Glutamine metabolism is required for phenotypic modulation of smooth muscle to a myofibroblast-like extracellular matrix-producing state

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INTRODUCTION

- Rupture or erosion of unstable atherosclerotic plaques is the underlying cause of heart attack or stroke, which are the leading causes of death worldwide.[1]
- Post-mortem human studies concluded that atherosclerotic plaque stability depends more on the cellular and extracellular matrix (ECM) composition of the protective fibrous cap than on lesions size.[2]
- The major source of ECM-producing myofibroblasts (MF) that populate the fibrous cap include smooth muscle cells (SMC) (~60% of all ACTA2$^+$ cells in the fibrous cap area). *(Newman at el. manuscript in review)*
- Deletion of platelet-derived growth factor receptor-β (PDGFRB) in SMC of Western diet-fed mice prevented SMC investment into the fibrous cap and resulted in eventual decreased collagen content and increased intraplaque hemorrhage. *(Newman at el. manuscript in review)*
- SMC transition to a myofibroblast (MF) phenotype is promoted by transforming growth factor-β (TGFβ) signaling pathway and atherosclerotic lesions of anti-TGFβ-treated mice showed decreased collagen content and increased infiltration of inflammatory cells.[3-4]
- Analysis of 150 human carotid endarterectomies showed that glutamine was among the most significantly changed metabolites between stable and unstable plaques.[5]
- Glutamine (Gln) is converted to glutamate by glutaminase (Gls).[6]

HYPOTHESIS

Glutamine availability is a critical requirement for SMC-to-MF transitions within atherosclerotic lesions, not only as a **catabolic** substrate for the energetically demanding process of ECM production but also as an **anabolic** source of proline, which comprises nearly 30% of the amino acid composition of collagen

MODEL

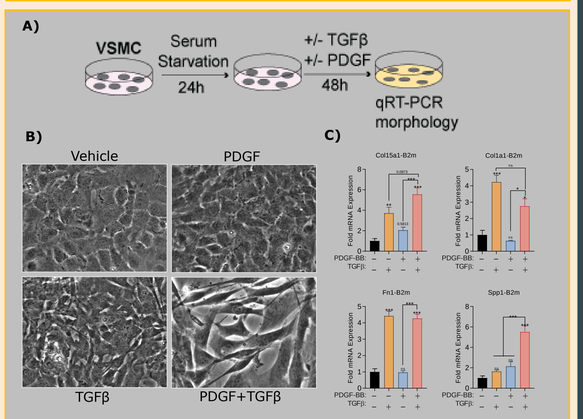

Figure 1. PDGF and TGFβ together phenotypically modulate SMC to a MF-like state. a) Schematic showing the experimental design. 8x10^4 cultured murine aortic smooth muscle cells (mASMCs) were serum-starved for 24-72 hours prior induction of the SMC-to-MF system. mASMCs were treated for 48h with 50ng/mL mouse platelet-derived growth factor BB (mPDGF-BB), and 50ng/mL mouse transforming growth factor-β (mTGFβ1), followed by morphological assessment and transcriptome studies. b) Representative phase contrast images and c) gene expression showing PDGF and TGFβ induced the phenotypic transition of SMC to a myofibroblast state. Graphs were analyzed using one-way ANOVA with Tukey's correction for post-hoc analysis with n≥ 3, error bars represent mean ±SEM. ***p<0.001, **p<0.01, *p<0.05

RESULTS

1. Bulk RNA-seq data from advanced brachiocephalic artery (BCA) lesions of *Pdgfrb*$^{SMC-\Delta/\Delta}$ *versus* *Pdgfrb*$^{SMC-wt/wt}$ mice, showed the greatest induction in bioenergetic pathways

Upregulated in PDGFBR KO vs. WT	Adj. P-val
Oxidative Phosphorylation	2.2E-12
Cirtrate cycle (TCA cycle)	2.7E-08
Valine, leucine and isoleucine degradation	1.5E-05
Propanoate metabolism	1.9E-05
Pyruvate metabolism	2.6E-04
Fatty acid metabolism	8.1E-04
Butanoate metabolism	2.2E-03
Peroxisome metabolism	2.4E-03
PPAR signaling pathway	2.6E-03
Glyoxylate and dicarboxylate metabolism	1.0E-02

Table1. Major transcriptional changes in energy metabolism underlie loss of SMC-PDGFRB signaling. GSEA pathway analysis showed substrate utilization and energy production pathways comprised the top 10 upregulated pathways in *Pdgfrb*$^{SMC-\Delta/\Delta}$ compared to *Pdgfrb*$^{SMC-WT/WT}$ mice fed WD for 18 weeks.

2. SMC treated with PDGF/TGFβ experience a 3-fold increase in oxidative phosphorylation compared to control, 32% of which is glutamine-derived

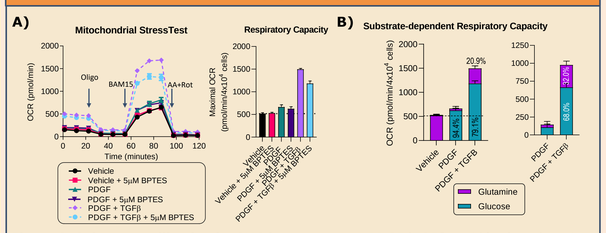

Figure 2. Substrate utilization by SMC in response to PDGF/TGFβ. 4x10^4 cultured murine aortic smooth muscle cells (mASMCs) were serum-starved for 24-72 hours prior to experiments. mASMCs were treated for 24 hours in basal medium with vehicle (4μmM HCl in PBS), 10ng/mL PDGF-BB, 10ng/mL TGFβ. **A)** Cellular respiratory capacity was assessed with a mitochondrial stress test measuring oxygen consumption rate (OCR) using DMEM media (20mM glucose; 2mM glutamine; with DMSO vehicle or 5μM glutaminase inhibitor BPTES) serially injected with ATP-synthase inhibitor oligomycin (1μM), mitochondrial uncoupler BAM15 (2μM), and mitochondrial complex III and I inhibitors antimycin A (10μM) and rotenone (1μM). **B)** The contribution of glutamine to respiratory capacity was determined by subtracting the X + BPTES condition's respiratory capacity from the X + Vehicle condition, where X is vehicle, PDGF, or TGFβ. The contribution of glucose was determined as the respiratory capacity of the X + BPTES condition. The substrate contribution to the enhanced respiratory capacity (over vehicle control) of the PDGF and TGFβ conditions is shown. Statistical significance was calculated by One-way ANOVA followed by post-hoc multiple comparison tests. (***p < 0.05*; ****p < 0.01*)

3. The glutaminase inhibitors BPTES and CB-839 decreased expression of ECM-associated genes in the SMC-to-MF system

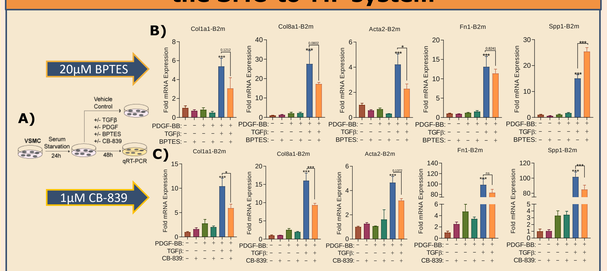

Figure 3. Transcriptional changes of SMC in response to PDGF/TGFβ and inhibitors of glutamine metabolism. A) Schematic showing the experimental design. Briefly, mSMC were plated, serum starved, and treated with recombinant PDGF-BB/TGFβ with or without *Gls*-inhibitors: BPTES, and CB-839 for 48 hours, followed by transcriptome studies. **B)** mRNA expression of ECM-associated genes after treatment with BPTES, and **C)** CB-839 (. Graphs were analyzed using one-way ANOVA with Tukey's correction for post-hoc analysis with n≥ 3, error bars represent mean ±SEM. ***p<0.001, **p<0.01, *p<0.05

4. Absence of glutamine results in nearly complete reduction of ECM-related gene expression

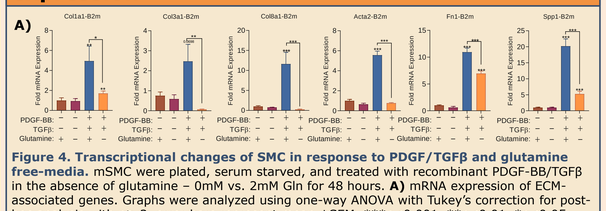

Figure 4. Transcriptional changes of SMC in response to PDGF/TGFβ and glutamine free-media. mSMC were plated, serum starved, and treated with recombinant PDGF-BB/TGFβ in the absence of glutamine – 0mM vs. 2mM Gln for 48 hours. **A)** mRNA expression of ECM-associated genes. Graphs were analyzed using one-way ANOVA with Tukey's correction for post-hoc analysis with n≥ 3, error bars represent mean ±SEM. ***p<0.001, **p<0.01, *p<0.05

5. Genetic knockdown of glutaminase did not change expression of *Col1a1*, *Col8a1* and *Spp1*

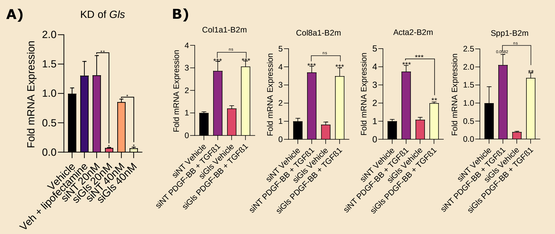

Figure 5. Transcriptional changes of SMC in response to PDGF/TGFβ and siRNA of glutaminase. A) mSMC were plated, treated with 20nM and 40nM of siRNA (targeting *Gls* – siGls and non-targeting RNA - siNT) and serum starved, followed by transcriptome studies. Gene expression of *Gls* showed > 90% reduction of expression compared to vehicle controls (vehicle, vehicle + lipofectamine, siNT). **B)** mSMC were plated, serum starved, treated with 20nM of siRNA for 24 hours, next treated with PDGF/TGFβ for 48h. Graphs represent mRNA expression of ECM-associated genes after treatment with siRNA and PDGF/TGFβ. Graphs were analyzed using one-way ANOVA with Tukey's correction for post-hoc analysis with n≥ 3, error bars represent mean ±SEM. ***p<0.001, **p<0.01, *p<0.05

6. Transglutaminase 2 (Tgm2) may convert glutamine to glutamate in lesion SMC

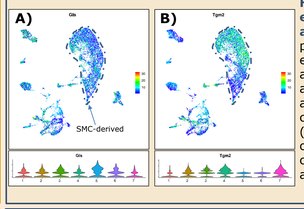

Figure 6. Glutamine-to-glutamate enzymes are differentially expressed among SMC clusters *in vivo*. UMAP presentation of scRNA-seq data showing gene expression of *Gls* vs. *Tgm2* in *Pdgfrb*$^{SMC-WT/WT}$ and *Pdgfrb*$^{SMC-\Delta/\Delta}$. scRNA-seq analysis of advanced BCA lesions from *Pdgfrb*$^{SMC-WT/WT}$ and *Pdgfrb*$^{SMC-\Delta/\Delta}$ mice identified 19 distinct cell clusters. Clusters 1-7 are *eyfp* positive (SMC-derived). **(top) A)** UMAP representation of *Gls* expression and **B)** *Tgm2* within all 19 clusters. **(bottom)** Violin plots showing *Gls* and *Tgm2* in SMC-clusters (1-7).

PROPOSED MODEL

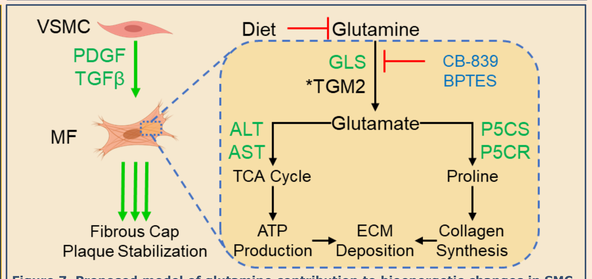

Figure 7. Proposed model of glutamine contribution to bioenergetic changes in SMC-to-MF model, in response to PDGF/ TGFβ. Glutamine is converted to glutamate via glutaminase (*Gls*) and/or transglutaminase 2 (*Tgm2*). Contribution of glutamine to ECM deposition can be regulated by BPTES **(Fig. 3B),** CB-839 **(Fig. 3C)** or/and by diet **(Fig. 4).** Genes involved in glutamine-to-proline conversion (anabolism), include P5CS (pyrroline-5 carboxylase synthase) and P5CR (pyrroline-5 carboxylase reductase). Genes involved in ATP-production (catabolism) include ALT (alanine aminotransferase) and AST (aspartate aminotransferase) and will be targeted in future studies to further asses glutamine contribution to SMC-to-MF transition and plaque stability in atherosclerosis.

SUMMARY

- Using oxygen consumption rate (OCR) as a measure for mitochondrial respiration, we found that 32% of the PDGF/TGFβ-induced oxidative phosphorylation in SMC is glutamine-dependent - **Fig. 2**
- Perturbation of glutamine metabolism with CB-839 and BPTES markedly reduced PDGF-TGFβ -induced SMC-to-MF transition – **Fig. 3**
- Absence of glutamine led to a complete reduction of *Col8a1* and *Col3a1* (~95%) and a significant reduction of *Col1a1* and *Fn1* - 65% and 36%, respectively - **Fig. 4**
- siRNA knockdown of glutaminase in mSMC have not affect SMC-to-MF transitions as shown by ECM expression at the mRNA level of *Col1a1, Col8a1 and Spp1* – **Fig. 5**
- Glutamine-to-glutamate enzymes are differentially expressed among SMC clusters *in vivo* – **Fig. 6**

CONCLUSIONS

1. Glutamine is essential in the phenotypic modulation of SMC to an ECM-producing myofibroblast-state (SMC-to-MF), which forms and maintains stable fibrous caps
2. Manipulation of SMC-derived ECM-production by energetic reprogramming is of great clinical interest. We propose to alter SMC bioenergetics in vivo through genetic (**Fig. 5**) and pharmacologic approaches (**Fig. 3**), or through manipulation of substrate availability (**Fig. 4**)
3. We propose that SMC glutamine metabolism is necessary for atherosclerotic plaque stability with the ultimate goal of identifying new therapies and dietary approaches to lower the risk of thrombotic events

FUTURE DIRECTIONS

Question: Can glutamine metabolism augmentation or depletion impact the pathogenesis in late state atherosclerotic lesion *in vivo*?

- **Approach 1:** Systemic inhibition of *Gls* through administration of CB-839 after lesion development
  a. Initially feed Myh11-Cre^{ERT2}-RAD/tdTomato$^{+/+}$/*Apoe*$^{-/-}$ mice Western diet for 18-26 weeks
  b. Expectations: decreased indices of lesion stability, including decreased fibrous cap thickness and reduced collagen content
- **Approach 2:** Dietary augmentation of glutamine with normal versus high glutamine levels
  a. Initially feed Myh11-Cre^{ERT2}-RAD/tdTomato$^{+/+}$/*Apoe*$^{-/-}$ mice custom Western diet with with normal (2.8%) versus high (10%) glutamine level for 18-26 weeks
  b. Expectations: Improved indices of lesion stability, including increased fibrous cap thickness and higher collagen content

LIMITATIONS

1. Our preliminary data relied on small number of genes but with bulk RNA-seq as a readout of ECM-related genes we will be able to assess how glutamine contribute to SMC-to-MF
2. Genetic manipulation of glutaminase with and without si*Gls* resulted in similar expression of ECM-related genes, suggesting a compensation driven by other glutamine-to-glutamate enzymes which needs to be verified via si*Tgm2* and similar approaches as shown here

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