Title: FAK Activation Promotes SMC Dedifferentiation via Increased DNA Methylation in Contractile Genes

In an effort to promote greater transparency in peer review, the authors and reviewers of this *Circulation Research* article have opted to post the original decision letter with reviewer comments to the authors and the authors' response to reviewers for each significant revision.

March 18, 2021

Prof. Ssang-Taek Steve Lim
University of South Alabama College of Medicine
Biochemistry and Molecular Biology
5851 North USA Dr.
Biochemistry, MSB2320
Mobile, Alabama 36688

RE: CIRCRES/2021/319066: FAK activation promotes SMC dedifferentiation via increased DNA methylation in contractile genes by stabilizing DNMT3A

Dear Dr. Lim:

Your manuscript has been carefully evaluated by 3 external reviewers and the editors as a Regular Article. Although of potential interest, the paper is not acceptable for publication in Circulation Research in its present form.

As you will gather from the reviews, the referees identified a number of substantive conceptual and methodological problems. The editors concur. Major issues include concerns about insufficient mechanistic insight, especially related to the in vivo situation and function of smooth muscle cells, and lack of important controls and data quantification.

Given the extensive new data that would be required for a responsive revision, we would understand if you were to decide to submit the paper elsewhere. Nevertheless, the editors see this manuscript as potentially important and would be willing to evaluate a revised version if you feel that you can effectively address the reviewers' concerns and are willing to perform the new experiments required. The paper would be reviewed again, with no assurance of acceptance.

As detailed in the reviewers' critiques, a responsive revision would require a substantial amount of new data. In particular, the editors feel that additional data would be necessary to strengthen the links between the mechanistic in vitro studies and the in vivo studies. Additional experiments on how FAK affects smooth muscle function would also be needed. In addition, all data should be quantified from a sufficient number of independent experiments, the results should be subjected to statistical analyses, and the requested controls should be included.

To read the comments to authors from the reviewers, please see below.

Please note that revised and resubmitted manuscripts are not assured of publication, and that fewer than 15% of all papers submitted to Circulation Research are eventually published.

Our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted; the editors may also choose to obtain additional opinions from new reviewers. If you need more than 90 days to submit a revised paper, please notify the editorial

office. In general, extensions over the revision time limit will not be granted except under special circumstances at the editors' discretion.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Each page of the revised manuscript should be numbered in the top right corner, using your manuscript number followed by /R1 to denote a first revision.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Revisions that do not conform to the current limits on numbers of words (8000 total) will be returned to the authors for abbreviation. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

We wish to thank you for having submitted this manuscript to Circulation Research.

AHA Scientific Sessions 2020: One World. Together for Science.
Even though #AHA20 is over, you can still register to access OnDemand through 2021.
https://professional.heart.org/en/meetings/scientific-sessions.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

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REVIEWER COMMENTS TO AUTHORS:

Reviewer #1:

In this manuscript, Dr. Jeong and colleagues reported that FAK inhibition promoted gene expression of smooth muscle contractile proteins in isolated smooth muscle cells (SMCs) and wire-injured mouse arteries. The authors demonstrated that nuclear FAK specifically suppressed DNMT3A protein stability which subsequently reduced methylation of the contractile genes. The authors provided convincing results that supported the critical role of DNMT3A-mediated transcription silencing of contractile genes in injured arteries. The work is thought to be impactful because the loss of contractile proteins is a major characteristic of smooth muscle cell de-differentiation that takes place in major vascular disorders. However, the work did not address several important mechanistic questions such as how FAK only binds to DNMT3A and increases its ubiquitination. The specific concerns and questions are in the following.

*1 (major). The recent publication from the group (ref 28) showed that FAK inhibition causes GATA4 degradation thus inhibits smooth muscle proliferation. Because increased smooth muscle proliferation and loss of contractile proteins are both observed in injured arteries, what role GATA4 may play in the suppression of contractile genes? Similarly, does diminished DNMT3A contribute to proliferation suppression caused by FAK inhibition?

*2 (major). In Fig. 1B and E, the changes of MYH11 protein levels were not all impressive. Please include quantifications of multiple Western blots to strengthen the claim that FAK inhibition increased levels of contractile proteins. Similarly, quantifications should be performed for other Western blots particularly those in Fig. 6A and B.

*3 (major). Fig. 1A&D showed that FAK inhibition increased the number of spindle shaped cells from ~10% to 40%. In other words, FAK inhibition failed to change cell shape in 60% of cells. It would be interesting to investigate whether contractile protein levels change in the cells that appeared unchanged.

*4 (major). Fig. 3C. As the authors correctly stated, DNMT3A and DNMT3B share a high degree of similarity, through what mechanism that FAK interacts with one but not the other? Furthermore, how does FAK binding cause ubiquitination of DNMT3A?

*5 (minor). The authors concluded that "FAK inhibitors may provide a new treatment option to block SMC phenotypic switching during vascular remodeling and atherosclerosis". However, FAK inhibitors also affect smooth muscle cell proliferation and migration and thus may adversely affect fibrous caps. The authors should consider all of the potential consequences of FAK inhibition and tune down this statement.

Reviewer #2:

This is a very elegant study on the role of FAK/DNMT3A regulation of SMC genes associated with phenotypic switching in SMC associated with atheromas. The data are novel, the studies are very well designed and the results are clearly delineated. This should be of great interest to clinicians and scientists interested in this topic. My suggestions to further increase the impact of the work are:

1. Functional assessment of SMCs: The phenotyping of the SMCs obtained from transgenic mice and with FAK inhibition are based on cell morphology and gene expression but there is no evidence that cell behavior is affected. I would request that the authors include contractility studies (gel contraction would be acceptable) as well as proliferation/survival/motility assay. In particular, be clear regarding the substrate used since fibronectin/gelatin/collagen or plastic can influence integrin dependent FAK activity. Finally, studies should also be done with human SMCs (healthy cells from femoral or carotid).

2. I am surprised that EMT genes were not affected by changes in FAK activity given the association between integrin/FAK signaling and EMT. While I recognize that the focus of the paper is on contraction genes, the phenotypic switch under study may also involves changes in lineage markers. This should be explored on the dataset and as part of the discussion.

3. Figure 8: In addition to brachiocephalic arteries samples, it would be important to show atheroma samples from other vascular beds to establish that this is a global mechanism of action.

4. The major question for me here is: How is the FAK nuclear pool being generated? There is FAK associated with integrin complexes which is activated via mechanotransduction (outside-in) but how are changes in the ECM triggering the redistribution of FAK into the nucleus? This should be explained and correlated with the studies suggested in (1).

Reviewer #3:

The authors present a detailed mechanistic study of the activation of the FAK-DNMT3A axis and its impact on SMC phenotype switching in vitro and in vivo. In vivo models are using the femoral injury and an athero model. An association is also assessed in a human cohort, but this data appears somewhat weaker.

Major comments:
1. While the experiments are broad and detailed, there is an over-reliance on interpretation of n=1 WB analysis across the entire manuscript, unless this is representative and the authors have all replicates for all blots. Further, there is a reliance on the individual immunostains and controls and replicates are not presented. This will be important to rectify.
2. In a similar manner, the KD in the model with the shRNA is not presented - how efficient in the delivery and KD in this setting?
3. The RNAseq analysis is from adventitia and the SMC layer. Why? While vessel wall plasticity is important, this paper focuses on SMC, so is there a contribution to other cells types? i.e. adventitial cells?
4. The introduction to the role of SMC in disease looks rather generic and dated. Please improve this first para. In a similar manner, it is not convincing from a clinical perspective about the extensive use of the femoral model. What is the clinical correlate to this injury that has unmet need? The athero aspect is far less detailed in the paper, but far more clinically relevant, although this is muddied by the complex mechanistic role of SMC in atheroma. The use of the human athero samples to match the mouse femoral model is a stretch as the mechanisms of SMC function in those settings is so vastly different. This needs re-positioning in the paper to have more relevance across mouse and human. This is a major weakness of the manuscript.
5. The reporting of the injury in the femoral model is superficial and could be improved further to assess vessel wall parameters in more detail and accuracy.
6. Can the authors show the FAK:DNMT3A interaction from the in vivo samples?

RESPONSES TO THE REVIEWERS

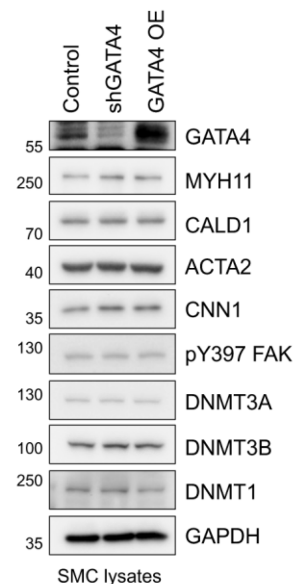
We are very grateful to the reviewers for their insightful comments and questions. In the revised manuscript, we have addressed all the reviewers' concerns by performing additional experiments and by rewriting the manuscript. We believe that our revised manuscript is much improved after the revision. Please note that changes in the main text were marked in blue.

Reviewer #1:

In this manuscript, Dr. Jeong and colleagues reported that FAK inhibition promoted gene expression of smooth muscle contractile proteins in isolated smooth muscle cells (SMCs) and wire-injured mouse arteries. The authors demonstrated that nuclear FAK specifically suppressed DNMT3A protein stability which subsequently reduced methylation of the contractile genes. The authors provided convincing results that supported the critical role of DNMT3A-mediated transcription silencing of contractile genes in injured arteries. The work is thought to be impactful because the loss of contractile proteins is a major characteristic of smooth muscle cell de-differentiation that takes place in major vascular disorders. However, the work did not address several important mechanistic questions such as how FAK only binds to DNMT3A and increases its ubiquitination. The specific concerns and questions are in the following.

1. The recent publication from the group (ref 28) showed that FAK inhibition causes GATA4 degradation thus inhibits smooth muscle proliferation. Because increased smooth muscle proliferation and loss of contractile proteins are both observed in injured arteries, what role GATA4 may play in the suppression of contractile genes?

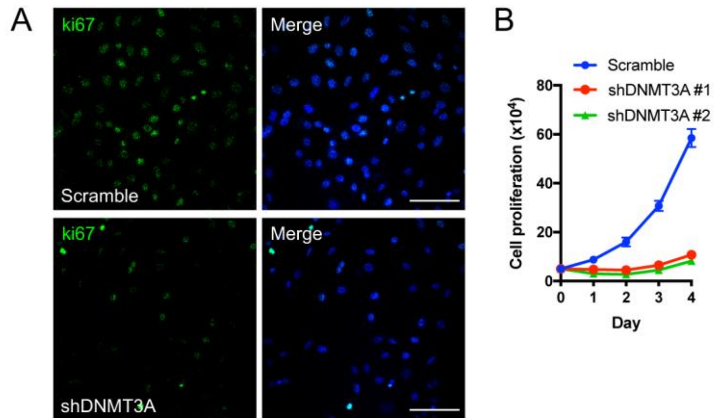
>We thank for the reviewer's thoughts regarding the role of GATA4 in contractile gene expression. Actually, we initially looked to see if GATA4 also regulated contractile gene expression. However, shGATA4 knockdown or GATA4 overexpression in SMCs had no effect contractile gene expression. This led us to conclude that increased GATA4 expression following injury does not suppress contractile genes. This led us to investigate other mechanism by which nuclear FAK promoted contractile gene expression. The new GATA4 data have been included in the results section (See Page 5) and as new Online Figure V.



Online Figure V. Changes in GATA4 levels do not alter contractile gene expression in SMCs. SMCs were infected either lentiviral shRNA GATA4 or GATA4 overexpressing plasmid and the cell lysates were subjected to immunoblotting as indicated antibodies (n=3). GAPDH was used a loading control.

2. Similarly, does diminished DNMT3A contribute to proliferation suppression caused by FAK inhibition?

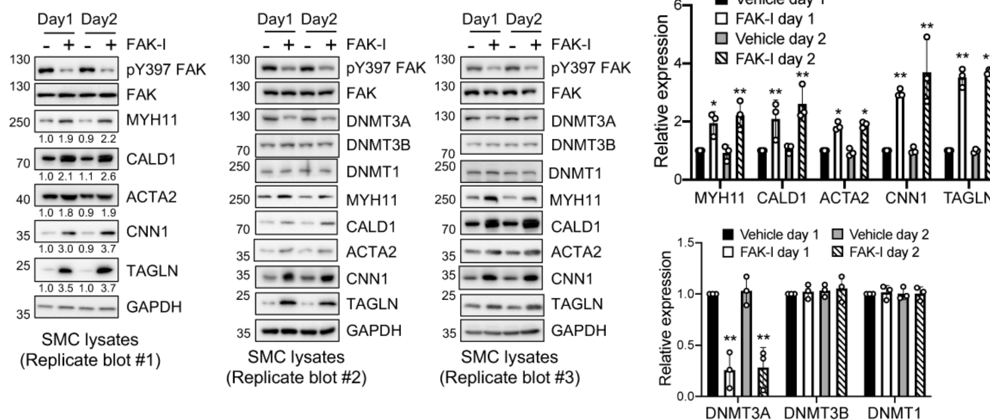
>To verify the contribution of DNMT3A expression to cell proliferation, we have performed proliferation assay and Ki67 staining using shRNA DNMT3A SMCs. We found that knockdown of DNMT3A significantly reduced SMC proliferation. In our shDNMT3A RNA-Seq dataset cell cycle genes such as cyclin D and E were among the top differentially changed genes compared to control. However, more studies are needed to determine how DNMT3A may promote SMC proliferation. We included these data as new Online Figure XXIII and stated in the result section (page 18).



Online Figure XXIII. Knockdown of DNMT3A slows SMC proliferation. SMCs were infected lentiviral shRNA DNMT3A for 2 days. Representative immunostaining of Ki-67 **(A)** and cell proliferation assays **(B)** were performed using two shRNA-mediated knockdown of DNMT3A SMCs (n=3). Scale bars: 100 μm

3. In Fig. 1B and E, the changes of MYH11 protein levels were not all impressive. Please include quantifications of multiple Western blots to strengthen the claim that FAK inhibition increased levels of contractile proteins. Similarly, quantifications should be performed for other Western blots particularly those in Fig. 6A and B.

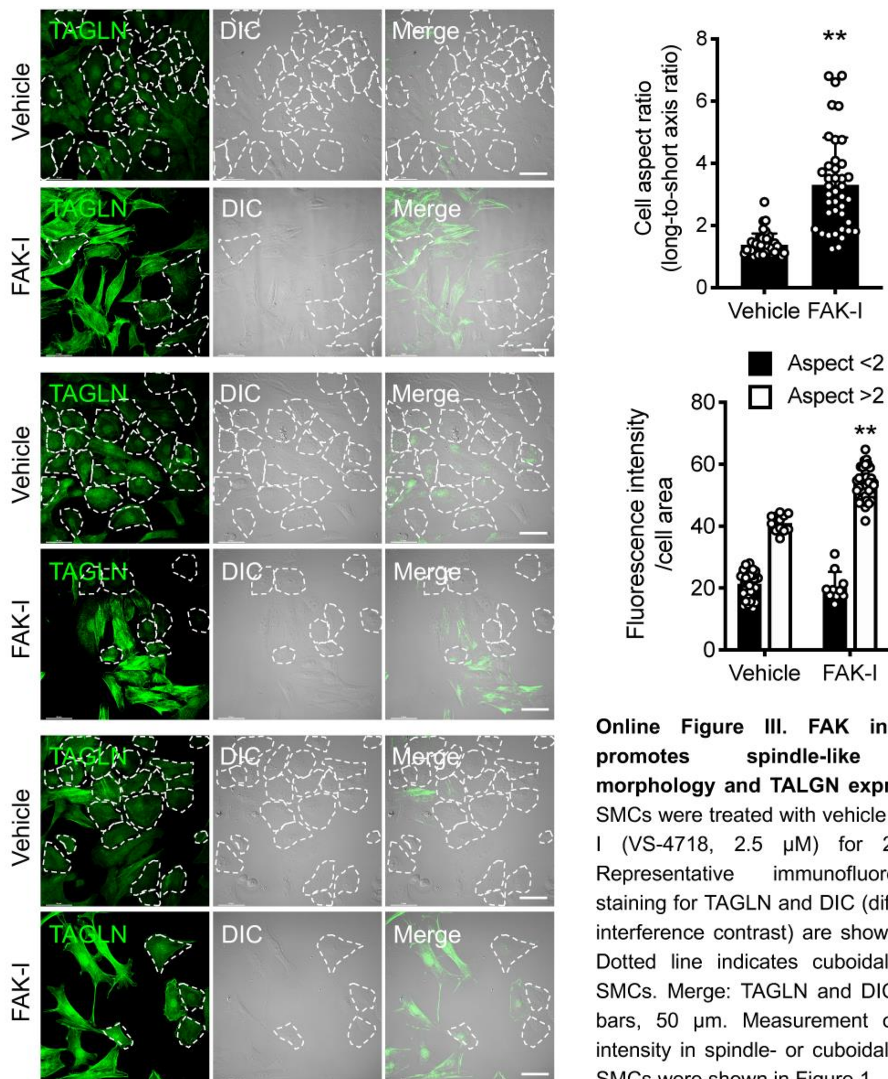
>As per the reviewer suggestion, we quantified the band density from multiple blots and indicated the average relative density levels in all blots presented in main figures and online figures. Also, we have shown one representative blot in the main figure and replicate blots in Online figures. Fig 1C is shown as an example here.



Online Figure II. FAK inhibition induces SMC specific contractile gene expression. FAK-I (VS-4718, 2.5 μM) was treated in SMCs for 2 days. Shown are replicates (#1, #2 and #3) of immunoblots of lysates of SMCs treated with or without FAK-I for active FAK (pY397 FAK), total FAK, DNMT3A, DNMT3B, DNMT1, MYH11, CALD1, ACTA2, CNN1, TAGLN, and GAPDH as loading control. Blots were quantified and plotted relative to GAPDH (n=3) and relative blot intensity were depicted in Figure 1 (replicate #1).

4. Fig. 1A&D showed that FAK inhibition increased the number of spindle shaped cells from ~10% to 40%. In other words, FAK inhibition failed to change cell shape in 60% of cells. It would be interesting to investigate whether contractile protein levels change in the cells that appeared unchanged.

>To compare the contractile protein levels in spindle- or cuboidal-shaped SMCs, we analyzed the correlation between cell shape visualized from DIC images and TAGLN fluorescence intensity in SMCs. First, we observed that cell aspect ratio (cell long and short axis ratio) in FAK-I group was significantly higher than that of vehicle SMCs, indicating that FAK inhibition altered SMC shape towards more elongated spindle-like morphology (aspect ratio over 2 was set as spindle-like morphology). Next, we found that the fluorescence intensity of TAGLN was significantly higher in FAK-I-treated spindle-liked SMCs. In summary, FAK inhibition promotes a more spindle shaped morphology by increasing SMCs contractile gene expression. We have included the data in Figure 1D and E, and Online Figure III.



Online Figure III. FAK inhibition promotes spindle-like SMC morphology and TALGN expression. SMCs were treated with vehicle or FAK-I (VS-4718, 2.5 μM) for 2 days. Representative immunofluorescence staining for TAGLN and DIC (differential interference contrast) are shown (n=4). Dotted line indicates cuboidal-shaped SMCs. Merge: TAGLN and DIC. Scale bars, 50 μm. Measurement of Tagln intensity in spindle- or cuboidal-shaped SMCs were shown in Figure 1.

5. Fig. 3C. As the authors correctly stated, DNMT3A and DNMT3B share a high degree of similarity, through what mechanism that FAK interacts with one but not the other?

>Although both DNMT3A and DNMT3B contain highly conserved amino acid sequences in their PWWP domains, ADD (cysteine-rich) domains, and carboxyl-terminal catalytic domains, the homology between the two proteins at the N-terminal variable region is only 28% (Okano and Li, Nat Genet. 1998;19:219-20, and Xie et al., Gene. 1999;236:87-95). Therefore, we predicted that FAK interaction sequence would be in a.a. 1-70 of DNMT3A which is not present in DNMT3B. To determine whether DNMT3A N-terminal domain associates with FAK, we overexpressed Myc-tagged DNMT3A full-length (WT), N-term 1-70, or N-term deletion constructs (71-912). We identified FAK indeed interacts with DNMT3A through DNMT3A 1-70 N-terminal domain. The data has been included in Figure 4E.

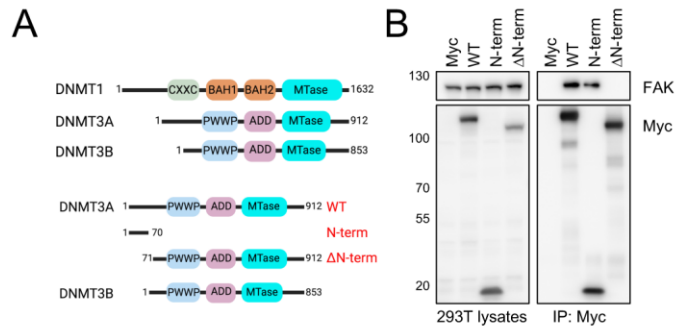


Figure 4E and Online Figure XXI. FAK interacts N-term of DNMT3A. (A) Schematic representation of DNMT1, DNMT3B, DNMT3A and its different deletion mutants. **(B)** 293T cells were transfected with Myc-tagged DNMT3A mutants and subjected to immunoprecipitation with anti-Myc antibody and subjected to immunoblotting with indicated antibodies (n=3).

6. Furthermore, how does FAK binding cause ubiquitination of DNMT3A?

>We have shown that scaffolding function of nuclear FAK promotes protein degradation by recruiting a target protein and its E3 ligases (e.g., p53 and Mdm-2, GATA4 and CHIP). To determine which E3 ligase accelerate DNMT3A turnover, we overexpressed potential E3 ligases for DNMT3A including TRAF6 (Yu et al., J Clin Invest. 2018;128:2376-2388), UHRF1 and 2 (Jia et al., Cell Discov. 2016;2:16007.) in 293T cells. Overexpression of TRAF6 greatly reduced DNMT3A levels compared to UHRF1 or 2. Thus, we further tested the possibility that TRAF6 might be the E3 ligase for DNMT3A ubiquitination via nuclear FAK in SMCs. FAK coimmunoprecipitation revealed the formation of ternary complex of TRAF6, DNMT3A, and FAK was observed only upon FAK inhibition condition, and this association was further increased by MG132. We have included the data in Figure 4D and Online Figure XXIA.

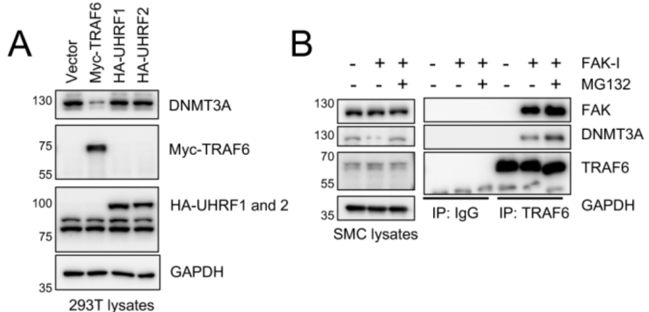


Figure 4D and Online Figure XXI. E3 ligase TRAF6 regulates DNMT3A protein expression. (A) 293T cells were transfected Myc-tagged TRAF6, and HA-tagged UHRF1 or UHRF2. The cell lysates were subjected to immunoblotting with as indicated antibodies (n=3). **(B)** SMCs were treated with FAK-I only or together with MG132 (20 μM) for 6 h. Lysates were immunoprecipitated with control IgG, or anti-TRAF6, and subjected to immunoblotting with indicated antibodies (n=3).

7. The authors concluded that "FAK inhibitors may provide a new treatment option to block SMC phenotypic switching during vascular remodeling and atherosclerosis". However, FAK inhibitors also affect smooth muscle cell proliferation and migration and thus may adversely affect fibrous caps. The authors should consider all of the potential consequences of FAK inhibition and tune down this statement.

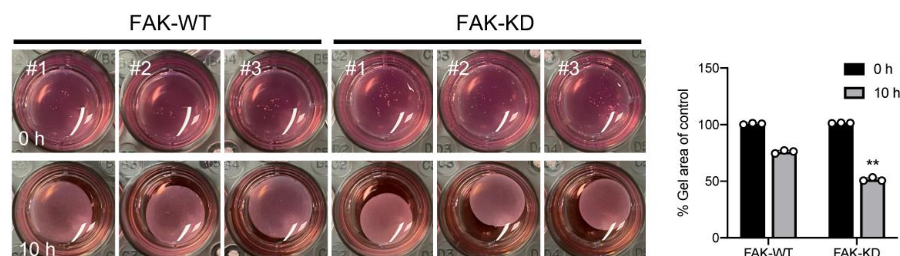
>We revised the statement as below by considering other potential limitations in using FAK inhibitor on the fibrous cap stability.
“FAK inhibitors may provide a new treatment option to block SMC dedifferentiation during vascular remodeling and atherosclerosis. Although blocking SMC dedifferentiation in atherosclerosis would be beneficial to reduce intimal thickening, FAK inhibitors also affect smooth muscle cell proliferation and migration and it may adversely affect the formation of a stable fibrous cap.”

Reviewer #2:

This is a very elegant study on the role of FAK/DNMT3A regulation of SMC genes associated with phenotypic switching in SMC associated with atheromas. The data are novel, the studies are very well designed and the results are clearly delineated. This should be of great interest to clinicians and scientists interested in this topic. My suggestions to further increase the impact of the work are:

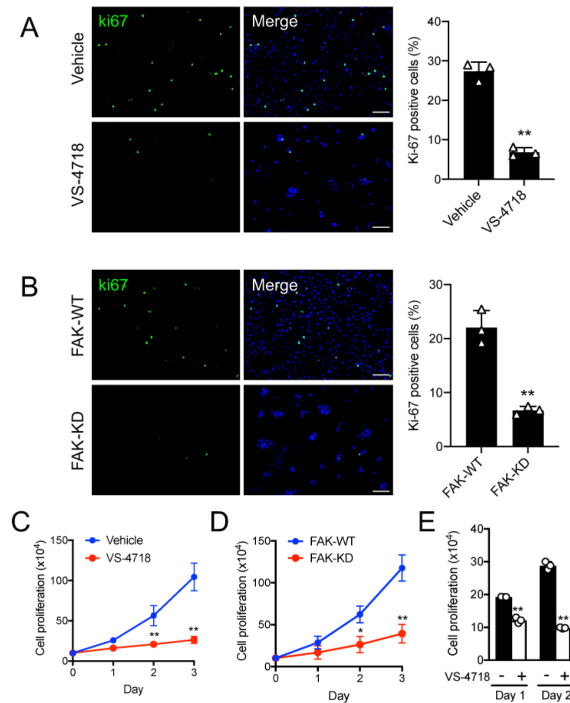
1. Functional assessment of SMCs: The phenotyping of the SMCs obtained from transgenic mice and with FAK inhibition are based on cell morphology and gene expression but there is no evidence that cell behavior is affected. I would request that the authors include contractility studies (gel contraction would be acceptable) as well as proliferation/survival/motility assay.

>We have performed collagen gel contraction assay using FAK-WT and FAK-KD SMCs isolated from FAK genetic mouse. FAK-WT SMCs showed a minimal contraction (by measuring the area of the floating gel). However, interestingly FAK-KD SMCs induced 2-fold faster contraction compared with FAK-WT. This matches with the low levels of DNMT3A in FAK-KD (Figure 3E) and with the high levels of contractile proteins in FAK-KD (Figure 1G).



Online Figure VI. Loss of FAK activity and DNMT3A induces collagen gel contraction. FAK-WT or FAK-KD SMCs were embedded in collagen gel for 10 h. Representative images (left panel) from the collagen gel contraction are shown. The relative collagen gel areas (right) were calculated and plotted by dividing the initial gel areas (±SEM, n=3, **P<0.005 vs control group, two-way ANOVA followed by Sidak multiple comparisons test).

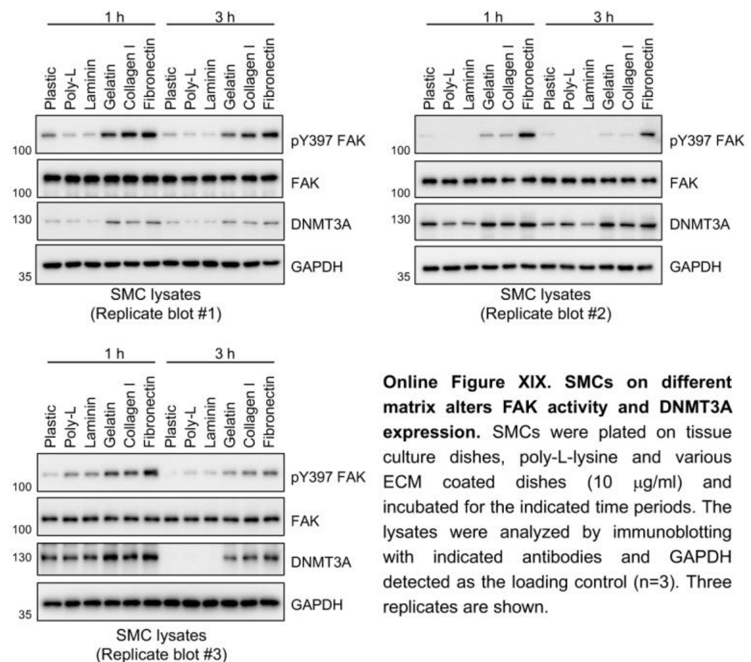
We also previously performed cell proliferation (Jeong et al., Circ Res. 2019;125:152-166, and Jeong et al., Cardiovasc Res. 2021;cvab132), and migration assays. FAK inhibition reduced both mouse SMC and human SMC proliferation and migration.



Reviewer only Figure. FAK inhibition reduces mouse SMCs and human coronary artery smooth muscle cell (hCASMC) proliferation. Representative immunostaining of Ki-67 were performed with/without FAK inhibitor (VS-4718, 2.5 μM) for 48 h **(A)** or genetic FAK-WT and -KD mouse SMCs **(B)**. The percentage of Ki-67 positive cells was calculated for each square separately (± SEM, n=3, **P<0.005 vs. vehicle, paired t-test). Mouse SMCs with FAK inhibitor (VS-4718, 2.5 μM) **(C)**, FAK-WT and FAK-KD mouse SMCs **(D)**, or hCASMC **(E)** counted on the indicated days (± SD, n=3, *P<0.01, **P<0.005, two-way ANOVA followed by Sidak multiple comparisons test).

In particular, be clear regarding the substrate used since fibronectin/gelatin/collagen or plastic can influence integrin dependent FAK activity.

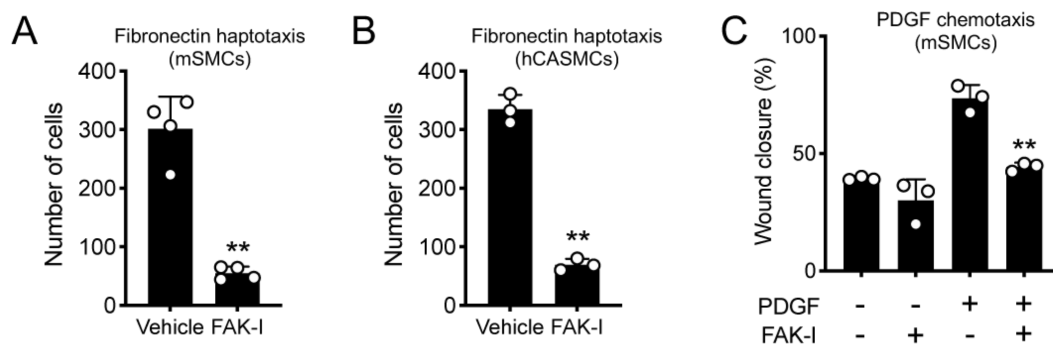
>We have used various matrix to evaluate FAK activity in SMCs. While we found that the levels of FAK activation was lower on laminin, collagen, or gelatin, compared to fibronectin matrix. In addition, the higher degree of FAK activation is correlated with a lower DNMT3A expression. We have included the data in Online Figure XIX.



Online Figure XIX. SMCs on different matrix alters FAK activity and DNMT3A expression. SMCs were plated on tissue culture dishes, poly-L-lysine and various ECM coated dishes (10 μg/ml) and incubated for the indicated time periods. The lysates were analyzed by immunoblotting with indicated antibodies and GAPDH detected as the loading control (n=3). Three replicates are shown.

Finally, studies should also be done with human SMCs (healthy cells from femoral or carotid).

>As we have already evaluated the effect of FAK inhibition on proliferation and migration human SMCs (healthy human coronary arterial SMCs), we have added the unpublished haptotaxis data using human SMCs in Online Figure IV.

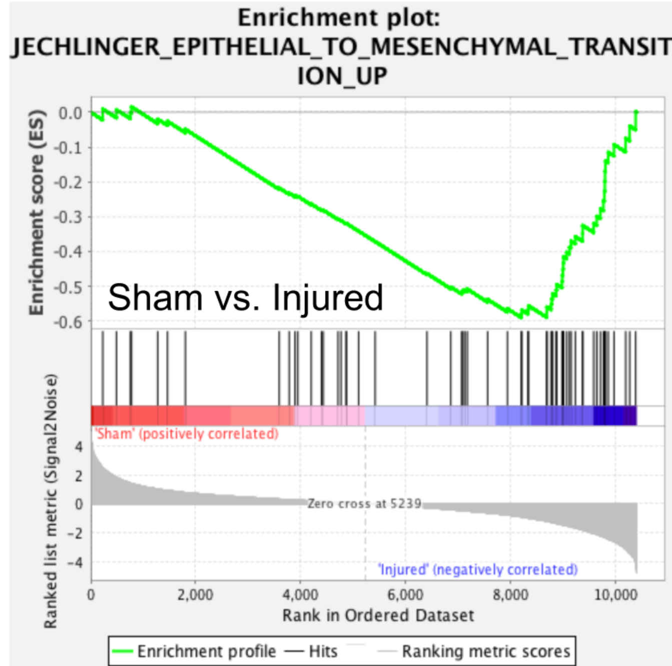


Online Figure IV. FAK inhibition reduces SMC migration. Number of mouse SMCs **(A)** or hCASMCs **(B)** that migrated toward fibronectin (10 ng/μl) with or without FAK-I (VS-4718, 2.5 μM) for 6 h were enumerated (±SD, n=3 or 4, unpaired t-test, ** p<0.005 vs vehicle). **(C)** Shown is percent wound closure after 24 h of mouse SMCs stimulated with PDGF-BB (20 ng/ml) with or without FAK-I (±SEM, n=3, **P<0.005 vs PDGF, two-way ANOVA followed by Sidak multiple comparisons test).

2. I am surprised that EMT genes were not affected by changes in FAK activity given the association between integrin/FAK signaling and EMT. While I recognize that the focus of the paper is on contraction genes, the phenotypic switch under study may also involves changes in lineage markers. This should be explored on the dataset and as part of the discussion.

>We only showed top five changes (up or down) in our KEGG analysis from RNA seq data. Indeed, the gene pathway analysis revealed that EMT gene sets (*Dab2*, *Lamb1*, *Col3a1*, *Mmp2*, *Mmp12*, *Ccl2*, and *Cxcl2*) were significantly decreased upon FAK inhibition. Now we have mentioned these genes in the results section.

A

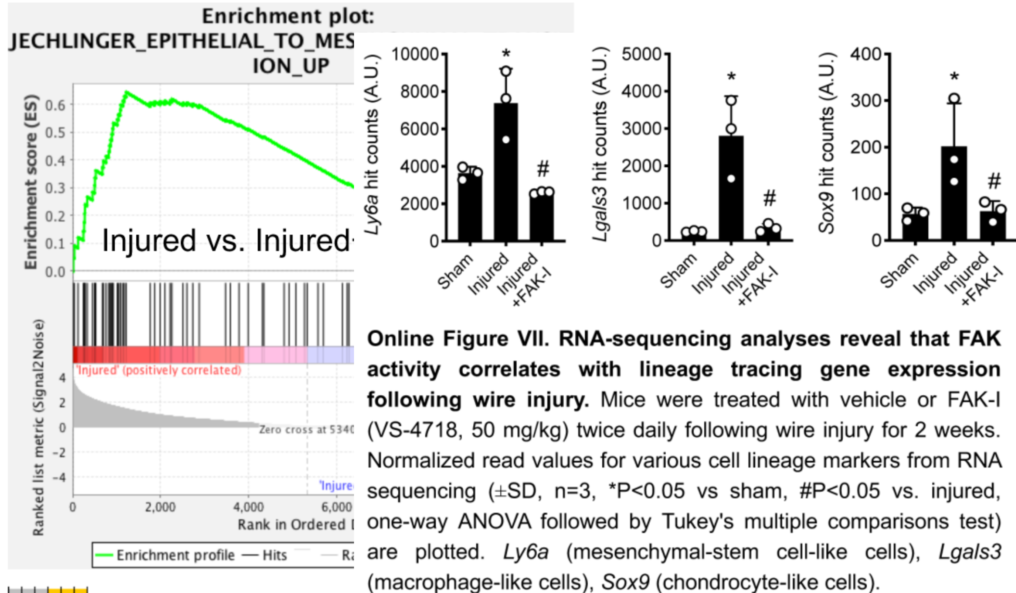


Veh_Un_1 Veh_Un_2 Veh_Un_3 Veh_In_1 Veh_In_2 Veh_In_3

SampleName		
VLDLR	VLDLR	very low density lipoprotein receptor
RRAS	RRAS	related RAS viral (r-ras) oncogene homolog
VIM	VIM	vimentin
CCK	CCK	cholecystokinin
TNXB	TNXB	tenascin XB
HTRA1	HTRA1	HtrA serine peptidase 1
SRM	SRM	spermidine synthase
CFH	CFH	complement factor H
GALK1	GALK1	galactokinase 1
CYP1B1	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
ADA	ADA	adenosine deaminase
SDC2	SDC2	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)
TNC	TNC	tenascin C (hexabrachion)
MTHFD2	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
PDGFRB	PDGFRB	platelet-derived growth factor receptor, beta polypeptide
FMO1	FMO1	flavin containing monooxygenase 1
PMP22	PMP22	peripheral myelin protein 22
COL6A2	COL6A2	collagen, type VI, alpha 2
DDR2	DDR2	discoidin domain receptor family, member 2
INHBA	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)
COL6A1	COL6A1	collagen, type VI, alpha 1
HIF1A	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
GBP4	GBP4	guanylate binding protein 4
PTGS1	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
SERPINH1	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
SLC3A2	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
PCOLCE	PCOLCE	procollagen C-endopeptidase enhancer
CXCL2	CXCL2	chemokine (C-X-C motif) ligand 2
CDH15	CDH15	cadherin 15, M-cadherin (myotubule)
GAS1	GAS1	growth arrest-specific 1
PHGDH	PHGDH	phosphoglycerate dehydrogenase
S100A8	S100A8	S100 calcium binding protein A8
ASNS	ASNS	asparagine synthetase
CTSZ	CTSZ	cathepsin Z
MMP13	MMP13	matrix metallopeptidase 13 (collagenase 3)
B2M	B2M	beta-2-microglobulin
SLPI	SLPI	secretory leukocyte peptidase inhibitor
ISG15	ISG15	ISG15 ubiquitin-like modifier
SPARC	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
CDH2	CDH2	cadherin 2, type 1, N-cadherin (neuronal)
PROCR	PROCR	protein C receptor, endothelial (EPCR)
STAT1	STAT1	signal transducer and activator of transcription 1, 91kDa
PDGFRA	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
IFIT3	IFIT3	interferon-induced protein with tetratricopeptide repeats 3
IRF7	IRF7	interferon regulatory factor 7
MMP12	MMP12	matrix metallopeptidase 12 (macrophage elastase)
IFITM3	IFITM3	interferon induced transmembrane protein 3 (1-8U)
SDC1	SDC1	syndecan 1
CCL2	CCL2	chemokine (C-C motif) ligand 2
IL11	IL11	interleukin 11
DCN	DCN	decorin
MMP2	MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
PTPN22	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
DAB2	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
COL3A1	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
LAMB1	LAMB1	laminin, beta 1
UPP1	UPP1	uridine phosphorylase 1
BCL3	BCL3	B-cell CLL/lymphoma 3
PLA2G7	PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
PPIC	PPIC	peptidylprolyl isomerase C (cyclophilin C)
SNAI1	SNAI1	snail homolog 1 (Drosophila)
C4B	C4B	complement component 4B (Childo blood group)
CD68	CD68	CD68 molecule

Speaking of the lineage marker changes, we identified mesenchymal stem cell (Ly6a/Sca1), macrophage (Lgal3), and chondrocyte (Sox9) lineage marker as top differentially expressed genes upon FAK inhibitor treated group compared to injured samples. We

B



Online Figure VII. RNA-sequencing analyses reveal that FAK activity correlates with lineage tracing gene expression following wire injury. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. Normalized read values for various cell lineage markers from RNA sequencing (±SD, n=3, *P<0.05 vs sham, #P<0.05 vs. injured, one-way ANOVA followed by Tukey's multiple comparisons test) are plotted. *Ly6a* (mesenchymal-stem cell-like cells), *Lgals3* (macrophage-like cells), *Sox9* (chondrocyte-like cells).

Veh_In_1 Veh_In_2 Veh_In_3 FAK_I_In_1 FAK_I_In_2 FAK_I_In_3

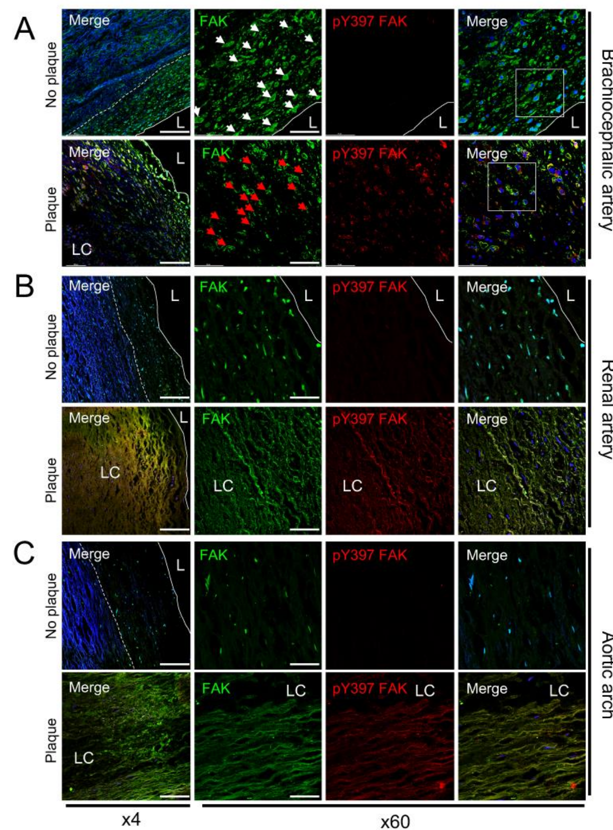
SampleName		
CD68	CD68	CD68 molecule
C4B	C4B	complement component 4B (Childo blood group)
SNAI1	SNAI1	snail homolog 1 (Drosophila)
UPP1	UPP1	uridine phosphorylase 1
PPIC	PPIC	peptidylprolyl isomerase C (cyclophilin C)
DAB2	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
DCN	DCN	decorin
LAMB1	LAMB1	laminin, beta 1
COL3A1	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
PLA2G7	PLA2G7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)
IL11	IL11	interleukin 11
MMP2	MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
ASNS	ASNS	asparagine synthetase
PHGDH	PHGDH	phosphoglycerate dehydrogenase
IFITM3	IFITM3	interferon induced transmembrane protein 3 (1-8U)
GAS1	GAS1	growth arrest-specific 1
PDGFRA	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
BCL3	BCL3	B-cell CLL/lymphoma 3
CTSZ	CTSZ	cathepsin Z
CCL2	CCL2	chemokine (C-C motif) ligand 2
SDC1	SDC1	syndecan 1
IFIT3	IFIT3	interferon-induced protein with tetratricopeptide repeats 3
SPARC	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
CDH2	CDH2	cadherin 2, type 1, N-cadherin (neuronal)
STAT1	STAT1	signal transducer and activator of transcription 1, 91kDa
PCOLCE	PCOLCE	procollagen C-endopeptidase enhancer
MMP12	MMP12	matrix metallopeptidase 12 (macrophage elastase)
PROCR	PROCR	protein C receptor, endothelial (EPCR)
PTPN22	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
MMP13	MMP13	matrix metallopeptidase 13 (collagenase 3)
CDH15	CDH15	cadherin 15, M-cadherin (myotubule)
IRF7	IRF7	interferon regulatory factor 7
SERPINH1	SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)
SLPI	SLPI	secretory leukocyte peptidase inhibitor
CXCL2	CXCL2	chemokine (C-X-C motif) ligand 2
GBP4	GBP4	guanylate binding protein 4
ISG15	ISG15	ISG15 ubiquitin-like modifier
FMO1	FMO1	flavin containing monooxygenase 1
PMP22	PMP22	peripheral myelin protein 22
SLC3A2	SLC3A2	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2
COL6A1	COL6A1	collagen, type VI, alpha 1
PTGS1	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
DDR2	DDR2	discoidin domain receptor family, member 2
CFH	CFH	complement factor H
COL6A2	COL6A2	collagen, type VI, alpha 2
GALK1	GALK1	galactokinase 1
HIF1A	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
CYP1B1	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
SRM	SRM	spermidine synthase
PDGFRB	PDGFRB	platelet-derived growth factor receptor, beta polypeptide
B2M	B2M	beta-2-microglobulin
S100A8	S100A8	S100 calcium binding protein A8
SDC2	SDC2	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)
ADA	ADA	adenosine deaminase
TNC	TNC	tenascin C (hexabrachion)
MTHFD2	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase
CCK	CCK	cholecystokinin
INHBA	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)
HTRA1	HTRA1	HtrA serine peptidase 1
TNXB	TNXB	tenascin XB
VIM	VIM	vimentin
RRAS	RRAS	related RAS viral (r-ras) oncogene homolog
VLDLR	VLDLR	very low density lipoprotein receptor

Reviewer only Figure. FAK inhibition alters epithelial mesenchymal transition related genes expression post wire injury. Gene Set Enrichment Analysis (GSEA) shows enrichment of EMT (epithelial mesenchymal transition) related genes of RNAseq data among sham vs. injured **(A)** or Injured vs. Injured+FAK-I **(B)**. GSEA validated enhanced activity Jechlinger epithelial to mesenchymal transition up.

have included the data in Online Figure VIIE.

3. Figure 8: In addition to brachiocephalic arteries samples, it would be important to show atheroma samples from other vascular beds to establish that this is a global mechanism of action.

>We have tested FAK localization and FAK activity in different vascular beds of human atherosclerosis samples including renal arteries and aortic arch. We have verified that nuclear FAK localization is consistent in all various healthy arteries, but not in the diseased arteries. The new data have been included in Online Figure XVI.



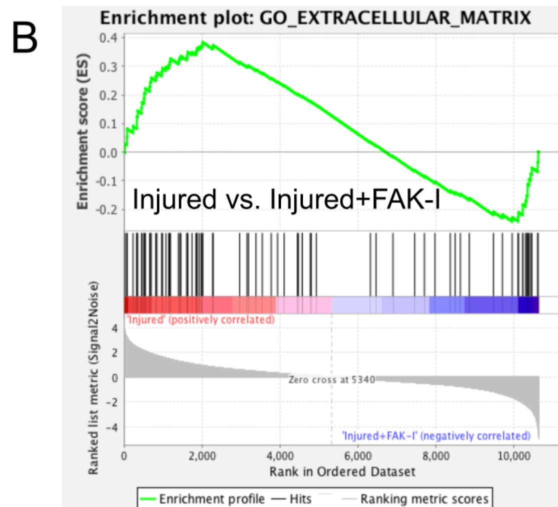
Online Figure XVI. Cytoplasmic and active FAK is observed in human atherosclerotic specimens from different vessel beds compared to healthy samples. Human brachiocephalic artery **(A)**, renal artery **(B)**, aortic arch **(C)** were excised postmortem, and frozen sections were made for immunostaining. Representative immunostainings for FAK, pY397 FAK, and DAPI are shown (n=5). White arrows: nuclear FAK. Red arrows: cytoplasmic FAK. Merge: Green, red, and DAPI (blue). Dashed line, boundary between media and diffuse intima. White line indicates endothelial layer. L, lumen; LC, lipid core. Scale bars: x4, 100 μm; or x60, 50 μm. Boxed areas are shown in **Figure 3C**.

4. The major question for me here is: How is the FAK nuclear pool being generated? There is FAK associated with integrin complexes which is activated via mechanotransduction (outside-in) but how are changes in the ECM triggering the redistribution of FAK into the nucleus? This should be explained and correlated with the studies suggested in (1).

>We thank the reviewer for bringing up this important point. Our long-term goal is to elucidate why FAK is predominantly in the nucleus in vivo and how it translocates to the cytoplasm. We would like to reframe the reviewer's question to discuss FAK subcellular localization based on our current knowledge and recent findings. We found that FAK is predominantly inactive and localized in nucleus of SMCs of healthy arteries (Jeong et al., Circ Res. 2019;125:152-166). We think that vessel injury activates FAK by increasing the ECM matrix production which causes a high stiffness that will activate integrin FAK or by promoting synthetic ECM such as fibronectin which is a much stronger activator compared to other ECM. We also found that vessel injury induces many ECM components (*Fn1*, *Col1a1*, *Col2a1*, *Col3a1*, *Col5a1*, and *Lamb1* etc.) compared to healthy vessel (See below). As we demonstrated above with regard to your suggestion that different ECM components may affect FAK activation in Question 1, some ECM proteins, such as fibronectin, more readily activated FAK and increased DNMT3A expression. Based on these findings, we think that increased integrin-ECM (outside-in) signaling actually causes FAK translocation from the nucleus to the cytoplasm, and these activated transmembrane receptors keep FAK in the cytoplasm to facilitate outside-in signaling. On the other hand, it is likely that the absence of integrin activation triggers to generate nuclear FAK pool.

A Enrichment plot: GO_EXTRACELLULAR_MATRIX

B



FGF10	FGF10	fibroblast growth factor 10
COL8A1	COL8A1	collagen, type VIII, alpha 1
COL5A3	COL5A3	collagen, type V, alpha 3
COL2A1	COL2A1	collagen, type II, alpha 1 (primary osteoarthritis, spondyloepiphyseal dysplasia, congenital)
LAMB1	LAMB1	laminin, beta 1
COL24A1	COL24A1	collagen, type XXIV, alpha 1
COL8A2	COL8A2	collagen, type VIII, alpha 2
COL3A1	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
COL16A1	COL16A1	collagen, type XVI, alpha 1
COL1A1	COL1A1	collagen, type I, alpha 1
MMP2	MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
MMP27	MMP27	matrix metallopeptidase 27
COL1A2	COL1A2	collagen, type I, alpha 2
COL7A1	COL7A1	collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)
MMP3	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
COL9A3	COL9A3	collagen, type IX, alpha 3
MMP14	MMP14	matrix metallopeptidase 14 (membrane-inserted)
COL5A2	COL5A2	collagen, type V, alpha 2
COL10A1	COL10A1	collagen, type X, alpha 1(Schmid metaphyseal chondrodysplasia)
CDH2	CDH2	cadherin 2, type 1, N-cadherin (neuronal)
COL12A1	COL12A1	collagen, type XII, alpha 1
MMP12	MMP12	matrix metallopeptidase 12 (macrophage elastase)
COL27A1	COL27A1	collagen, type XXVII, alpha 1
COL28A1	COL28A1	collagen, type XXVIII, alpha 1
COL5A1	COL5A1	collagen, type V, alpha 1
COL14A1	COL14A1	collagen, type XIV, alpha 1 (undulin)
COL11A1	COL11A1	collagen, type XI, alpha 1
MMRN2	MMRN2	multimerin 2
MMP11	MMP11	matrix metallopeptidase 11 (stromelysin 3)
FBN2	FBN2	fibrillin 2 (congenital contractural arachnodactyly)
MMP19	MMP19	matrix metallopeptidase 19
MMP13	MMP13	matrix metallopeptidase 13 (collagenase 3)
LAMC2	LAMC2	laminin, gamma 2
FBN1	FBN1	fibrillin 1
FGF9	FGF9	fibroblast growth factor 9 (glia-activating factor)
COL6A3	COL6A3	collagen, type VI, alpha 3
MMP25	MMP25	matrix metallopeptidase 25
MMP15	MMP15	matrix metallopeptidase 15 (membrane-inserted)
FN1	FN1	fibronectin 1
LAMB3	LAMB3	laminin, beta 3
MMP8	MMP8	matrix metallopeptidase 8 (neutrophil collagenase)
MMRN1	MMRN1	multimerin 1
ELN	ELN	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)
LAMA3	LAMA3	laminin, alpha 3
COL6A1	COL6A1	collagen, type VI, alpha 1
TGFB1	TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
COL20A1	COL20A1	collagen, type XX, alpha 1
MMP9	MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
COL23A1	COL23A1	collagen, type XXIII, alpha 1
LAMA2	LAMA2	laminin, alpha 2 (merosin, congenital muscular dystrophy)
LAMC1	LAMC1	laminin, gamma 1 (formerly LAMB2)
VTN	VTN	vitronectin
COL6A2	COL6A2	collagen, type VI, alpha 2
MMP16	MMP16	matrix metallopeptidase 16 (membrane-inserted)
ECM1	ECM1	extracellular matrix protein 1
COL15A1	COL15A1	collagen, type XV, alpha 1
COL4A1	COL4A1	collagen, type IV, alpha 1
LAMA4	LAMA4	laminin, alpha 4
MMP28	MMP28	matrix metallopeptidase 28
COL4A2	COL4A2	collagen, type IV, alpha 2
COL22A1	COL22A1	collagen, type XXII, alpha 1
TGFB3	TGFB3	transforming growth factor, beta 3
COL11A2	COL11A2	collagen, type XI, alpha 2
TGFB2	TGFB2	transforming growth factor, beta 2
CDH13	CDH13	cadherin 13, H-cadherin (heart)
COL13A1	COL13A1	collagen, type XIII, alpha 1
ECM2	ECM2	extracellular matrix protein 2, female organ and adipocyte specific
LAMA5	LAMA5	laminin, alpha 5
LAMB2	LAMB2	laminin, beta 2 (laminin S)
COL4A4	COL4A4	collagen, type IV, alpha 4
FGF1	FGF1	fibroblast growth factor 1 (acidic)
FGFR2	FGFR2	ffer syndrome, Jackson-Weiss syndrome)
COL4A3	COL4A3	collagen, type IV, alpha 3 (Goodpasture antigen)
COL18A1	COL18A1	collagen, type XVIII, alpha 1
TGFB1I1	TGFB1I1	transforming growth factor beta 1 induced transcript 1
MMP17	MMP17	matrix metallopeptidase 17 (membrane-inserted)
COL4A5	COL4A5	collagen, type IV, alpha 5 (Alport syndrome)
COL19A1	COL19A1	collagen, type XIX, alpha 1
LAMC3	LAMC3	laminin, gamma 3
COL4A6	COL4A6	collagen, type IV, alpha 6

ase)

Reviewer only Figure. FAK inhibition alters extracellular matrix related genes expression post wire injury. Gene Set Enrichment Analysis (GSEA) shows enrichment of extracellular matrix related genes of RNAseq data among sham vs. injured **(A)** or Injured vs. Injured+FAK-I **(B)**.

sive)
ase)
congenital)

COL8A1 COL8A1 collagen, type VIII, alpha 1
COL5A3 COL5A3 collagen, type V, alpha 3

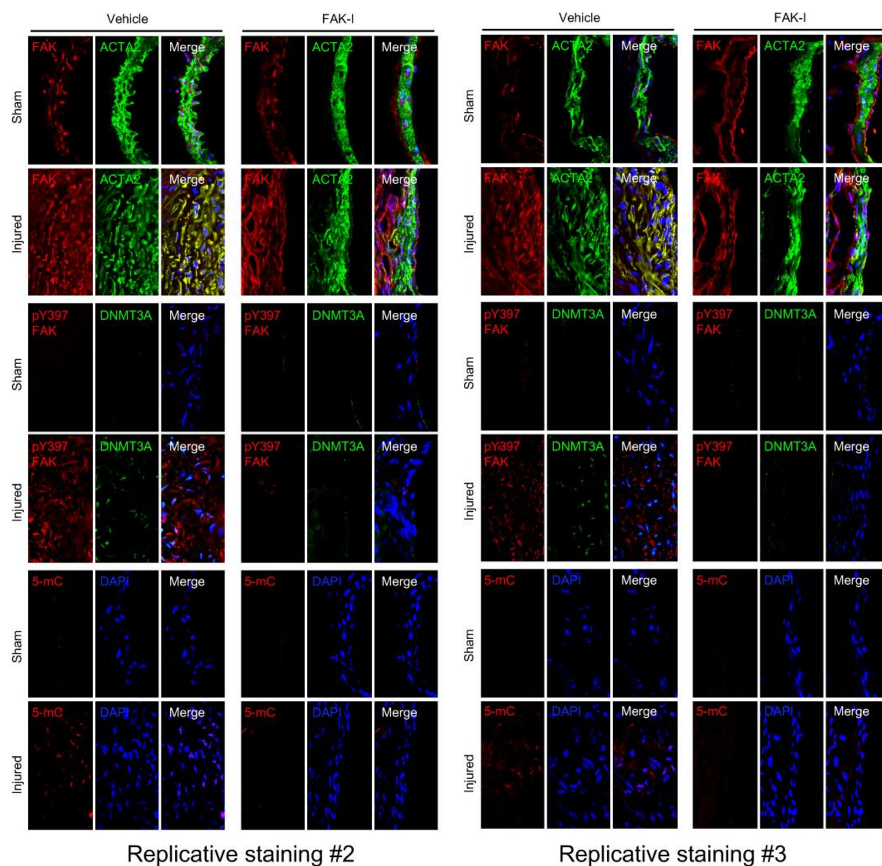
Reviewer #3:

The authors present a detailed mechanistic study of the activation of the FAK-DNMT3A axis and its impact on SMC phenotype switching in vitro and in vivo. In vivo models are using the femoral injury and an athero model. An association is also assessed in a human cohort, but this data appears somewhat weaker.

Major comments:

1. While the experiments are broad and detailed, there is an over-reliance on interpretation of n=1 WB analysis across the entire manuscript, unless this is representative and the authors have all replicates for all blots. Further, there is a reliance on the individual immunostains and controls and replicates are not presented. This will be important to rectify.

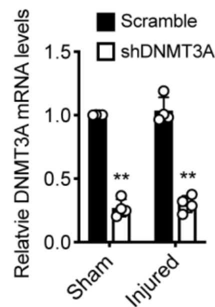
>We indeed performed all the Western blots at least 3 times to draw our conclusion and included all blot replicates in Online Figures. As the reviewer suggested, we measured the band density from at least three replicates, calculated the average of relative density levels and indicated them under the representative blots. For immunostaining, we used species-specific IgG or secondary antibodies for negative control and we now mentioned in the method section. We also performed all the staining experiments at least 3 times as an example shown here.



Reviewer only Figure. Pharmacological FAK inhibition reduced injury-induced upregulation of DNMT3A and 5-mC preventing neointima formation in mice. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. Shown are representative immunofluorescence stainings of frozen section from vehicle or FAK-I-treated femoral arteries for FAK, pY397 FAK, DNMT3A, 5-mC, and ACTA2. Red, green, and blue (DAPI) were merged.

2. In a similar manner, the KD in the model with the shRNA is not presented - how efficient in the delivery and KD in this setting?

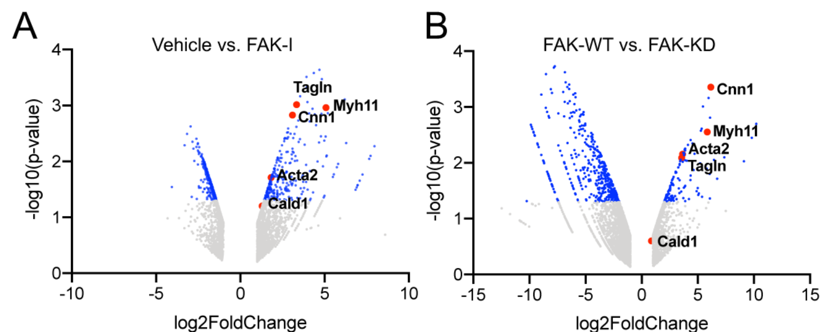
>We have verified the effectiveness of DNMT3A shRNA delivery in vivo by using mCherry marker (Figure 8) and also confirmed that both DNMT3A RNA levels are suppressed upon shDNMT3A lentivirus delivery in vivo. We now have included the data in Online Figure XXVIIB.



Online Figure XXVII. shDNMT3A lentivirus is efficient to knockdown of DNMT3A mRNA level. Femoral arteries were coated with shDNMT3A lentivirus immediately following wire injury. Relative DNMT3A mRNA expression in femoral arteries harvested 2 weeks postinjury were measured by RT-qPCR (±SEM; **p< 0.005 vs. sham, two-way ANOVA followed by Sidak multiple comparisons test).

3. The RNAseq analysis is from adventitia and the SMC layer. Why? While vessel wall plasticity is important, this paper focuses on SMC, so is there a contribution to other cells types? i.e. adventitial cells?

We should have indicated “a minimal residual adventitia” as we cleaned up most of adventitia layers. We corrected the sentence in the methods. We don’t think this significantly affects gene expression profile because in vivo RNA seq data were comparable to RNA-seq data from SMC in culture (the contractile gene data is shown below).



Reviewer only Figure. RNA sequencing results using SMCs. Vehicle vs. FAK-I **(A)** and FAK-WT vs. –KD **(B)** identified VSMC contractile genes as a top transcriptome.

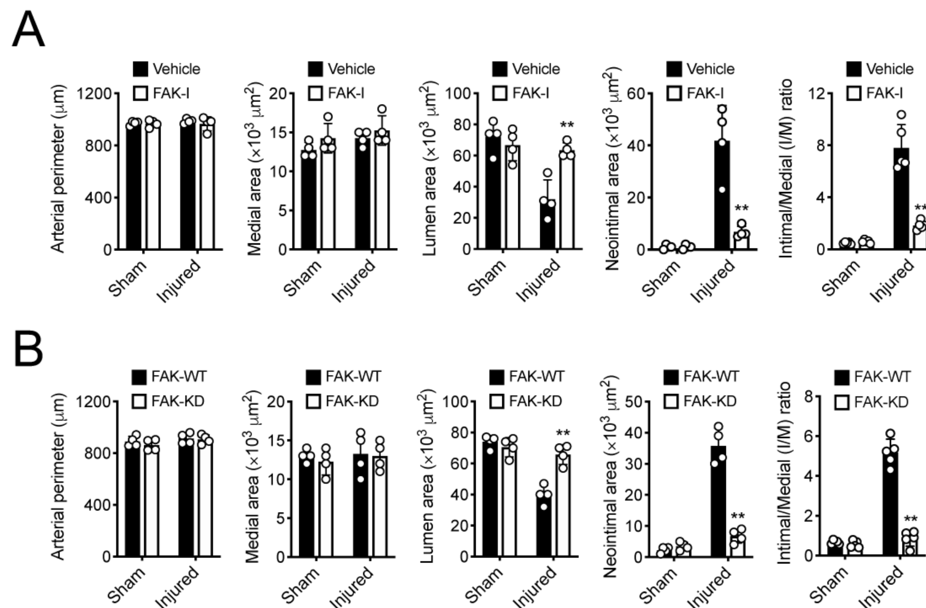
4. The introduction to the role of SMC in disease looks rather generic and dated. Please improve this first para. In a similar manner, it is not convincing from a clinical perspective about the extensive use of the femoral model. What is the clinical correlate to this injury that has unmet need? The athero aspect is far less detailed in the paper, but far more clinically relevant, although this is muddied by the complex mechanistic role of SMC in atheroma. The use of the human athero samples to match the mouse femoral model is a stretch as the mechanisms of SMC function in those settings is so vastly different. This needs re-positioning in the paper to have more relevance across mouse and human. This is a major weakness of the manuscript.

>We have modified the introduction with regards to SMCs in disease which are in the first paragraph of the introduction. We have expanded the atherosclerosis aspect of our manuscript with regards to human samples and mentioned these findings earlier in the manuscript. While we agree the mechanisms of SMC function during atherosclerosis and restenosis differ, it appears that there may be common pathways that give rise SMC dedifferentiation and proliferation under both circumstances (i.e., increased FAK activation and DNMT3A stability). We chose the femoral wire injury model as it allows rapid and reproducible SMC neointimal

hyperplasia which mimics restenosis following vascular interventions. Of particular importance is the need for better therapeutic options for patients with occlusion of the femoropopliteal artery. While stents and balloon angioplasties have increased long term patency 1-3 years post-intervention, there are still issues that arise from these procedures (A. Diamantopoulos and K. Katsanos *Semin Intervent Radio* 2014 Dec; 31(4): 345–352; K.J. Ho and C.D. Owens *J Vasc Surg* 2017 Feb;65(2):545-557). Stents can be used to prevent the elastic recoil observed in balloon angioplasty; however, stents can become cracked, promote artery kinking, and result in pseudoaneurysm due to the mechanical nature of the leg (J.M.C. dos Reis et al., *J Surg Case Rep* 2019 Nov; 2019(11): rjz312.; Y. Tsuji et al., *Ann Vasc Surg* 2011 Aug;25(6):840.e5-8; S. Adlakha et al., *J Interv Cardio.* 2010 Aug;23(4):411-9). It was also reported that balloon angioplasty following venous bypass of a lesion in femoropopliteal artery resulted in several pseudoaneurysms (H. Bergenfeldt et al., *Ann Vasc Surg* 2021 Apr;72:665.e5-665). Overall, the femoral wire injury model allows us to evaluate pathways and mechanisms which regulate SMC restenosis and find new therapeutic targets that could be used in a systemic manner allowing for increased patency following percutaneous transluminal angioplasty.

5. The reporting of the injury in the femoral model is superficial and could be improved further to assess vessel wall parameters in more detail and accuracy.

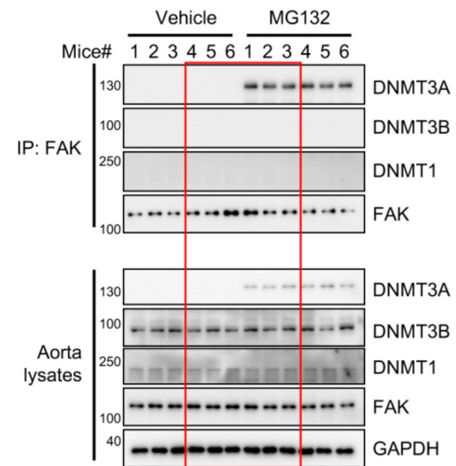
>We have added detailed multipoint measurement of vessel parameters including arterial perimeter, medial area, lumen area, and neointimal area. We included the new data in Online Figure I.



Online Figure I. FAK inhibition reduces neointima formation following wire injury. Vessel parameters including arterial perimeter, medial area, lumen area, neointimal area, and Intima/media ratio are measured and plotted (±SD, n=5 for FAK-I or n=4 for FAK-KD, **P<0.005 vs vehicle injured, two-way ANOVA followed by Sidak multiple comparisons test).

6. Can the authors show the FAK:DNMT3A interaction from the in vivo samples?

>We thank the reviewer for asking for this experiment as we were wondering if we could detect FAK-DNMT3A interaction in vivo. To facilitate this, we treated mice with MG132 (0.3 mg/kg) at 24, 6, and 1 h post euthanasia. Treatment with MG132 blocked DNMT3A degradation within healthy arteries, allowing us to detect FAK-DNMT3A interactions. To test FAK-DNMT3A interaction from the in vivo samples, lysates of aorta were subjected to FAK immunoprecipitation (IP). MG132 treatment increased DNMT3A expression compared to none in control and showed the interaction of FAK and DNMT3A on aorta, indicating that nuclear FAK binds with DNMT3A in vivo to regulate the levels. We included new IP data in Figure 4F and Online Figure XXIC.



Online Figure XXI. FAK interacts DNMT3A *in vivo*. Mice were treated with MG132 (0.3 mg/kg) by intraperitoneal injection for 24, 6, and 1 h before euthanasia. Isolated aorta lysates were immunoprecipitated with anti-FAK antibody and FAK-IPs were subjected to immunoblotting with indicated antibodies (n=6). Boxed areas are shown in **Figure 4F**.

August 26, 2021

Prof. Ssang-Taek Steve Lim
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Biochemistry and Molecular Biology
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Biochemistry, MSB2320
Mobile, Alabama 36688

RE: CIRCRES/2021/319066R1: FAK activation promotes SMC dedifferentiation via increased DNA methylation in contractile genes by stabilizing DNMT3A

Dear Dr. Lim:

Your manuscript has been carefully evaluated by 5 external reviewers and the editors as a Regular Article. We regret to inform you that the paper is not acceptable for publication in its present form.

As you will gather from the reviews, the referees identified a number of conceptual and methodological problems. The editors concur. Major issues include statistical and technical problems. Please address all of these as well as reviewer #3's remaining minor concern.

Despite these concerns, the editors see this paper as potentially important and wish to encourage revision. If you would like to revise the manuscript in accordance with the suggestions of the reviewers and editors, we would be willing to evaluate a new version. The manuscript would be reviewed again, with no assurance of acceptance.

The Editors strongly encourage you to adhere to the journal's Statistical Reporting Recommendations in your revision, which can be found here: https://www.ahajournals.org/statistical-recommendations.

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are required to upload the Table at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

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We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****
Reviewer comments to the Authors:

Reviewer #1:
The new experiments conducted by the authors significantly strengthened the manuscript. The current version of manuscript provides an interesting mechanism underlying smooth muscle phenotypic regulation. This reviewer has no additional concerns or questions.

Reviewer #2:
All my comments have been addressed.

Reviewer #3:
There manuscript has been improved and solid response to the comments from this reviewer and other reviewers. Mechanistic insight is strong. Important to please include the western blots and the immune stains in the response to me in the manuscript and not simply for the reviewer.

Statistical Reviewer:
1) The statistical tests used assume a normal distribution. Please state the statistical property or test of normality used to meet this assumption. If the data are not normal please use a non-parametric alternative. If N is too small to determine normality (<6) or use a non-parametric alternative.

2) Please ensure error bars go in both directions (black on black cannot be seen)

3) Please provide precise p-values (rather than P<0.0x). This can be obtained in GraphPad by increasing significant digits on the "Options" tab. Scientific notation with 2 significant figures is strongly encouraged.

4) For each presented p-value make sure it is clear what test it is derived from. If it is adjusted specify what (and how many tests) it is adjusted for.

5) How were representative images selected for inclusion?

6) Consider correcting for multiple testing across the entire body of work. The more tests done the higher the chance of observing a false association. If you chose not to, state this as a weakness of the study.

7) You cannot make claims of no change. You can say no statistical difference was observed.

8) Relative expression needs to be clearly defined (e.g. figure 4c). What is it relative to?

9)How do you account for violation of the assumption of independent sampling (e.g. when using multiple cells/samples from the same animal).

10) color scales should be labeled including units.

Technical Reviewer:

Comments to Authors on Rigor Checklists:
The current study was carefully evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified some items that were either omitted or not adequately addressed in the text. Please see below for details:

In vitro checklist items:
1) Ensure that manufacturer catalog numbers are provided for all antibodies employed in experiments (including secondary antibodies, isotype controls, etc.). This information should be provided in both the methods section and "Major Resources Table."
2) In the "Cell culture" section, please specify at what passage or passage range primary cell lines were utilized in experiments.
3) Please add units (in kDa) for molecular weight markers in all presented immunoblots.
4) All methods should be of sufficient detail to allow replication, even for those procedures that may be considered routine. Referring to previously published procedures or manufacturer protocols is accepted; however, any deviations should be detailed in the text. Please carefully review for adherence to these guidelines. Some examples are shown below:
a. Immunoblotting: provide speed (RCF: xg) and duration of lysate centrifugation steps. Specify the gels used to resolve proteins (e.g., % acrylamide/bis-acrylamide). Specify reagents used for blocking membranes.
b. Immunoprecipitation: procedural details are somewhat vague; provide additional details so that these experiments may be reproduced (or refer to a manufacturer protocol).
c. RNA extraction and quantitative real-time quantitative polymerase chain reaction: Provide thermocycle conditions used in amplification (i.e., temperature and duration of denaturation, annealment, and extension). Specify method of analysis (e.g., ΔΔCt method, etc.) used for reporting relative mRNA expression.
In vivo checklist items:
1) In the manuscript, please indicate whether any animals were excluded from analyses, and if so, based on what criteria these exclusions were made.
2) In the manuscript, provide statements regarding author disclosures/conflicts of interest.
3) In the methods section, please specify the source of the animals used in the study (e.g., vender or laboratory).

Other:
1) Per the Journal's requirements, please complete and submit a "Major Resources Table." Please refer to the website for formatting instructions.
2) Please submit unedited immunoblots for review.

Comments to the Authors on Research Guidelines and Reporting:
Authors need to indicate whether RNA sequencing data generated in the study has been made available in a public data repository.

Reviewer comments to the Authors:

Reviewer #1:

The new experiments conducted by the authors significantly strengthened the manuscript. The current version of manuscript provides an interesting mechanism underlying smooth muscle phenotypic regulation. This reviewer has no additional concerns or questions.
>We are grateful to the reviewer for the valuable comments.

Reviewer #2:

All my comments have been addressed.
>We appreciate the reviewer for the thoughtful comments.

Reviewer #3:

There manuscript has been improved and solid response to the comments from this reviewer and other reviewers. Mechanistic insight is strong. Important to please include the western blots and the immune stains in the response to me in the manuscript and not simply for the reviewer.
>Thank you for the critical comments on our work. According to the Reviewer's suggestion, we have included the replicates of western blots and immunostainings in new Online Figure IX, XV, XXII, XXIII, XXVI, XXVII, XXIX, XXX, XXXIV, and XXXV.

Statistical Reviewer:

1) The statistical tests used assume a normal distribution. Please state the statistical property or test of normality used to meet this assumption. If the data are not normal please use a non-parametric alternative. If N is too small to determine normality (<6) or use a non-parametric alternative.
>We have tested the normality of data distribution with a Shapiro-Wilk test. For western blots, RNA-seq, qPCR, ChIP, migration assay, and gel contraction experiments, as each data set is an average from a large number of cells, we assumed the data was normally distributed by the central limit theorem. For 2-group studies, statistical analysis was performed using t-test. For >2 group comparisons, we performed one-way ANOVA with Bonferroni multiple comparisons test.

For data with more than 2 variables, analysis was performed using two-way ANOVA with Bonferroni multiple comparisons test. All tests used are described in the methods section as well as in the appropriate figure legends.

2) Please ensure error bars go in both directions (black on black cannot be seen)
>We have changed the error bar colors to visualize them more easily going in both directions.

3) Please provide precise p-values (rather than P<0.0x). This can be obtained in GraphPad by increasing significant digits on the "Options" tab. Scientific notation with 2 significant figures is strongly encouraged.
>We have added the exact p-values on all graphs.

4) For each presented p-value make sure it is clear what test it is derived from. If it is adjusted specify what (and how many tests) it is adjusted for.
>We have included which test was used for each p-value in the Materials and Methods section and in the corresponding figure legends. If multiple comparisons were performed, we presented the adjusted p-value and these are indicated in the figure legend (test and number of comparisons adjusted for).

5) How were representative images selected for inclusion?
>We have selected the representative images with the result close to the average value. We also included all replicates in Online supplemental data.

6) Consider correcting for multiple testing across the entire body of work. The more tests done the higher the chance of observing a false association. If you chose not to, state this as a weakness of the study.
>When multiple testing was performed, we corrected as such based on the number of comparisons evaluated. The number of comparisons for appropriate analysis are indicated in the figure legends.

7) You cannot make claims of no change. You can say no statistical difference was observed.
>We have amended these statements to no statistical difference observed.

8) Relative expression needs to be clearly defined (e.g. figure 4c). What is it relative to?

>We have now clarified the relative expression on Figure 4C graph.

9)How do you account for violation of the assumption of independent sampling (e.g. when using multiple cells/samples from the same animal).

>To avoid violation of the assumption of independent sampling, SMCs isolated from mice were pooled together from the aorta of 5 mice each time so that we avoided potential mouse to mouse differences. Additionally, immunoblots and immunostainings of in vivo samples were evaluated in multiple mice.

10) color scales should be labeled including units.

>We have added units to the color scales.

Technical Reviewer:

Comments to Authors on Rigor Checklists:

The current study was carefully evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified some items that were either omitted or not adequately addressed in the text. Please see below for details:

In vitro checklist items:

1) Ensure that manufacturer catalog numbers are provided for all antibodies employed in experiments (including secondary antibodies, isotype controls, etc.). This information should be provided in both the methods section and "Major Resources Table."

>We have generated a Major Resources Table and included it in the supplemental files.

2) In the "Cell culture" section, please specify at what passage or passage range primary cell lines were utilized in experiments.

>We have now mentioned the details in Materials and Methods sections

3) Please add units (in kDa) for molecular weight markers in all presented immunoblots.

>We have added kDa units in all immunoblots.

4) All methods should be of sufficient detail to allow replication, even for those procedures that may be considered routine. Referring to previously published procedures or manufacturer protocols is accepted; however, any deviations should be detailed in the text. Please carefully review for adherence to these guidelines. Some examples are shown below:
a. Immunoblotting: provide speed (RCF: xg) and duration of lysate centrifugation steps. Specify the gels used to resolve proteins (e.g., % acrylamide/bis-acrylamide). Specify reagents used for blocking membranes.
>We have added centrifugation speed units and specified the gel and blocking reagents.

b. Immunoprecipitation: procedural details are somewhat vague; provide additional details so that these experiments may be reproduced (or refer to a manufacturer protocol).
>We have added the detailed protocol for immunoprecipitation.

c. RNA extraction and quantitative real-time quantitative polymerase chain reaction: Provide thermocycle conditions used in amplification (i.e., temperature and duration of denaturation, annealment, and extension). Specify method of analysis (e.g., ΔΔCt method, etc.) used for reporting relative mRNA expression.
>We have added details regarding thermocycle conditions and method of analysis on Materials and Methods

In vivo checklist items:
1) In the manuscript, please indicate whether any animals were excluded from analyses, and if so, based on what criteria these exclusions were made.
>We have indicated that no animals were excluded from analyses.

2) In the manuscript, provide statements regarding author disclosures/conflicts of interest.
>We have included the statements regarding conflicts of interest.

3) In the methods section, please specify the source of the animals used in the study (e.g., vender or laboratory).
>We have included the animal sources from in both the Methods and Materials section and in the Major Resources Table.

Other:

1) Per the Journal's requirements, please complete and submit a "Major Resources Table." Please refer to the website for formatting instructions.

>We have now attached Major Resources Table

2) Please submit unedited immunoblots for review.

>We have now attached unedited immunoblots.

Comments to the Authors on Research Guidelines and Reporting:

Authors need to indicate whether RNA sequencing data generated in the study has been made available in a public data repository.

>We have now deposited our RNA sequencing data (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE183143) and mentioned in the Materials and Methods section as well as in the Major Resources Table.

September 21, 2021

Dr. Ssang-Taek Steve Lim
University of South Alabama College of Medicine
Biochemistry and Molecular Biology
5851 North USA Dr.
Biochemistry, MSB2320
Mobile, AL 36688

RE: CIRCRES/2021/319066R2: FAK activation promotes SMC dedifferentiation via increased DNA methylation in contractile genes by stabilizing DNMT3A

Dear Dr. Lim:

Your revised manuscript has been carefully evaluated by the editors as a Regular Article. While we are interested in your paper, further minor revision is required before we can accept the manuscript for publication in Circulation Research. Specifically, there are several minor formatting issues that need to be addressed. Please submit your revision by SEPTEMBER 20.

The Editors strongly encourage you to adhere to the journal's Statistical Reporting Recommendations in your revision, which can be found here: https://www.ahajournals.org/statistical-recommendations.

Please ascertain that your revised manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Accepted manuscripts are published online ahead of print, usually within 24 hours of acceptance. Therefore, when submitting the final files of the manuscript and figures, please ensure you have made any essential changes or corrections to content, grammar, and formatting. Please also ensure that author information provided in the online submission system is correct, including author order, proper names, and institutions. Once published ahead of print, you will be unable to make any revisions to the manuscript until you receive your author proofs from the publisher and any changes made to proofs will be reflected in the final print and online journal version of your article.

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Figures:
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- Recent studies have shown that active engagement in social media is beneficial in advancing your science. Circulation Research encourages all authors to provide their twitter handles, if possible.

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We look forward to receiving the final revised version of your manuscript as soon as possible. Thank you for contributing to Circulation Research.

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Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal