Beneficial Effects of Moderate Hepatic Activin A Expression on Metabolic pathways, Inflammation, and Atherosclerosis

Huan Liu, PhD, Margaret Hallauer Hastings, PhD, Robert Kitchen, PhD, Chunyang Xiao, Ph.D., Justin Ralph Baldovino Guerra, B.S., Alexandra Kuznetsov, B.A., Anthony Rosenzweig, M.D.*

Short title: Beneficial Effects of Activin A in Atherosclerosis

Total Word Count:7343

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

*Correspondence to:

        Anthony Rosenzweig, MD
        Corrigan Minehan Heart Center
        Division of Cardiology,
        Massachusetts General Hospital, GRB810,
        55 Fruit St, Boston, MA 02114
        Email: arosenzweig@partners.org

1

BACKGROUND: Atherosclerosis is an inflammatory vascular disease marked by hyperlipidemia and hematopoietic stem cell (HSC) expansion. Activin A, a member of the Activin/GDF/TGFβ/BMP family is broadly expressed and increases in human atherosclerosis, but its functional effects *in vivo* in this context remain unclear.

METHODS: We studied LDLR$^{-/-}$ mice on a Western diet for 12 weeks and used adeno-associated viral vectors with a liver-specific thyroxine binding globulin (TBG) promoter to express Activin A or GFP (control). Atherosclerotic lesions were analyzed by oil red staining. Blood lipid profiling was performed by HPLC (High Performance Liquid Chromatography), and immune cells were evaluated by flow cytometry. Liver RNA-sequencing was performed to explore the underlying mechanisms.

RESULTS: Activin A expression decreased in both livers and aortae from LDLR$^{-/-}$ mice fed a Western diet compared with chow. AAV-TBG-Activin A increased Activin A hepatic expression (~10-fold at 12-weeks, $p<0.0001$) and circulating Activin A levels (~2000pg/ml vs ~50pg/ml, $p<0.001$, compared with controls). Hepatic Activin A expression decreased plasma total and low-density lipoprotein (LDL) cholesterol (~60% and ~40%, respectively), reduced inflammatory cells in aortae and proliferating hematopoietic stem cells (HSC) in bone marrow, and reduced atherosclerotic lesion area in the aortic arch by ~60%. Activin A also attenuated liver steatosis and expression of the lipogenesis genes, Srebp1 and Srebp2. RNA sequencing revealed Activin A not only blocked expression of genes involved in hepatic *de novo* lipogenesis but also fatty acid uptake, and liver inflammation. In addition, Activin A expressed in the liver also reduced white fat tissue accumulation, decreased adipocyte size, and improved glucose tolerance.

CONCLUSIONS: Our studies reveal hepatic Activin A expression reduces inflammation, HSC expansion, liver steatosis, circulating cholesterol, and fat accumulation, which likely all contribute to the observed protection against atherosclerosis. The reduced Activin A observed in LDLR$^{-/-}$ mice on a Western diet appears maladaptive and deleterious for atherogenesis.

Key words: Activin A, atherosclerosis, HSC, inflammation, cholesterol

2

47   **Non-standard Abbreviations and Acronym**

48   TBG-thyroxine binding globulin

49   CVD-cardiovascular diseases

50   LDL-low density lipoprotein

51   VLDL-very low-density lipoprotein

52   oxLDL- (oxidized- LDL)

53   AAV-adeno-associated virus

54   FST-Follistatin

55   HSC- hematopoietic stem cell

56   LtHSC-long-term hematopoietic stem cell

57   StHSC-short-term hematopoietic stem cell

58   MPP-multipotential progenitor cells

59   PMN- polymorphonuclear cells

60   DEG- differentially expressed genes

61   FAs-fatty acids

62

63

64

65

66

67

68

69

70

Introduction

71

72   Cardiovascular diseases (CVD) are the leading cause of death worldwide[1]. Atherosclerosis is a

73   progressive CVD characterized by accumulation of lipid plaques in arterial walls with

74   inflammation and hematopoietic stem cell (HSC) expansion [2, 3], and often driven by increased low

75   density lipoprotein (LDL) cholesterol[4]. Understanding better the mechanisms of atherosclerosis

76   and identifying potential new therapeutic approaches to this condition has important clinical

77   implications.

78   The liver is central to lipid metabolism including involves in uptake, esterification, oxidation,

79   and secretion of fatty acids (FAs) with important downstream effects on systemic inflammation

80   and metabolism. The liver is also responsible for synthesis, uptake, storage, and efflux of

81   cholesterol, essential for multiple biological processes[5-7]. Cholesterol, triglycerides, and other

82   lipids are assembled in the liver into lipoproteins, which are transported to peripheral tissues

83   through the circulation[5, 6]. Hepatic free cholesterol can be esterified, incorporated into very low-

84   density lipoprotein (VLDL) particles, secreted into the bloodstream, further converted into smaller

85   LDL particles, and then oxidized and taken up by macrophages to form foam cells, which promote

86   the formation of atherosclerotic plaques[5, 6]. Thus, hepatic lipid metabolism and cholesterol

87   synthesis are essential for health of the organism but also have potential deleterious effects, such

88   as atherosclerosis.

89   Hepatic sterol regulatory element binding proteins (SREBPs) play important roles in

90   atherosclerosis. These include the transcription factors, Srebp1a, Srebp1c (alternative splicing

91   products of Srebp1, also known as Srebf1), which are involved in synthesis of cholesterol, fatty

92   acids, and triglycerides (TGs), and Srebp2 (Srebf2), which regulates expression of HMG-CoA (3-

93   hydroxy-3-methylglutaryl-CoA) reductase, the target of statins, and the LDL receptor (LDLR)[8].

4

94   Hepatic Srebp1c expression increases atherogenic lipoprotein profiles and accelerates

95   atherosclerosis in LDLR^{-/-} mice [9]. Concordantly, an inhibitor of Srebp processing reduces

96   atherosclerosis[10]. Similarly, GPR146 deficiency reduces hepatic Srebp2 activity and protects

97   against atherosclerosis[11].

98   Activin A is a member of Activin/GDF/TGFβ/BMP protein family. Many members of this

99   family, including Activin A, drive a metabolic switch from an anabolic to catabolic state. Activin

100   A signals through type I (ACTR1B [ALK4], ACTR1C [ALK7] or TGFBR1) and type II

101   (ACVR2A, ACVR2B, and BMPR2) receptors to induce Smad2/3 phosphorylation and nuclear

102   translocation regulating gene expression[12, 13]. Follistatin (FST) is induced by Activin signaling

103   and antagonizes its ACVR receptor binding site[14], forming a negative feed-back loop[15]. Activin

104   A can also form a non-signaling complex with type I (ACVR1A, ALK2) and type II receptors[16-

105   18].

106   The role of Activin A in atherosclerosis is unclear. On one hand, Activin A is expressed in the

107   intima in early atherosclerotic lesions and elevated further in lesions of advanced atherosclerotic

108   patients[19, 20]. Plasma Activin A is increased in angina patients (~500 pg/ml) compared with

109   controls (~300 pg/ml)[19-21]. These observations suggest Activin A could contribute to

110   atherosclerosis progression. On the other hand, *in vitro* studies demonstrate that Activin A inhibits

111   foam cell formation in Thp1 and Raw 264.7 macrophages[22, 23], thought to model an early step in

112   atherogenesis, while FST promotes foam cell formation in Thp1 macrophages[24, 25]. Exogenous

113   Activin A has also been reported to reduce neointimal hyperplasia in a femoral artery cuff murine

114   model *in vivo*[26]. These studies suggest Activin A may be anti-atherogenic and focus on its role in

115   vessel wall constituents. Thus, we lack an understanding of the *in vivo* effects of Activin in

116   atherosclerosis and the role of different sites of expression.

117   Here we find that Activin A is highly expressed in aorta and liver but decreases in both in

118   LDLR$^{-/-}$ mice fed a Western diet compared with those fed chow. Hepatic Activin A expression

119   mediated by an adeno-associated viral vector in LDLR$^{-/-}$ atherosclerotic mice increased circulating

120   Activin A levels (~2000 pg/ml vs ~50pg/ml, p<0.0001) and protected against atherosclerosis by

121   reducing cholesterol, decreasing inflammation, and reducing proliferating HSCs. Mechanistically,

122   hepatic Activin A reduced liver Srebp1/Srebp2 expression, pathways involved in hepatic

123   lipogenesis and fatty acid uptake, as well as liver steatosis. In addition, hepatic Activin A reduced

124   body fat accumulation and improved glucose tolerance. We conclude that the reduction in hepatic

125   Activin A observed on a Western diet is maladaptive and could represent an evolutionary vestige

126   perhaps valuable in calorie-poor environment but potentially maladaptive amidst caloric

127   abundance. Whether AAV8-TBG-Activin A has therapeutic potential in atherosclerosis and

128   related metabolic diseases may warrant further investigation.

129

130   **Methods**

131   The data, methods, and study materials used to conduct the research will be available from the

132   corresponding author upon reasonable request.

133   **Animal Studies**

134   All mouse experiments were performed in accordance with the NIH Guide for the Care and Use

135   of Laboratory Animals and approved by the Institutional Animal Care and Use Committee

136   (IACUC) of Massachusetts General Hospital.

137   For the atherosclerosis model, 8-week-old male LDLR$^{-/-}$ (#002207, body weight ~24-25g)

138   obtained from Jackson Laboratory were injected via tail vein with AAV8-TBG-GFP or AAV8-

6

139   TBG-Activin A viruses at 2×10^{10} genome copies/mouse. After 1 week, mice were fed a high

140   cholesterol Western diet (Envigo Teklad, TD.96121) or standard chow diet for 12 weeks. Mice

141   were fasted overnight before euthanasia.

142   A detailed description of the methods and supporting data are available in the Online

143   Supplemental materials.

144   **Statistical Analysis**

145   Data are expressed as mean±SEM and analyzed using GraphPad Prism 8 (GraphPad Software)

146   by unpaired Student t-tests. $p < 0.05$ was considered significant. $*p < 0.05$, $**p < 0.005$, and

147     $***p < 0.001$.

148

149   **Results**

150   **Activin A mRNA decreases in aorta and liver in LDLR$^{-/-}$ mice fed a Western diet**

151   We examined plasma Activin A levels as well as the expression of the Activin A transcript in

152   liver and aorta, and how these were affected by hyperlipidemia. Plasma Activin A levels were

153   increased in LDLR$^{-/-}$ mice fed a Western diet compared with chow (Figure 1A) while plasma

154   Follistatin (FST) levels were similarly undetectable in all mice (data not shown). Baseline Activin

155   A mRNA levels measured by QPCR were high in aorta and liver relative to other organs tested

156   (Figure S1A). Surprisingly, given previous reports of increased Activin A expression in

157   atherosclerosis[19-21], Activin A mRNA levels decreased in both aortae and livers from LDLR$^{-/-}$ mice

158   fed a Western diet compared with chow (Figure 1B-1C). In contrast, FST was expressed most

159   highly in muscle and aorta, and at lower levels in liver (Figure S1B) and was unaffected by diet

160   (Figure 1B-1C). Consistent with these observations, we found that oxidized LDL (oxLDL)

161 decreased Activin A but not FST mRNA in primary peritoneal macrophages and the Hepa 1-6

162 hepatic carcinoma cell line (Figure 1D-1E). Together these observations suggest the Activin

163 pathway is suppressed in the liver and aorta, and this may be a direct effect of oxLDL.

Activin A protects against atherosclerosis *in vivo*

165 We generated AAV8 vectors using the TBG promoter to drive liver-specific[27, 32-34] expression

166 of Activin or GFP. We confirmed TBG-driven Activin A mRNA expression in HepG2 cells and

167 protein in the media, as well as induction of Smad2/3 phosphorylation that was blocked by FST-

168 315, the circulating isoform of FST (Figure S2A). Similar to recombinant Activin A protein[24, 25],

169 Activin A from HepG2 conditioned media also reduced oxLDL uptake by peritoneal macrophages

170 *in vitro* (Figure 2A-2B). Thus, the AAV8-TBG-Activin A vector effectively induces Activin A

171 expression and functional protein secretion.

172 *In vivo* studies confirmed that TBG-driven GFP expression was specific to the liver (Figure

173 S2B) and TBG also mediated hepatic Activin A expression that induced Smad2/3 phosphorylation,

174 and increased circulating plasma Activin A (Figure S2C-S2E). LDLR$^{-/-}$ mice were injected with

175 AAV8-TBG-GFP (control) or AAV8-TBG-Activin A, and then fed a western diet for 12 weeks to

176 induce early-stage atherosclerosis (Figure 2C). Hepatic Activin A expression induced Smad2/3

177 phosphorylation and expression of the downstream target gene FST in the liver but not aorta

178 (Figure 2D-2F and S2F). Notably, Activin receptors, ACVR1b and ACVR2b, dramatically

179 decreased and ACVR2a showed a nonsignificant trend toward decreasing in Activin-expressing

180 mice compared with controls (Figure S2G). Plasma Activin A increased in AAV8-TBG-Activin

181 A treated mice compared to controls (Figure 2G). Of note, plasma Activin A levels in control mice

182 fed the Western diet were higher than chow diet (Figure 1A). However, plasma Activin A levels

183 seen in Activin A-expressing mice fed a Western diet were even higher (Figure 2G). The molecular

8

184 weight of the FST band detected in the liver (<35kd) was smaller than FST315 (>35kd), suggesting

185 it may correspond to the FST288 isoform, which is limited to tissue and not secreted into the

186 circulation. Indeed, plasma FST levels remained at background levels (data not shown), suggesting

187 the ratio of plasma Activin A/FST was increased in the Activin A expressing mice compared with

188 controls. Surprisingly, Activin A did not induce liver injury and plasma ALT (or SGPT for

189 Serum Glutamic-Pyruvic Transaminase) decreased in Activin-expressing mice compared with

190 controls (Figure 2H).

191 Oil red staining demonstrated that Activin A expression dramatically decreased atherosclerotic

192 plaques in the aortic arch by ~60% (Figure 2I-2J). In addition, flow cytometry revealed that Ly-

193 6C[high] monocytes, F4/80+ macrophages and Ly-6G+ neutrophils were all substantially decreased

194 in aortae from Activin A-expressing mice compared with controls (Figure 2K-2L), suggesting both

195 lesion size and inflammation were reduced by Activin A. Hepatic Activin A expression

196 dramatically decreased plasma total cholesterol (>50%), LDL (~40%), and triglycerides (>80%)

197 (Figure 2M-2O), without altering HDL (Figure 2P); thus, reducing the LDL/HDL ratio (Figure

198 2Q). Taken together, hepatic Activin A protected against atherosclerosis *in vivo* and this was

199 associated with a substantial decline in circulating atherogenic lipid levels (LDL-C and TGs)

200 without a change in HDL or increased liver injury.

201 We next used flow cytometry to investigate the effect of Activin A on HSCs. Activin A

202 decreased the absolute number of bone marrow LSK (Lin-CD45+Sca1+c-Kit+) cells, the

203 population that includes HSCs[35], by ~60% (Figure S2H-S2I). In the LSK population, long-term

204 HSCs (LtHSC, CD135-/CD150+/CD48- LSK(Lin-Sca1+c-Kit+)) differentiate into short-term

205 HSCs (StHSC, CD135-/CD150-/CD48-LSK) and then multipotent progenitor cells (MPPS)

206 including MPP2 (CD135-/CD150+/CD48+LSK), MPP3 (CD135-/CD150-/CD48+ LSK) and

9

207   MPP4 (CD135+/CD150-/CD48+/- LSK)[36]. There was an >80% decrease in LtHSC and StHSC

208   and ~60% decrease in MPP2, MPP3 and MPP4 cells in atherosclerotic mice expressing Activin A

209   compared with controls (Figure S2H-S2I).

210   To determine whether the decrease in LSK cells is due to an effect on HSC proliferation, we

211   assessed BrdU incorporation by flow cytometry. LSK cells were dramatically decreased overall

212   and BrdU$^+$ LSK cells were reduced ~60% in Activin A-expressing atherosclerotic mice versus

213   controls (Figure 2R-2S). Thus, Activin A decreased the actively proliferating and the total number

214   of HSC in atherosclerotic mice.

215   **Activin A decreases liver steatosis and inflammation in atherosclerotic mice**

216   We next investigated the effects of AAV8-TBG-Activin A on the liver itself. The accumulation

217   of excessive lipid droplets is a pathological change known as hepatic steatosis[7]. H&E and oil red

218   staining revealed accumulation of much smaller lipid droplets in Activin A-expressing livers

219   compared with controls (Figure 3A-3B). The accumulation of free cholesterol contributes to liver

220   injury, ER stress, mitochondrial dysfunction, fibrosis inflammation, as well as cholesterol

221   crystallization in lipid droplets and consequent hepatocyte cell death[37-40]. Activin A expression

222   decreased hepatic total and free cholesterol by 30% without changing triglycerides, a common and

223   "safe storage" for fatty acids (Figure 3C-3E). In addition, QPCR demonstrated that Activin A

224   expression reduced expression of Srebp1 and Srebp2 (Figure 3F), suggesting that Activin A could

225   inhibit hepatic lipogenesis. Picrosirius Red staining (collagen I and III) demonstrated that fibrosis

226   was primarily located in lipid droplet-rich regions in control livers but around veins in Activin A

227   expressing livers (Figure 3A). There was no difference in overall fibrotic area in the two groups

228   (Figure 3H). Although the Activin A pathway was activated, as indicated by increased FST protein

229   and Smad2/3 phosphorylation (Figure 2E-2F), fibrosis marker genes Col1a1, Col1a2, Col3a1, and

230 MMP12 were significantly decreased (Figure 3G). In addition, there was a trend toward decreased

231 expression of the hepatic stellate cell marker gene, Acta2, in Activin A expressing livers compared

232 with controls (Figure 3G). Immunohistochemical staining for CD45 showed that inflammatory

233 cells were dramatically decreased in Activin A-expressing livers compared with control (Figure

234 3A and 3I). Furthermore, mRNA levels of inflammatory marker genes TNFα and F4/80 were

235 decreased in Activin A-expressing liver versus controls (Figure 3J). Collectively, these findings

236 suggest that Activin A decreased liver steatosis and inflammation without inducing fibrosis in

237 atherosclerotic mice.

238 **Activin A reduced expression of pathways involved in fatty acid uptake, *de novo* lipogenesis**

239 **and lipid associated macrophage recruitment**

240 To identify the mechanisms responsible for the effects of hepatic Activin A, we performed

241 RNA-sequencing in Activin A-expressing compared with control liver samples. Differential

242 expression analysis confirmed upregulation of Activin A (also known as Inhba) and its target gene

243 FST in Activin A-expressing mice compared with controls (Figure 4A). Overrepresentation

244 analysis of the differentially expressed genes (DEG) identified "Fatty acid metabolic process"

245 ranks 1 among the GO Biological Process and "lipid and atherosclerosis" ranks 8 among the

246 KEGG pathways, and (Figure 4B and S3A), likely contributing to the decreased lipid accumulation

247 seen in Activin A expressing livers.

248 A heatmap generated based on the DEG list from the "fatty acid metabolic process" GO term

249 and "lipid and atherosclerosis" KEGG pathway revealed decreases in multiple genes related to *de*

250 *novo* lipogenesis and fatty acid uptake in Activin A-expressing livers (Figure 4C). Srebp1 is a

251 pivotal transcription factor for triglyceride metabolism and *de novo* lipogenesis, which directly

252 activates the key desaturases for the biosynthesis of monounsaturated fatty acids (MUFA) and

11

253 polyunsaturated fatty acid (PUFAs), including Scd1(stearoyl-CoA desaturase 1, primarily in liver),

254 Scd2 (stearoyl-CoA desaturase 2), and fatty acid desaturase (Fads2) expression[41-43]. By QPCR, we

255 verified that Scd1/2 and Fads2 were significantly decreased in Activin A-expressing livers

256 compared with controls (Figure 4D). FABPs are essential for intracellular binding and transport of

257 fatty acids, as well as cholesterol and phospholipid metabolism[44]. Fabp1 is the major hepatic

258 FABP, and its deficiency attenuates both diet-induced hepatic steatosis and fibrogenesis[45]. mRNA

259 levels of Fabp1, Fabp2, Fabp5, and the atherogenic scavenger receptor and fatty acid transporter,

260 CD36[46, 47], were all decreased in Activin A-expressing liver compared with controls (Figure 4E),

261 as were the corresponding protein levels (Figure 4G-4H). Consistently, Activin A also inhibits

262 Srebp1, Srebp2, Fabp5 in Hepa 1-6 cells with oxLDL treatment *in vitro*. (Figure S3B). Oleic acid

263 (OA, a monounsaturated omega-9 fatty acid) is a potent inducer of triglyceride synthesis and

264 storage. Activin A reduced OA-induced -fatty acid uptake in Hepa 1-6 cells *in vitro*, while this

265 effect was rescued with a neutralizing Activin A antibody (Figure S3C-S3D).   Multiple

266 differentially-regulated genes have been reported to play roles in lipid metabolism or to promote

267 atherosclerosis, such as GDF15[48, 49], IRF7[50], Cyba[51], Cybb[52], Fas[53, 54], Vcam1[55] and TLR2[56]

268 (Figure 4C). Expression of these genes was dramatically reduced in Activin A-expressing liver

269 (Figure 4F). Thus, Activin A likely blocks both *de novo* lipogenesis and fatty acid uptake to protect

270 against liver steatosis, free cholesterol accumulation, and associated atherosclerosis (Figure 6F).

271   A recent report showed that adenoviral expression of the glycoprotein NMB (Gpnmb, also

272 known as DC-HIL) in hepatocytes promotes lipogenesis in white adipose tissue (WAT) and

273 exacerbates obesity and insulin resistance[57]. Gpnmb is also a core component of Trem2^{high}CD9+

274 lipid associated- macrophages (LAM) in the liver and is induced in trans-fat containing AMLN

275 (amylin liver non-alcoholic steatohepatitis, NASH) diet-induced nonalcoholic steatohepatitis

276   (NASH) liver[58], while Elafibranor, an agonist of PPARα and PPARδ, reverses NASH and

277   dramatically decreases Gpnmb protein levels, as well as Trem2, CD9, and Gpnmb mRNA levels

278   in mice[58] Interestingly, hepatic Gpnmb mRNA was decreased in Activin A-expressing mice as

279   (Figure 4A). Interestingly, we found that hepatic Gpnmb mRNA and protein expression were

280   induced by the Western diet (Figure S3E-S3F) and dramatically decreased in Activin A-expressing

281   mice (Figure 4A, 4G-4H, 4K). Activin A also attenuated Gpnmb expression and secretion in Hepa

282   1-6 cells *in vitro* (Figure S3G), suggesting this is a direct effect. Immunofluorescent staining

283   showed that Gpnmb was colocalized with CD45+ and F4/80+ but not hepatocytes, confirming

284   Gpnmb expression in a macrophage lineage (Figure 4I-4J). Notably, our RNA-seq data also

285   indicated downregulation of Trem2 (Figure 5A). We next focused on LAM. The LAM marker

286   genes Trem2 and CD9 are also decreased in Activin A-expressing livers compared with controls

287   (Figure 4K). Moreover, we confirmed that Activin A expression led to a >3-fold decrease in LAM

288   Trem2^{high} CD9+ populations in the liver by flow cytometry (Figure 4L-4M). Together, these data

289   suggest that Activin A expression decreased genes involved in fatty acid uptake, *de novo*

290   lipogenesis and lipid- associated- macrophage recruitment in atherosclerotic liver, and thus

291   decreased liver steatosis.

292   **Activin A decreased inflammatory gene expression**

293   We next explored how Activin A protects against inflammation. In overrepresentation analysis

294   of DEG, "cytokine-cytokine receptor interaction" ranked 1 and "chemokine signaling pathway"

295   ranked 4 among the KEGG pathways, and "cytokine-mediated signal pathway" ranked 2,

296   "phagocytosis" ranked 5, "myeloid cell differentiation" ranked 10, and "leukocytes migration"

297   ranked 11 among the GO Biological Process (Figure 4B and S3A). In addition, a heatmap

298   generated for the DEG list from those pathways revealed downregulation of many genes involved

13

299   in leukocyte migration (GO) and cytokine-cytokine receptor interactions, such as

300   monocyte/macrophage trafficking/migration chemokines CCL2 (also known as MCP1), CCL3,

301   CCL4, CCL5, CCL6, CCL9[59], macrophage markers CD68, CCR2 (the receptor of CCL2) and

302   Lgals3, monocytes marker Ly-6C2, hepatic steatosis/ NASH associated lymphocyte antigen

303   Ly6D, monocyte/macrophage homeostasis chemokine CXCL16, neutrophil trafficking chemokine

304   CXCL1, as well as other pro-inflammatory chemokines CXCL9, CXCL10 and CXCL11 (Figure

305   5A). Meanwhile, liver hematopoietic lineage markers, including CD44, CD48, Fcgr1 and Csf3r

306   were decreased in Activin A-expressing mice compared with control (Figure 5A). Next, we

307   confirmed by QPCR that the mRNA levels of all these genes were decreased in Activin A-

308   expressing livers compared with controls (Figure 5B-5C). We performed flow cytometry on liver

309   samples and found that Activin A expression leads to a ~50% decrease in F4/80+ macrophages,

310   ~60% decrease in Ly-6C^{high} monocytes, >50% decrease in PMN (polymorphonuclear cells), PMO

311   (patrolling monocytes), as well as MDM (monocytes-derived macrophages) (Figure 5D-5G). In

312   addition, eosinophils were also decreased in Activin A-expressing livers (Figure 5E, 5G).

313   However, MM (mature macrophages or Kupffer cells) did not significantly change (Figure 5E,

314   5G). These data suggest that Activin A expression decreased liver inflammation and recruitment

315   of inflammatory monocytes/macrophages and PMNs to the liver.

316   **Activin A inhibits fat accumulation and improves glucose tolerance**

317   Cardiovascular diseases, including atherosclerosis, are highly correlated with obesity and

318   diabetes[60], and Activin A regulated genes Scd1 may also play crucial roles in obesity and diabetes.

319   This led us to probe whether Activin A affects WAT lipogenesis and glucose tolerance. H&E

320   staining showed that Activin A led to ~2-fold decrease in epididymal, and inguinal WAT (Figure

321   6A-6B). In addition, adipocyte size was dramatically decreased in epididymal and inguinal WAT)

14

322   by hepatic Activin A expression liver compared with controls (Figure 6A, 6C). Moreover, fasting

323   glucose levels were substantially decreased by hepatic Activin A expression compared with

324   controls (Figure 6D). Furthermore, Activin A expression in liver resulted in improved glucose

325   tolerance (Figure 6E). Overall, these data demonstrate that hepatic Activin A inhibited fat

326   accumulation and improved glucose tolerance in atherosclerotic mice.

327

Discussion

329   Here we report that Activin A exogenously expressed in the liver protects against

330   atherosclerosis. AAV8-TBG-Activin A treatment resulted in a ~40% decrease in plasma LDL

331   cholesterol, ~50% decrease in plasma total cholesterol, ~60% decrease in atherosclerotic lesions

332   in the aortic arch, and fewer inflammatory cells infiltrating aortas and proliferating HSC in bone

333   marrow. Hepatic Activin A not only reduced liver steatosis by inhibiting Srebp1 and Srebp2

334   expression and hepatic *de novo* lipogenesis, as well as exogenous fatty acid uptake, but also

335   alleviated liver inflammation and reduced monocytes, macrophages, and neutrophil infiltration. In

336   addition, Activin A expression in liver also reduced white fat tissue accumulation and adipocyte

337   size as well as improving glucose tolerance. Taken together these data suggest the reduction in

338   hepatic Activin A observed in mice on the Western diet is maladaptive and demonstrate that

339   hepatic Activin A expression has a host of favorable effects in this context that culminate in

340   reduced atherosclerosis and hepatic steatosis, phenotypes that cause significant morbidity and

341   mortality throughout the world.

342   Increased Activin A has previously been reported in vascular lesions and plasma of patients

343   with atherosclerosis[19], but whether it contributed to disease pathogenesis or played a compensatory

344   protective role has not been firmly established. While plasma Activin A was indeed increased in

345 hyperlipidemic mice in our study, we observed a decrease in Activin A expression in the aorta and

346 liver of these animals. Together with the finding that increasing Activin A in the liver and

347 circulation attenuates the development of atherosclerosis, these observations suggest that the

348 increase in Activin A reported in patient plasma and lesions may reflect a compensatory change.

349 While previous studies have identified anti-atherosclerotic effects of Activin A signaling on

350 cellular processes within the vessel wall such as foam cell formation and vascular smooth muscle

351 cell differentiation *in vitro*, our data suggest that important primary contributors to Activin's anti-

352 atherosclerotic effects in the LDLR$^{-/-}$ murine model lie outside the vessel. In line with the dramatic

353 decrease in plasma LDL and total cholesterol observed in Activin A-treated mice, our liver RNA-

354 seq studies were consistent with anti-atherogenic changes in liver lipid metabolism, and reduced

355 Srebp1 and Srebp2. Subsequent validation studies confirmed downregulation of key transcription

356 factors and enzymes involved in cholesterol and triglyceride metabolism and fatty acid uptake

357 (Srebp1 and Srebp2, Scd1/ Scd2, Fads2, Fabp1 and CD36) (Figure 6F), and reduced liver total

358 cholesterol and free cholesterol. The reduction in cholesterol synthesis and fatty acid uptake likely

359 contribute to the reduced inflammation seen in both the aorta and liver. Consistently, knockout of

360 fatty acid desaturase (Fads2) reduces cholesterol and triglycerides and protects against Western

361 diet-induced atherosclerosis[61]. In addition, bone marrow transplantation of HSC from Fabp5

362 deficiency mice protects against atherosclerosis in LDLR$^{-/-}$ mice[62]. Here, Activin A had other

363 beneficial effects including a reduction in proliferating HSCs, reduction in WAT, and improved

364 glucose tolerance. Some of these effects may be secondary to the observed reduction in total and

365 LDL cholesterol but other primary mechanisms may also be contributing and will be of interest

366 for future studies.

16

367   In broad terms, many effects of Activin A (and other GDF/BMP proteins) can be seen as

368   toggling tissues between catabolic and anabolic states. Throughout evolution in calorie scarce

369   environments, downregulation of Activin may have been an adaptive response that conserved

370   energy and activated useful anabolic processes. However, in calorie rich settings, exemplified

371   here by the Western diet, the downregulation of Activin A appears maladaptive, promoting

372   atherogenesis and hepatic steatosis, major causes of morbidity and mortality in the modern world.

373   Other members of the Activin/GDF/TGFβ/BMP family have also been reported to have roles

374   in atherosclerosis. GDF11, another ligand for ACVR receptors, protected against atherosclerosis

375   in ApoE^{-/-} mice by ameliorating inflammation and endothelial cell injury[63]. Knockout of the

376   TGFβ receptor, TGFβRII, in dendritic cells, smooth muscle cells and T cell was reported to

377   accelerate atherosclerosis while knockout of TGFβRII in endothelial cells protected against

378   atherosclerosis[64-67]. Neutralization of TGFβ with an antibody (anti–hTGF-β1, -β2, -β3 2G7

379   monoclonal) increased atherosclerosis and inflammation[68]. Interestingly, TGFβ1 was also reported

380   to induce HSC quiescence and cell cycle arrest by upregulation of p57, a member of the cyclin-

381   dependent kinase inhibitor family[69, 70]. Bone marrow transplantation studies showed that the

382   hematopoietic stem/progenitor population was reduced in bone marrow from TGFβ1KO pups and

383   bone marrow reconstitution was impaired compared with TGFβ1^{+/+} pups [71]. However, TGFβ1

384   receptor I (TGFβRI) deficient mice showed normal HSC self-renewal and regeneration ability[72].

385   This is partially because TGFβ2, another isoform of TGFβ, supports LSK cell proliferation[73]. It

386   will be interesting to probe the role of Activin A, its receptors, as well as other ligands in Activin

387   pathway in HSC self-renewal, regeneration, expansion, and atherosclerosis.

388   Interestingly, we did not see pro-fibrotic or pro-apoptotic effects of hepatic Activin A

389   expressions despite prior reports of such effects with other members of this protein family. Rather,

17

390 we instead saw a robust decrease in fibrosis markers and no alteration in apoptosis in the liver

391 (data not shown) in hyperlipidemic mice treated with Activin A. Unlike the classic TGFβ signaling

392 pathways, Activin A is regulated by a negative feedback loop. FST is not only a direct target but

393 also a strong antagonist of these pathway. In addition, Activin A also forms a non-signaling

394 complex with the type I receptors (ACVR1a, ALK2) and type II receptor. These self-limiting

395 mechanisms may preserve homeostasis and prevent overactivation of the Activin A pathway. The

396 expression level of Activin A is likely a key determinant of whether these self-limiting pathways

397 are activated or not, with beneficial or harmful consequences for the liver. Here we delivered a

398 very low dosage of AAV8-TBG-Activin A (2×10^{10} genome copies/mice, ~2000 pg/ml in plasma,

399 compared to the common dose of $1-2 \times 10^{11}$ genome copies/mouse[32-34]). This level of expression

400 activated downstream signaling, as indicated by an increase in phosphorylation of Smad2/3, as

401 well as increased FST. The Activin A receptors ACVR1b, ACVR2a, and ACVR2b were also

402 decreased or showed a nonsignificant trend toward decrease. Interestingly, levels of the liver injury

403 marker plasma ALT greatly decreased in Activin A expressing liver compared with control,

404 suggesting a low dosage of AAV8-TBG-Activin A may be beneficial for the liver. This is

405 consistent with a previous study showing low-dose AAV2/8-smad3 (2×10^{9} genome copies/mice)

406 attenuated aortic atherosclerosis without increasing fibrosis[74].

407 The beneficial effects of hepatic Activin A expression raise the intriguing possibility that

408 Activin A expression could have therapeutic potential. However, reasonable concerns could be

409 raised about this approach. We have previously shown that Activin A levels increase with age in

410 humans and contribute to multiple models of cardiac dysfunction and heart failure[75]. Activin A

411 was sufficient to cause cardiac dysfunction in healthy mice but importantly, that was at higher

412 concentrations of Activin A (~10,000 pg/ml) by adenovirus delivery without special diet[75]. Here,

18

413 the levels of Activin expression used were lower and echocardiography did not reveal alterations

414 in cardiac function (data not shown). Still there could be legitimate concerns about therapeutic

415 windows particularly given the challenges of fine-tuning concentrations achieved with gene

416 therapy approaches. Finally, while the growing number of treatments available for primary and

417 secondary prevention of atherosclerosis may undermine interest in that setting, no drugs have been

418 approached for hepatic steatosis or the closely related non-alcoholic liver disease suggesting there

419 may be greater enthusiasm for evaluation of Activin A's ability to address this important unmet

420 clinical need.

421 Activin A also reduced WAT and improved glucose tolerance, suggesting a potential beneficial

422 role for Activin A in obesity and diabetes, similar with hepatic Activin E[76]. However, this may

423 seem paradoxical given that *deletion* of a closely related protein, myostatin (GDF8) has been

424 shown to reduce obesity and protect against diabetes in mice[77]. It seems possible that deletion and

425 overexpression could culminate in similar phenotypes through different mechanisms. For example,

426 the dramatic skeletal muscle growth seen in myostatin knockout mice likely mediates multiple

427 metabolic benefits while the catabolic state induced by overexpression of Activin A may reduce

428 fat storage and insulin resistance. In this context, it would be interesting in future studies to

429 examine the effects of genetic or pharmacological inhibition of these pathways in atherosclerosis

430 as well.

431 In summary, the data presented here reveal for the first time that Activin A expressed in the

432 liver protects against atherosclerosis, as well as liver steatosis and inflammation, reduces fat

433 accumulation and improves glucose tolerance. These findings have implications for our

434 understanding of the role of this pathway and the regulation of processes central to atherogenesis,

435 while suggesting the therapeutic potential of moderate hepatic Activin A expression in

436 atherosclerotic vascular disease and hepatic disorders, such as obesity and non-alcoholic liver

437 disease, warrants further investigation.

438

452

453 **Disclosures:** The authors declare that they have no conflicts of interest with the contents of this

454 article.

455

456 **Supplemental Material**

457 Supplemental Materials and Methods

458 Supplemental Table 1

459 Supplemental Figure S1, S2, and S3

460   Supplemental References 29-31

461

462

References

1. <Hannah Ritchie and Max Roser (2018) - "Causes of Death". Published online at OurWorldInData.org. Retrieved from: 'https://ourworldindata.org/causes-of-death' [Online Resource].

2. Gistera A, Hansson GK. The immunology of atherosclerosis. *Nat Rev Nephrol*. 2017;13:368-380

3. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, et al. Ly-6chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. *J Clin Invest*. 2007;117:195-205

4. Shiomi M, Ito T, Tsukada T, Yata T, Watanabe Y, Tsujita Y, et al. Reduction of serum cholesterol levels alters lesional composition of atherosclerotic plaques. Effect of pravastatin sodium on atherosclerosis in mature whhl rabbits. *Arterioscler Thromb Vasc Biol*. 1995;15:1938-1944

5. Abe RJ, Abe JI, Nguyen MTH, Olmsted-Davis EA, Mamun A, Banerjee P, et al. Free cholesterol bioavailability and atherosclerosis. *Curr Atheroscler Rep*. 2022;24:323-336

6. Li H, Yu XH, Ou X, Ouyang XP, Tang CK. Hepatic cholesterol transport and its role in non-alcoholic fatty liver disease and atherosclerosis. *Prog Lipid Res*. 2021;83:101109

7. Gluchowski NL, Becuwe M, Walther TC, Farese RV, Jr. Lipid droplets and liver disease: From basic biology to clinical implications. *Nat Rev Gastroenterol Hepatol*. 2017;14:343-355

8. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest*. 1998;101:2331-2339

9. Karasawa T, Takahashi A, Saito R, Sekiya M, Igarashi M, Iwasaki H, et al. Sterol regulatory element-binding protein-1 determines plasma remnant lipoproteins and accelerates atherosclerosis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2011;31:1788-1795

10. Tang JJ, Li JG, Qi W, Qiu WW, Li PS, Li BL, et al. Inhibition of srebp by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques. *Cell Metab*. 2011;13:44-56

11. Yu H, Rimbert A, Palmer AE, Toyohara T, Xia Y, Xia F, et al. Gpr146 deficiency protects against hypercholesterolemia and atherosclerosis. *Cell*. 2019;179:1276-1288 e1214

12. Morianos I, Papadopoulou G, Semitekolou M, Xanthou G. Activin-a in the regulation of immunity in health and disease. *J Autoimmun*. 2019;104:102314

13. Yadin D, Knaus P, Mueller TD. Structural insights into bmp receptors: Specificity, activation and inhibition. *Cytokine Growth Factor Rev*. 2016;27:13-34

14. Thompson TB, Lerch TF, Cook RW, Woodruff TK, Jardetzky TS. The structure of the follistatin:Activin complex reveals antagonism of both type i and type ii receptor binding. *Dev Cell*. 2005;9:535-543

15. Blount AL, Vaughan JM, Vale WW, Bilezikjian LM. A smad-binding element in intron 1 participates in activin-dependent regulation of the follistatin gene. *J Biol Chem*. 2008;283:7016-7026

16. Aykul S, Corpina RA, Goebel EJ, Cunanan CJ, Dimitriou A, Kim HJ, et al. Activin a forms a non-signaling complex with acvr1 and type ii activin/bmp receptors via its finger 2 tip loop. *Elife*. 2020;9

17. Macias-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL. Specific activation of smad1 signaling pathways by the bmp7 type i receptor, alk2. *J Biol Chem*. 1998;273:25628-25636

508   18.   Hatsell SJ, Idone V, Wolken DM, Huang L, Kim HJ, Wang L, et al. Acvr1r206h receptor mutation
509   causes fibrodysplasia ossificans progressiva by imparting responsiveness to activin a. *Sci Transl*
510   *Med*. 2015;7:303ra137
511   19.   Engelse MA, Neele JM, van Achterberg TA, van Aken BE, van Schaik RH, Pannekoek H, et al.
512   Human activin-a is expressed in the atherosclerotic lesion and promotes the contractile
513   phenotype of smooth muscle cells. *Circ Res*. 1999;85:931-939
514   20.   Oklu R, Hesketh R, Wicky S, Metcalfe J. Tgfbeta/activin signaling pathway activation in intimal
515   hyperplasia and atherosclerosis. *Diagn Interv Radiol*. 2011;17:290-296
516   21.   Smith C, Yndestad A, Halvorsen B, Ueland T, Waehre T, Otterdal K, et al. Potential anti-
517   inflammatory role of activin a in acute coronary syndromes. *J Am Coll Cardiol*. 2004;44:369-375
518   22.   Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell*. 2011;145:341-355
519   23.   Kojima Y, Weissman IL, Leeper NJ. The role of efferocytosis in atherosclerosis. *Circulation*.
520   2017;135:476-489
521   24.   Kozaki K, Akishita M, Eto M, Yoshizumi M, Toba K, Inoue S, et al. Role of activin-a and follistatin
522   in foam cell formation of thp-1 macrophages. *Arterioscler Thromb Vasc Biol*. 1997;17:2389-2394
523   25.   Wang H, Zhang P, Chen X, Liu W, Fu Z, Liu M. Activin a inhibits foam cell formation and up-
524   regulates abca1 and abcg1 expression through alk4-smad signaling pathway in raw 264.7
525   macrophages. *Steroids*. 2021;174:108887
526   26.   Engelse MA, Lardenoye JH, Neele JM, Grimbergen JM, De Vries MR, Lamfers ML, et al.
527   Adenoviral activin a expression prevents intimal hyperplasia in human and murine blood vessels
528   by maintaining the contractile smooth muscle cell phenotype. *Circ Res*. 2002;90:1128-1134
529   27.   Yan Z, Yan H, Ou H. Human thyroxine binding globulin (tbg) promoter directs efficient and
530   sustaining transgene expression in liver-specific pattern. *Gene*. 2012;506:289-294
531   28.   Kimura T, Ferran B, Tsukahara Y, Shang Q, Desai S, Fedoce A, et al. Production of adeno-
532   associated virus vectors for in vitro and in vivo applications. *Sci Rep*. 2019;9:13601
533   29.   Wang X, Xu BL, Chen XW. Acute gene inactivation in the adult mouse liver using the crispr-cas9
534   technology. *STAR Protoc*. 2021;2:100611
535   30.   McAlpine CS, Kiss MG, Rattik S, He S, Vassalli A, Valet C, et al. Sleep modulates haematopoiesis
536   and protects against atherosclerosis. *Nature*. 2019;566:383-387
537   31.   Stijlemans B, Sparkes A, Abels C, Keirsse J, Brys L, Elkrim Y, et al. Murine liver myeloid cell
538   isolation protocol. *Bio-protocol*. 2015;5:e1471
539   32.   Zhao H, Li Y, He L, Pu W, Yu W, Li Y, et al. In vivo aav-crispr/cas9-mediated gene editing
540   ameliorates atherosclerosis in familial hypercholesterolemia. *Circulation*. 2020;141:67-79
541   33.   Wang X, Cai B, Yang X, Sonubi OO, Zheng Z, Ramakrishnan R, et al. Cholesterol stabilizes taz in
542   hepatocytes to promote experimental non-alcoholic steatohepatitis. *Cell Metab*. 2020;31:969-
543   986 e967
544   34.   Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, et al. In vivo genome editing using
545   staphylococcus aureus cas9. *Nature*. 2015;520:186-191
546   35.   King KY, Goodell MA. Inflammatory modulation of hscs: Viewing the hsc as a foundation for the
547   immune response. *Nat Rev Immunol*. 2011;11:685-692
548   36.   Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic
549   stem cells. *Nat Rev Mol Cell Biol*. 2011;12:643-655
550   37.   Teratani T, Tomita K, Suzuki T, Furuhashi H, Irie R, Hida S, et al. Free cholesterol accumulation in
551   liver sinusoidal endothelial cells exacerbates acetaminophen hepatotoxicity via tlr9 signaling. *J*
552   *Hepatol*. 2017;67:780-790
553   38.   Teratani T, Tomita K, Suzuki T, Oshikawa T, Yokoyama H, Shimamura K, et al. A high-cholesterol
554   diet exacerbates liver fibrosis in mice via accumulation of free cholesterol in hepatic stellate
555   cells. *Gastroenterology*. 2012;142:152-164 e110

556 39. Ioannou GN. The role of cholesterol in the pathogenesis of nash. *Trends Endocrinol Metab*.
557   2016;27:84-95
558 40. Horn CL, Morales AL, Savard C, Farrell GC, Ioannou GN. Role of cholesterol-associated
559   steatohepatitis in the development of nash. *Hepatol Commun*. 2022;6:12-35
560 41. Iizuka K, Takao K, Yabe D. Chrebp-mediated regulation of lipid metabolism: Involvement of the
561   gut microbiota, liver, and adipose tissue. *Front Endocrinol (Lausanne)*. 2020;11:587189
562 42. Nakamura MT, Nara TY. Structure, function, and dietary regulation of delta6, delta5, and delta9
563   desaturases. *Annu Rev Nutr*. 2004;24:345-376
564 43. Tabor DE, Kim JB, Spiegelman BM, Edwards PA. Identification of conserved cis-elements and
565   transcription factors required for sterol-regulated transcription of stearoyl-coa desaturase 1 and
566   2. *J Biol Chem*. 1999;274:20603-20610
567 44. Li B, Hao J, Zeng J, Sauter ER. Snapshot: Fabp functions. *Cell*. 2020;182:1066-1066 e1061
568 45. Chen A, Tang Y, Davis V, Hsu FF, Kennedy SM, Song H, et al. Liver fatty acid binding protein (l-
569   fabp) modulates murine stellate cell activation and diet-induced nonalcoholic fatty liver disease.
570   *Hepatology*. 2013;57:2202-2212
571 46. Febbraio M, Guy E, Silverstein RL. Stem cell transplantation reveals that absence of macrophage
572   cd36 is protective against atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2004;24:2333-2338
573 47. Hajri T, Han XX, Bonen A, Abumrad NA. Defective fatty acid uptake modulates insulin
574   responsiveness and metabolic responses to diet in cd36-null mice. *J Clin Invest*. 2002;109:1381-
575   1389
576 48. de Jager SC, Bermudez B, Bot I, Koenen RR, Bot M, Kavelaars A, et al. Growth differentiation
577   factor 15 deficiency protects against atherosclerosis by attenuating ccr2-mediated macrophage
578   chemotaxis. *J Exp Med*. 2011;208:217-225
579 49. Wang J, Wei L, Yang X, Zhong J. Roles of growth differentiation factor 15 in atherosclerosis and
580   coronary artery disease. *J Am Heart Assoc*. 2019;8:e012826
581 50. Wang XA, Zhang R, Zhang S, Deng S, Jiang D, Zhong J, et al. Interferon regulatory factor 7
582   deficiency prevents diet-induced obesity and insulin resistance. *Am J Physiol Endocrinol Metab*.
583   2013;305:E485-495
584 51. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Functional effect of the
585   c242t polymorphism in the nad(p)h oxidase p22phox gene on vascular superoxide production in
586   atherosclerosis. *Circulation*. 2000;102:1744-1747
587 52. Li JM, Newburger PE, Gounis MJ, Dargon P, Zhang X, Messina LM. Local arterial nanoparticle
588   delivery of sirna for nox2 knockdown to prevent restenosis in an atherosclerotic rat model. *Gene*
589   *Ther*. 2010;17:1279-1287
590 53. Martin-Ventura JL, Blanco-Colio LM, Munoz-Garcia B, Gomez-Hernandez A, Arribas A, Ortega L,
591   et al. Nf-kappab activation and fas ligand overexpression in blood and plaques of patients with
592   carotid atherosclerosis: Potential implication in plaque instability. *Stroke*. 2004;35:458-463
593 54. Item F, Wueest S, Lemos V, Stein S, Lucchini FC, Denzler R, et al. Fas cell surface death receptor
594   controls hepatic lipid metabolism by regulating mitochondrial function. *Nat Commun*.
595   2017;8:480
596 55. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, et al. A major role for vcam-1, but not
597   icam-1, in early atherosclerosis. *J Clin Invest*. 2001;107:1255-1262
598 56. Lee GL, Yeh CC, Wu JY, Lin HC, Wang YF, Kuo YY, et al. Tlr2 promotes vascular smooth muscle cell
599   chondrogenic differentiation and consequent calcification via the concerted actions of
600   osteoprotegerin suppression and il-6-mediated rankl induction. *Arterioscler Thromb Vasc Biol*.
601   2019;39:432-445

602 57. Gong XM, Li YF, Luo J, Wang JQ, Wei J, Wang JQ, et al. Gpnmb secreted from liver promotes
603   lipogenesis in white adipose tissue and aggravates obesity and insulin resistance. *Nat Metab*.
604   2019;1:570-583
605 58. Xiong X, Kuang H, Ansari S, Liu T, Gong J, Wang S, et al. Landscape of intercellular crosstalk in
606   healthy and nash liver revealed by single-cell secretome gene analysis. *Mol Cell*. 2019;75:644-
607   660 e645
608 59. Cao S, Liu M, Sehrawat TS, Shah VH. Regulation and functional roles of chemokines in liver
609   diseases. *Nat Rev Gastroenterol Hepatol*. 2021;18:630-647
610 60. Tokgozoglu L, Libby P. The dawn of a new era of targeted lipid-lowering therapies. *Eur Heart J*.
611   2022
612 61. Stoffel W, Binczek E, Schmidt-Soltau I, Brodesser S, Wegner I. High fat / high cholesterol diet
613   does not provoke atherosclerosis in the omega3-and omega6-polyunsaturated fatty acid
614   synthesis-inactivated delta6-fatty acid desaturase-deficient mouse. *Mol Metab*. 2021;54:101335
615 62. Babaev VR, Runner RP, Fan D, Ding L, Zhang Y, Tao H, et al. Macrophage mal1 deficiency
616   suppresses atherosclerosis in low-density lipoprotein receptor-null mice by activating
617   peroxisome proliferator-activated receptor-gamma-regulated genes. *Arterioscler Thromb Vasc*
618   *Biol*. 2011;31:1283-1290
619 63. Mei W, Xiang G, Li Y, Li H, Xiang L, Lu J, et al. Gdf11 protects against endothelial injury and
620   reduces atherosclerotic lesion formation in apolipoprotein e-null mice. *Mol Ther*. 2016;24:1926-
621   1938
622 64. Chen PY, Qin L, Li G, Malagon-Lopez J, Wang Z, Bergaya S, et al. Smooth muscle cell
623   reprogramming in aortic aneurysms. *Cell Stem Cell*. 2020;26:542-557 e511
624 65. Chen PY, Qin L, Li G, Wang Z, Dahlman JE, Malagon-Lopez J, et al. Endothelial tgf-beta signalling
625   drives vascular inflammation and atherosclerosis. *Nat Metab*. 2019;1:912-926
626 66. Robertson AK, Rudling M, Zhou X, Gorelik L, Flavell RA, Hansson GK. Disruption of tgf-beta
627   signaling in t cells accelerates atherosclerosis. *J Clin Invest*. 2003;112:1342-1350
628 67. Lievens D, Habets KL, Robertson AK, Laouar Y, Winkels H, Rademakers T, et al. Abrogated
629   transforming growth factor beta receptor ii (tgfbetarii) signalling in dendritic cells promotes
630   immune reactivity of t cells resulting in enhanced atherosclerosis. *Eur Heart J*. 2013;34:3717-
631   3727
632 68. Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R, et al. Inhibition of
633   transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable
634   plaque phenotype in mice. *Circ Res*. 2001;89:930-934
635 69. Scandura JM, Boccuni P, Massagué J, Nimer SD. Transforming growth factor beta-induced cell
636   cycle arrest of human hematopoietic cells requires p57kip2 up-regulation. *Proc Natl Acad Sci U S*
637   *A*. 2004;101:15231-15236
638 70. Blank U, Karlsson S. Tgf-beta signaling in the control of hematopoietic stem cells. *Blood*.
639   2015;125:3542-3550
640 71. Capron C, Lacout C, Lecluse Y, Jalbert V, Chagraoui H, Charrier S, et al. A major role of tgf-beta1
641   in the homing capacities of murine hematopoietic stem cell/progenitors. *Blood*. 2010;116:1244-
642   1253
643 72. Larsson J, Blank U, Helgadottir H, Bjornsson JM, Ehinger M, Goumans MJ, et al. Tgf-beta
644   signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in
645   vivo despite increased proliferative capacity in vitro. *Blood*. 2003;102:3129-3135
646 73. Langer JC, Henckaerts E, Orenstein J, Snoeck HW. Quantitative trait analysis reveals
647   transforming growth factor-beta2 as a positive regulator of early hematopoietic progenitor and
648   stem cell function. *J Exp Med*. 2004;199:5-14

649 74. Zhu H, Cao M, Figueroa JA, Cobos E, Uretsky BF, Chiriva-Internati M, et al. Aav2/8-hsmad3 gene
650   delivery attenuates aortic atherogenesis, enhances th2 response without fibrosis, in ldlr-ko mice
651   on high cholesterol diet. *J Transl Med*. 2014;12:252

652 75. Roh JD, Hobson R, Chaudhari V, Quintero P, Yeri A, Benson M, et al. Activin type ii receptor
653   signaling in cardiac aging and heart failure. *Sci Transl Med*. 2019;11

654 76. Hashimoto O, Funaba M, Sekiyama K, Doi S, Shindo D, Satoh R, et al. Activin e controls energy
655   homeostasis in both brown and white adipose tissues as a hepatokine. *Cell Rep*. 2018;25:1193-
656   1203

657 77. McPherron AC, Lee SJ. Suppression of body fat accumulation in myostatin-deficient mice.
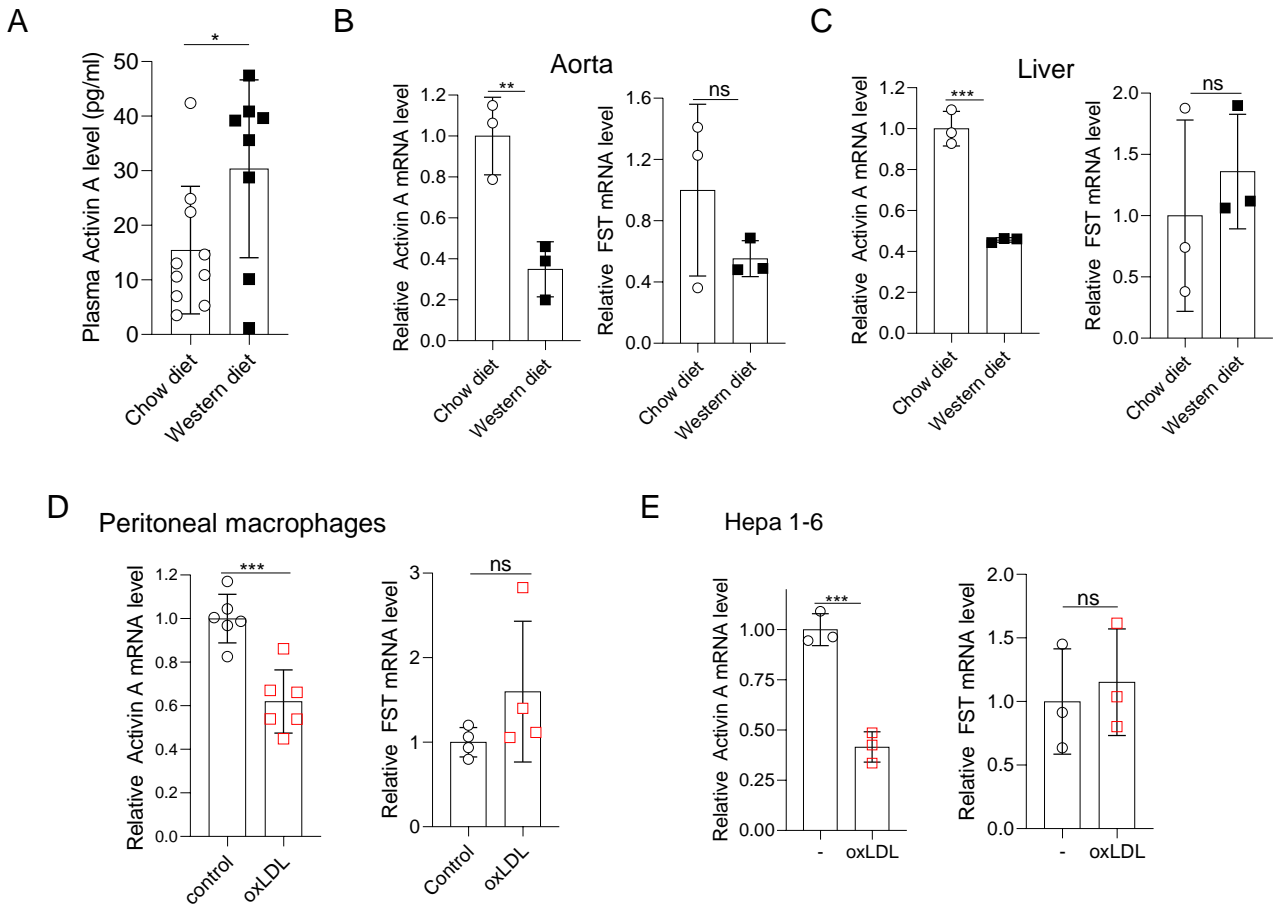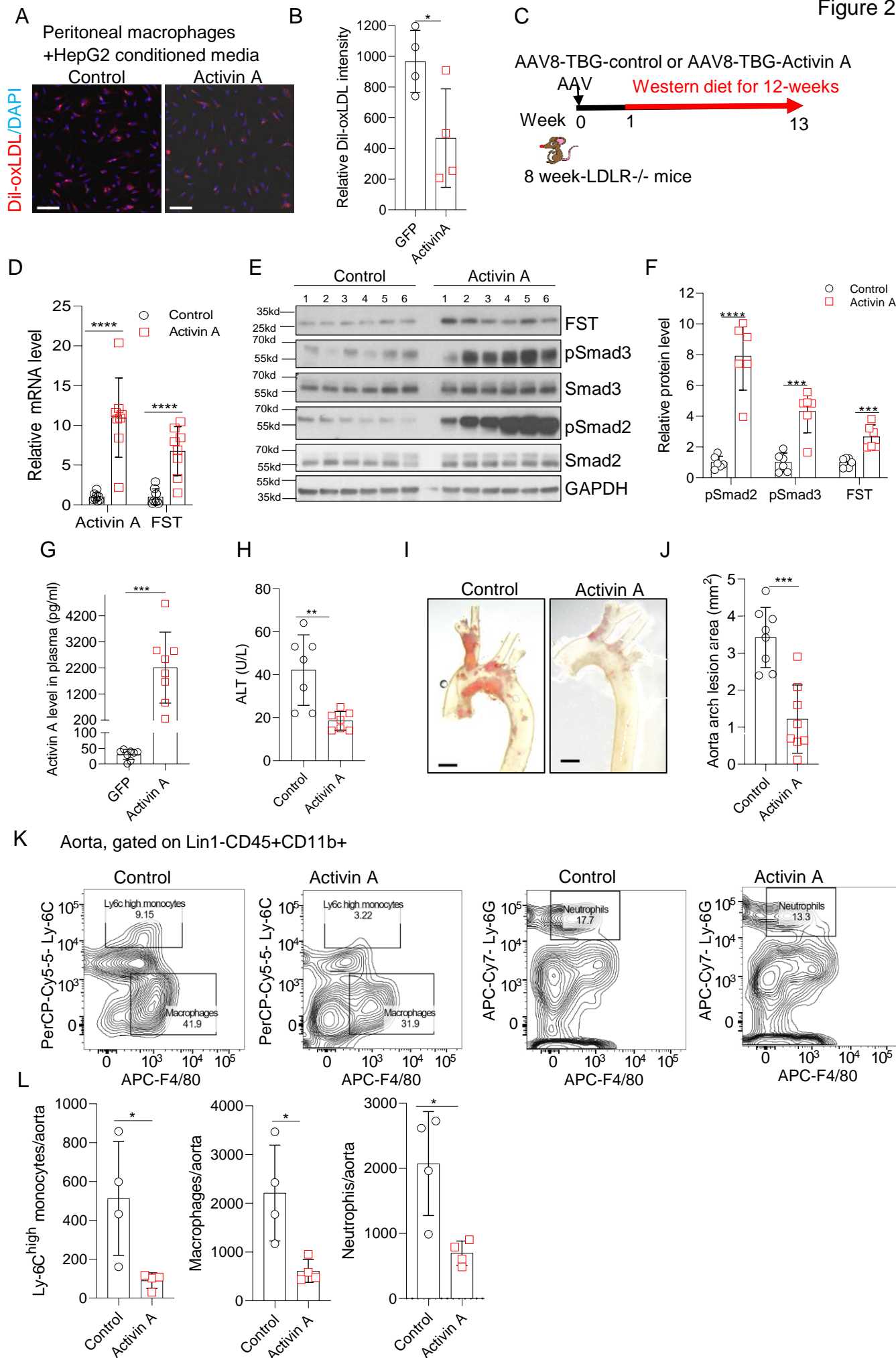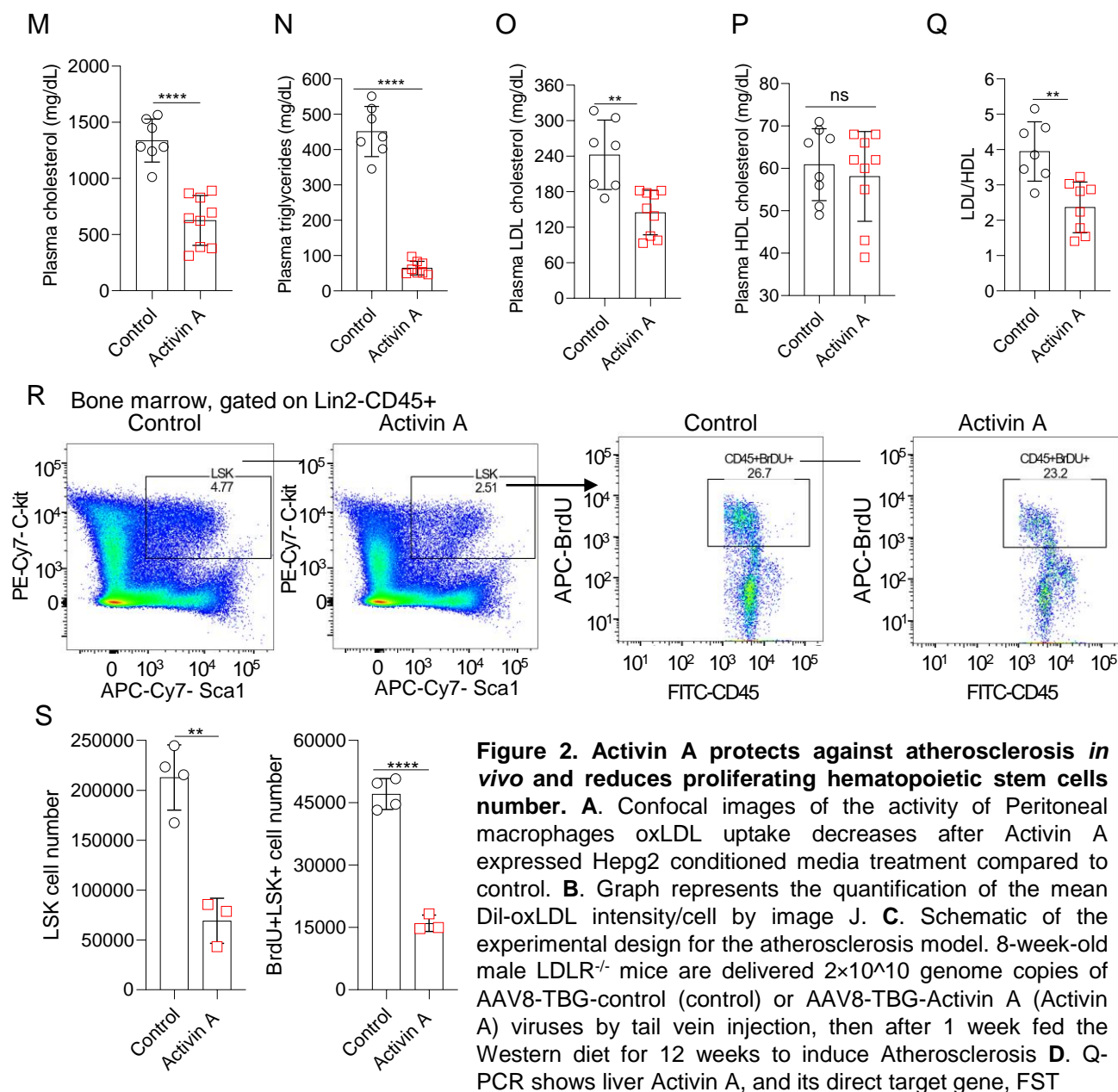658   *Journal Of Clinical Investigation.* 2002;109:595-601

659

660

Figure 1

Figure 1. Activin A mRNA decreases in aorta and liver in western diet fed mice, oxLDL treated macrophages and Hepa 1-6 cells. A. ELISA shows plasma Activin A level in LDLR-/- mice fed with western diet for 12 weeks compared to chow diet. Plasma FST levels are close to baseline (0) and not shown here. N=8-10 mice. **B-C**. Q-PCR shows the Activin A but not FST mRNA decreases in the aorta and liver in LDLR-/- mice fed the western diet for 12 weeks compared to chow diet. N=3 mice. **D**.Q-PCR shows Activin A but not FST mRNA decreases in peritoneal macrophages after oxLDL treatment. Peritoneal macrophages were treated with and without 10µg/ml oxLDL in 0.5%BSA (fatty acid free)/RPMI at 37°C for 24h. **E.** Q-PCR shows Activin A but not FST mRNA decreased in oxLDL treated Hepa 1-6 cells. Hepa 1-6 cells were treated with and without 10 µg/ml oxLDL in 0.5%BSA (fatty acid free)/RPMI 1640 at 37°C for 24h.

Figure 2

A

Peritoneal macrophages
+HepG2 conditioned media

Control   Activin A

Dil-oxLDL/DAPI

B

C

AAV8-TBG-control or AAV8-TBG-Activin A

AAV   Western diet for 12-weeks

Week 0   1   13

8 week-LDLR-/- mice

D

E

Control   Activin A

1 2 3 4 5 6   1 2 3 4 5 6

35kd
25kd   FST
70kd
55kd   pSmad3
70kd
55kd   Smad3
70kd
55kd   pSmad2
70kd
55kd   Smad2
55kd
35kd   GAPDH

F

G

H

I

Control   Activin A

J

K   Aorta, gated on Lin1-CD45+CD11b+

Control   Activin A   Control   Activin A

L

Figure 2

M Plasma cholesterol (mg/dL) — Control vs Activin A, ****

N Plasma triglycerides (mg/dL) — Control vs Activin A, ****

O Plasma LDL cholesterol (mg/dL) — Control vs Activin A, **

P Plasma HDL cholesterol (mg/dL) — Control vs Activin A, ns

Q LDL/HDL — Control vs Activin A, **

R Bone marrow, gated on Lin2-CD45+

Control / Activin A — PE-Cy7- C-kit vs APC-Cy7- Sca1; LSK 4.77 / LSK 2.51

Control / Activin A — APC-BrdU vs FITC-CD45; CD45+BrDU+ 26.7 / CD45+BrDU+ 23.2

S LSK cell number — Control vs Activin A, **; BrdU+LSK+ cell number — Control vs Activin A, ****

Figure 2. Activin A protects against atherosclerosis *in vivo* and reduces proliferating hematopoietic stem cells number. A. Confocal images of the activity of Peritoneal macrophages oxLDL uptake decreases after Activin A expressed Hepg2 conditioned media treatment compared to control. **B**. Graph represents the quantification of the mean Dil-oxLDL intensity/cell by image J. **C**. Schematic of the experimental design for the atherosclerosis model. 8-week-old male LDLR$^{-/-}$ mice are delivered 2×10^10 genome copies of AAV8-TBG-control (control) or AAV8-TBG-Activin A (Activin A) viruses by tail vein injection, then after 1 week fed the Western diet for 12 weeks to induce Atherosclerosis **D**. Q-PCR shows liver Activin A, and its direct target gene, FST mRNA were induced in Activin A treated atherosclerotic LDLR-/- mice compared with control. N=8 mice. **E**. Western blotting shows the Activin signaling pathway is activated in Activin A expressing liver compared with control. Antibodies for Smad2, pSmad2, Smad3, pSmad3 and the Activin signaling pathway direct target gene FST were used, and GAPDH as the loading control. **F**. Quantification of relative pSmad2, pSmad3, and FST protein level. **G**. ELISA shows Activin A protein circulating in the plasma of atherosclerotic LDLR-/- mice after AAV8-TBG-Activin A treated and western diet for 12 weeks. N=8 mice. **H.** Graph represents plasma ALT (SGPT) decreases in Activin A treated atherosclerotic mice compared with control. **I.** Images show the oil red staining of atherosclerotic plaques in the aortic arch in control and Activin A expressing atherosclerotic mice. Scale bar, 1mm. **J.** Quantification of lesion area in the aortic arch. N=8 mice. **K.** Assessment and gating strategy of aortic immune cells by flow cytometry. Lin1: Ter119/NK1.1/CD19/CD90.2/CD3. Ly-6C^{high}monocytes (CD45+Lin1−CD11b+F4/80−Ly-6C^{high}), macrophages (CD45+Lin1−CD11b+F4/80+Ly-6C^{low}) and neutrophils (CD45+Lin1−CD11b+Ly-6G+F4/80−). **L**. Quantification of Ly-6C^{high} monocytes, macrophages, and neutrophils in aorta. N=4 mice. **M-Q.** Graphs represent the plasma lipid profiles including cholesterol, triglycerides, LDL, HDL and LDL/HDL in control and Activin A-expressing atherosclerotic mice. N=7-9 mice. **R.** Assessment and gating strategy of hematopoietic stem cells (HSC) proliferation by flow cytometry. Lin2: CD3/Ly-6G/Ly-6C/ B220/CD11B/Ter119/CD127. LSK: Lin2-CD45+Sca1+c-Kit+. **S.** Quantification of the of LSK cells and BrdU+ LSK cells. HSC were from 2 tibia and 2 femurs of each atherosclerotic mouse. 1mg BrdU was injected into mice 2 hours before euthanasia. N=3-4 mice. Similar results were obtained from 3 independent experiments.
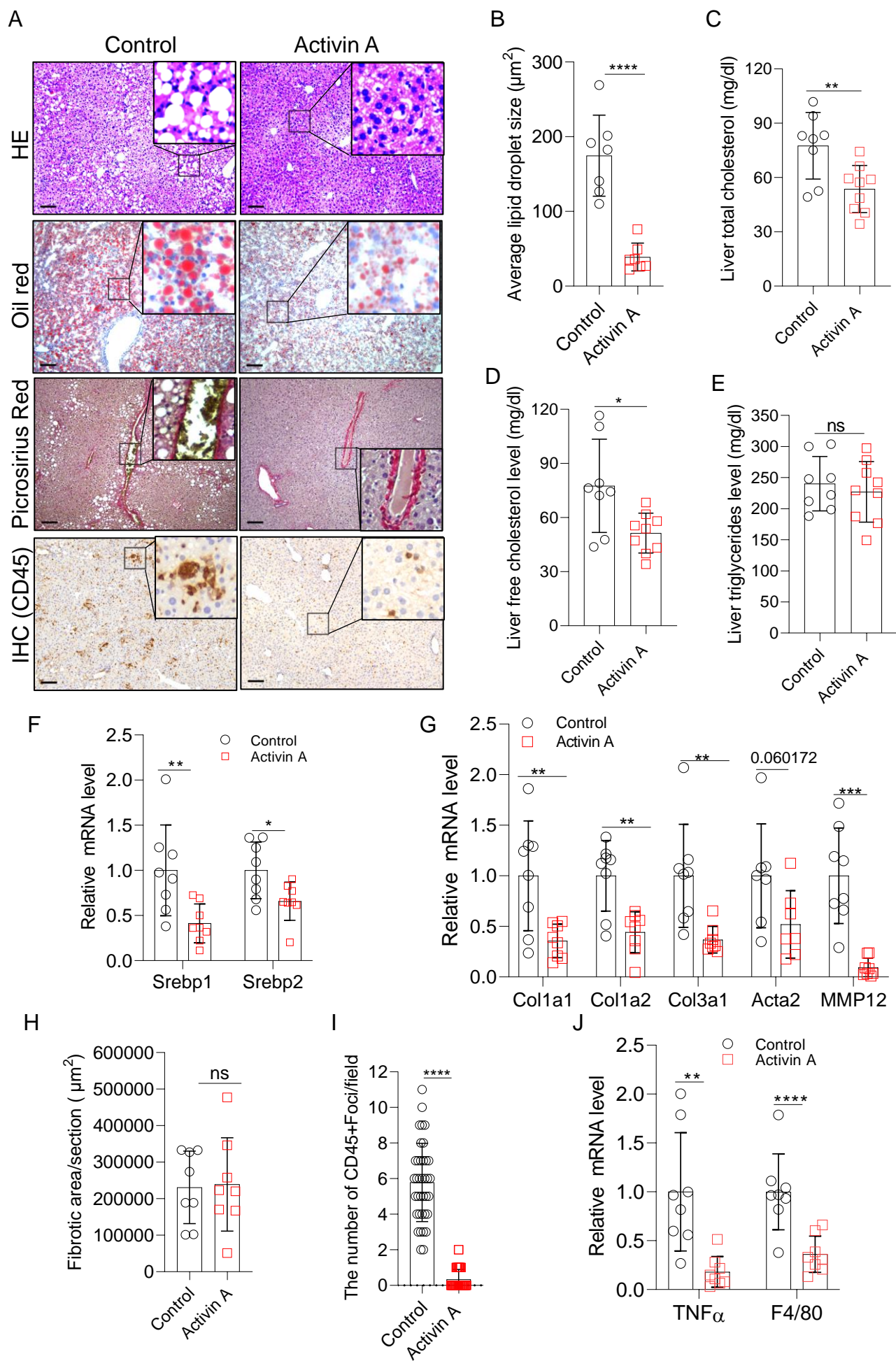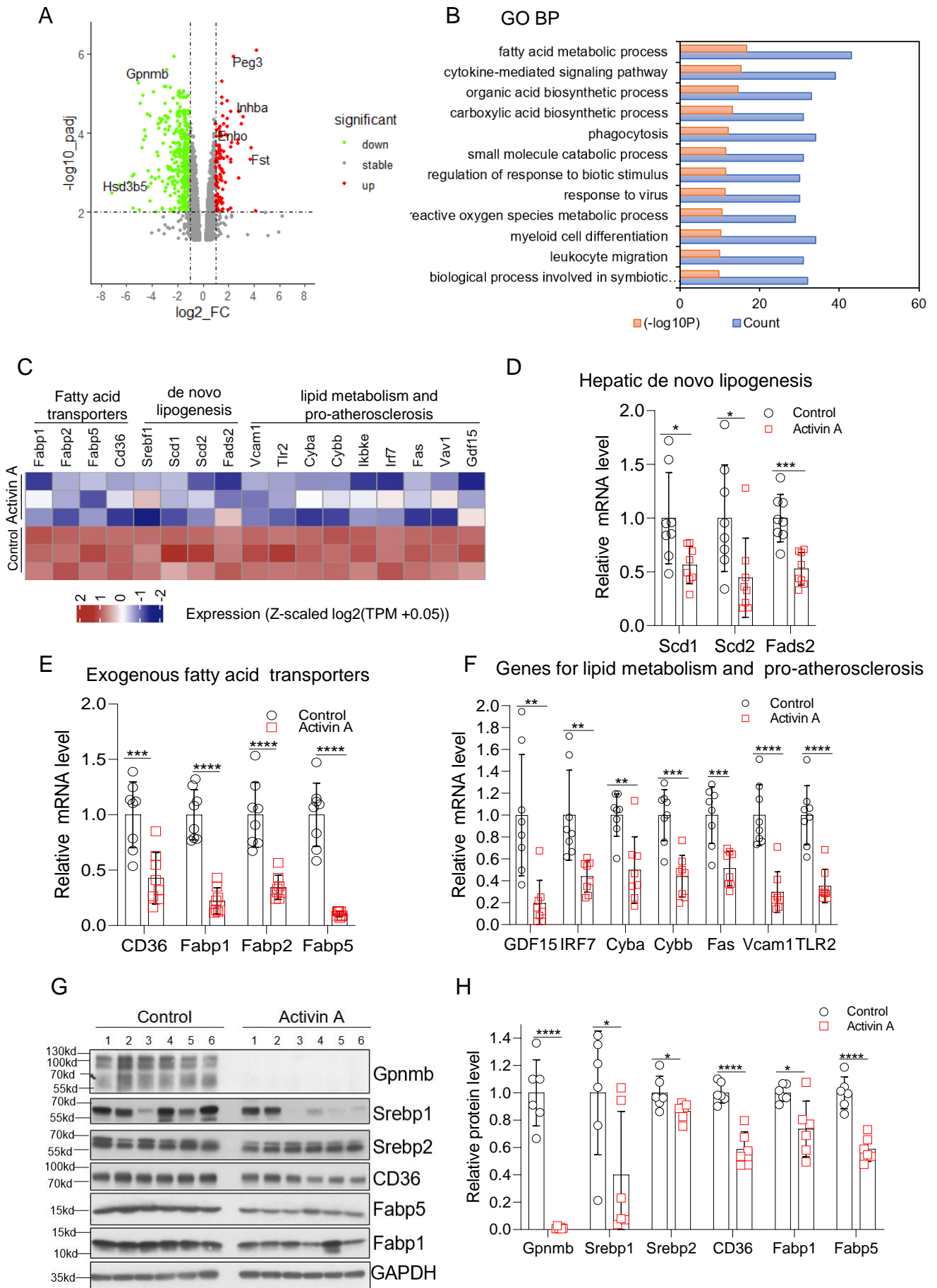
Figure 3

Figure 3

Figure 3. Activin A decreases liver steatosis and inflammation in atherosclerotic mice. **A**. Representative Hematoxylin and Eosin staining, oil red staining (lipid), picrosirius red staining (collagen I and III), and immunohistochemistry staining of leukocytes (with anti-CD45 antibody) in liver samples from control and Activin A treated –atherosclerotic mice. N=7-8 mice in each staining. **B**. Quantitation of the lipid droplet size based on oil red staining in A. N=8 mice. **C-E**. Graph represent the liver total cholesterol, free cholesterol level and triglycerides in atherosclerotic mice. N=7-9 mice. **F**. Q-PCR showing the mRNA level of Srebp1 and Srebp2, the key transcription factors for cholesterol synthesis and triglycerides in control and Activin A- expressing liver. N=8 mice. **G**. Q-PCR showing the mRNA level of fibrosis marker genes (Col1a1, Col1a2, Col3a1 and MMP12) and hepatic stellate cell marker gene (Acta2) in control and Activin A-expressing liver. N=8 mice. **H**. Quantification of fibrotic area of in the right lateral lobe based on picrosirius red staining in A. N=8 mice. **I**. Quantification of CD45+ foci based on IHC staining in A. CD45+ foci was defined as the sites with > 8 CD45 positive cells. The number of CD45+ foci/field (7-8 field/mice) in the right lateral lobe was quantified. N=8 mice. **J**. Q-PCR showing the mRNA level of inflammatory marker genes TNFα, and F4/80 in control and Activin A expressing- atherosclerotic liver. N=8 mice. Mean values for each mouse were used for quantification.

Figure 4

A

B GO BP

C Fatty acid transporters / de novo lipogenesis / lipid metabolism and pro-atherosclerosis

Expression (Z-scaled log2(TPM +0.05))

D Hepatic de novo lipogenesis

E Exogenous fatty acid transporters

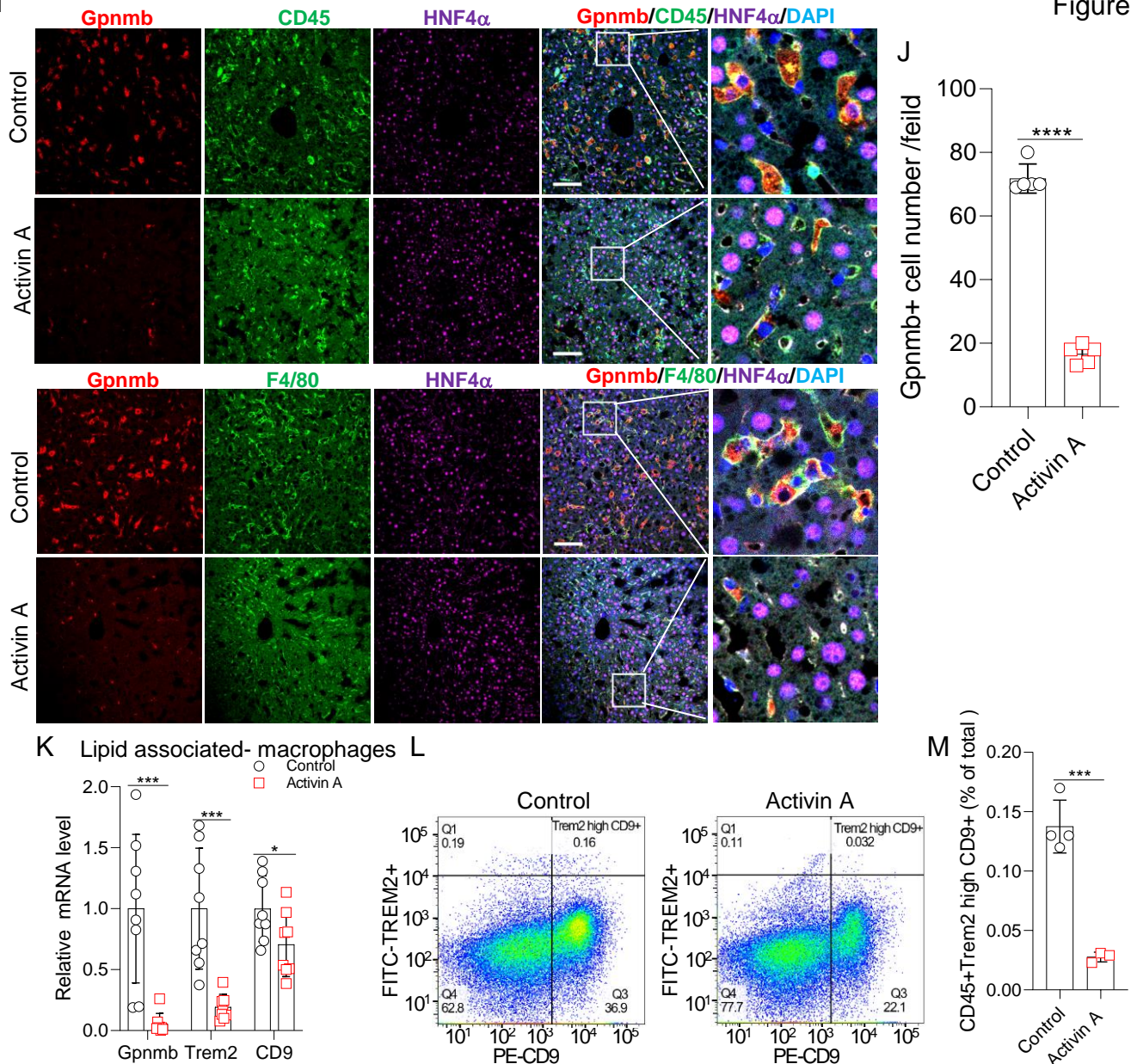**F** Genes for lipid metabolism and pro-atherosclerosis

G

H

Figure 4. Activin A decreases expression of genes involved in fatty acid uptake, and *de novo* lipogenesis, and lipid associated- macrophage markers in atherosclerotic liver. A. Representative volcano plot of differentially regulated genes in RNA-seq analysis of Activin A expressed liver compared with control. Triplicates per group. TPM (control) or TPM (Activin A) ≥ 5. Significant: |log2Fc|>=1, padj<0.01. Downregulation: green, upregulation: red, and stable: grey. Labeling genes, padj<0.01, TPM>=10, log2_Fc>=3 or log2_Fc<=-5. **B**. Top 12 GO biological processes dramatically changed in RNA-seq analysis of Activin A-expressing liver compared with control, based on overrepresentation analysis. Cluster Profiles package was used. TPM (control) or TPM (Activin A) ≥ 5, p<0.05, |log2Fc|>=1. **C.** Heatmap shows differentially regulated featured genes in fatty acid metabolic process, lipid metabolism and atherosclerosis. Gene list is from GO and KEGG enrichment. TPM (control) or TPM (Activin A) ≥ 5, p<0.05, |log2Fc|>=1, padj<0.01. Relative gene expression values (Z-scaled log2(TPM +0.05)) were represented. **D-E.** Q-PCR confirmation of decreased transcript expression of genes involved in hepatic *de novo* lipogenesis and exogenous fatty acid uptake in Activin A-expressing liver compared with control. N=8 mice. **F.** Q-PCR confirmation of decreased transcript expression of genes involved in lipid metabolism and atherosclerosis in Activin A expressing liver compared with control . N=8 mice. **G**. Western blotting showing decreased Gpnmb, Srebp1, Srebp2, CD36, Fabp1, Fabp5 protein in Activin A- expressing liver compared with control. Antibodies for Gpnmb, Srebp1, Srebp2, CD36, Fabp1, and Fabp5 antibodies were used, GAPDH as the loading control. **H.** Quantification of relative Gpnmb, Srebp1, Srebp2, CD36 and Fabp5 protein level. **I.** Images show the immunofluorescence staining of Gpnmb colocalization with CD45+ and F4/80+ cells, but not hepatocytes in atherosclerotic liver. CD45 (green), leukocytes marker, F4/80 (green), macrophages marker, Gpnmb (red), hepatocyte marker, HNF4α, (purple, locates in nuclei), and nuclear stain DAPI (blue). Scale bar, 100μm. **J.** Quantification of Gpnmb+ cells in I. 4-5 random fields /section. N=5 mice. **K**. Q-PCR shows decreased mRNA levels of lipid-associated-macrophages markers Trem2, Gpnmb and CD9 in Activin A-expressing liver compared with control. N=8 mice. **L-M**. Assessment, gating strategy and quantification of lipid associated- macrophages (Trem2 high CD9+). Gpnmb antibodies did not work for flow. N=3-4 mice.
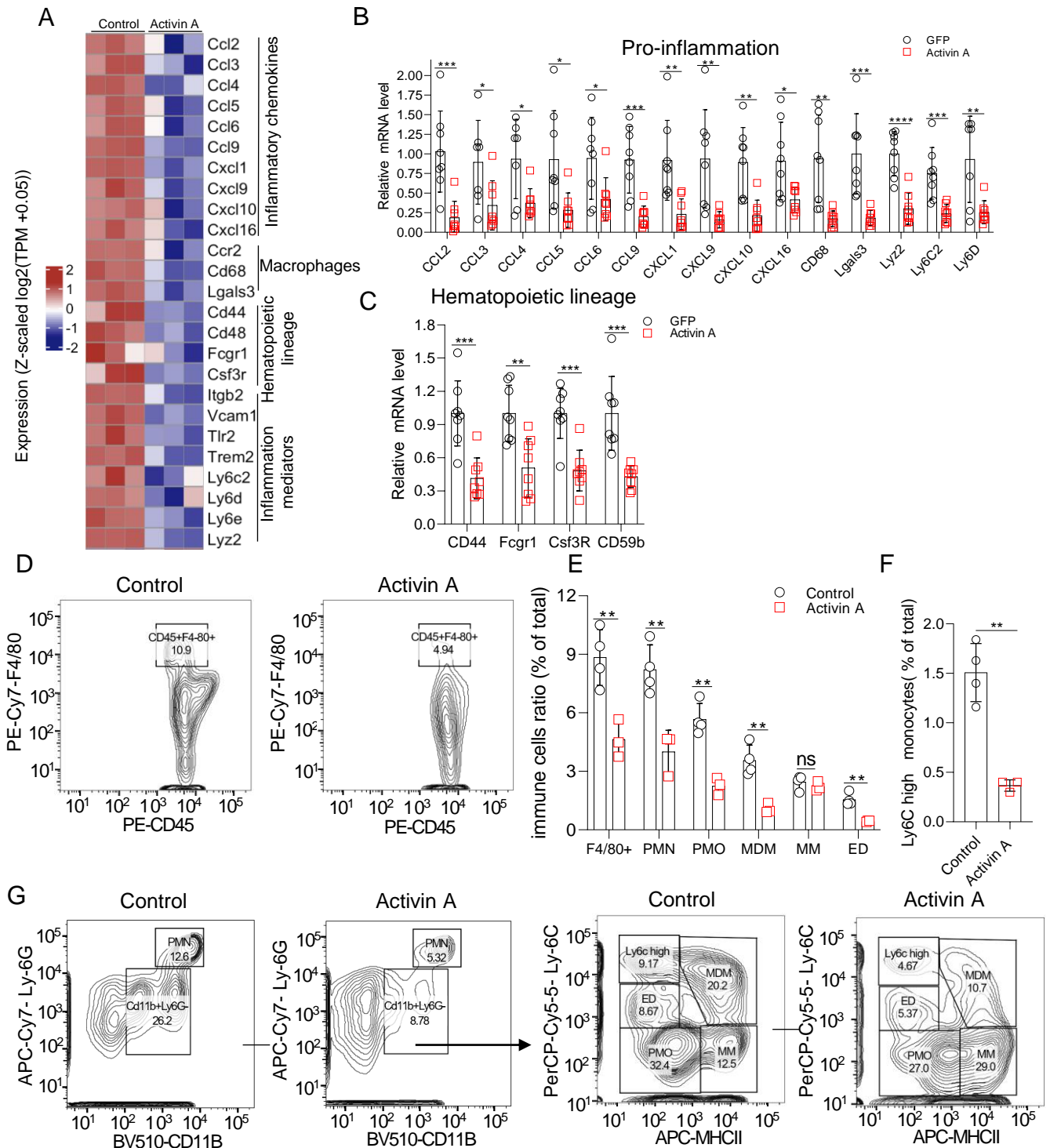
Figure 5

Figure 5. Activin A decreases liver macrophages, Ly-6C^{high} monocytes and polymorphonuclear cells (PMN) infiltration in Atherosclerotic mice. A. Heatmap shows differentially regulated featured genes in leukocytes migration pathway. Gene list is from GO enrichment. Relative gene expression values (Z-scaled log2(TPM +0.05)) for the 25 genes related to pro-inflammation. TPM (control) or TPM (Activin A) ≥ 5. p<0.05, |log2Fc|>=1, padj<0.01. **B-C**. Q-PCR confirmation of RNA-seq data in A. **D.** Assessment of CD45+F4-80+ populations by flow cytometry. **E-F**. Quantification of immune cells from D and G. 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), Ly-6C^{high} monocytes (CD45+CD11b+ Ly-6G-Ly-6C^{high}/MHCII-). N=3-4 mice. Repeat for 3 times. **G**. Assessment and gating strategy of immune cells in atherosclerotic liver by flow cytometry in E and F.
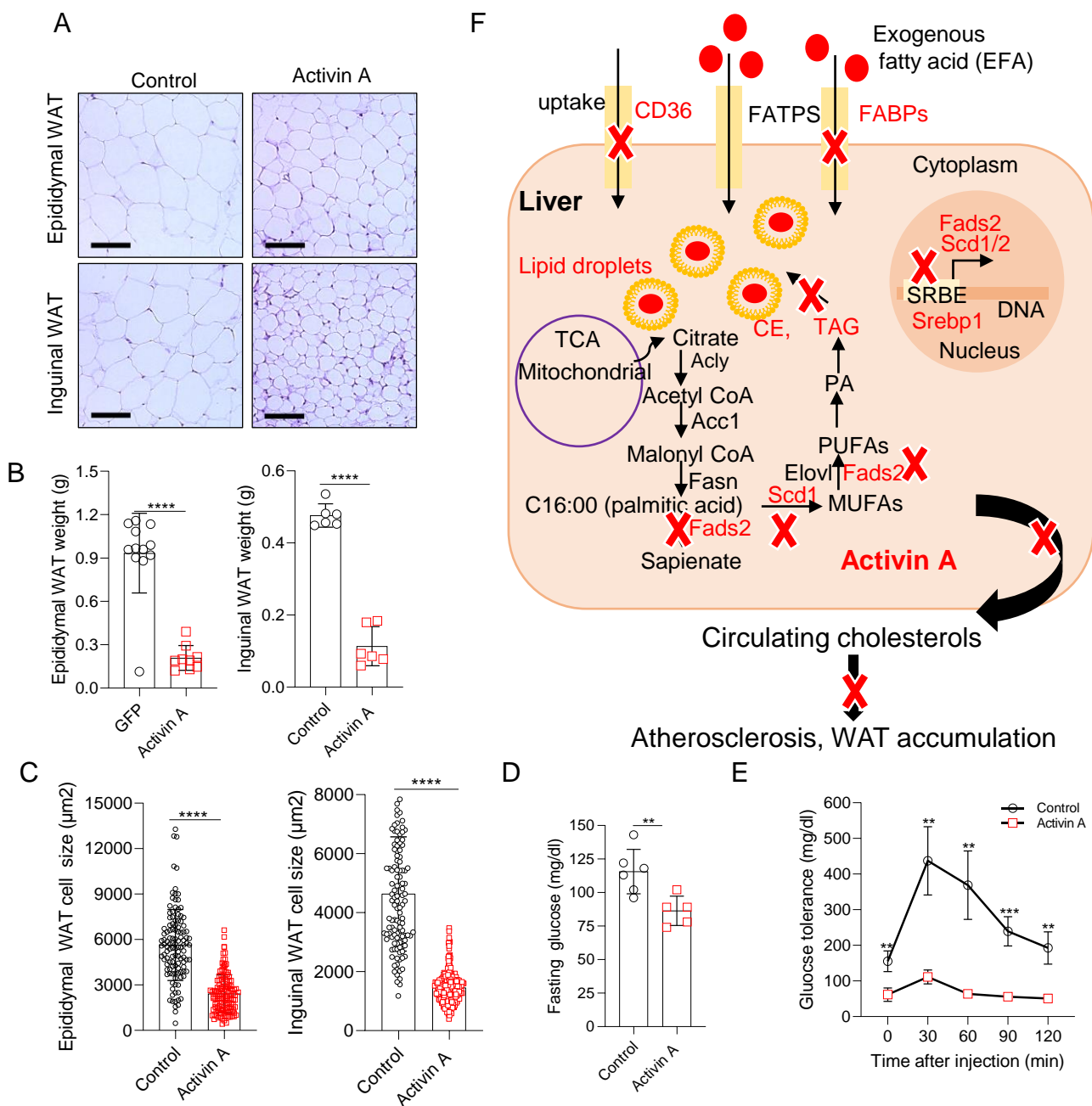
Figure 6

Figure 6. Activin A inhibits fat accumulation and improves glucose tolerance. A. Representative Hematoxylin and Eosin staining of epididymal White Fat Tissue (WAT) and inguinal WAT in Activin A treated mice compared with Control. N=7 for Inguinal WAT and N=11-12 in epididymal WAT. Scale bar: 50μm. **B.** Quantification of the WAT weight in Activin A delivered mice compared with Control. Unilateral fat was calculated. N=6-12 mice. **C.** Quantification of inguinal WAT and epididymal WAT adipocyte areas. N=7 mice. **D.** Fasting glucose level was measured by a glucose meter and strips. Mice were fasted for 5 hours before the measurement. N=5-6 mice. **E.** Glucose tolerance was measured by a glucometer. Overnight-fasted mice were injected intraperitoneally with glucose (2 g per kg body weight). Blood glucose levels were measured at the basal level and at 15, 30, 60, 90 and 120 min after glucose administration using a blood glucometer. N=3-4 mice. **F**. A schematic model of the role of Activin A in regulating the metabolic processing of fatty acid in the liver, and effects on circulating cholesterol levels and atherosclerosis. Activin A not only blocks genes involved in fatty acid uptake including CD36 and FABPS, but also attenuates expression of genes involved in fatty acid *de novo* lipogenic pathways including Srebp1-Scd1/Fads2. Pathways expected to be downregulated by Activin A are indicated with a red X. Scd1/Scd2 and Fads2 expression are regulated by Srebp1 at the transcriptional level. ACLY, ATP–citrate lyase; ACC, acetyl-CoA carboxylase; FASN, fatty acid synthase; ELOVLs, elongation of very long-chain fatty acid protein; PA, phosphatidic acid; TAG, triacylglycerol; CE, cholesterol ester.
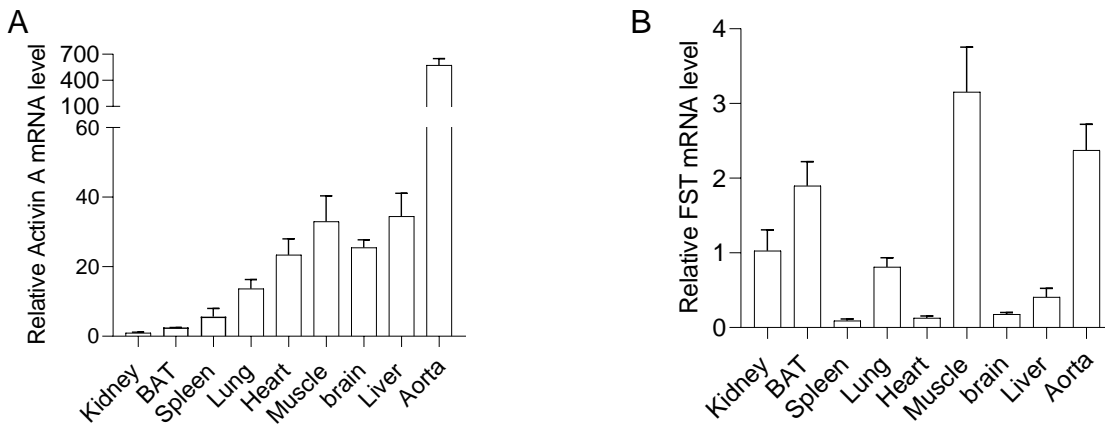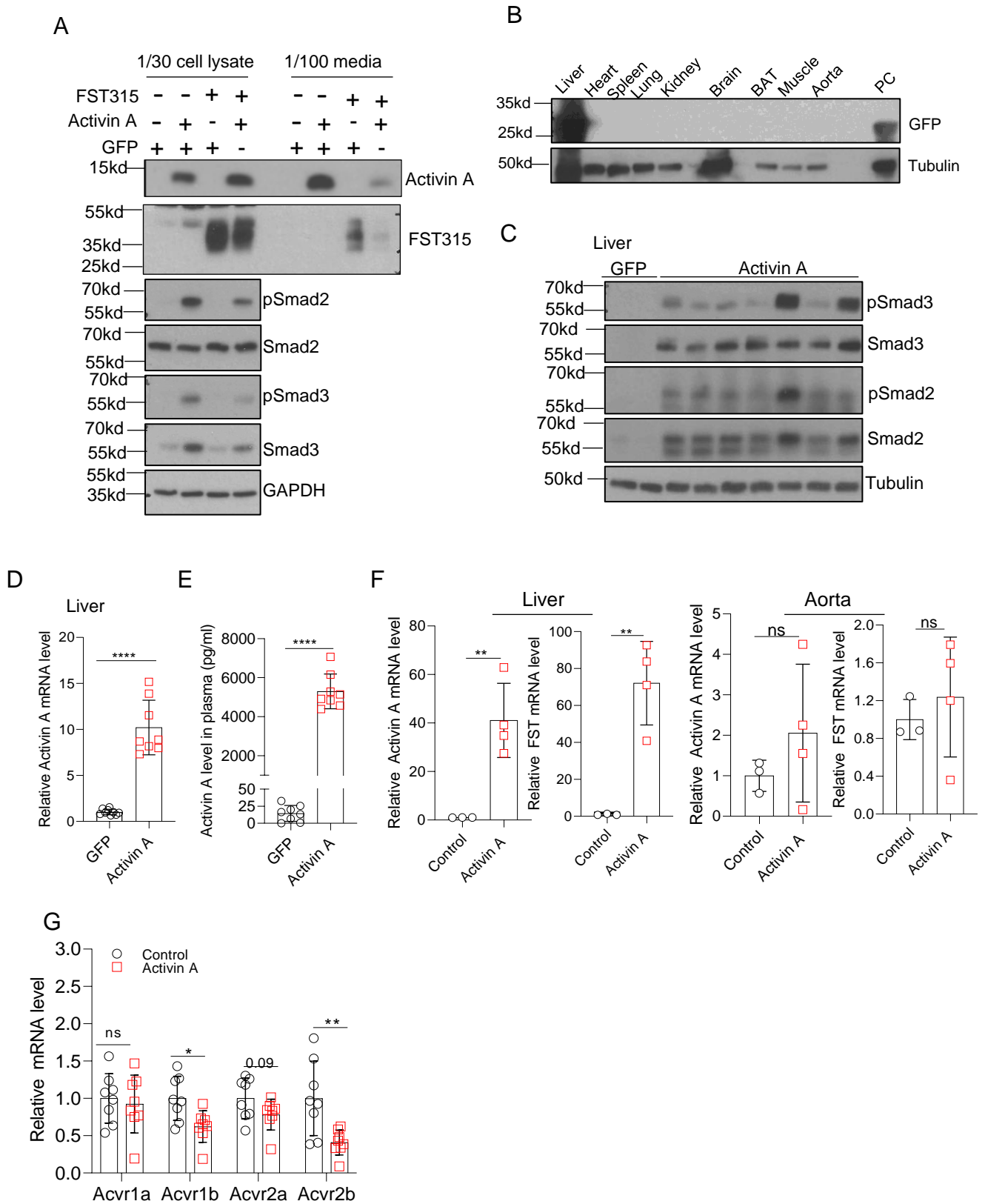
A

B

Figure S1, related to Figure 1. A-B. mRNA levels of Activin A and FST measured by QPCR in different tissues in mice.

H Bone marrow, Gated on Lin-CD45+

Control — Activin A

PE-Cy7- c-Kit vs APC-Cy7- Sca1
- Control: LSK 3.14
- Activin A: LSK 1.98

APC-CD135 vs PerCP-Cy5-5- CD150
- Control: MPP4 24.0, CD135- 36.7
- Activin A: MPP4 21.7, CD135- 37.4

BV510-CD48 vs PerCP-Cy5-5- CD150
- Control: MPP3 81.0, MPP2 6.78, StHSC 10.4, LtHSC 1.78
- Activin A: MPP3 89.7, MPP2 5.68, StHSC 4.03, LtHSC 0.63

I
- LSK cell number (Control vs Activin A) **
- MPP4 Cell number **
- LtHSC Cell number ****
- StHSC Cell number ***
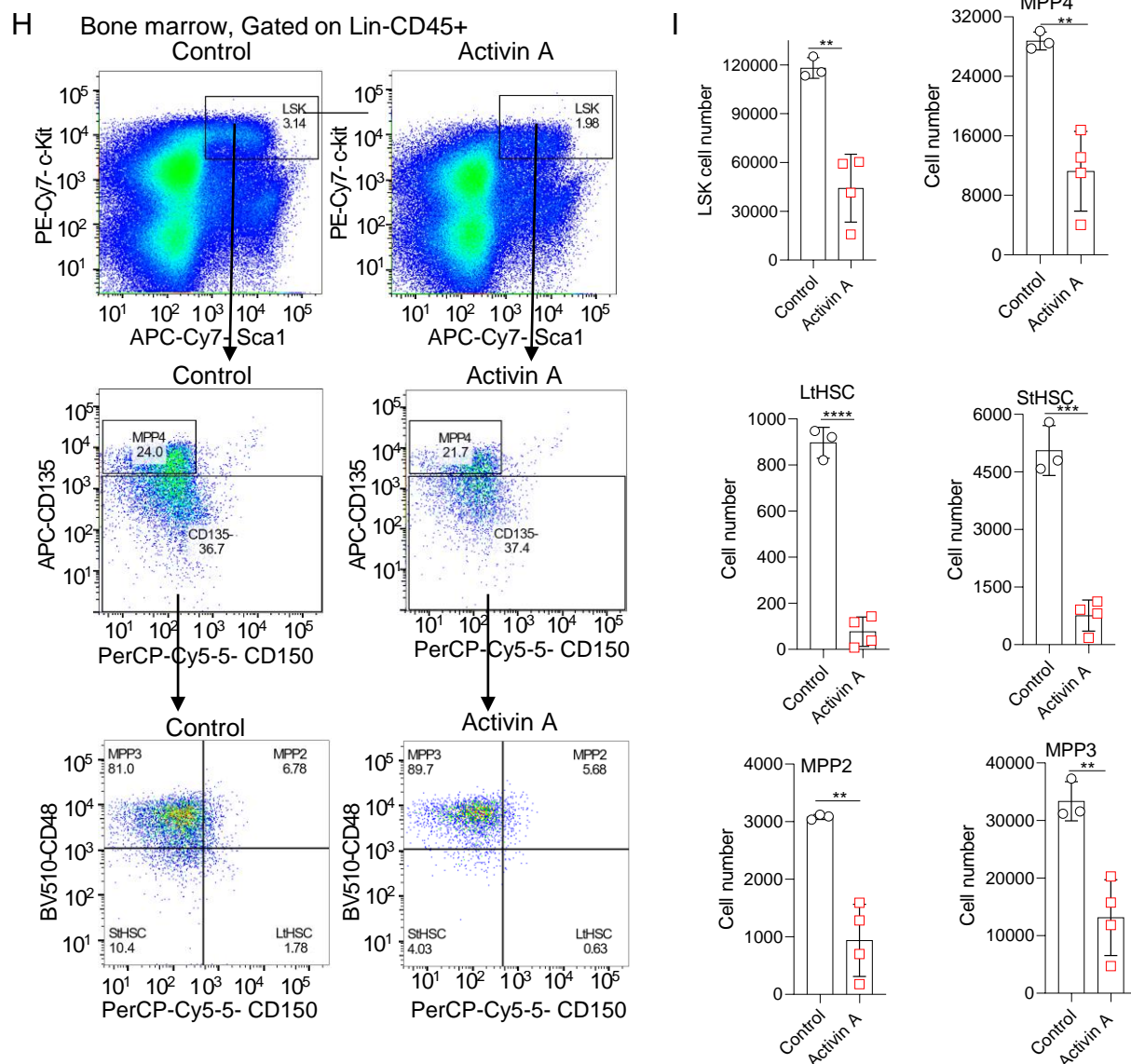- MPP2 Cell number **
- MPP3 Cell number **

Figure S2, related to Figure 2. A. Western blotting showing that AAV8-TBG-Activin A is expressed in HepG2 cells, activates Smad2/Smad3 phosphorylation, and circulates in the media, while AAV8-TBG-FST315 blocks the activity of Activin A. The indicated plasmids were transfected into HepG2 cells and cultured for 48 h. **B.** Western blotting shows AAV8-TBG-GFP was highly expressed in liver but no other tissue after tail vein injection. 6-week-old male C57/BL6 mice were I.V. injected with 5×10^{10} AAV8-TBG-GFP, and then sacrificed after 4 weeks. Protein levels were quantified and normalized by BCA kit (pierce), and target proteins were detected by western blotting using anti-GFP antibody. HepG2 cell lysates with GFP expression were used as the positive control (PC). 18.74 µg protein /sample. **C-D.** Western blotting and Q-PCR show AAV8-TBG-Activin A was expressed and activated the Activin pathway. 6-week-old male C57/BL6 mice were I.V. injected with 5×10^{10} genome copies AAV8-TBG-GFP, and then scarified after 4 weeks. Protein level was quantified by BCA kit (pierce) and target proteins detected by western blotting. **E**. ELISA shows liver expressed Activin A circulates in plasma. Mice from H were used. Plasma FST levels are close to baseline (0) and not shown here. **F.** Q-PCR shows AAV8-TBG-Activin A is expressed and induces expression of its direct target gene FST in the liver but not aorta in atherosclerotic LDLR-/- mice. Control, n=3 and Activin A, n=4. **G.** Q-PCR shows the mRNA levels of Activin pathway receptors in control and Activin A-expressing atherosclerotic liver. N=8 mice. Acvr1a, Acvr1b, Acvr1c, Acvr2a and Acvr2b primers were used for Q-PCR, and Acvr1c was undetectable. **H**. Assessment and gating strategy of hematopoietic stem cells (HSC) populations by flow cytometry. LSK: Lin2-CD45+Sca1+c-Kit+. Long-term HSC (LtHSC), short-term HSC (StHSC) and multipotent progenitors (MPP2, MPP3 and MPP4). StHSC (CD135-/CD150-/CD48- LSK), LtHSC (CD135-/CD150+/CD48- LSK) MPP2 (CD135-/CD150+/CD48+ LSK), MPP3 (CD135- /CD150-/CD48+ LSK), and MPP4(CD135+/CD150-/CD48+/- LSK). **I.** Quantification of the number of LSK, LtHSC, StHSC and multipotent progenitors in control and Activin A-expressing atherosclerotic mice. N=3-4 mice. Similar results were obtained from 3 independent experiments.

A   KEGG

B

Hepa 1-6

C

BODIPY 500/510-Fatty acid/DAPI

D

Fatty acid uptake
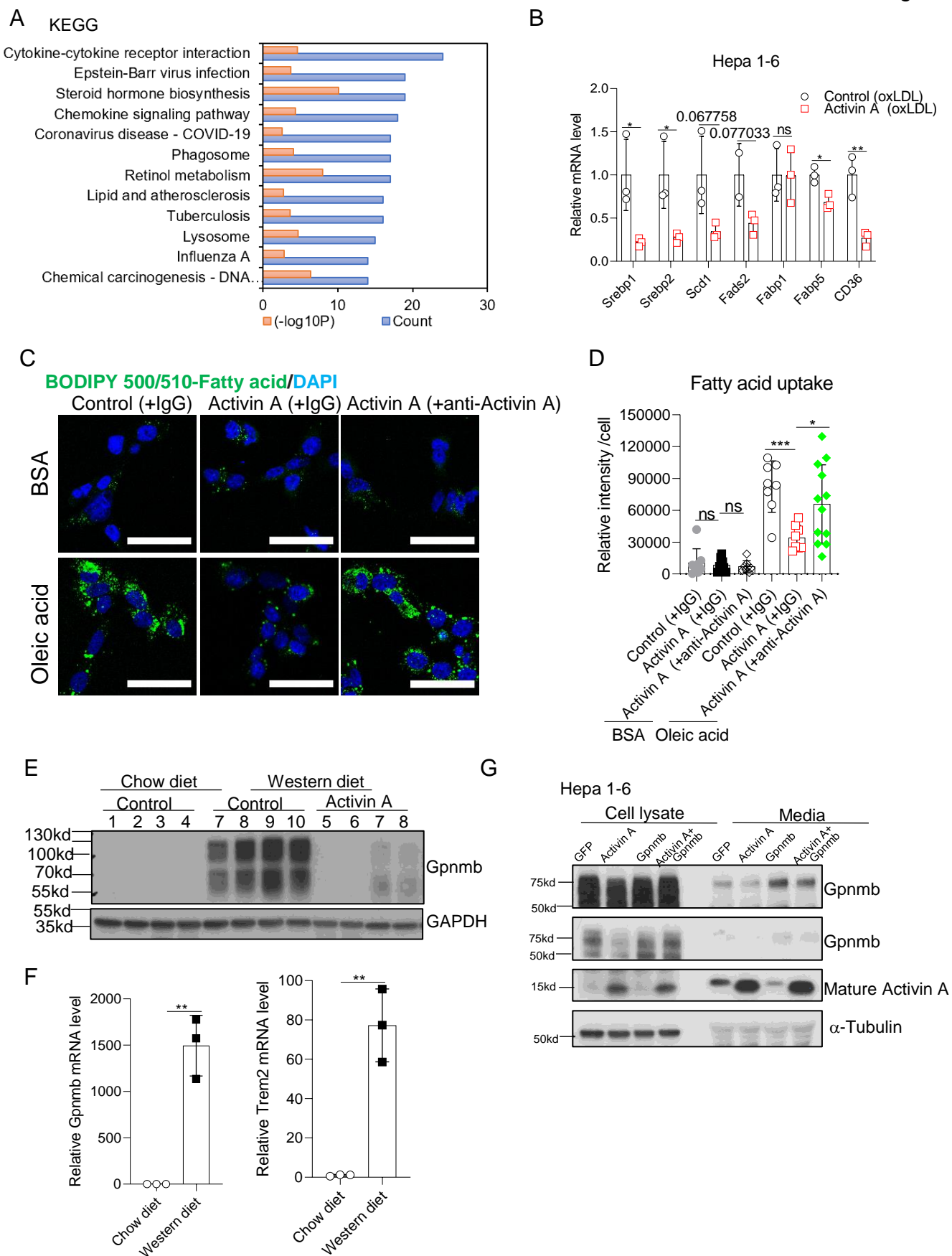

E

G

Hepa 1-6

F

Figure S3, related to Figure 4. A. KEGG enrichment of top 12 significantly changed pathways in RNA-seq analysis of Activin A-expressing liver compared with control. TPM (control) or TPM (Activin A) ≥ 5, p<0.05, |log2Fc|≥ 1. **B.** Q-PCR shows the mRNA levels of genes for hepatic *de novo* lipogenesis and exogenous fatty acid uptake decrease in Hepa 1-6 cells treated with Activin A-expressing conditioned media and 10 μg/ml oxLDL compared with control. Hepa 1-6 cells were transfected with TBG-GFP or TBG-Activin A plasmid, and 24h later were changed into 0.5%BSA (fatty acid free)/RPMI at 37°C for 24h. The conditioned media were collected and filtered through a 0.22 μm filter, diluted twice with fresh 0.5%BSA/RPMI1640, and then added into fresh Hepa 1-6 cells together with 10 μg/ml oxLDL for 24 hours. **C-D.** Confocal images and quantification of fatty acid uptake. Hepa 1-6 cells were transfected with TBG-GFP or TBG-Activin A plasmid, and 24h later were changed into 0.5%BSA (fatty acid free)/RPMI at 37°C for 24h. The conditioned media were collected and filtered with a 0.22 μm filter, diluted twice with fresh 0.5%BSA/RPMI1640, and then added into fresh Hepa 1-6 cells, with or without 30 μM Oleic acid (OA), with 1μg/mL Mouse IgG1 Isotype Control or neutralizing anti-Activin A antibody for 24 hours. Then cells were washed with PBS, stained with 2 μM BODIPY 500/510-fatty acid, fixed with 4%PFA, and stained with DAPI. Scale bar, 50 μm. **E.** Western blotting shows Gpnmb is expressed in western diet-fed LDLR-/- control mice compared with chow but decreases in Activin A-expressing western diet-fed LDLR-/- mice. **F.** Q-PCR shows Gpnmb and Trem2 mRNA increase in western diet-fed LDLR-/- mice compared with chow diet. **G.** Western blotting shows Activin A decreases Gpnmb protein levels in cell lysate and media. α-Tubulin as the loading control.