Supplemental Material

Generation of Quiescent Cardiac Fibroblasts from Human Induced Pluripotent Stem Cells for *in vitro* Modeling of Cardiac Fibrosis

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SUPPLEMENTAL MATERIALS AND METHODS

Human primary skin, cardiac, lung, bladder fibroblast, and brain pericyte cultures. Human primary cardiac fibroblasts were isolated and cultured as follows: a piece of fresh right atrial biopsy was weighed, minced into small pieces (1-2 mm^3), and digested in 6-well peri-dishes as described[1]. Human primary cardiac fibroblasts were grown and maintained in a fibroblast growth medium (C-23025, Promocell) supplemented with 1% penicillin-streptomycin (15140-122, Gibco) in a humidified atmosphere (95% air, 5% CO_2) at 37 °C. The medium was renewed every 2–3 days. Cells were passaged using standard trypsinization techniques at 80–90% confluency. The same protocol was also used to isolate and culture fibroblasts from human skin and lung tissues. Primary bladder fibroblasts were purchased from Lifeline (FC-0050) and maintained in the same fibroblast growth medium. Human brain pericytes were purchased from Cell systems (ACBRI 498) and cultured in SmGM-2 medium (Lonza, CC-3182). All experiments were carried out using cells at low cell passages (<P4) and cells were serum-starved for 24 hrs before treatment.

Quantitative real-time PCR. Total RNA was isolated using the Qiagen miRNeasy Mini Kit (Qiagen Sciences, Inc, Germantown, MD) following the manufacturer's instruction. For reverse transcription, a High Capacity RNA-to-cDNA kit (Life Technologies) was used, and the cDNA template was synthesized based on 1 μg of total mRNA. After mixing 0.2 μl of the cDNA template, 1 μl of TaqMan® primer sets (Life Technologies), 10 μl of TaqMan® Master Mix (Life Technologies), and 8.8 μl of ddH_2O in the reaction system, real-time quantitative PCR was performed on the StepOne™ Real-Time PCR System (Life Technologies). Each reaction was run in triplicate. Expression values were normalized to the average expression of *GAPDH*. Taqman

assay IDs were listed in Online Table IV.

Immunoblotting and quantification. Cultured cells were homogenized in the RIPA buffer with proteinase and phosphatase inhibitor cocktails (78440, ThermoFisher), and quantified by Pierce™ BCA Protein Assay Kit (Pierce Biotechnology Inc.). A total of 20 μg protein was separated on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (Invitrogen, Carlsbad, CA). After being transferred to Amersham™ Hybond™ Blotting Membranes (GE Healthcare), proteins were incubated with primary antibodies against α-SMA (ab7817, Abcam), periostin (ab14041, Abcam), collagen III (ab7778, Abcam), fibronectin (ab6328, Abcam), MYH7 (sc-53089, Santa Cruz), and GAPDH (MA5-15738, Invitrogen). Band intensities for target bands were analyzed and quantified by the ImageJ Fuji program.

Immunofluorescent staining. Cells were fixed using 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS (Sigma-Aldrich) for 10 min, and blocked with 3% bovine serum albumin (BSA). Cells were incubated overnight at 4 °C with primary antibody or isotype controls diluted in 3% BSA. Cells were incubated with Alexa Fluor™ secondary antibodies (1:500 dilution) at room temperature for 1 hr, and mounted with mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Primary antibodies against NANOG2 (ab109250, Abcam), SSEA-4 (ab16287, Abcam), NKX2.5 (MAB2444, R&D), WT1 (ab89901, Abcam), ZO1 (40-2200, Invitrogen), GATA4 (sc-25310, Santa Cruz), DDR2 (sc-81707, Santa Cruz), COLI (ab90395, Abcam), VIM (V6630, Sigma-Aldrich), POSTN (ab14041, Abcam), TBX20 (hpa008192, Sigma-Aldrich), TCF21 (hpa013189, Sigma-Aldrich), α-SMA (ab7817,

Abcam), CNN1 (ab46794, Abcam), SMTN (ab204305, Abcam), and TAGLN (ab14106, Abcam) were used. Labeled cells were imaged by a confocal microscope (Carl Zeiss, LSM 510 Meta, Göttingen, Germany).

Fluorescence-activated cell sorting (FACS) analysis. Cells were dissociated into singlets by TrypLE™ (12605, Gibco,) for 10 min at 37 °C, and the cell suspension was filtered through a 40-μm cell strainer (BD Falcon, San Diego) to remove cell clumps. Single cells were fixed and permeabilized by BD Cytofix/Cytoperm™ buffer (BD 554722) for 20 min at 4 °C. Next, cells were incubated with primary antibodies followed by Alexa Fluor™ secondary antibodies (Invitrogen). Primary antibodies against COLI (ab90395, Abcam), DDR2 (sc-81707, Santa Cruz), TCF21 (hpa013189, Sigma-Aldrich), α-SMA (ab7817, Abcam), and WT1 (ab89901, Abcam) were used. The percentage of fibroblasts was calculated as the ratio of $TCF21^+$, $DDR2^+$, and $COL\text{-}I^+$ cells in the whole population, respectively. The percentage of myofibroblasts was calculated as the ratio of $\alpha\text{-}SMA^+$ cells in the whole population.

Differentiation of human induced pluripotent stem cell-derived cardiomyocytes. All of the protocols for this study were approved by the Stanford University Human Subjects Research Institutional Review Board (IRB). Human induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) differentiation protocol was used as previously described.[2] Briefly, human iPSC lines were maintained on Matrigel-coated plates (CB-40234, BD Biosciences, San Jose, CA) in Essential 8™ Medium (A1517001, Gibco®, Life Technologies). Human iPSCs were treated with 6 μM of CHIR99021 (S2924, Selleck chemicals) for 2 days, recovered in RPMI medium (11875-

119, Gibco®, Life Technologies) with B27 supplements minus insulin (A1895601, Gibco®, Life Technologies) (RPMI+B27-insulin) for 24 hrs, treated with 5 μM of IWR-1 (I0161, Sigma) for 2 days, and then placed in RPMI+B27-insulin for another 2 days, and finally switched to RPMI medium with B27 supplements (17504044, Gibco®, Life Technologies). Beating cells were observed at days 9-11 after differentiation. Human iPSC-CMs were re-plated and purified with the glucose-free RPMI medium (11879020, Gibco®, Life Technologies) for 2-3 rounds. Typically, the purity of human iPSC-CMs ($TNNT2^+$) is >90% after purification. Cultures were maintained in a humidified atmosphere (95% air, 5% CO_2) at 37 °C.

Differentiation of human iPSC-derived cardiac fibroblasts. Human iPSCs were treated with 6 μM of CHIR99021 for 2 days, recovered in RPMI+B27-insulin for 24 hrs, treated with 5 μM of IWR-1 (I0161, Sigma) for 2 days, and then RPMI+B27-insulin for another 24 hrs. On day 6, human induced pluripotent stem cell-derived cardiac progenitor cells (iPSC-CPCs) were re-plated at a density of 20,000 cells/cm^2 in advanced DMEM medium (12634028, Gibco®, Life Technologies). On day 7 or 8, cells were treated with 5 μM of CHIR99021 and 2 μM of retinoic acid (R2625, Sigma-Aldrich) for 2 days, and recovered in advanced DMEM for 3 days. Typically, the purity of human induced pluripotent stem cell-derived epicardial cells (iPSC-EPCs) is > 90% ($WT1^+$). Human iPSC-EPCs were re-plated and treated with 10 μM of FGF2 (100-18B, PeproTech) and 10 μM of SB431542 (S1067, Selleck chemicals) in a fibroblast growth medium for another 6 days. >90% purity of iPSC-CFs ($COL\text{-}I^+/DDR2^+/TCF21^+$) can be achieved as assessed by FACS. Cultures were maintained in a humidified atmosphere (95% air, 5% CO_2) at 37 °C.

Differentiation of human iPSC-derived cardiac smooth muscle cells. Human iPSC-EPCs were plated in a chemically defined medium and treated with PDGF-BB (10 ng/ml, 100-14B, PeproTech) and TGFβ1 (2 ng/ml, 100-2, Peprotech) for 12 days.[3] Over 90% purity of human induced pluripotent stem cell-derived smooth muscle cells (iPSC-SMCs) ($SMTN^+$/$TAGLN^+$) can be achieved as assessed by immunofluorescent staining. Cultures were maintained in a humidified atmosphere (95% air, 5% CO_2) at 37 °C.

Drug treatment. Unless otherwise stated, cells were treated with all drugs for 48 hrs. We used 5 ng/ml of TGFβ1 (240-B-010, R&D) to activate iPSC-CFs. Due to the short half-life, BNP (CYT-369, Prospec) was added into the medium three times a day. We used 20 μM of sacubitril (SML1380, Sigma) to inhibit ANP/BNP degradation. We used pirfenidone (S2907, Selleck Chemicals) and doxorubicin (25316-40-9, Selleck Chemicals) to test their anti-fibrotic and pro-fibrotic effects on iPSC-CFs, respectively.

Indirect and direct contact co-culture. Transwells (353102, Corning) containing fibroblasts were suspended over the iPSC-CMs in the plastic bottom dish, so that the base of the Transwell sat within the culture medium on the iPSC-CMs but did not touch the base of the dish. As a control, iPSC-CMs were co-cultured with iPSC-CMs in the transwells. The co-cultures were incubated at 37 °C and 5% CO_2 before analysis. For direct contact co-culture, we first labeled fibroblasts and iPSC-CMs with CFSE (C34554, Thermo Fischer) and Far cell trace red (C34564, Thermofisher) dyes, respectively. Then, iPSC-CFs were seeded on top of the iPSC-CM monolayer at a ratio of 1:5, allowing for direct cell-cell contact. After 5 days of co-culture, the two cell types were

separated by FACS sorting for downstream molecular analyses. The medium used for co-culture is the mixture of RPMI+B27 and fibroblast growth medium at a ratio of 3:1.

Colorimetric assays. The cells were treated in the absence or presence of TGF-β for 48 hrs. Quantification of total secreted collagen in the cell culture supernatant was performed using a Sirius red collagen detection kit (9062, Chondrex). The colorimetric assay was performed according to the manufacturer's protocol.

Gel contraction assay. Collagen gel contraction assays were performed in three independent lines of iPSC-CFs (2.0×10^5 cells per well) using a Cell Contraction Assay kit (CBA5020, Cell Biolabs) as per the manufacturer's protocol. The cells were treated in the absence or presence of TGF-β ± a TGF-β inhibitor SB431542 (SB). The gels were imaged after 24 hrs and gel area was quantified using ImageJ software (version 1.49).

***In vitro* wound-healing assay.** The migration of human iPSC-CFs and primary CFs was determined using a Cytoselect™ 24-Well Wound Healing Assay Kit (CBA-120, Cell Biolabs). Briefly, after confluent monolayers of CFs were formed, cells were synchronized in a low serum medium (0.5% FBS) for 24 hrs. After the wound-generating scaffold was removed from each well, cells were washed in PBS to remove residual cells in the wounded area. Cells were treated with vehicle or PDGF-BB (25 ng/ml), and images were captured using a light microscope in identical areas at baseline, 12 hrs, and 24 hrs post-treatment. The cell-free areas at each time point were measured by Image-Pro Plus (version 6.0, Media Cybernetics). For quantification purposes, the

wound area generated at 0 hr was set as S_0, the uncovered areas captured at 12 hrs, and 24 hrs were recorded as S_i (i=12 or 24). The wound closure rate was then calculated by the following equation: wound closure rate (%)=$\frac{(S_0-S_i)}{S_0}\times 100$.

Bioinformatics analysis. We downloaded all single-cell RNA sequencing (scRNA-seq) data from the *Tabula Muris* database.[4] Specifically, Seurat objects were downloaded from Figshare (https://figshare.com/account/home#/projects/27733) and cell annotations were downloaded from the *Tabula Muris* Github website (https://github.com/czbiohub/tabula-muris). We filtered out cells with fewer than 500 genes expressed or 50,000 reads covered with "FilterCells" function. Next, raw counts data were normalized using "Normalize Data" function and further regressed against the number of total reads ("nRead") and mitochondrial proportion ("percent.mito"). The dimensional reduction was performed on genes with dispersion >0.5 using Principal Component Analysis (PCA) algorithm implemented in "RunPCA" function. We ran t-distributed stochastic neighbor embedding (t-SNE) algorithm using top 20 PCs for all FB and top 10 PCs for heat FB with "RunTSNE" function. We detected differentially expressed genes (DEGs) among organs and between sexes using the Wilcoxon rank sum test under adjusted *P*-value <0.01, fold-change ≥ 2, and >25% cells expressed in either group, and only positive markers were reported. All the above steps were performed with Seurat R package.[5] We performed pathway enrichment analysis on DEGs with a hypergeometric test using geneAnswers R package under FDR <0.05 and visualized the results using Cytoscape software v3.6.[6] The ligand-receptor analysis was performed following the steps previously[7] and visualized with Cytoscape software.

Statistical analyses. For statistical analyses, the Student's t-test was used to compare two normally distributed data sets. One-way or two-way ANOVA was used, where appropriate, to compare multiple data sets, and Bonferroni multiple comparison after-tests were used for all pairwise comparisons, depending on the properties of the data sets. A P value <0.05 was considered to be statistically significant. All data are shown as mean ± SEM.

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SUPPLEMENTAL TABLES

Online Table I. Specifically expressed genes in tissue-specific fibroblasts. Cut off: pct.1≥25%, FDR<1%; Wilcoxon rank sum test. p_val: p-value, avg_logFC: log-scaled fold change, pct.1: proportion of adipose fibroblasts with gene expression>0, pct.2: proportion of other fibroblasts with gene expression>0, p_val_adj: FDR adjusted p-value. (Supplemental Table 1.xlsx).

Online Table II. Specifically expressed transcription factors in tissue-specific fibroblasts. Cut off: pct.1≥25%, FDR<1%; Wilcoxon rank sum test. p_val: p-value, avg_logFC: log-scaled fold change, pct.1: proportion of adipose fibroblasts with gene expression>0, pct.2: proportion of other fibroblasts with gene expression>0, p_val_adj: FDR adjusted p-value. (Supplemental Table 1.xlsx).

Online Table III. Complementary ligand-receptor genes between fibroblasts and a diverse array of cell types. The threshold for defining a ligand/receptor expressed in a cluster is ≥ 25% with gene expression > 0. Clusters1: clusters express ligand, Clusters2: clusters express receptor. (Supplemental Table 3.xlsx).

Online Table IV. Human primers/probe and assay IDs for TaqMan real-time PCR

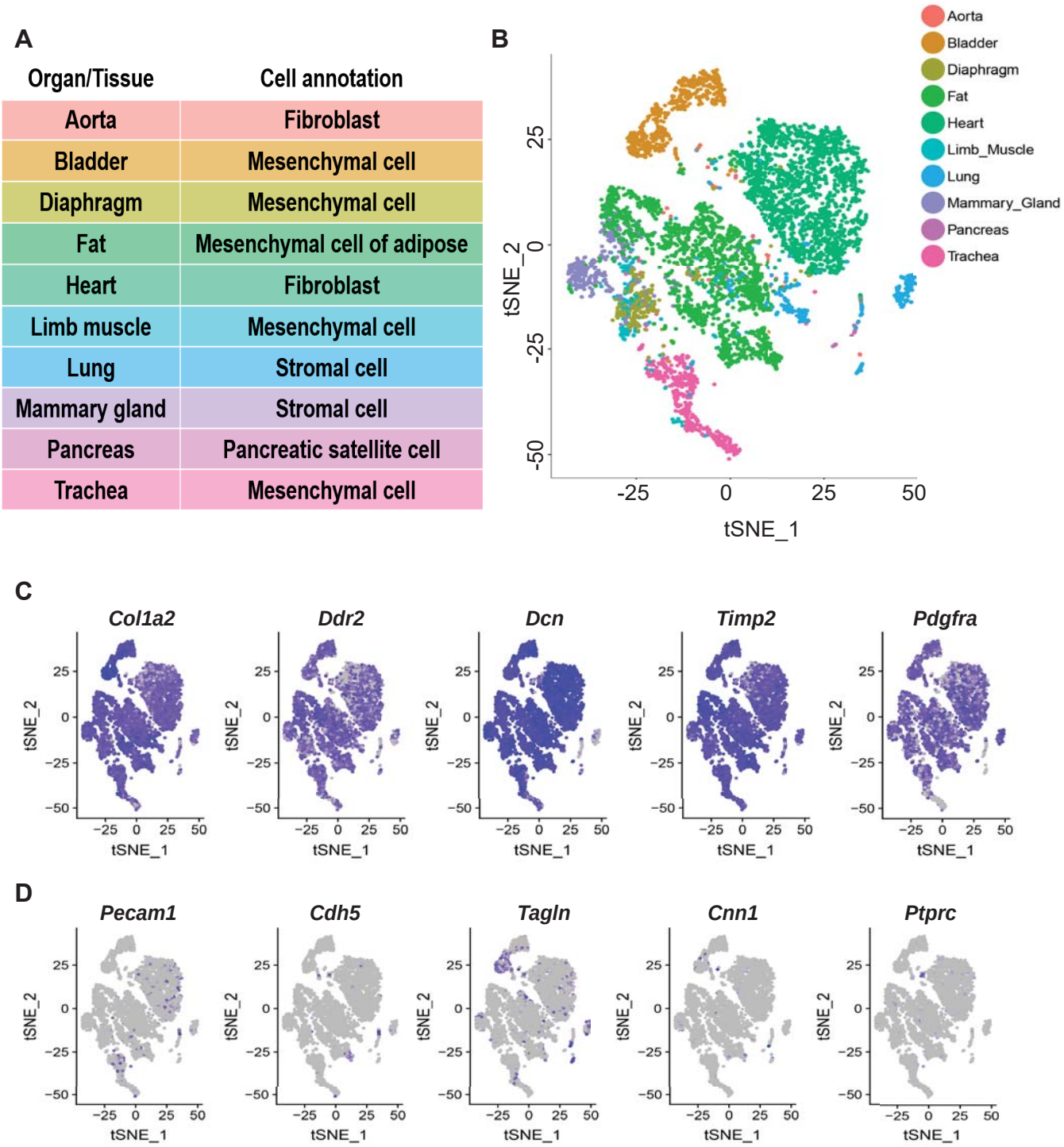
Gene	Assay ID
GAPDH	Hs02786624_g1
GATA4	Hs00171403_m1
TBX20	Hs00396596_m1

HOXA5	Hs00430330_m1
TBX4	Hs01057581_m1
HOXA11	Hs00194149_m1
ISL1	Hs00158126_m1
NKX2-5	Hs00231763_m1
TBX5	Hs00361155_m1
WT1	Hs01103751_m1
TBX18	Hs01385457_m1
TCF21	Hs00162646_m1
COL1A1	Hs00164004_m1
DDR2	Hs01025957_m1
POSTN	Hs01566750_m1
VIM	Hs00958111_m1
ACTA2	Hs00426835_g1
CNN1	Hs00959434_m1
MYH11	Hs00975796_m1
TAGLN	Hs01038777_g1
PDGFRB	Hs01019589_m1
CSPG4	Hs00361541_g1
MCAM	Hs00174838_m1
COL3A1	Hs00943809_m1

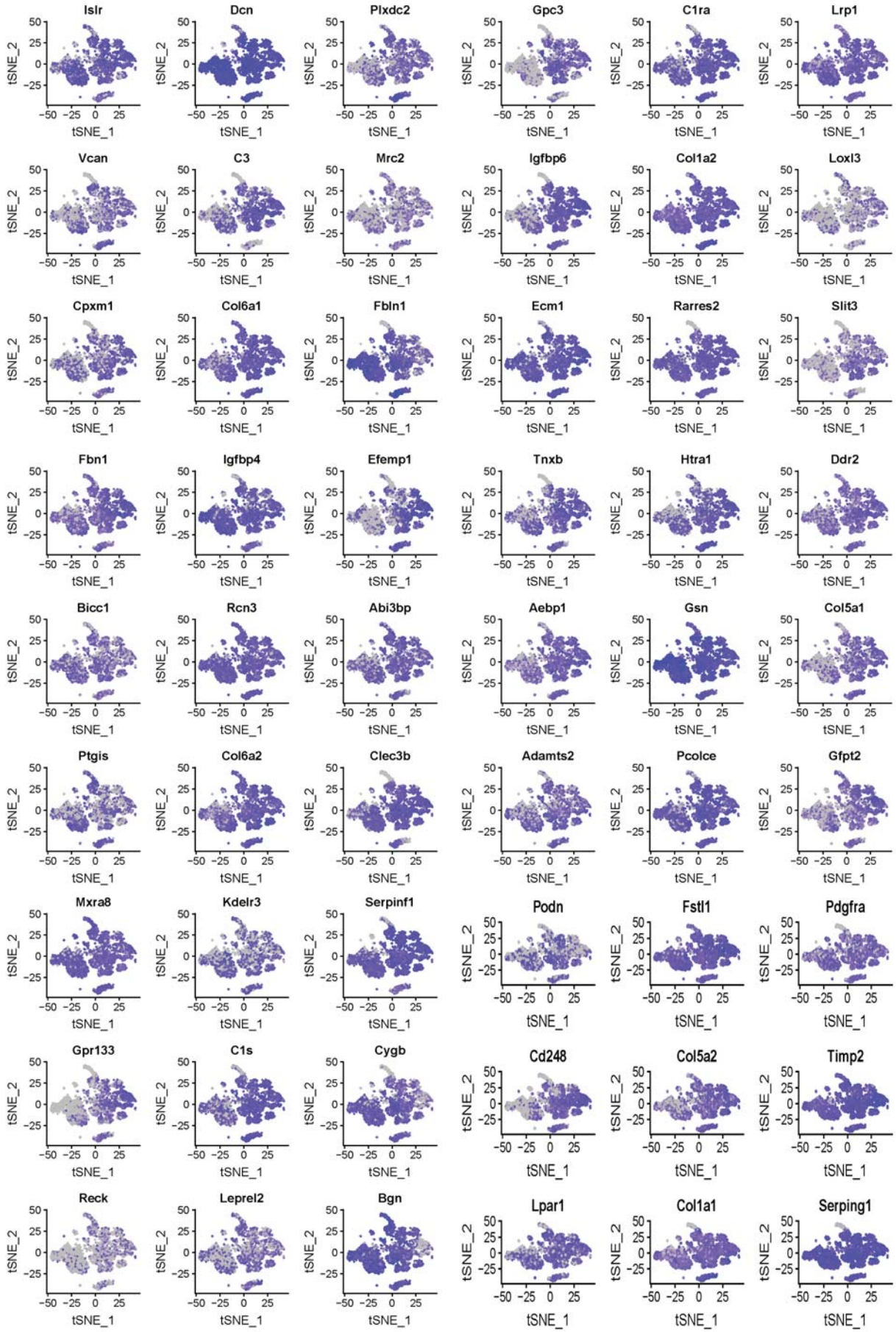
NPPA	Hs00383230_g1
NPPB	Hs00173590_m1
NPR1	Hs01099745_m1
ELK1	Hs00901847_m1
KLF4	Hs00358836_m1
Extracellular matrix and adhesion molecule array	4414133, Taqman

A

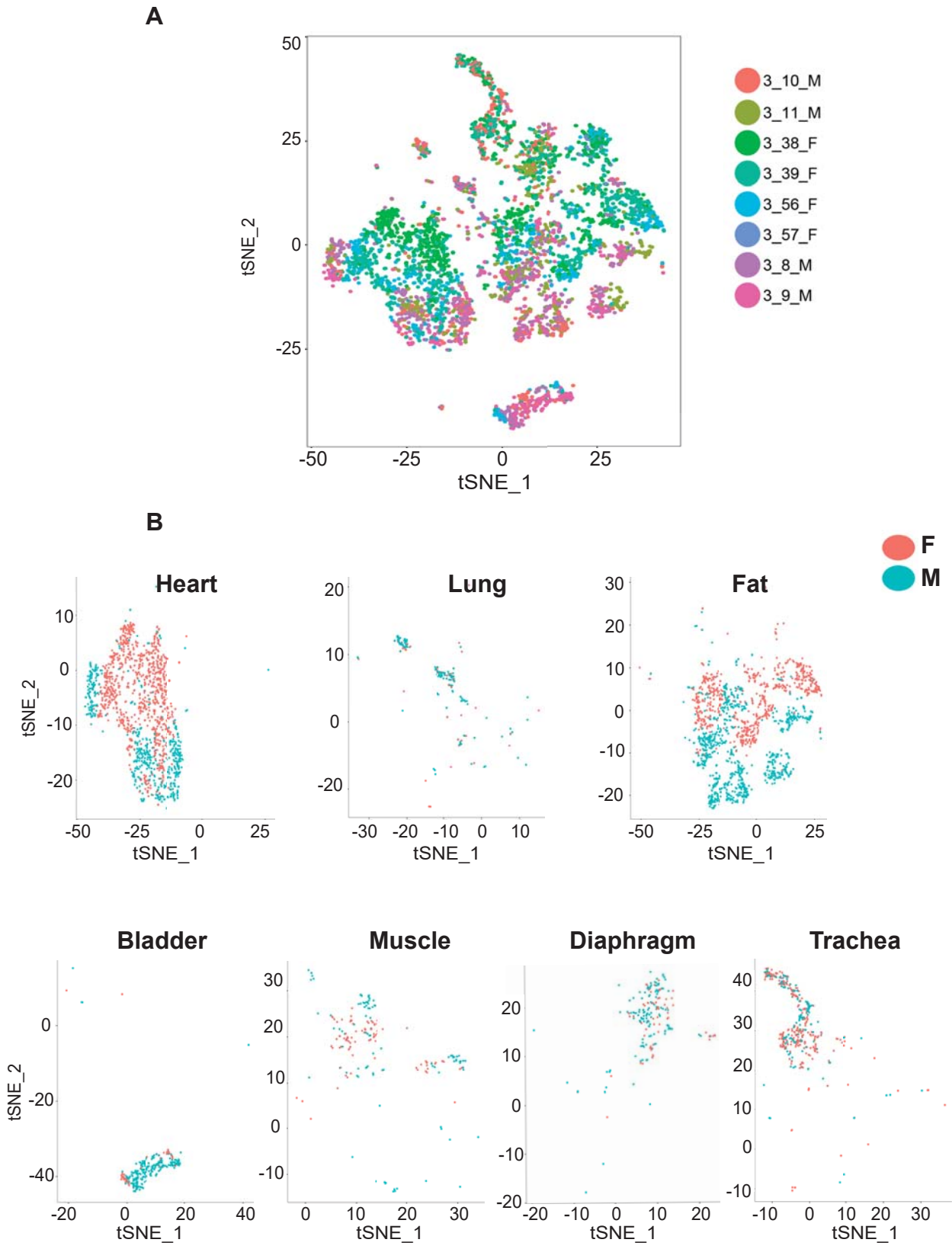
Organ/Tissue	Cell annotation
Aorta	Fibroblast
Bladder	Mesenchymal cell
Diaphragm	Mesenchymal cell
Fat	Mesenchymal cell of adipose
Heart	Fibroblast
Limb muscle	Mesenchymal cell
Lung	Stromal cell
Mammary gland	Stromal cell
Pancreas	Pancreatic satellite cell
Trachea	Mesenchymal cell



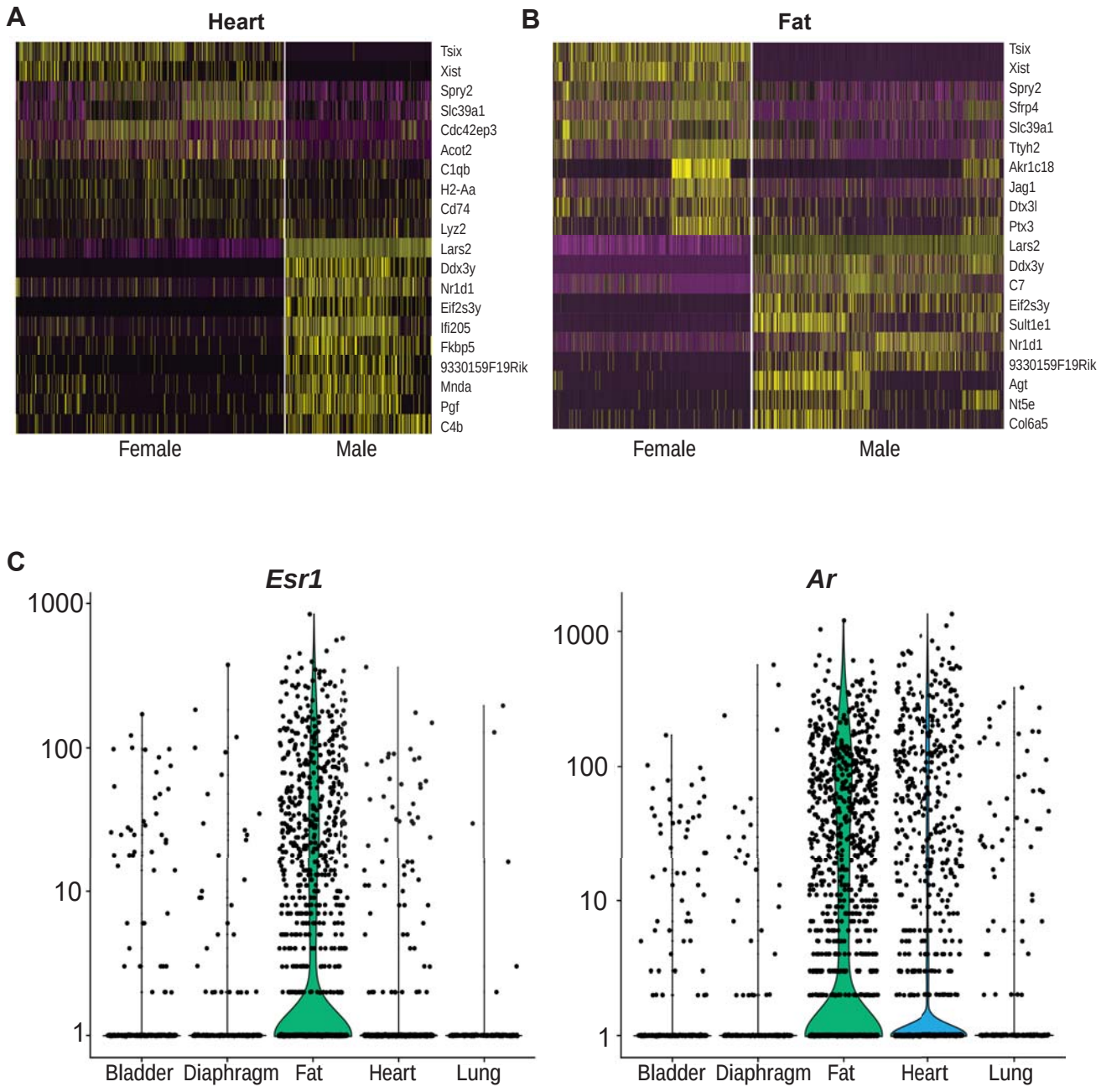
Online Figure I. Generation tissue-specific fibroblast subpopulations from *Tabula Muris*. A, Originally assigned annotations to fibroblast-containing cell clusters derived from different tissues and organs of healthy adult mice in the *Tabula Muris* database. **B,** A t-SNE (t-distributed stochastic neighbor embedding) plot showing the distribution pattern of the cell clusters in (A). **C,** t-SNE plots showing cells positive for *Col1a2*, *Ddr2*, *Dcn*, *Timp2*, and *Pdgfra* (highlighted in purple) in each cluster. **D,** t-SNE plots showing cells positive for endothelial markers (*Pecam1* and *Cdh5*), smooth muscle cell markers (*Tagln* and *Cnn1*), or an immune cell marker (*Ptprc*).



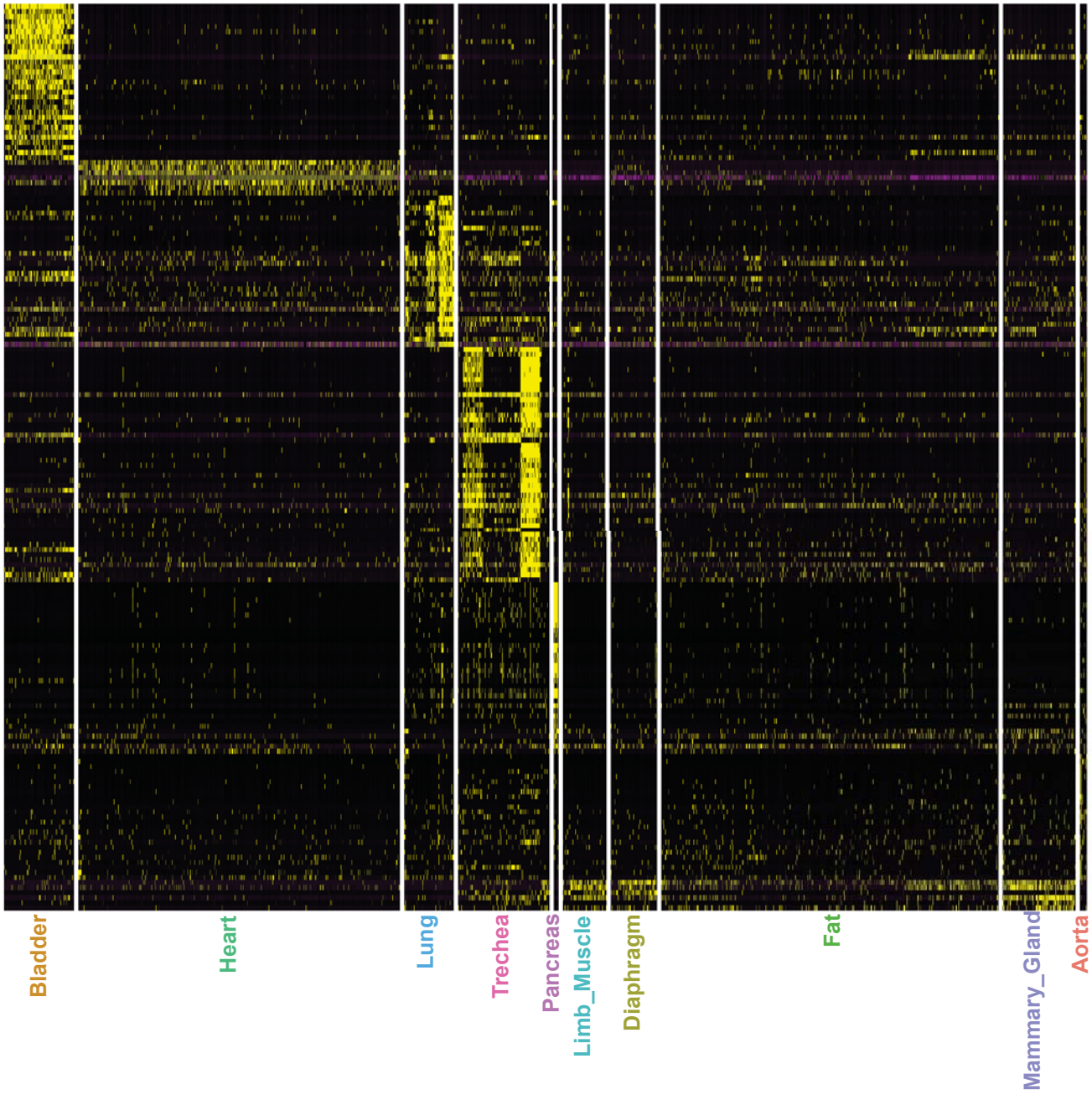
Online Figure II. Commonly expressed genes in tissue-specific fibroblast subpopulations. These genes are detected by comparing all generated tissue-specific fibroblast subpopulations with other cell types with the cutoff: ≥25% fibroblasts expressed, logFC≥log2, FDR adjusted P-value <1% under the Wilcoxon rank sum test.



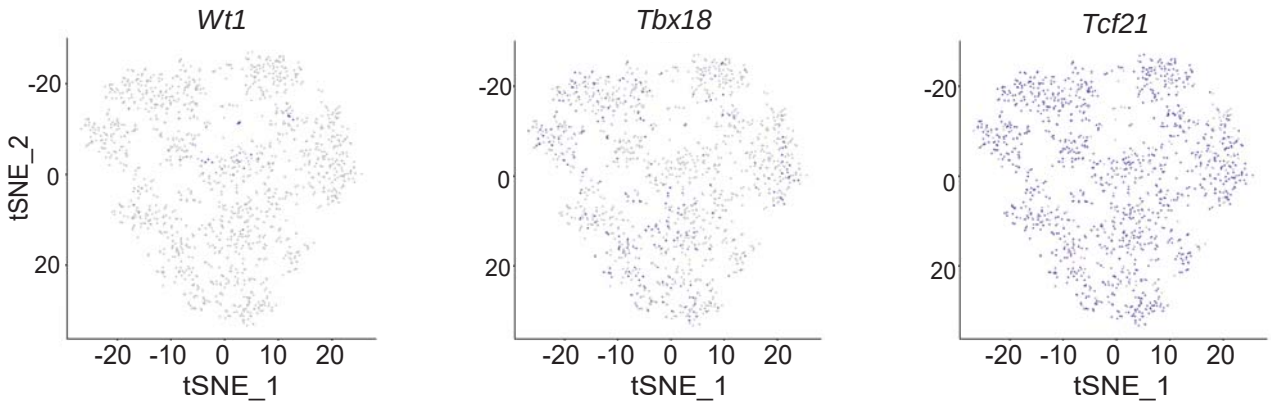
Online Figure III. Donor and sex effects on tissue-specific fibroblast clustering. A, A t-SNE (t-distributed stochastic neighbor embedding) plot showing a donor-independent distribution pattern of tissue-specific fibroblasts from different mice (*n* = 4 for M and 4 for F). F, female; M, male. **B,** A series of t-SNE plots showing thedistribution patterns of tissue-specific fibroblasts in various organs of male versus female mice.



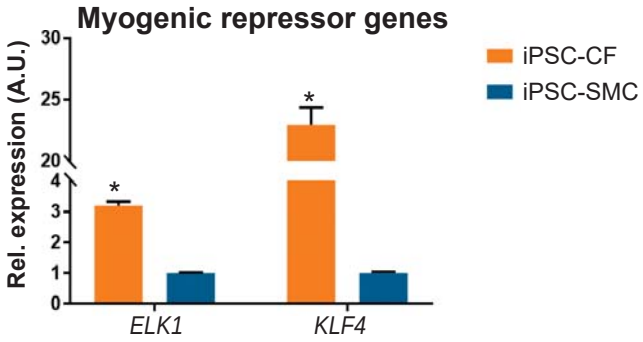
Online Figure IV. Genes in fibroblasts from the heart and fat tissues showing a sex-dependent expression pattern. A, A heatmap comparing top 10 specifically expressed genes in cardiac fibroblasts from male versus female mice. **B,** A heatmap comparing top 10 specifically expressed genes in fibroblasts of fat tissues from male versus female mice. **C,** Violin plots showing the expression levels of estrogen receptor (*Esr1*) and androgen receptor (*Ar*) among fibroblasts of various tissue origins.



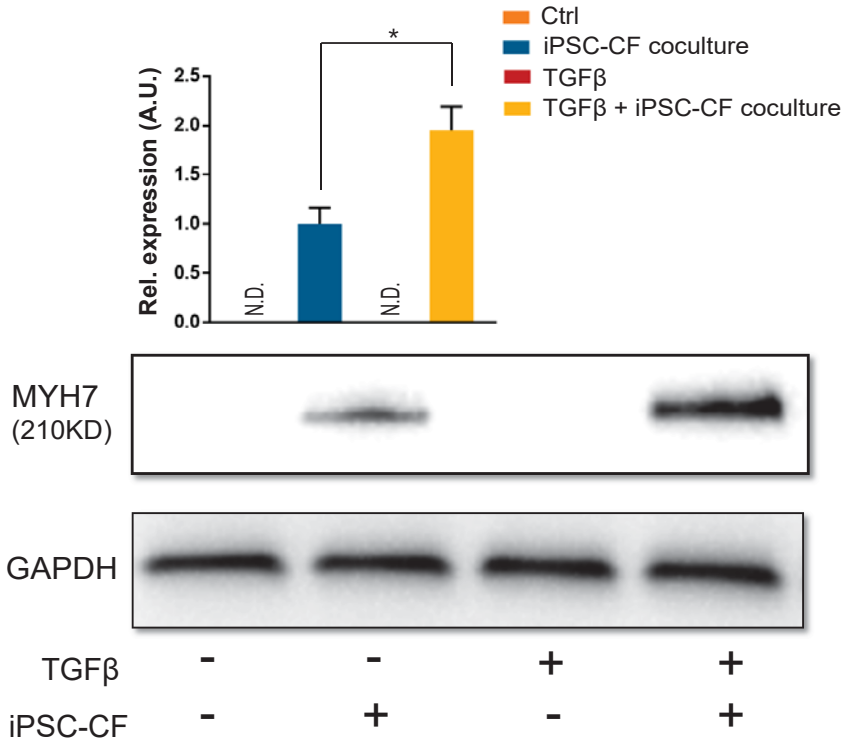
Online Figure V. The most specifically expressed genes in tissue-specific fibroblasts. The numbers of tissue-specific fibroblasts used to generate this heatmap are 314 (bladder), 1444 (heart), 220 (lung), 411 (trachea), 19 (pancreas), 194 (limb muscle), 206 (diaphragm), 1523 (fat), 323 (mammary gland), and 31 (aorta). The top expressed genes in each tissue-specific fibroblast subpopulations are enriched by the cutoff: ≥25% fibroblasts expressed, logFC≥2.5 and FDR adjusted *P*-value <1%; Wilcoxon rank sum test.



Online Figure VI. Expression levels of epicardial genes in mouse cardiac fibroblasts. t-SNE (t-distributed stochastic neighbor embedding) plots showing the distribution patterns of epicardial markers (*Wt1*, *Tbx18*, and *Tcf21*) in adult mouse cardiac fibroblasts (highlighted in purple). These data are generated from the *Tabula Muris* dataset.



Online Figure VII. Expression levels of myogenic repressor genes in human iPSC-derived cardiac fibroblasts and smooth muscle cells. Gene expression levels of myogenic repressor genes (*ELK1* and *KLF4*) in human iPSC-derived cardiac fibroblasts versus smooth muscle cells of the epicardial lineage. iPSC, induced pluripotent stem cell; CF, cardiac fibroblast; SMC, smooth muscle cell. *P<0.05 by one-way ANOVA followed by Bonferroni multiple comparisons. Data were generated based on three independent differentiations, and are presented as mean ± SEM.



Online Figure VIII. Human iPSC-derived cardiac fibroblasts upregulate the expression of MYH7 in iPSC-derived cardiomyocytes in the direct contact coculture condition. A representative immunoblot showing the levels of MHY7 (myosin heavy chain 7) in human iPSC-derived cardiomyocytes after cocultured with or without iPSC-derived CFs in the absence or presence of TGF-β. Densitometric quantifications are shown above the immunoblots. N.D., not detected; iPSC, induced pluripotent stem cell; CF, cardiac fibroblast; TGF-β, transforming growth factor-β. *P<0.05 by one-way ANOVA followed by Bonferroni multiple comparisons. Data were generated based on three independent differentiations, and are presented as mean ± SEM.