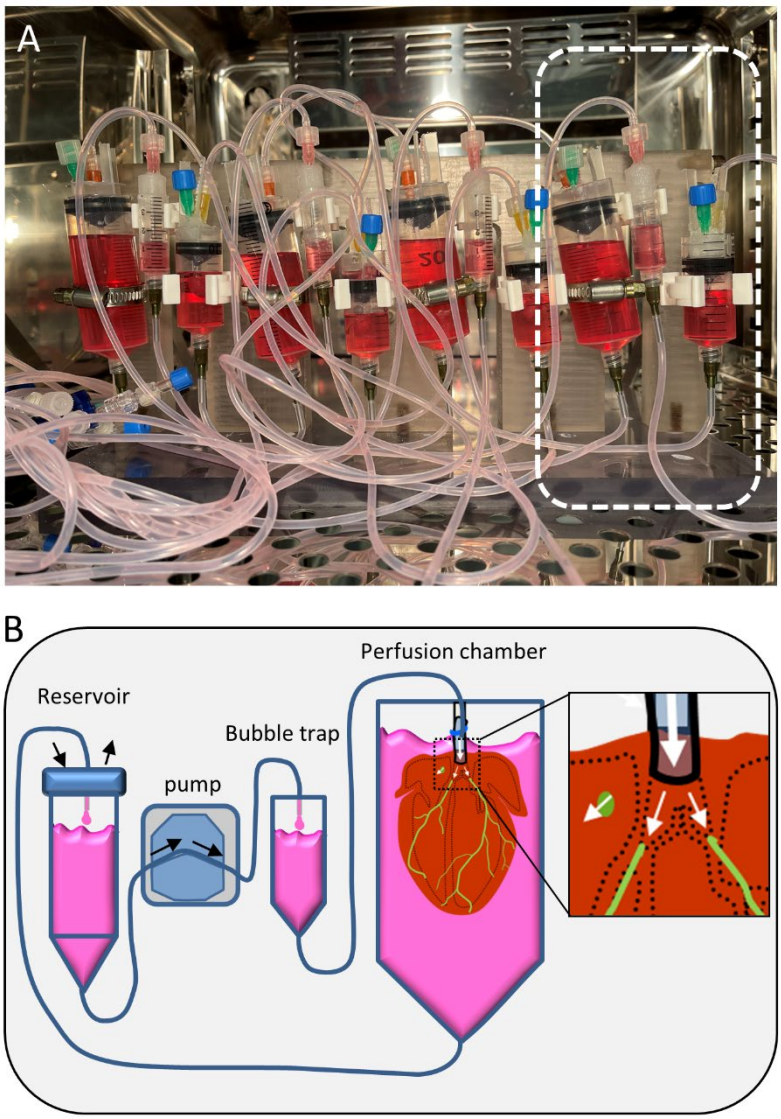
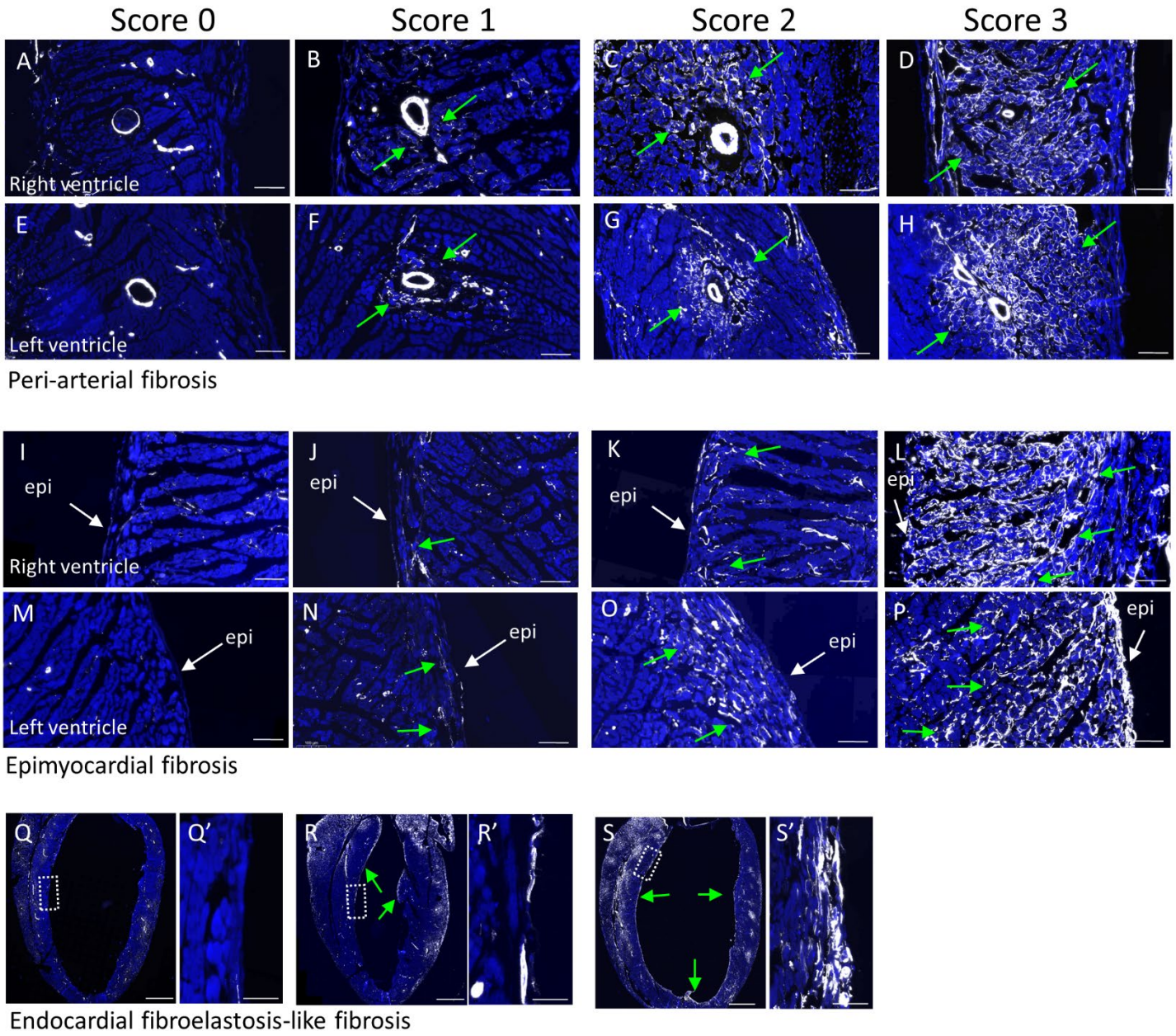
Supplemental Appendix:

Supplemental data:

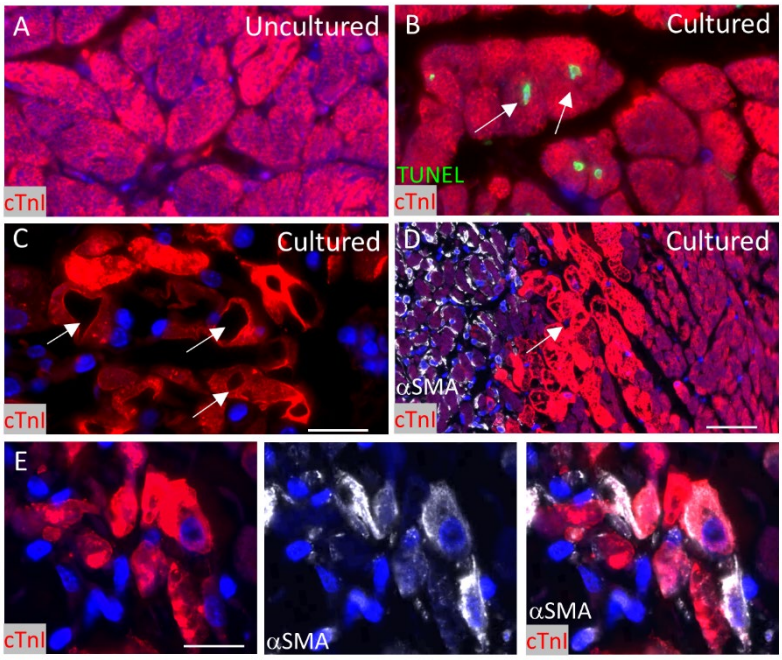
To determine whether the extent of fibrosis is related to the age of the mouse heart, the extent of each type of fibrosis was determined and correlated to the age of the mice. The expression of the myofibroblast marker αSMA was used as a measure of fibrosis as it is an early hallmark of fibrosis. The αSMA-positive area and scores were applied as described in material & methods sections with examples in supplemental figure 2. No significant correlation (Pearson's correlation for linear association) was found between the age (n=36; age range 2-15 months) and the percentage of αSMA-positive area of the ventricle (r=0,39;p=0,07), atria (r=0,27;p=0,23), ventricles and atria (r=0,38;p=0,08) or the scores for peri-arterial fibrosis (r=0,27; p=0,10), epicardial fibrosis (r=-0,14;p=0,42), endocardial fibroelastosis-like fibrosis (r=-0,26; p=0,12) or overall fibrosis (r=0,19; p=0,26), indicating that the extent of fibrosis in the *ex vivo* cultured hearts is not age-dependent.



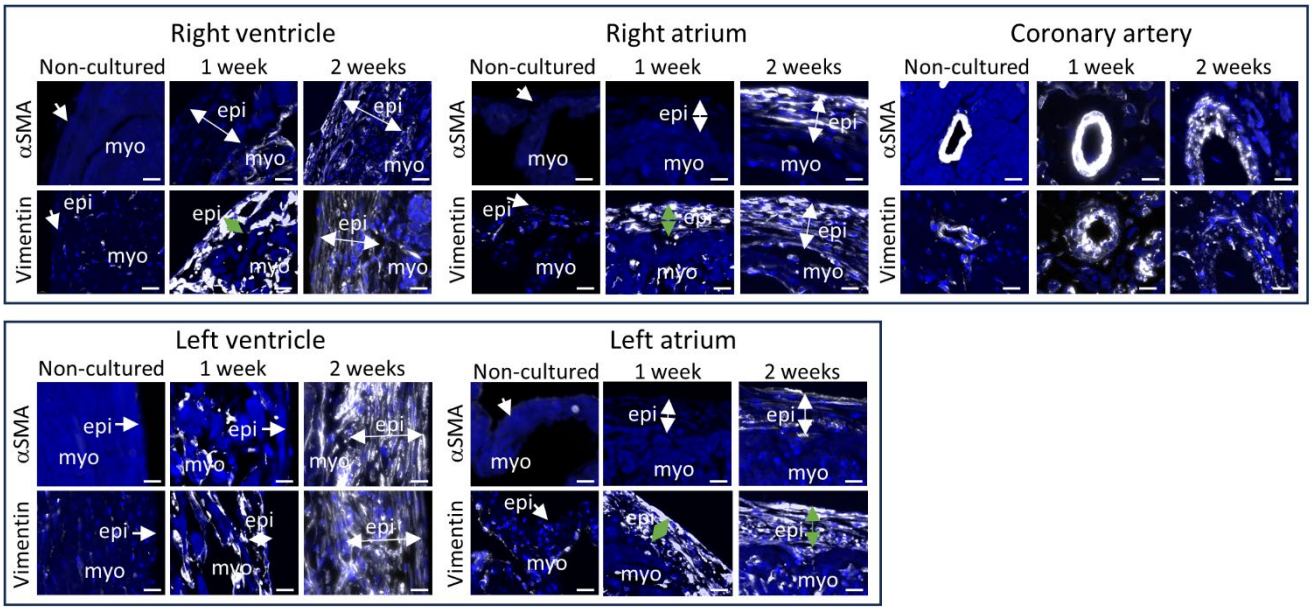
Supplemental Figure 1. The Miniature Tissue Culture System (MTCS). A) picture of the MTCS in action perfusing 4 hearts simultaneously in separate closed flow circuits. B) graphic representation of 1 flow circuit as outlined in A consisting of a reservoir, bubble trap and perfusion chamber through which medium is directed using a pump (Adapted from Kruithof et al[1]).



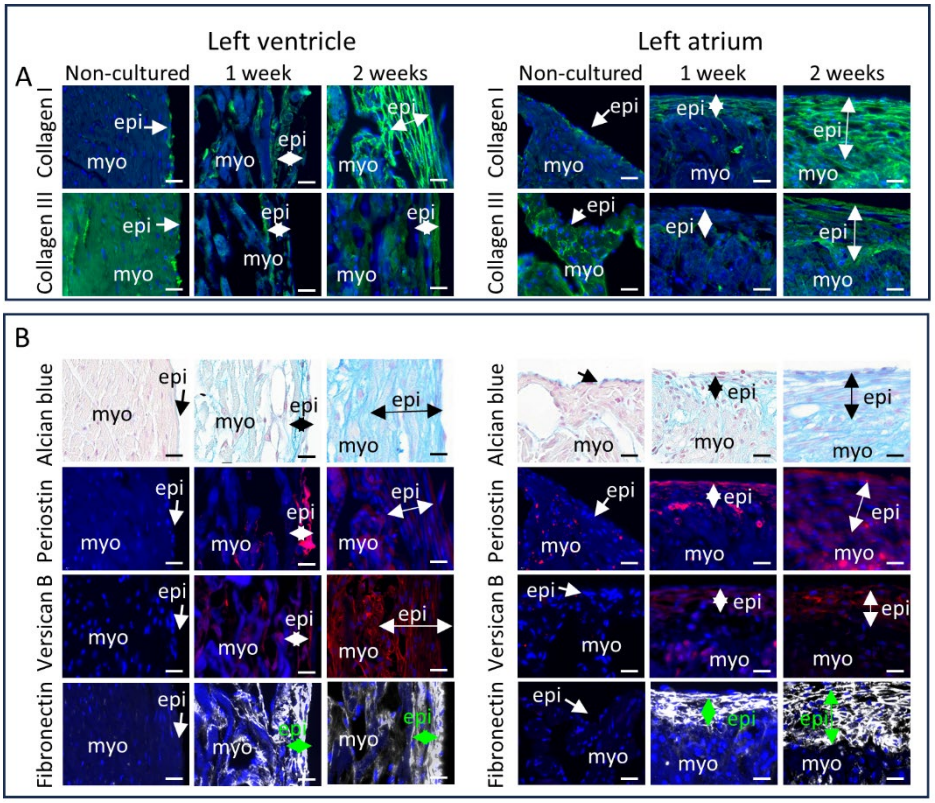
Supplemental Figure 2. Examples scoring system fibrosis in *ex vivo* cultured hearts. Representative pictures of αSMA stainings (white) counterstained with DAPI (blue) indicating peri-arterial fibrosis in the right (A-D) and left ventricle (E-H), epimyocardial fibrosis in the right (I-L) and left ventricle (M-P), and endocardial fibroelastosis-like fibrosis in the left ventricle (Q-S) given the scores 0 (A,E,I,M,Q), 1 (B,F,J,N,R), 2 (C,G,K,O,S) and 3 (D,H,L,P). DAPI is used as counterstain in all pictures. Green arrows indicate the extend of fibrosis. Epi: epicardium. Scalebar A-P: 100 µm; Q-S: 1000 µm; Q'-S':50 µm.



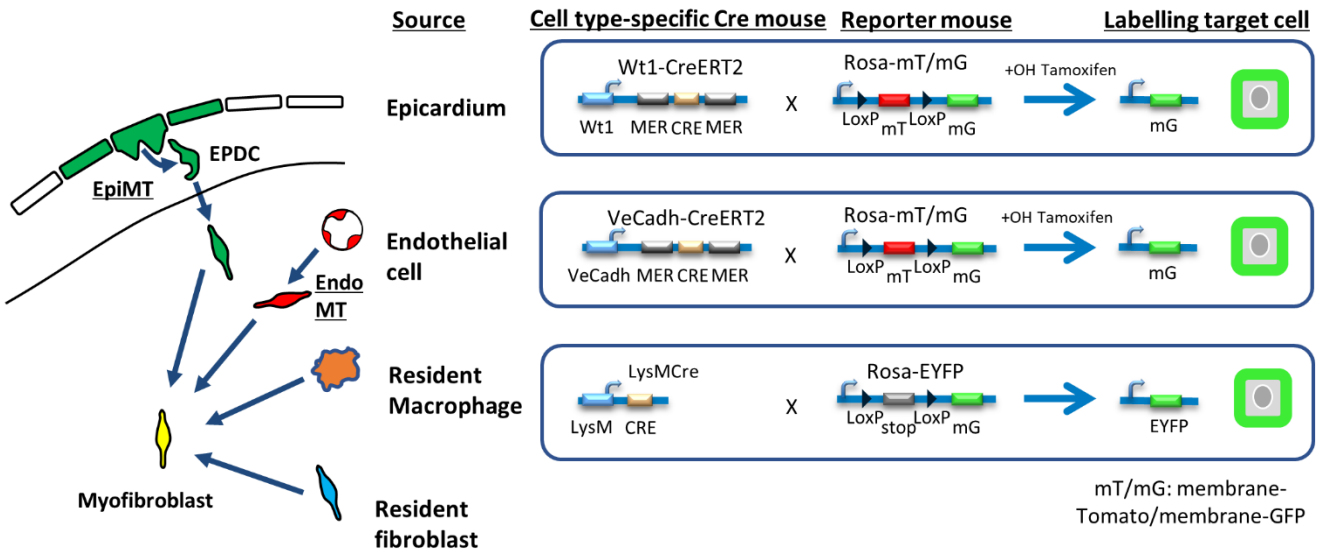
Supplemental Figure 3. Myocardial phenotypes in *ex vivo* cultured hearts. Representative pictures of uncultured and cultured hearts stained for cardiac Troponin I (cTnI; A,C), cTnI and TUNEL (B), cTnI and αSMA (D,E). Scalebar A-C,E: 20 µm; D: 50 µm. DAPI is used as counterstain in all pictures.



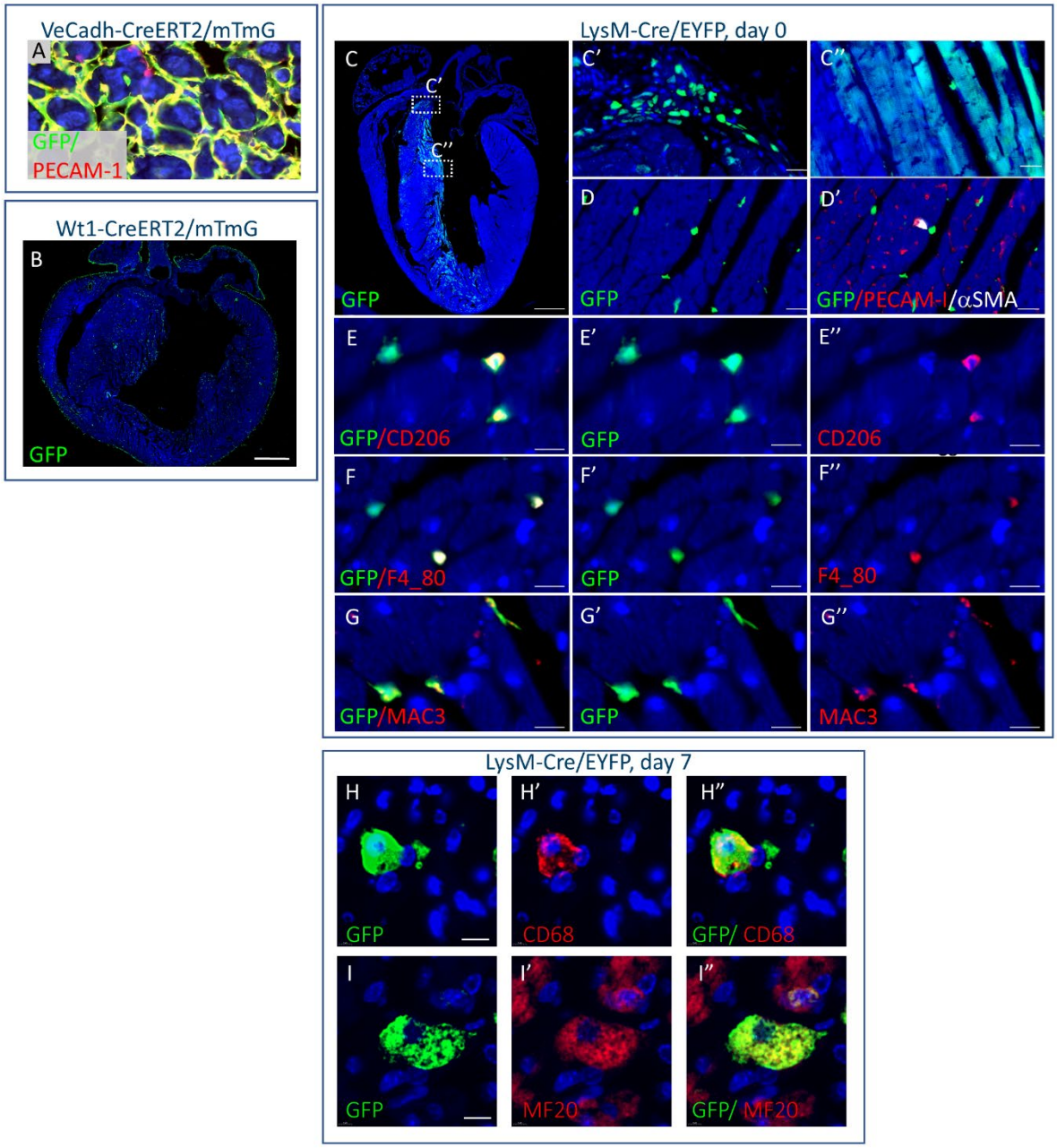
Supplemental Figure 4. Differential timing of αSMA and vimentin expression in *ex vivo* cultured hearts. Representative pictures of the right ventricle, right atrium, coronary artery, left ventricle and left atrium in non-cultured mouse hearts and hearts cultured for 1 or 2 weeks stained for αSMA and vimentin. DAPI is used as counterstain in all pictures. Epi: epicardium; myo: myocardium. Scalebar: 20 µm.



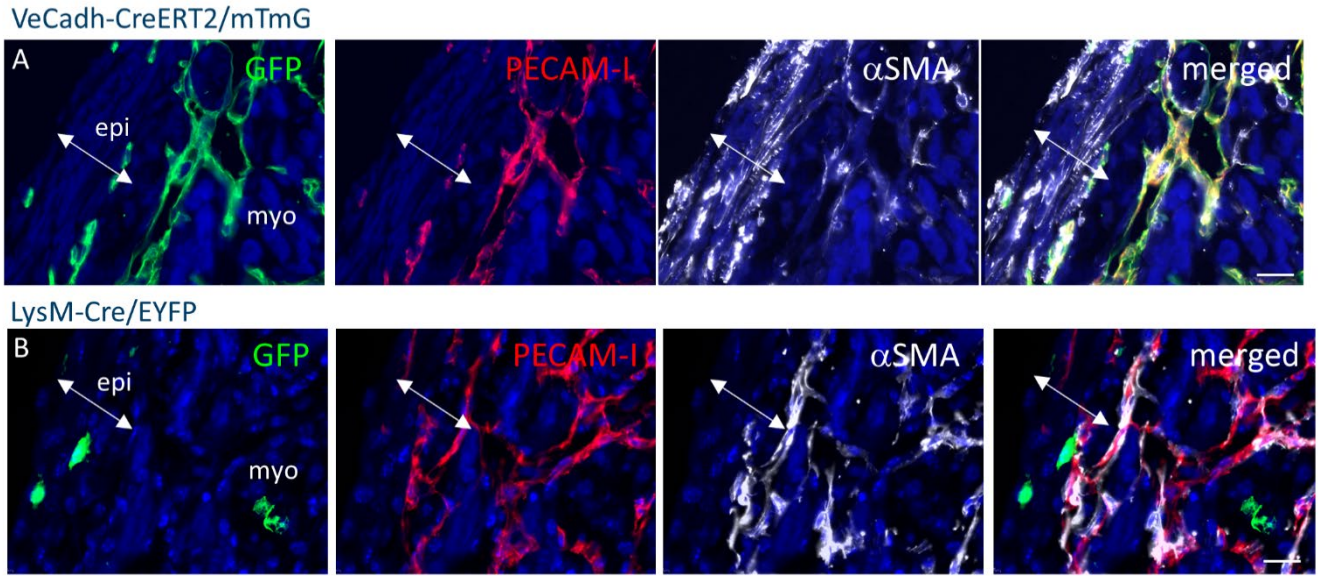
Supplemental Figure 5. Extracellular matrix dynamics in ex vivo cultured hearts. Representative pictures of the left ventricle and left atrium in non-cultured mouse hearts and hearts cultured for 1 or 2 weeks stained for collagen I, collagen III (A), alcian blue, periostin, versican B and fibronectin (B). DAPI is used as counterstain in all fluorescent pictures. Epi: epicardium; myo: myocardium. Scalebar: 20 µm.



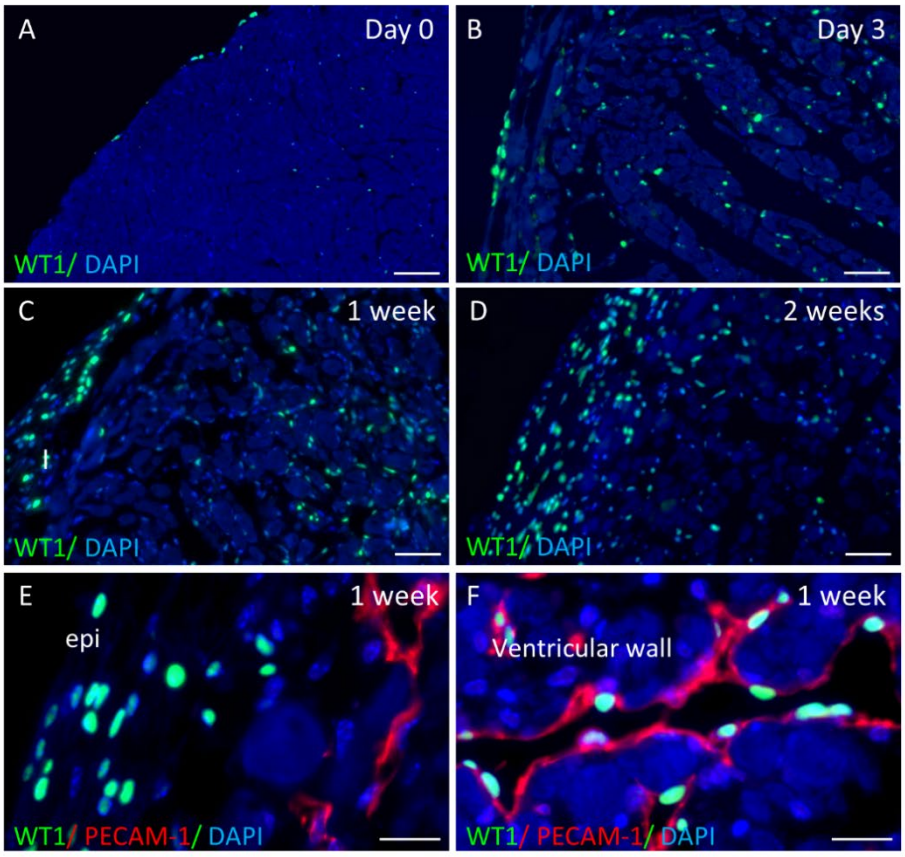
Supplemental Figure 6. Lineage trace models. Schematic drawing of the potential sources of the myofibroblast in the *ex vivo* cultured hearts and the lineage trace models used. The Wt1-CreERT2 and VeCadh-CreERT2 mice were crossed with the Rosa-mT/mG reporter mice. Exposure to hydroxytamoxifen (OH Tamoxifen) at the start of the culture results in the labelling of epicardial and endothelial cells respectively. The LysM-Cre mice were crossed with Rosa-EYFP reporter mice resulting in the constitutive labelling of macrophages. EndoMT: Endothelial to mesenchymal transformation; EPDC: epicardium-derived cells; EpiMT: Epicardial tot mesenchymal transformation; EYFP: Enhanced Yellow Fluorescent Protein; LysM: Lysozyme M; mT; membrane-tomato; mG: membrane Green Fluorescent Protein; VeCadh: VE-cadherin; Wt1: Wilms' tumor-1.



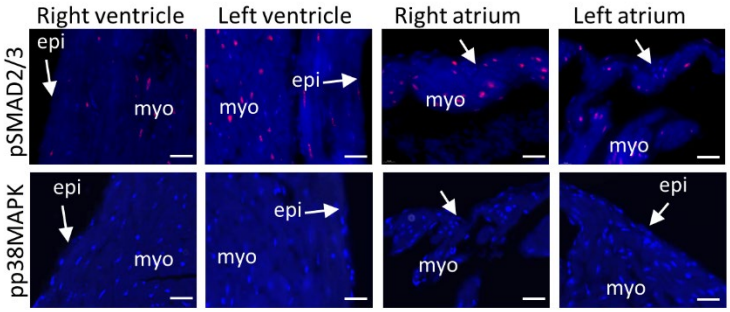
Supplemental Figure 7. Labelling efficiency and specificity of lineage traced cells. The VeCadh-CreERT2/mTmG (A), Wt1-CreERT2/mTmG (B) and LysM-Cre/EYFP (C-I) mice were used to trace the fate of endothelial cells, epicardial cells and macrophages, respectively. A) Representative picture of GFP and PECAM-1 stainings in VeCadh-CreERT2/mTmG hearts cultured for 7 days in the presence of tamoxifen. B) Representative picture of GFP staining in Wt1-CreERT2/mTmG heart cultured for 7 days in the presence of tamoxifen depicting the GFP-positive epicardial layer. C-I) Representative pictures of stainings for GFP (C) and co-stainings of GFP/PECAM-1/αSMA (D), GFP/CD206 (E), GFP/F4_80 (F) and GFP/MAC3 (G) in a non-cultured LysM-Cre/EYFP heart and GFP/CD68 (H), GFP/MF20 (I) in a LysM-Cre/EYFP heart cultured for 7 days. DAPI is used as counterstain in all pictures. Scalebar A: 50 µm; B,C: 1000 µm; C',C'',D,D':20 µm: E-I'': 10 µm.



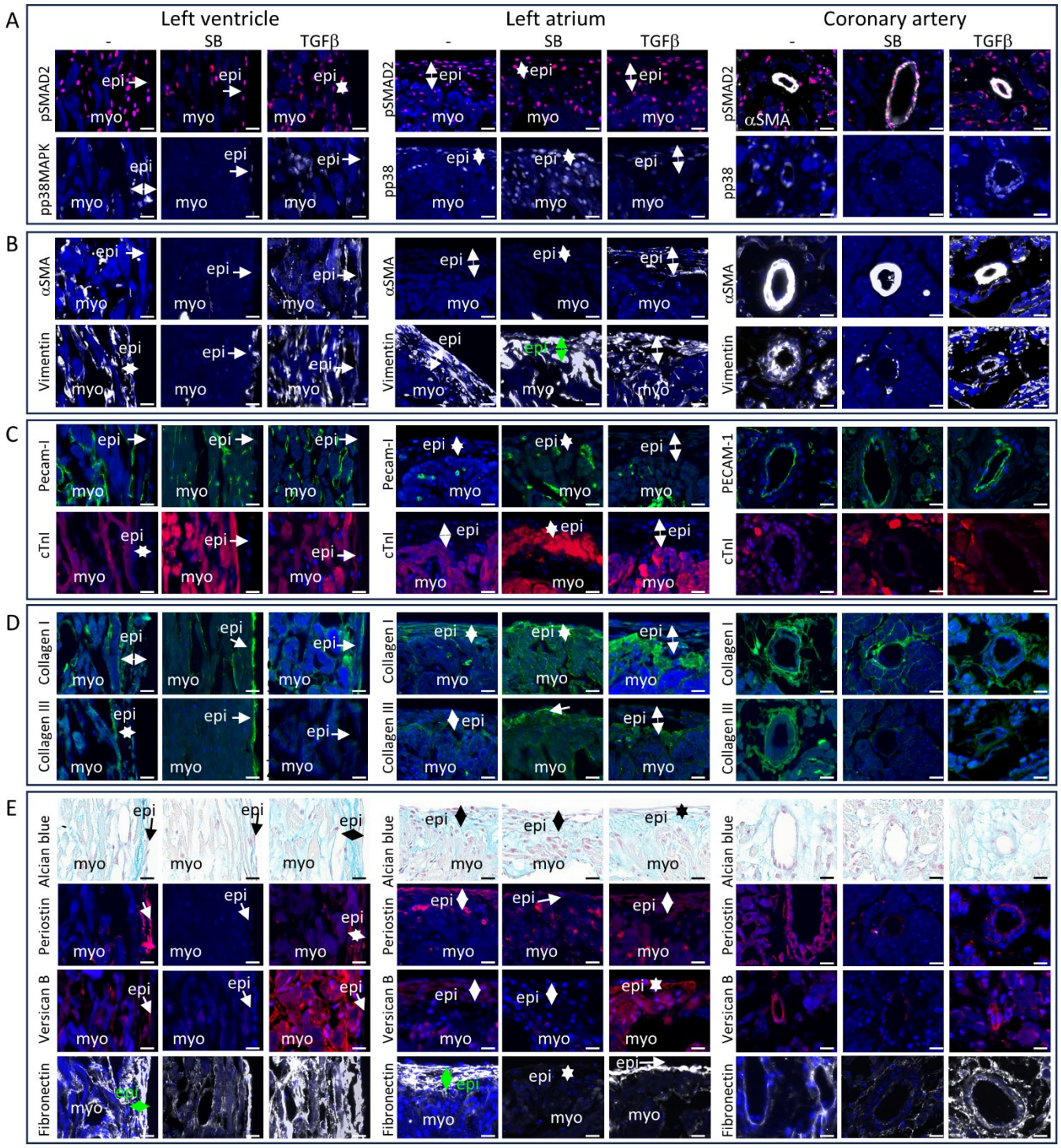
Supplemental Figure 8. Contribution of endothelium-derived cells and macrophages to the subepicardium of *ex vivo* cultured hearts. Representative pictures of GFP, PECAM-1 and αSMA stainings in VeCadh-CreERT2/mTmG hearts (A) and LysM-Cre/EYFP heart (B) cultured for 7 days in the presence of tamoxifen. DAPI is used as counterstain in all pictures. Epi: epicardium; myo: myocardium. Scalebar: 20 µm.



Supplemental Figure 9: Wt1 expression in *ex vivo* cultured hearts. A-D) Representative pictures of Wt1 stainings on mouse hearts cultured for 0 days (A), 3 days (B), 1 week (C), and 2 weeks (D) and stainings of Wt1 and PECAM-1 in the epicardial layer (epi; E) and ventricular wall (F) after 1 week of culture. DAPI is used as counterstain in all pictures. Scalebar A,D: 50 µm; E,F: 20 µm.



Supplemental Figure 10. pSMAD2/3 and pp38MAPK expression in non-cultured mouse hearts. Representative pictures of pSMAD2/3 and pp38MAPK stainings on non-cultured hearts. DAPI is used as counterstain in all pictures. Epi: epicardium; myo: myocardium. Scalebar:20 µm.



Supplemental Figure 11. Molecular regulation of fibrosis in *ex vivo* cultured hearts; left ventricle, left atrium, coronary artery. Representative pictures of the left ventricle, the left atrium and coronary arteries of hearts cultured for 7 days under standard conditions (-), in the presence of SB431542 (SB, SB1, SB2) or TGFβ stained for A) pSMAD2/3 and pp38MAPK, B) αSMA and vimentin, C) PECAM-1 and cTnI, D) collagen I and collagen III, E) Alcian blue, periostin, versican B, fibronectin. DAPI is used as counterstain in all fluorescent pictures. Epi: epicardium; myo: myocardium. Scalebar: 20 µm.

1. Kruithof BPT, van de Pol V, Los T et al. New calcification model for intact murine aortic valves. J Mol Cell Cardiol 2021;156:95-104.