SUPPLEMENTAL MATERIAL

Roles of Activin A and Gpnmb in metabolic dysfunction-associated steatotic liver disease (MASLD)

Huan Liu[1,2], Armen Yerevanian[3], Maria Westerhoff[4], Margaret H. Hastings[1,2], Justin Ralph Baldovino Guerra[1,2], Meng Zhao[5,6,7], Katrin J. Svensson[5,6,7], Bishuang Cai[8] Alexander A. Soukas[3], Anthony Rosenzweig[1,2]*

[1]Cardiovascular Research Center, Massachusetts General Hospital, and Harvard Medical School, Boston, MA, 02114, USA.

[2]Institute for Heart and Brain Health, University of Michigan Medical Center, Ann Arbor, MI, 48109, USA.

[3]Department of Medicine, Diabetes Unit and Center for Genomic Medicine, Massachusetts General Hospital, and Harvard Medical School, Boston, MA, 02114, USA.

[4]Department of Pathology, University of Michigan, Ann Arbor, MI, 48109, USA.

[5]Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305, USA.

[6]Stanford Diabetes Research Center, Stanford University School of Medicine, Stanford, CA 94305, USA

[7]Stanford Cardiovascular Institute, Stanford University School of Medicine, CA 94305, USA

[8]Division of Liver Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

*Correspondence: Anthony Rosenzweig, MD, Institute for Heart and Brain Health, University of Michigan Medical Center, North Campus Research Complex, 2800 Plymouth Road, 2791(3)/Bldg 25 NCRC Ann Arbor, MI 48109. Email anthros@med.umich.edu.

Detailed Research Methods

AAV Production

AAV production, purification and titration were performed as described (1). Briefly, HEK293 cells were transfected with pΔF6 (Addgene Plasmid #112867), pAAV2/8 (Addgene Plasmid #112864) helper plasmids and AAV8-TBG-GFP or AAV8-TBG-Activin A or AAV8-shRNA Scramble (SC) or AAV8-shRNA-Gpnmb transfer plasmids by PEI. Viruses were purified using iodixanol gradient ultracentrifugation using OptiPrep™ Density Gradient Medium (Sigma D1556), concentrated with columns（Fisher scientific UFC910024）, and titrated using droplet digital PCR.

Cell culture

Hepa 1-6 cells (ATCC), L929 cells (ATCC) and BMDM cells were prepared and cultured as previously described (1). Hepa 1-6 cells (ATCC, CRL1830, lot#70031235) were cultured in DMEM medium (Gibco) supplemented with 10% FBS (Gibco) and 50 μg/ml Penicillin/ Streptomycin (Gibco). L929 cells (ATCC, CCL-1, lot# 70026472) were cultured in RPMI1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 50 μg/ml Penicillin/ Streptomycin (Gibco). BMDMs were isolated from mice bone marrow and cultured in RPMI1640 medium supplemented with 10% FBS (Gibco), 50 μg/ml Penicillin/ Streptomycin (Gibco) and 30% L929 conditioned medium for 7 days to induce differentiation. For the hepatic Activin A conditioned media treatment, Hepa 1-6 cells were transfected with TBG-GFP or TBG-Activin A plasmid by Lipofectamine 3000 (Thermo Fisher Scientific) or siRNA Activin A by Lipofectamine RNAiMax (Thermo Fisher Scientific) at 37°C for 48h. The conditioned media were collected and filtered with a 0.22 μm filter, diluted twice with fresh RPMI1640 medium supplemented with 10% FBS (Gibco), 50 μg/ml Penicillin/ Streptomycin (Gibco), with 10 μg/ml oxLDL and 1μg/mL Mouse IgG1 Isotype Control or neutralizing anti-Activin A antibody for 24 hours and then added into

mature PBS washed BMDM cells For the recombinant Activin A protein treatment, BMDMs were treated with or without 20ng/ml, 50ng/ml or 100ng/ml Activin A protein, with or without 100 ng/ml LPS and 50ng/ml IFN-γ, with or without 200 μM Palmitic acid, 100 μM cholesterol, or 30 μM Oleic acid for 24 hours.

Animal studies

Only males were used because males have more marked metabolic changes on FPC diet than females. 8-10 week-old male C57BL/6J mice (Jackson Laboratory) were injected via tail vein with AAV8-TBG-GFP or AAV8-TBG-Activin A (2×10^{10} genome copies/mouse), or AAV8-H1-scramble control or AAV8-H1-shRNA Activin A or AAV8-H1-shRNA Gpnmb viruses (2×10^{11} genome copies/mouse), and then fed FPC diet (Envigo) and sugar water containing 23.1g/L fructose and 18.9g/L glucose for 16 weeks as described (2). Diet and sugar water were refilled twice weekly. Body weight, food weight/cage and water volume/bottle were measured weekly. Mice were sacrificed 16 weeks after treatment unless otherwise indicated.

Blood/ Plasma Analyses

Fasting blood glucose was measured using a glucometer (Clarity) in mice after fasting for 5hrs with free access to water. Overnight fasting plasma was collected in 5 mM EDTA. Plasma Activin A and FST ELISA Kit (R&D) and Plasma ALT and AST/Got (Teco Diagnostics) were analyzed according to manufacturer's instructions.

Glucose tolerance and insulin resistance

Mice were fasted overnight (glucose tolerance) or for 5h (insulin resistance) and then injected intraperitoneally with 2g/kg d-glucose (Sigma) or 0.5 U/kg insulin (Sigma) for glucose and insulin

tolerance testing, respectively. Blood glucose was measured by glucometer before,15, 30, 60, 90 and 120 min after.

Quantitative RT-PCR

Total RNA was extracted from liver using Trizol (Thermo Fisher Scientific)/chloroform/isopropyl alcohol. cDNA was synthesized using PrimeScript RT Master Mix (Takara) and subjected to RT-PCR with SYBR Green (Applied Biosystems). Relative mRNA abundance was calculated by normalization to hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA. For RT-PCR primers, see Supplemental Table 2.

Immunoblotting

Liver protein was extracted using RIPA/SDS buffer (Thermo Fisher Scientific, #89900), and protein concentration was quantified by a BCA assay (Thermo Fisher Scientific, #23227). Proteins were resolved by electrophoresis on 4-20% Tris gels (Bio-Rad) and transferred to PVDF membranes (Millipore Sigma). Membranes were blocked for 1hr at room temperature in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% (wt/vol) nonfat milk and incubated with primary antibodies in the same buffer at 4°C overnight, using 1:1000 dilution. Antibodies used in this paper are listed in Supplemental Table 1. Image Studio was used for quantification.

Liver senescent cells staining

Liver senescent cells were stained by Senescence Cells Histochemical Staining Kit (Sigma) using frozen section according to manufacturer's instructions at 37 °C overnight. β-gal+ cells were imaged by Keyence and quantified using Image J at the same setting.

Flow cytometry

Flow cytometry was performed blindly by authors with a flow core technician as described (1). Livers were excised after PBS (Thermo Fisher Scientific) perfusion, minced, and digested with 450Uml/L collagenase I (Worthington Biochemical), 125Uml/L collagenase XI, 60Uml/L DNase I and 60Uml/L hyaluronidase (Sigma-Aldrich) in PBS at 37°C for 20 min, and then passed through a 70μm-filter. Red blood cells were lysed with RBC lysis buffer. Total viable cell numbers were counted using counting beads (Thermo Fisher Scientific). Single-cell suspensions were stained in PBS supplemented with 2% FBS and 0.5% BSA. DAPI was used for staining live/dead cells. Monoclonal antibodies used for flow cytometry are listed in the Supplemental Table 1.

Liver immune cells were defined as described (4): PMN (polymorphonuclear cells, CD45+CD11b+Ly-6G+, including neutrophils, basophils, and mast cells), PMO (patrolling monocytes, CD45+CD11b+Ly-6G-Ly-6C- MHC-II-), MDM (monocytes-derived macrophages, CD45+CD11b+Ly-6G-Ly-6C^{high} MHC-II^{high}), MM (mature macrophages, also known as Kupffer cells, CD45+CD11b+Ly-6G-Ly-6C$^{neg/low}$ MHC-II ^{high}), ED (eosinophils, CD45+CD11b+Ly-6G-Ly-6C^{low} MHCII-), Ly-6C^{high} monocytes (CD45+CD11b+Ly-6G-Ly-6C^{high} MHCII-).

RNA-seq and Bioinformatic Analyses

Total RNA was isolated from liver using Trizol (Thermo Fisher Scientific)/chloroform/isopropyl alcohol. RNA-sequencing was performed using NovaSeq6000 as described (1) . Differential gene expression was analyzed and normalized with R package DESeq2. Count cut off: mean normalized count >=10 in >=1 group. Genes with adjusted p-value (padj) <0.05. Significant upregulation: log2FoldChange>=1, padj<0.01. Significant downregulation: log2FoldChange<=-1, padj<0.01. Else is considered stable. Heatmap z-scaled (log10(normalized count + 0.05) and Principal Components Analysis (PCA) scaled (log10(RNA-

seq Matrix + 0.01) were used. R-packages are listed in the Supplemental Table 1. RNA-seq raw data are available in GEO (accession number GSE221443).