Development of a lentivirus shRNA delivery system to elucidate the cellular mechanism of the butyrate-FFAR3 activation pathway in endothelial cells

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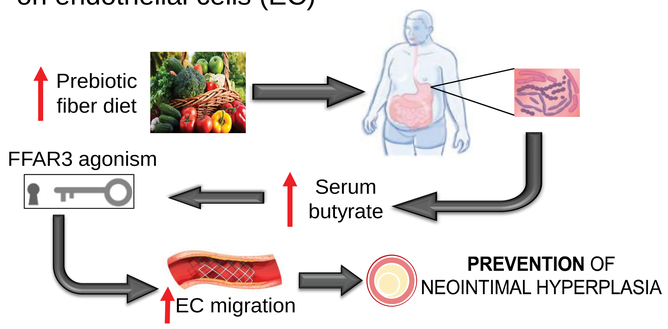
Background

- Gut microbial fermentation of dietary fiber to butyrate reduces susceptibility to neointimal hyperplasia after vascular surgery[1]
- Butyrate, a short-chain fatty acid, attenuates neointimal hyperplasia via activation of free fatty acid receptor 3 (FFAR3) on endothelial cells (EC)[1]



- The mechanism of the butyrate-FFAR3 activation pathway in EC is unknown
- A major hinderance to *in vitro* studies has been the resistance of primary EC to gene transfer[2]
- Lentivirus-mediated transduction of short hairpin RNA (shRNA) is an effective way to generate stable gene knockdown in “hard-to-transfect” cells

Hypothesis

Lentiviral-induced shRNA-FFAR3 (shFFAR3) knockdown in EC will maintain key endothelial characteristics while reducing FFAR3 production levels

Methods

- shFFAR3 clones were selected from the Broad Institute Genetic Perturbation Platform (GPP)
- Lentivirus were produced from HEK293T cells transfected with pLKO-based lentiviral constructs containing shRNA clones, packaging plasmid psPAX2 and envelope plasmid pMD2.G using TransIT-LT1 (MirusBio)
- Human umbilical vein endothelial cells (HUVEC) were transduced with harvested lentiviral particles and 10mg/ml polybrene
- Successfully transduced HUVEC (shFFAR3-HUVEC) were selected with 1.5mg/ml Puromycin
- Gene expression and protein production were analyzed to determine shFFAR3-HUVEC phenotypes

Results

shFFAR3 clones target different regions of FFAR3 and have high predicted performance scores

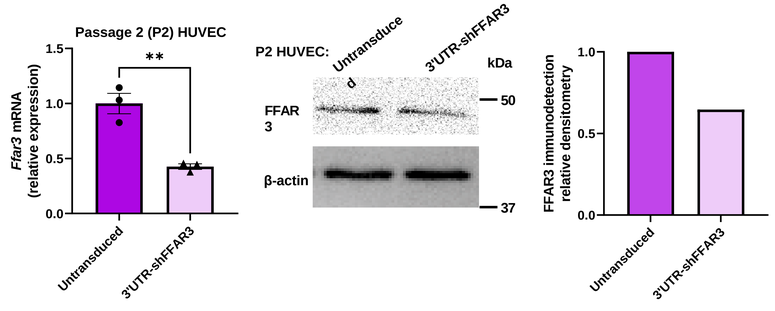
Selected shFFAR3 clones (bold-highlighted) target 3’-untranslated region (3’UTR) and coding sequence (CDS) of FFAR3 and are predicted to perform well compared to other clones based on Intrinsic and Adjust GPP scores

	Clone ID	Target Seq	Vector	Matching Transcripts for Gene	Match Regions	SDR Match	Intrinsic Score	Adjusted Score	Matches other Human Gene	Orig. Target Gene
1	**TRCN0000358282**	**CTAAGGGTATGC GCGCTAAAG**	**pLKO_005**	**NM_005304.5, XM_011526858.2**	**3UTR**	**100%**	**10.800**	**8.640**	**N**	**FFAR3**
2	TRCN0000014473	GTAGACATCTAG CCTCCCTAA	pLKO.1	NM_005304.5, XM_011526858.2	3UTR	100%	4.050	2.835	N	FFAR3
3	TRCN0000232049	GTCCCATGTCGT GGGCTATAT	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	13.200	6.600	Y	GPR42
4	**TRCN0000358283**	**TCCCATGTCGTG GGCTATATC**	**pLKO_005**	**NM_005304.5, XM_011526858.2**	**CDS**	**100%**	**13.200**	**6.600**	**Y**	**FFAR3**
5	TRCN0000232048	AGATGGCTGTGG TCCTCTTTG	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	10.800	5.400	Y	GPR42
6	TRCN0000232045	TCGGTGTACCTTC TCACTTTC	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	10.800	5.400	Y	GPR42
7	TRCN0000232046	TCTTCTTCACCACC ATCTATC	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	10.800	5.400	Y	GPR42
8	TRCN0000232047	AGCGTGGTCTAC GTCATAGAA	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	5.625	2.813	Y	GPR42
9	TRCN0000014474	GCGTGGTCTACGT CATAGAAT	pLKO.1	NM_005304.5, XM_011526858.2	CDS	100%	5.625	2.813	Y	FFAR3
10	TRCN0000358281	CAGCGTGGTCTAC GTCATAGA	pLKO_005	NM_005304.5, XM_011526858.2	CDS	100%	4.950	2.475	Y	FFAR3

First ten results of shRNA constructs with 100% match to *Ffar3* from the Broad Institute Genetic Perturbation Platform (GPP)

FFAR3 gene expression and protein production is reduced in early passages of 3’UTR-shFFAR3-HUVEC

Initial trial of HUVEC treated with lentivirus encoding shRNA targeting 3’UTR of FFAR3 produced 54-62% knockdown of FFAR3 gene expression and attenuated protein production after two passages (P2) relative to untransduced cells

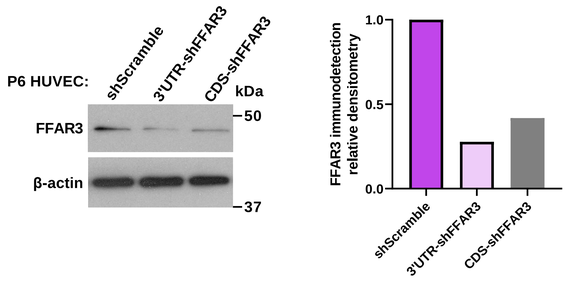


Left panel: *Ffar3* expression relative to GAPDH. *n*=3 per group **p = 0.004, unpaired t-test. Middle panel and right panel: Immunoblot and immunodetection intensity measurements from whole cell extracts of P2 Untransduced and 3’UTR-shFFAR3-HUVECs.

Results

Stable FFAR3 knockdown in 3’UTR and CDS-targeted HUVEC after consecutive passages

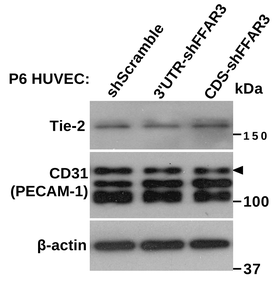
FFAR3 protein attenuation was sustained in P6 HUVEC treated with lentivirus encoding shRNA against 3’UTR and CDS of FFAR3 compared to non-targeting scramble shRNA



Immunoblot and immunodetection intensity measurements from whole cell extracts of P6 HUVEC treated with lentivirus encoding shRNA against non-targeting sequence (shScramble), 3’UTR and CDS-regions of FFAR3

shFFAR3-HUVEC maintain endothelial identity

Markers of endothelial identity (Tie-2 & CD31) were relatively unchanged in HUVEC transduced with lentiviral constructs



Immunoblot from whole cell extracts of P6 HUVEC treated with lentivirus encoding shRNA against non-targeting sequences (shScramble), 3’UTR and CDS-regions of FFAR3

Conclusion

- **An shRNA-lentivirus system generated targeted knockdown of FFAR3 in HUVEC over multiple passages**
- **HUVEC maintained endothelial identity despite stable suppression of FFAR3**
- The lentivirus system will be utilized to elucidate cellular mechanism of the butyrate-FFAR3 activation pathway in EC and other vascular cell types

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.

2. Hunt MA, Currie MJ, Robinson BA, Dachs GU. Optimizing transfection of primary human umbilical vein endothelial cells using commercially available chemical transfection reagents. J Biomol Tech. 2010;21(2):66-72.