The Chromatin Remodeler Brg1 Regulates Adventitial Progenitor Cell Myofibroblast Differentiation and Pathological Vascular Remodeling

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Background

- Cardiovascular diseases cause blood vessels to become excessively stiff. This leads to diminished vascular function and decreased quality of life.
- We discovered a unique population of stem/progenitor cells that reside in the outer adventitial layer of the blood vessel. These cells are derived from smooth muscle cells (SMCs) and express the stem marker Sca1 (**AdvSca1-SM cells**).
- In disease states, AdvSca1-SM cells differentiate into myofibroblasts and secrete extracellular matrix proteins and contribute to vascular stiffening.
- Brahma-related gene 1 (**Brg1**) is a chromatin remodeling protein that can displace histones to regulate DNA accessibility for transcription. Our previous data identified Brg1 being upregulated in AdvSca1-SM cells after acute vascular injury.

Hypothesis

Brg1 is activated in response to acute vascular injury and modulates chromatin to preferentially drive AdvSca1-SM cells towards the myofibroblast phenotype. Inhibition of Brg1 will block AdvSca1-SM cell myofibroblast differentiation and decrease pathologic vascular fibrosis.

Materials and Methods

AdvSca1-SM Reporter Mice

- The sonic-hedgehog transcriptional regulator, **Gli1**, is uniquely expressed by AdvSca1-SM cells as compared to other adventitial populations. Taking advantage of this, we developed a lineage-mapping system to permanently label AdvSca1-SM cells with the fluorescent reporter YFP enabling reliable tracking of AdvSca1-SM cells *in situ* (**Figure1**).
- To induce vascular remodeling, complete carotid ligation (**Figure 1**) was performed on the left carotid artery (CA). The carotid ligation is a well-characterized model to generate vascular lesions such as neointima formation, adventitial expansion, and vascular fibrosis. The right carotid artery is left uninjured and serves as an internal control.

Tissue Preparation for Immunofluorescence Microscopy

- Tissues were fixed in 4% paraformaldehyde and sectioned at 6μm. Incubation with primary antibodies was at 4C overnight. Samples were visualized with a Keyence Immunofluorescence microscope.

RNA Extraction and qPCR Experiments

- Total RNA was extracted from purified AdvSca1-SM cells. Sequence-specific primers were designed, and Quantitative real-time PCR was performed with a BioRad CFX96 Real Time System ThermoCycler.

Rigor and Reproducibility

- qPCR experiments were performed on 3 independent biological samples, with each sample containing RNA pooled from at least 10 mice (M and F).
- Power analyses are performed to determine the number of animals needed for *in vivo* studies for statistical significance. Descriptive statistics were calculated by ANOVA or Student t tests.

Results

AdvSca1-SM Cells significantly expand in the adventitia and contribute to pathological vascular remodeling after acute carotid ligation.

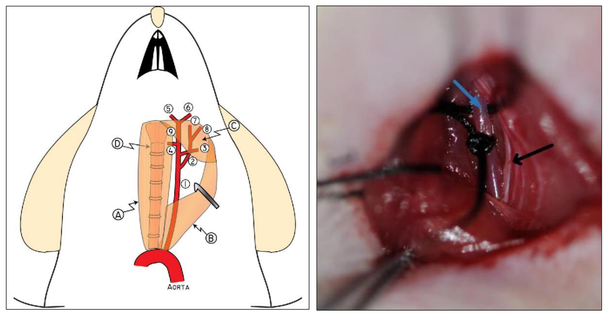


Figure 1. Carotid ligation is a model of acute vascular injury and induces AdvSca1-SM expansion. Inducible AdvSca1-SM-specific YFP reporter mice were treated with tamoxifen to induce YFP expression and subjected to complete ligation of the left CA. Arteries are harvested 4 weeks later for histological analysis. AdvSca1-SM cells expand in the adventitia in response to carotid ligation.

Pharmacologic inhibition of Brg1 attenuates injury-induced pathological vascular remodeling

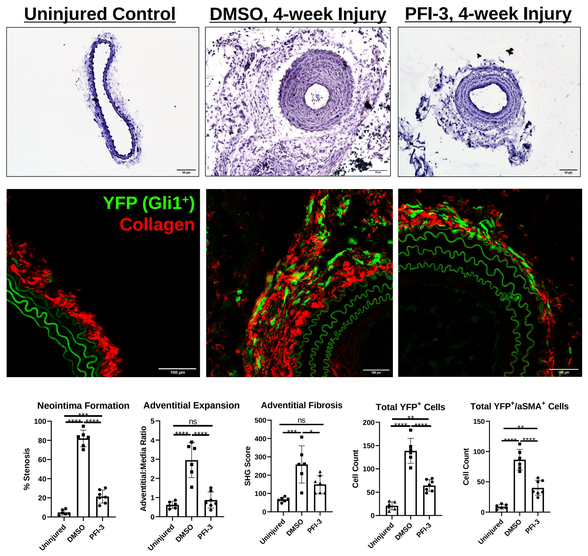


Figure 2. The Brg1 bromodomain inhibitor PFI-3 decreases adventitial expansion, neointima formation, and vascular fibrosis. AdvSca1-SM-specific YFP reporter mice were subject to carotid ligation injury and separated into 2 groups: control animals received a vehicle solution of 10% DMSO in corn oil, and experimental animals received 50mg/kg PFI-3 via oral gavage. Animals were treated every 4 days and vessels were harvested four weeks after ligation. Tissues were stained with hematoxylin and label-free second harmonic generation imaging was performed to label perivascular collagen deposition. *3 independent experiments were analyzed, and male and female mice were used equally. ANOVA was used to test for differences.*

Brg1 inhibition blunts TGF-β induced myofibroblast differentiation of AdvSca1-SM cells

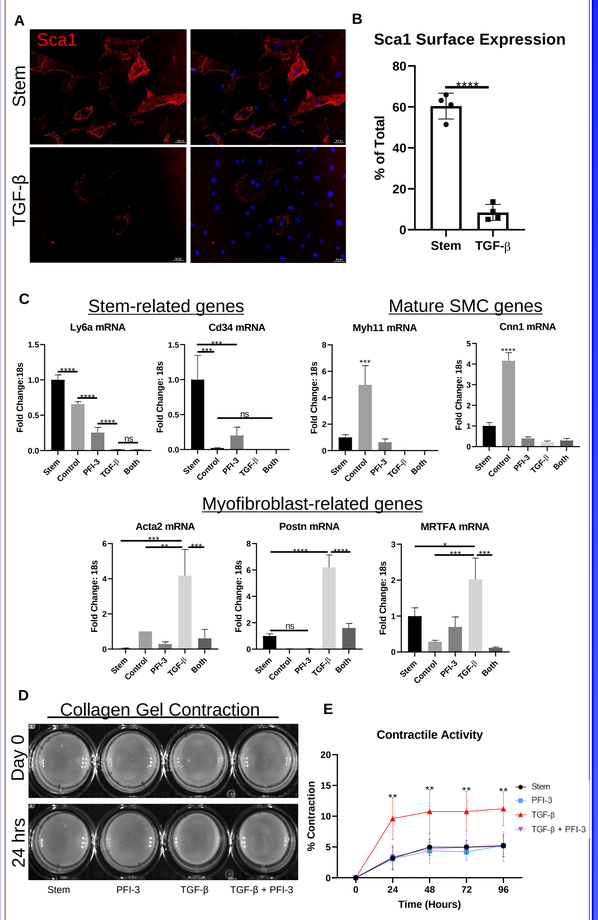


Figure 3. Brg1 inhibition blocks expression of myofibroblast related genes and contraction induced by TGF-β in cultured AdvSca1-SM cells.

AdvSca1-SM cells were cultured in stem cell media, stimulated with TGF-β (5ng/mL), or stimulated with TGF-β and PFI-3 (50μM) for 72hrs. Sca1 surface expression was examined by immunofluorescence microscopy and quantified (A, B). In separate studies, total RNA was harvested and subject to qPCR analysis (C). AdvSca1-SM cells were plated on compressible collagen gel matrices and stimulated with TGF-β and PFI-3. Images of collagen matrices were taken every 24 hours (D). Gel area for each well was determined using ImageJ and data are reported as percent contraction (E).

RNA sequencing of AdvSca1-SM cells reveals Brg1 bromodomain inhibition blunts TGF-β inducible genes related to fibrosis.

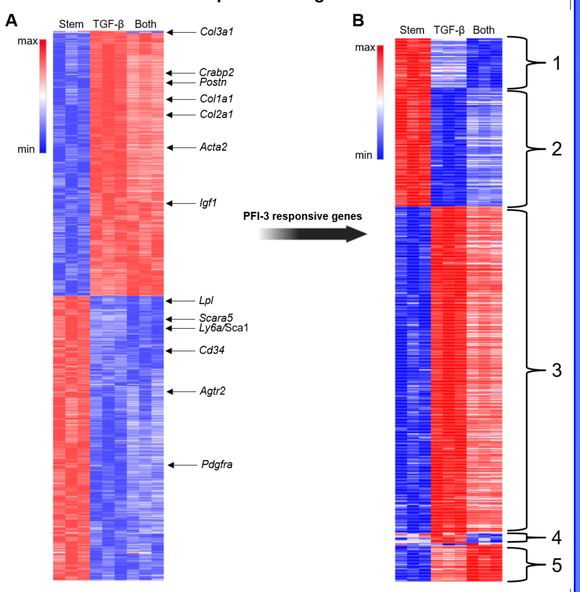


Figure 4. RNA sequencing of AdvSca1-SM cells reveals Brg1 bromodomain inhibition blunts TGF-β inducible genes related to fibrosis.
AdvSca1-SM cells were cultured in stem-cell media ("Stem"), treated with 50μM PFI-3 ("PFI-3"), 5ng/mL TGF-β ("TGF-β"), or TGF-β + PFI-3 ("Both") for 72 hours. RNA was harvested for whole RNA sequencing. Three independent replicates per condition were included for analysis. The heatmap shows all differentially expressed genes across experimental groups (A). The top 100 TGF-β induced genes most downregulated by PFI-3 were ranked and represented by the two-column heatmap (B). Gene Ontology Analysis of TGF-β_1 induced genes most downregulated by PFI-3 returned GO terms including "extracellular matrix structural constituent" and "collagen binding".

TGF-β induces Brg1 redistribution to fibrosis gene promoters, Brg1 recruitment is blocked by PFI-3

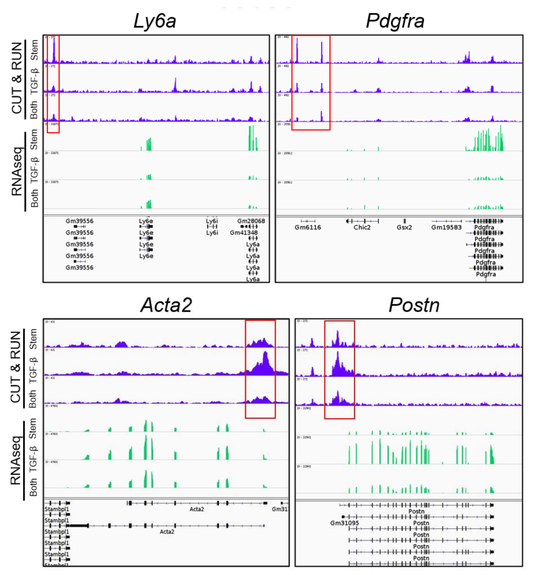
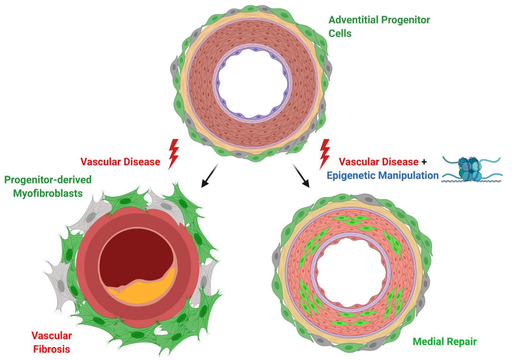


Figure 5. TGF-β induces Brg1 redistribution from stem-related loci to fibrosis-related gene promoters;PFI-3 blocks Brg1 recruitment to fibrosis-related gene promoters.

CUT&RUN Assays were performed to isolate and sequence DNA fragments bound to Brg1 to interrogate Brg1:DNA interactions in AdvSca1-SM cells at basal conditions, stimulated with TGF-β, or stimulated with TGF-β plus PFI3 for 72 hours. The data show that TGF-β stimulation dramatically decreases Brg1 occupancy on distal regulator elements of stem-related genes while Brg1 occupancy on fibrosis-related gene promoters significantly increased. The presence of PFI-3 led to a decreased amplitude of Brg1 signal at fibrosis-related gene promoters, suggesting PFI-3 is inhibiting Brg1 recruitment to fribrosis related genes, concordant with decreased gene expression measured by qPCR.

Conclusions



- **Pharmacological inhibition of Brg1 bromodomain attenuates ligation-induced vascular remodeling.**
- **Brg1 inhibition blunts TGF-β mediated induction of myofibroblast genes and TGF-β mediated contractile function *in vitro and* leads to downregulation of gene sets enriched for GO terms associated with fibrosis.**
- **Brg1 facilitates AdvSca1-SM-to-myofibroblast differentiation by redistributing from distal regulatory loci for stem-related genes to promoters of fibrosis-related genes.**

Targeting vascular progenitor cell differentiation may confer promising strategies to treat patients who are affected by chronic vascular fibrosis.

- **Future Directions**
  To test Brg1 inhibition *specifically* in AdvSca1-SM cells *in vivo*, shRNA constructs against Brg1 will be delivered locally to the adventitia of ligated carotid arteries.
  Identify the overlapping genes in the RNAseq and CUT&RUN datasets to identify Brg1-dependent genes.

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