The profibrotic transition of vascular smooth muscle cells-derived resident vascular adventitial progenitor cells contributes to Angiotensin II-induced cardiac fibrosis.

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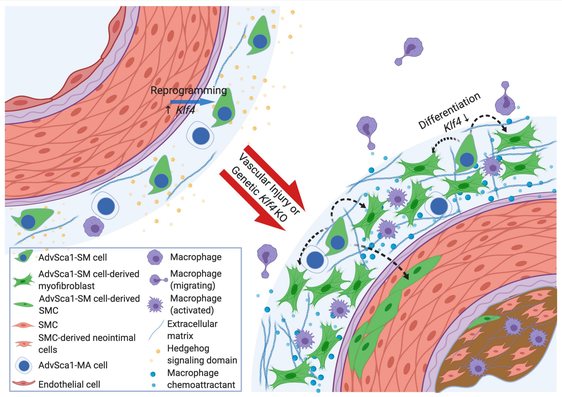
Background

- Cardiovascular fibrosis is an important end-stage pathology that characterizes most cardiovascular diseases. Although fibrotic tissue facilitates the maintenance of organ integrity, excessive deposition of extracellular matrix (ECM) in cardiac tissue significantly disrupts normal function of the heart.
- Activated cardiac myofibroblasts are the major contributors to ECM deposition in pathological fibrosis. However, due to potential heterogeneity of myofibroblasts, the origin of these cells remains controversial.
- Resident vascular adventitial progenitor cells express the stem cell marker Sca1 (AdvSca1), exhibit multilineage differentiation potential and play an important role in vascular injury and remodeling.
- Using highly specific smooth muscle cell lineage-tracing mouse models, our laboratory discovered the smooth muscle cell origin of a unique subpopulation of AdvSca1 cells, which we termed AdvSca1-SM cells.
- Our recent published bulk RNA-Seq data identified a specific gene signature of active hedgehog/WNT/beta-catenin/KLF4 signaling in AdvSca1-SM cells.
- Leveraging the specific expression of *Gli1* gene by AdvSca1-SM cells, we validated a *Gli1*-Cre^{ERT2}-ROSA26-YFP reporter mouse model to be a faithful lineage tracing system for AdvSca1-SM cells.
- Using the *Gli1* lineage tracing system, we reported that AdvSca1-SM cells lose their progenitor phenotype, rapidly proliferate and adopt myofibroblast phenotype in response to acute vascular injury.
- Similarly, AdvSca1-SM cell-specific genetic ablation of *Klf4* gene induces differentiation and proliferation of AdvSca1-SM cells and promotes spontaneous adventitial remodeling.
- However, the function of AdvSca1-SM cells in cardiac diseases and its contribution to myofibroblasts is unknown.



Central role for Klf4-dependent AdvSca1-SM cell contribution to pathological vascular remodeling and fibrosis. AdvSca1-SM cells express a unique gene signature that supports a role for hedgehog/WNT/beta-catenin/KLF4 signaling in regulating SMC-to-AdvSca1-SM cell reprogramming and AdvSca1-SM progenitor cell phenotype and survival. Injury-mediated and/or genetic downregulation of KLF4 disrupts this local progenitor cell niche and promotes activation of AdvSca1-SM cells, as assessed by downregulation of a stemness phenotype. AdvSca1-SM cell activation promotes upregulation of a profibrotic gene signature, thereby facilitating differentiation toward pathological myofibroblasts that contribute to pathological vascular remodeling and fibrosis (created with BioRender.com). (*Weiser-Evans, Circ. Res. 2017; Lu et al. JCI Insight. 2020*).

Hypothesis

Cardiac AdvSca1-SM cells adopt a myofibroblast phenotype and contribute to cardiac fibrosis in the setting of Angiotensin II-induced cardiac hypertrophy.

Materials and Methods

➢ **Animals**.

- *Gli1*-Cre^{ERT2}-ROSA26-YFP reporter mice (*Gli1*-Cre^{ERT}-YFP) were injected with 1 mg tamoxifen daily for 12 consecutive days to induce YFP reporter knockin. After a 5-day washout period, the mice received Angiotensin II (AngII; 1 μg/kg/min) or vehicle (saline) infusion for 14 or 28 days through subcutaneous osmotic pump implantation. Cardiac tissues were harvested and fixed with 4% PFA and embedded in OCT for imaging studies or enzymatically digested into single cell suspension for subsequent analysis.

➢ **Second harmonic generation (SHG) imaging**

- Cardiac tissue sections from Saline/AngII treated mice were labelled with FITC-conjugated anti-GFP antibody and processed for label-free second harmonic generation (SHG) imaging at the Advanced Light Microscopy core to examine collagen deposition and AdvSca1-SM cells.

➢ **Single cell RNA-sequencing (scRNA-seq)**

- Saline/AngII treated mice were euthanized and the heart tissues were harvested for single cell suspension preparation. Cells were labelled with APC conjugated anti-CD31 antibody and CD31$^+$ endothelial cells and CD31$^-$ non-endothelial population were sorted using fluorescence activated cell sorting (FACS) at the CU Cancer Center Flow Cytometry Shared Resource core facility. Sorted cell populations were counted with a hemocytometer and the endothelial cell populations were mixed with non-endothelial at 1:9 ratio for scRNA-seq library preparation and sequencing at the Genomics Shared Resource at the University of Colorado Cancer Center. A total of 5000 cells per treatment condition were captured and sequenced at the depth of 5000 reads per cell using the 10x Genomics platform.
- Sequencing data were processed through the Cell Ranger pipeline with custom build reference genome (Ensembl GRCm39 release 104) containing eYFP ORF sequence. Scanpy and scVelo were used for the analysis of scRNA-seq data.

➢ **Visium Spatial Gene Expression**

- Cardiac tissues from AngII/Saline treated mice were fresh frozen embedded in OCT. 10 μm sections were collected on Visium spatial tissue optimization slide for permeabilization optimization. 12 min of permeabilization gave the strongest fluorescence signal with the lowest signal diffusion and was therefore used for experiments. Cardiac tissue sections captured on Visium spatial gene expression slide were fixed in methanol at -20 ° C for 30 min and labelled with anti-αSMA-Cy3 antibody for 30 min. Stained slide was imaged with Keyence BZ-X710 fluorescence microscope at 10x on DAPI and Cy3 channels. Tiled images were stitched with imageJ to create the complete tissue image with fiducial frame. Tissue permeabilization, reverse transcription and second strand synthesis & Denaturation were performed according to manufacturer protocols. cDNA samples were submitted to Genomics Shared Resource for QC, library construction and sequencing.
- Manual image alignment was performed with Loupe Browser. Fastq files were aligned to reference genome (Ensembl GRCm39 release 104 with the addition of eYFP gene) using Space Ranger. Downstream QC, analysis and visualizations were performed with Scanpy.

➢ **Statistics**. Data were analyzed using PRISM 9 (GraphPad Software, Inc.). Column statistics and D'Agostino and Pearson omnibus normality tests were performed to determine the mean, standard deviation, and validate the normality of the data. One-way ANOVA was used to determine significance of the overall P value followed by Tukey's post-hoc to determine differences between the groups.

Results

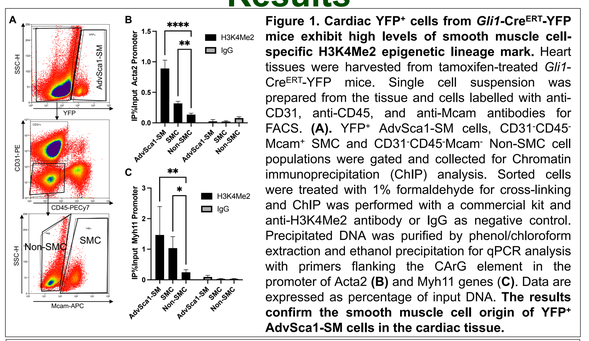


Figure 1. Cardiac YFP$^+$ cells from *Gli1*-Cre^{ERT}-YFP mice exhibit high levels of smooth muscle cell-specific H3K4Me2 epigenetic lineage mark. Heart tissues were harvested from tamoxifen-treated *Gli1*-Cre^{ERT}-YFP mice. Single cell suspension was prepared from the tissue and cells labelled with anti-CD31, anti-CD45, and anti-Mcam antibodies for FACS. **(A).** YFP$^+$ AdvSca1-SM cells, CD31$^-$CD45$^-$ Mcam$^+$ SMC and CD31$^-$CD45$^-$Mcam$^-$ Non-SMC cell populations were gated and collected for Chromatin immunoprecipitation (ChIP) analysis. Sorted cells were treated with 1% formaldehyde for cross-linking and ChIP was performed with a commercial kit and anti-H3K4Me2 antibody or IgG as negative control. Precipitated DNA was purified by phenol/chloroform extraction and ethanol precipitation for qPCR analysis with primers flanking the CArG element in the promoter of Acta2 **(B)** and Myh11 genes **(C)**. Data are expressed as percentage of input DNA. **The results confirm the smooth muscle cell origin of YFP$^+$ AdvSca1-SM cells in the cardiac tissue.**

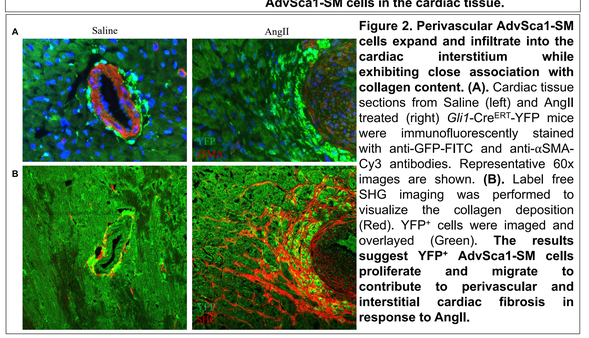


Figure 2. Perivascular AdvSca1-SM cells expand and infiltrate into the cardiac interstitium while exhibiting close association with collagen content. (A). Cardiac tissue sections from Saline (left) and AngII treated (right) *Gli1*-Cre^{ERT}-YFP mice were immunofluorescently stained with anti-GFP-FITC and anti-αSMA-Cy3 antibodies. Representative 60x images are shown. **(B).** Label free SHG imaging was performed to visualize the collagen deposition (Red). YFP$^+$ cells were imaged and overlayed (Green). **The results suggest YFP$^+$ AdvSca1-SM cells proliferate and migrate to contribute to perivascular and interstitial cardiac fibrosis in response to AngII.**

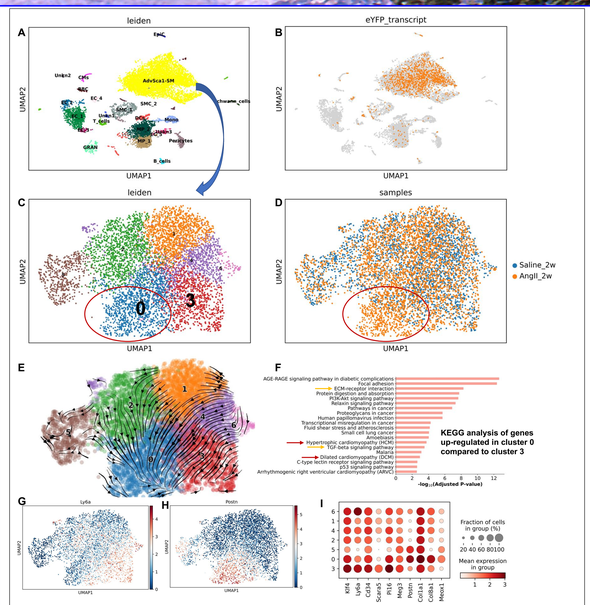


Figure 3. scRNA-seq analysis reveals profibrotic differentiation trajectory of AdvSca1-SM cells in response to AngII treatment. scRNA-seq experiment was performed with cardiac cells sorted from Saline/AngII treated *Gli1*-Cre^{ERT}-YFP mice as described in the Materials and Methods. (**A**). Uniform Manifold Approximation and Projection (UMAP) visualization of the scRNA-seq data set. **(B).** eYFP$^+$ AdvSca1-SM cells were selectively visualized. **(C)**. AdvSca1-SM cluster (yellow in **A**) was selected for sub-clustering. **(D).** Cells from Saline (blue) and AngII (orange) treated samples were shown in the UMAP plot. **(E).** Stream plot of RNA velocity embedded in UMAP. The length of each vector represents RNA velocity along the pseudotime trajectory, and the width is proportional to the number of cells at a given position. **(F).** KEGG pathway enrichment was performed with the genes up-regulated in cluster 0 compared to cluster 3. Pathways related to fibrosis (orange arrows) and cardiac hypertrophy (red arrows) were highlighted. Expressions of stemness gene Ly6a **(G)** and myofibroblast gene Postn **(H)** were plotted in the sub-cluster UMAP. **(I).** Additional differentially expressed genes between cluster 0 and cluster 3 are shown in dotplot. **The scRNA-seq data support that AdvSca1-SM cells, in response to AngII stimulation, differentiate along a profibrotic trajectory, which is characterized by loss of expression of Klf4 and stemness genes and up-regulation of a myofibroblast gene signature.**

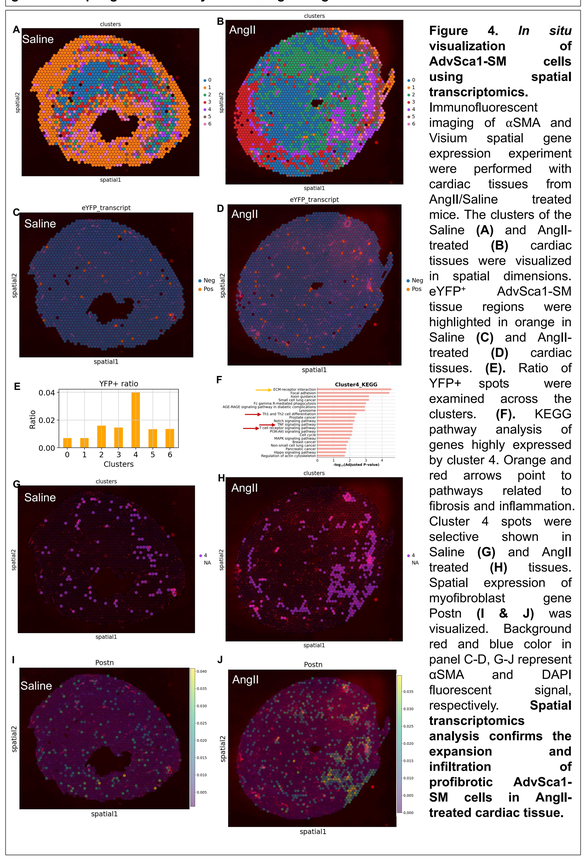


Figure 4. *In situ* visualization of AdvSca1-SM cells using spatial transcriptomics. Immunofluorescent imaging of αSMA and Visium spatial gene expression experiment were performed with cardiac tissues from AngII/Saline treated mice. The clusters of the Saline **(A)** and AngII-treated **(B)** cardiac tissues were visualized in spatial dimensions. eYFP$^+$ AdvSca1-SM tissue regions were highlighted in orange in Saline **(C)** and AngII-treated **(D)** cardiac tissues. **(E).** Ratio of YFP+ spots were examined across the clusters. **(F).** KEGG pathway analysis of genes highly expressed by cluster 4. Orange and red arrows point to pathways related to fibrosis and inflammation. Cluster 4 spots were selective shown in Saline **(G)** and AngII treated **(H)** tissues. Spatial expression of myofibroblast gene Postn **(I & J)** was visualized. Background red and blue color in panel C-D, G-J represent αSMA and DAPI fluorescent signal, respectively. **Spatial transcriptomics analysis confirms the expansion and infiltration of profibrotic AdvSca1-SM cells in AngII-treated cardiac tissue.**

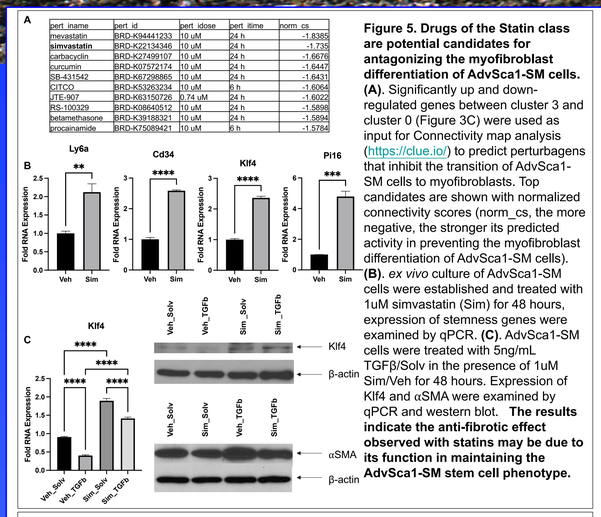


Figure 5. Drugs of the Statin class are potential candidates for antagonizing the myofibroblast differentiation of AdvSca1-SM cells. (A). Significantly up and down-regulated genes between cluster 3 and cluster 0 (Figure 3C) were used as input for Connectivity map analysis (https://clue.io/) to predict perturbagens that inhibit the transition of AdvSca1-SM cells to myofibroblasts. Top candidates are shown with normalized connectivity scores (norm_cs, the more negative, the stronger its predicted activity in preventing the myofibroblast differentiation of AdvSca1-SM cells). **(B)**. *ex vivo* culture of AdvSca1-SM cells were established and treated with 1uM simvastatin (Sim) for 48 hours, expression of stemness genes were examined by qPCR. **(C)**. AdvSca1-SM cells were treated with 5ng/mL TGFβ/Solv in the presence of 1uM Sim/Veh for 48 hours. Expression of Klf4 and αSMA were examined by qPCR and western blot. **The results indicate the anti-fibrotic effect observed with statins may be due to its function in maintaining the AdvSca1-SM stem cell phenotype.**

pert_iname	pert_id	pert_idose	pert_itime	norm_cs
mevastatin	BRD-K04441233	10 uM	24 h	-1.8385
simvastatin	BRD-K22134346	10 uM	24 h	-1.735
carbacyclin	BRD-K27499107	10 uM	24 h	-1.6678
curcumin	BRD-K07572174	10 uM	24 h	-1.6447
SB-431542	BRD-K67298865	10 uM	24 h	-1.6431
CITCO	BRD-K53263234	10 uM	6 h	-1.6064
JTE-907	BRD-K63150726	0.74 uM	24 h	-1.6022
RS-100329	BRD-K08640512	10 uM	24 h	-1.5898
betamethasone	BRD-K39188321	10 uM	24 h	-1.5894
procainamide	BRD-K75089421	10 uM	6 h	-1.5784

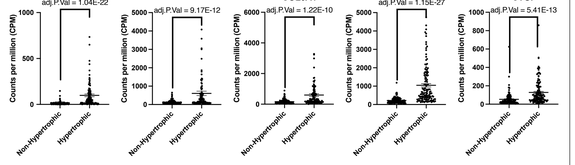


Figure 6. The progenitor and myofibroblasts gene signature of AdvSca1-SM cells were observed in bulk RNA-seq data of left ventricular tissue of non-hypertrophic and hypertrophic human subjects, respectively. Data were downloaded from the Genotype-Tissue Expression (GTEx) project and the samples were ranked by their Natriuretic Peptide B (NPPB) expression level and 30% subjects with highest and lowest NPPB expression in each sex/age group were designated as hypertrophic and non-hypertrophic samples, respectively **(A)**. Lima-Voom was used to examine the differentially expressed genes. Genes up-regulated in non-hypertrophic group exhibit significant overlap with genes selectively expressed by AdvSca1-SM progenitor cells (Cluster 3 in Figure 3C). The normalized count data of select overlapping genes were shown in **(B)**. Genes up-regulated in the hypertrophic group exhibit significant overlap with genes selectively expressed by myofibroblast transitioned AdvSca1-SM cluster (Cluster 0 in Figure 3C). The normalized count data of select overlapping genes were graphed in **(C)**. **The results strongly support the translational relevance of the AdvSca1-SM-myofibroblast transition in human cardiac hypertrophy and fibrosis.**

Conclusions

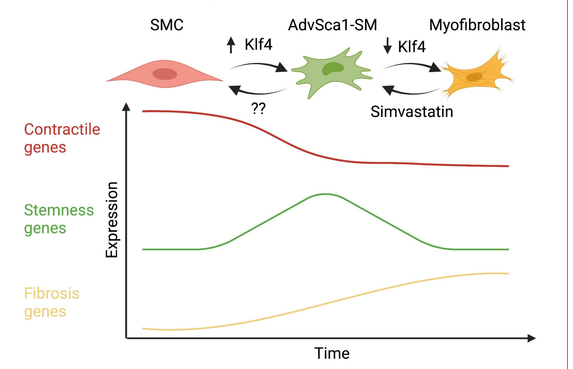


Figure 7. The reprogramming and myofibroblast transition of AdvSca1-SM cells.

References

1. MW Majesky, H Horita, A Ostriker, JN Regan, XR Dong, J Poczobutt, RA Nemenoff, and MCM Weiser-Evans (2017) Differentiated smooth muscle cells generate a subpopulation of resident vascular progenitor cells in the adventitia regulated by Klf4. *Circulation Research*, 120:296-311. PMC5250562

2. S Lu, A Jolly, K Strand, AM Dubner, MF Mutryn, K.S. Moulton, R.A. Nemenoff, M.W. Majesky, MCM Weiser-Evans (2020) Smooth muscle-derived progenitor cell myofibroblast differentiation through KLF4 downregulation promotes arterial remodeling and fibrosis. *J Clinical Investigation Insight*, 5(23):e139445. PMC7714399

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