The BET bromodomain inhibitor, (+)-JQ1, inhibits neointima formation following acute vascular injury via PTEN upregulation

Keith Strand, Sizhao Lu, Marie Mutryn, Allison Dubner, Austin Jolly,
Raphael Nemenoff, Maria Cavasin, Timothy McKinsey, Mary Weiser-Evans
Division of Renal Diseases and Hypertension, Department of Medicine
Consortium for Fibrosis Research and Translation, SOM
University of Colorado Anschutz Medical Campus

Background

- Pathological conditions of the cardiovascular system are characterized by significant vascular remodeling.
- In response to injury or disease, smooth muscle cells (SMCs) de-differentiate and take on a proliferative, pro-inflammatory, pro-fibrotic phenotype and play a key role in mediating the pathological remodeling processes.
- PTEN is a dual-specificity lipid and protein phosphatase that suppresses numerous signaling networks including pro-inflammatory and pro-fibrotic signaling pathways. In SMCs, nuclear PTEN also acts as an essential transcriptional co-factor to promote a differentiated, anti-inflammatory SMC phenotype.
- SMC-specific PTEN deletion exacerbates injury induced neointima formation and atherosclerosis, but systemically overexpressing PTEN protects against atherosclerosis and angiotensin II induced vascular remodeling (Figure 1-3).
- There are few compounds known to induce PTEN upregulation. Novel compounds that increase PTEN expression could be pursued as therapeutics to treat pathological vascular remodeling.
- JQ1 is an acetyl-lysine mimic that competitively inhibits BET bromodomain proteins (BRD2, BRD3, **BRD4** and BRD-T) from binding to acetylated lysine residues.
- BRD4 has been shown to directly interact with p300 and acetylated RelA/p65 to activate NF-κB transcription.

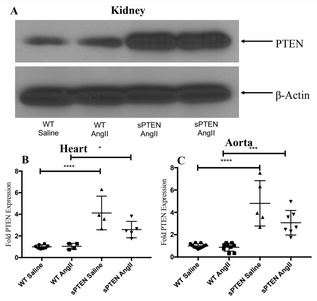


Figure 1. PTEN is systemically overexpressed in sPTEN mice. WT and sPTEN mice were subjected to AngII or saline treatment through subcutaneously implanted osmotic minipumps for 28 days. Mice were sacrificed and protein lysates were prepared from kidneys **(A)**, and RNAs extracted from the heart **(B)** and aortic tissues **(C)**. The expression of PTEN at protein level and mRNA level was examined by western blotting **(A)** and qPCR **(B&C)**. **The results confirm that sPTEN mice exhibit increased PTEN expression in various tissues.**

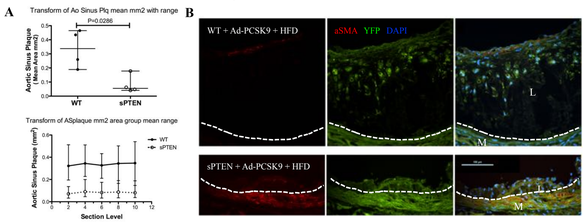


Figure 2. Repression of atherosclerosis lesion formation in sPTEN mice. (A) Atheroprone WT or sPTEN mice were fed a high fat diet for 20 weeks. Plaque areas were measured at 5 levels in the aortic sinus and averaged. **(B)** Tissues were stained for αSMA and YFP (SMC reporter). **SMC-derived intimal and medial cells (YFP+) lose expression of SMC markers in WT mice, but SMC phenotype is preserved in sPTEN and is associated with decreased lesion formation.** L = lesion, M = medial layer.

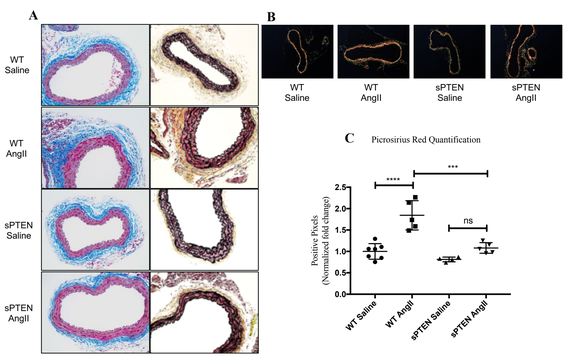


Figure 3. AngII-induced aortic collagen deposition is blunted in sPTEN mice. WT and sPTEN mice were treated with saline or AngII for 28 days. **(A)** Aortic tissues were stained with Masson's trichrome (left; blue stain) and Movat's pentachrome (right; yellow stain) to detect collagen deposition. Representative 10x images are shown. **(B)** PSR staining was performed and polarized images quantified with ImageJ and normalized to the aortic media outer perimeter. **(C)** Area of positive stain was expressed as fold change from saline-treated WT mice. **Results indicate that AngII promotes extensive aortic fibrosis in WT mice, but sPTEN mice are protected.**

Hypothesis

Pharmacologic PTEN upregulation is a novel therapeutic approach to treat vascular disease.

Methods and Materials

- **Inducible SMC-Specific PTEN Knockout Mice and JQ1 Injections**
  - 8-10 week old PTEN$^{flox/flox}$-*Myh11*-Cre^{ERT2};Rosa26-YFP (**PTEN iKO**) or control (**WT**) PTEN$^{+/+}$-*Myh11*-Cre^{ERT2};Rosa26-YFP mice received 1.5 mg tamoxifen injections i.p. for 7 consecutive days to induce PTEN knockout and YFP knock-in.
  - After 7 day tamoxifen washout, carotid artery ligation injury was performed and JQ1 injections began at time of injury
  - JQ1 was prepared in 100% DMSO, then diluted to 50 mg/mL in sterile saline + 10% 2-hydroxypropyl-β-cyclodextrin immediately prior to injection, then injected i.p. at 50 mg/kg 5x weekly for 3 weeks
  - Only male mice were used for *in vivo* experiments, as the *Myh11*-Cre^{ERT2} BAC transgene inserted onto the Y chromosome.
- **Inducible SMC-Specific BRD4 Knockout Mice**
  - 8-10 week old Brd4$^{fl/fl\#2}$;*Myh11*-Cre^{ERT2}-Rosa26-YFP (**BRD4 KO**) mice, BRD4 heterozygotes (**BRD4 Het**) Brd4$^{fl/+\#2}$;*Myh11*-Cre^{ERT2}-Rosa26-YFP or control wild type (**WT**) mice expressing *Myh11*-Cre^{ERT2};Rosa26-YFP, but WT for BRD4 mice received 1.5 mg tamoxifen injections i.p. for 7 consecutive days to induce BRD4 knockout and YFP knock-in.
  - Only male mice were used for *in vivo* experiments, as the *Myh11*-Cre^{ERT2} BAC transgene inserted onto the Y chromosome.
  - Carotid ligation was performed as described above.
- **P300 Chromatin Immunoprecipitation**
  - Primary rat aortic SMCs were maintained in MEM containing 10% FCS and plated at 1.8x10^6 cells in a 10 cm dish and allowed to adhere for 24 hr. Media was then changed to MEM containing 0.1% FCS for 48 hrs, then cells were stimulated with PDGF-BB (20 ng/mL) or vehicle for 24 hrs. Chromatin immunoprecipitation was performed using anti-p300 or mouse IgG.
  - Samples were prepared for qPCR then qPCR was performed using nine different primer sets that spanned a ~4000 bp region of the PTEN promoter.
- ***In vitro* JQ1 studies**
  - Primary rat aortic SMCs were stimulated with 1 μM JQ1 for 24-96 hrs prior to mRNA or protein analysis
  - Control or PTEN shRNA SMCs were pretreated with 1 μM JQ1 then co-stimulated with 1 μM JQ1 and PDGF-BB (20 ng/mL) for 24 hrs prior to RNA extraction

Results

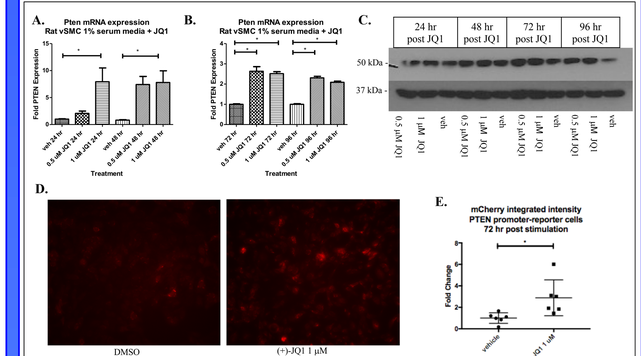


Figure 4. PTEN expression and PTEN promoter activity in JQ1-stimulated SMCs. **(A)** PTEN mRNA expression levels in SMCs stimulated with DMSO, 0.5 μM or 1 μM JQ1 for 24-48 hrs; Paired T test, *p<0.05. **(B)** PTEN mRNA expression levels in SMCs stimulated with DMSO, 0.5 μM or 1 μM JQ1 for 72-96 hrs; Paired T test, *p<0.05. **(A+B)** Data from three independent experimental replicates. **(C)** PTEN protein expression in SMCs stimulated with DMSO, 0.5 μM or 1 μM JQ1 for 24-96 hrs, with samples harvested every 24 hrs following JQ1 treatment. β-actin is used as a loading control. **(D)** Representative images showing endogenous mCherry signal intensity in PTEN promoter-reporter SMCs stimulated with DMSO or 1 μM JQ1 for 72 hrs. **(E)** Quantification of mCherry signal intensity in PTEN promoter-reporter SMCs after 72 hrs stimulation with vehicle or 1 μM JQ1, each point represents the average mCherry signal intensity calculated from all GFP+ cells in a single microscope field; Paired T test, *p<0.05.

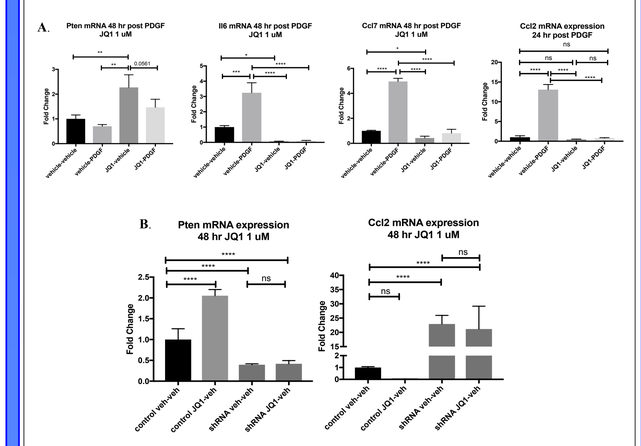


Figure 5. JQ1 inhibits SMC inflammation in response to de-differentiating stimuli in a PTEN-dependent manner. (A) mRNA expression of numerous inflammatory genes known to be induced during SMC phenotypic modulation, including *Pten*, *Il6*, *Ccl7* and *Ccl2* following co-stimulation with JQ1 and PDGF for 24 hrs; *p<0.05, **p<0.01; ***p<0.001, ****p<0.0001. **(B)** mRNA expression of *Pten* and *Ccl2* in PTEN shRNA or control shRNA SMCs treated with JQ1; ****p<0.0001.

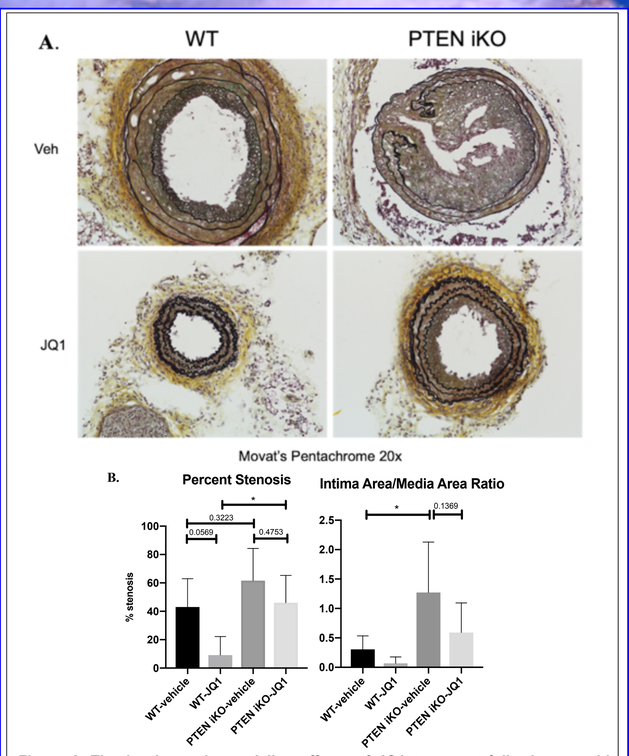


Figure 6. The *in vivo* anti-remodeling effects of JQ1 treatment following carotid ligation are blunted in mice with SMC-specific PTEN depletion. (A) Representative images of injured carotid arteries taken from WT or PTEN iKO mice treated with vehicle (top) or JQ1 50 mg/kg (bottom) for 3 weeks following surgery (see Methods). Representative images of injured carotid arteries stained with Movat's pentachrome stain are shown. **(B)** Quantification of percent stenosis (left) and I/M ratio (right) based on morphological analysis of injured carotid arteries in WT or PTEN iKO mice following 3 weeks of JQ1 or vehicle treatment. N=7 (WT vehicle), N=4 (WT JQ1), N=7 (PTEN iKO vehicle), N=7 (PTEN iKO JQ1). ANOVA with Bonferroni post hoc. *p<0.05.

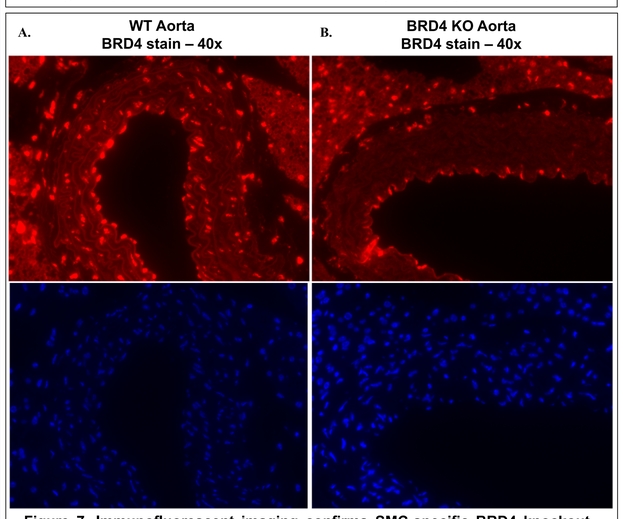


Figure 7. Immunofluorescent imaging confirms SMC-specific BRD4 knockout. BRD4$^{fl/fl}$-*Myh11*-CreER^{T2};Rosa26-YFP (BRD4 KO) or BRD4$^{+/+}$-*Myh11*-CreER^{T2};Rosa26-YFP (WT) were injected daily for one week with 1.5 mg tamoxifen to induce BRD4 knockout selectively in SMCs. **(A)** Paraffin embedded WT aorta sections stained for BRD4. **(B)** Paraffin embedded aorta from BRD4 KO mice stained for BRD4. DAPI images (bottom row) shown to visualize nuclei.

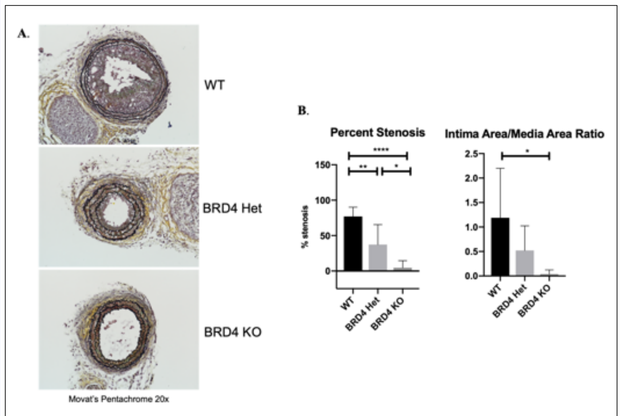


Figure 8. SMC-specific BRD4 deletion mimics the effects of JQ1 treatment and blocks neointima formation following carotid injury. (A) Representative images of injured carotid arteries taken from WT (top), BRD4 Het (middle) and BRD4 KO (bottom) mice 3 weeks after carotid ligation. Representative images from each group are shown using Movat's pentachrome staining. **(B)** Quantification of percent stenosis (left) and I/M ratio (right) based on morphological analysis of injured carotid arteries in WT, BRD4 Het and BRD4 KO mice 3 weeks post carotid ligation. N=7 (WT), N=6 (BRD4 Het), N=6 (BRD4 KO). ANOVA with Bonferroni post hoc. *p<0.05, **p<0.01, ****p<0.0001.

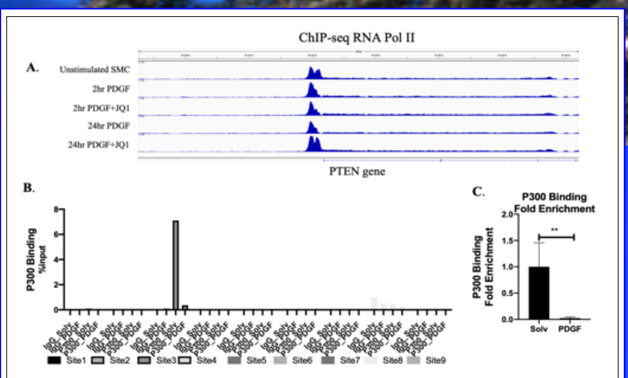


Figure 9. RNA Pol II and p300 occupancy at the PTEN promoter in PDGF stimulated SMCs as assessed by chromatin immunoprecipitation. (A) Analysis of publicly available ChIP-seq data (GSE111712) revealed that *in vitro* stimulation of SMCs with PDGF led to reduced docking and pausing of RNA Pol II on PTEN promoter, which was restored by JQ1 treatment. Graph represents the density of RNA Pol II reads per million mapped reads per bp. **(B)** 9 primer sets were designed to cover a 4000 bp region of the PTEN promoter for p300 ChIP-qPCR assay. The presence of site specific DNA eluted using a p300-specific antibody or IgG control antibody is expressed as a percent of total DNA input The results indicated that p300 interacts specifically at Site 3 of PTEN promoter and the binding is significantly reduced in PDGF-treated cells. **(C)** Quantification of p300 occupancy at site 3 of the PTEN promoter in SMCs treated with vehicle or PDGF. Fold enrichment was calculated as a percent of basal p300 interaction in solvent stimulated SMCs. Paired T test, **p<0.01.

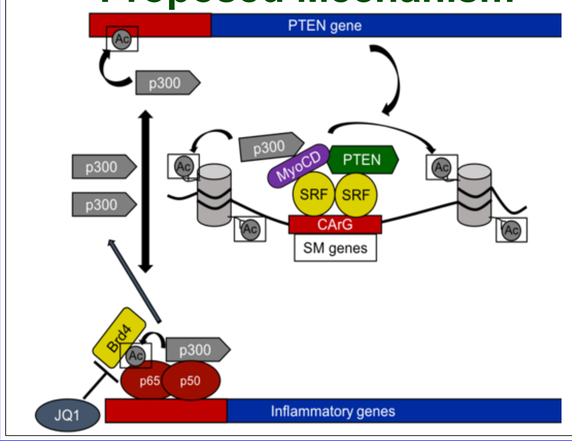
Conclusions

JQ1-mediated BET bromodomain inhibition results in PTEN upregulation and exerts PTEN-dependent anti-inflammatory effects in SMCs that inhibit neointimal development following acute vascular injury. Similarly, SMC-specific BRD4 deletion results in reduced neointimal development, highlighting the crucial role of BRD4 activity during pathological vascular remodeling. Our preliminary results suggest that JQ1 treatment inhibits the formation of p300/RelA/BRD4 complex, resulting in increased p300 interaction with the PTEN promoter to enhance promoter activity and upregulate PTEN expression.

Future Directions

- ❖ Continue investigation of BRD4-p300-RelA complex formation and p300 occupancy at PTEN promoter using co-immunoprecipitation and ChIP.
- ❖ Examine PTEN expression and inflammatory gene expression in BRD4 depleted SMCS.
- ❖ Generate SMC-specific PTEN-BRD4 double knockout mice, with the expectation that loss of PTEN will reverse the protective effects of BRD4 deletion.

Proposed Mechanism



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