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

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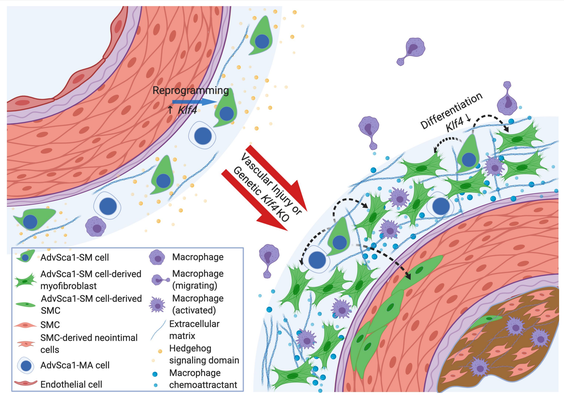
Background

- 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.
- **Flow cytometry**
- Flow cytometry analysis was performed with the Gallios Flow Cytometer at the CU Cancer Center Flow Cytometry Shared Resource core facility to examine the phenotype of 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 containing YFP ORF sequence. Seurat and Monocle3 R packages were used for the analysis of scRNA-seq data.
- **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

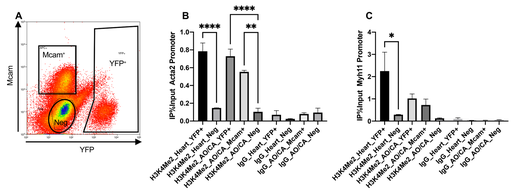


Figure 1. YFP$^+$ cells from *Gli1*-Cre^{ERT}-YFP mice exhibit high levels of smooth muscle cell-specific H3K4Me2 epigenetic lineage mark. Heart tissues were harvested from tamoxifen-treated *Gli1*-Cre^{ERT}-YFP mice. Single cell suspension was prepared from the tissue and cells labelled with APC-conjugated anti-Mcam antibody for FACS. Aortic and Carotid Artery (AO/CA) tissue were collected as smooth muscle cell rich positive control **(A).** YFP$^+$, Mcam$^+$ and double negative (Neg) 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.**

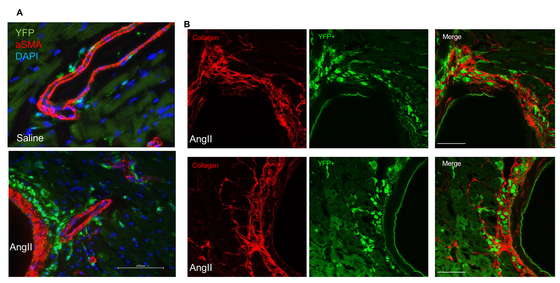


Figure 2. Perivascular AdvSca1-SM cells expand and infiltrate into the cardiac interstitium while exhibiting close association with collagen content. (A). Cardiac tissue sections from Saline (top) and AngII (bottom) treated *Gli1*-Cre^{ERT}-YFP mice were immunofluorescently stained with anti-GFP-FITC and anti-αSMA-Cy3 antibodies. Representative images were obtained with Keyence Microscope. Scale bar: 100 μm. **(B).** Label free SHG imaging was performed to visualize the collagen deposition (Red). YFP$^+$ cells were imaged and overlayed (Green). Scale bar: 50 μm. **The results suggest YFP$^+$ AdvSca1-SM cells proliferate and migrate to contribute to perivascular and interstitial cardiac fibrosis in response to AngII.**

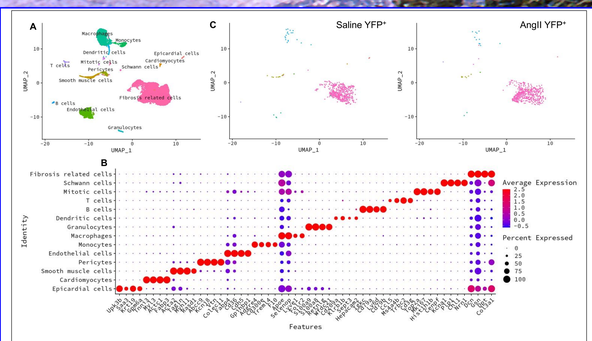


Figure 3. The great majority of AdvSca1-SM cells cluster with fibrosis related cells and exhibit significant phenotypic modulation upon AngII treatment. scRNA-seq experiment was performed with cardiac cells sorted from Saline/AngII treated *Gli1*-Cre^{ERT}-YFP mice as described in the Materials and Methods. (**A**). Uniform Manifold Approximation and Projection (UMAP) visualization of the scRNA-seq data set. **(B).** Dotplot of the top four expressed genes defining each type of cell cluster. **(C).** UMAP visualization of YFP$^+$ AdvSca1-SM cells in the scRNA-seq data from saline-treated (left) and AngII-treated (right) sample. **The results indicate YFP$^+$ AdvSca1-SM cells predominately exhibit phenotypes related to fibrosis. AngII treatment induces significant changes in the gene expression profile of AdvSca1-SM cells as indicated by the altered distribution of YFP$^+$ cells in the UMAP plot.**

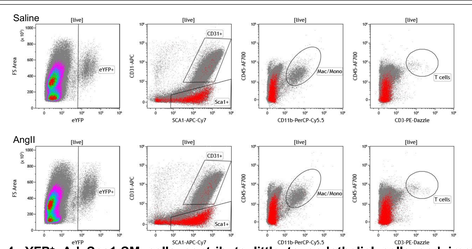


Figure 4. YFP$^+$ AdvSca1-SM cells contribute little to endothelial cells and immune cell populations. Single cell suspensions from cardiac tissue of Saline (top panel) and AngII (bottom panel) treated mice were stained with an antibody cocktail containing anti-Sca1-APC-Cy7, anti-CD31-APC, anti-CD45-A700, anti-CD11b-PerCP-Cy5.5 and anti-CD3-PE-Dazzle antibodies. YFP$^+$ cells were gated (left panels) and highlighted with red color. The majority of YFP$^+$ cell (red) are Sca1$^+$, CD31$^-$ (middle left panels), CD45$^-$, CD11b$^-$ (middle right panels) and CD3$^-$ (right panels). **The flow cytometry results agree with the transcriptomics data and altogether support the conclusion that AdvSca1-SM cells predominantly contribute to fibrosis related processes.**

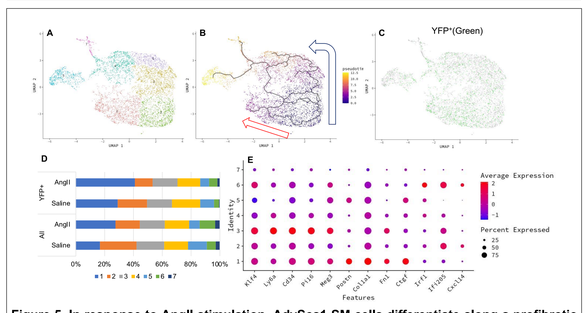


Figure 5. In response to AngII stimulation, AdvSca1-SM cells differentiate along a profibrotic trajectory, which is characterized by loss of expression of Klf4 and stemness genes and up-regulation of a myofibroblast gene signature. Cells in the Fibrosis related cell cluster (see Figure 3A) were selected for sub-clustering and trajectory analysis with the R package Monocle3. **(A).** UMAP visualization of sub-clusters of fibrosis related cells. **(B).** Cells were ordered along the pseudotime trajectory. Two main branches of the trajectory were identified originating from cluster 3 (red box arrow, from cluster 3 to 1, blue box arrow, from cluster 3 down the path of cluster 2, 6, 4, 5 and 7). **(C).** UMAP visualization of the cell clusters highlighting YFP$^+$ AdvSca1-SM cells in green. **(D).** Column graph showing percentage of cells in each cluster among all cells and YFP+ AdvSca1-SM cells in Saline/AngII treated samples. **(E).** Dotplot showing expression of select genes. **The trajectory analysis demonstrated the differentiation of AdvSca1-SM cells (cluster 3) to myofibroblasts (cluster 1) induced by AngII.**

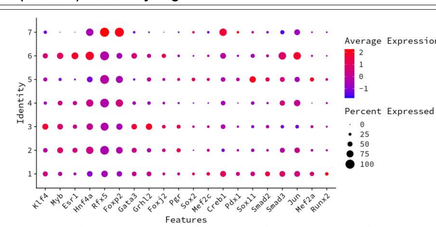
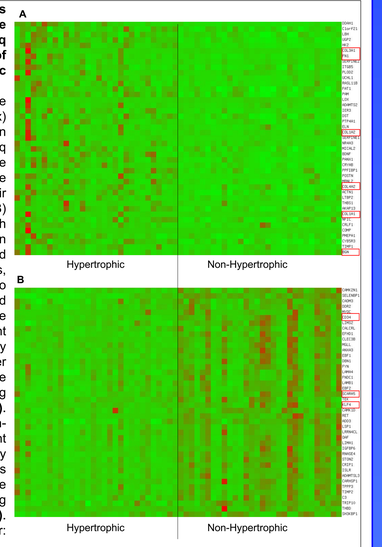


Figure 6. Transcription factor activity analysis reveals the key regulators dictating the myofibroblast differentiation of AdvSca1-SM cells. The R package DoRothEA was employed to infer transcription factor activity from the scRNA-seq gene expression data. Top transcription factors exhibiting significantly different activity between the clusters are shown in the Dotplot. **The results suggest a role for the transcription factors Klf4 and Smad2/3 in maintaining the progenitor phenotype of AdvSca1-SM cells and driving the myofibroblast transition, respectively.**

pert_iname	pert_id	pert_idose	pert_itime	norm_cs
mevastatin	BRD-K94441233	10 uM	24 h	-1.8385
simvastatin	BRD-K22134346	10 uM	24 h	-1.735
carbacyclin	BRD-K27499107	10 uM	24 h	-1.6676
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 7. Drugs of the Statin class, including Mevastatin and Simvastatin, are potential candidates for antagonizing the myofibroblast differentiation of AdvSca1-SM cells. Significantly up and down-regulated genes between cluster 3 and cluster 1 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.). **The results indicate the anti-fibrotic effect observed with statins may be due to its function in maintaining the AdvSca1-SM stem cell phenotype.**

Figure 8. The AdvSca1-SM cells and myofibroblasts gene signature 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 male subjects between the age of 50 and 59 with RNA-seq data from left ventricular tissues were selected for the analysis. The samples were ranked by their Natriuretic Peptide B (NPPB) expression level and 30 subjects with highest and lowest NPPB expression were designated as hypertrophic and non-hypertrophic samples, respectively. EdgeR was used to examine the differentially expressed genes. Genes up-regulated in the hypertrophic group exhibit significant overlap with genes selectively expressed by myofibroblast cluster (Cluster 1 in Figure 5A). The normalized count data of overlapping genes were shown as heatmap in **(A)**. Genes up-regulated in non-hypertrophic group exhibit significant overlap with genes selectively expressed by AdvSca1-SM cells (Cluster 3 in Figure 5A). The normalized count data of overlapping genes were shown as heatmap in **(B)**. Red: high expression, Green color: low expression.



The results strongly support the translational relevance of the AdvSca1-SM-myofibroblast transition in human cardiac hypertrophy and fibrosis.

Conclusions

- *Gli1*-CreERT-YFP mouse model specifically tracks smooth muscle cell-derived AdvSca1-SM progenitor cells in cardiac tissue.
- Cardiac AdvSca1-SM cells expand and are associated with perivascular and interstitial fibrosis in heart tissue of AngII-treated mice.
- In response to AngII stimulation AdvSca1-SM cells differentiate along a profibrotic trajectory, characterized by loss of stemness gene expression and up-regulation of myofibroblast gene signature. Transcription factors, such as Klf4 and Smad2/3, orchestrate the maintenance and myofibroblast differentiation of AdvSca1-SM cells.
- The AngII-induced profibrotic transcriptomic changes of AdvSca1-SM cells are recapitulated in human cardiac tissues exhibiting a gene signature of cardiac hypertrophy, emphasizing the translational significance of this phenotypic transition.
- Based on the scRNA-seq data, statins are predicted to inhibit the myofibroblast differentiation of AdvSca1-SM cells and therefore inhibit or reverse cardiac fibrosis.

References

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