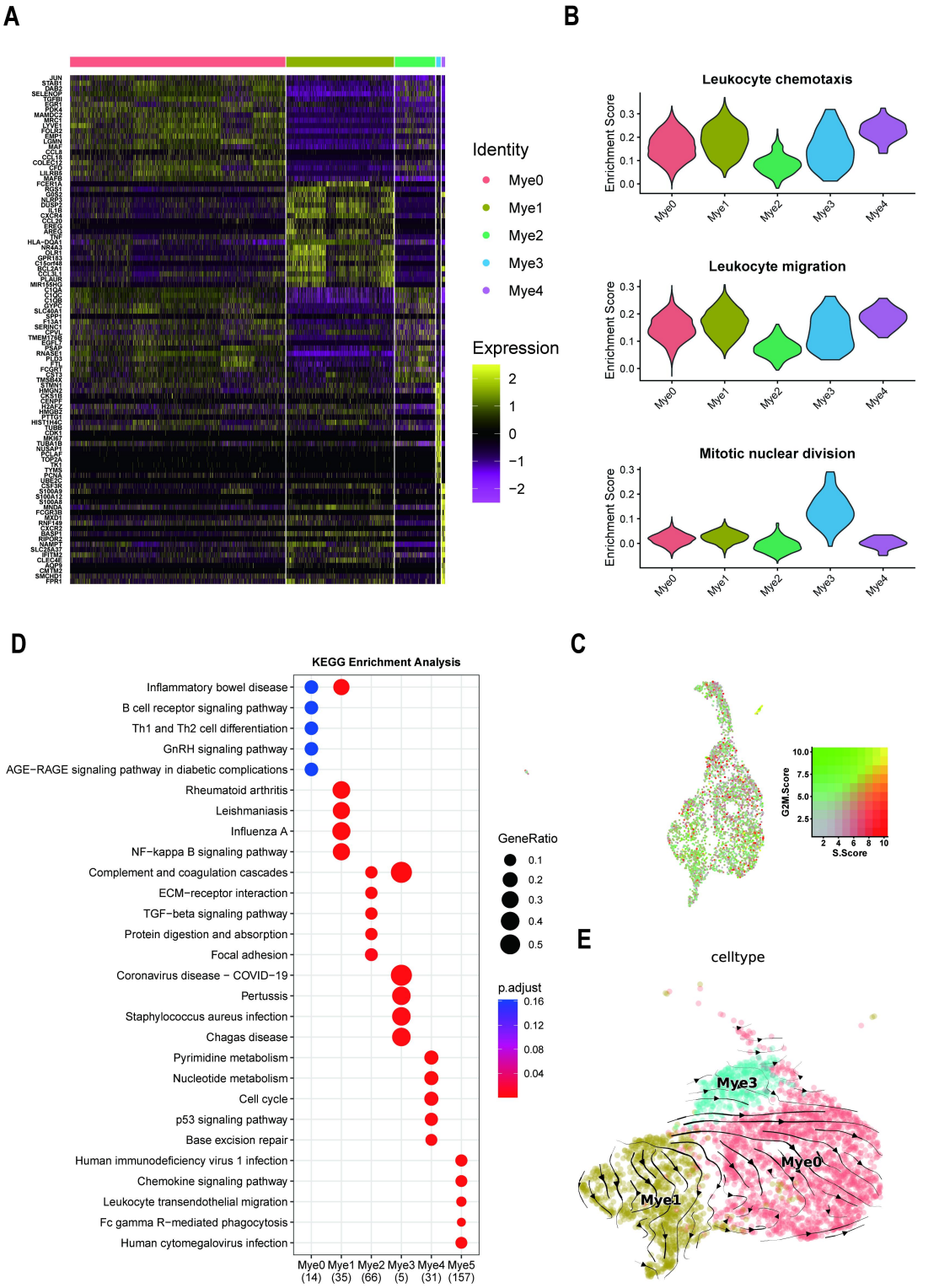

Figure S8. Supplementary figures on myeloid analysis. A. Heatmap showing top 20 differentially expressed genes with a Log2 (Fold change) of >0.5 between one Myeloid cluster and other Myeloid clusters. **B.** The enrichment scores of four

different biological processes in each Myeloid subcluster. **C.** The cell cycle phases of individual cells in each population predicted using Seurat CellCycleScoring. UMAP plot showing the cell cycle phase of myeloid. Red and green points correspond to cells at S phase and G2/M phase, respectively. **D.** The enriched KEGG terms of each Myeloid cluster. **E.** RNA velocity-based cell lineage of Myeloid.

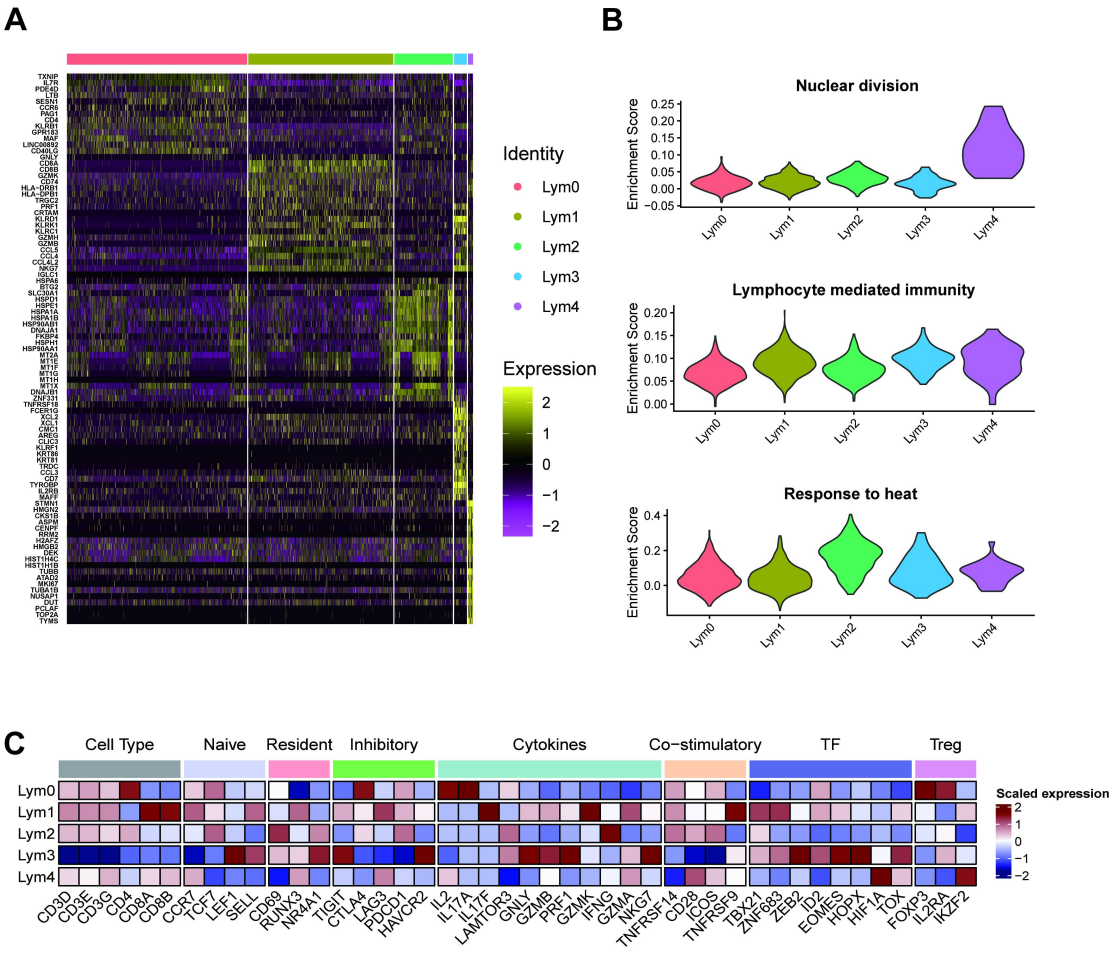

Figure S9. Supplementary figures on Lymphocyte analysis. A. Heatmap showing top 20 differentially expressed genes with a Log2 (Fold change) of >0.5 between one Lymphocyte cluster and other Lymphocyte clusters. **B.** The enrichment scores of four different biological processes in each Lymphocyte subclusters. **C.** Markers of cell types, naive T cells, Resident T cells, inhibitory T cells, cytokines, co-stimulatory chemokines, TF and Treg in each cluster was delivered in heatmap.

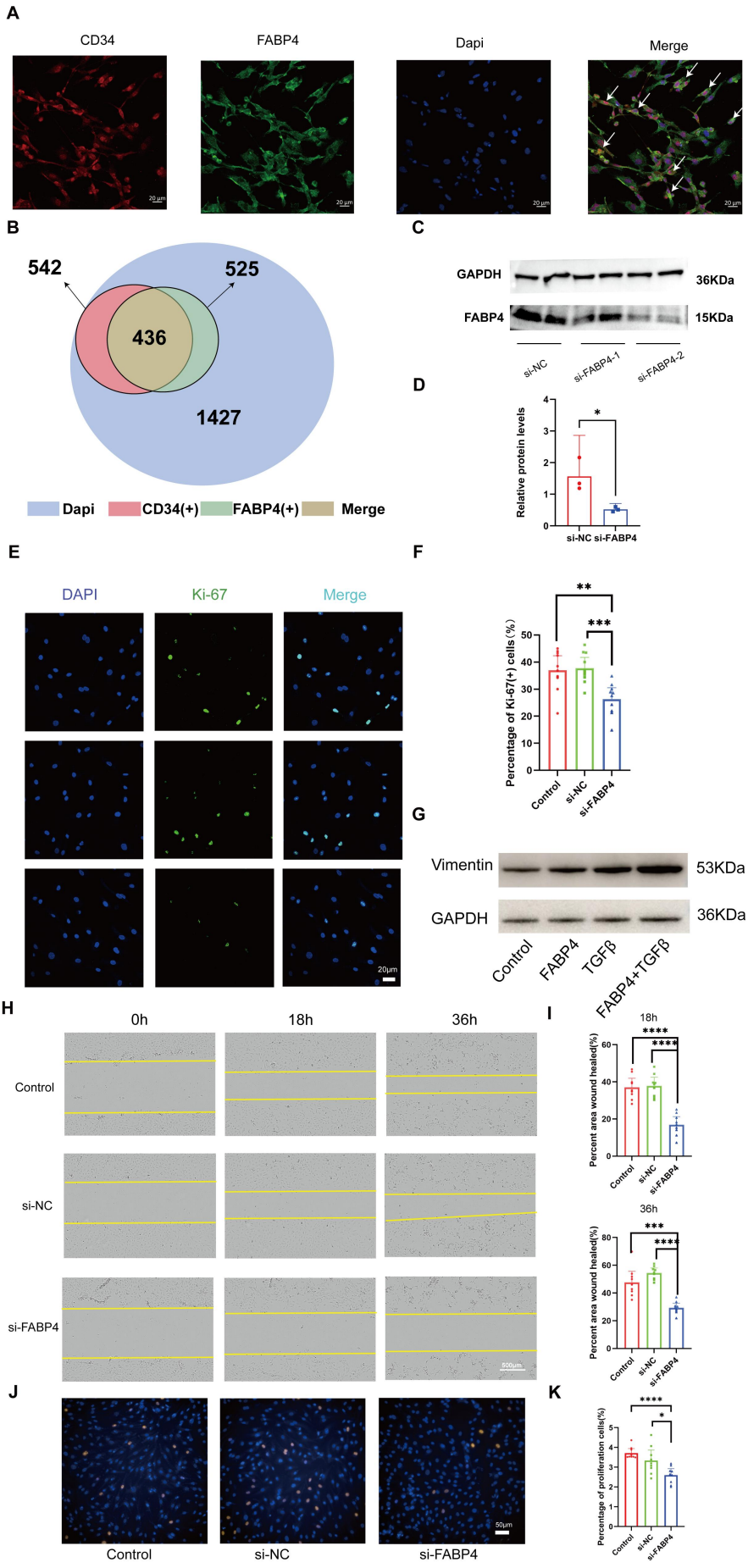
A
CD34
FABP4
Dapi
Merge
B
542
525
436
1427
Dapi
CD34(+)
FABP4(+)
Merge
C
GAPDH
36KDa
FABP4
15KDa
si-NC
si-FABP4-1
si-FABP4-2
D
Relative protein levels
si-NC si-FABP4
E
DAPI
Ki-67
Merge
20μm
F
Percentage of Ki-67(+) cells (%)
Control
si-NC
si-FABP4
G
Vimentin
53KDa
GAPDH
36KDa
Control
FABP4
TGFβ
FABP4+TGFβ
H
0h
18h
36h
Control
si-NC
si-FABP4
I
18h
Percent area wound healed(%)
36h
J
Control
si-NC
si-FABP4
K
Percentage of proliferation cells(%)

Figure S10. Supplementary figures on function of FABP4 in VEC

A. Immunostaining images showing the colocalization of CD34 and FABP4 in human VECs. **B.** A Venn diagram depicting the proportions of various positive cell types in cultured cells. **C, D** Western blot and quantity results of FABP4 and GAPDH **E.** Ki-67 proliferation assay of VECs. **F.** Quantitative analysis of the results of the Ki-67 proliferation assay **G.** Western blot of Vimentin and GAPDH between groups. **H.** Representative images of human VECs from the cell scratch assay. **I**. Quantitative analysis of the results of the cell scratch assay. J. Representative images of EdU staining **K.** Quantitative analysis of the results of EdU staining. (**A**). Scale bar, 20 μm. (**E**). Scale bar, 20 μm. (**H**). Scale bar, 500 μm. (**J**). Scale bar, 50 μm. Points in each group in **D, F, I**, **K** represent 3, 10, 9 and 9 biological replicates, respectively. The data were normally distributed and had equal variance. One-way ANOVA followed by Tukey's multiple comparison test was used for multiple groups. A value of $P<0.05$ was considered statistically significant.