Science Advances
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Supplementary Materials for

Integrated single-cell RNA-seq analysis reveals mitochondrial calcium signaling as a modulator of endothelial-to-mesenchymal transition

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Other Supplementary Material for this manuscript includes the following:

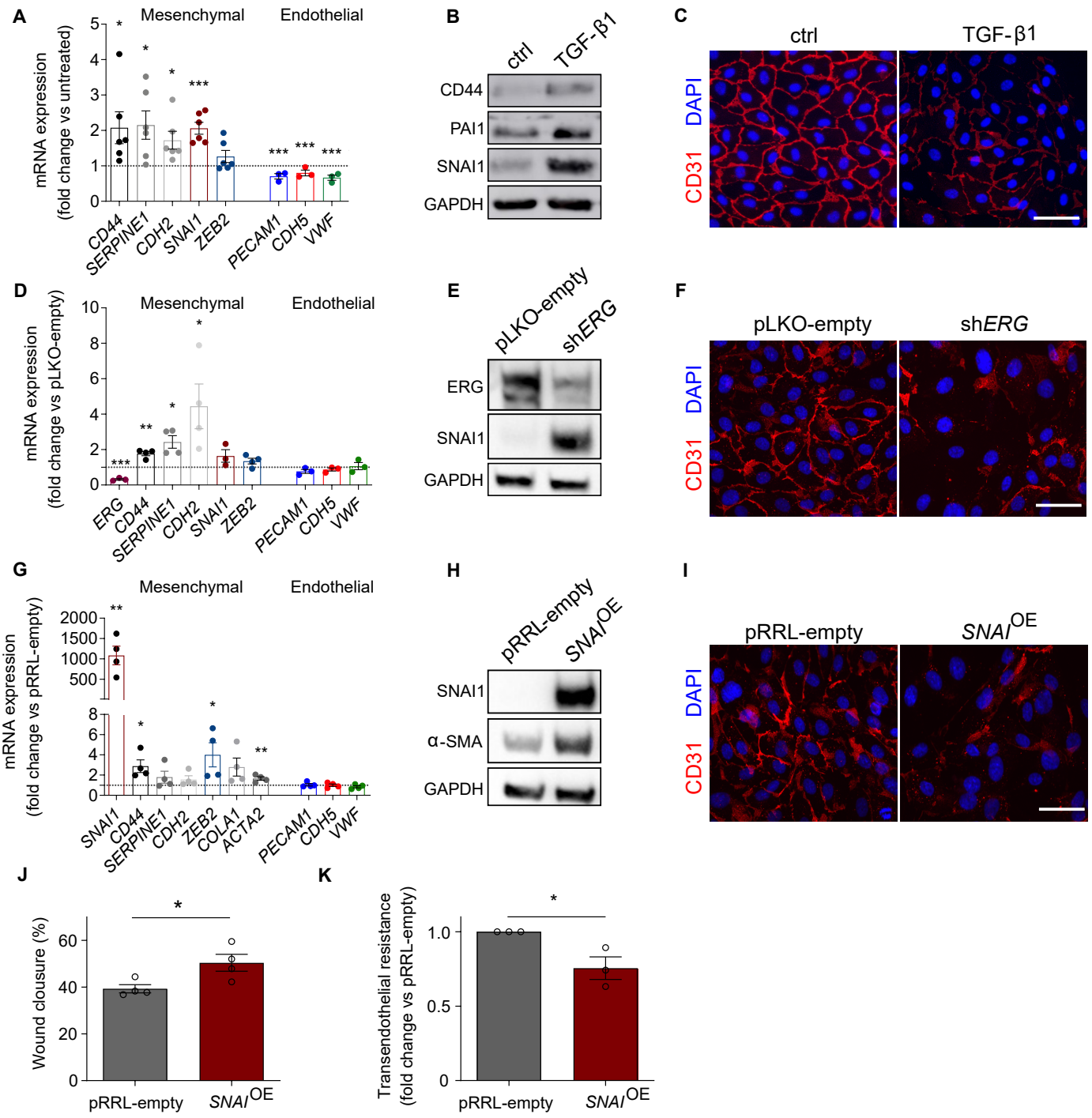
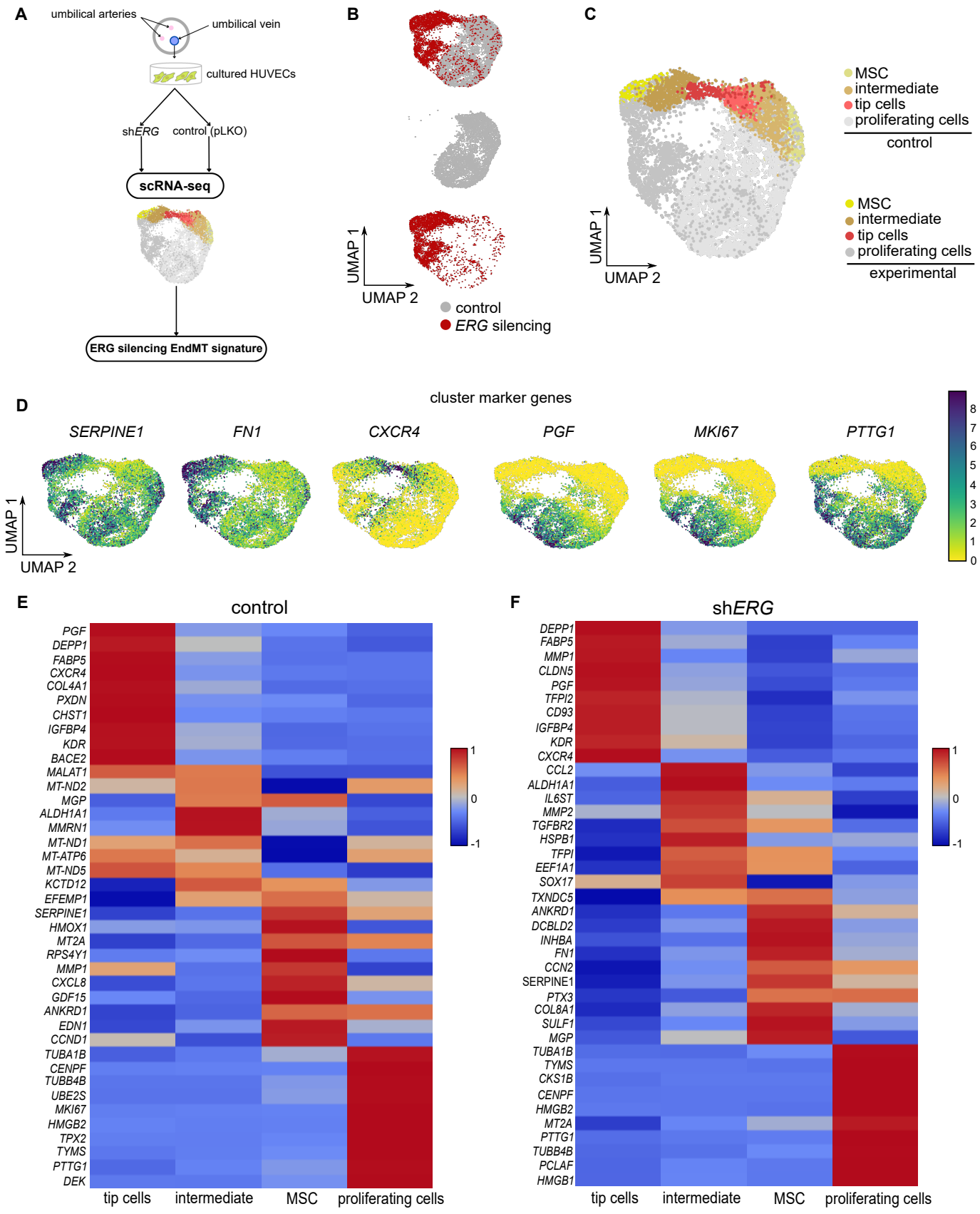


Fig. S1. In vitro EndMT induction. (**A**) RT-PCR analysis of mRNA expression levels of mesenchymal and endothelial genes in TGF-β-treated ECs compared to control ECs. Expression values were normalized to 18S mRNA levels and they are relative to untreated control (N= 3-6). Dotted line: expression levels in untreated ECs. *P value < 0.05, ***P < 0.001. (**B**) Representative immunoblots for CD44, PAI, SNAI1 and GAPDH in cytosolic fractions in control and TGF-β1-treated ECs. (**C**) CD31 staining of control and TGF-β1-treated ECs. Nuclei are counterstained with DAPI (blue). Scale bar, 50 µm. (**D**) RT-PCR analysis of mRNA expression levels of mesenchymal and endothelial and genes in sh*ERG* compared to non-silenced control ECs (pLKO-empty). Expression values were normalized to 18S mRNA levels and they are relative to non-silenced control (N= 3-4). Dotted line: expression levels in pLKO-empty. *P value < 0.05, **P < 0.01, ***P < 0.001. (**E**) Representative immunoblots for ERG, SNAI1 and GAPDH in cytosolic fractions in sh*ERG* and non-silenced control (pLKO-empty). (**F**) CD31 staining of sh*ERG* and non-silenced control (pLKO-empty). Nuclei are counterstained with DAPI (blue). Scale bar, 50 µm. (**G**) RT-PCR analysis of mRNA expression levels of mesenchymal and endothelial genes in *SNAI*^{OE} compared to control ECs (pRRL-empty). Expression values were normalized to 18S mRNA levels and they are relative to non-overexpressing control (N= 4). Dotted line: expression levels in pRRL-empty. *P value < 0.05, **P < 0.01. (**H**) Representative immunoblots for SNAI1, α-SMA and GAPDH in cytosolic fractions in SNAI^{OE} and control (pRRL-empty). (**I**) CD31 staining of *SNAI*^{OE} and control (pRRL-empty). Nuclei are counterstained with DAPI (blue). Scale bar, 50 µm. (**J**) Quantification of scratch wound assay using mitomycin C-treated control (pRRL-empty) and *SNAI*^{OE} (N= 4). *P < 0.05. (**K**) Transendothelial electrical resistance of control (pRRL-empty) and *SNAI*^{OE} EC monolayers (N= 3). *P value < 0.05.


A
umbilical arteries
umbilical vein
cultured HUVECs
shERG
control (pLKO)
scRNA-seq
ERG silencing EndMT signature
B
UMAP 1
UMAP 2
control
ERG silencing
C
MSC
intermediate
tip cells
proliferating cells
control
MSC
intermediate
tip cells
proliferating cells
experimental
UMAP 1
UMAP 2
D
cluster marker genes
SERPINE1
FN1
CXCR4
PGF
MKI67
PTTG1
UMAP 1
UMAP 2
8
7
6
5
4
3
2
1
0
E
control
PGF
DEPP1
FABP5
CXCR4
COL4A1
PXDN
CHST1
IGFBP4
KDR
BACE2
MALAT1
MT-ND2
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MT-ND5
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EFEMP1
SERPINE1
HMOX1
MT2A
RPS4Y1
MMP1
CXCL8
GDF15
ANKRD1
EDN1
CCND1
TUBA1B
CENPF
TUBB4B
UBE2S
MKI67
HMGB2
TPX2
TYMS
PTTG1
DEK
1
0
-1
tip cells
intermediate
MSC
proliferating cells
F
shERG
DEPP1
FABP5
MMP1
CLDN5
PGF
TFPI2
CD93
IGFBP4
KDR
CXCR4
CCL2
ALDH1A1
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tip cells
intermediate
MSC
proliferating cells

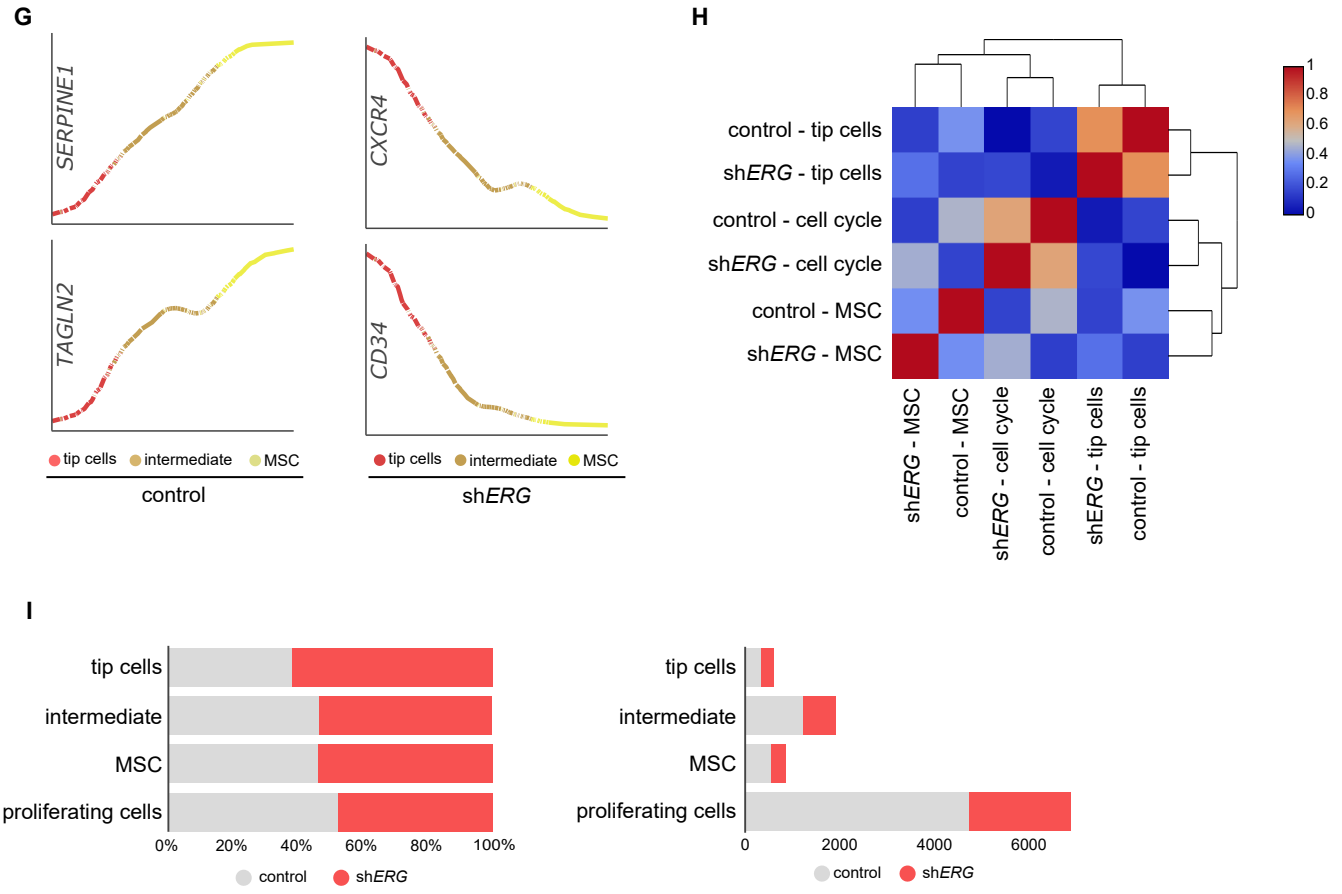
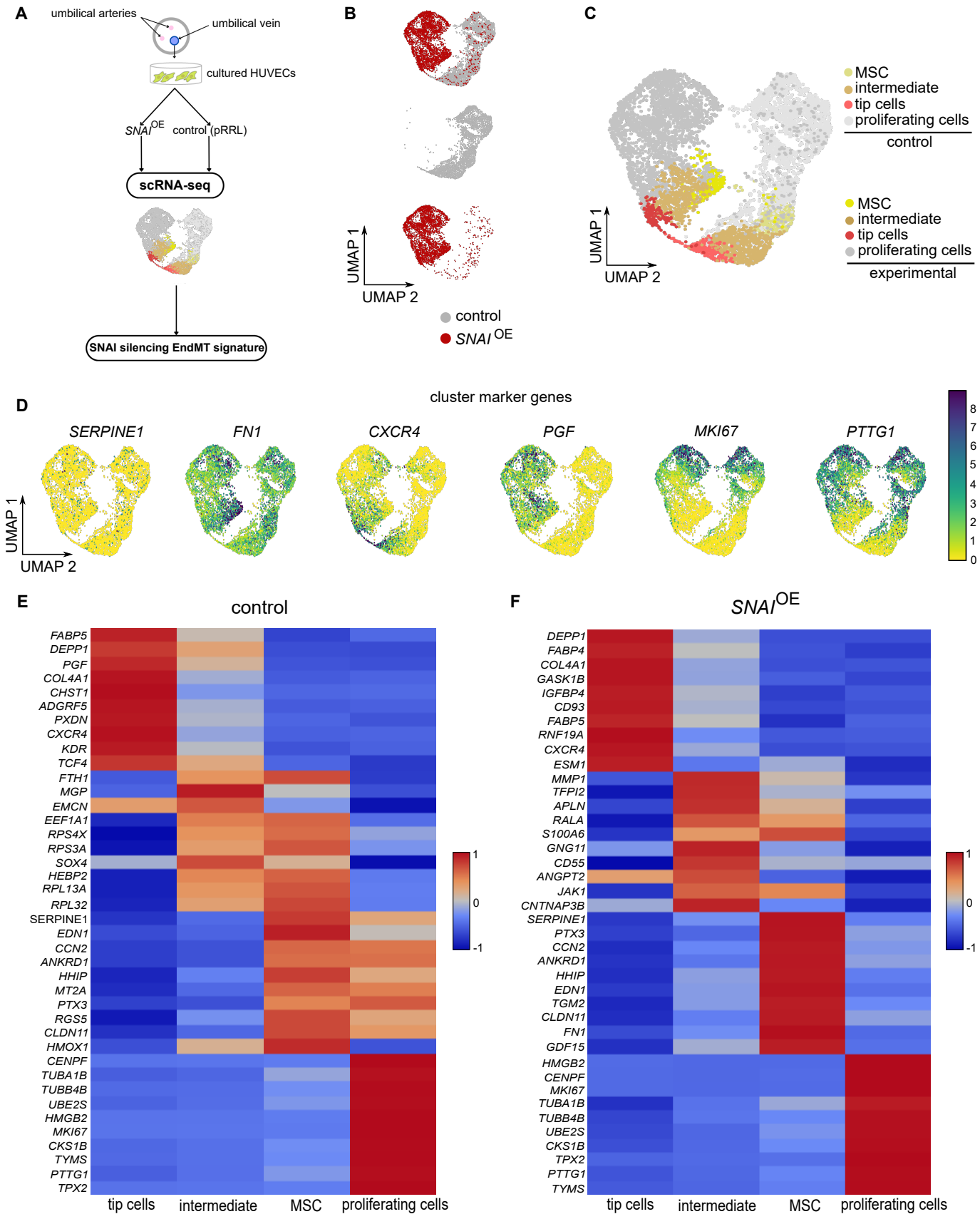


Fig. S2. scRNA-seq analysis for the shERG model. (**A**) Graphical representation of the experimental design for the *ERG* silencing model. (**B**) UMAP showing separation of cells based on condition. (**C**) UMAP analysis of 10,225 cultured HUVECs from the *ERG* silencing experiment. (**D**) UMAP plots color-coded for the indicated marker genes. (**E**) Heatmap analysis of top 10 uniquely upregulated cluster marker genes in control (pLKO-empty) and sh*ERG* (**F**). (**G**) Pseudotime analysis of canonical tip and MSC genes. (**H**) Dendrogram visualization of hierarchical clustering analysis on gene signature correlations of top 10 annotated clusters marker genes. Note: marker genes for the intermediate cluster were not calculated since it represents a transitional phase between tip cells and MSC. (**I**) Cell type quantification per annotated cluster and condition expressed in relative (top) and absolute (bottom) numbers.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umbilical arteries
umbilical vein
cultured HUVECs
SNAI OE
control (pRRL)
scRNA-seq
SNAI silencing EndMT signature
B
UMAP 1
UMAP 2
control
SNAI OE
C
MSC
intermediate
tip cells
proliferating cells
control
MSC
intermediate
tip cells
proliferating cells
experimental
UMAP 1
UMAP 2
D
cluster marker genes
SERPINE1
FN1
CXCR4
PGF
MKI67
PTTG1
UMAP 1
UMAP 2
8
7
6
5
4
3
2
1
0
E
control
FABP5
DEPP1
PGF
COL4A1
CHST1
ADGRF5
PXDN
CXCR4
KDR
TCF4
FTH1
MGP
EMCN
EEF1A1
RPS4X
RPS3A
SOX4
HEBP2
RPL13A
RPL32
SERPINE1
EDN1
CCN2
ANKRD1
HHIP
MT2A
PTX3
RGS5
CLDN11
HMOX1
CENPF
TUBA1B
TUBB4B
UBE2S
HMGB2
MKI67
CKS1B
TYMS
PTTG1
TPX2
1
0
-1
tip cells
intermediate
MSC
proliferating cells
F
SNAI OE
DEPP1
FABP4
COL4A1
GASK1B
IGFBP4
CD93
FABP5
RNF19A
CXCR4
ESM1
MMP1
TFPI2
APLN
RALA
S100A6
GNG11
CD55
ANGPT2
JAK1
CNTNAP3B
SERPINE1
PTX3
CCN2
ANKRD1
HHIP
EDN1
TGM2
CLDN11
FN1
GDF15
HMGB2
CENPF
MKI67
TUBA1B
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tip cells
intermediate
MSC
proliferating cells

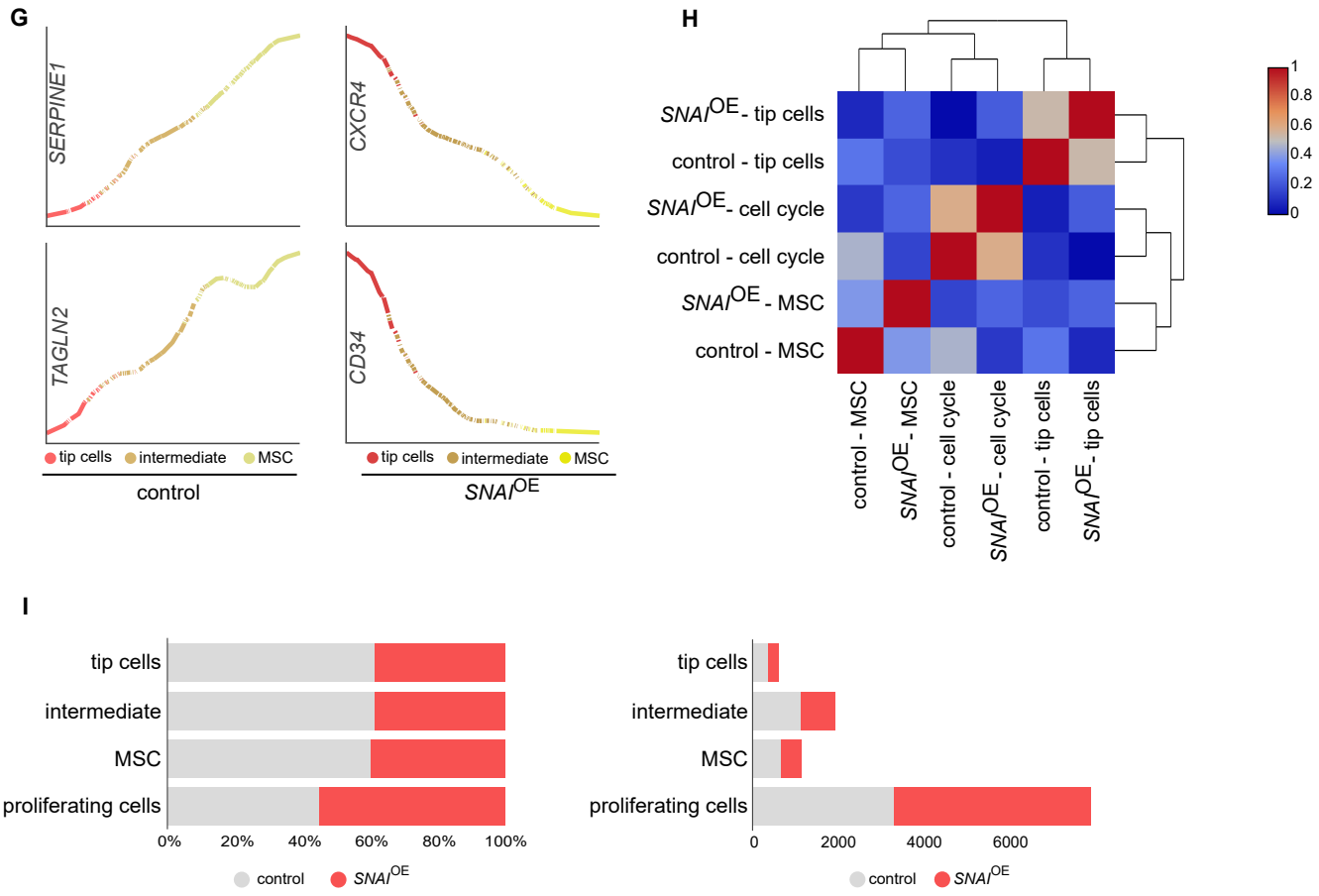
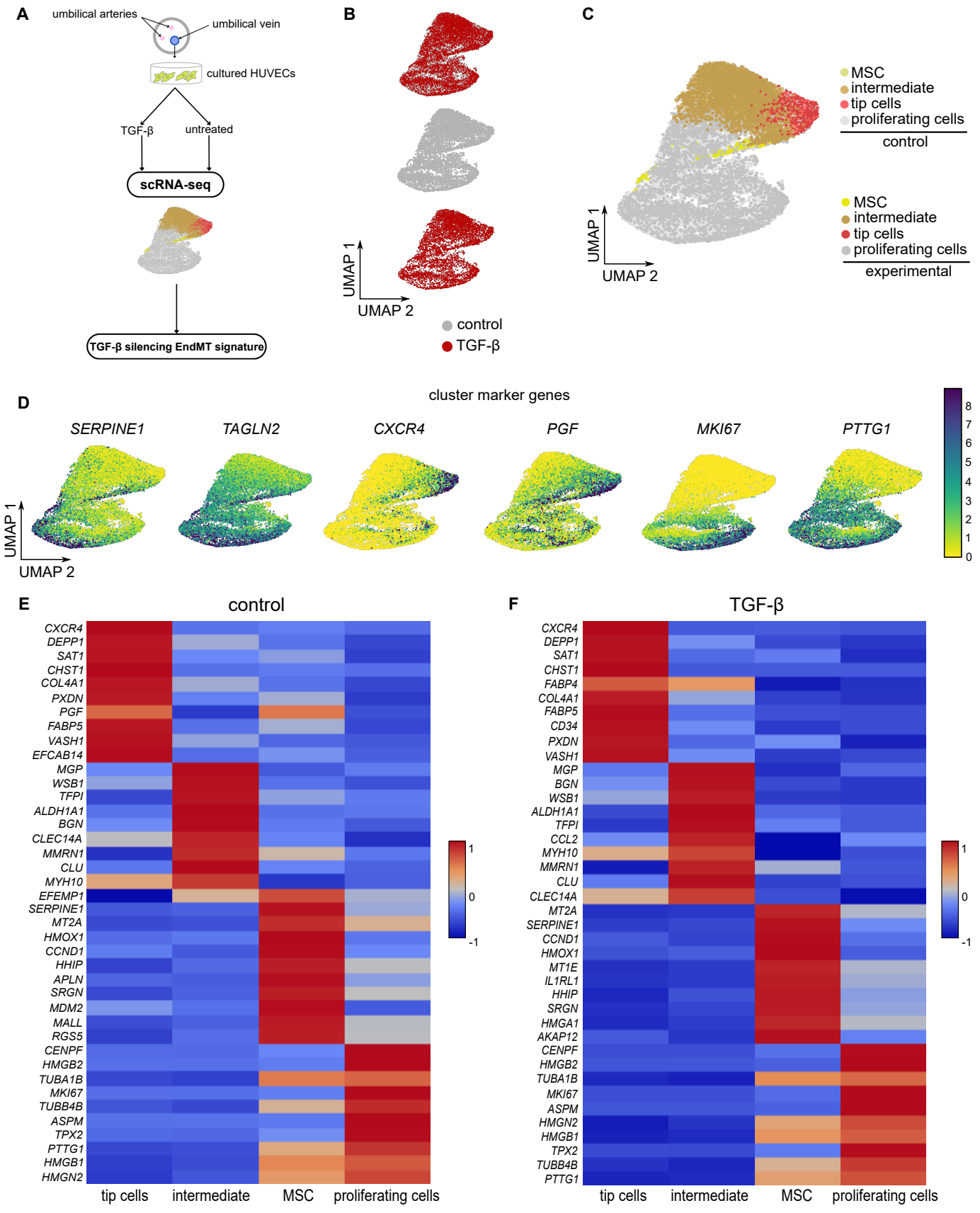


Fig. S3. scRNA-seq analysis for the SNAI^{OE} model. (**A**) Graphical representation of the experimental design for the *SNAI* overexpression model. (**B**) UMAP showing separation of cells based on condition. (**C**) UMAP analysis of 7,298 cultured HUVECs from the *SNAI* overexpression experiment. (**D**) UMAP plots color-coded for the indicated marker genes. (**E**) Heatmap analysis of top 10 uniquely upregulated cluster marker genes in control (pRRL-empty) and *SNAI*^{OE} (**F**). (**G**) Pseudotime analysis of canonical tip and MSC genes. (**H**) Dendrogram visualization of hierarchical clustering analysis on gene signature correlations of top 10 annotated clusters marker genes. Note: marker genes for the intermediate cluster were not calculated since it represents a transitional phase between tip cells and MSC. (**I**) Cell type quantification per annotated cluster and condition expressed in relative (top) and absolute (bottom) numbers.


A
umbilical arteries
umbilical vein
cultured HUVECs
TGF-β
untreated
scRNA-seq
TGF-β silencing EndMT signature
B
UMAP 1
UMAP 2
control
TGF-β
C
MSC
intermediate
tip cells
proliferating cells
control
MSC
intermediate
tip cells
proliferating cells
experimental
UMAP 1
UMAP 2
D
cluster marker genes
SERPINE1
TAGLN2
CXCR4
PGF
MKI67
PTTG1
UMAP 1
UMAP 2
8
7
6
5
4
3
2
1
0
E
control
CXCR4
DEPP1
SAT1
CHST1
COL4A1
PXDN
PGF
FABP5
VASH1
EFCAB14
MGP
WSB1
TFPI
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BGN
CLEC14A
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MYH10
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SERPINE1
MT2A
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CCND1
HHIP
APLN
SRGN
MDM2
MALL
RGS5
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TUBB4B
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HMGN2
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tip cells
intermediate
MSC
proliferating cells
F
TGF-β
CXCR4
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SAT1
CHST1
FABP4
COL4A1
FABP5
CD34
PXDN
VASH1
MGP
BGN
WSB1
ALDH1A1
TFPI
CCL2
MYH10
MMRN1
CLU
CLEC14A
MT2A
SERPINE1
CCND1
HMOX1
MT1E
IL1RL1
HHIP
SRGN
HMGA1
AKAP12
CENPF
HMGB2
TUBA1B
MKI67
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proliferating cells

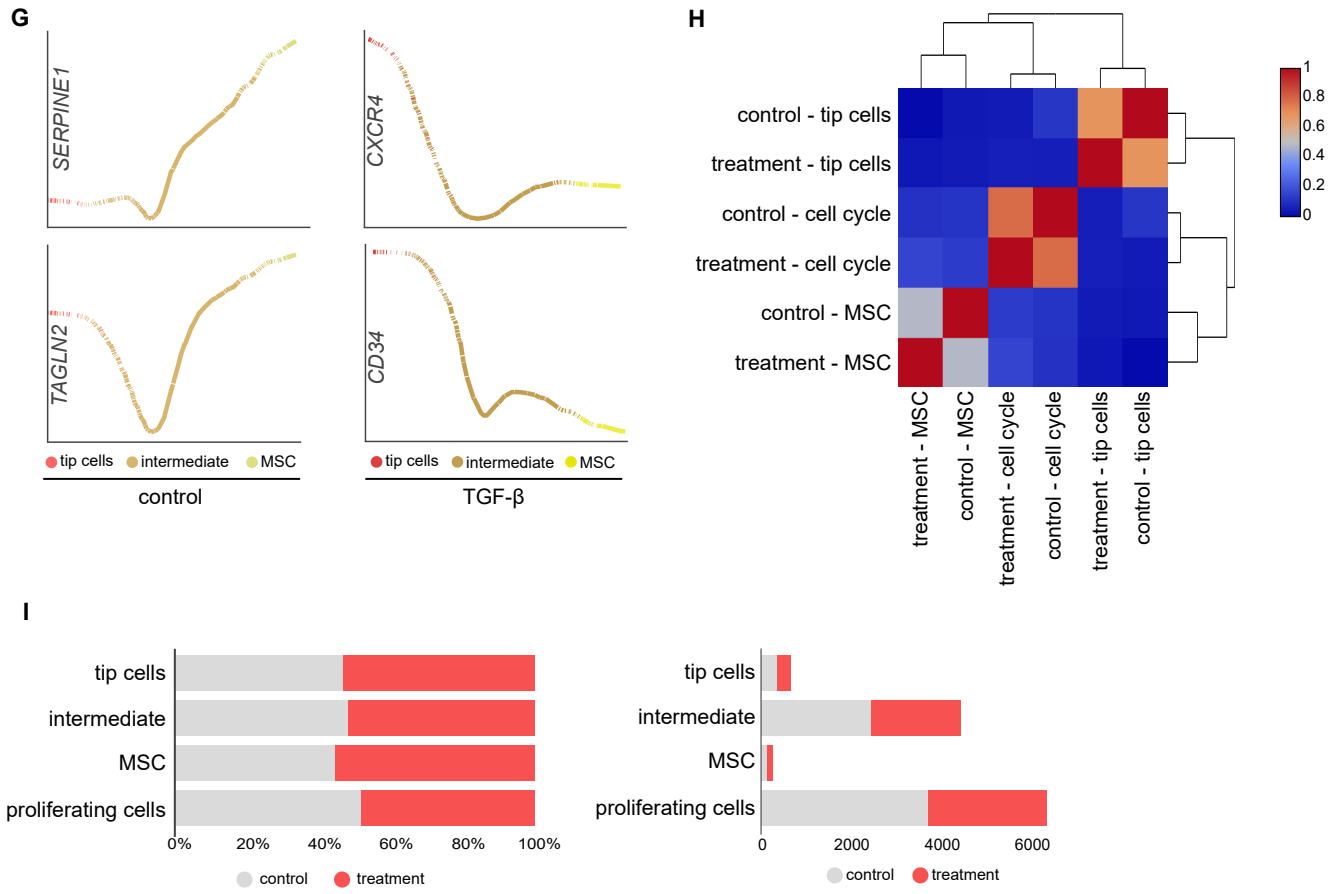
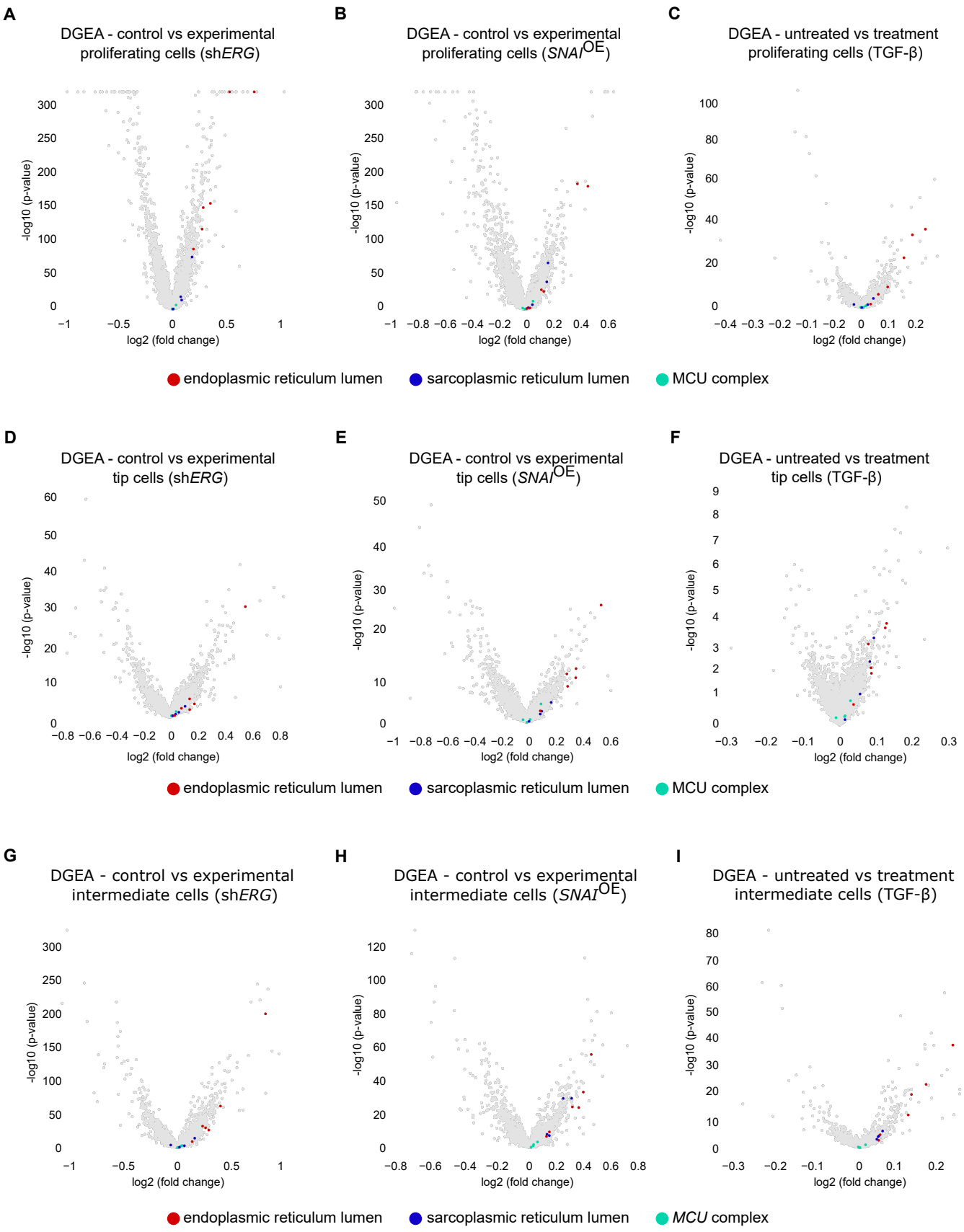


Fig. S4. scRNA-seq analysis for the TGF-β model. (**A**) Graphical representation of the experimental design for the TGF-β model. (**B**) UMAP showing separation of cells based on condition. (**C**) UMAP analysis of 14,602 cultured HUVECs from the TGF-β experiment. (**D**) UMAP plots color-coded for the indicated marker genes. (E) Heatmap analysis of top 10 uniquely upregulated cluster marker genes in control (untreated) and TGF-β (**F**). (**G**) Pseudotime analysis of canonical tip and MSC genes. (**H**) Dendrogram visualization of hierarchical clustering analysis on gene signature correlations of top 10 annotated clusters marker genes. Note: marker genes for the intermediate cluster were not calculated since it represents a transitional phase between tip cells and MSC. (**I**) Cell type quantification per annotated cluster and condition expressed in relative (top) and absolute (bottom) numbers.


A
DGEA - control vs experimental proliferating cells (shERG)
B
DGEA - control vs experimental proliferating cells (SNAIOE)
C
DGEA - untreated vs treatment proliferating cells (TGF-β)
D
DGEA - control vs experimental tip cells (shERG)
E
DGEA - control vs experimental tip cells (SNAIOE)
F
DGEA - untreated vs treatment tip cells (TGF-β)
G
DGEA - control vs experimental intermediate cells (shERG)
H
DGEA - control vs experimental intermediate cells (SNAIOE)
I
DGEA - untreated vs treatment intermediate cells (TGF-β)
-log10 (p-value)
log2 (fold change)
endoplasmic reticulum lumen
sarcoplasmic reticulum lumen
MCU complex
MCU complex

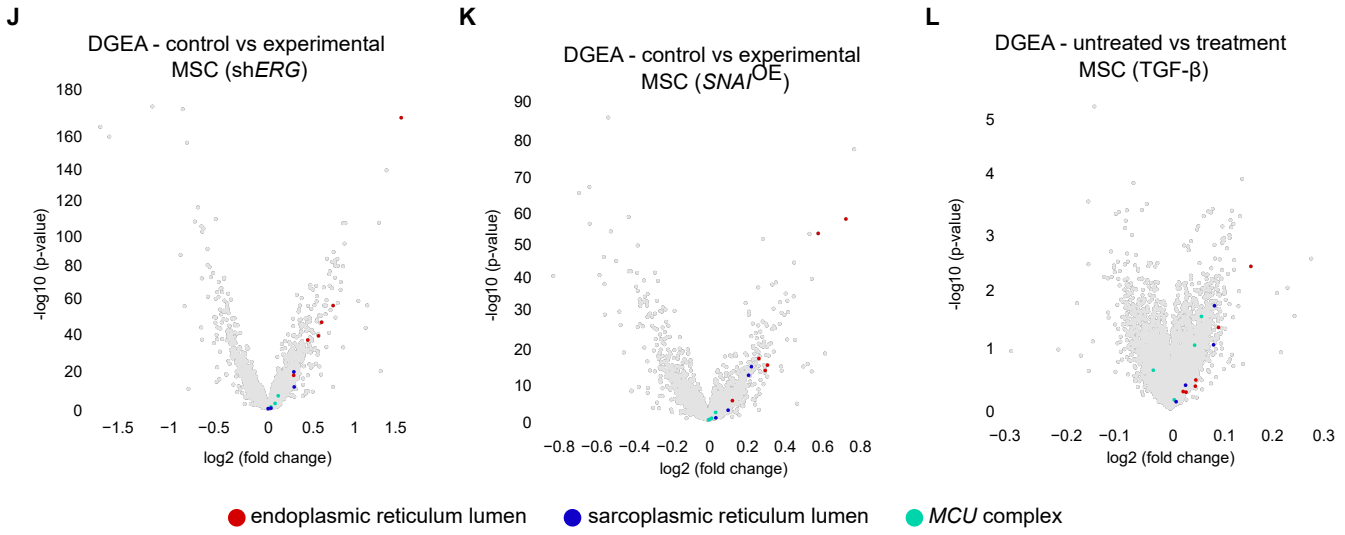
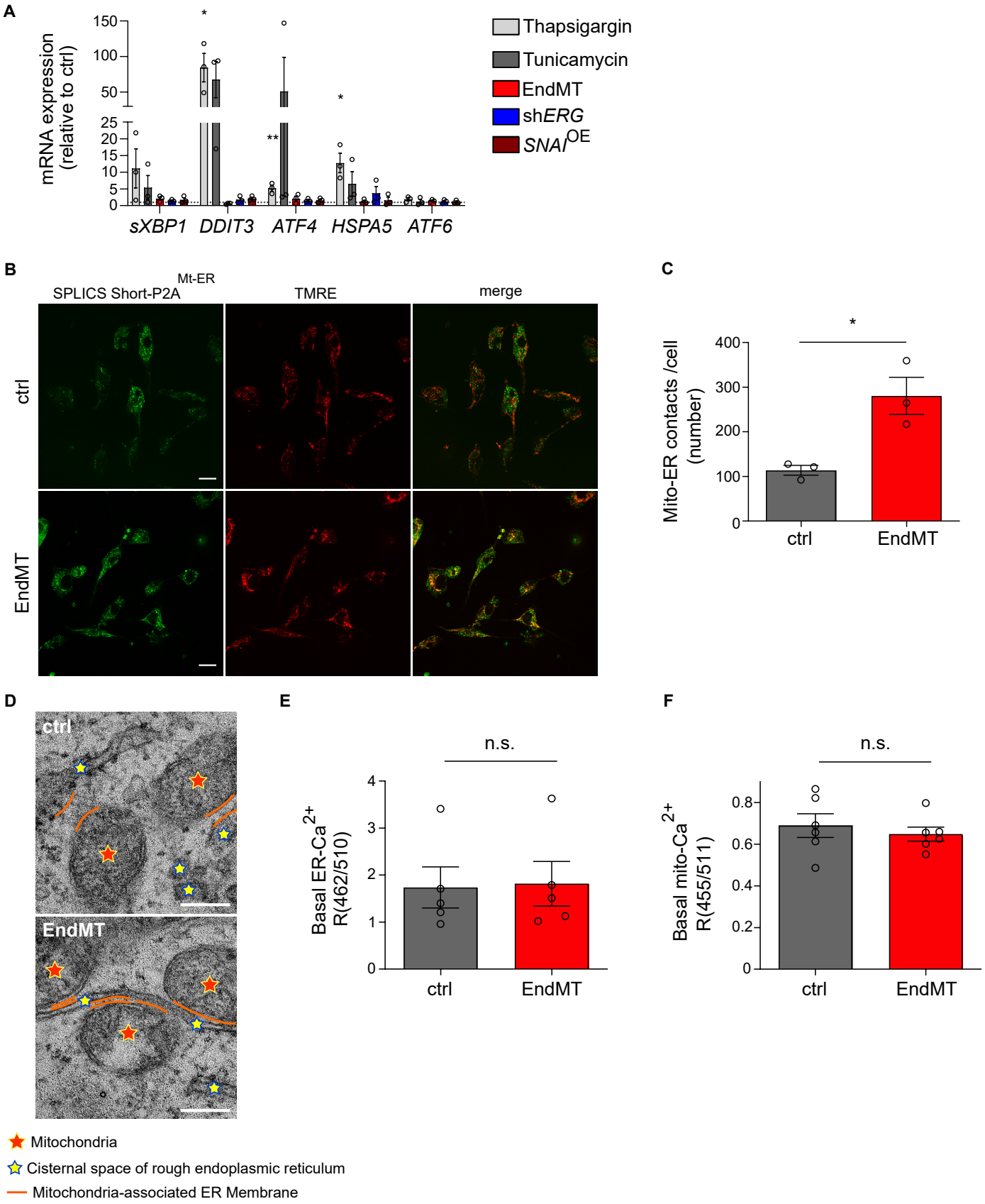


Fig. S5. Comparison between control and experimental condition (EndMT induction) on different levels. (**A**) Volcano plot of a differential analysis of control vs experimental condition for proliferating cells in sh*ERG* (**A**), *SNAI*^{OE} (**B**) and TGF-β treatment (**C**). Volcano plot of a differential analysis of control vs experimental condition for tip cells in sh*ERG* (**D**), *SNAI*^{OE} (**E**) and TGF-β treatment (**F**). Volcano plot of a differential analysis of control vs experimental condition for intermediate state in sh*ERG* (**G**), *SNAI*^{OE} (**H**) and TGF-β treatment (**I**). Volcano plot of a differential analysis of control vs experimental condition for MSC in sh*ERG* (**J**), *SNAI*^{OE} (**K**) and TGF-β treatment (**I**). The endoplasmic reticulum lumen genes are in red outline; the sarcoplasmic reticulum lumen genes are highlighted in blue; *MCU* complex genes are highlighted in green.


A
mRNA expression (relative to ctrl)
150
100
50
25
20
15
10
5
0
*
**
*
sXBP1
DDIT3
ATF4
HSPA5
ATF6
Thapsigargin
Tunicamycin
EndMT
shERG
SNAI OE
B
Mt-ER
SPLICS Short-P2A
TMRE
merge
ctrl
EndMT
C
Mito-ER contacts /cell (number)
400
300
200
100
0
*
ctrl
EndMT
D
ctrl
EndMT
Mitochondria
Cisternal space of rough endoplasmic reticulum
Mitochondria-associated ER Membrane
E
Basal ER-Ca2+ R(462/510)
4
3
2
1
0
n.s.
ctrl
EndMT
F
Basal mito-Ca2+ R(455/511)
0.8
0.6
0.4
0.2
0
n.s.
ctrl
EndMT

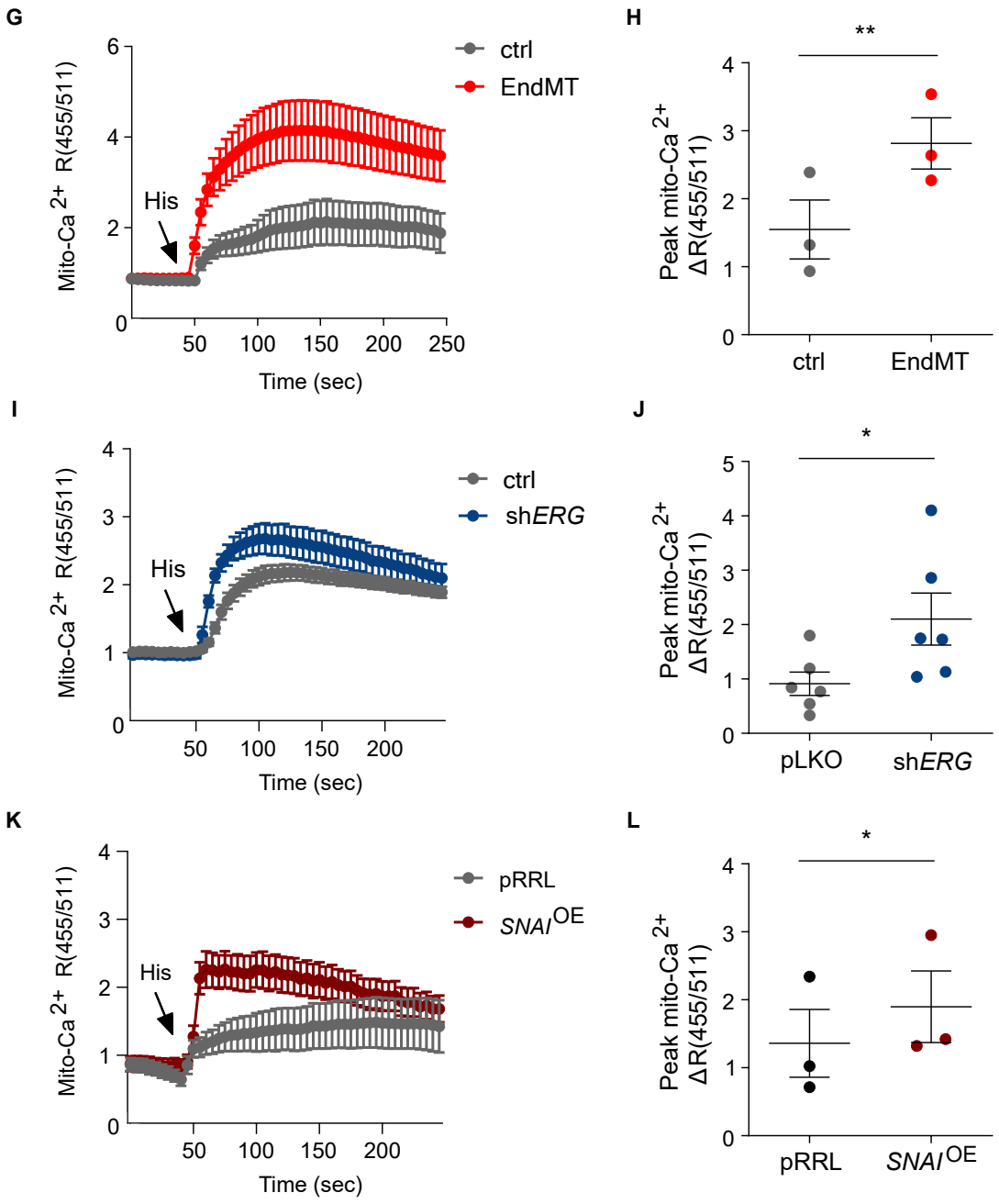
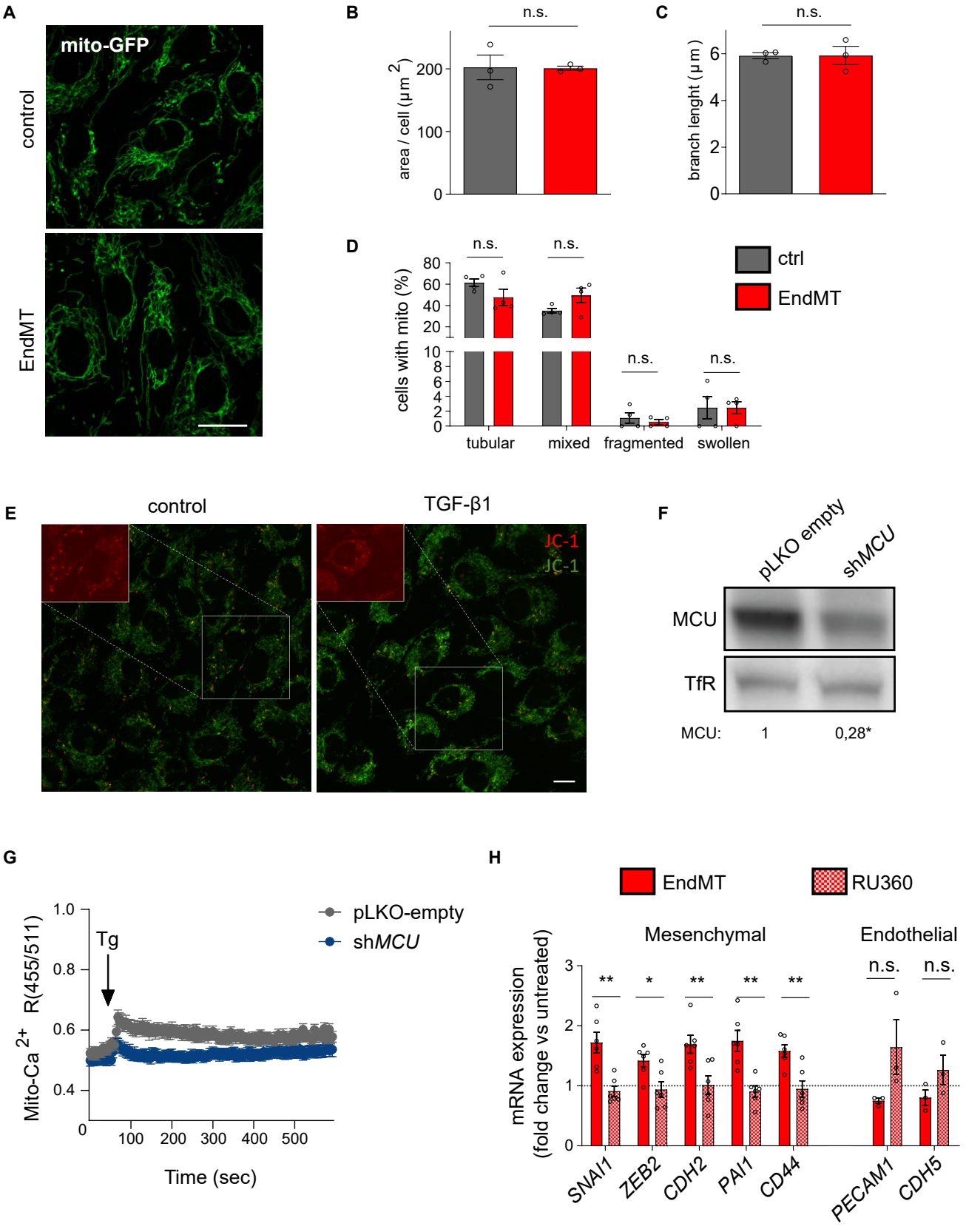


Fig. S6. EndMT is associated with increased mitochondrial Ca^{2+} uptake. (**A**) RT-PCR analysis of mRNA expression levels of ER stress genes in the three EndMT models. Thapsigargin (10 μM) and tunicamycin (5 μg/ml) were used as ER stressors over 4 hours (positive controls). Expression values were normalized to 18S mRNA levels and they are relative to control (for EndMT models) or untreated (for positive controls) (N= 3). Dotted line: expression levels in control ECs. *P value < 0.05, **P < 0.01. (**B**) Representative images of mitochondrial-ER contact sites in control and EndMT-derived cells (TGF-β1 stimulation) expressing the SPLICSS probe (SPLICS Short-P2A$^{Mt\text{-}ER}$ in green). Mitochondria were visualized by TMRE (red). (**C**) Quantification of B. The SPLICS dots were quantified from the 3D rendering of a complete z-stack (N= 3). *P value < 0.05. Scale bar 20 μm. (**D**) Electron micrographs of control and EndMT-derived cells (TGF-β1 stimulation) showing mitochondrial-associated ER membranes (orange lines). Red stars represent mitochondria, yellow stars cisternal space of rough ER. Scale bar, 200 nm. Quantification of basal ER-Ca^{2+} (**E**) and mito-Ca^{2+} (**F**) levels in control and EndMT-derived cells (TGF-β1 stimulation) transduced with the ER-targeted or mitochondria-targeted ratiometric Ca^{2+} sensors GEM-Cepia1er and GEM-Geco1mito, respectively and maintained in 0 mM extracellular Ca^{2+} solution (N= 5 for ER-Ca^{2+}; n=6 for mito-Ca^{2+}). n.s.: no significance. (**G-L**) Imaging traces and quantification of mito-Ca^{2+} levels in response to histamine (His, 50 μM) in control and EndMT-derived cells in 2 mM extracellular Ca^{2+} solution in the three models. Traces (**G-I-K**) represent the mean Geco ratio (455/511 nm) ± SEM of different cells from one representative experiment. The scatter dot plots (H-J-L) show the mean peak amplitude ± SEM of His-mediated mitochondrial Ca^{2+} uptake relative to three (TGF-β and *SNAI*^{OE}) or six (sh*ERG*) independent experiments. *P value < 0.05, **P value < 0.01.


A
mito-GFP
control
EndMT
B
n.s.
area / cell (µm 2)
200
100
0
C
n.s.
branch lenght (µm)
6
4
2
0
D
n.s.
n.s.
n.s.
n.s.
cells with mito (%)
80
60
40
20
10
8
6
4
2
0
ctrl
EndMT
tubular
mixed
fragmented
swollen
E
control
TGF-β1
JC-1
JC-1
F
pLKO empty
shMCU
MCU
TfR
MCU: 1 0,28*
G
pLKO-empty
shMCU
Tg
Mito-Ca 2+ R(455/511)
1.0
0.8
0.6
0.4
0
100
200
300
400
500
Time (sec)
H
EndMT
RU360
Mesenchymal
Endothelial
mRNA expression (fold change vs untreated)
3
2
1
0
**
*
**
**
**
n.s.
n.s.
SNAI1
ZEB2
CDH2
PAI1
CD44
PECAM1
CDH5

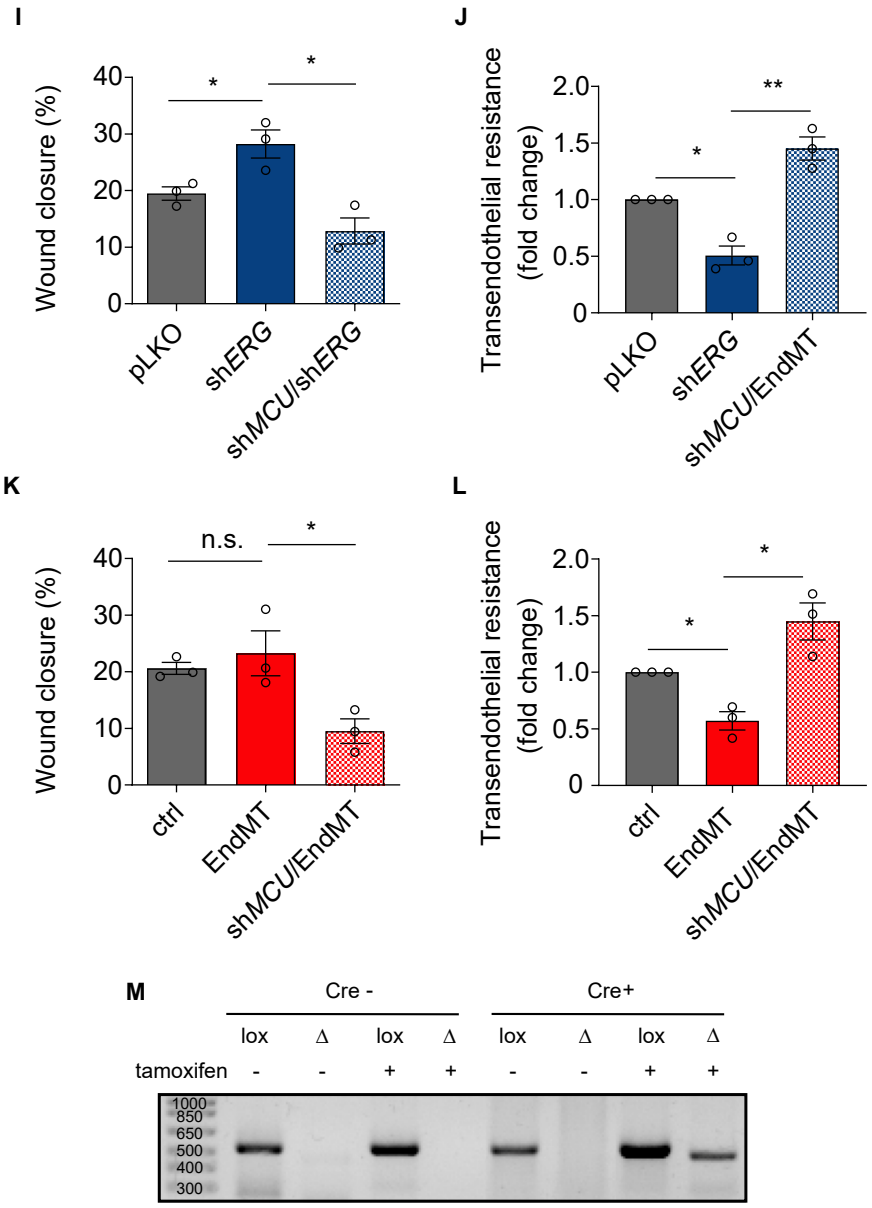


Fig. S7. MCU overexpression regulates EndMT. (**A-D**) Representative images and relative quantification of mitochondrial morphology in control and EndMT-derived cells (TGF-β1 stimulation) transduced with a mito-GFP reporter (N= 3-4). n.s.: no significance. Scale bar, 20 μm. (**E**) Representative images of control (ctrl) and TGF-β1-treated ECs stained for the JC-1 dye. Red fluorescence emission of the dye is indicative of potential-dependent accumulation of JC-1 in the mitochondria (associated with a fluorescence emission shift from green (520-550 nm) to red (579-633 nm)). Insets in the single channel JC-1 red panels show larger magnifications of the boxed areas to visualize presence of red aggregates. Scale bar, 10 μm. (F) Representative expression levels and relative quantification of immunoblot for MCU in control ECs (pLKO-empty) and ECs transduced with a shRNA targeting MCU (sh*MCU1*). Transferrin receptor (TfR) was used as loading control (N= 3). *P value < 0.05. (**G**) Mito-Ca^{2+} imaging traces in response to thapsigargin (Tg, 2 μM) in control cells (black trace) and in cells silenced for *MCU* (sh*MCU1*/sh*MCU2*, blue trace) maintained in 0 mM extracellular Ca^{2+} solution. Traces on the left represent the mean Geco ratio (455/511 nm) ± SEM of different cells from one representative experiment of N= 6. (**H**) RT-PCR analysis of mRNA expression levels of mesenchymal and endothelial genes in untreated vs ECs treated with RU360 (1 μM) prior EndMT induction by TGF-β1 stimulation. Expression values were normalized to 18S mRNA levels (N= 3-6). Dotted line: expression levels in untreated EndMT. *P value < 0.05, **P < 0.01, n.s.: no significance. (**I**) Quantification of scratch wound assay using mitomycin C-treated control (pLKO-empty) and sh*ERG* (N= 3). *P < 0.05. (**J**) Transendothelial electrical resistance of control (pLKO-empty) and sh*ERG* monolayers (N= 3). *P value < 0.05, **P < 0.01. (**K**) Quantification of scratch wound assay using mitomycin C-treated control (ctrl) and EndMT-derived cells (TGF-β1 stimulation) (N= 3). *P < 0.05, n.s.: no significance. (**L**) Transendothelial electrical resistance of control (ctrl) and EndMT-derived cells (TGF-β1 stimulation) (N= 3). *P < 0.05. (**M**) Correct recombination of the lox allele in WT (Cre-) and *Snai1*^{ECKO} (Cre+) mice upon tamoxifen (Tam) treatment, as assessed by genomic DNA PCR. PCR to amplify the loxP-flanked *Snai1* allele (lox) or the Cre-recombined allele (Δ) were run and loaded in separate reactions. The gel picture shown is representative of all WT and *Snai1*^{ECKO} mice used in this study.

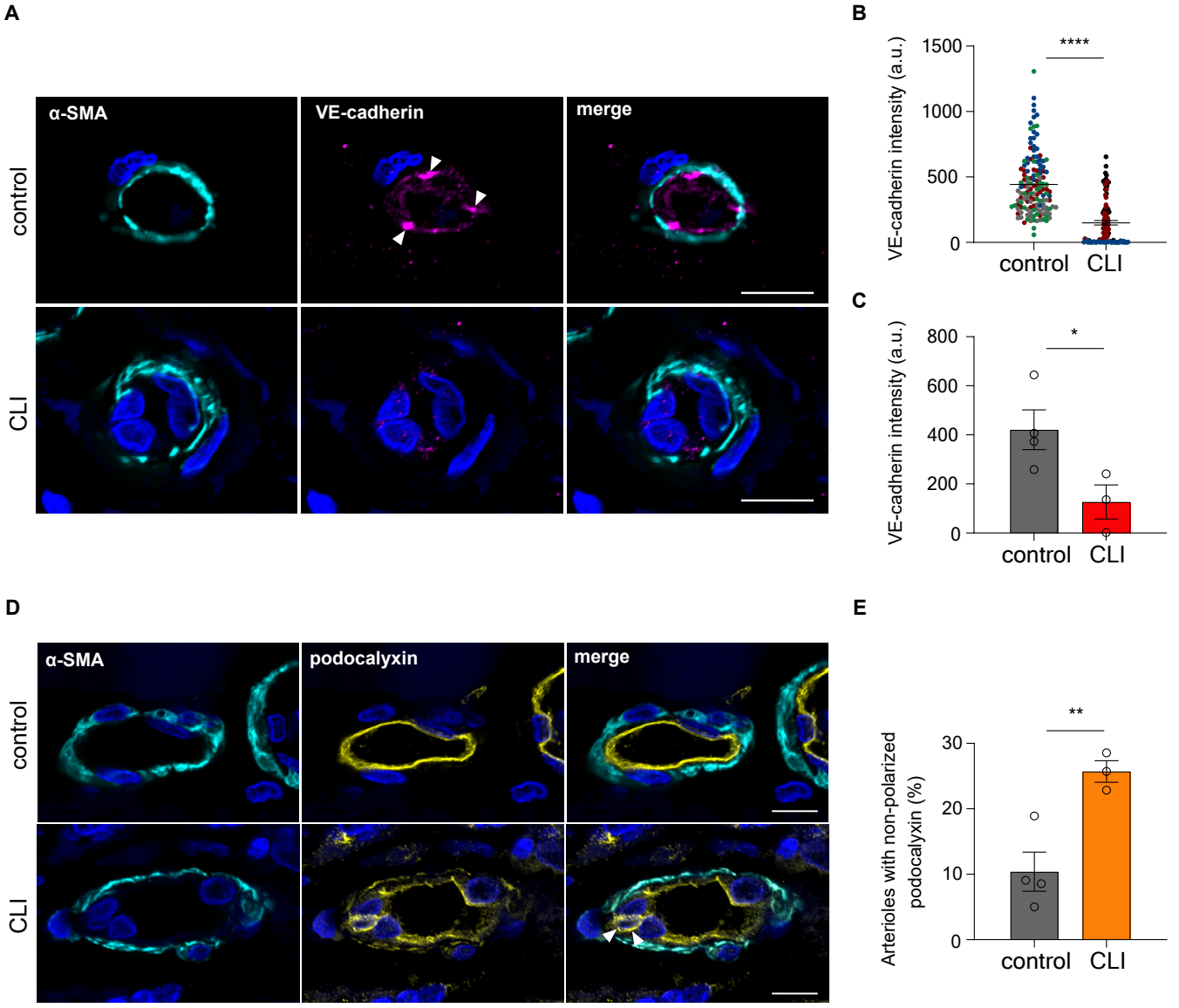


Fig. S8. ECs in CLI muscle display EndMT features. (**A**) Confocal micrographs in human skeletal muscle from control individuals and individuals with critical limb ischemia (CLI) stained for mural cells (α-SMA, cyan), VE-cadherin (magenta), and nuclei (DAPI, blue). Scale bars, 10 μm. Control arterioles show junctional enrichment of VE-cadherin (arrows), whereas CLI arterioles show weak and diffuse VE-cadherin signals. (**B**) Quantification of VE-cadherin in individual arterioles (median intensity and interquartile range, 140 and 105 arterioles, respectively) and per subject (**C**) (Mean ± SEM). *P < 0.05, ****P < 0.0001. (**D**) Confocal micrographs of small arterioles stained for mural cells (α-SMA, green), podocalyxin (yellow), and nuclei (DAPI, blue). Scale bars, 10 μm. CLI arterioles show ECs with variably located podocalyxin including at lateral and basal locations (arrows). (**E**) Quantification of A depicting the percent of arterioles per subject with non-polarized podocalyxin (N= 4 for control and N= 3 for CLI patients). Data are mean ± SEM. **P < 0.01.

Supplementary tables

Table S1. scRNA-seq quality control metrics.

Table S2 (related to Fig. 1B and Figures S2A-D, S3A-D and Fig. S4A-D). Uniquely upregulated cluster marker genes characterizing the four different EC subpopulations.

Table S3 (related to Fig. 1D-F). Congruent genes in the top 100 marker genes of proliferating, tip and MSC.

Table S4 (related to Fig. S5). Differential gene expression analyses comparing cell clusters in control *vs* experimental conditions. Gene lists are ranked according to log2 fold change and used as input for the cell cluster-independent meta-analysis.

Table S5 (related to Fig. 3A). Congruently upregulated genes in EndMT induction independent of the cell cluster.

Table S6 (related to Fig. 3B). Cell cluster-independent EndMT meta-analysis.

Table S7 (related to Fig. 4A-C). Results of GOrilla cellular component GO analysis.