Presenter Disclosure Information:

Name: Dr. Gary K. Owens

The presenter or their spouse/partner have had a financial interest/arrangement or affiliation with the organizations listed below. However, none of the data related to these relationships is being presented today.

Company Name	Relationship
Setagon, Inc.	Founder, CSO, acquired by Medtronic Nov. 2007
NanoMedical Systems, Inc.	Founder, CSO
AstraZeneca Pharmaceutical	PI, UVA-AZ Research Alliance

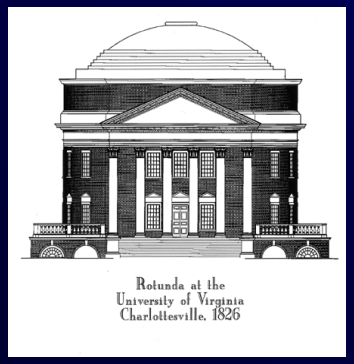
The Stem Cell Pluripotency Genes Klf4 and Oct4 Play a Key Role in Regulating the Phenotype-Functions of SMC-Pericytes in Normal and Diseased States

Dr. Gary K. Owens

Professor of Molecular Physiology and Biological Physics

Professor of Internal Medicine, CV Division

Director Robert M. Berne Cardiovascular Research Center



THE ROBERT M. BERNE CARDIOVASCULAR RESEARCH CENTER

Talk Outline:

I. The majority (>80%) of SMC within advanced atherosclerotic lesions lack expression of SMC marker genes and undergo transition to cells exhibiting characteristics of multiple other cell types including macrophages, MSC, and myofibroblasts (MFs).

II. The stem cell pluripotency genes Klf4 and Oct4 regulate phenotypic transitions of SMC critical in the pathogenesis of atherosclerosis. However, these transitions can be beneficial or detrimental depending on the nature of those changes.

III. IL1β has atheroprotective effects in late stage atherosclerotic lesions including being required for maintenance of a protective fibrous cap.

IV. Oct4 and Klf4 expression in SMC-P is critical in regulating angiogenesis, perivascular cell coverage, and the innate metabolic and inflammatory properties of adipose tissues.

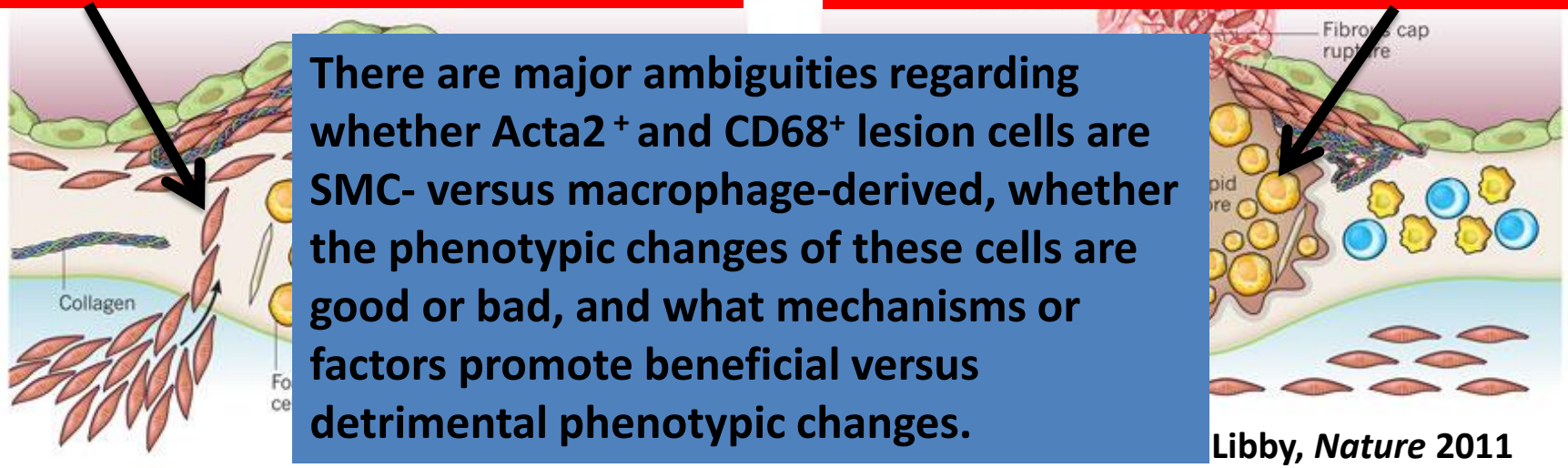
Established Dogma: Advanced Atherosclerotic Lesions with an Increased Ratio of SMC (Acta2^{+}) to Macrophages (LGALS3^{+} or CD68^{+}) Cells are More Stable

SMC PHENOTYPIC SWITCHING

- Reduced expression of SMC marker genes including Acta2 and Myh11
- Increased migration, proliferation, and ECM production
- Changes are believed to be atheroprotective
- Can undergo transition to a macrophage marker^{+} state with cholesterol loading *in vitro* (Rong et al. 2003 PNAS) but the role of this in lesion pathogenesis *in vivo* is unknown!

MACROPHAGE PHENOTYPIC SWITCHING

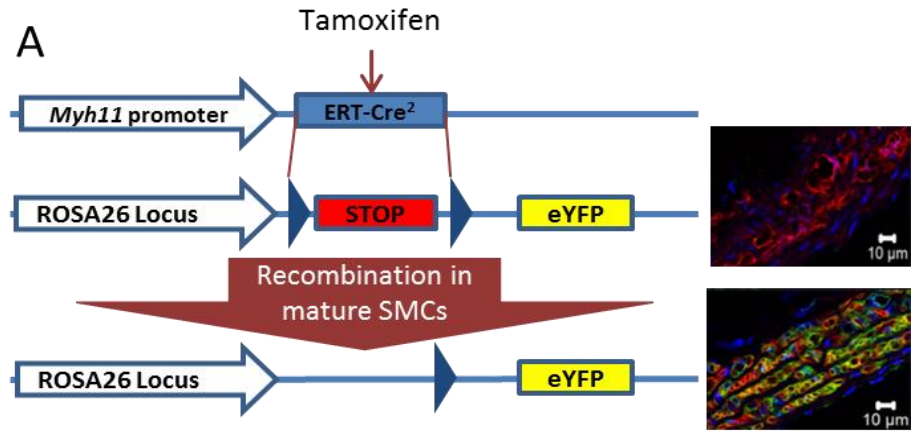
- Undergo complex phenotypic transitions
- Are the primary cell type giving rise to foam cells
- Generally regarded to exacerbate lesion pathogenesis
- Activation of Acta2 and other SMC genes in response to TGF_{β} or thrombin *in vitro*
- Some evidence they activate SMC markers *in vivo* but the role of this in lesion pathogenesis is unknown.

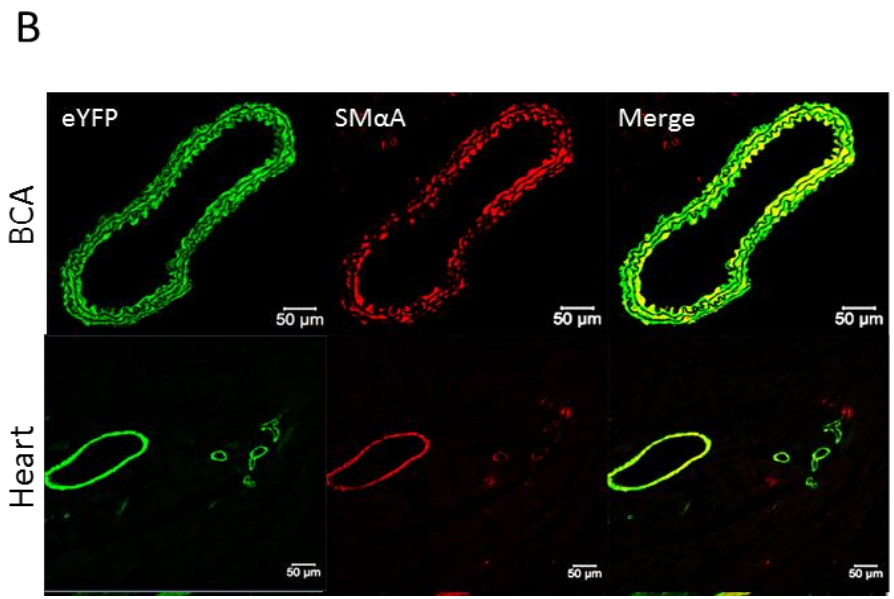


There are major ambiguities regarding whether Acta2^{+} and CD68^{+} lesion cells are SMC- versus macrophage-derived, whether the phenotypic changes of these cells are good or bad, and what mechanisms or factors promote beneficial versus detrimental phenotypic changes.

Libby, *Nature* 2011

Development of a Rigorous SMC Lineage Tracing Mouse Model and Use in Determining the Roles of these Cells in Atherosclerosis and Various Wound Healing Models





Gomez et al. *Nature Methods 10:171-177*, 2013.

Myh11 YFP mice show:

1. No detectable expression of YFP in the absence of tamoxifen.
2. High efficiency (>99%) labeling of arterial SMC (and NG2^{+} pericytes) following tamoxifen treatment between 6-8 weeks of age.
3. Complete SMC-pericyte specificity with no YFP labeling of any other cell type.

As such we can permanently lineage tag SMC-pericytes and determine what they or their progeny become irrespective of continued expression of Myh11 or other SMC-pericyte marker genes.

We have bred Myh11 YFP mice with ApoE$^{-/-}$ mice and various mice containing floxed alleles of various genes we postulate are involved in controlling SMC phenotypic transitions (Klf4, Oct4, PDGFRβ, IL1R1, Col15a).

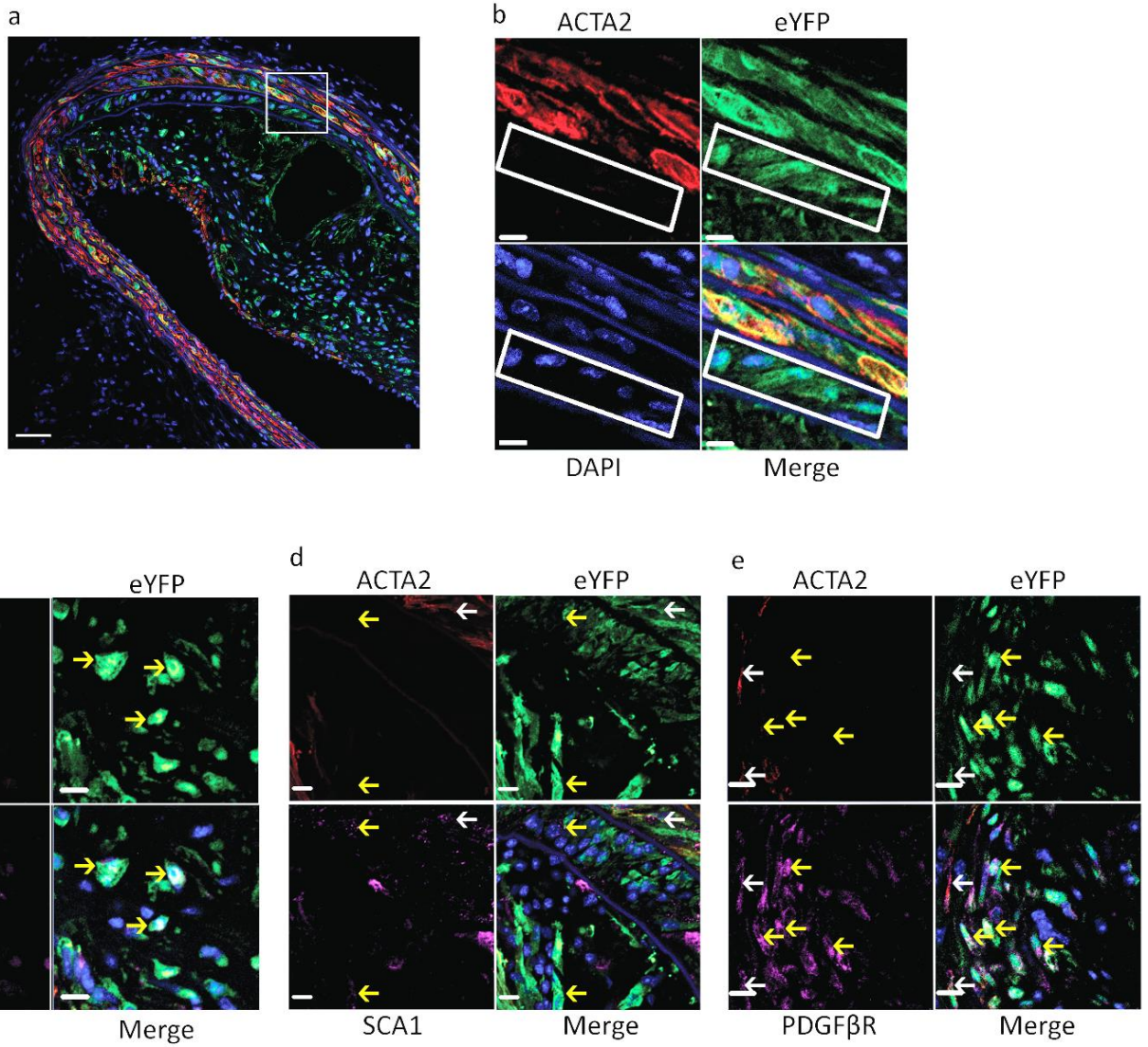
Overall Experimental Design: Myh11-ERT2 Cre eYFP ApoE$^{-/-}$ mice +/- floxed candidate genes

Analyses:

1. Lesion and lumen size (areas)
2. Remodeling indices (EEL, IEL lengths and areas)
3. SMC (eYFP^{+}) and non-SMC (eYFP^{-}) phenotypic transitions (IF high resolution z-stack confocal and flow cytometry)(SMC/leucocyte marker panels, novel marker panels from our in vivo genomic analyses of advanced BCA lesions)
4. Evaluation of Ki67 (DNA synthesis) and caspase3 (apoptotic indices) in the preceding SMC and non-SMC populations.
5. Sudan IV en face staining (fatty streaks)
6. Indices of plaque stability
   a. Fibrous cap thickness and area
   b. SMC/macrophage ratios (overall and in the fibrous cap)
   c. Collagen content and maturation (Picosirius red polarization)(overall and in the fibrous cap)
   d. Necrotic core size
   e. Intraplaque hemorrhage (Ter119)
   f. Lipid content (Oil Red O)

Blue = metrics analyzed in the majority of atherosclerosis studies

The Majority of SMC-Derived Cells within Advanced ApoE$^{-/-}$ Lesions Lack Detectable SMC Markers and have Activated Markers of Macrophages, MSC and Myofibroblasts



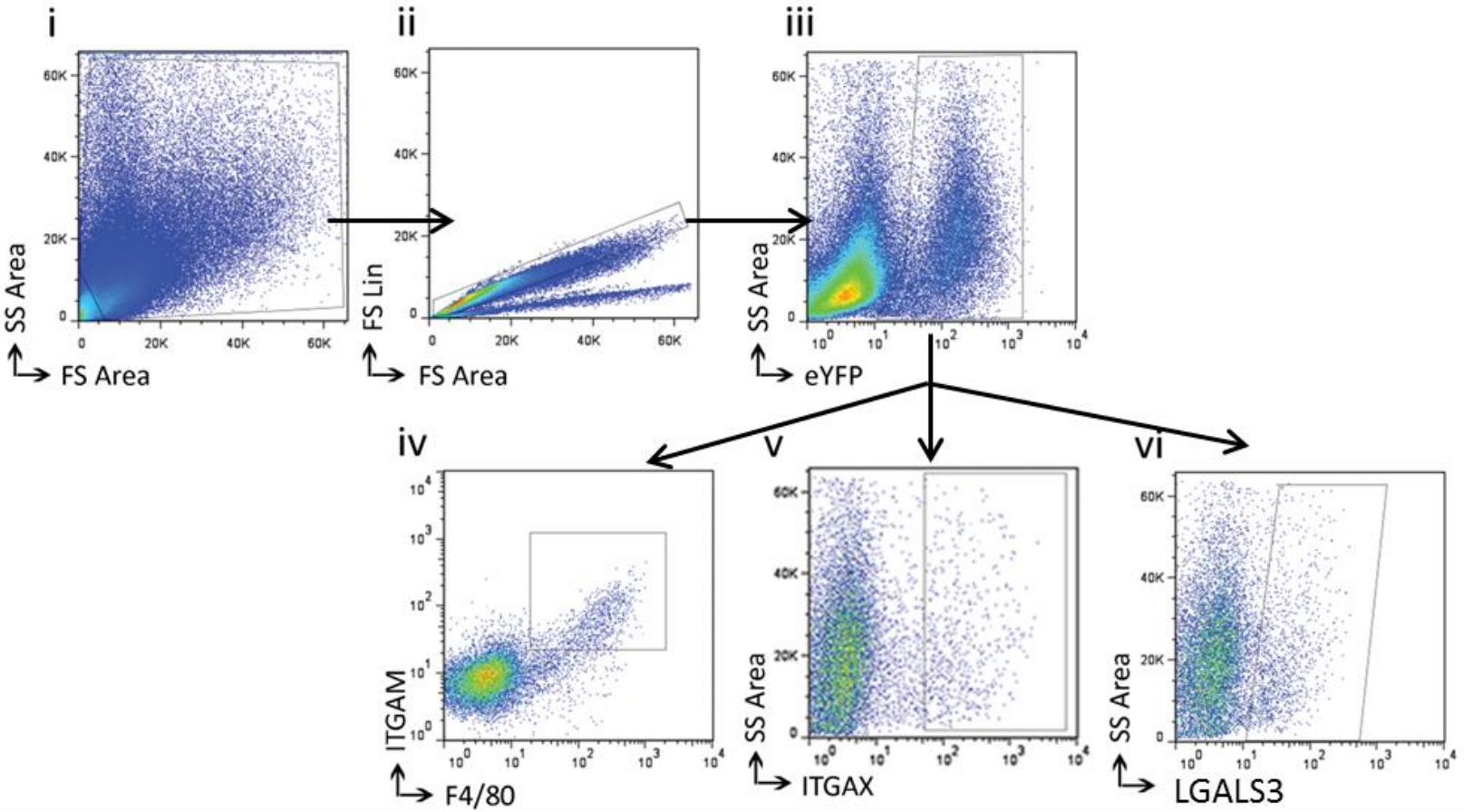
Shankman et al….Owens, 2015 *Nature Medicine*

Summary of Quantitative Confocal Microscopic Analyses of Intimal SMC Derived Cells in our Myh11-YFP ApoE-/- Mice Following 18 Weeks of Western Diet Feeding

Cell Populations Within the Lesion	% of YFP+	S.E.M.
% of eYPF+ cells that are ACTA2+	16.1	±1.6
% of eYPF+ cells that are ACTA2-	82.4	±2.4
% of eYPF+ cells that are LGALS3+	30.5	±4.2
% of eYPF+ cells that are Sca1+	7.3	±3.0
% of eYFP+ cells that are PDGFβ receptor+	12.7	±2.0
% of LGALS3+ Cells that are eYFP+	36.0	±4.2
Percent of eYFP+ Cells of Unknown Function	32-51	

Shankman et al….Owens, 2015 *Nature Medicine*

SMC-Derived Cells From 18-Week Western Diet Fed ApoE KO Mouse Aortas Express Multiple Macrophage Markers



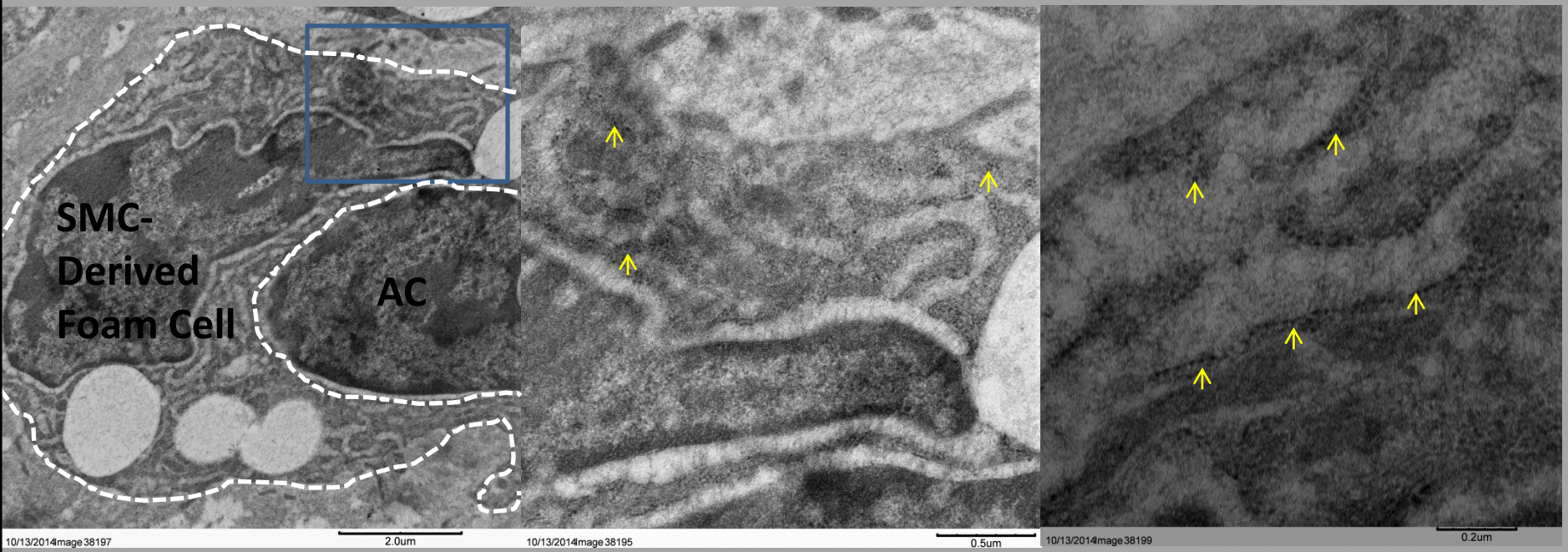
ITGAM = integrin alpha-M, CD11b, macrophage adhesion and phagocytosis
F4/80 = Ly71, Gpf480, mature macrophages
ITGAX = CD11c, dendritic cells
LGALS3 = Mac2
PTPRC = CD45, leucocyte common antigen, protein tyrosine phosphatase receptor c

Shankman et al.....Owens, 2015 *Nature Medicine*

What are the functional properties of SMC derived macrophage-like cells?

Are they phagocytic?

Immuno-EM Analyses of our SMC Lineage Tracing ApoE-/- Mice Provide Evidence SMC-Derived YFP+ Cells are Phagocytic In Vivo



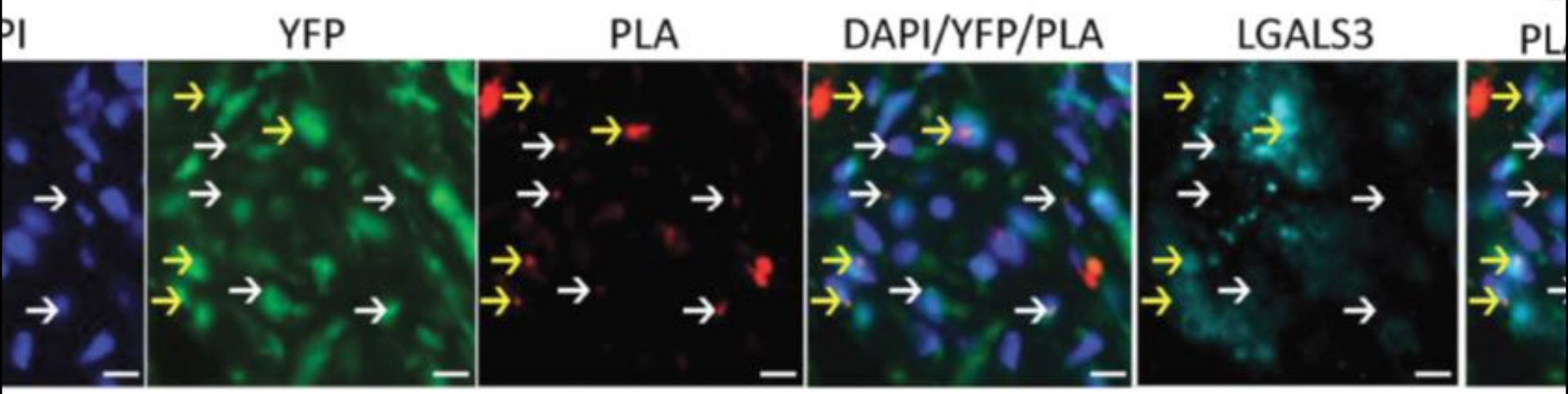
↑ Yellow arrows depict YFP immunogold particles

AC = apoptotic cell nucleus

Shankman et al., 2015 *Nature Medicine*

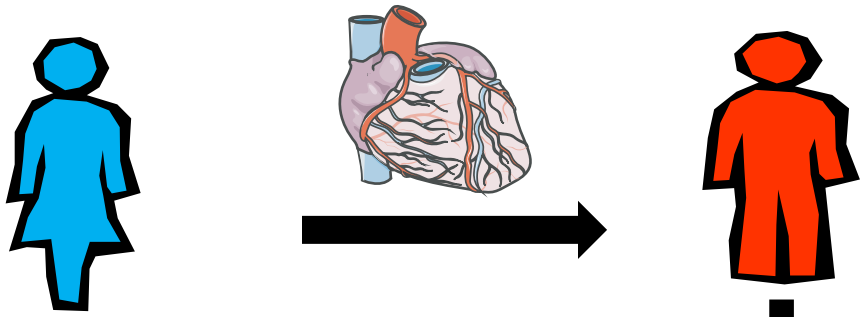
Do SMC transition to a macrophage-like state within human lesions?

SMC-Derived Macrophage-like Cells within Advanced ApoE$^{-/-}$ Lesions Retain Their Unique Myh11 H3K4diMe Epigenetic Signature

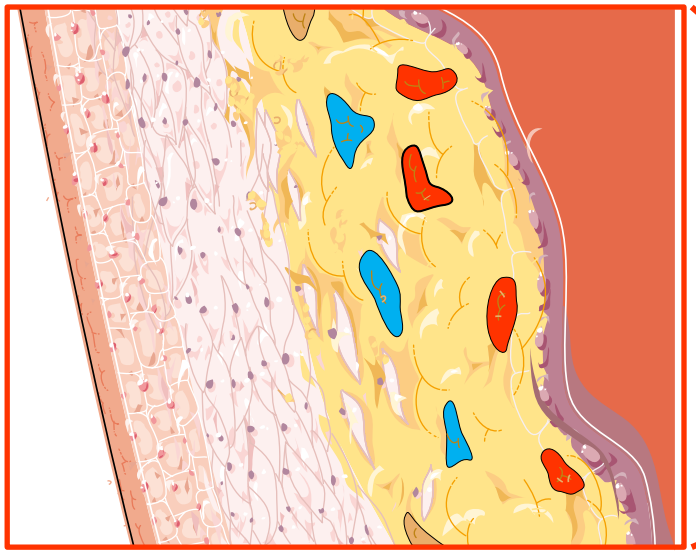


Shankman et al….Owens, 2015 *Nature Medicine*

Cross-gender Heart transplant



After X years: Death
&
Atherosclerotic lesion within coronary arteries



Macrophage-like cells Y chr + Y chr -

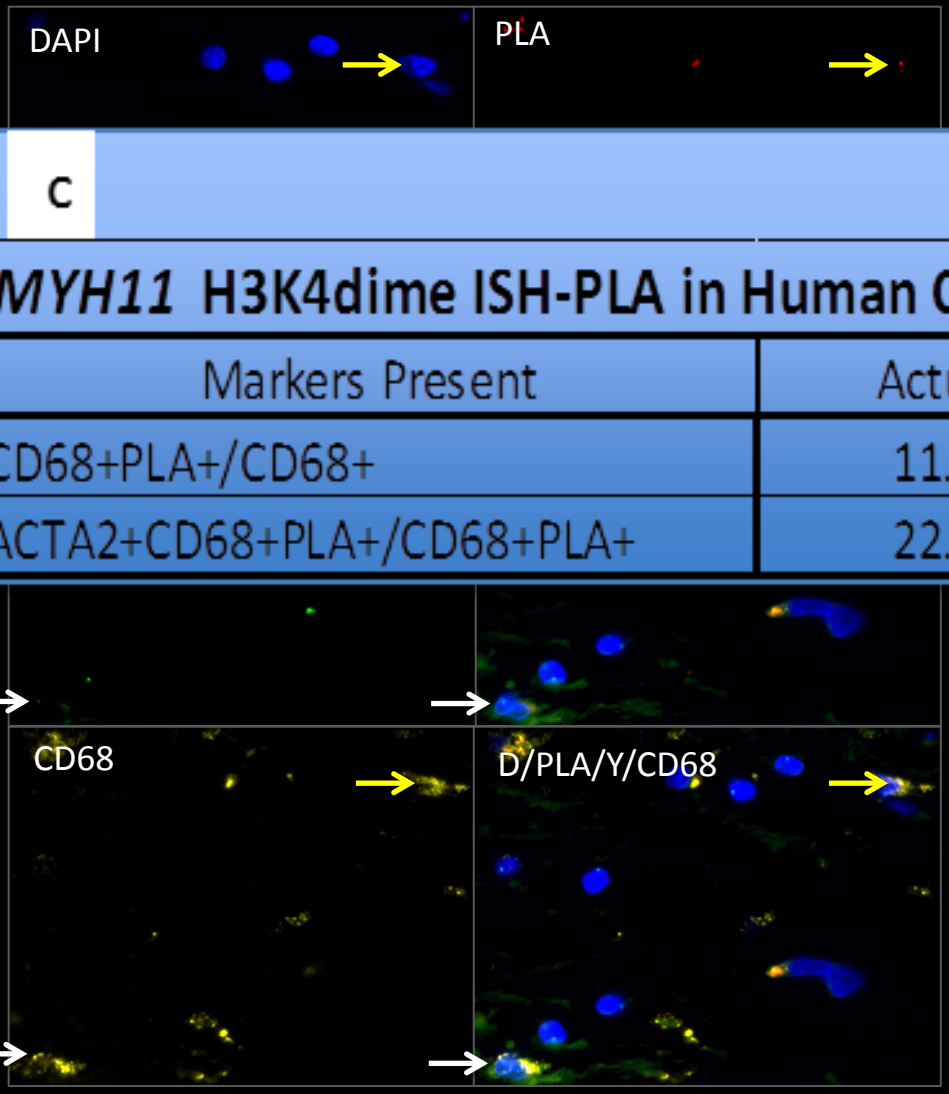
Coronary arteries from heart-transplanted patient
Donor = female
Recipient = male

Vascular cells = XX
Myeloid cells = XY

FISH
Y chr probe + IF/PLISA…

Female Donor Heart into a Male Recipient – Evidence for SMC Derived Mac-Like Cells in Human Coronary Artery Atherosclerotic Lesions

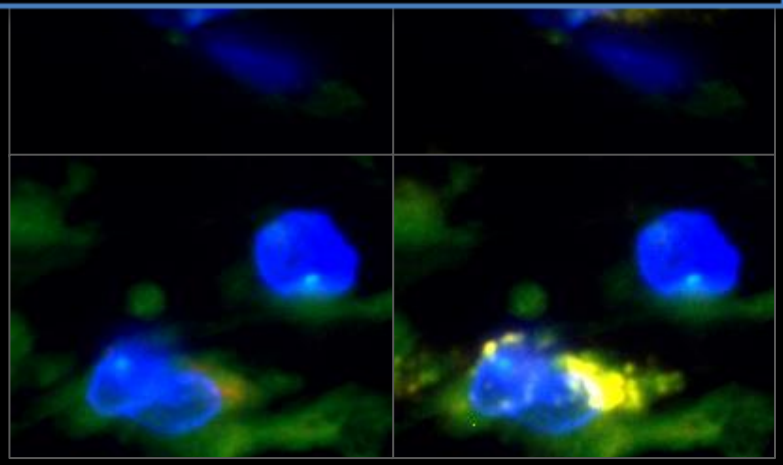
SMC in coronary arteries are XX; Myeloid cells are XY



C

***MYH11* H3K4dime ISH-PLA in Human Coronary Artery Atherosclerosis**

Markers Present	Actual Count	Correction Efficiency
CD68+PLA+/CD68+	11.68 ± 1.5	17.96 ± 2.4
ACTA2+CD68+PLA+/CD68+PLA+	22.47 ± 5.9	34.57 ± 9.1



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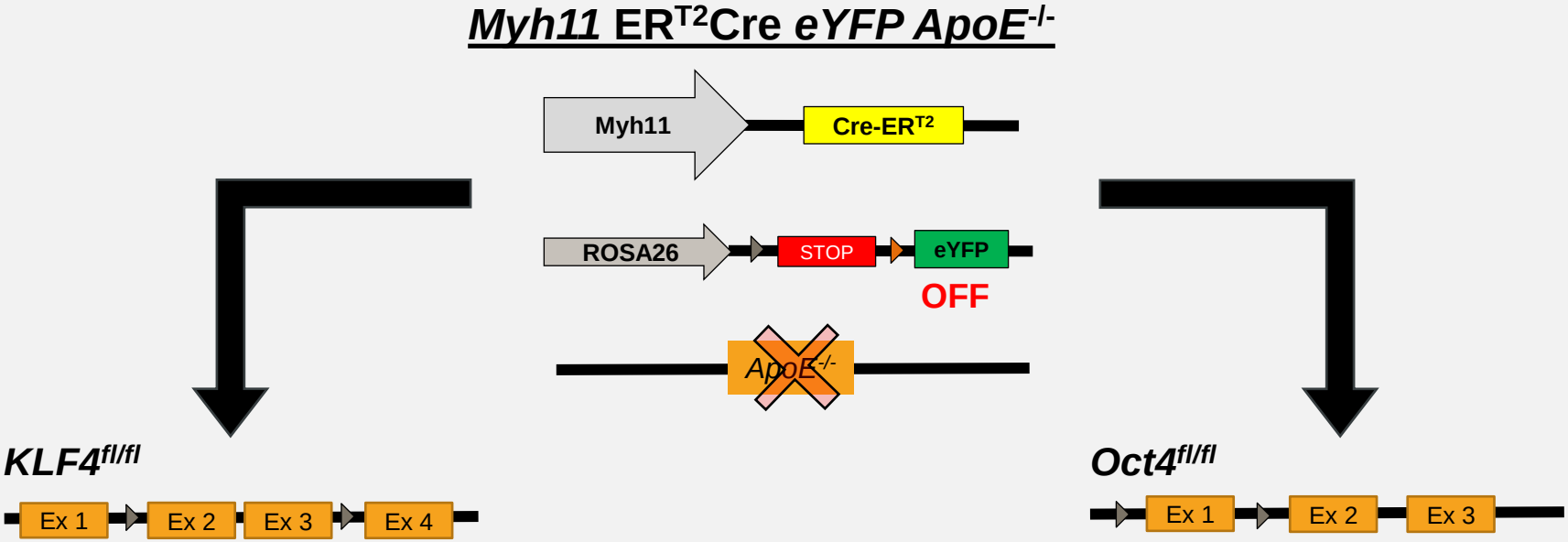
IV. Oct4 and Klf4 expression in SMC-P is critical in regulating angiogenesis, perivascular cell coverage, and the innate metabolic and inflammatory properties of adipose tissues.

SMC-specific conditional knockout of pluripotency factors, KLF4 and Oct4, demonstrate a critical role of SMC in late stage lesion pathogenesis



nature medicine

KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis

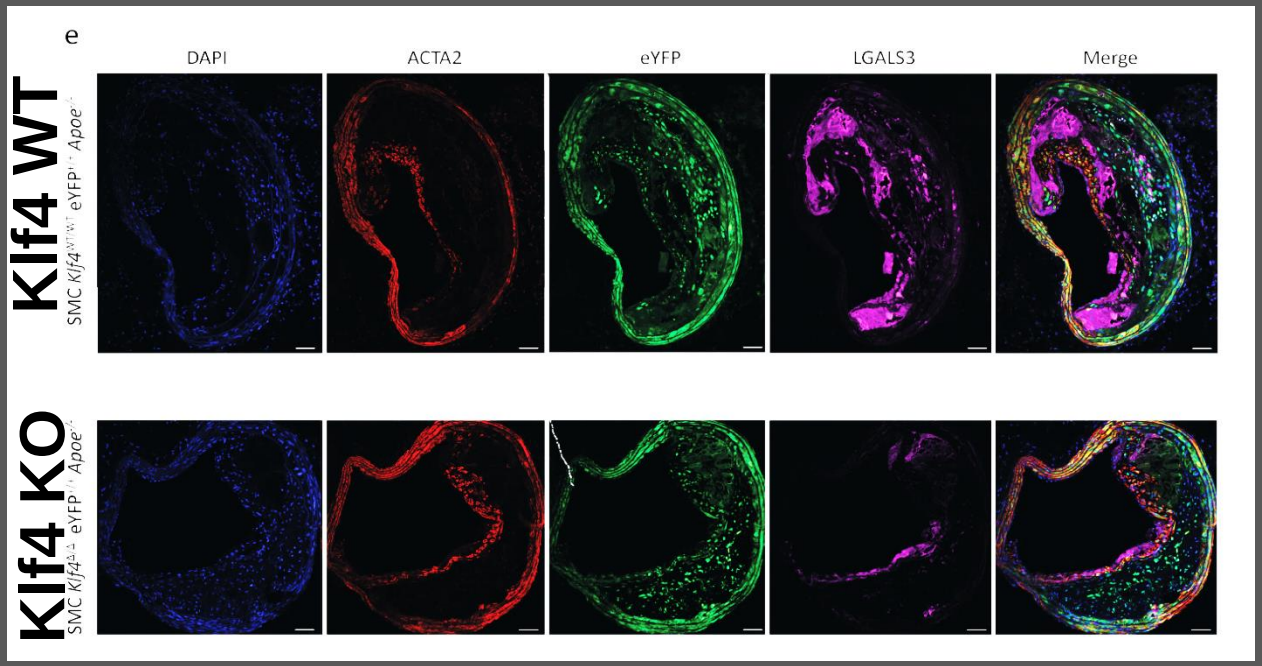
Laura S Shankman[1,2], Delphine Gomez[1], Olga A Cherepanova[1], Morgan Salmon[3], Gabriel F Alencar[1,4], Ryan M Haskins[1,5], Pamela Swiatlowska[1,6], Alexandra A C Newman[1,4], Elizabeth S Greene[1], Adam C Straub[7], Brant Isakson[1,2], Gwendalyn J Randolph[8] & Gary K Owens[1,2]

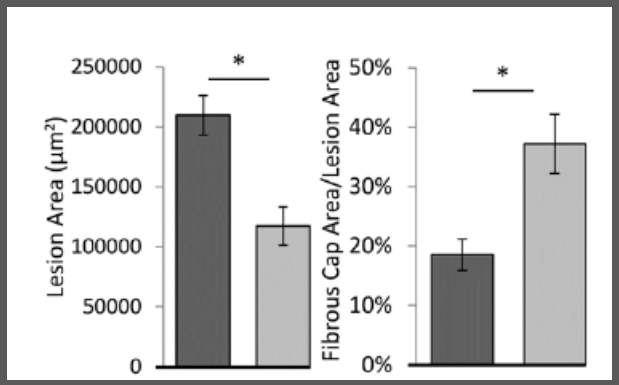
nature medicine

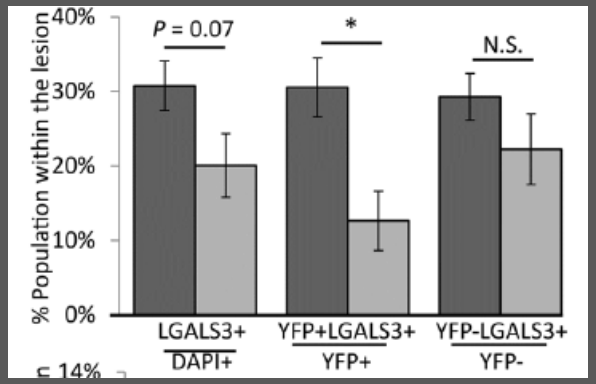
Activation of the pluripotency factor OCT4 in smooth muscle cells is atheroprotective

Olga A Cherepanova[1], Delphine Gomez[1,2], Laura S Shankman[1,2], Pamela Swiatlowska[1,3], Jason Williams[4], Olga F Sarmento[5], Gabriel F Alencar[1,6], Daniel L Hess[1,6], Melissa H Bevard[1], Elizabeth S Greene[1], Meera Murgai[1,7], Stephen D Turner[8], Yong-Jian Geng[4], Stefan Bekiranov[6], Jessica J Connelly[1,9], Alexey Tomilin & Gary K Owens[1,2]

SMC Specific Conditional KO of the Stem Cell Pluripotency Factor KLF4 Resulted in Markedly Reduced Lesion Size but Increases in Multiple Indices of Plaque Stability

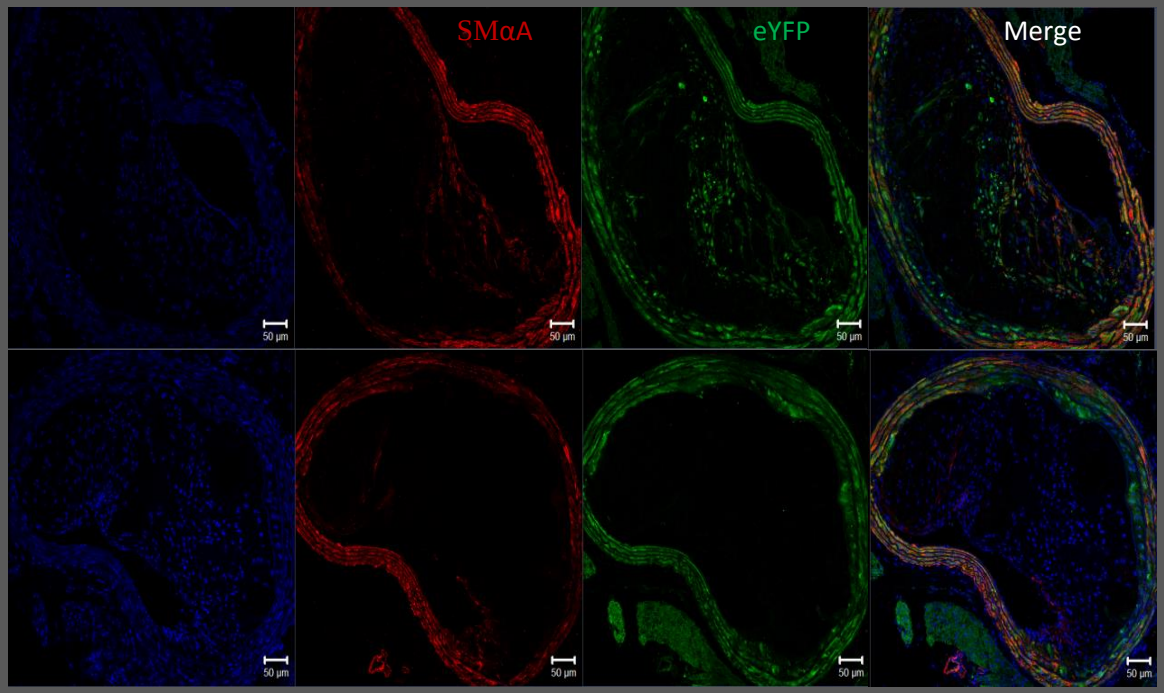


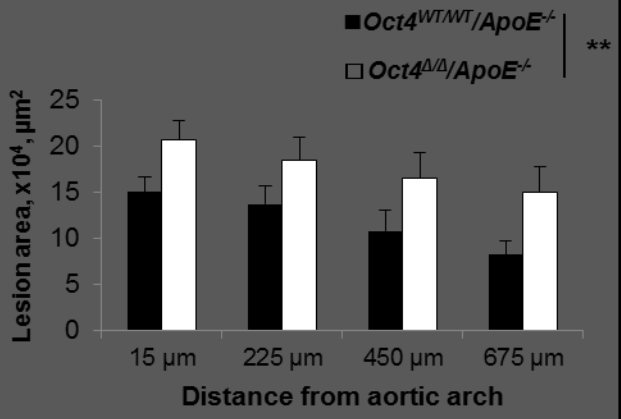


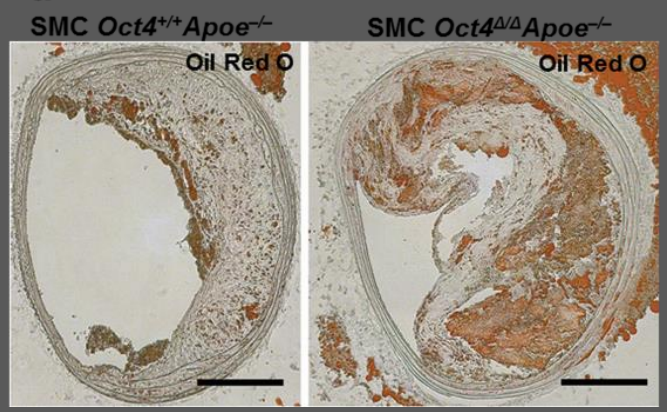


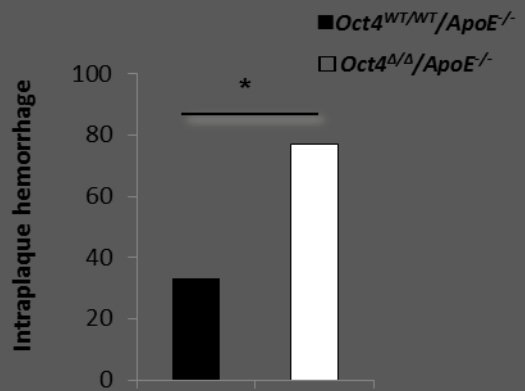
Shankman and Owens, 2015 *Nature Medicine*

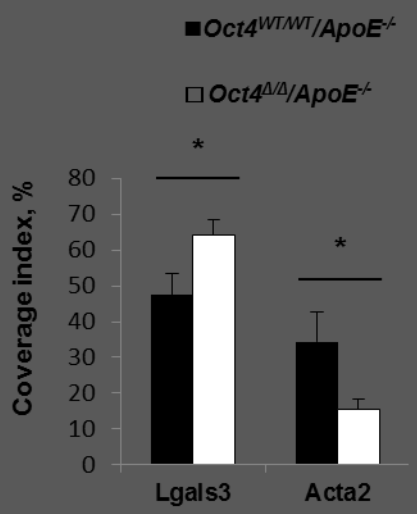
SMC-Specific Conditional KO of the Stem Cell Pluripotency Factor Oct4 Resulted in Lesions Virtually Devoid of SMC and which were Larger and Exhibited Multiple Features of Reduced Plaque Stability









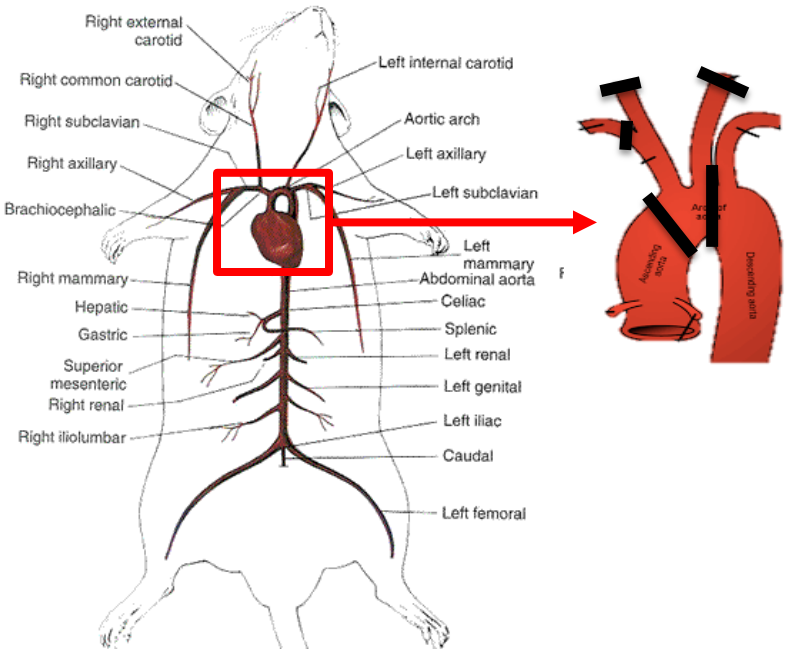


Cherepanova et al, *Nature Medicine* 2016

What are the critical Oct4 and Klf4 target genes that explain why their loss in SMC has profoundly different effects on lesion pathogenesis?

Can we exploit these differences to better define different SMC phenotypes that confer atheroprotective versus atheropromoting effects, and to identify novel therapeutic targets for promoting plaque stability?

In vivo genomic analyses of advanced BCA lesions from WT versus SMC Oct4 or Klf4 KO ApoE-/- mice fed a WD for 18 weeks



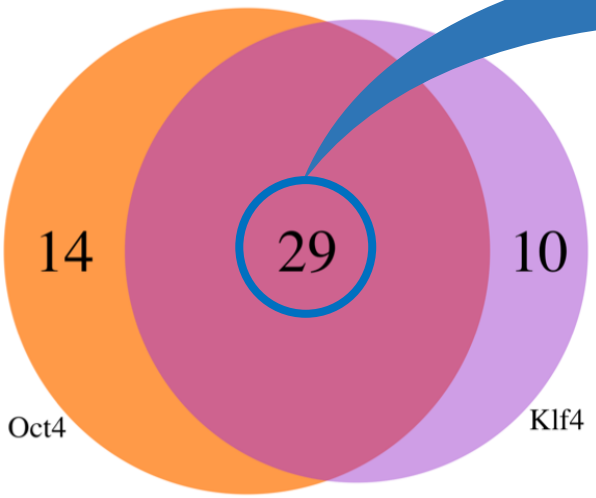
Genotype	ChIP-seq	RNA-seq
Klf4 KO	n = 1 (pooled from ~15 animals)	n = 5
Klf4 WT	n = 1 (pooled from ~15 animals)	n = 4

Genotype	ChIP-seq	RNA-seq
Oct4 KO	n = 1 (pooled from ~15 animals)	n = 4
Oct4 WT	n = 1 (pooled from ~15 animals)	n = 4

Multiplex Genomic Analyses of Advanced BCA Lesions from SMC Conditional Oct4 and Klf4 KO Mice Exhibit Profoundly Different Molecular Signatures

Genotype	# of Up-Regulated KEGG Pathways (FDR=0.05)	# of Down-Regulated KEGG Pathways (FDR=0.05)
Klf4 KO vs WT	0	39
Oct4 KO vs WT	43	0

Common Differentially Regulated Pathways



Klf4 and Oct4 log Fold-change of examples of genes present in *Klf4* ChIP-seq and common Pathways (n=54)

Gene	Klf4 KO logFC	Oct4 KO logFC
CD36	-0.499	0.495
Calr	-0.175	0.289
Csf2ra	-0.074	0.256
Cxcr4	-0.169	0.416
Itgax (CD11c)	-0.372	0.663
Stat3	-0.151	0.059
Tubb2b	0.556	-0.108

- logFC + logFC

Klf4 and Oct4 log Fold-change of examples of genes present in *Oct4* ChIP-seq and common Pathways (n=32)

Gene	Klf4 KO logFC	Oct4 KO logFC
CD9	-0.074	0.140
H2-M1	0.132	-0.146
Il7r	-0.311	0.641
Itga4 (CD49D)	-0.218	0.236
Ly96	-0.248	0.281
Tgfbr2	-0.208	0.121
Tlr4	-0.206	0.410

- logFC + logFC

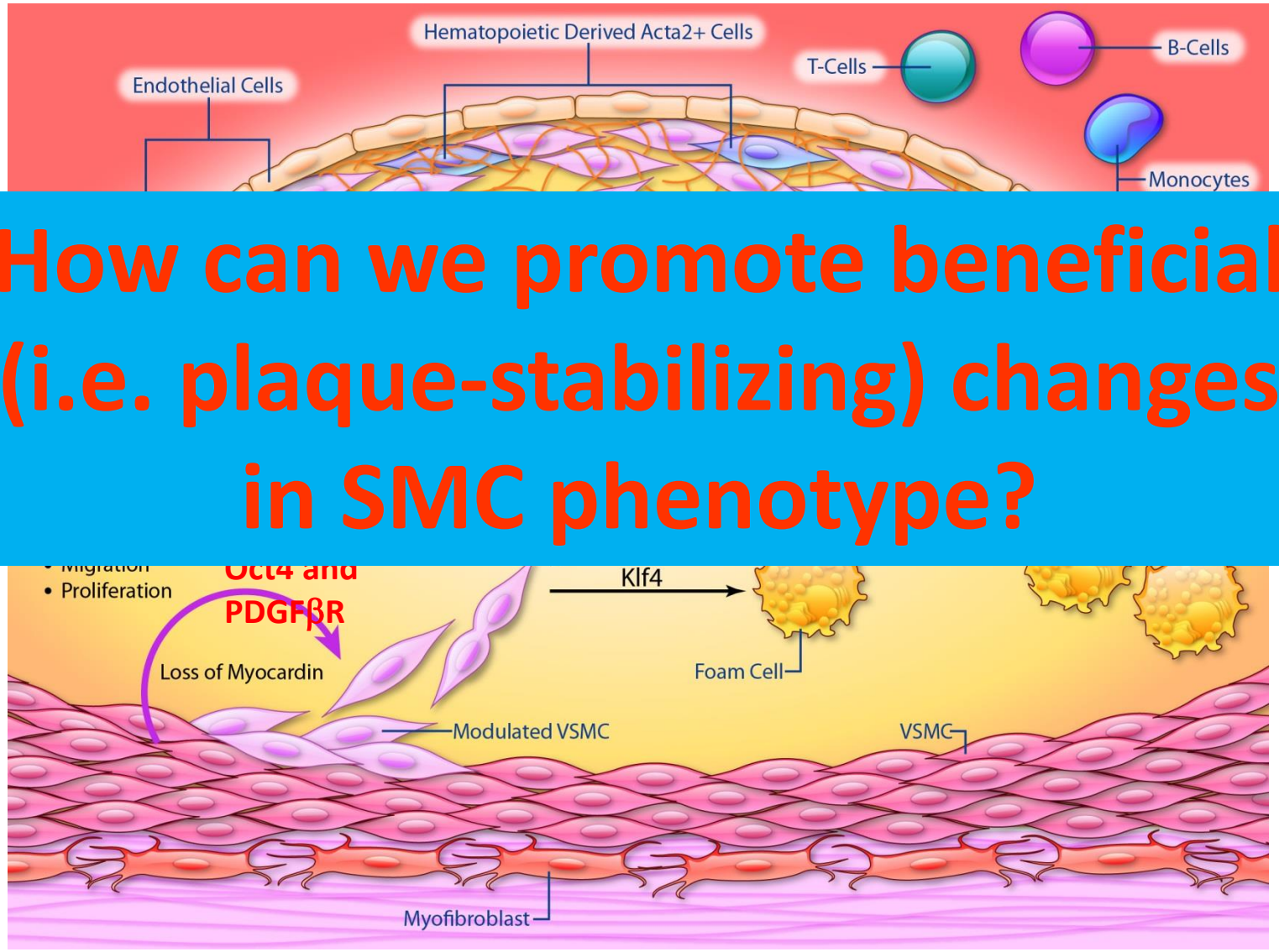
Gabriel Alencar, Stefan Bekiranov, and Gary K. Owens, manuscript in preparation

Of 94 human genes shown to be associated with increased CAD risk, 29 were identified as SMC Klf4 and/or Oct4 target genes in our mouse advanced BCA lesion genomic analyses including the following:

CAD/MI risk gene	OCT4 target	KLF4 target
PHACTR1	x	x
ATP2B1	x	x
LRP1	x	x
APOE-APOC1	x	x
ZEB2	x	
MAD2L1	x	
EDNRA	x	
TRIB1	x	
SCARB1	x	
RHOA		x
UMPS-ITGB5		x
FURIN-FES		x
SMG6		x

Collaboration with Jeanette Erdmann (U Lubeck) and Heri Schunkert (U Munich)

SMC Derived Cells within Advanced Lesions Can Exhibit Athero-Protective or Athero-Promoting Phenotypes



How can we promote beneficial (i.e. plaque-stabilizing) changes in SMC phenotype?

Bennett, Sinha, and Owens, *Circulation Research* 2016

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IV. Oct4 and Klf4 expression in SMC-P is critical in regulating angiogenesis, perivascular cell coverage, and the innate metabolic and inflammatory properties of adipose tissues.

Ongoing clinical trial treating high-risk patients with **established**, symptomatic atherosclerosis with Anti-IL1β Ab

CANTOS Trial: phase III, randomized, placebo-controlled trial

1. **Intervention**: Administering Anti-IL1β Ab or placebo

2. **Cohort**: 10,065 patients

3. **High-risk Patients**:
   a) Already survived one myocardial infarction
   b) Elevated inflammatory biomarkers despite standard of care therapy

4. **Endpoints**: recurrent MI, stroke, or cardiovascular death

5. **Cost**: 776 million dollars over 6 years

Hypothesis: global suppression of inflammation will promote plaque stability and/or induce beneficial cardiac remodeling and thereby reduce the probability of cardiovascular death due to MI, HF, stoke, or other causes.

A Key Pre-clinical Study for the Novartis CANTOS Trial Was a Prevention Study and Did not Examine Indices of Plaque Stability

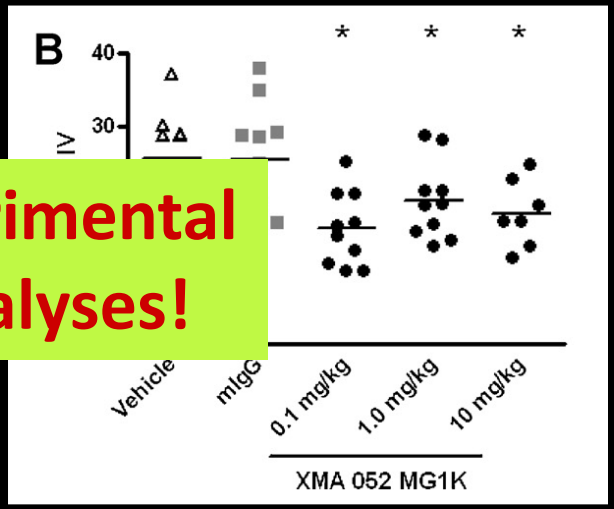
Prevention

Apoe$^{-/-}$ Il1b Ab: 0.1-10mg/kg

Apoe$^{-/-}$ IgG

0 8 26 34

We believe this is the wrong experimental design and method for lesion analyses!



Bhaskar et al. *Atherosclerosis* 216:313, 2011

Our Approach – Reversal (intervention)

SMC YFP Apoe$^{-/-}$ Il1b Ab: 10mg/kg

SMC YFP Apoe$^{-/-}$ IgG

Age (weeks)

Il1b Ab

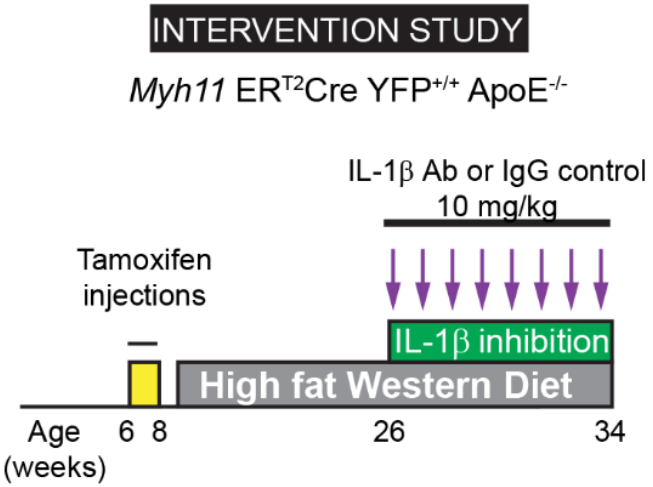
High Fat (Western) Diet

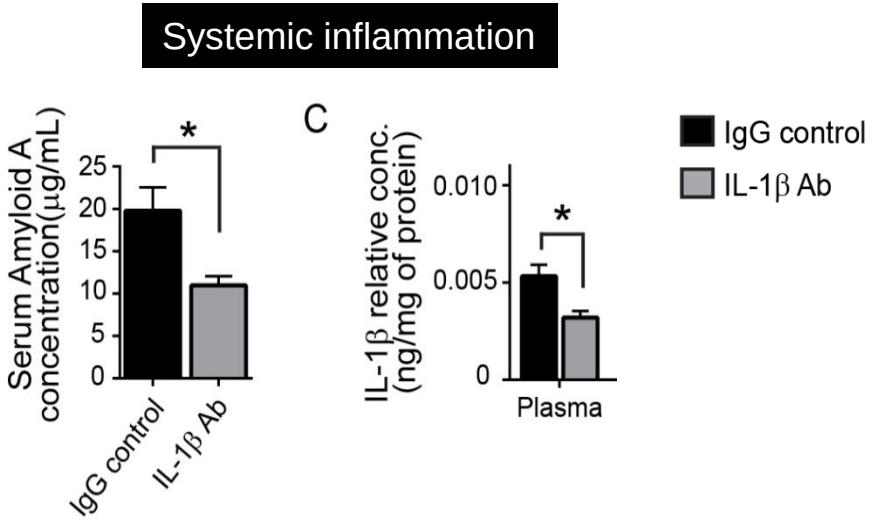
0 8 26 34

What is the impact of IL1β antibody treatment of mice with established lesions including evaluation of lesion size, cell composition and phenotypes, outward remodeling, and indices of plaque stability?

Gomez and Owens, 2016, manuscript in preparation; see our Viewpoint Paper Baylis et *Circ Res* 2016

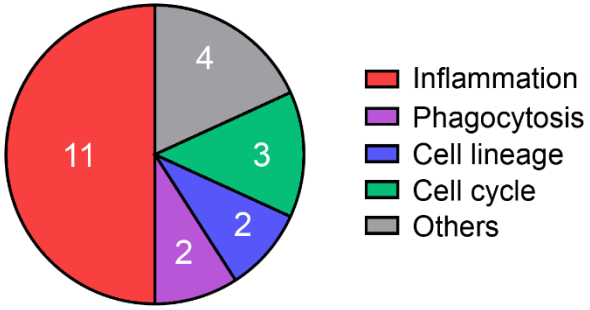
IL-1β neutralizing antibody administration in 18 week WD fed mice induced a marked repression of systemic and lesion inflammation



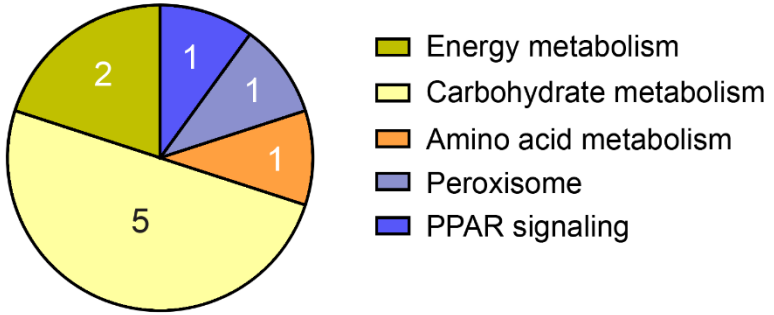


Local inflammation: RNA-seq on atherosclerotic lesion (BAC and aortic arch)

Down-regulated pathways by IL-1β Ab

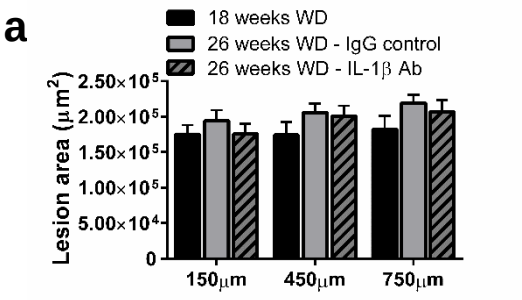


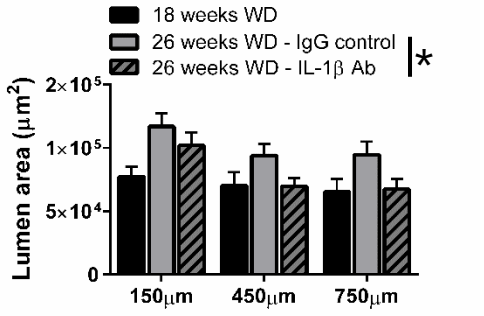
Up-regulated pathways by IL-1β Ab

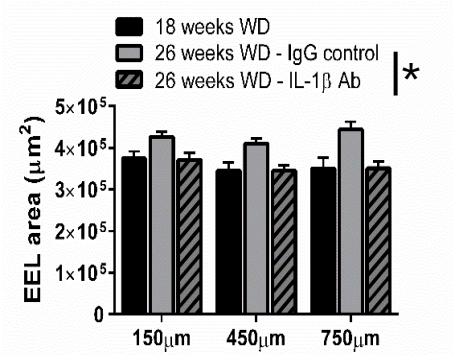


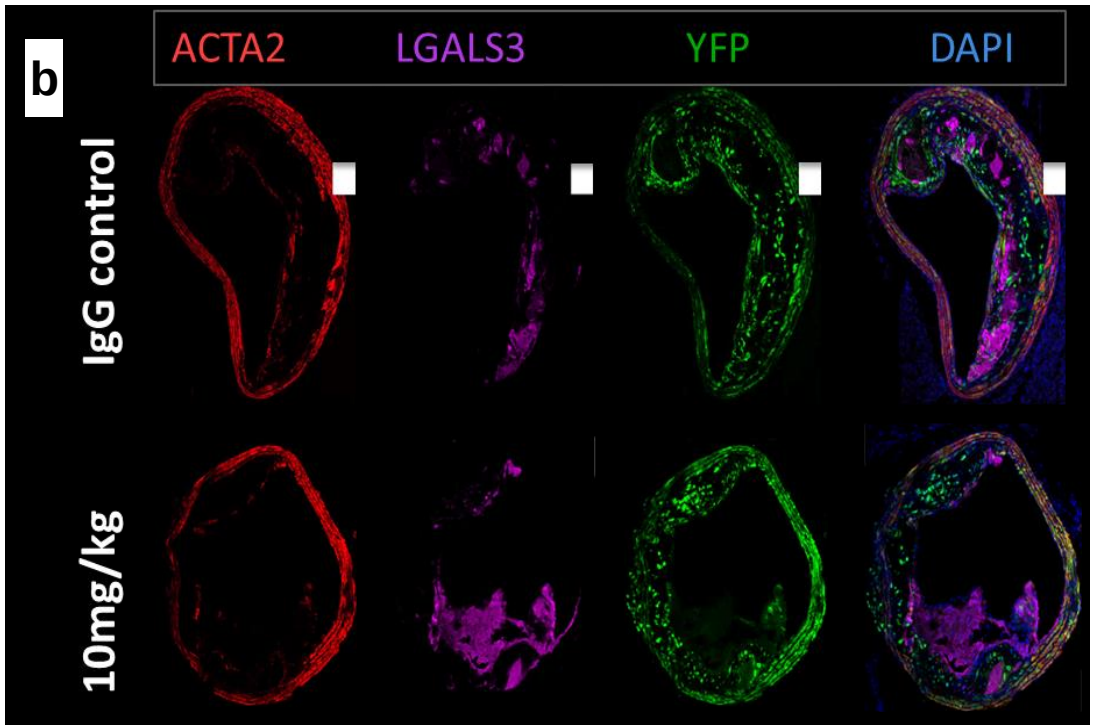
Gomez et al. In preparation

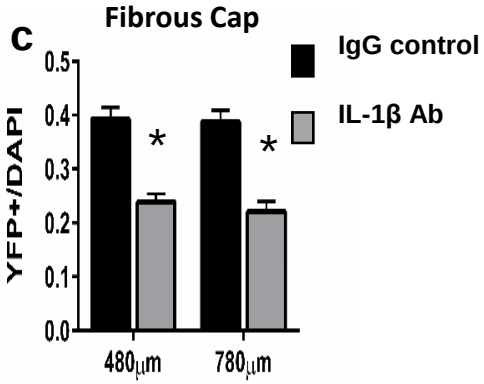
Treatment of our Myh11 eYFP ApoE-/- Mice with the Novartis Anti-IL1β Antibody Failed to Reduce Lesion Size, Impaired Beneficial Outward Remodeling, and Reduced Indices of Plaque Stability

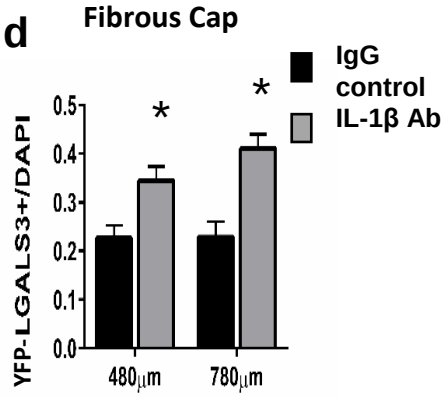


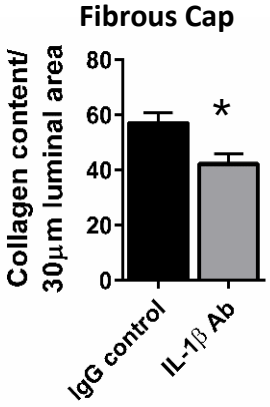




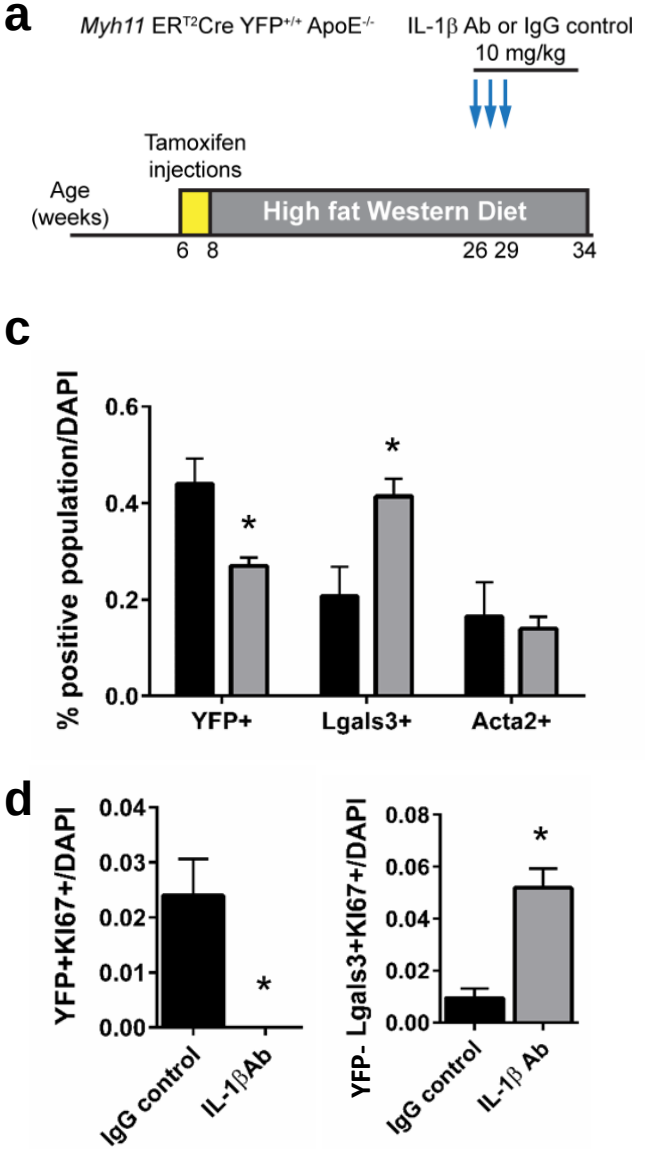


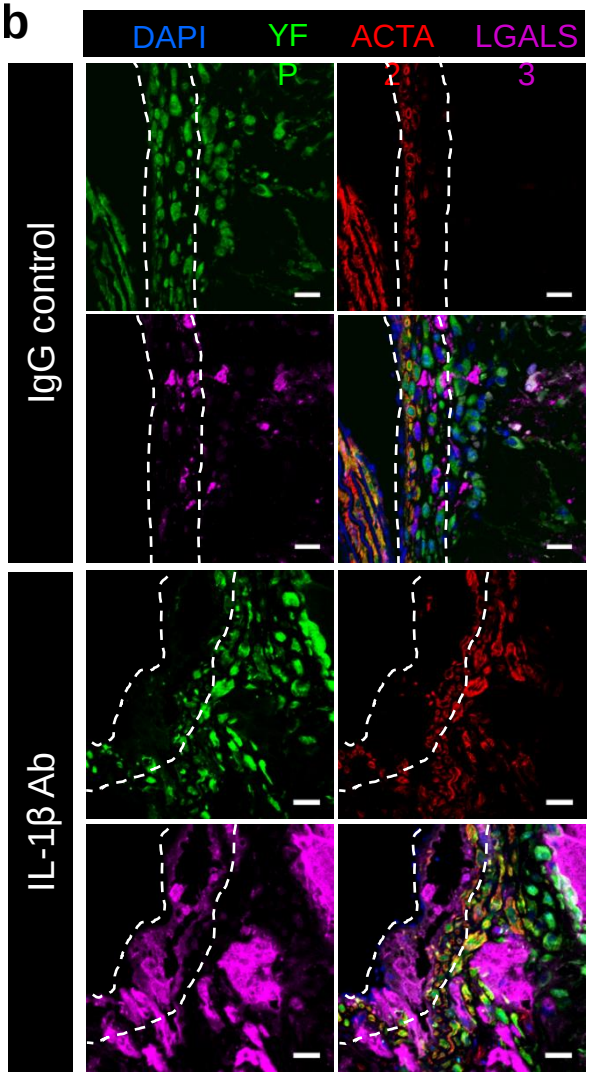






IL-1β Neutralization Induced Macrophage Accumulation and Loss of SMC within the Fibrous Cap is Due at Least in Part to Reduced SMC but Increased Macrophage Proliferation



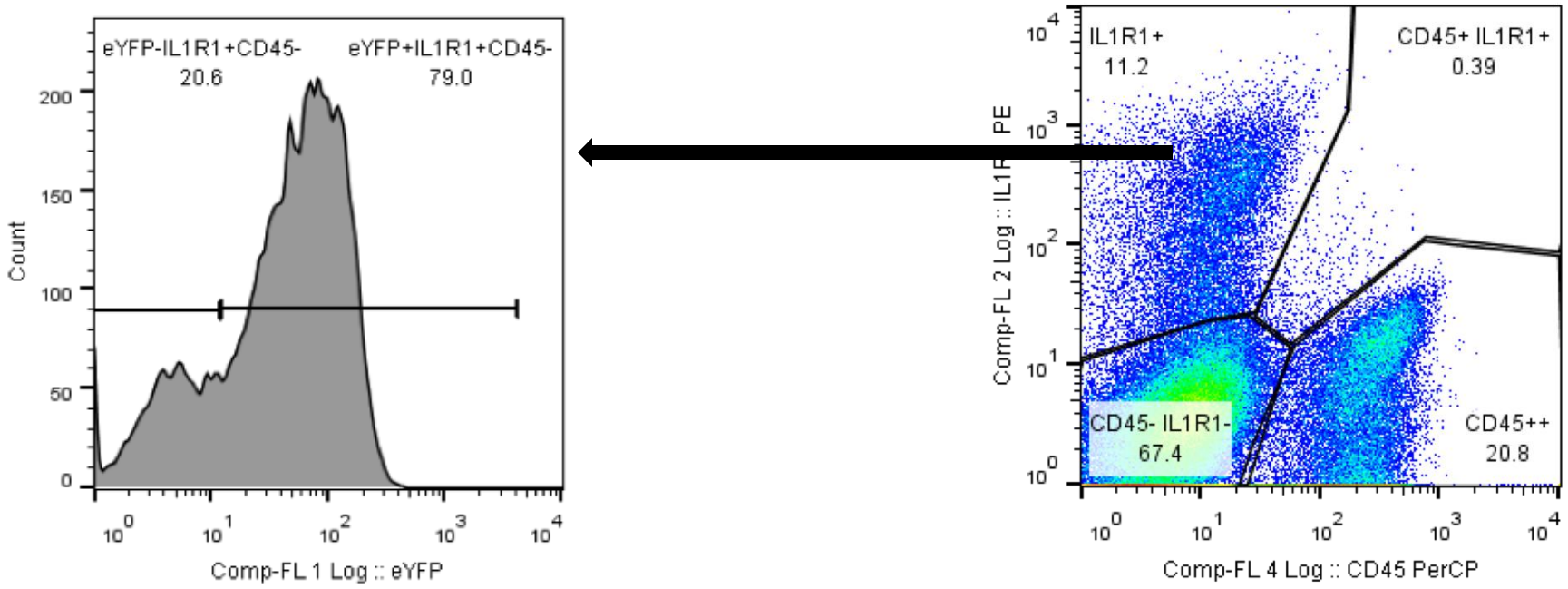


Gomez and Owens, 2016, manuscript in preparation

Key Unresolved Questions:

1. **Are these unexpected detrimental effects of IL1β neutralization due to inactivation of IL1β within the lesions themselves, reductions in systemic inflammation, a combination thereof, or some unknown alternative mechanism?**
2. **Are effects mediated through impaired IL1R1 signaling in macrophages, SMC, EC, some other cell type within lesions, or a combination thereof?**
3. **What are the underlying mechanisms whereby IL1β confers beneficial effects within advanced lesions?**
4. **Can we negate or counteract the detrimental effects of IL1β neutralization by some complementary therapy?**

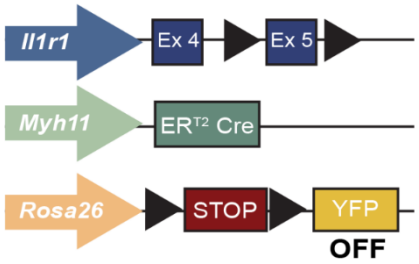
Nearly 80% of IL1R1 Receptor^{+} Cells within the Aorta of 18 week WD-Fed Myh11eYFP ApoE$^{-/-}$ Mice are of SMC Origin



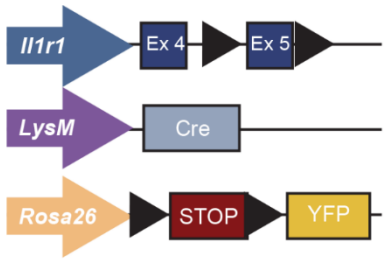
Baylis, Gomez, and Owens; manuscript in preparation

Generation of SMC Specific and Myeloid Selective IL1R1 KO Mice

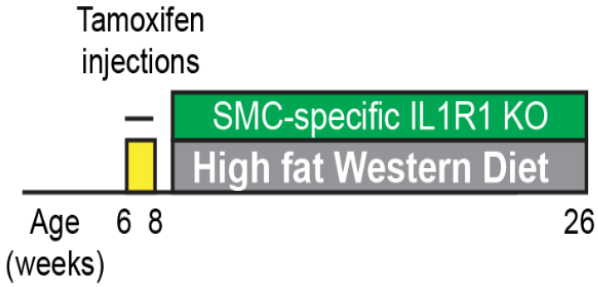
Myh11 ER^{T2} Cre YFP ApoE$^{-/-}$ IL1R1^{flox}



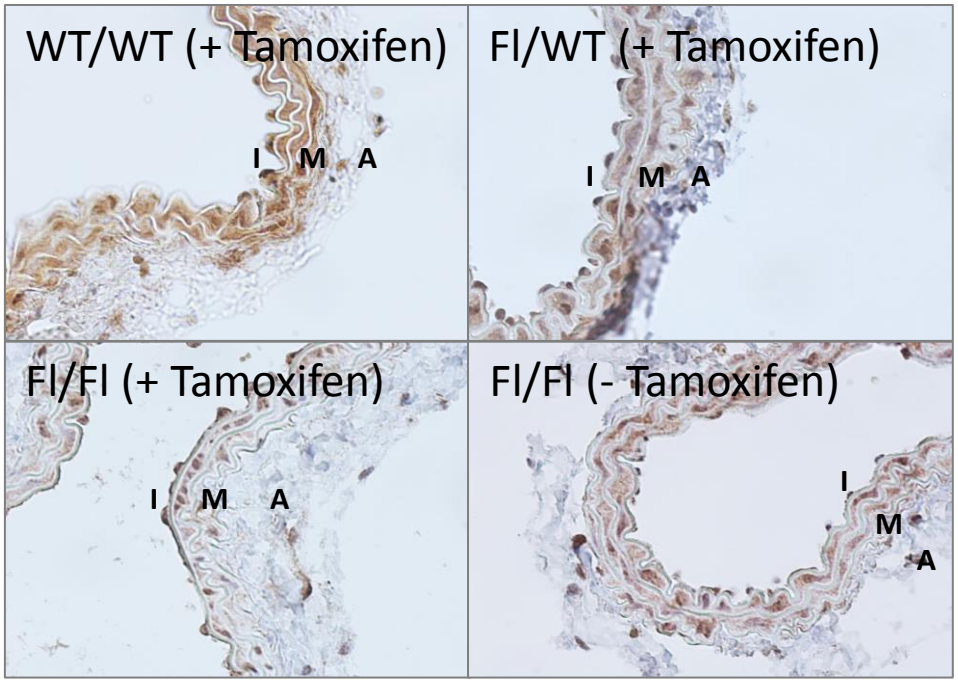
LysM Cre YFP ApoE$^{-/-}$ IL1R1^{flox}



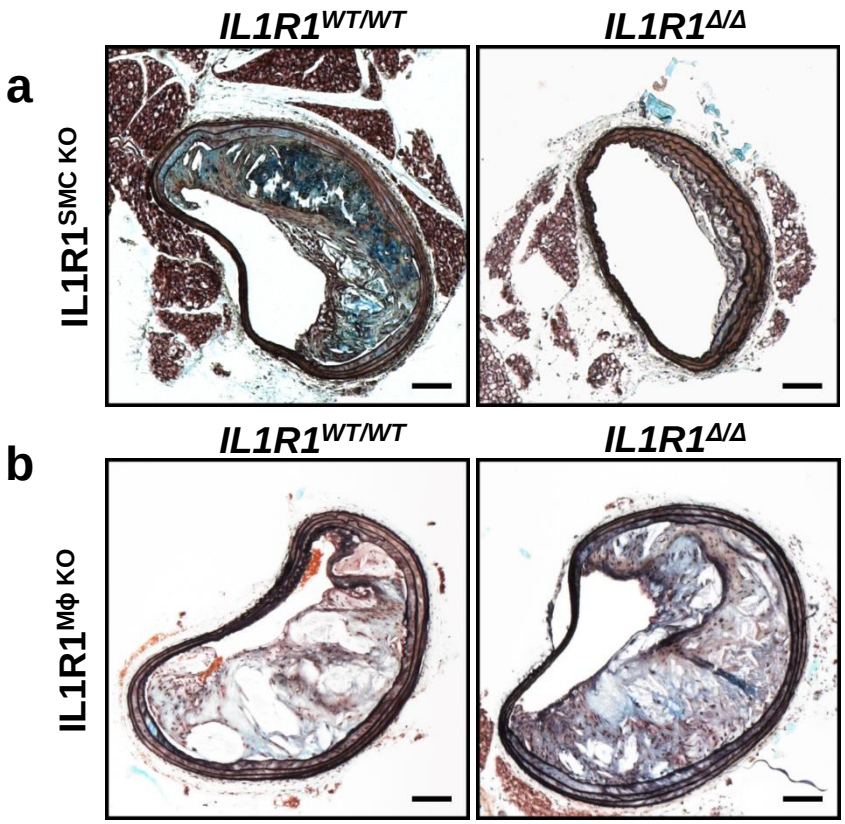
SMC KO Experimental Design

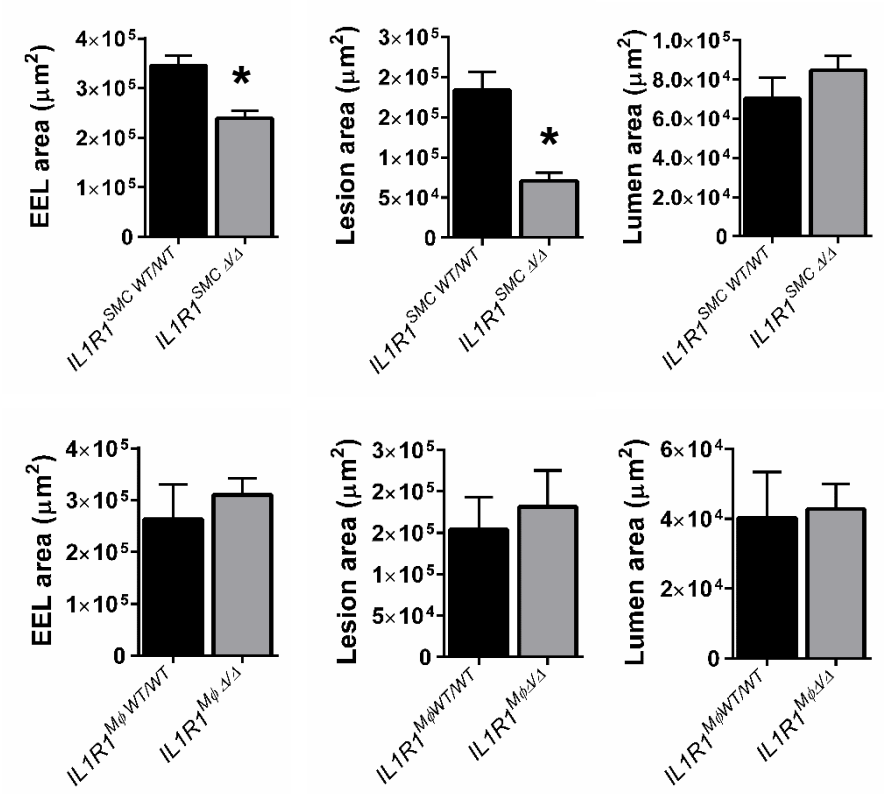


SMC IL1R1 Immunostaining



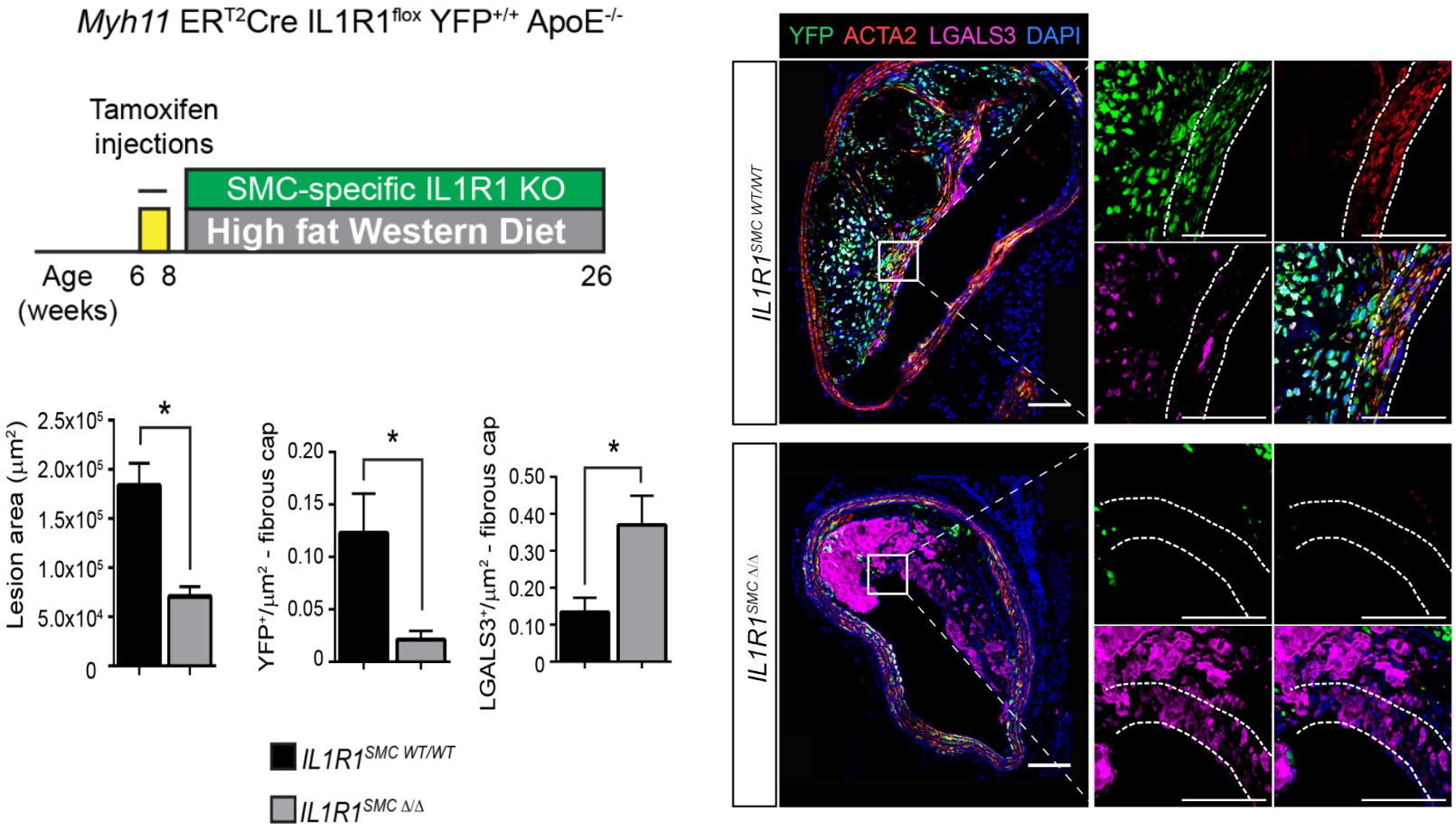
IL1R1-Mediated Exacerbation of Atherosclerosis Development is Mediated by SMC not Myeloid Cells





Gomez et al. manuscript in preparation

IL1R1 Signaling in SMC is Required for Investment of SMC into Lesions Including Formation of a Protective Fibrous Cap



Gomez et al. manuscript in preparation

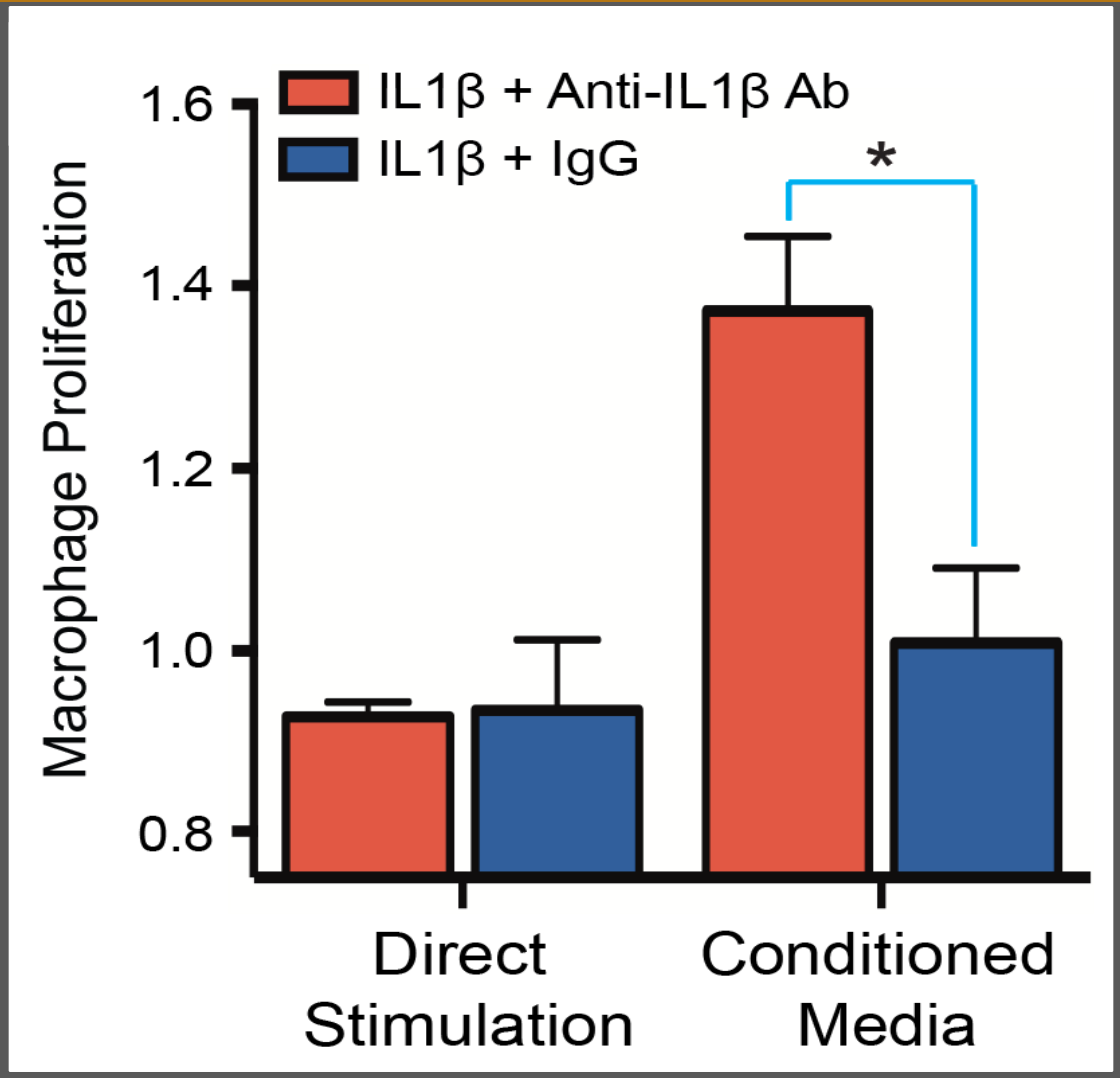
What are the underlying mechanisms whereby IL1β confers beneficial effects within advanced lesions?

Hypotheses:

1. **IL1β promotes increased stabilization of advanced lesions at least in part by inducing fibrous cap SMC to secret macrophage chemo-repulsants and anti-proliferative cytokines as well as extracellular matrix components.**

2. **IL1β neutralization in the setting of advanced atherosclerosis induces a false sense of inflammation resolution leading to dissolution of the fibrous cap in part by loss of SMC and influx and/or proliferation of resident M2 macrophages.**

Cultured SMC stimulated by IL1β secrete a factor or factors that inhibits Mϕ proliferation



Richard Baylis and Delphine Gomez, studies in progress

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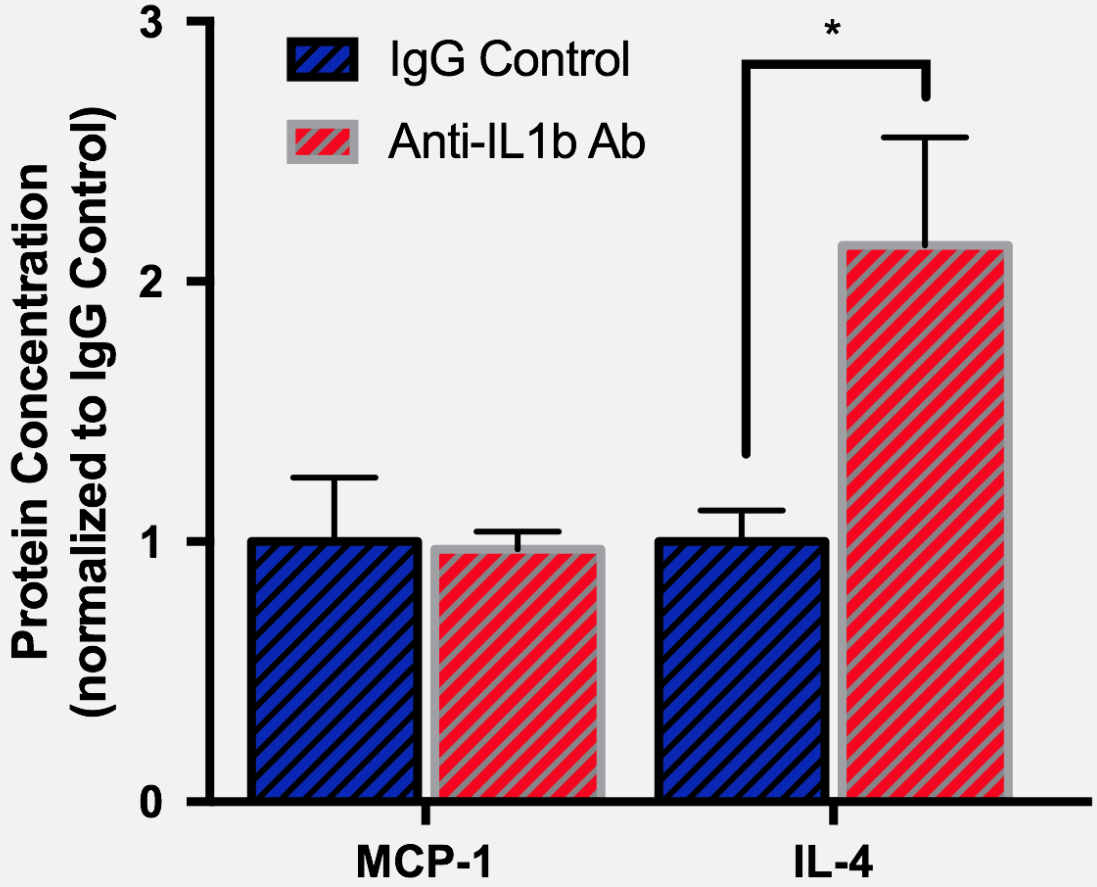
IL1β Treatment of SMC eYFP ApoE-/- Mice with Advanced BCA Lesions Induced Increased IL-4 Expression



IL-4 has been shown to inhibit SMC proliferation but promote proliferation of macrophages as well as their transition to an M2 state (Hawker et al., 1998 AJP; Jenkins et al., 2013 JEM; Moore and Tabas, 2011 *Cell* .

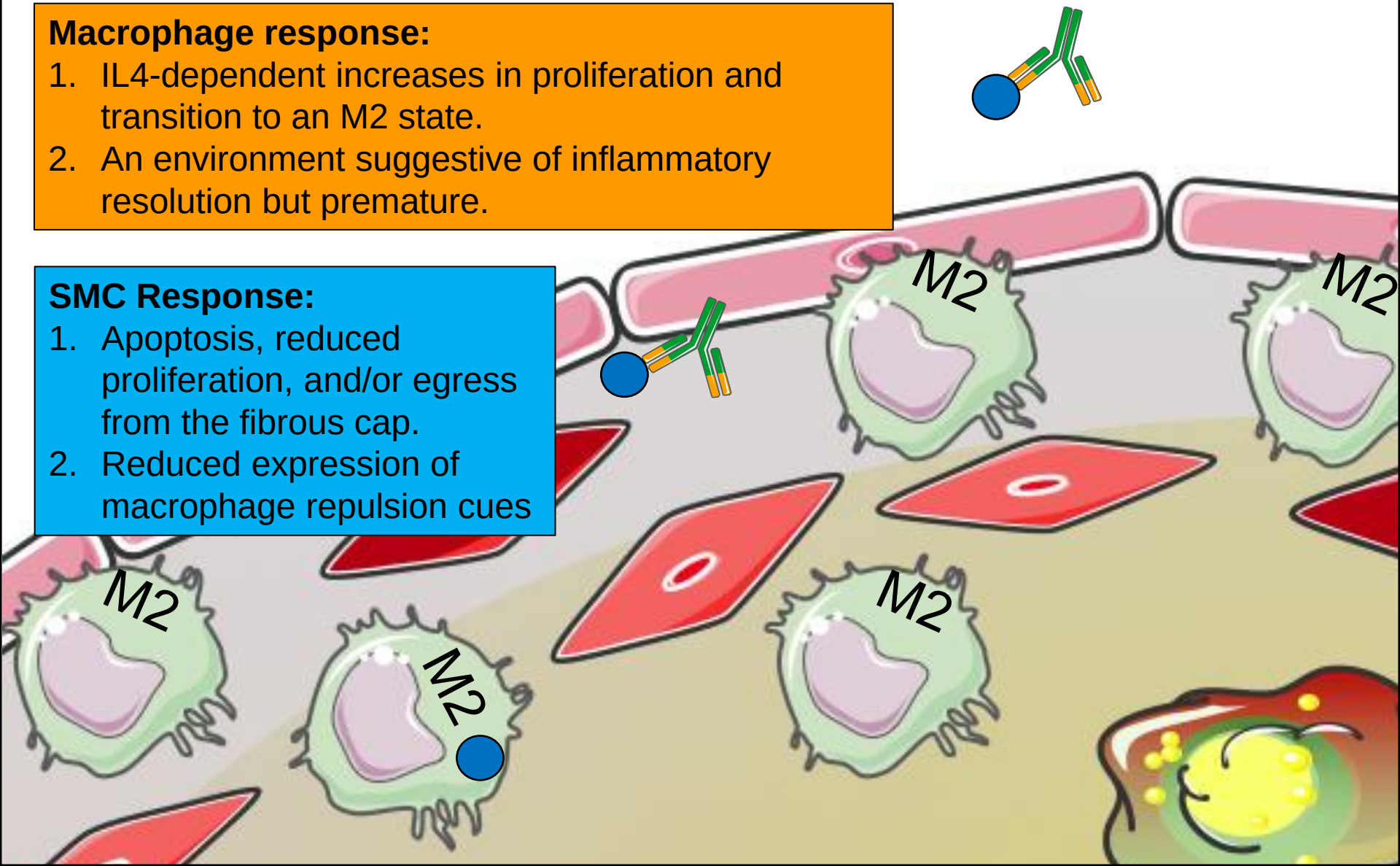
Hypothesis: IL1β Antibody Neutralization Induces a False Sense of Inflammation Resolution

Macrophage response:

1. IL4-dependent increases in proliferation and transition to an M2 state.
2. An environment suggestive of inflammatory resolution but premature.

SMC Response:

1. Apoptosis, reduced proliferation, and/or egress from the fibrous cap.
2. Reduced expression of macrophage repulsion cues



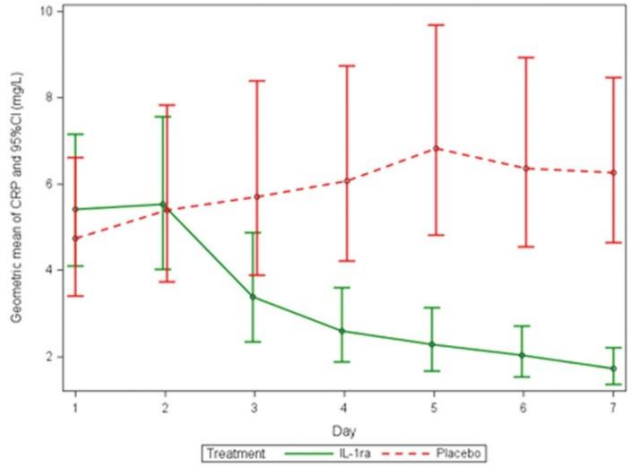
IL1β Study Conclusions:

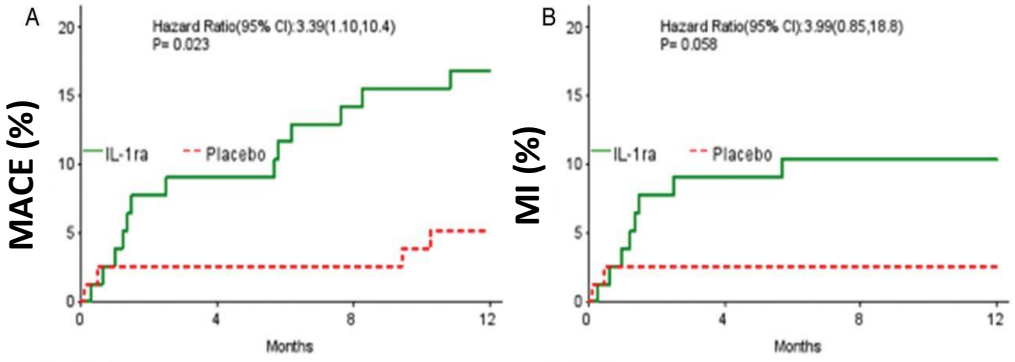
1. The cellular composition of the fibrous cap appears to be far more plastic than has generally been appreciated.
2. IL-1β has an unexpected atheroprotective role in late stage lesions by promoting a SMC-rich macrophage-deficient fibrous cap.
3. Inhibition of IL-1β is associated with marked decreases in SMC proliferation and increased macrophage proliferation within the fibrous cap but no change in monocyte trafficking.
4. It is unclear if these effects are due to direct effects of IL-1β on SMC, macrophages, and/or another cell type. However, by far the majority of IL1R1+ cells within lesions are of SMC origin, and we have evidence that fibrous cap SMC may produce factors that inhibit macrophage recruitment and proliferation within the fibrous cap through an IL1β-dependent process.
5. We found that IL-1β -dependent enhancement of development of atherosclerosis is dependent on SMC not myeloid cells.
6. Whereas our mouse studies certainly do not predict outcomes in the CANTOS trial several recent studies suggest that they may.

Evidence that suppression of IL-1 signaling increases rather than decreases cardiovascular risk in humans

The effect of interleukin-1 receptor antagonist therapy on markers of inflammation in non-ST elevation acute coronary syndromes: the MRC-ILA Heart Study 2014

Morton et al. European Heart Journal 2014.

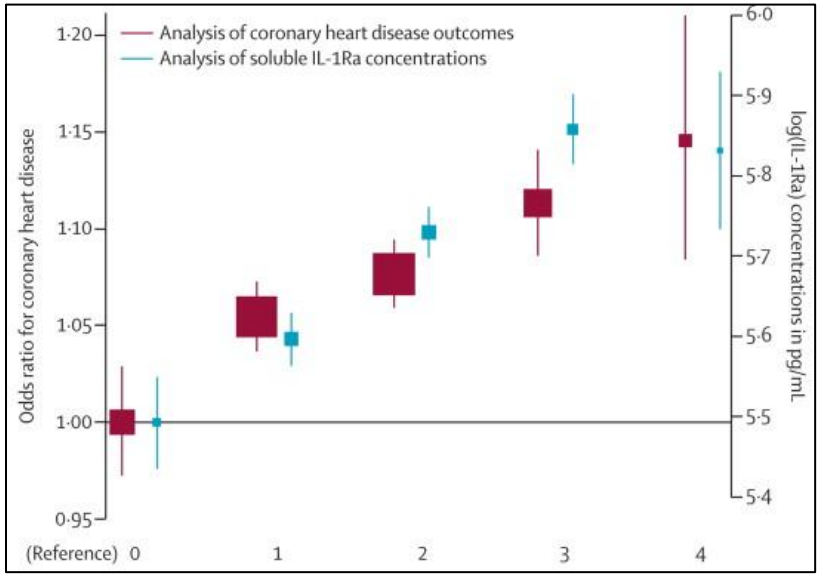




Cardiometabolic effects of genetic upregulation of the interleukin 1 receptor antagonist: a Mendelian randomisation analysis 2015

*The Interleukin 1 Genetics Consortium**

The Interleukin 1 Genetics Consortium. Lancet. 2015.



Talk Outline:

I. The majority (>80%) of SMC within advanced atherosclerotic lesions lack expression of SMC marker genes and undergo transition to cells exhibiting characteristics of multiple other cell types including macrophages, MSC, and myofibroblasts (MFs).

II. The stem cell pluripotency genes Klf4 and Oct4 regulate phenotypic transitions of SMC critical in the pathogenesis of atherosclerosis. However, these transitions can be beneficial or detrimental depending on the nature of those changes.

III. IL1β has atheroprotective effects in late stage atherosclerotic lesions including being required for maintenance of a protective fibrous cap.

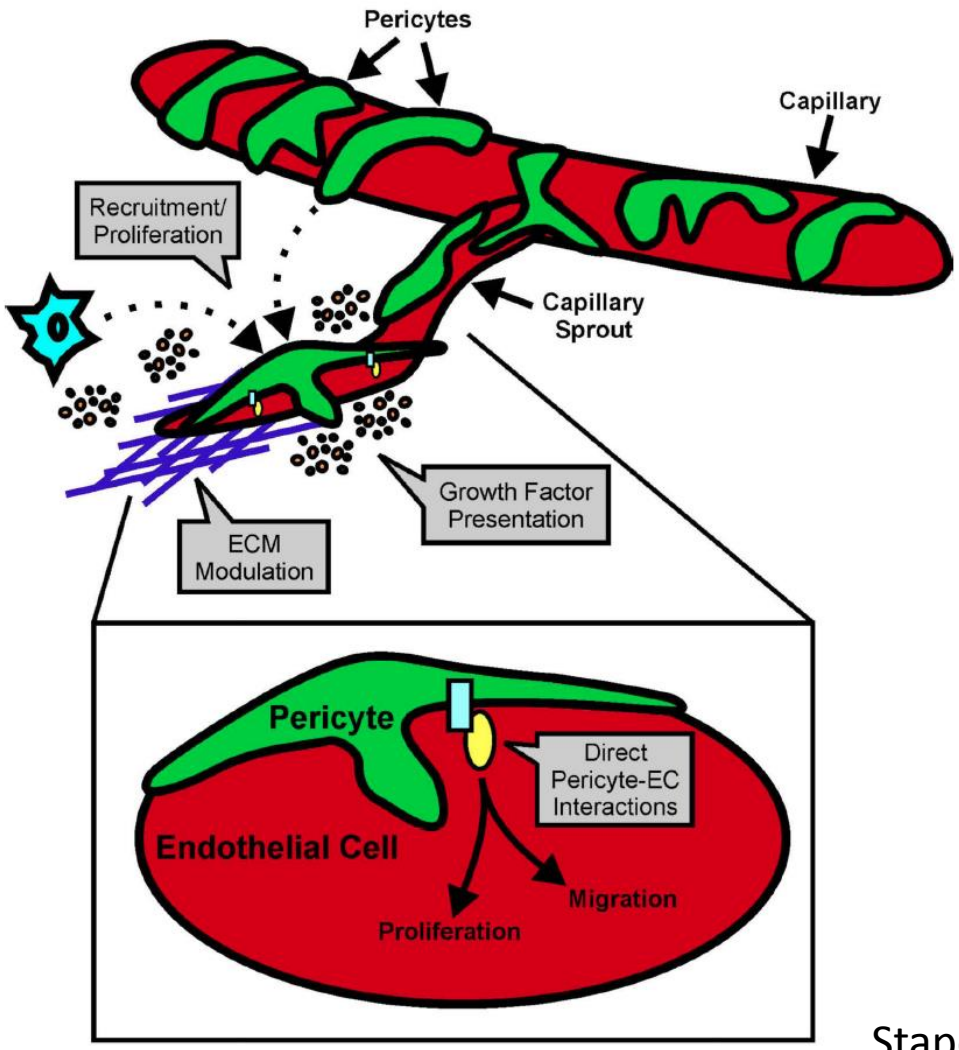
IV. Oct4 and Klf4 expression in SMC-P is critical in regulating angiogenesis, perivascular cell coverage, and the innate metabolic and inflammatory properties of adipose tissues.

What is the normal function of the stem cell pluripotency genes Oct4 and Klf4 in SMC and pericytes?

1. It is not related to their roles in atherosclerosis because the clinical complications of this disease kill us well after our reproductive years - as such there has been little if any evolutionary selection on this basis.

2. Rather, we hypothesize that Oct4 and Klf4 play a critical role in regulating the plasticity of SMC and pericytes during generation of new blood vessels as well as in the repair and remodeling of existing blood vessels - processes that are essential for growth, survival, and reproduction of all organisms.

Angiogenesis occurs during both physiologic and pathologic growth, and requires tightly regulated and coordinated movement of SMC-P and ECs



1) Angiogenesis: new blood vessels sprouting from pre-existing vessels
2) Is thought to be driven primarily by endothelial cells.
3) However, it is well established that appropriate perivascular cell investment is required for formation of stable functional vascular networks.
4) Dysregulation of angiogenesis plays a role in multiple disease pathologies:
   A. Myocardial infarction, stroke, peripheral arterial disease (PAD)
   B. Cancer, eye diseases e.g. diabetic retinopathy, corneal neovascularization, etc.

Stapor et al, 2014

Multiple signaling pathways are believed to be important for SMC-P investment of EC tubes.

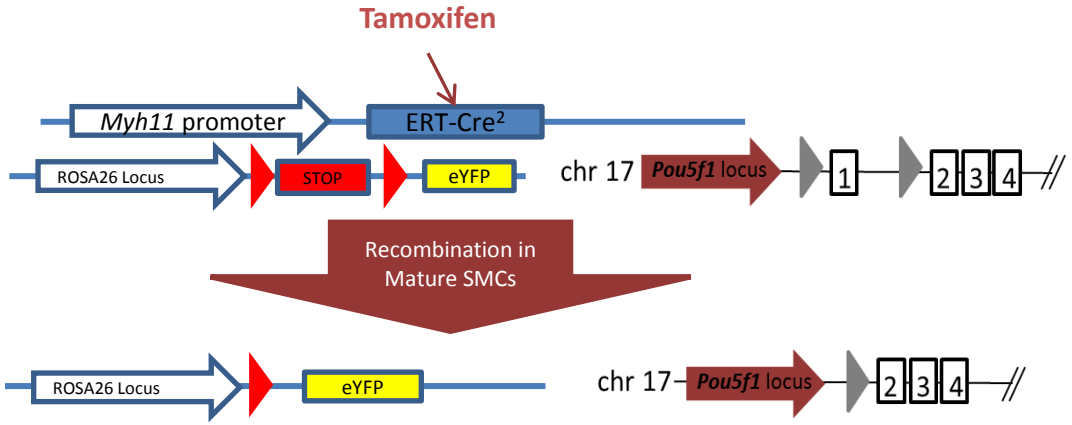
1. Loss of function of TGFBR2 causes vessel fragility due to impaired perivascular cell development (Pardali et al, 2010).

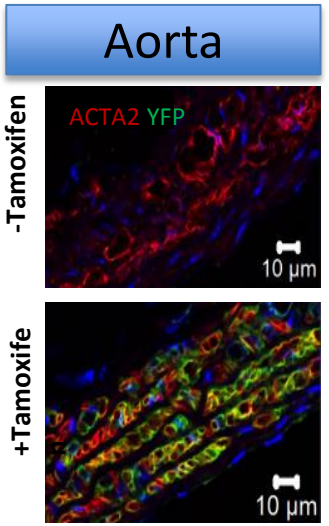
2. Inactivation or heterozygous loss of PDGFB or PDGFBR leads to pericyte deficiency, leaky vasculature, and BBB defects (Armulik et al, 2010).

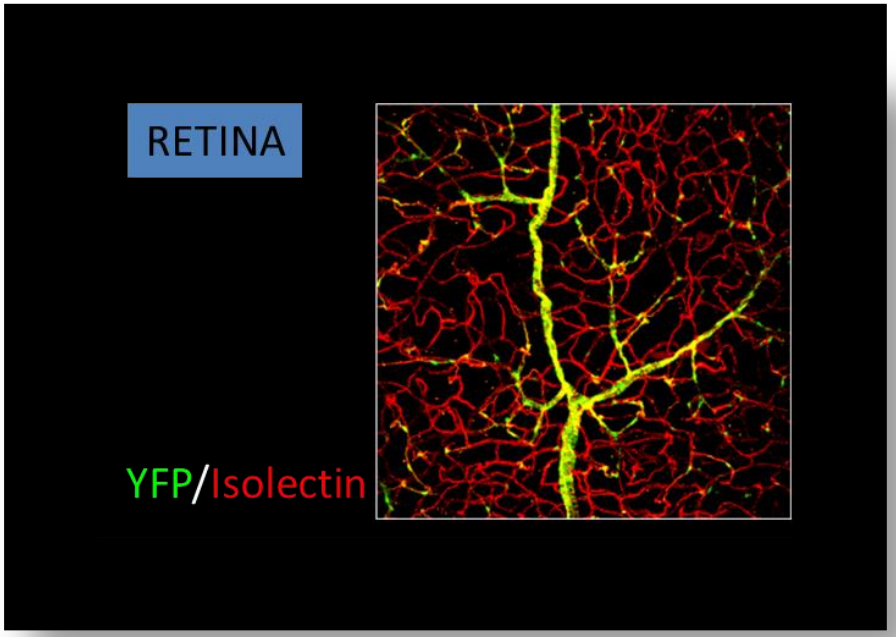
3. EphrinB2 deficiency leads to impaired perivascular cell migration and vascular defects (Pitulescu and Adams, 2010).

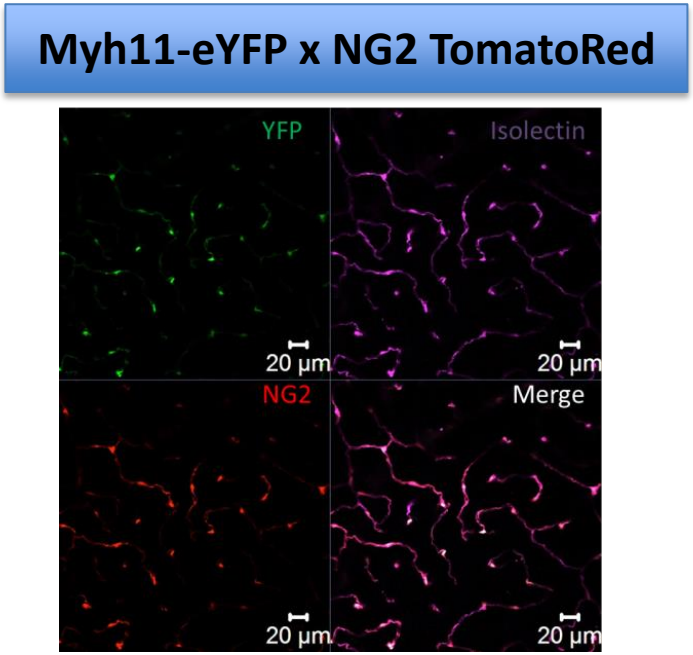
However, this evidence is based on global rather than SMC-P specific KO studies so effects may be indirect. As such, there is currently NO direct evidence that SMC-P can play a rate-limiting role in control of angiogenesis or showing that this process is dependent on a particular regulatory pathway.

The Myh11-eYFP Mouse Provides Definitive Lineage Tracing and Conditional Knockout of Genes (e.g. Oct4) in SMC and NG2^{+} Pericytes

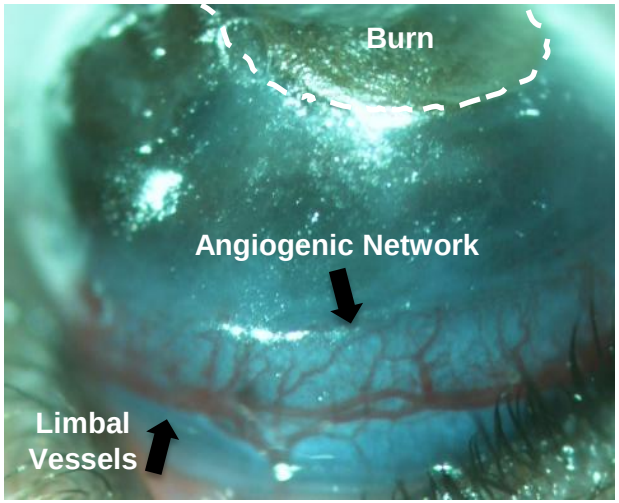


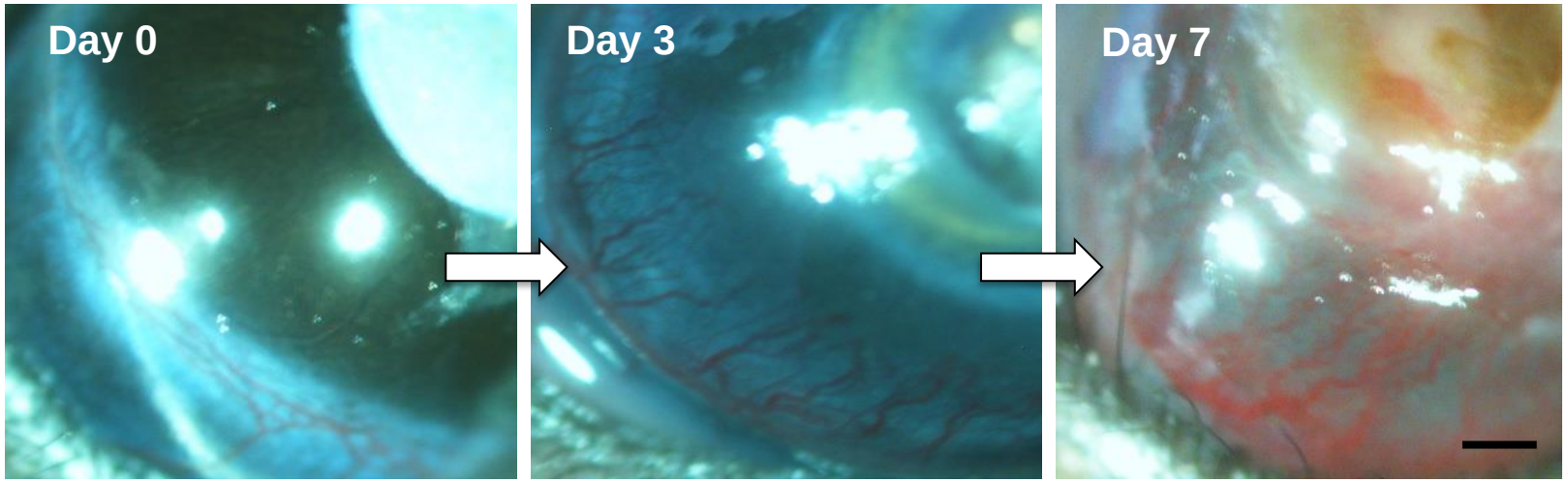






Corneal alkali burn injury: Application of silver nitrate to the mouse cornea causes angiogenesis from the limbus in to the cornea

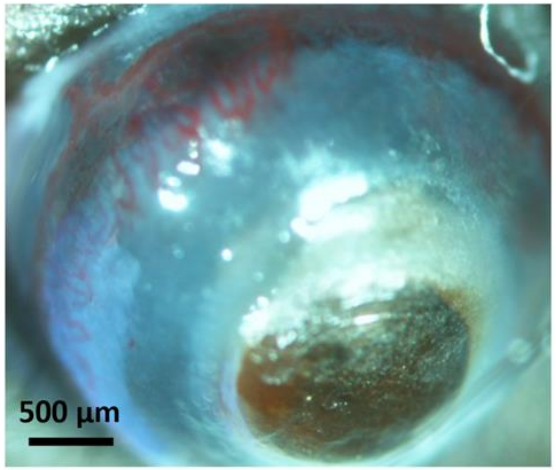




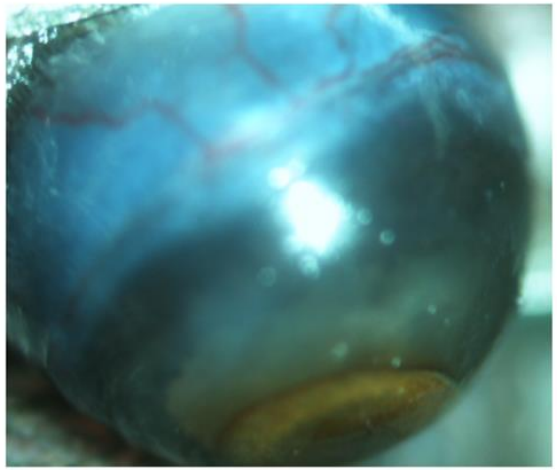
Molly Kelly-Goss

Bright Field Imaging Shows Impaired Angiogenesis in SMC-P Oct4 KO Mice 48 Hours Post-Corneal Burn

(Wild Type) Myh11+YFP+/+

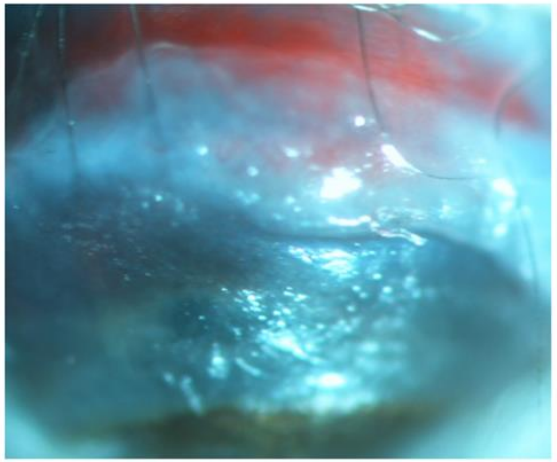


Oct4 -/- Myh11+YFP+/+



- Very little sprouting
- Very little corneal tissue remodeling

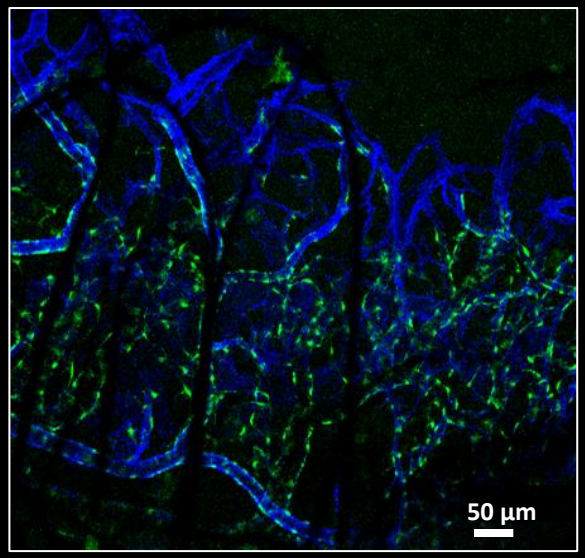
Oct4 +/- Myh11+YFP+/+



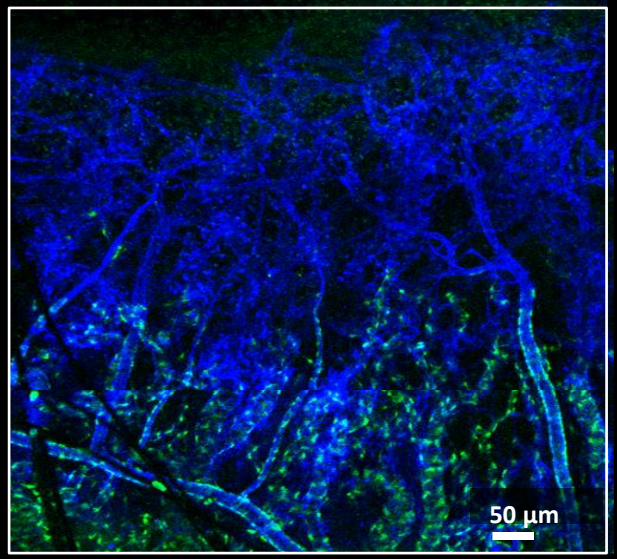
- Similar rate of angiogenesis (distance from limbus)
- Highly leaky vasculature
- “Thicker” remodeling of cornea

SMC-P Specific Conditional KO of Oct4 Resulted in Profound Impairment of Perivascular Cell Investment of Neovessels in a Corneal Burn Model (a 7 day time point is shown)

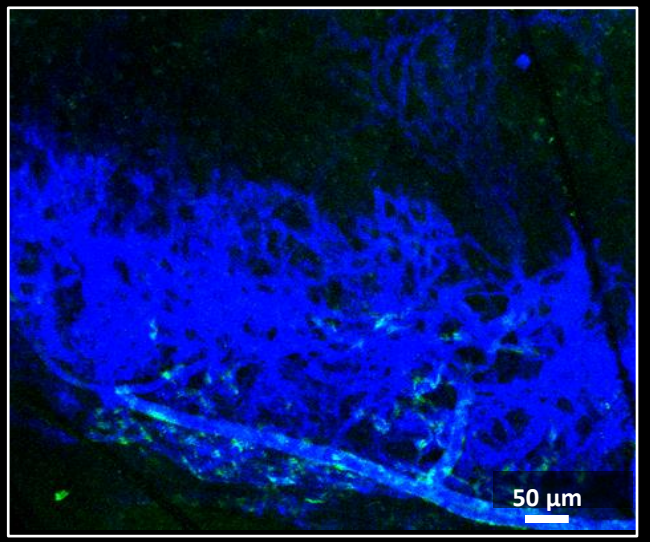
Myh11-eYPF+ Wild Type



Myh11-eYFP+ Oct4 +/- (Heterozygous KO)

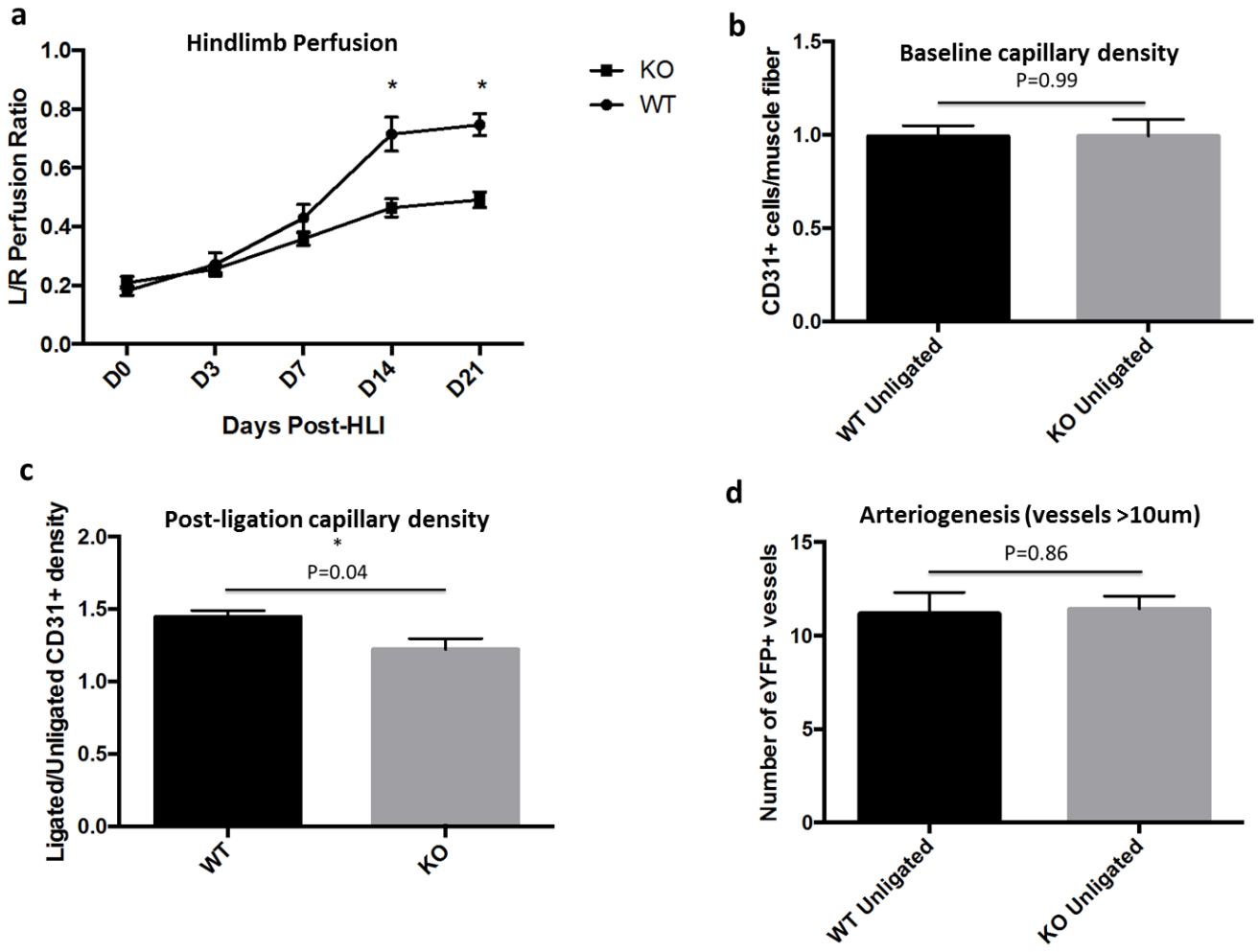


Myh11-eYFP+ Oct4 -/- (Homozygous KO)



Myh11-eYFP (SMC-P derived cells
Perfused Lectin

SMC-P specific KO of Oct4 impaired perfusion recovery following Hind Limb Ischemia due in part to impaired angiogenesis



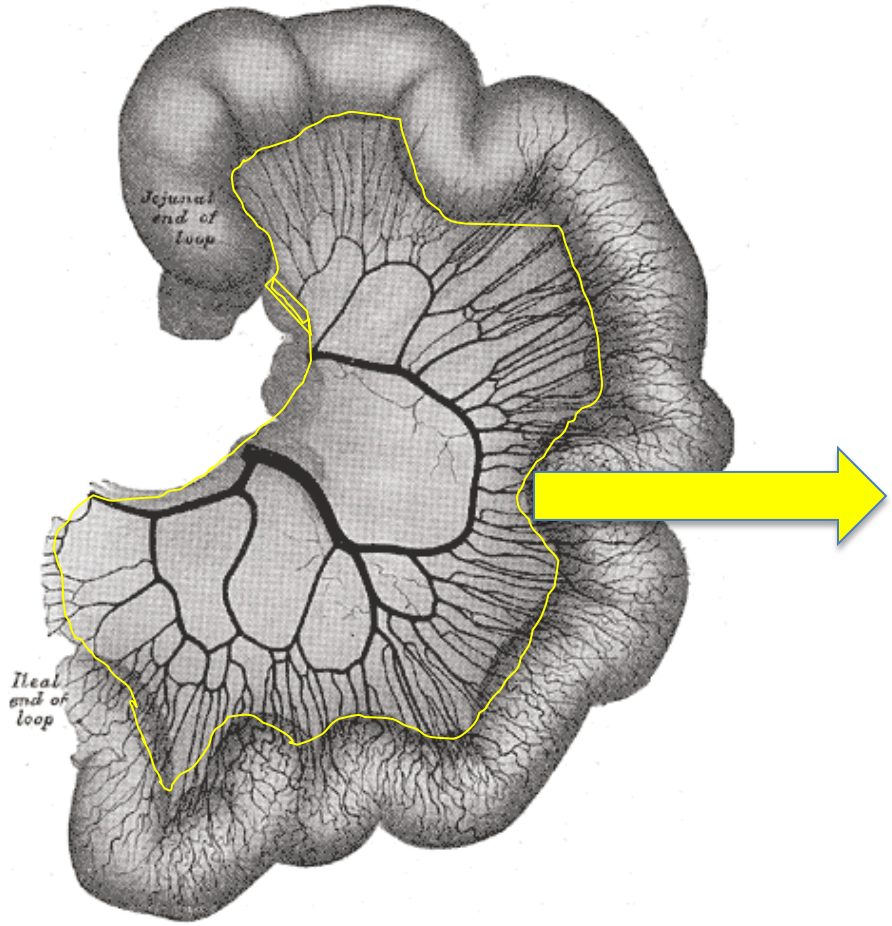
Dan Hess, M Kelly-Goss, ……S Peirce, and Owens, manuscript in preparation

Does Klf4 also have a functional role in microvascular SMC-P?

1. Based on our large vessel studies, we initially hypothesized that it would not have a role in normal maintenance of blood vessels but would regulate dedifferentiation of these cells during vessel injury-repair, angiogenesis, and/or remodeling.

2. We also postulated that Klf4 might regulate pro-inflammatory changes in SMC-P in during development of microvascular dysfunction associated with Type 2 diabetes and metabolic disease.

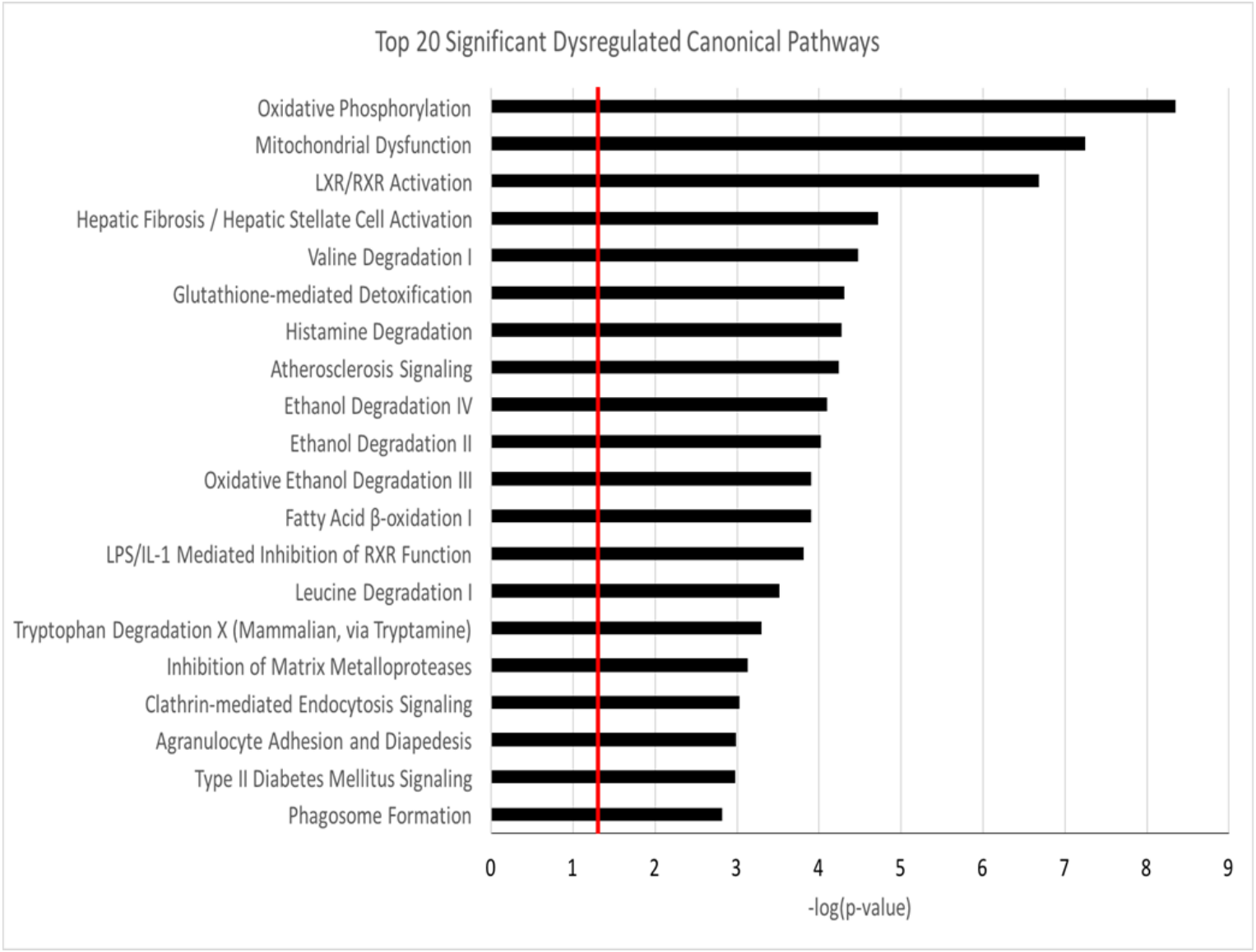
Genomic analysis of mesenteric resistance vessels and the associated perivascular adipose tissue +/- SMC-P specific conditional KLF4 KO



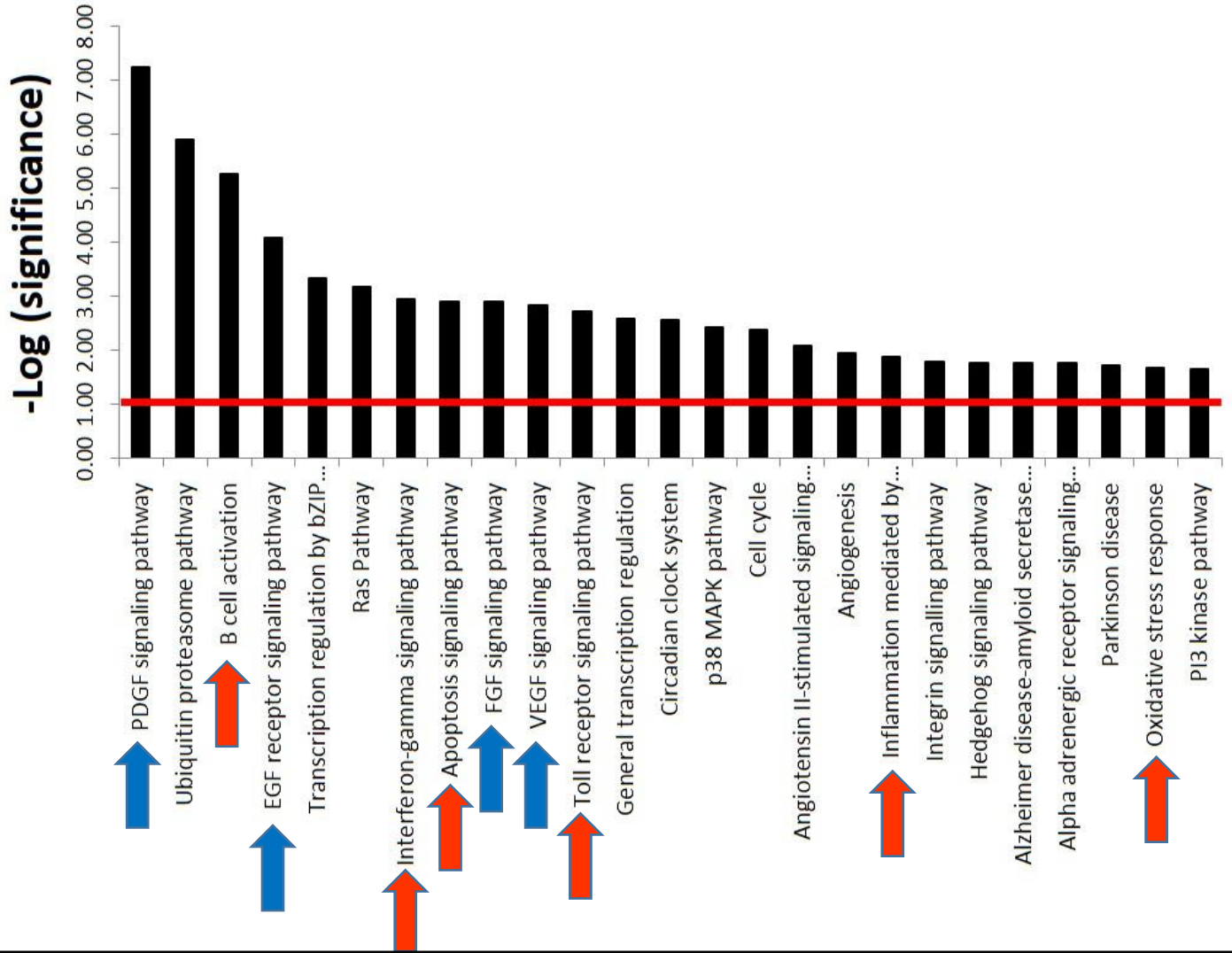
Klf4 CHiPseq and RNAseq +/- SMC-P specific Klf4 KO at 2 weeks post-tamoxifen

Ryan Haskins, Gabe Alencar, Anh Nguyen and Owens, 2017, manuscript in preparation

SMC-P Specific Knockout of Klf4 Resulted in Profound Changes in Genomic Expression Patterns within the Mesenteric Microvasculature and Surrounding Adipose Tissue Including Numerous Metabolic Gene Pathways

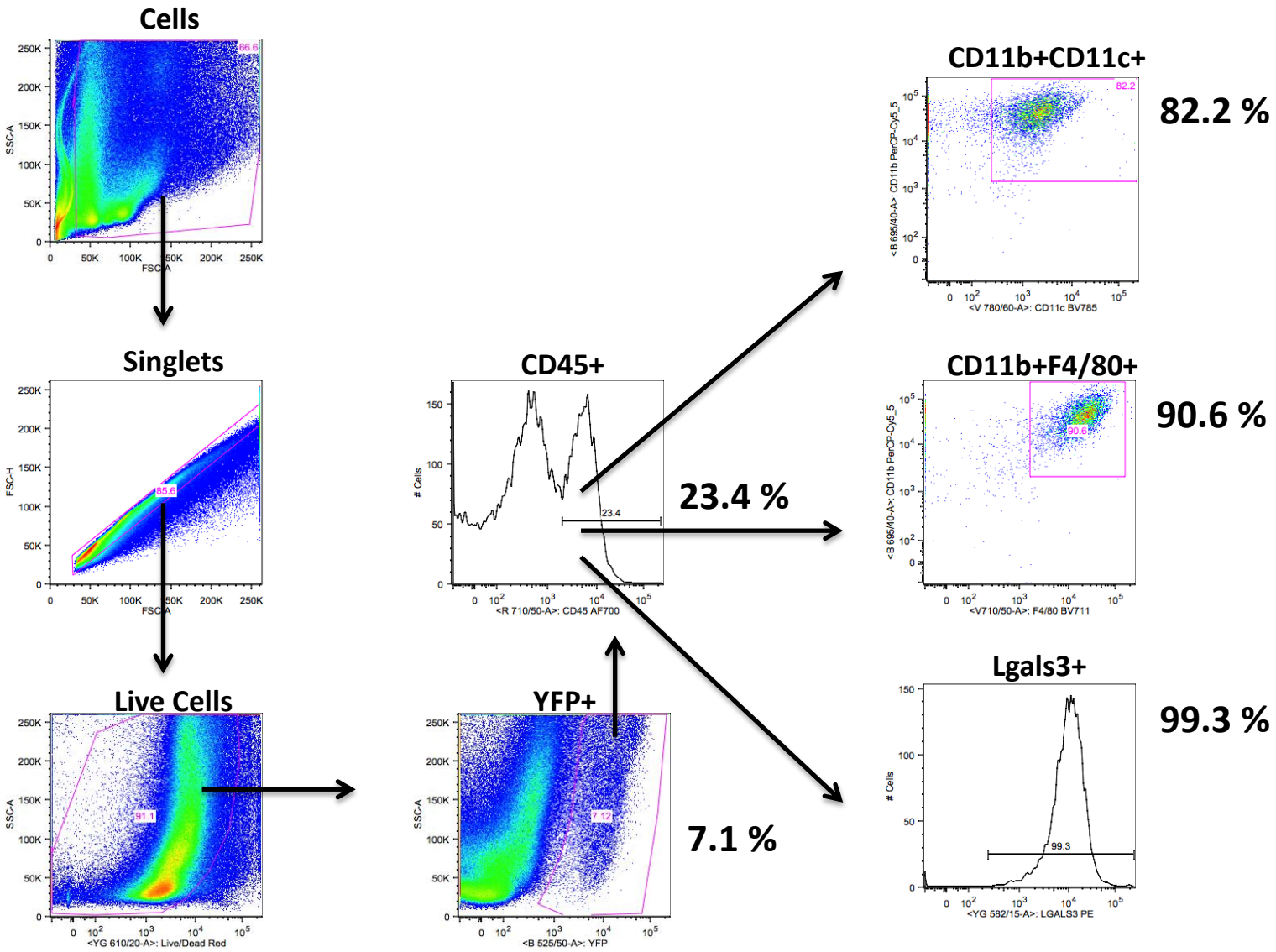


In vivo Klf4 CHiPseq Analysis of Genomic DNA from the Mesenteric Microvasculature and Surrounding Adipose Tissue of SMC-P Specific Klf4 KO Mice Identified a Large Cohort of Putative SMC-P Klf4 Target Genes Associated with Vascular Maturation and Inflammation



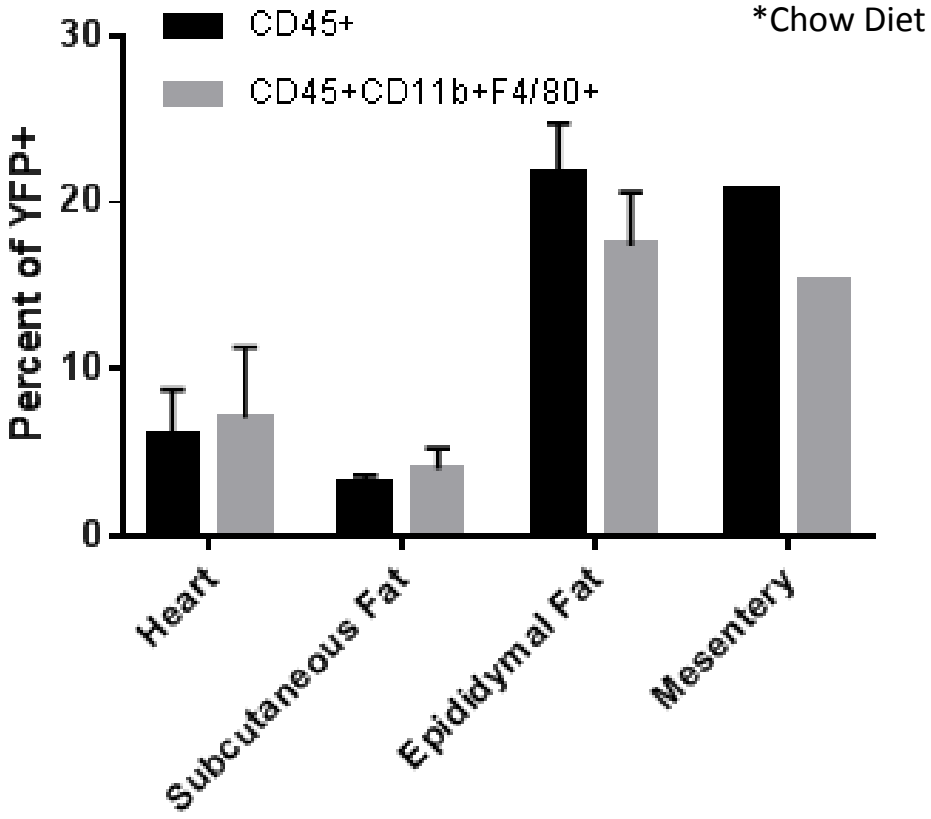
A large fraction of SMC-P within the stromovascular fraction (SVF) of adipose tissue express multiple markers of macrophages

Epididymal Fat – 10 week old Myh11 eYFP mice on chow diet



Anh Nguyen and Owens, 2017

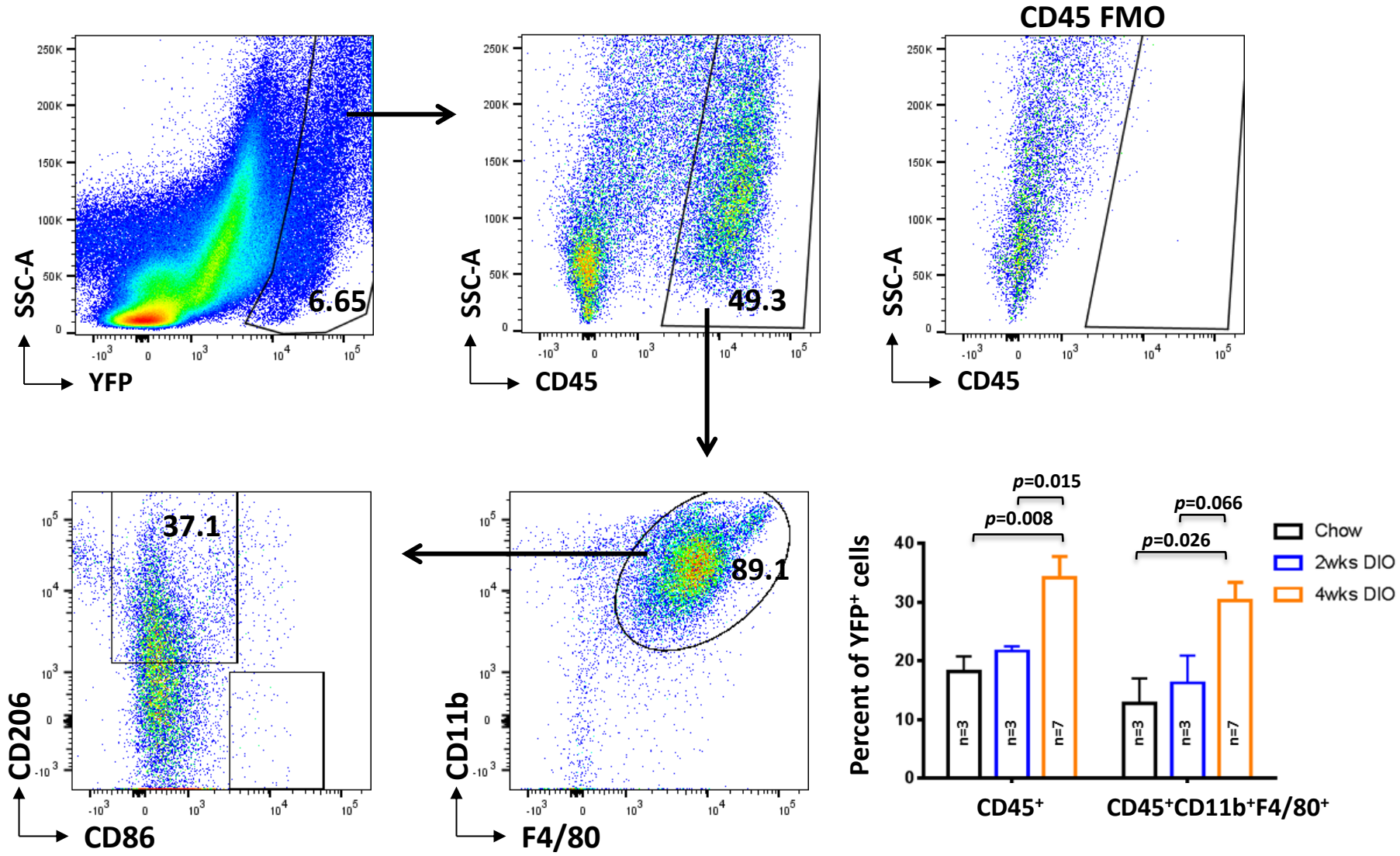
The Fraction of SMC-P Derived Cells That Express Macrophage Markers is Much Higher in Pathological Versus Non-Pathological Adipose Tissue and Other Microvascular Beds



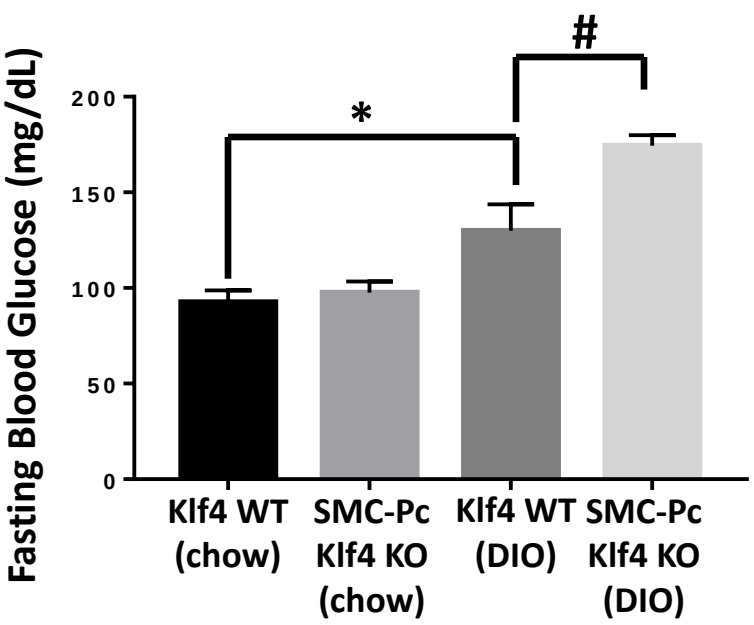
Number of animals per tissue:
Heart – 4
Subcutaneous Fat – 3
Epididymal Fat – 7
Mesentery - 1

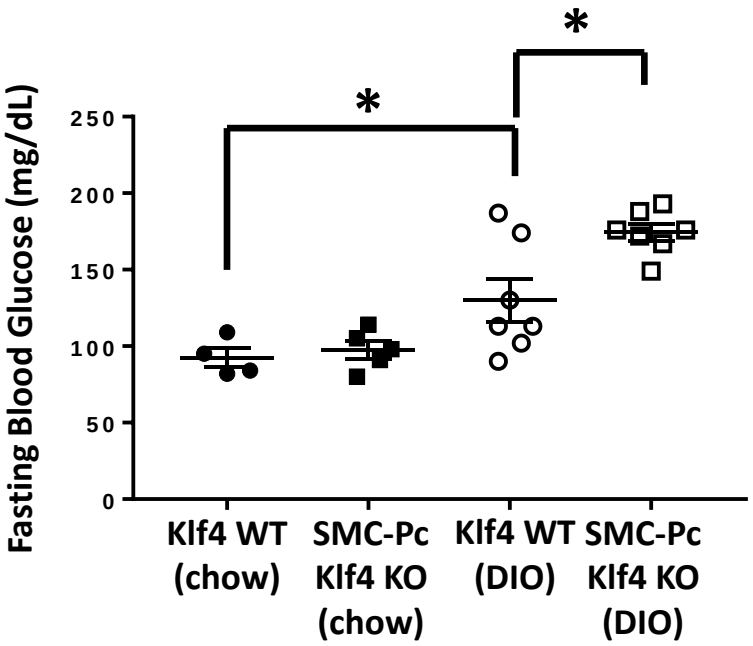
Anh Nguyen and Owens, 2017

SMC-Pc derived macrophage-like cells within epididymal fat exhibit an M2 not an M1 phenotype whose frequency increases with Diet Induced Obesity (DIO)



SMC-Pc Specific Conditional Klf4 KO mice show exacerbation of DIO-dependent hyperglycemia

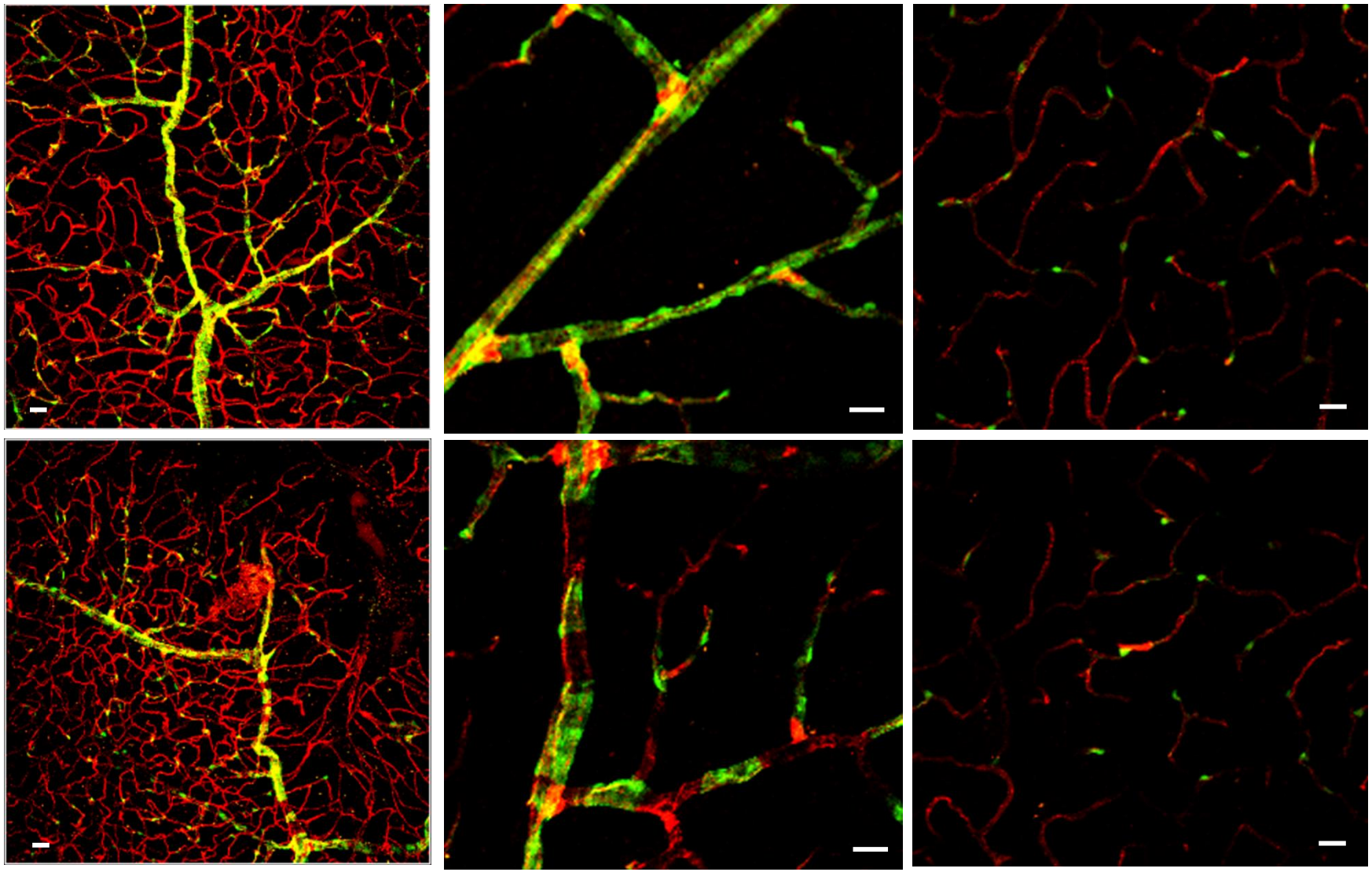




Anh Nguyen and Owens, 2017

Arterioles in SMC-P Conditional KLF4 KO Are Poorly Invested in YFP+ Perivascular Cells

A



Ryan Haskins, Anh Nguyen and Owens, 2017, manuscript in preparation

Overall Summary and Conclusions:

1. SMC play a far greater role in atherosclerotic lesion pathogenesis than has been generally appreciated but can have a beneficial or detrimental role depending on the nature of their phenotypic transitions. As such, there is a critical need to identify factors, genetic mechanisms, and therapeutic approaches that promote beneficial (plaque stabilizing) changes in SMC phenotype.
2. You cannot distinguish SMC- and macrophage-derived cells by marker panels alone.
3. The stem cell pluripotency genes Oct4 and Klf4 SMC-P regulate phenotypic transitions-functions critical for maintenance of perivascular cell coverage of microvessels, and mediating adaptive responses during hypoxia and injury-repair.
4. A significant fraction of resident “macrophages” within adipose tissues are SMC-P derived and their frequency increases with DIO.
5. SMC-P specific loss of Klf4 was associated with profound changes in gene expression within the microvasculature and surrounding adipose tissue including marked dysregulation of metabolic and inflammatory pathways, and increased sensitivity to development of hyperglycemia associated with DIO.
6. Thus, Klf4 appears to play a protective role within SMC-P in the normal microvasculature, whereas Klf4-dependent transitions of SMC to macrophage marker+ foam cells in advanced atherosclerotic lesions is maladaptive.

2016 Owens Lab

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Former graduate student

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Hermann Gram (Novartis)

Jason Williams and Yong-Jian Geng, UT Houston

Alexey Tomilin, Russian Academy of Science

Gwen Randolph (Wash U)

Sheila Francis (Sheffield) and Emmanuel Pinteaux (U Manchester)

Michelle Bendeck (U Toronto)

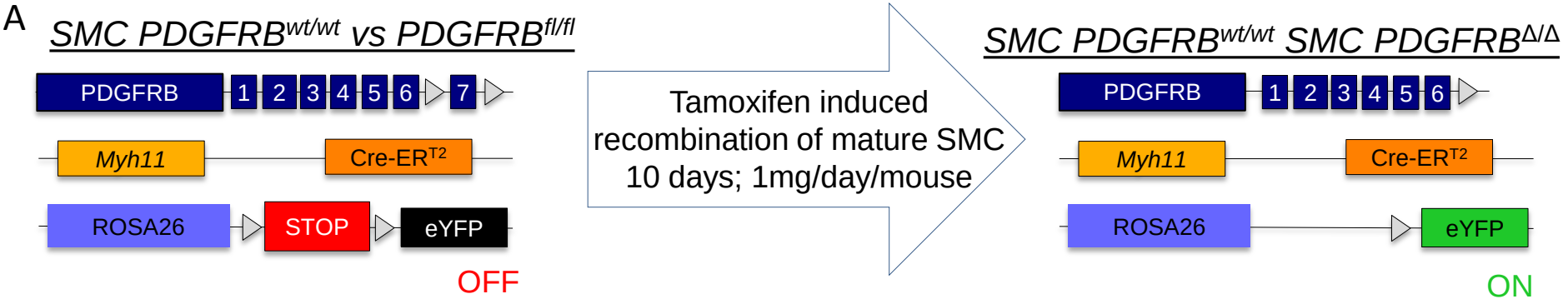
Heri Schunkert (U Munich) and Jeanette Erdmann (Luebeck)

Please contact me if interested in post-doctoral and fellowship training positions in my lab and others at the University of Virginia Cardiovascular Research Center.
http://training.cvrc.virginia.edu/

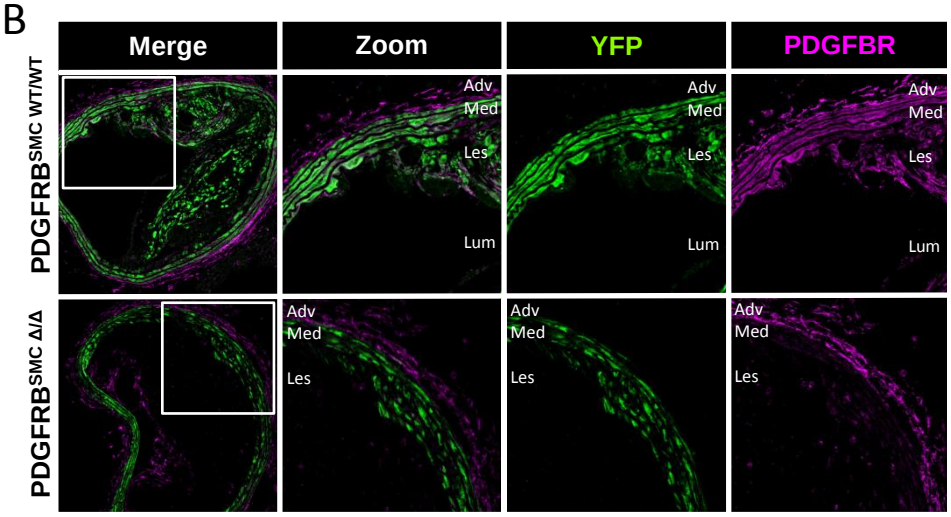
THE ROBERT M. BERNE CARDIOVASCULAR RESEARCH CENTER

Hypothesis: PDGFβR-dependent changes in SMC phenotype are athero-protective and are required for stable investment and ECM production within the fibrous cap.

A

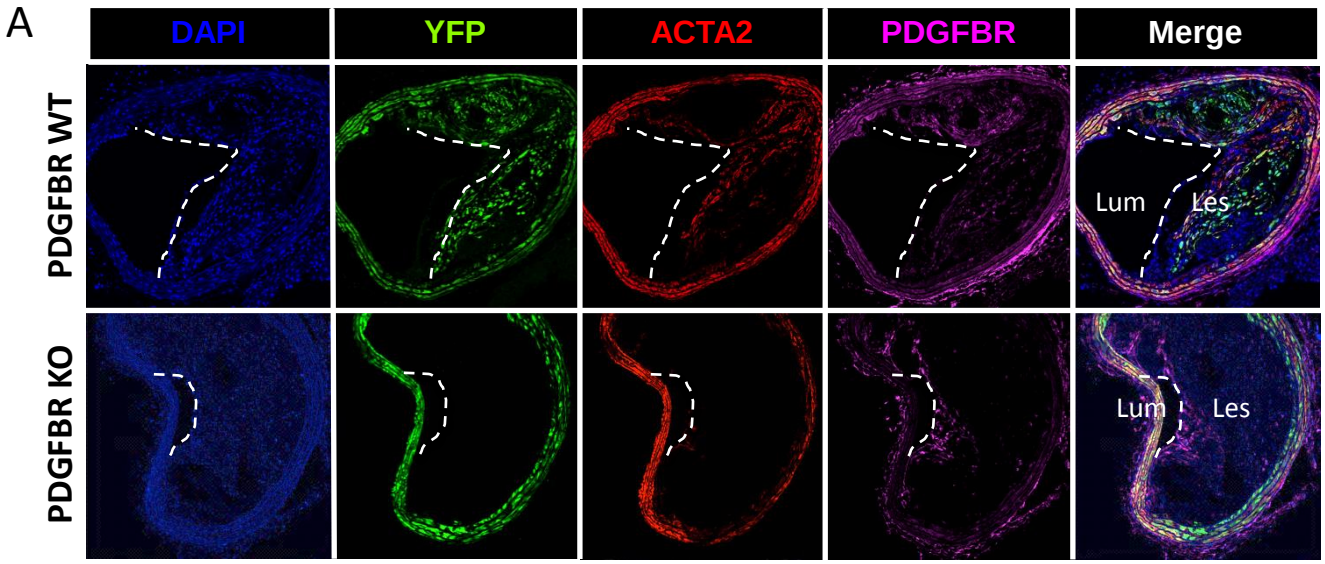


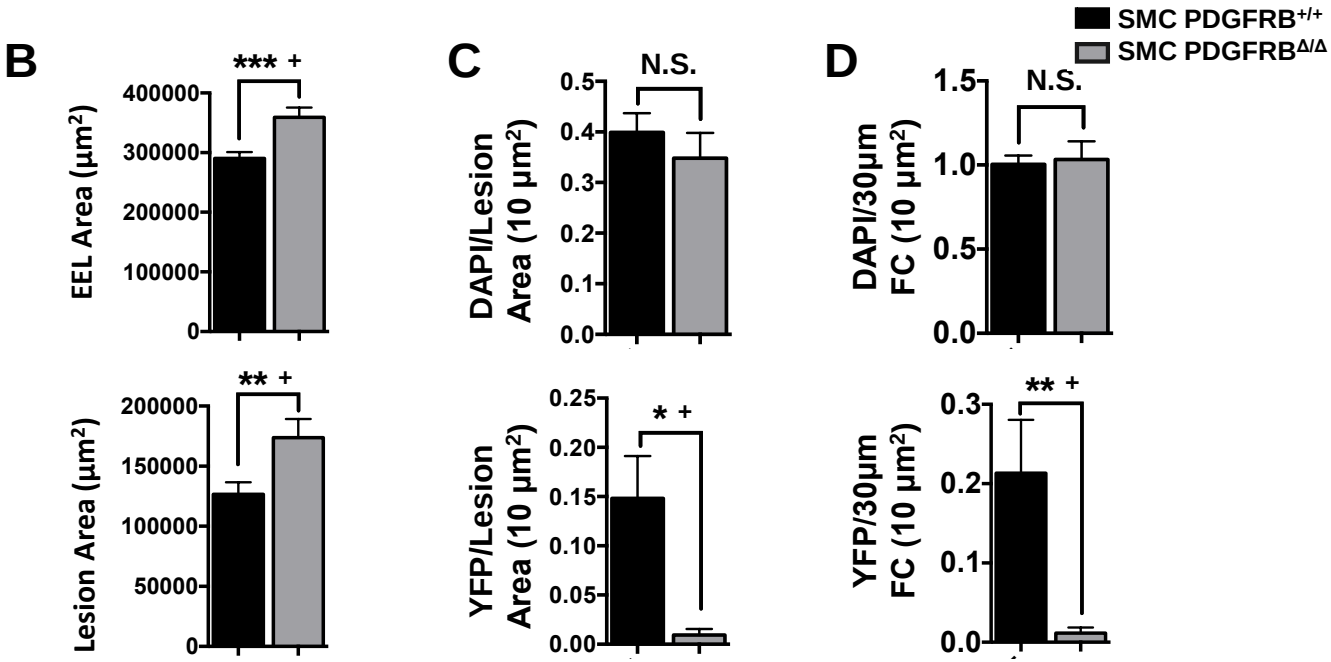
B



See Poster #W159 by Alexandra Newman, Wednesday 12-2PM, Atherosclerosis II, Liberty Ballroom

SMC-specific conditional KO of the PDGFBR within ApoE$^{-/-}$ mice fed a WD for 18 weeks resulted in larger lesions virtually devoid of YFP^{+} SMC

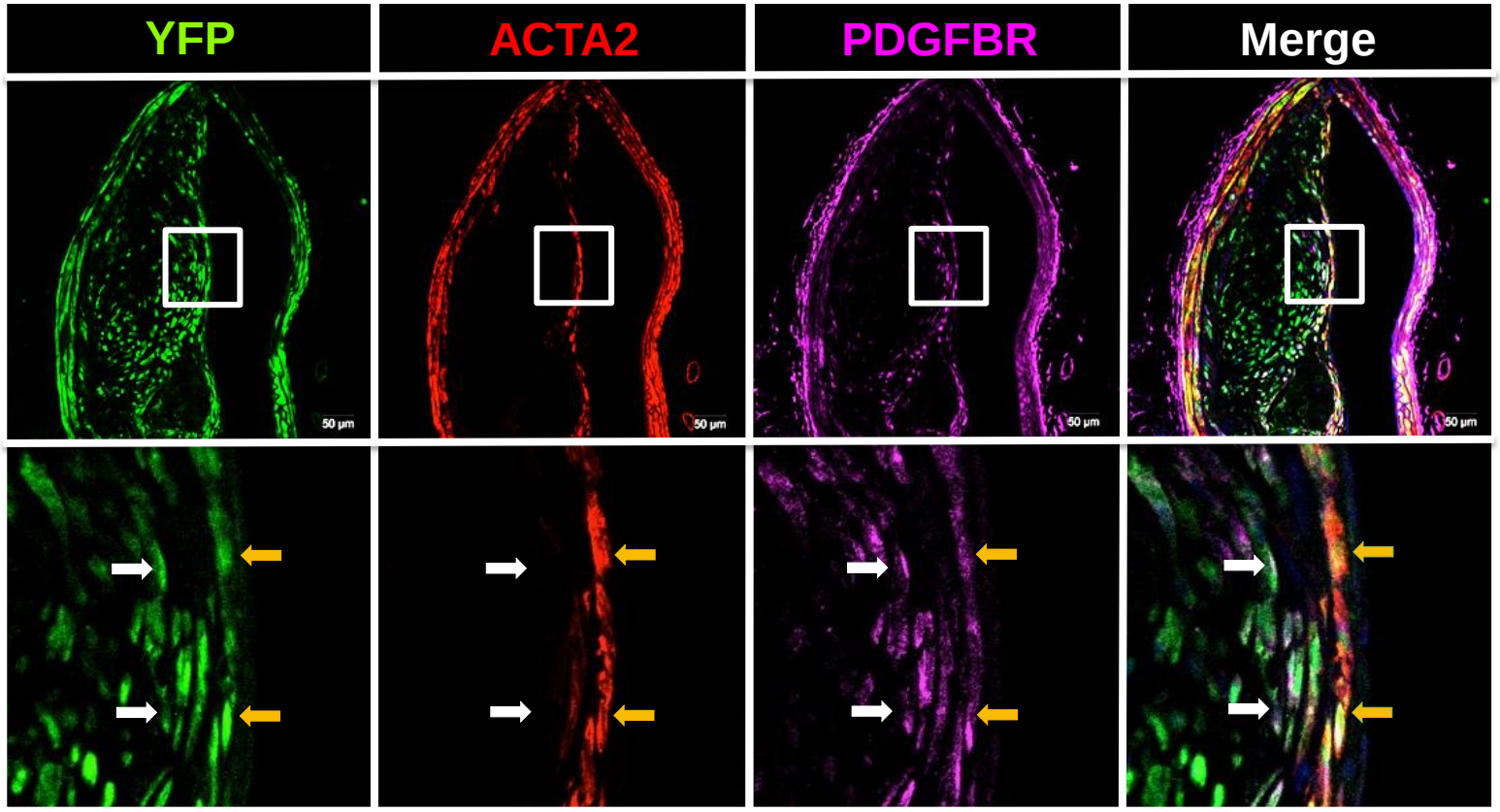




* p<0.04
** p<0.03
*** p<0.008
Unpaired Student T test
+ with Welch's Correction

PDGFβR is expressed by YFP+ SMC within the fibrous cap of advanced BCA lesions after 18 weeks of Western diet

SMC YFP ApoE-/- mice

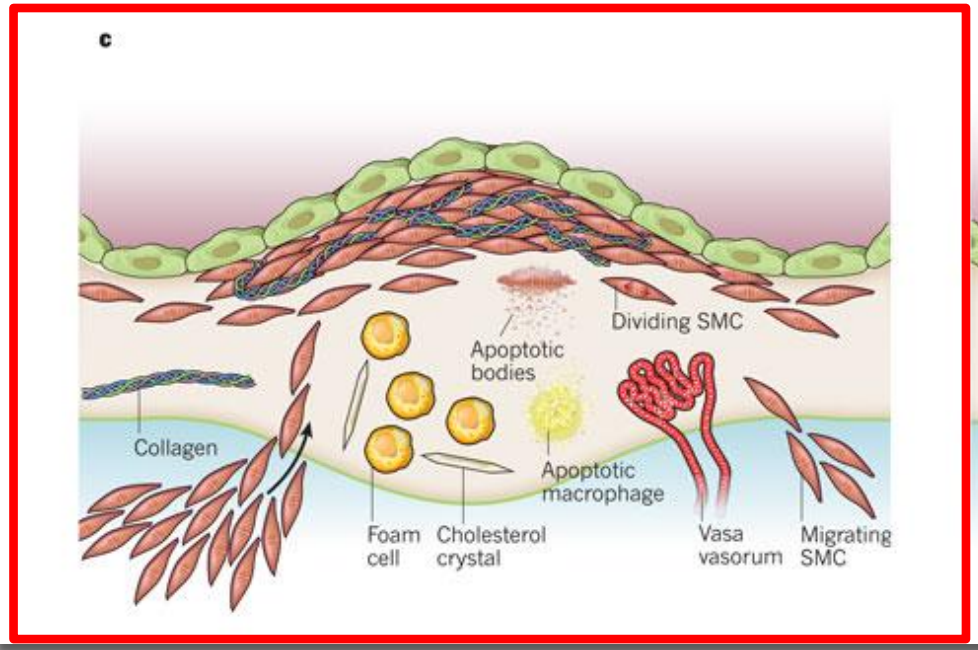


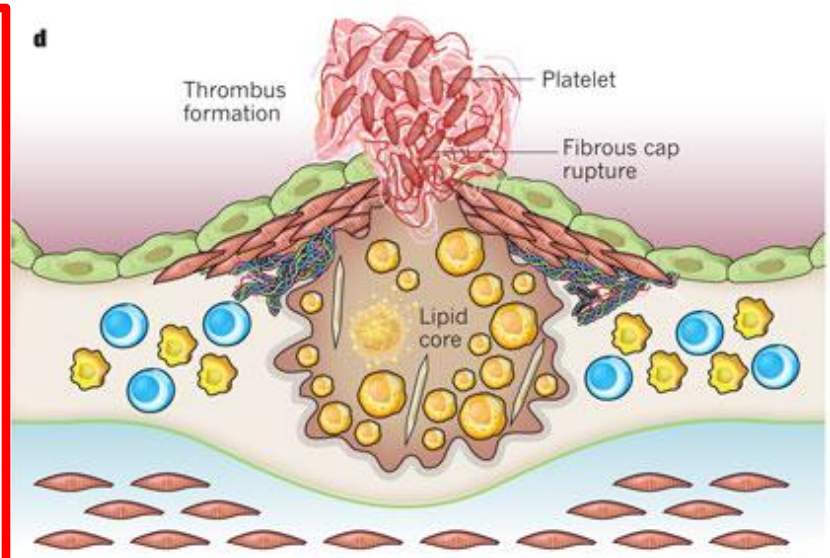
eYFP+ PDGFβR+ Acta2+ →
eYFP+ PDGFβR+ Acta2- →

Alexandra Newman and Owens, manuscript in preparation

The established dogma is that SMC are the principal source of ECM components in lesions and that this promotes plaque stabilization

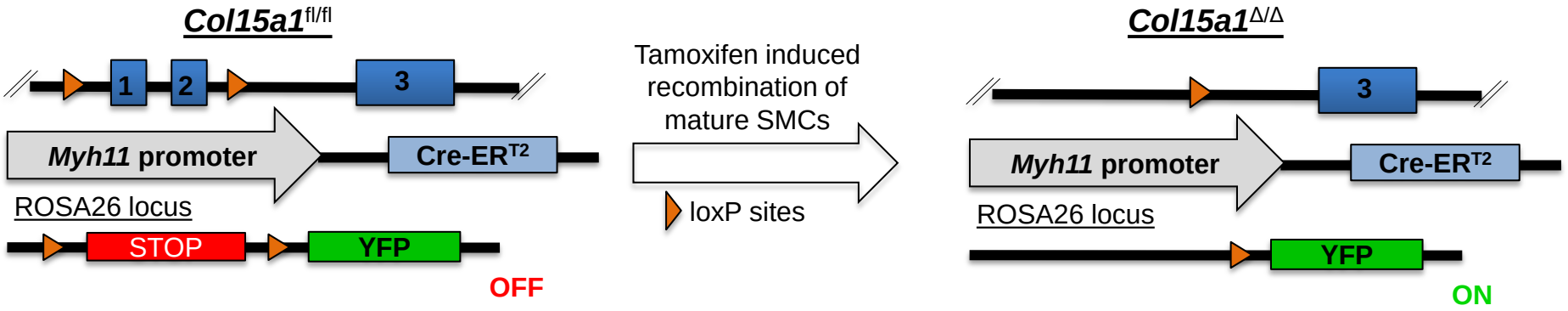
However, there is no direct evidence that this is the case. Rather it has been inferred from *in vitro* studies showing that cultured SMC can produce ECM, and correlative evidence showing the presence of ACTA2+ cells in ECM rich lesion areas.



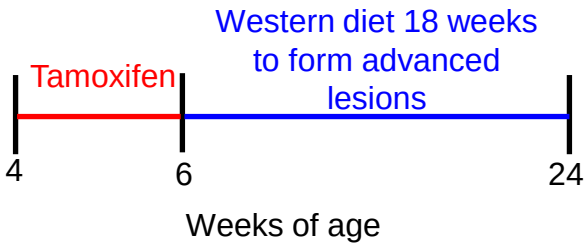


Libby, *Nature* 2011

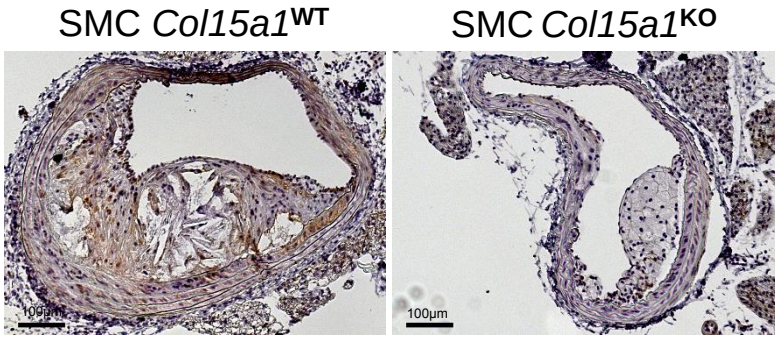
Hypothesis: SMC derived Col15a1, which is known to be involved in organization of collagen fibers and extracellular matrix scaffolds, plays a critical role in late stage lesion pathogenesis by promoting lesion collagen fibril organization within the protective fibrous cap. **Prediction**: SMC Col15a1 KO ApoE$^{-/-}$ mice will exhibit late stage plaque destabilization.

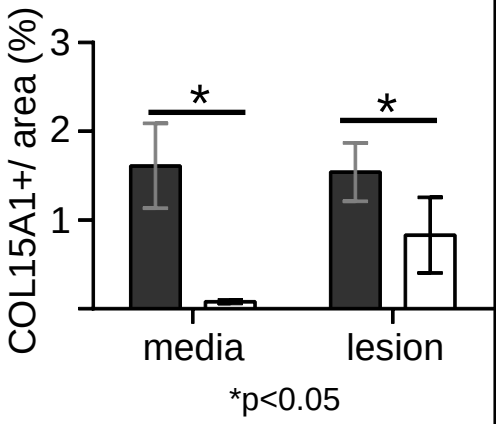


Experimental Design



Validation of SMC *Col15a1* knockout after 18 weeks Western Diet



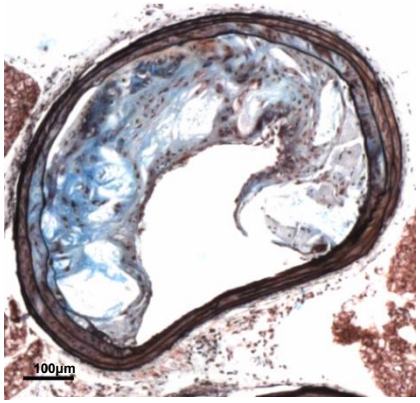


*p<0.05

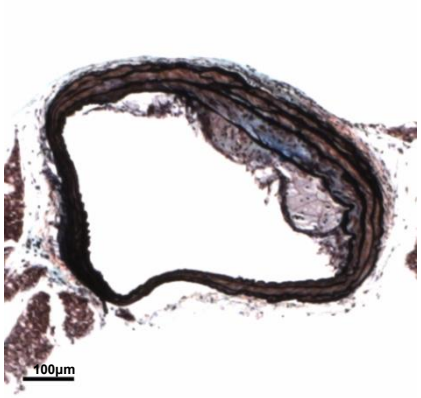
Durgin et al., manuscript in revision

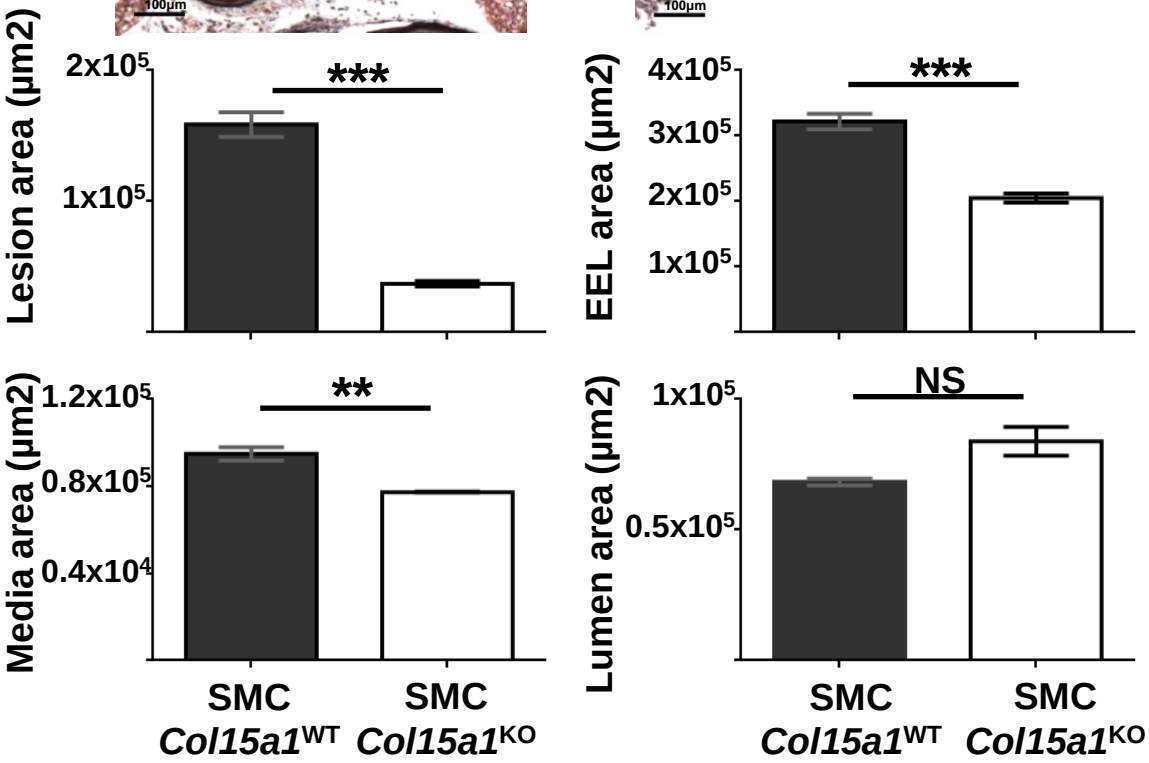
SMC specific *Col15a1* deletion resulted in an 80% reduction in BCA lesion size but no change in luminal Sudan IV Staining

SMC *Col15a1*^{WT}

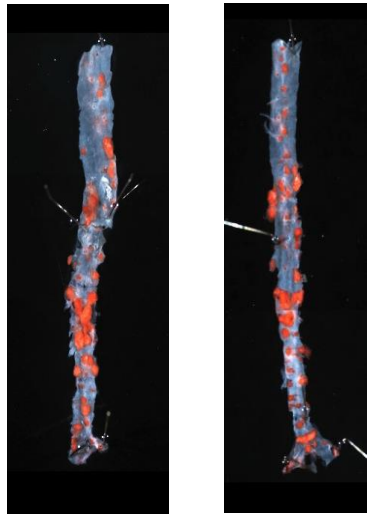


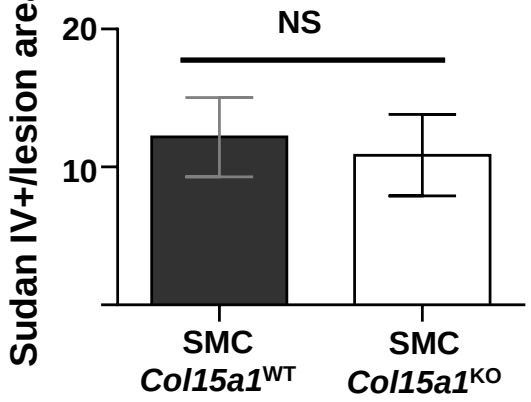
SMC *Col15a1*^{KO}





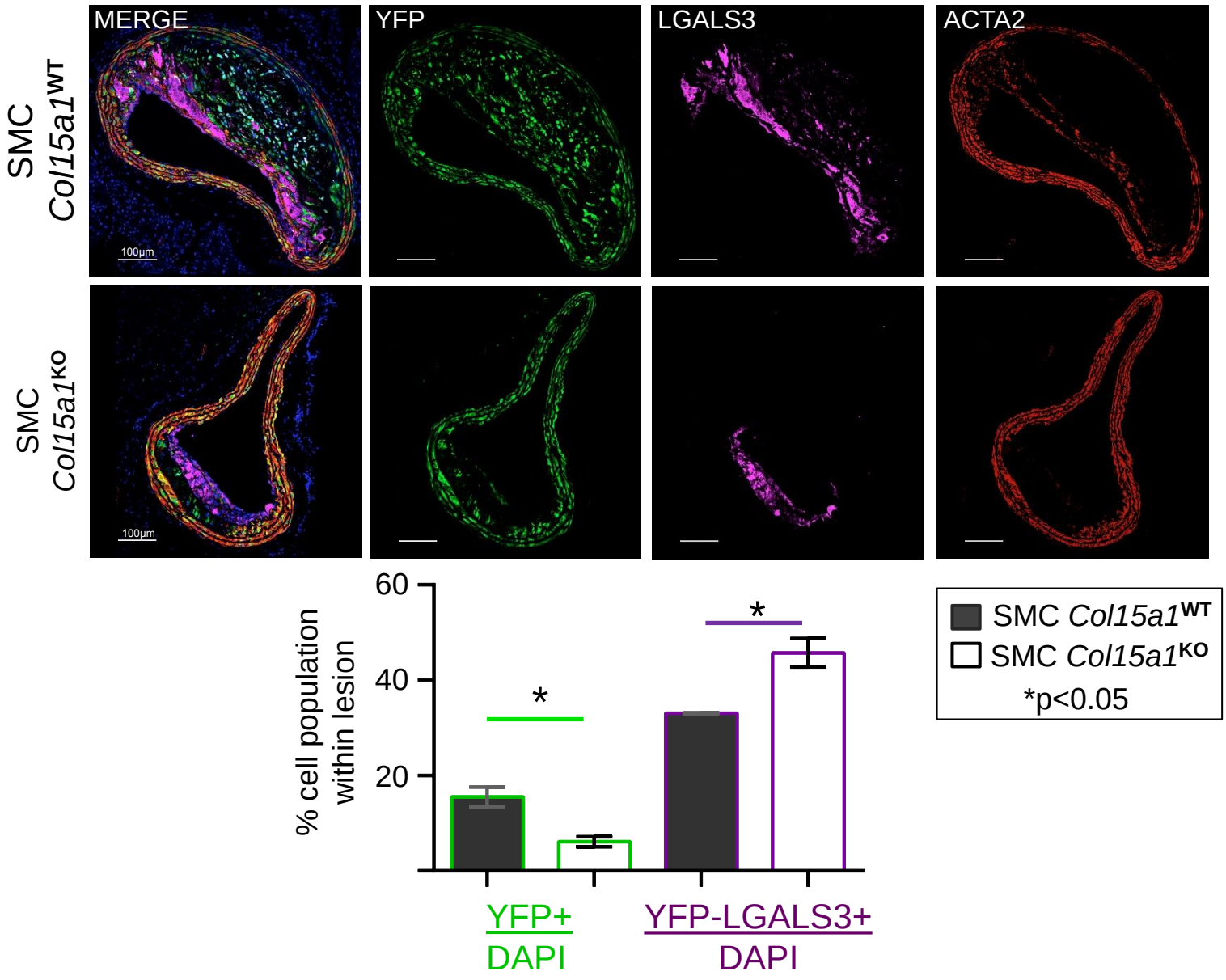
SMC *Col15a1*^{WT} SMC *Col15a1*^{KO}





*** p<0.001, **p<0.01

SMC *Col15a1* deletion leads to a reduction in SMC (YFP+) and an increase in YFP-LGALS3+ cells populating the lesion



Take Home Messages:

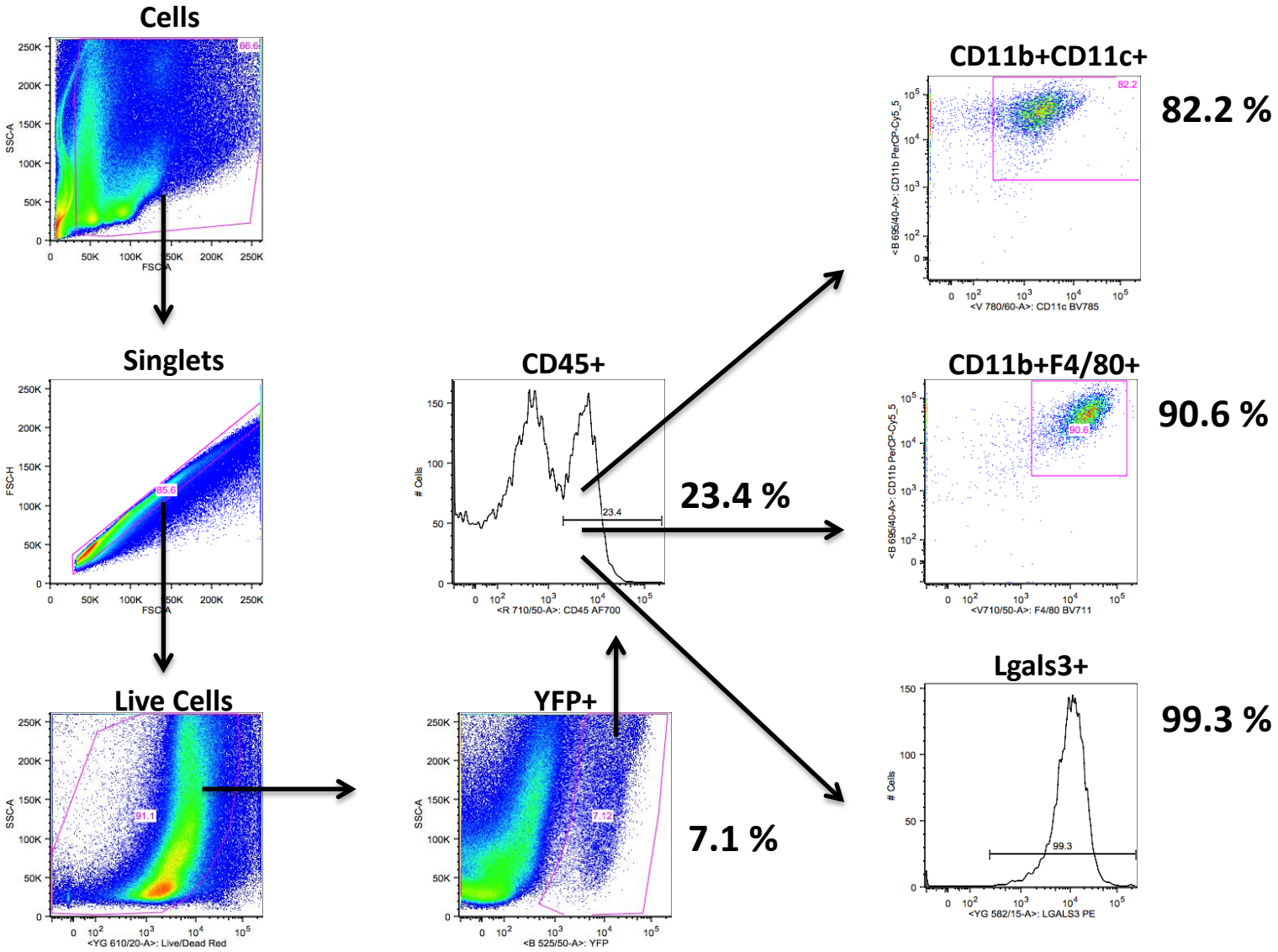
1. SMC play a far greater role in atherosclerotic lesion pathogenesis than has been generally appreciated but can play either an atheroprotective or atheropromoting role depending on the nature of their phenotypic transitions.
2. You cannot distinguish SMC- and macrophage-derived cells within advanced lesions by use of marker panels alone.
3. The cellular composition of the fibrous cap appears to be far more plastic than has generally been appreciated.
4. IL-1β has an unexpected atheroprotective role in late stage lesions by promoting a SMC-rich macrophage-deficient fibrous cap.
5. We have evidence that fibrous cap SMC may produce factors that inhibit macrophage recruitment and proliferation in the fibrous cap through an IL1β-dependent process.
6. IL1R1 signaling in SMC not in myeloid cells contributes to enhanced lesion development.
7. Surprisingly, loss of SMC expression of the fibrillar collagen organizer Col15a appears to be critical for development of advanced atherosclerotic lesions.
8. Our over-riding hypothesis is that detrimental reprogramming of SMC and/or other ECM-producing lesion cells is a critical determinant of plaque destabilization.
9. There is a critical need to identify factors, genetic mechanisms, and therapeutic approaches that promote changes in the phenotype of SMC and other major cell types present within lesions that are beneficial in promoting plaque stabilization.

Does Klf4 also have a functional role in microvascular SMC-P?

Original Hypothesis: Klf4-mediated transitions of microvascular SMC and pericytes to a pro-inflammatory state contribute to widespread microvascular dysfunction associated with type 2 diabetes and metabolic disease.

Surprisingly we observed that a large fraction of SMC-P within the microvasculature of adipose tissue expressed multiple markers of macrophages

Epididymal Fat – Chow Diet 2wks



ATVB 2016;36 (in press)

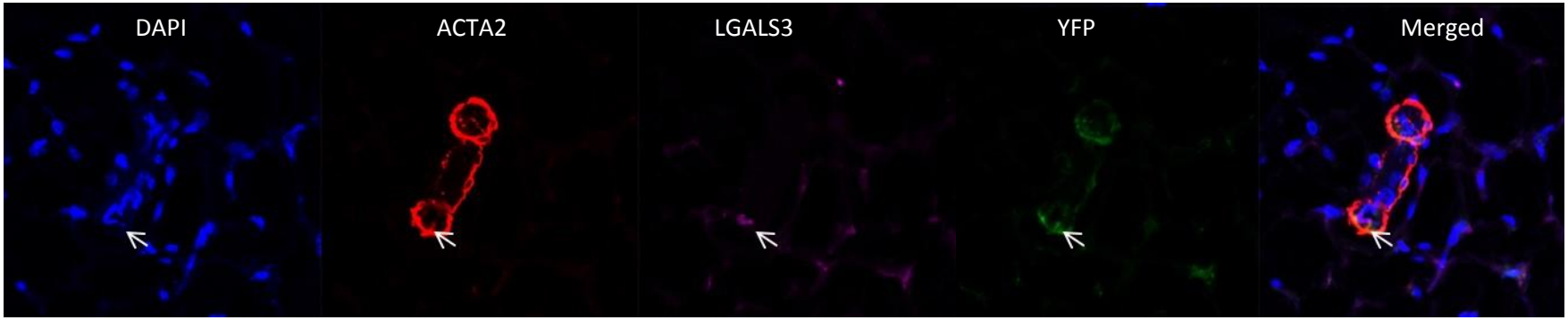
Original Research

Perivascular Macrophages Limit Permeability

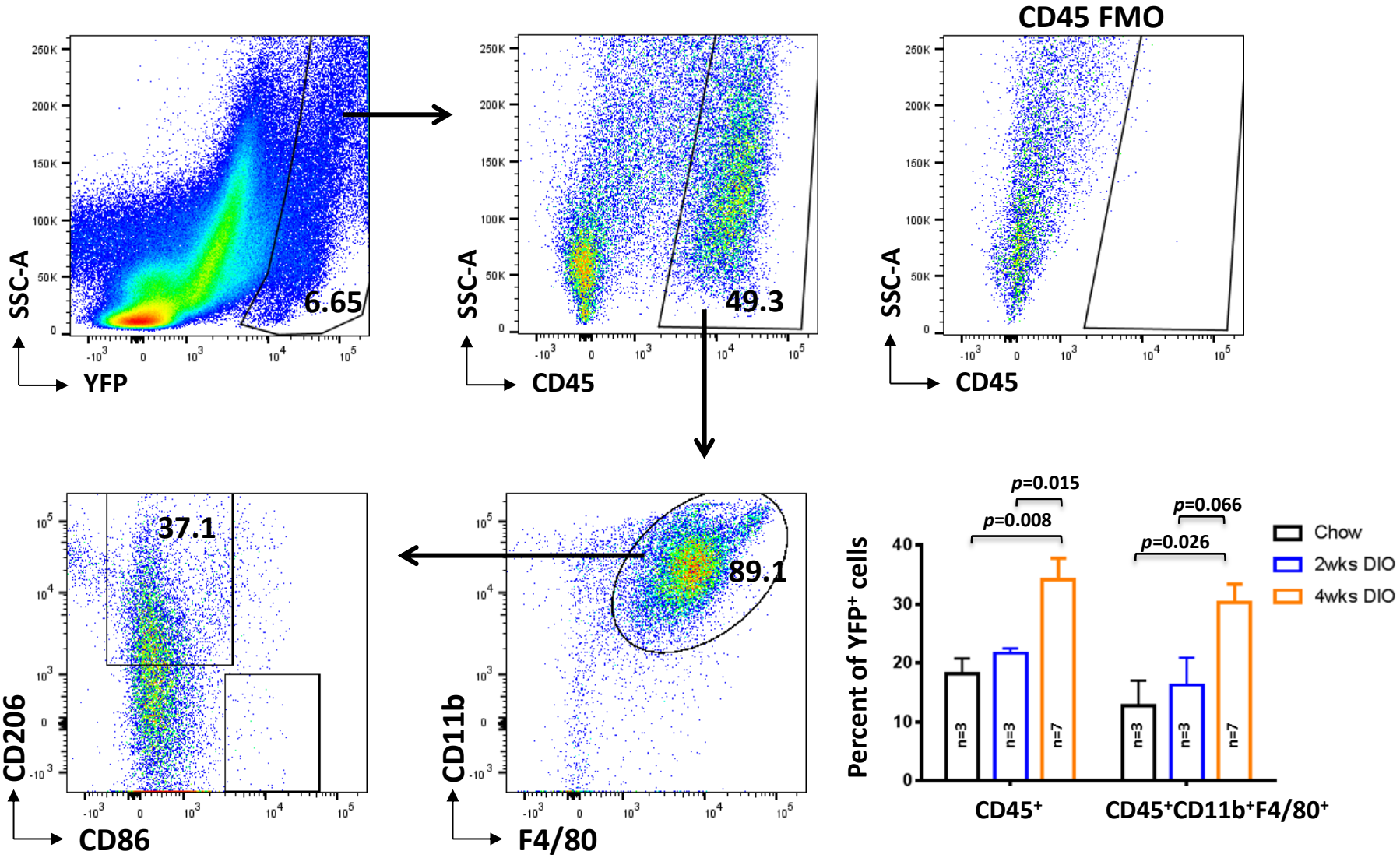
Huanhuan He, Julia J. Mack, Esra Güç, Carmen M. Warren, Mario Leonardo Squadrito, Witold W. Kilarski, Caroline Baer, Ryan D. Freshman, Austin I. McDonald, Safiyyah Ziyad, Melody A. Swartz, Michele De Palma, M. Luisa Iruela-Arispe

1. Major claim is that perivascular M2 macrophages are critical in maintenance of EC barrier function and regulation of microvascular permeability.
2. However, they did no lineage tracing to clearly establish these as myeloid derived macrophages, the markers they used are all expressed by SMC-P derived macrophage like cells (e.g. Lgals3, Mac1/CD11b, F4/80) and the methods used to deplete them, clonodate liposomes and an antibody to CSF1R, would also target SMC-P derived cells.
3. As such, we believe that a significant subset of the cells they are describing, if not all, are of SMC-P origin.

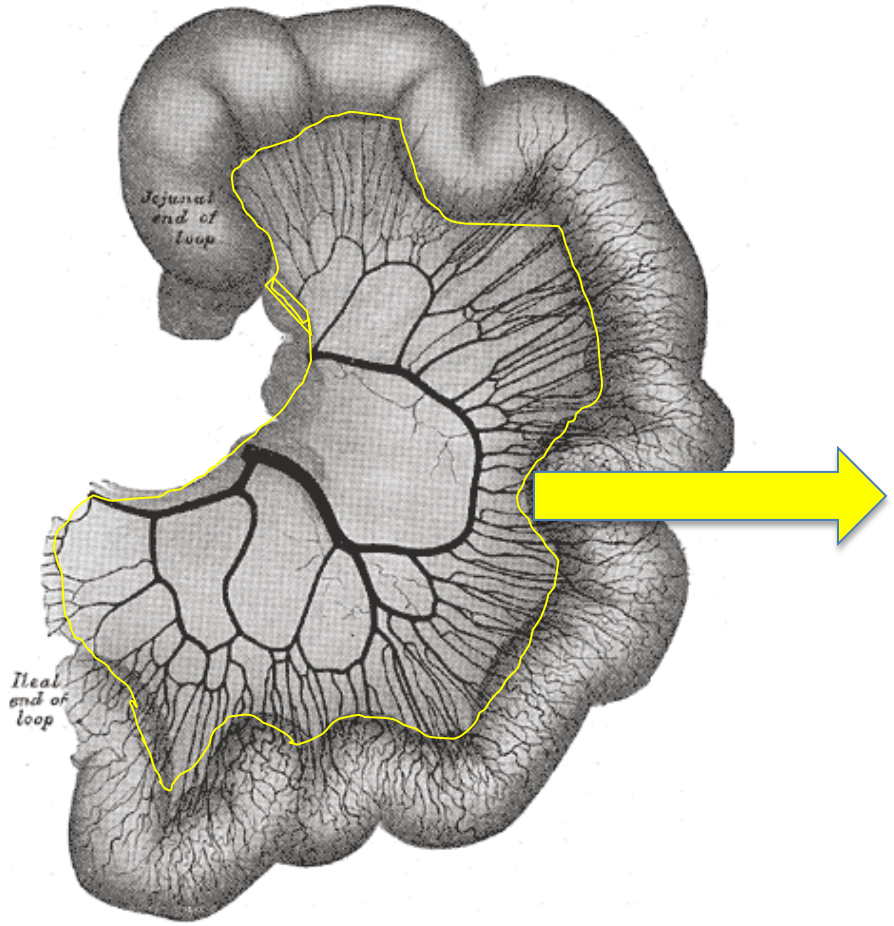
SMC-P derived Lgals3+ cells were mainly located in a perivascular position



DIO is associated with an increased frequency of SMC-Pc derived macrophage-like cells within epididymal fat including many that express the M2 marker CD206

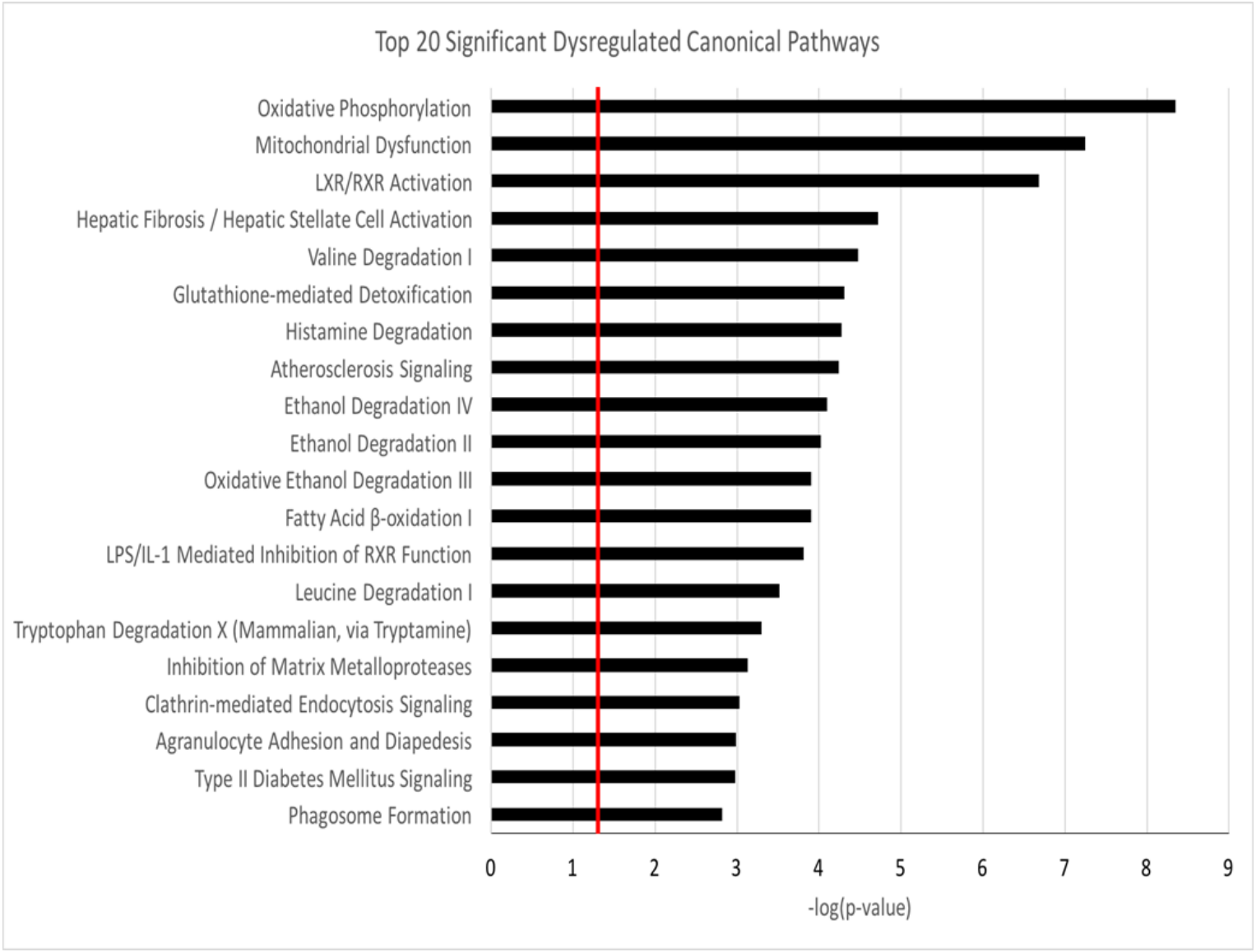


Genomic analysis of mesenteric resistance vessels and the associated perivascular adipose tissue +/- DIO

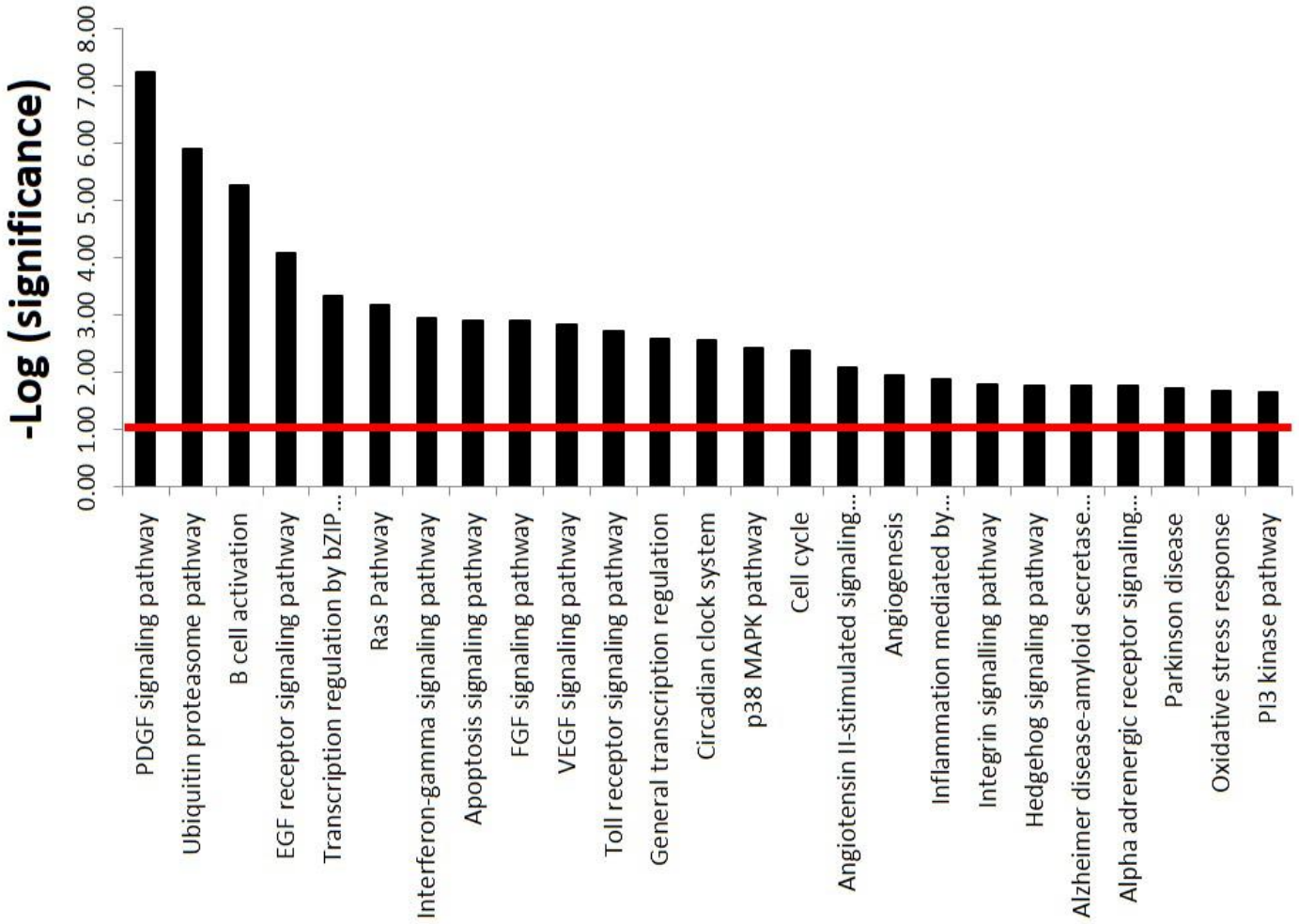


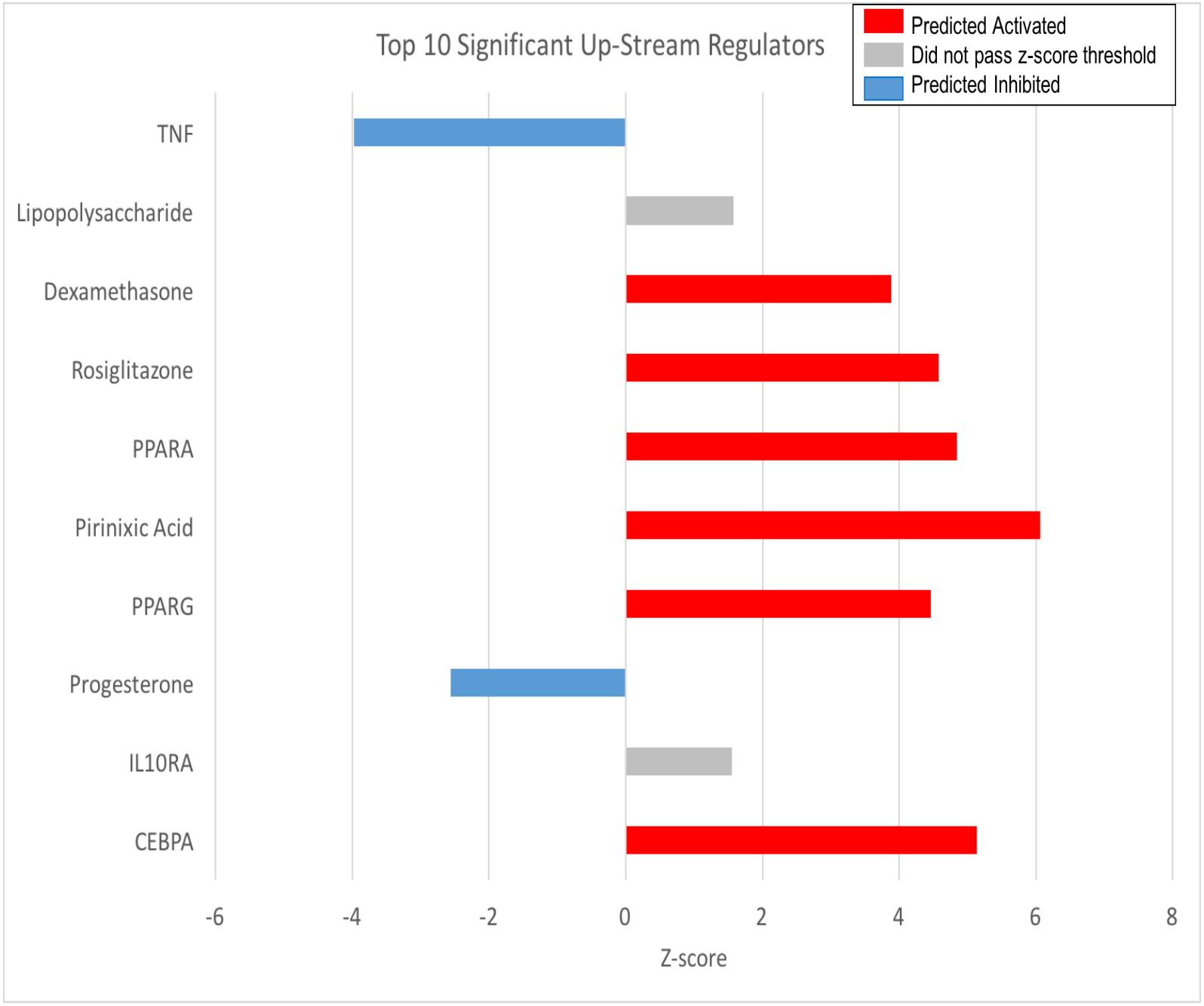
Klf4 CHiPseq and RNAseq +/- SMC-pericyte specific Klf4 KO +/- DIO

SMC-P Specific Knockout of Klf4 Resulted in Profound Changes in Genomic Expression Patterns within the Mesenteric Microvasculature and Surrounding Adipose Tissue Including Numerous Metabolic Gene Pathways



In vivo Klf4 CHiPseq Analysis of Genomic DNA from the Mesenteric Microvasculature and Surrounding Adipose Tissue of SMC-P Specific Klf4 KO Mice Identified a Large Cohort of Putative SMC-P Klf4 Target Genes Including Those Associated with Vascular Maturation and Inflammation





p-value
1.63E-26
1.17E-25
2.14E-23
3.31E-23
9.62E-22
4.34E-20
6.33E-19
3.92E-18
5.18E-18
9.64E-18

Arterioles in SMC-P Conditional KLF4 KO Are Poorly Invested in YFP+ Perivascular Cells

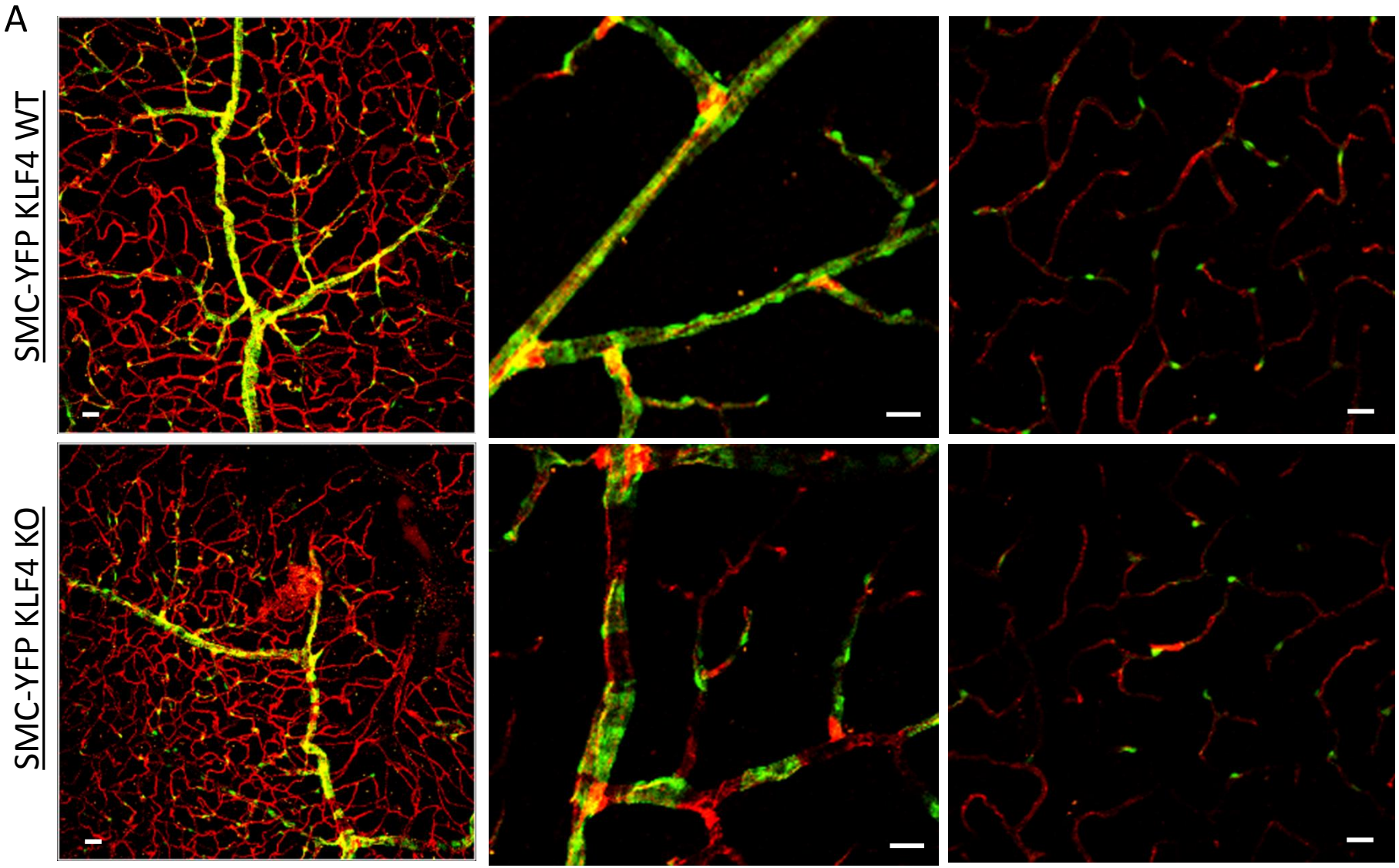


Figure 4.

Myocardial Arterioles in SMC-P Conditional KLF4 KO Are Poorly Invested in YFP+ Perivascular Cells

B

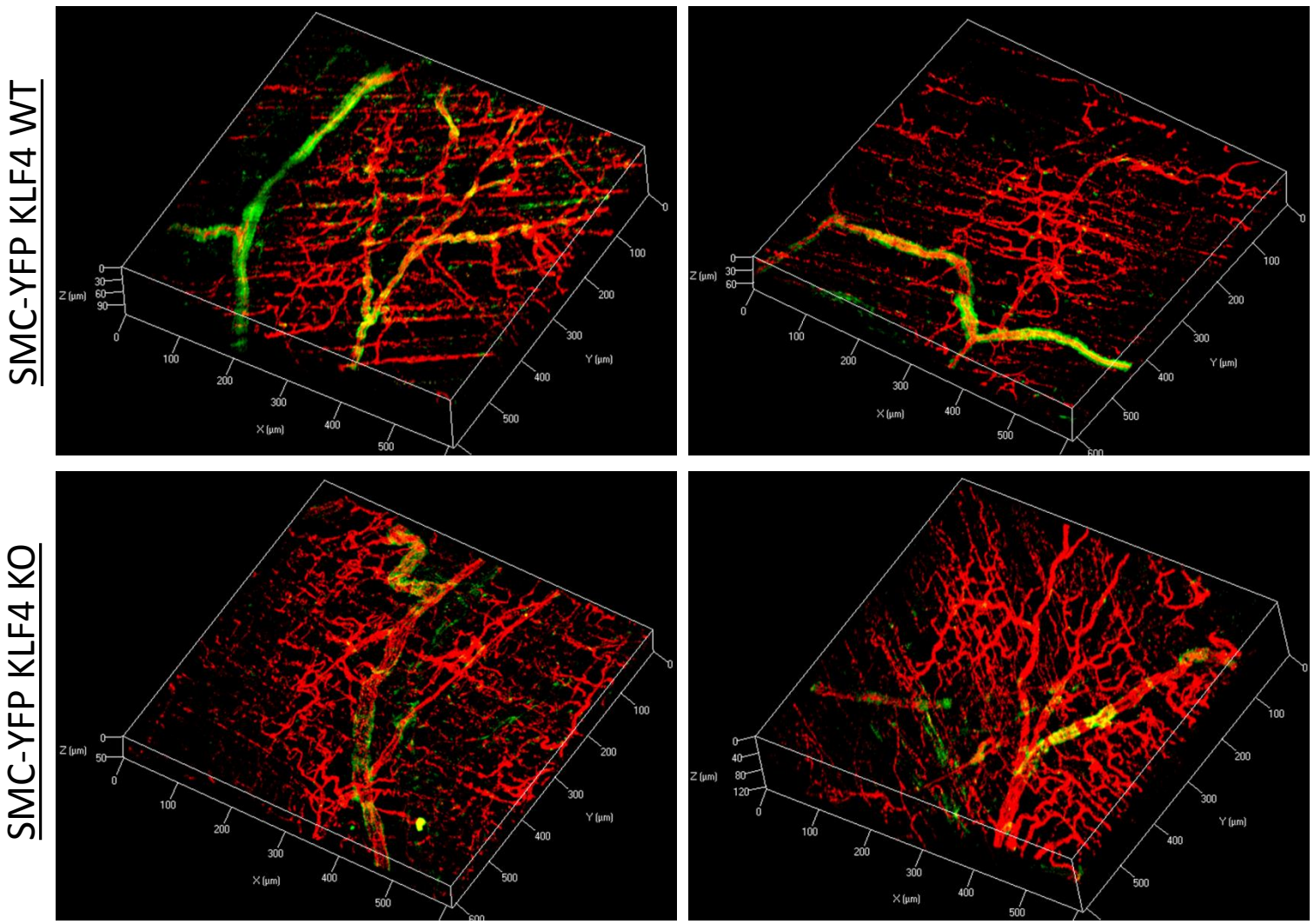
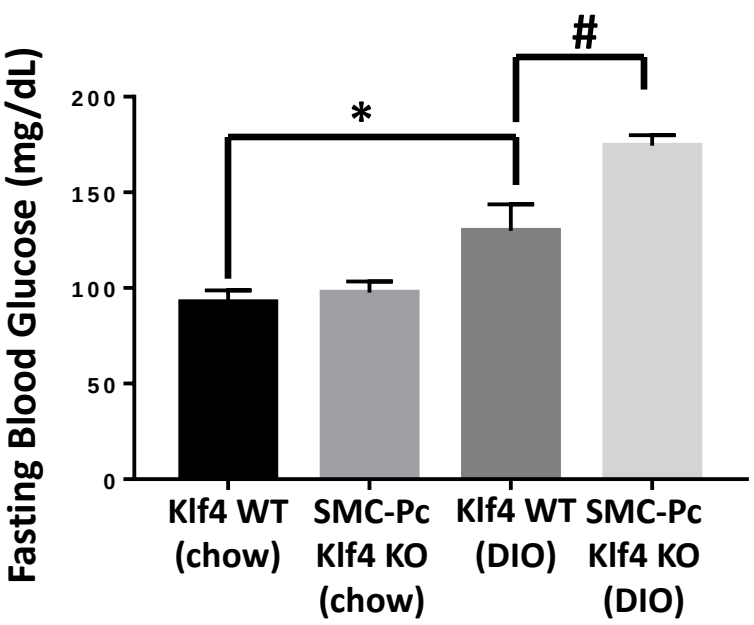
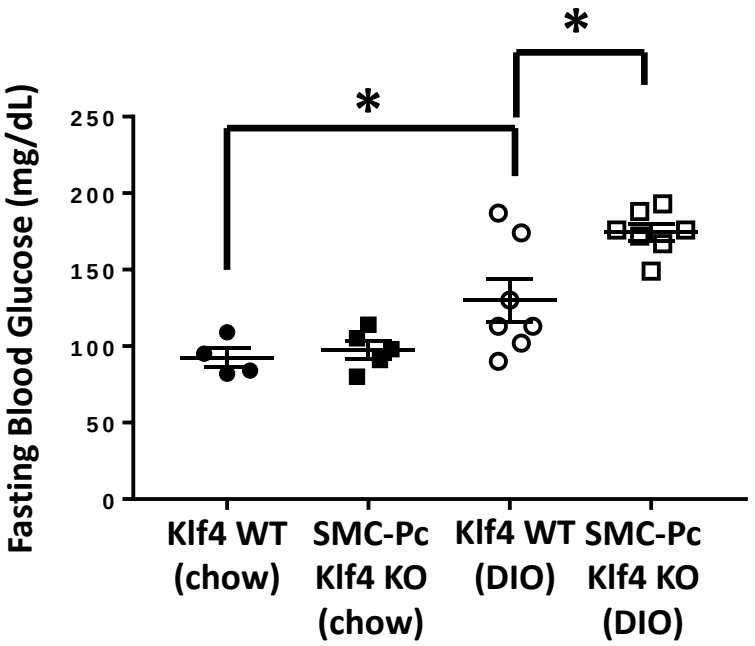


Figure 4.

SMC-Pc Specific Conditional Klf4 KO mice show exacerbation of DIO-dependent hyperglycemia



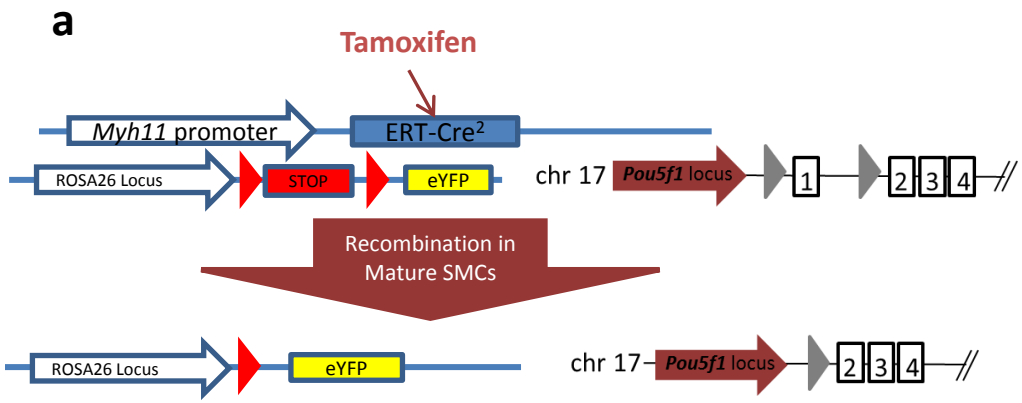


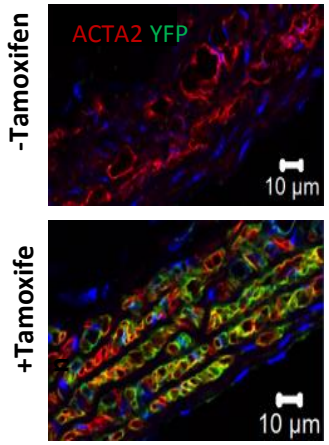
Hypothesis 1: Klf4 expression within microvascular SMC and pericytes (Pc) plays a critical protective role in regulating multiple aspects of microvascular function within adipose tissue including maintenance of vascular permeability and perivascular cell coverage-vessel integrity. We believe this is the role of Klf4 within SMC-Pc that has been conserved through evolution because it confers a survival advantage.

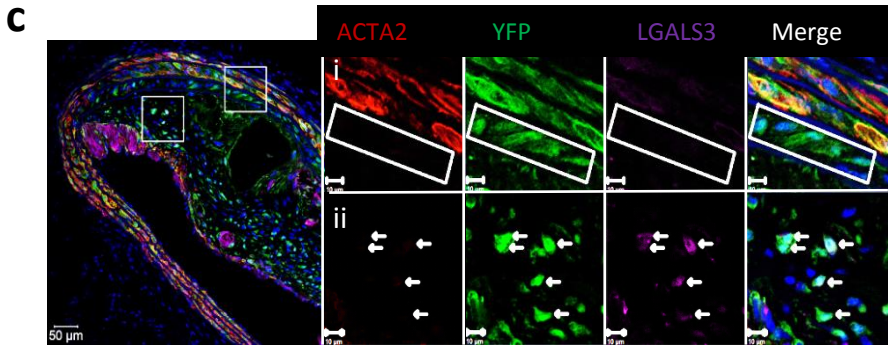
Hypothesis 2: Loss of Klf4 within perivascular cells results in increased permeability and alterations in tissue P02 that in turn result in dysregulation of multiple metabolic and inflammatory pathways even in young lean chow fed mice.

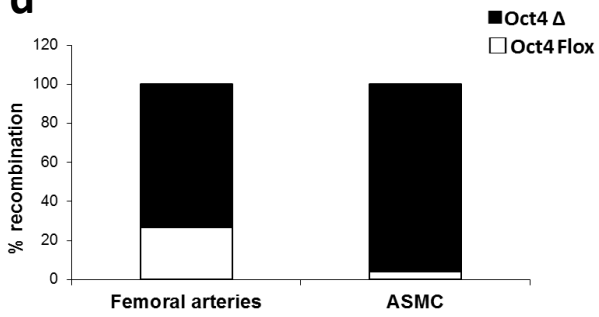
Hypothesis 3: Widespread microvascular dysfunction associated with DIO-induced metabolic disease and diabetes, including within non-adipose tissues is mediated at least in part by loss of protective Klf4 functions and/or Klf4-dependent activation of pro-inflammatory genes within microvascular SMC-Pc. We believe these latter changes are maladaptive as are Klf4-dependent transition of SMC to a macrophage-like foam cell within advanced atherosclerotic lesions.

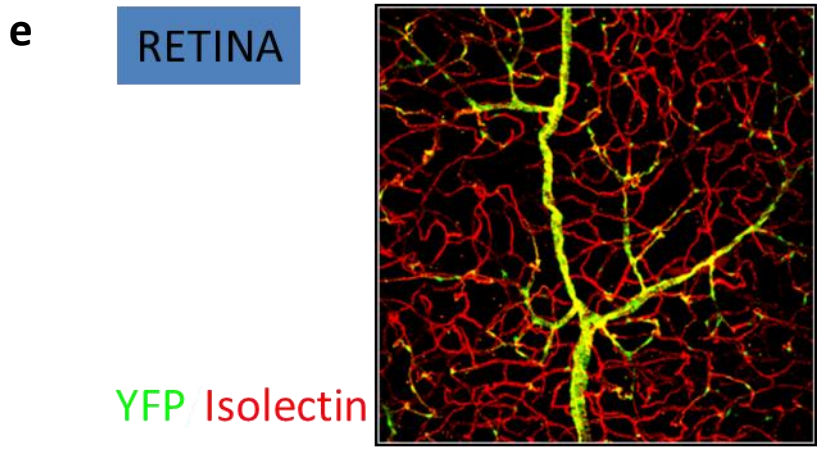
Hypothesis 4: whereas Klf4 itself is not a viable therapeutic target gene, we believe that genomic analysis of the microvasculature and surrounding adipose tissue +/- DIO +/- SMC-pericyte specific Klf4 KO will identify novel therapeutic targets for treating or preventing development of widespread microvascular dysfunction associated with metabolic disease.

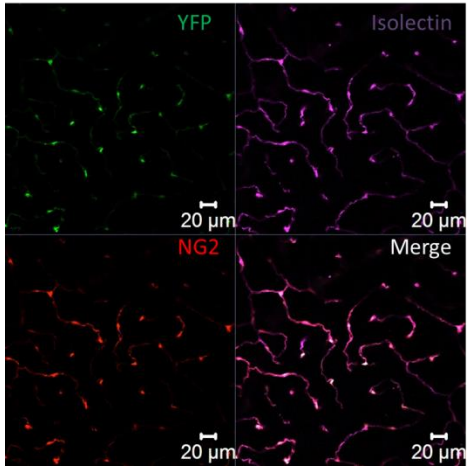

a
Tamoxifen
Myh11 promoter
ERT-Cre2
ROSA26 Locus
STOP
eYFP
chr 17
Pou5f1 locus
1
2
3
4
Recombination in Mature SMCs
ROSA26 Locus
eYFP
chr 17
Pou5f1 locus
2
3
4


b
-Tamoxifen
ACTA2 YFP
10 µm
+Tamoxife
10 µm


c
ACTA2
YFP
LGALS3
Merge
i
ii
50 µm


d
Oct4 Δ
Oct4 Flox
% recombination
120
100
80
60
40
20
0
Femoral arteries
ASMC

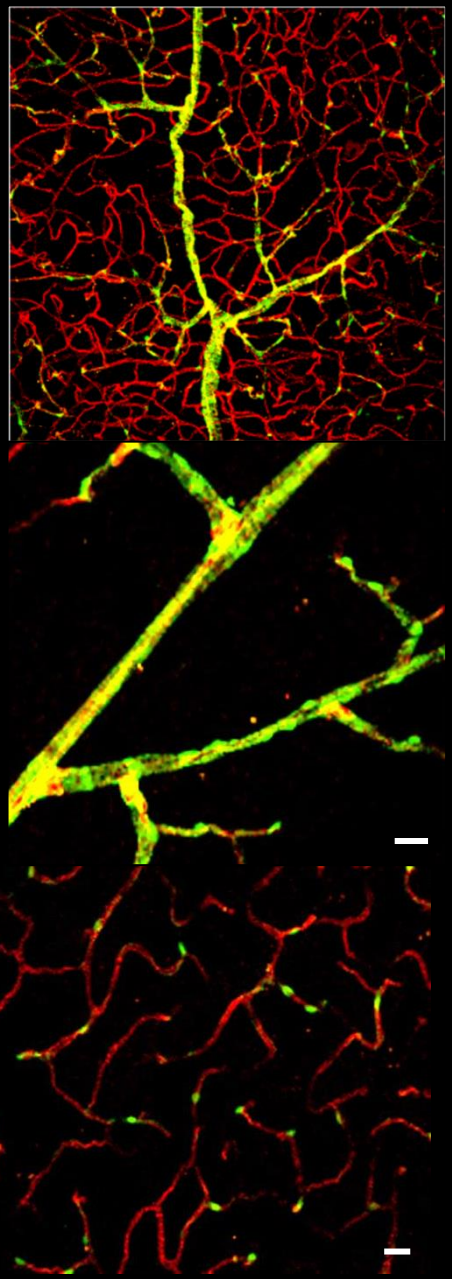

e
RETINA
YFP Isolectin

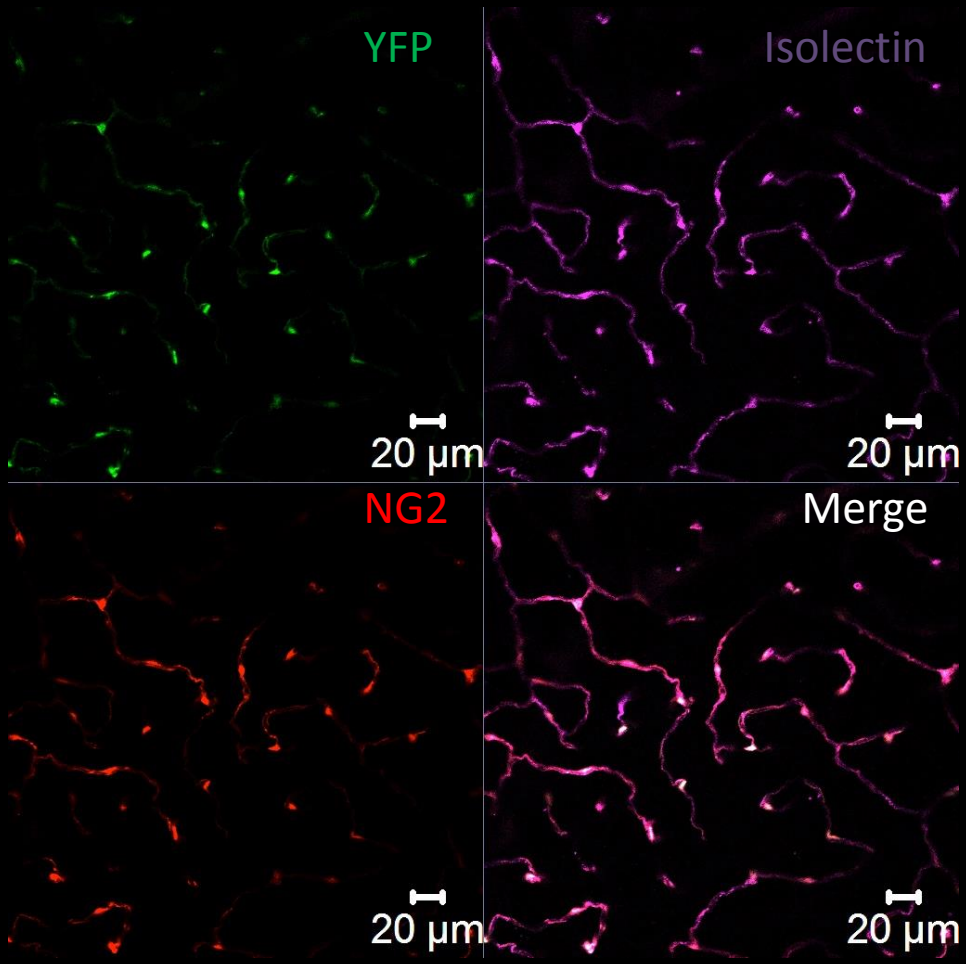

f
YFP
Isolectin
20 µm
20 µm
NG2
Merge
20 µm
20 µm

The Myh11-eYFP Mouse Also Provides Definitive Lineage Tracing of NG2+ Pericytes

RETINA

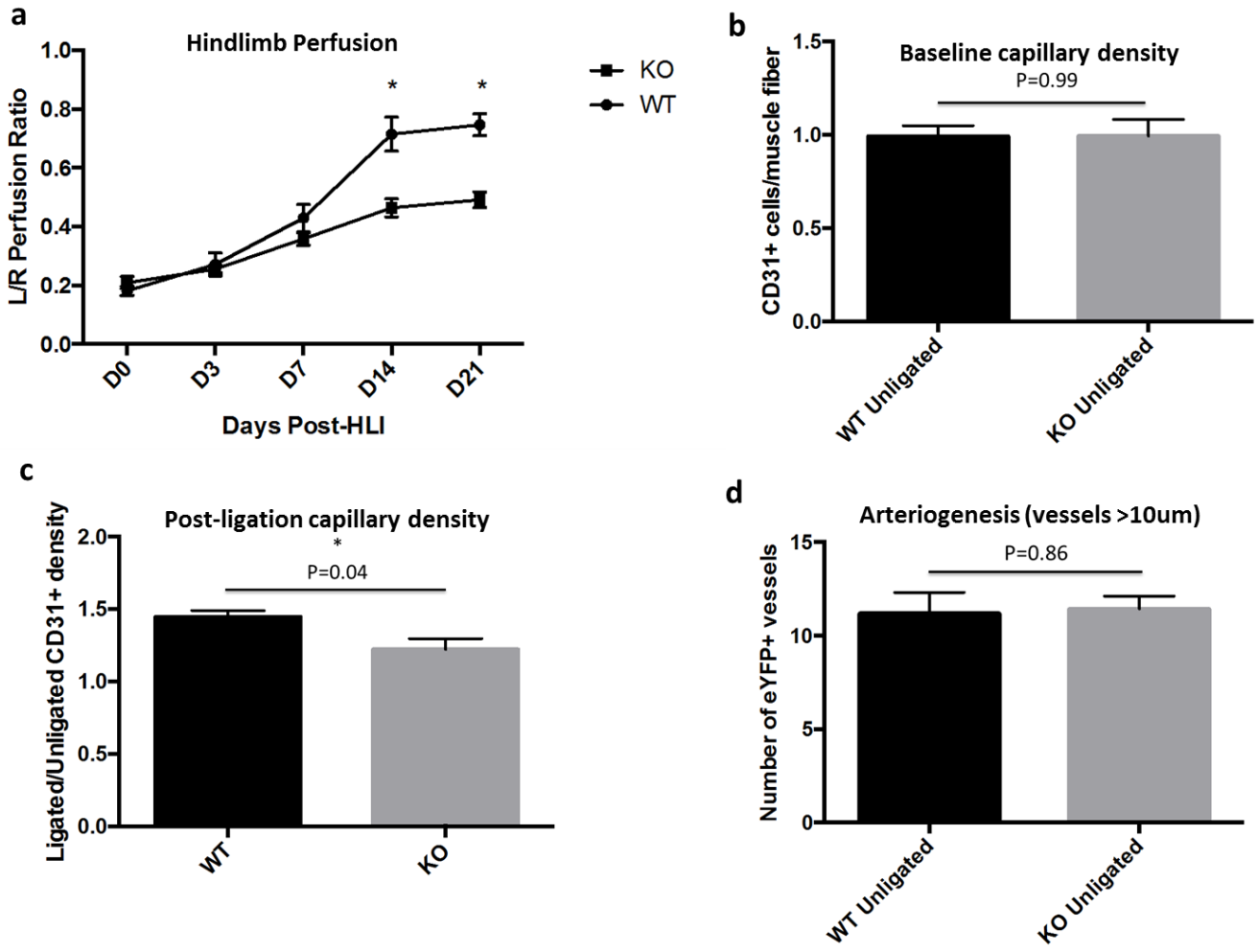
YFP/Isolectin





Haskins, Nguyen…Owens, manuscript in preparation

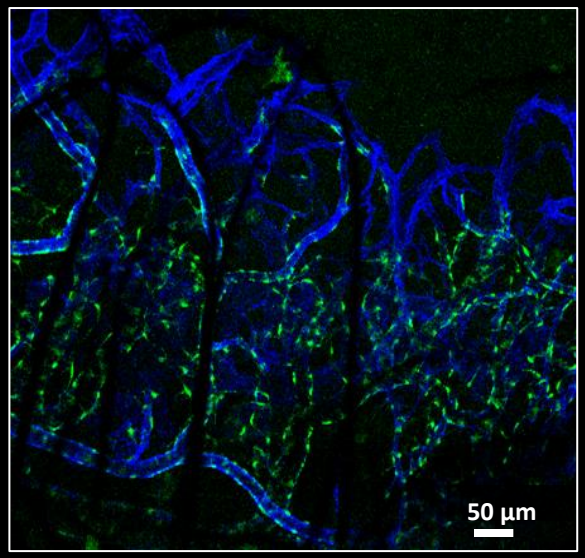
SMC-P specific KO of Oct4 impaired perfusion recovery following HLI due in part to impaired angiogenesis



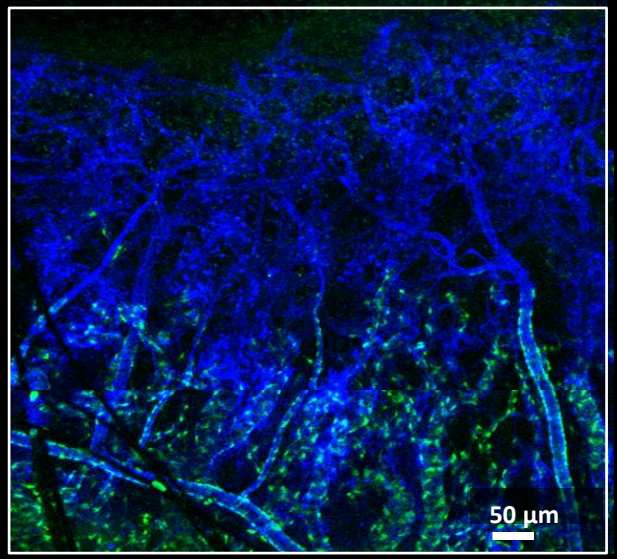
Dan Hess, M Kelly-Goss, ……S Peirce, and Owens, manuscript in preparation

SMC-P Specific Conditional KO of Oct4 Resulted in Profound Impairment of Perivascular Cell Investment of Neovessels in a Corneal Burn Model (7 days)

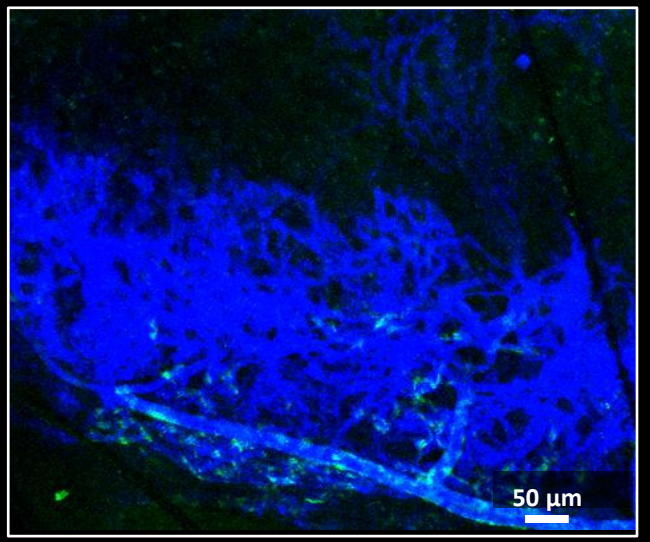
Myh11-eYPF+ Wild Type



Myh11-eYFP+ Oct4 +/- (Heterozygous KO)



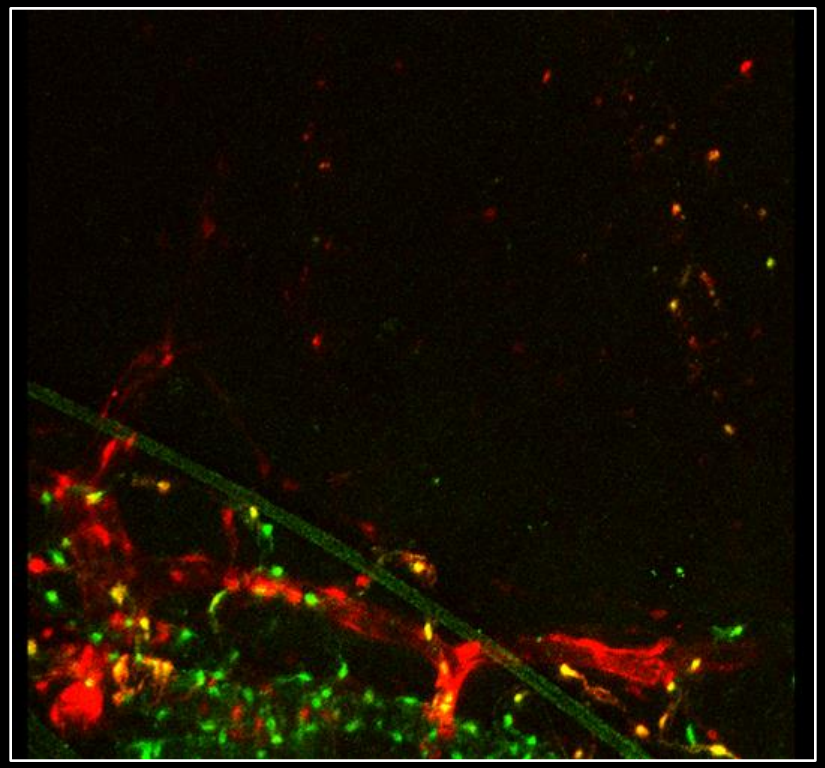
Myh11-eYFP+ Oct4 -/- (Homozygous KO)



Myh11-eYFP (SMC-P derived cells
Perfused Lectin

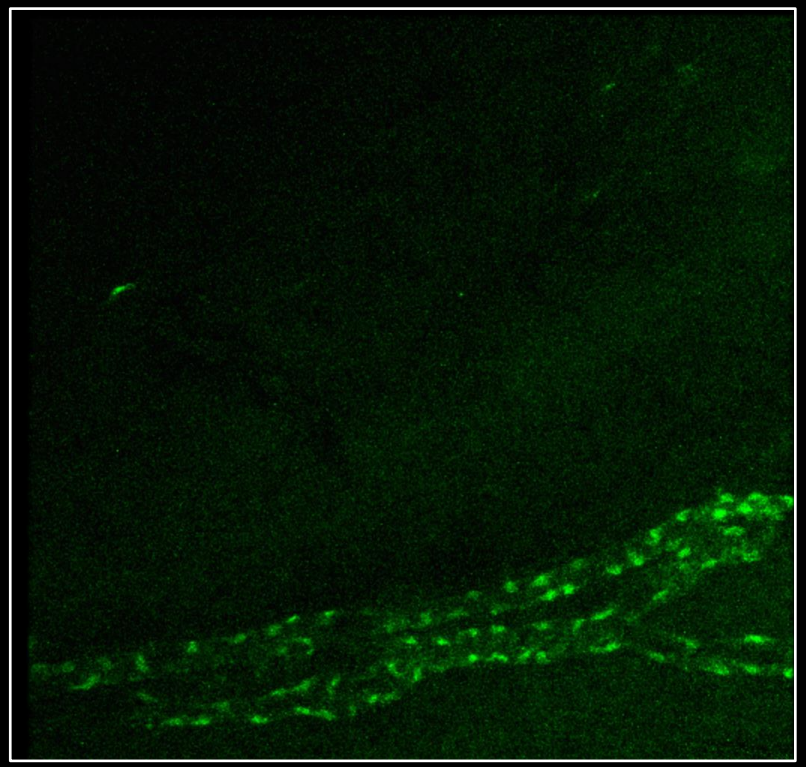
Intravital Microscopic Evaluations In Vivo Show Impaired Migration of eYFP+ Perivascular Cells from the Limbus in SMC-P Oct4 KO mice within 12 hours of the Corneal Burn

Myh11-eYFP



NG2-DsRed Myh11-YFP

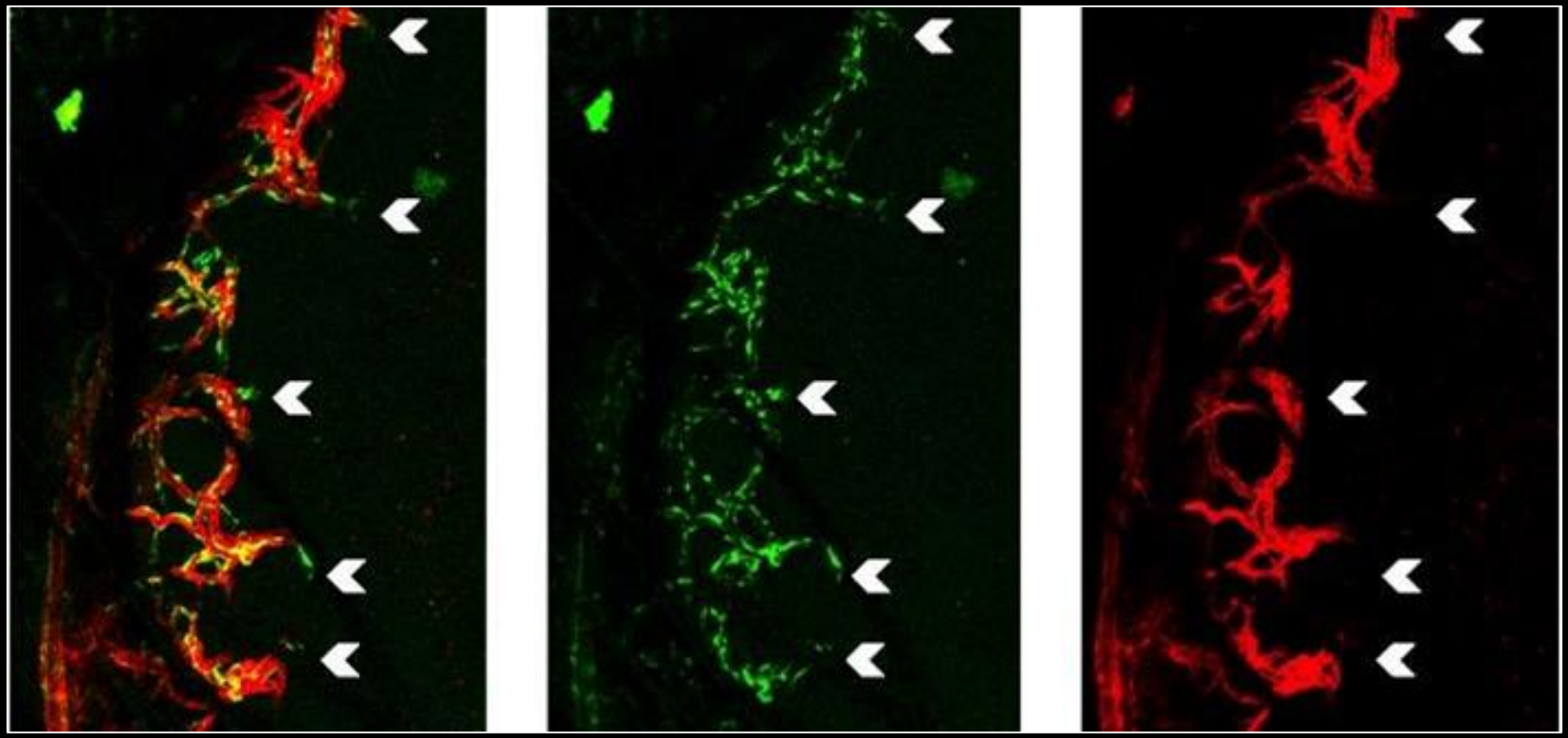
Oct4 -/- Myh11-eYFP
(Homozygous)



Myh11-YFP

50 µm

Pericytes of Wild Type Myh11+ cell-lineage extend past the perfused lumen of the vasculature

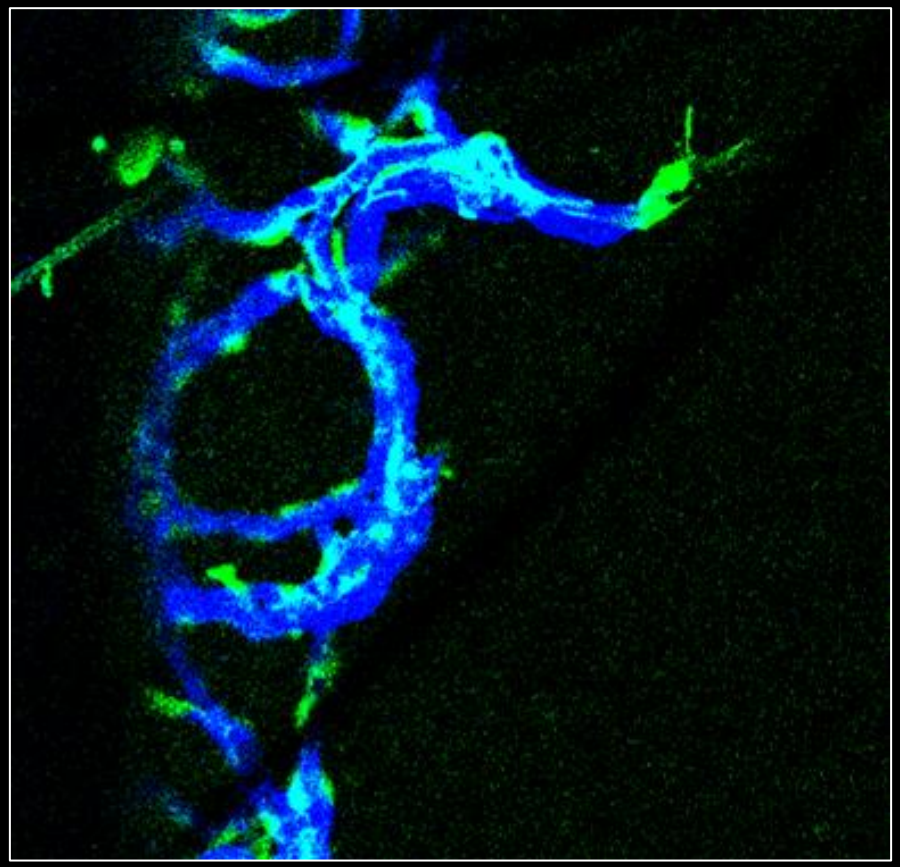


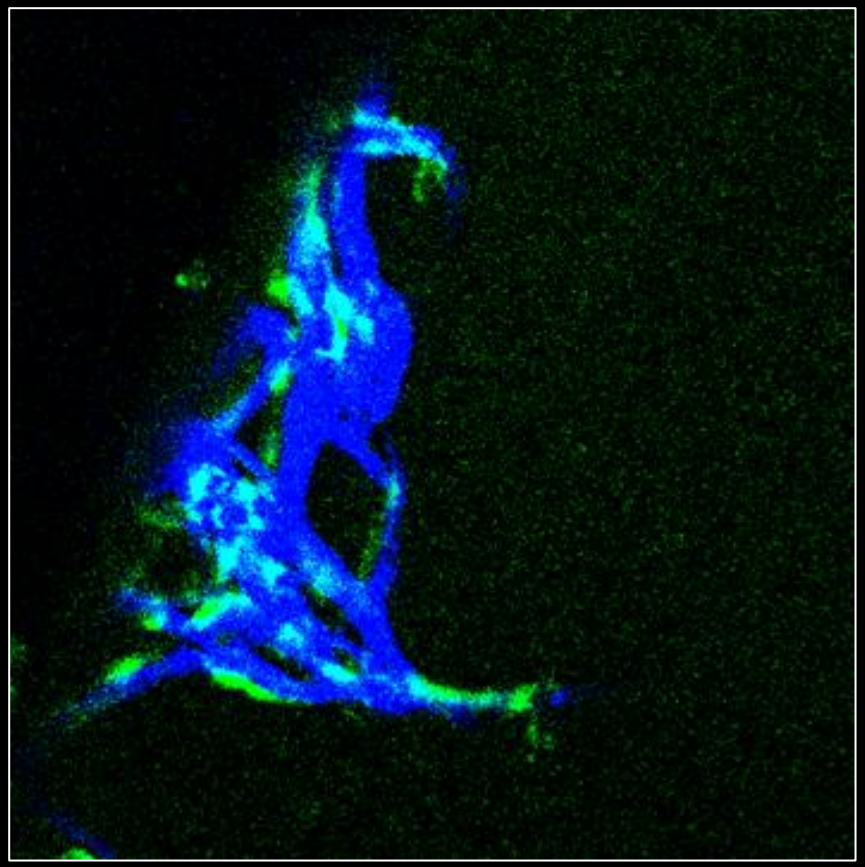
Myh11-YFP, Perfused Lectin

50 µm

10X

Pericytes of Myh11+ cell-lineage extend past the perfused lumen of the vasculature





Oct4-GFP, Perfused Lectin

50 µm

20X