Microbe-derived butyrate activation of free fatty acid receptor-3 reduces neointimal hyperplasia after arterial injury by regulating immune response transcriptional networks in endothelial cells

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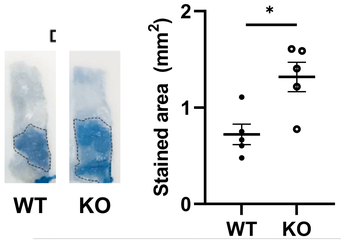
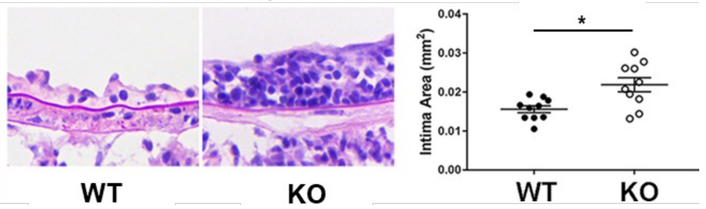
Background

- Promoting endothelial recovery is one strategy to mitigate neointimal hyperplasia after vascular surgery
- Butyrate, a short-chain fatty acid, attenuates neointimal hyperplasia via activation of free fatty acid receptor 3 (FFAR3) on endothelial cells (EC)[1]
- Denudation of the vascular intima in FFAR3 knockout (KO) mice results in delayed endothelial recovery
- The mechanism of the FFAR3 activation pathway in endothelial cells is unknown

FFAR3 loss increases neointimal hyperplasia after injury[1]



Left panel: Representative Evans blue staining from arteries from wildtype (WT) and FFAR3 KO mice. Right panel: Quantification of Evans blue staining area indicating delayed re-endothelialization in FFAR3 KO mice compared to WT.



Left panel: Representative arterial sections from WT and FFAR3 KO mice 4 weeks after femoral artery injury. Right panel: Morphometric analysis of intimal area 4 weeks post-injury indicating a 60% increase in neointimal hyperplasia in FFAR3 KO mice compared to WT. *n*=10 mice/group, *p = 0.03

Hypothesis

FFAR3 regulates endothelial cell (EC) migration and proliferation by modulating transcriptional responses

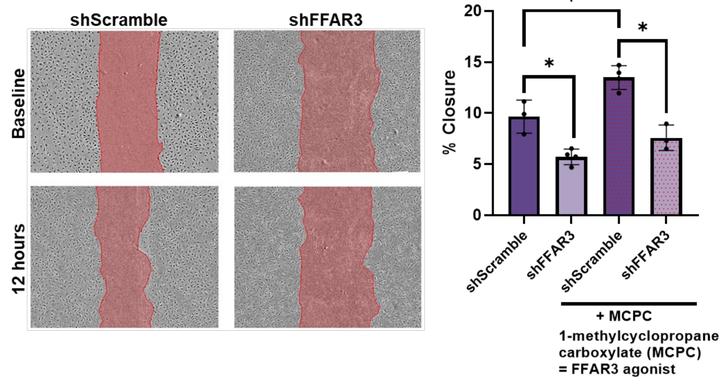
Methods

- Primary human umbilical vein endothelial cells (HUVEC) were infected with lenti-virus expressing shRNA against FFAR3 (shFFAR3) and non-targeting sequence (shScramble)
- In scratch assays, confluent HUVEC monolayers were subjected to a longitudinal "scratch" which was quantified at 0 and 12 hours as ΔArea/Initial Area.
- Proliferation (Ki67) and cell cycle (propidium iodide, PI) analysis was assayed by flow cytometry and qPCR
- Bulk RNA-seq was performed on HUVEC and endothelial fractions of FFAR3 WT and KO mouse aortas. Gene set enrichment analysis (GSEA) was used to identify transcriptional pathways affected by FFAR3.

Results

shFFAR3 knockdown inhibits EC migration

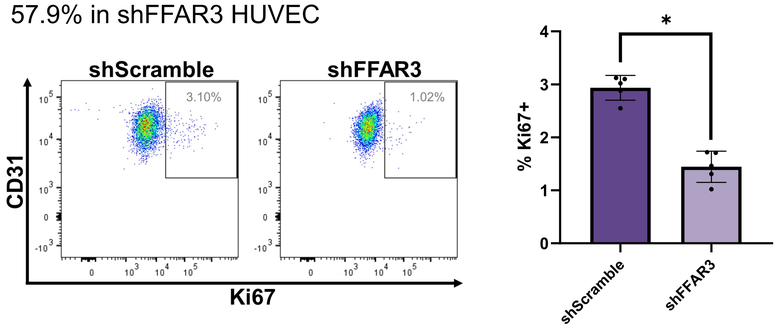
40.8% reduction in endothelial migration observed in shFFAR3 HUVEC was not rescued by the FFAR3 agonist, 1-methylcycloproaone carboxylate (MCPC)



Left panel: Representative images of scratch assay at t=0 and t=12 hr. Right panel: Quantification of scratch assay. *n* = 3 per group, *p < 0.05.

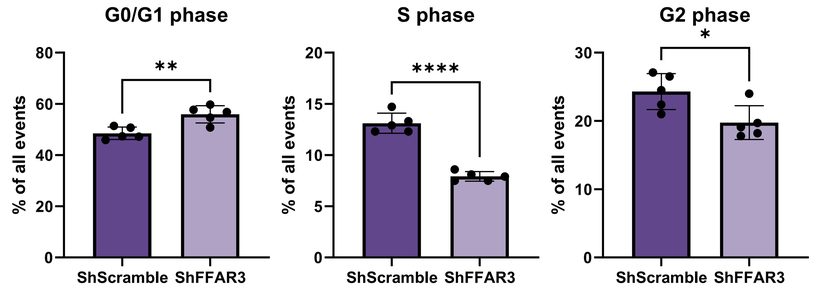
shFFAR3 knockdown inhibits EC proliferation and cell cycle progression

Active cell proliferation as indicated by Ki67 staining is reduced by 57.9% in shFFAR3 HUVEC



Left panel: Representative flow cytometry plots of CD31+Ki67+ double positive events. Right panel: Quantification of CD31+Ki67+ cells *n* = 5 per group, *p < 0.05.

FFAR3 knockdown in HUVEC increased the proportion of cells in G0/G1 phase by 7.42% and reduced the proportion of cell is S and G2 phase by 4.98% and 4.54%, respectively

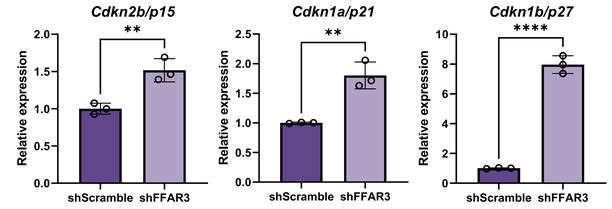


Quantification of HUVEC in different phases of the cell cycle as indicated by PI staining. *n* = 5 per group, *p < 0.05, **p < 0.01, ****p < 0.0001.

Results

shFFAR3 knockdown cells upregulate markers of cellular senescence

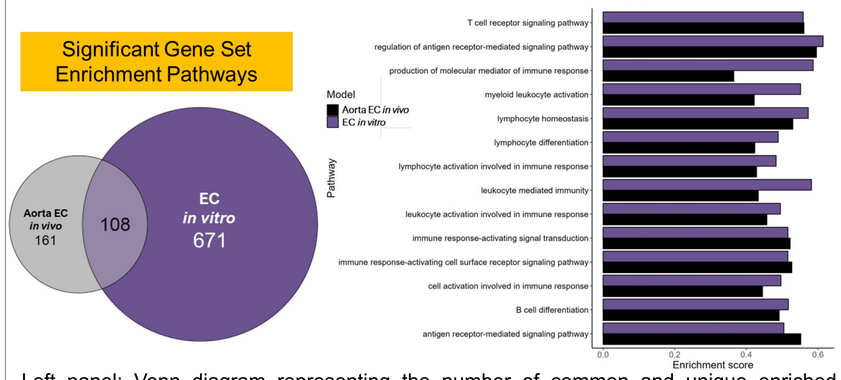
Markers of senescence and cyclin inhibitors p15, p21 and p27 are significantly upregulated.



Gene expression analysis of markers of senescence relative to *GAPDH*. *n* = 3 per group, *p <0.01, ****p < 0.0001.

FFAR3 knockdown/knockout in alters similar inflammatory pathways *in vitro* and *in vivo*

GSEA analysis of ECs isolated from aortae of mice and HUVEC reveals 108 transcriptional pathways that are enriched between both cell types when FFAR3 is attenuated. The most enriched pathways are related to inflammation. Implicated inflammatory pathways have similar enrichment scores in *in vivo* and *in vitro* ECs



Left panel: Venn diagram representing the number of common and unique enriched transcriptional pathways in HUVEC and aortic EC identified by GSEA. Right panel: Enrichment scores of inflammation-related transcriptional pathways.

Conclusion

- **FFAR3 knockdown inhibits EC migration and proliferation**
- **Increasing endothelial FFAR3 activity or targeting inflammatory pathways downstream of FFAR3 may mitigate neointimal hyperplasia after arterial injury**
- Understanding FFAR3-related signaling and transcriptional networks in EC and assessing the effects of FFAR3 overexpression or FFAR3 agonists may lead to the development of novel FFAR3-based therapeutics to target neointimal hyperplasia

References

1. Nooromid M, Chen EB, Xiong L, Shapiro K, Jiang Q, Demsas F, et al. Microbe-Derived Butyrate and Its Receptor, Free Fatty Acid Receptor 3, But Not Free Fatty Acid Receptor 2, Mitigate Neointimal Hyperplasia Susceptibility After Arterial Injury. J Am Heart Assoc. 2020;9(13):e016235.