FoxO1 is a critical regulator of hepatocyte lipid deposition in chronic stress mice

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FoxO1 is involved in lipid metabolisms. However, its role in chronic stress-related nonalcoholic fatty liver disease (NAFLD) is unclear. The scientific premise of our study was based on the finding that FoxO1 expression is increased in liver of mice after chronic stress. It is important to understand the mechanisms involved in the activation of FoxO1 and how its function affects the liver lipid deposition. We employed a murine chronic stress model, in which mice were treated by plantar electrical stimulation and restraint for 6 weeks, and a cellular model, in which Hepa 1-6 cells were treated with corticosterone. We also used a pharmacologic approach as1842856, a highly specific FoxO1 inhibitor. Lipid metabolism related genes levels were measured by qRT-PCR and the lipid levels by biochemical detection. We show that the level of FoxO1 is significantly elevated in the liver of chronic stress mice. Transcription factor FoxO1 regulates a lipid synthesis phenotype of hepatocyte that is involved in the development and progression of NAFLD. We have shown that inhibition of FoxO1 induced phenotypic conversion of hepatocytes and down-regulates lipid synthesis genes expression by hepatocytes, which contribute to lipid deposition in NAFLD. At the cellular level, the inhibitor of FoxO1 as1842856 can also attenuated the lipid deposition of Hepa1-6 cells induced by corticosterone. Targeting FoxO1 is a novel therapeutic target for chronic stress-related NAFLD.

1 **FoxO1 is a Critical Regulator of Hepatocyte Lipid Deposition in chronic stress mice**

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18 **Abstract**

19 Forkhead box O1 (FoxO1) is a potent transcriptional regulator of pancreatic β-cell function and

20 lipid metabolism. However, its role in chronic stress-related nonalcoholic fatty liver disease

21 (NAFLD) is unclear. The scientific premise of our study was based on the finding that FoxO1

22 expression is increased in the livers of mice after chronic stress. It is important to understand the

23 effect of FoxO1 activation in chronic stress-induced liver lipid deposition. We employed a

24 murine chronic stress model, in which mice were treated by plantar electrical stimulation and

25 restraint for 6 weeks, and a cellular model, in which Hepa 1-6 cells were treated with

26 corticosterone. We also used a pharmacologic approach, with as1842856, a highly specific

27 FoxO1 inhibitor. Lipid metabolism-related gene levels were measured by quantitative reverse

28 transcription polymerase chain reaction (qRT-PCR) and the lipid levels were measured by

29 biochemical detection. We show that the level of FoxO1 is significantly elevated in the liver of

30 chronic stress mice. Transcription factor FoxO1 regulates a lipid synthesis phenotype of

31 hepatocytes that is involved in the development and progression of NAFLD. We have shown that

32 inhibition of FoxO1 induced phenotypic conversion of hepatocytes and downregulates lipid

33 synthesis gene expression by hepatocytes, which contribute to lipid deposition in NAFLD. At the

34 cellular level, the inhibitor of FoxO1 as1842856 can also attenuate the lipid deposition of Hepa1-

35 6 cells induced by corticosterone. Targeting FoxO1 is a novel therapeutic target for chronic

36 stress-related NAFLD.

Introduction

37 **Introduction**

38   The incidence of nonalcoholic fatty liver disease (NAFLD) is 20-30% globally (Henao-

39   Mejia et al., 2012). The lipid deposition of the liver, especially the abnormal deposition of

40   triglycerides (TG), is the basis of the pathogenesis of NAFLD (Piccinin et al., 2018). The

41   correlation between the occurrence of NAFLD and chronic stress has received increasing

42   attention in recent years (Ashtari et al., 2015). Our previous result and others' studies have

43   confirmed that chronic stress could lead to liver lipid deposition and inflammation, which lead to

44   the development of NAFLD (Fu et al., 2010; Liu et al., 2014). However, the specific mechanism

45   of chronic stress-induced liver lipid deposition is not clear.

46   FoxO1 is a member of the forkhead O family and plays a key role in lipid metabolism (Li et

47   al., 2017). Activation of FoxO1 was detected during lipid deposition in the liver (Kim et al.,

48   2016); activated FoxO1 was also found to lead to an increase in the output of very low-density

49   lipoprotein (triglyceride-rich particles) in the liver, resulting in hypertriglyceridemia (Kim et al.,

50   2011); the expression and activity of FoxO1 were increased in patients with NAFLD and

51   correlated positively with the severity of the disease (Valenti et al., 2008). Matsumoto et al.

52   found that the transfection of FoxO1 in the liver increased the TG content of the liver

53   (Matsumoto et al., 2006). Thus, we hypothesized that chronic stress leads to liver lipid deposition

54   by activation of FoxO1. Our previous study demonstrated that chronic stress induces liver lipid

55   deposition and FoxO1 activation in mice (Liu et al., 2014). However, the causal relationship

56   between FoxO1 and liver lipid deposition during chronic stress is unclear. The molecular

57   mechanisms of stress-induced lipid metabolism disorders are also unclear.

58   In this study, 6 weeks of plantar electrical stimulation and restraint were used to induce the

59   mouse chronic stress. FoxO1 activity inhibitor as1842856 was used to verify the role of FoxO1

60   in this model. After 6-week stress exposure, we performed lipid deposition tests and measured

61 TG synthesis genes and cholesterol metabolism-related genes to explore if and how FoxO1

62 influences the lipid deposition in a chronic stress animal model. It may provide new targets for

63 drug prevention and treatment of stress-related lipid metabolic disorders.

64

Materials & Methods

66 *Reagents and Antibodies*

67 FoxO1 inhibitor (as1842856) and Cortisol were purchased from Sigma-Aldrich (St. Louis,

68 MO, USA). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT, USA). Penicillin

69 and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). FoxO1, p-FoxO1, and β-

70 Actin antibodies were obtained from Abcam (Cambridge, MA, USA). IRDye 800CW donkey

71 anti-goat and IRDye 800CW goat anti-rabbit secondary antibodies were both purchased from LI-

72 COR Biosciences (LI-COR, Inc., Lincoln, NE, USA). The Real-time PCR reactions were

73 performed using SYBR Premix Ex Taq™ (Takara Biotechnology). Hematoxylin-eosin (H&E)

74 and oil red O were purchased from Nanjing Jiancheng Science and Technology Company.

75 *Animals and Chronic Stress Protocols*

76 C57BL/6J male mice, 5-7 weeks age, were purchased from Slaccas Laboratory Animal

77 Company (Shanghai, China). Mice were housed in plastic home-cages in a temperature

78 controlled room at 24°C, under a 12 h:12 h illumination cycle (lights on at 8:00 AM). To stress

79 animals, we used a modified version of the protocol described previously (Liu et al., 2014). For

80 chronic stress, each mouse was administered electric foot shock (10:00, 7.5s/2min, 15min, 25V)

81 and restraint stress (19:00-21:00) every day for 42 consecutive days before evaluations. Non-

82 stress mice (control group) remained in the home cage and kept isolated from stressed animals.

83 FoxO1 inhibitor group were administered as1842856 at 30mg/kg by gavage during the 42 days.

84 The control group was treated with equal volume solvent by gavage. Animal's weight and food

85 intake were measured every week. All animal procedures used in this study were approved by

86 the Institutional Animal Care and Use Committee of the Second Military Medical University

87 (No. 20161027, Shanghai, China) and Shanghai Science and Technology Committee (SYXK-

88 HU-2012-0003).

89   *Hematoxylin-Eosin (H&E) and oil red o staining*

90   Following fixation of the livers or cells with 10% formalin, livers were sliced and stained

91 with H&E for histological examination. Hepatic lipid content was also determined by staining

92 with Oil Red O (Sigma). Pictures were imaged with a Zeiss microscope (Carl Zeiss Microscopy,

93 Thornwood, NY, USA).

94   *Biochemical analysis*

95   The serum TG, total cholesterol (TC), and free fatty acid (FFA) were measured by an

96 automatic biochemistry analyzer (Hitachi 7170, Tokyo, Japan). Livers were homogenized at 4°C

97 in lysis buffer containing 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, and

98 0.5% sodium deoxycholate. Lipids from the total liver homogenate were extracted using the

99 chloroform/methanol method (2:1), evaporated, and dissolved in 2-propanol. Amounts of TC and

100 TG were measured by an automatic biochemistry analyzer (Hitachi 7170, Tokyo, Japan).

101   For glucose tolerance test (GTT), mice were fasted overnight and injected with 2 mg/g

102 glucose/body weight. For insulin tolerance test (ITT), mice were fasted for 4 hours and injected

103 with 1 U/Kg insulin/body weight. The blood glucose levels were monitored at 30 min intervals

104 for 2 h with an ACCU-CHEK Active Blood Glucose System (Roche) using tail tip blood

105 samples. Serum insulin was determined by mouse insulin ELISA kit which was purchased from

106 R&D, USA.

107   *Cell culture*

108   Mouse liver cancer Hepa 1-6 cell lines (ATCC CRL-1830) were cultured in Dulbecco's

109   Modified Eagle's Medium (DMEM) (Hyclone Laboratories, Logan, Utah, USA). The medium

110   contained 10% fetal bovine serum (FBS) and 100 unit/mL penicillin and 100 g/mL streptomycin

111   in a humidified atmosphere that contained 5% CO_2. Cells were further administrated in DMEM

112   containing 1 μM cortisol or 1 μM as1842856 for 48h.

113   *qRT-PCR*

114   Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was

115   synthesized using PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). Quantitative real-time

116   PCR was carried out using the One Step TB Green™ PrimeScript™ RT-PCR Kit (Takara)

117   according to manufacturer's instructions (Table 1). The $2^{-\Delta\Delta Ct}$ method was used to calculate the

118   relative expression level by normalizing to β-Actin levels (Zhao et al., 2019).

119   *Western blot analysis*

120   Protein samples were prepared by lysing cells or tissues in modified RIPA buffer [1×PBS,

121   1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail (Sigma-

122   Aldrich)]. Lysates (50-100 μg) were separated on a 10% SDS-PAGE and transferred to a

123   nitrocellulose membrane. The membrane was probed with the specific primary antibody and

124   secondary antibody and then quantified with Odyssey Infrared Imaging System (LI-COR, Inc.,

125   Lincoln, NE, USA) and Image J Software (National Institute of Mental Health, USA).

126   *Statistical analysis*

127   The data were presented as mean ± standard error (SEM) and differences considered

128   statistically significant only when *p*-values are less than 0.05. Data was mainly analyzed using a

129   two-way ANOVA followed by uncorrected Fisher's LSD post hoc test with GraphPad Prism 6

130   (GraphPad Software, Inc., La Jolla, CA, USA). Two-way ANOVA for repeated measures was

131 used to analyze the weight curves. Student's t-test was applied to detect significant difference

132 between two groups.

Results

134   *As1842856 inhibited FoxO1 activation in the liver of chronic stress mice*

135   The levels of FoxO1 protein and mRNA were significantly increased in the liver of mice

136 after 6 weeks of stress, while the FoxO1 inhibitor as1842856 did not affect FoxO1 expression

137 (Fig. 1A, B, and E). The ratio of *p*-FoxO1 protein to FoxO1 was significantly decreased in the

138 stress group (Fig. 1A, D) but significantly increased in the stress+as1842856 group.

139 Phosphorylated FoxO1 protein is the inactive form of FoxO1 (Pan et al., 2017). The changes in

140 the mRNA levels of PEPCK and G6Pase, the classical FoxO1 regulated target genes, were

141 consistent with the changes in the activity of FoxO1 (Fig. 1F, G).

142   *Effects of as1842856 on physiological index in mice after 6 weeks chronic stress*

143   To investigate the effects of as1842856 on indexes of glucose metabolism and food intake,

144 we compared these physiological indexes. During the stress period, the body weight and food

145 intake of the control group increased slowly and steadily, while the stress group did not increase

146 significantly. At the 6th week of stress, the body weight of the stress group was significantly

147 lower than that of the control group (Fig. 2A). The food intake at the 5th week of stress also

148 decreased significantly (Fig. 2B). There was no significant difference in body weight and food

149 intake between the control group and the as1842856 group. We also tested fasting blood glucose,

150 postprandial blood glucose, insulin, glucose tolerance, and insulin tolerance in 4 groups of mice

151 after 6 weeks of stress. The results showed no significant difference between the 4 groups (Fig.

152 2C-G).

153   *As1842856 reduced adipogenesis in the liver of chronic stress mice*

154   After 6 weeks of stress, hematoxylin and eosin (HE) staining of livers in mice showed

155   hepatocyte fatty degeneration: enlarged hepatocytes, loose and reticular cytoplasm, individual

156   cytoplasm was transparent, and shaped like a balloon (Fig. 3A). Oil red O staining showed

157   brownish red lipid droplet formation (Fig. 3A). FoxO1 inhibitor as1842856 significantly reduced

158   the lipid deposition induced by chronic stress (Fig. 3A). Furthermore, the morphology of

159   hepatocytes in the livers of mice fed with as1842856 still displayed normal architecture around

160   the central veins (Fig. 3A). We further quantified the TG and cholesterol levels in the livers of

161   mice. The results showed that FoxO1 inhibitors significantly reduced the liver TG content

162   induced by chronic stress, while cholesterol content had no significant change (Fig. 3B and C).

163   The levels of serum TG (Fig. 3D) and free fatty acid (FFA, Fig. 3E, $p<0.001$) were

164   significantly increased in the chronic stress group. Both TG and FFA were significantly reduced

165   in the stress+as1842856 group compared with the stress group (Fig. 3D and E). There was no

166   statistical difference in serum cholesterol after chronic stress and as1842856 treatment (Fig. 3F).

167   *Effects of as1842856 on the mRNA expression of lipoprotein metabolism-related genes in*

168   *mice after 6 weeks of chronic stress*

169   The mRNA expression of fatty acid synthase (FASN) was significantly increased in the

170   chronic stress group compared with the control group (Fig. 4A). However, the mRNA expression

171   of FASN in the stress+as1842856 group was significantly decreased compared with the stress

172   group (Fig. 4A). There were no significant differences between the 4 groups in ACC1 or SCD1

173   (Fig. 4B and C). The mRNA expression of fatty acid binding protein (FATP) and fatty acid

174   binding protein (FABP) in the chronic stress group was significantly increased compared with

175   the control group (Fig. 4D and E). As1842856 significantly decreased the mRNA expression of

176   FATP and FABP, which was elevated by the chronic stress (Fig. 4D and E). There were no

177 significant differences in the other synthetically-related gene, CD36 (Fig. 4F). We also found no

178 statistically significant differences in the mRNA expression of genes related to fatty acid

179 oxidation, such as PPARα, Acox1, Lcad, Mcad, Pdk4, Cpt1a, and Ucp2 (Fig. 4G-N).

180 *Effects of as1842856 on the mRNA expression of cholesterol metabolism-related genes in*

181 *mice after 6 weeks of chronic stress*

182 We found that the mRNA expression of HMG-CoAR and CYP7A1 was significantly

183 increased in the stress group. The mRNA expression of HMG-CoAR and CYP7A1 in the

184 stress+as1842856 group was significantly lower than that in the stress group (Fig. 5A and B).

185 However, there was no statistical difference in the synthetic-related gene cholesterol-regulating

186 element binding transcription factor 1C (SREBP-1c) and the cholesterol transport-related gene

187 ABCG1 (Fig. 5C and D).

188 *As1842856 inhibited FoxO1 activation in the Hepa1-6 cells after corticosterone treatment*

189 We also demonstrated the effect of as1842856 on FoxO1 activation and adipogenesis in the

190 Hepa1-6 cells. The levels of FoxO1 protein and mRNA were significantly increased in the

191 corticosterone group, compared with the control group (Fig. 6A-C). The *p*-FoxO1 protein and the

192 *p*-FoxO1/FoxO1 ratio were also significantly decreased after corticosterone treatment (Fig. 6A-

193 D). The FoxO1 inhibitor as1842856 significantly increased the *p*-FoxO1 protein and *p*-FoxO1/

194 FoxO1 value (Fig. 6A-D). The change of the classical target gene PEPCK regulated by FoxO1

195 was consistent with the change of FoxO1 activity (Fig. 6E and F).

196 *As1842856 reduced adipogenesis in the Hepa1-6 cells after corticosterone treatment*

197 After treatment with 1 μM of corticosterone for 48 h, oil red O staining indicated that most

198 Hepa1-6 cells showed obvious brown-red lipid droplets. The corticosterone+as1842856 group

199 showed no obvious lipid droplets (Fig. 7A).

200   FASN mRNA expression was significantly increased in the corticosterone group compared

201   with the control group (Fig. 7B). The corticosterone+as1842856 group showed a significant

202   decrease in FASN mRNA expression compared with the corticosterone group (Fig. 7B). Other

203   synthetically related genes, ACC1 and SCD1 showed no significant difference between the 4

204   groups (Fig. 7C and D).

205   **Discussion**

206   This study demonstrated that chronic stress induced lipid deposition in the livers of mice,

207   which were mainly TG. Liver TG synthesis genes and FFA uptake genes were significantly

208   upregulated in mRNA levels after chronic stress. These changes can be blocked by as1842856,

209   the inhibitor of FoxO1. Moreover, at the cellular level, the changes in lipid metabolism of

210   Hepa1-6 cells induced by corticosterone are basically the same as those in animal chronic stress

211   experiments. The inhibitor of FoxO1 as1842856 also attenuated the lipid deposition of Hepa1-6

212   cells induced by corticosterone. Our study confirms the important regulatory role of FoxO1 in

213   stress-induced lipid metabolism disorders.

214   Chronic stress refers to continuous physical or psychological forms of tension and pressure.

215   Macedo et al. (Macedo et al., 2012) found that the chronic stress model mice, created by a

216   narrow living environment and high-calorie diets, gained less weight than normal-fed mice.

217   However, there was no difference between the two groups on the ratios of body weight to body

218   length. It is suggested that mice fed a high-calorie diet may have abdominal obesity. Czech et al.

219   (Czech et al., 2013) found that chronic stress model mice caused by crowded communities have

220   significant oxidative stress and inflammatory response in the liver under normal feeding

221   conditions. Our previous study (Liu et al., 2014) found that liver index (total liver/body

222   weight×100) and liver TG levels increased significantly in chronic stress model (sustained foot

223   stimulation and tethered for 12 weeks) mice. Hepatic cell adipose degeneration was found by

224   liver section staining. Although the stress patterns and models described above are different, they

225   all suggest that stress is associated with hepatic steatosis and inflammatory response.

226   According to our previous results and related literature reports (Kim et al., 2017; Shin et al.,

227   2015; Yamamoto et al., 2002), we used a combination of plantar electrical stimulation and

228   restraint stress to construct a stress model. After 6 weeks of continuous stress, model mice liver

229   TG levels were significantly elevated. Liver fatty degeneration was also demonstrated by the

230   liver section staining. It indicated that chronic stress causes lipid deposition in the livers of mice,

231   and the model was successfully constructed. It is consistent with the results of our previous study

232   (Liu et al., 2014).

233   The possible mechanism of stress-induced hepatic lipid deposition is considered to be

234   related to long-term activation of the hypothalamic-pituitary-adrenal cortex (HPA) axis (van

235   Bodegom et al., 2017). The activated HPA axis releases glucocorticoids. On the one hand,

236   glucocorticoids enhance the decomposition of lipolysis hormones, promote the decomposition of

237   adipose tissue, and release a large amount of FFA into the blood. On the other hand,

238   glucocorticoids promote lipid formation and deposition of hepatocytes. At the same time, the

239   hippocampus has an inhibitory effect on HPA axis activation. Under long-term chronic stress, a

240   large number of hippocampal neurons undergo dysfunction or death by the chronically elevated

241   glucocorticoids, losing their inhibitory effect on the HPA axis, and leading to the massive release

242   of glucocorticoids (Kim et al., 2013; Sakamoto et al., 2015; Zhu et al., 2014). In our study, the

243   FFA content in the blood increased significantly after chronic stress. Corticosterone or cortisol is

244   the main hormone of the pituitary adrenocortical axis secreted by the adrenal cortex in response

245   to environmental challenges (McEwen, 2007; Quijije, 2015; Kant et al., 1987). At the same time,

246   we also investigated the effect of corticosterone in hepatocyte lipid deposition. Lipid droplets

247   were found in Hepa1-6 cells, which were treated with corticosterone for 48 hours. This suggests

248   that chronic stress may cause liver lipid deposition by elevating the corticosterone level.

249   Our previous experiments found that FoxO1 may be involved in stress-induced lipid

250   metabolism disorders. FoxO1 is a member of the FOXO transcription factor. FOXO is widely

251   expressed in a variety of tissues, including the liver, and is a key effector molecule in cell

252   homeostasis, metabolism, and stress response (Ma et al., 2018). It acts as a pre-transcriptional

253   regulator that binds to chromatin (Riedel et al., 2013; Zaret & Carroll, 2011) and initiates or

254   inhibits transcription (Furuyama et al., 2000; Ramaswamy et al., 2002; Webb et al., 2013).

255   FoxO1 is a typical forkhead protein transcription factor, a key signaling molecule downstream of

256   the insulin/ insulin-like growth factor-1 (IGF-1) signal and has been shown to be involved in the

257   regulation of glucose metabolism (O'Neill et al., 2016). FoxO1 binds to adjacent sites in the

258   insulin response elements (IREs) within the insulin-like growth factor binding protein 1

259   (IGFBP1) and glucose-6-phosphatase (G6Pase) promoters to initiate gene activation (Nakae et

260   al., 2001a; Matsumoto et al., 2007; Yeagley et al., 2001). Active nuclear FOXO1 also binds the

261   transcriptional coactivator peroxisome proliferative activated receptor-γ coactivator 1-α (PGC1α)

262   to coordinate a gluconeogenic transcriptional program involving increased expression of

263   glucose-6-phosphatase (*G6pc*) and cytosolic phospho*enol*pyruvate carboxykinase (*Pck1*)

264   (Puigserver et al., 2003; Nakae et al., 2001b). In addition, FOXOs mediate the effects of insulin

265   on adipocyte differentiation, neuropeptide transcription and processing, and β-cell health (Nakae

266   et al., 2003; Ren et al., 2012; Plum et al., 2009). In recent years, an increasing number studies

267   have shown that FoxO1 is also involved in the regulation of lipid metabolism in the liver. These

268   studies have found that activation of FoxO1 was observed in lipid deposition in the liver (Kim et

269   al., 2016); activated FoxO1 induced an increase in liver output of very low-density lipoprotein

270   (rich in TG particles) and hypertriglyceridemia (Kim et al., 2011). This study also indicated that

271   FoxO1 does participate in liver lipid deposition caused by chronic stress. On the one hand, we

272   observed changes in FoxO1 protein, mRNA, and phosphorylation levels in stressed mice; on the

273   other hand, liver lipid deposition due to chronic stress is alleviated after the treatment of FoxO1

274   activity inhibitor.

275   To further clarify how FoxO1 regulates liver lipid deposition, this experiment examined the

276   expression levels of genes involved in liver and lipid metabolism. Previous experiments have

277   confirmed that the main component of lipid deposition caused by chronic stress is TG, and the

278   amount of TG in the liver is inseparable from the uptake and synthesis of FFA (Liu et al., 2014).

279   FASN is a key enzyme for the de novo synthesis of endogenous fatty acids and is closely related

280   to lipid metabolism (Jones & Infante, 2015). FASN mRNA and protein levels were significantly

281   upregulated in fatty liver (Huang et al., 2013). Upregulation of FASN in rat liver is involved in

282   the formation of fatty liver. The FATP family and the FABP family are important protein

283   families that take up fatty acids and are primarily responsible for the transport of extracellular

284   FFA uptake. Studies have confirmed that fatty acid uptake is significantly reduced in hepatocytes

285   of FATP knockout mice (Falcon et al., 2010). Purified FATP1 exhibits long-chain and ultra-

286   long-chain fatty acyl-CoA synthetase activity (Hall et al., 2003). FABP can be activated by long-

287   chain FA, PPARs, and toll-like receptor (TLR) agonists, which are involved in sugar, lipid

288   metabolism, and inflammatory processes (Doege et al., 2006; Tan et al., 2002; Wolfrum et al.,

289   2001). This study found that FoxO1 increases fatty acid synthesis and intra-hepatic transport of

290   FFA by increasing the expression of FASN, FATP, and FABP and increasing liver TG

291   deposition. ACC1 and SCD1 are two other key enzymes involved in lipogenesis (29925265).

292   ACC1 and SCD1 showed no significant difference between the 4 groups. This suggested that the

293   expression of FASN, FTAP, and FABP, but not ACC1 or SCD1, are controlled by the

294   transcription factor FoxO1.We also found that FoxO1 did not affect the feeding of mice and fatty

295   acid oxidation in the liver. FoxO1 is a key molecule downstream of insulin, which regulates

296   glucose metabolism. However, chronic stress mice did not show significant glucose metabolism

297   abnormalities in this experiment. We speculate that mice may still be in a compensatory period

298   due to the relatively short stress time. This conclusion requires further experiments to prove by a

299   longer-term chronic stress test.

300   ## Conclusions

301   In summary, this study demonstrated that chronic stress induced FoxO1 activation and lipid

302   deposition in the livers of mice. Inhibition of FoxO1 attenuated the TG synthesis and fatty acid

303   oxidation induced by the chronic stress. Corticosterone may act as a mediator between FoxO1

304   and chronic stress. The present study indicated that inhibition of FoxO1 may have therapeutic

305   benefits for chronic stress relative fatty liver disease.

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438

Figure legend

439

440 **Figure 1**. FoxO1 specific inhibitor as1842856 enhances FoxO1 phosphorylation in mice liver

441 after 6 weeks. Chronic stress increased the protein (A and B) and mRNA lever (E) of FoxO1

442 expression and its downstream genes, G6Pase (F) and PEPCK (G), and decreased the lever of *p*-

443 FoxO1 protein(C) and *p*-FoxO1/FoxO1 (D), while as1842856 could reverse this effect except the

444 protein and mRNA lever of FoxO1 expression. *$p<0.05$,***$p<0.001$. Date presented as

445 mean±SEM, n=6.

446 **Figure 2**. As1842856 does not affect weight, food intake and glucose metabolic parameters in

447 mice after 6 weeks. (A) Changes in food intake. (B) Changes in body weight. (C) Profiles of

448 blood glucose concentration as function of time upon intraperitoneal injection of glucose (D)

449 Profiles of glucose concentration (percentage of initial value) as a function of time upon

450 intraperitoneal injection of insulin. (E-G) Blood insulin, postprandial blood glucose (PBG), and

451 fasting plasma glucose (FPG). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Date presented as mean±SEM,

452 n=6.

453 **Figure 3**. As1842856 protect mice against stress-induced liver lipid deposition. (A)

454 Representative slides showed hematoxylin and eosin (HE)-stain and Oil red O-stain liver

455 sections from four groups. (B and C) Liver TG and T-CHO concentrations were detected by

456 biochemical test in liver tissue homogenate. (D, E, and F) TG, FFA, and T-CHO concentrations

457 in serum. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Date presented as mean±SEM, n=6.

458 **Figure 4**. Effects of stress and as1842856 on liver lipid metabolism genes in mice after 6 weeks.

459 (A-C) Relative mRNA levels of genes involved in hepatic TG synthesis. (D-F) Relative mRNA

460 levels of genes involved in fatty acid transport. (G-N) Relative mRNA levels of genes involved

461 in fatty acid oxidation. *$p<0.05$, **$p<0.01$. Date presented as mean±SEM, n=6.

462   **Figure 5**. Effects of stress and as1842856 on cholesterol metabolism-related genes in mice after

463   6 weeks. Liver was harvested and cholesterol metabolism-related genes were determined by

464   realtime-PCR. *$p<0.05$, **$p<0.01$. Date presented as mean±SEM, n=6.

465   **Figure 6**. As1842856 protect Hepa1-6 cells against corticosterone-induced liver lipid deposition.

466   Effects of corticosterone and as1842856 on FoxO1 in Hepa1-6 cells after 48h. Corticosterone

467   (1μM) increased the protein (A and B) and mRNA lever (E) of FoxO1 expression and its

468   downstream gene, PEPCK (F), AND decreased the lever of *p*-FoxO1 protein (C) and *p*-

469   FoxO1/FoxO1 (D), while as1842856 (1μM) could reverse this effect except the protein and

470   mRNA lever of FoxO1 expression. *$p<0.05$,**$p<0.01$,***$p<0.0001$. Date presented as

471   mean±SEM, n=3.

472   **Figure 7**. Effects of corticosterone and as1842856 on lipid deposition and TG synthesis genes in

473   Hepa 1-6 cells after 48h. (A) Representative images showed above from four groups. After

474   treatment with 1μm Corticosterone or/and 1μm as1842856 for 48h, the cells were stained with

475   Oil red O. (B) Corticosterone increased the genes' expression of Fasn, while as1842856 could

476   reverse this effect. (C and D) There was no significant difference among the four groups in the

477   genes' expression of ACC1 and SCD1. ***$p<0.001$. Date presented as mean±SEM, n=3.

Sequences of primers used for qRT-PCR.

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1   **Table 1 Sequences of primers used for qRT-PCR**

Gene name	Sequence (5' – 3')
FoxO1-F1	AGTGGATGGTGAAGAGCGTG
FoxO1-R1	GAAGGGACAGATTGTGGCGA
Fasn-F1	CAAGTGTCCACCAACAAGCG
Fasn-R1	GGAGCGCAGGATAGACTCAC
Pepck-F1	TGCGGATCATGACTCGGATG
Pepck-R1	AGGCCCAGTTGTTGACCAAA
Scd1-F1	GGCTAGCTATCTCTGCGCTC
Scd1-R1	GAACTGCGCTTGGAAACCTG
HMGCoAR-F1	ACGATCCTTCCTTATTGGCGG
HMGCoAR-R1	CTCCGGATCTCAATGGAGGC
Fatp1-F1	GGCAAGCTCCAGCACAGGAT
Fatp1-R1	ACCCACGTACACACAGAACG
G6Pase-F1	CGACTCGCTATCTCCAAGTGA
G6Pase-R1	GTTGAACCAGTCTCCGACCA
SREBP1c-F1	GATGTGCGAACTGGACACAG
SREBP1c-R1	CATAGGGGGCGTCAAACAG
Cyp7a1-F1	GGGATTGCTGTGGTAGTGAGC
Cyp7a1-R1	GGTATGGAATCAACCCGTTGTC
Abcg1-F1	CTTTCCTACTCTGTACCCGAGG
Abcg1-R1	CGGGGCATTCCATTGATAAGG
Fabp1-F1	ATGAACTTCTCCGGCAAGTACC
Fabp1-R1	CTGACACCCCCTTGATGTCC
Cd36-F1	ATGGGCTGTGATCGGAACTG
Cd36-R1	GTCTTCCCAATAAGCATGTCTCC
Acc1-F1	ATGGGCGGAATGGTCTCTTTC
Acc1-R1	TGGGGACCTTGTCTTCATCAT
PPARα-F1	AGAGCCCCATCTGTCCTCTC
PPARα-R1	ACTGGTAGTCTGCAAAACCAAA
PPARγ-F1	TCGCTGATGCACTGCCTATG
PPARγ-R1	GAGAGGTCCACAGAGCTGATT
Pdk4-F1	AGGGAGGTCGAGCTGTTCTC
Pdk4-R1	GGAGTGTTCACTAAGCGGTCA
Cpt1a-F1	CTCCGCCTGAGCCATGAAG
Cpt1a-R1	CACCAGTGATGATGCCATTCT
Acox1-F1	TAACTTCCTCACTCGAAGCCA
Acox1-R1	AGTTCCATGACCCATCTCTGTC
Lcad-F1	TCTTTTCCTCGGAGCATGACA
Lcad-R1	GACCTCTCTACTCACTTCTCCAG

Mcad-F1	AGGGTTTAGTTTTGAGTTGACGG
Mcad-R1	CCCCGCTTTTGTCATATTCCG
Ucp2-F1	TGCACTCCTGTGTTCTCCTG
Ucp2-R1	GGGACCTTCAATCGGCAAGA
β-actin-F1	TTCTTGGGTATGGAATCCTGT
β-actin-R1	AGCACTGTGTTGGCATAGAG

Figure 1

FoxO1 specific inhibitor as1842856 enhances FoxO1 phosphorylation in mice liver after 6 weeks.

Chronic stress increased the protein (A and B) and mRNA lever (E) of FoxO1 expression and its downstream genes, G6Pase (F) and PEPCK (G), and decreased the lever of p-FoxO1 protein(C) and p-FoxO1/FoxO1 (D), while as1842856 could reverse this effect except the protein and mRNA lever of FoxO1 expression. *$p<0.05$□***$p<0.001$. Date presented as mean±SEM, n=6.
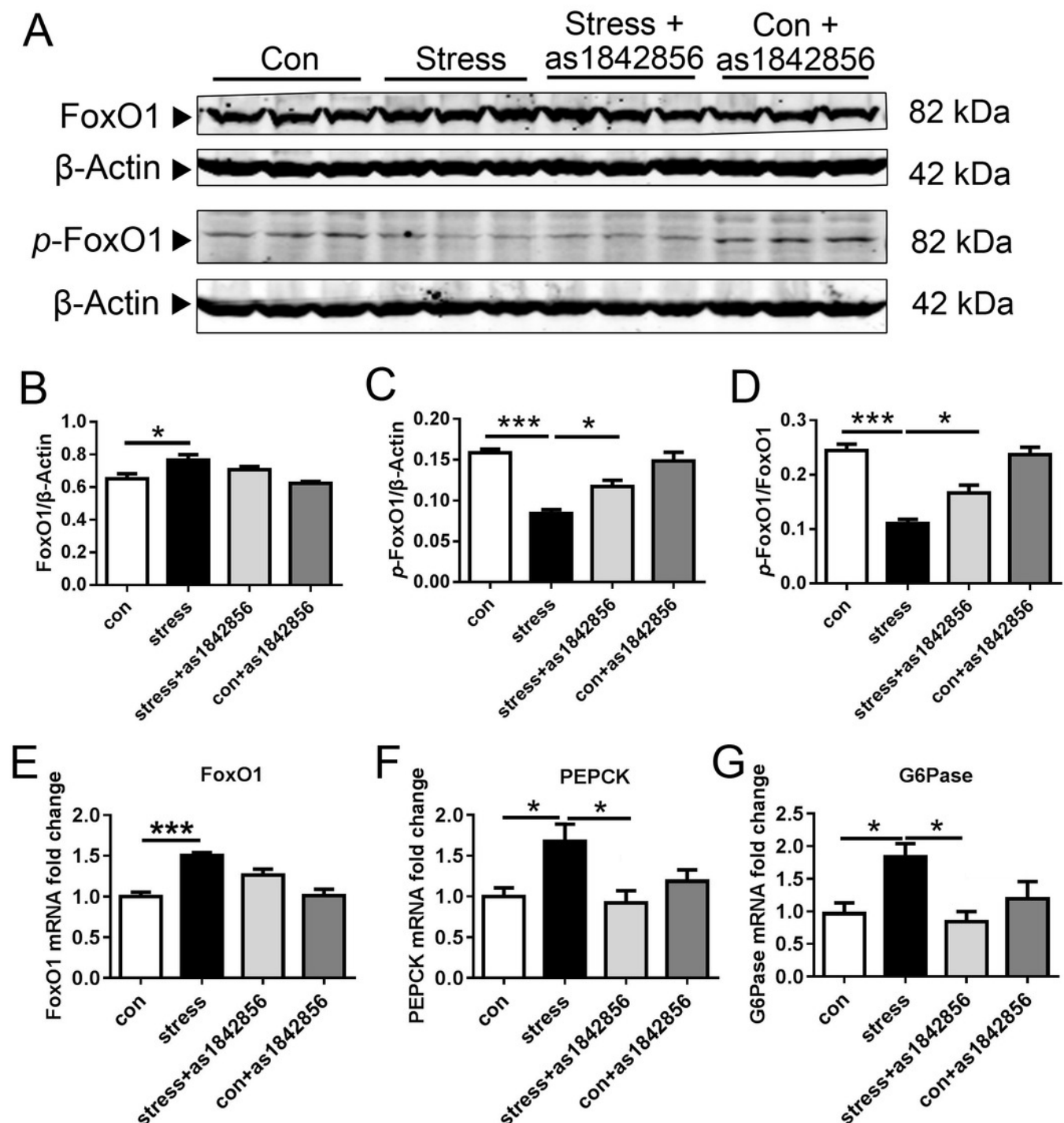
Figure 2

As1842856 does not affect weight, food intake and glucose metabolic parameters in mice after 6 weeks.

(A) Changes in food intake. (B) Changes in body weight. (C) Profiles of blood glucose concentration as function of time upon intraperitoneal injection of glucose (D) Profiles of glucose concentration (percentage of initial value) as a function of time upon intraperitoneal injection of insulin. (E-G) Blood insulin, postprandial blood glucose (PBG), and fasting plasma glucose (FPG). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Date presented as mean±SEM, n=6.
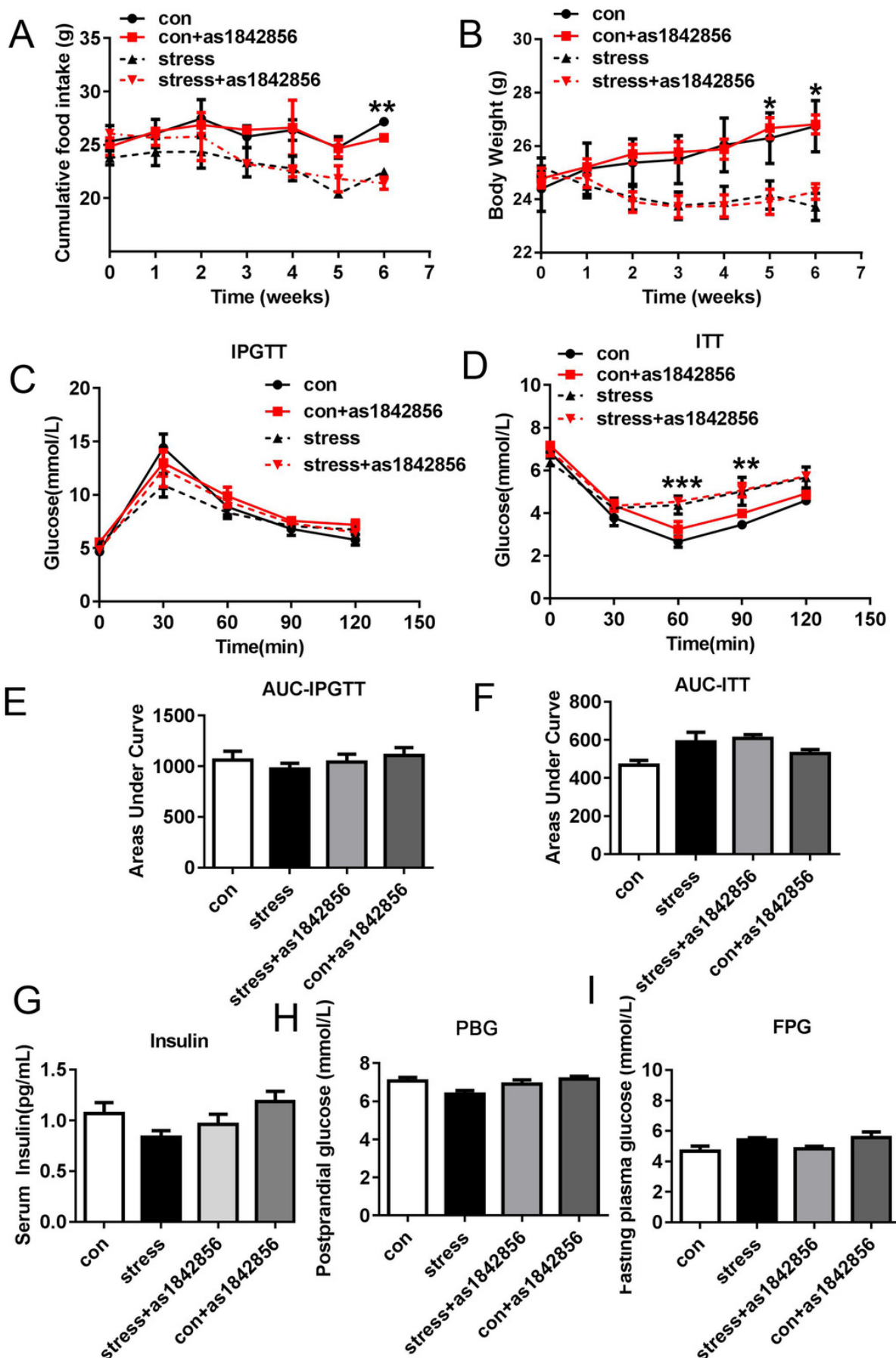
Figure 3

As1842856 protect mice against stress-induced liver lipid deposition.

(A) Representative slides showed hematoxylin and eosin (HE)-stain and Oil red O-stain liver sections from four groups. (B and C) Liver TG and T-CHO concentrations were detected by biochemical test in liver tissue homogenate. (D, E, and F) TG, FFA, and T-CHO concentrations in serum. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Date presented as mean±SEM, n=6.
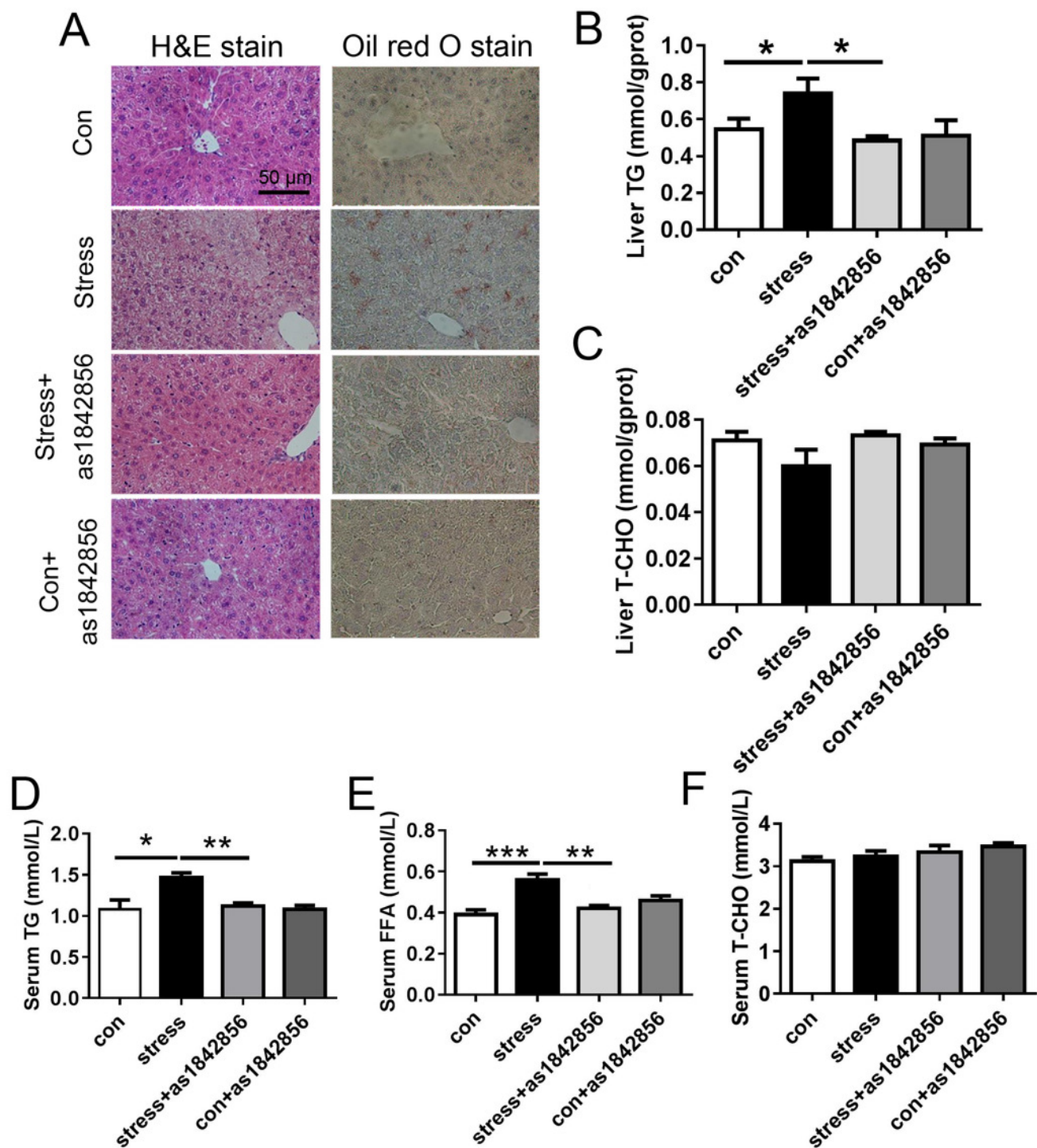
Figure 4

Effects of stress and as1842856 on liver lipid metabolism genes in mice after 6 weeks.

(A-C) Relative mRNA levels of genes involved in hepatic TG synthesis. (D-F) Relative mRNA levels of genes involved in fatty acid transport. (G-N) Relative mRNA levels of genes involved in fatty acid oxidation. *p<0.05, **p<0.01. Date presented as mean±SEM, n=6.
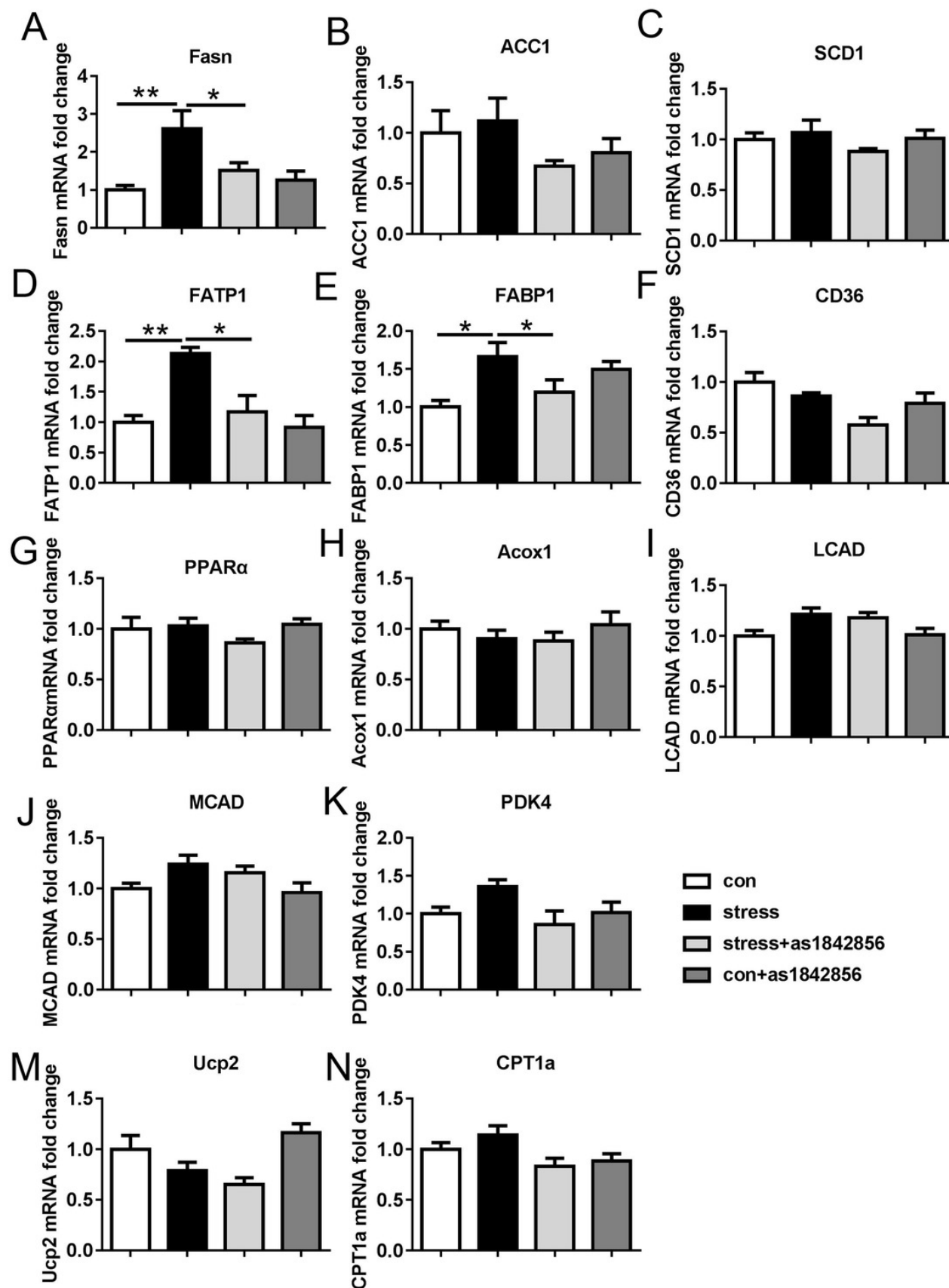
Figure 5

Effects of stress and as1842856 on cholesterol metabolism-related genes in mice after 6 weeks.

Liver was harvested and cholesterol metabolism-related genes were determined by realtime-PCR. *p*<0.05, **p*<0.01. Date presented as mean±SEM, n=6.
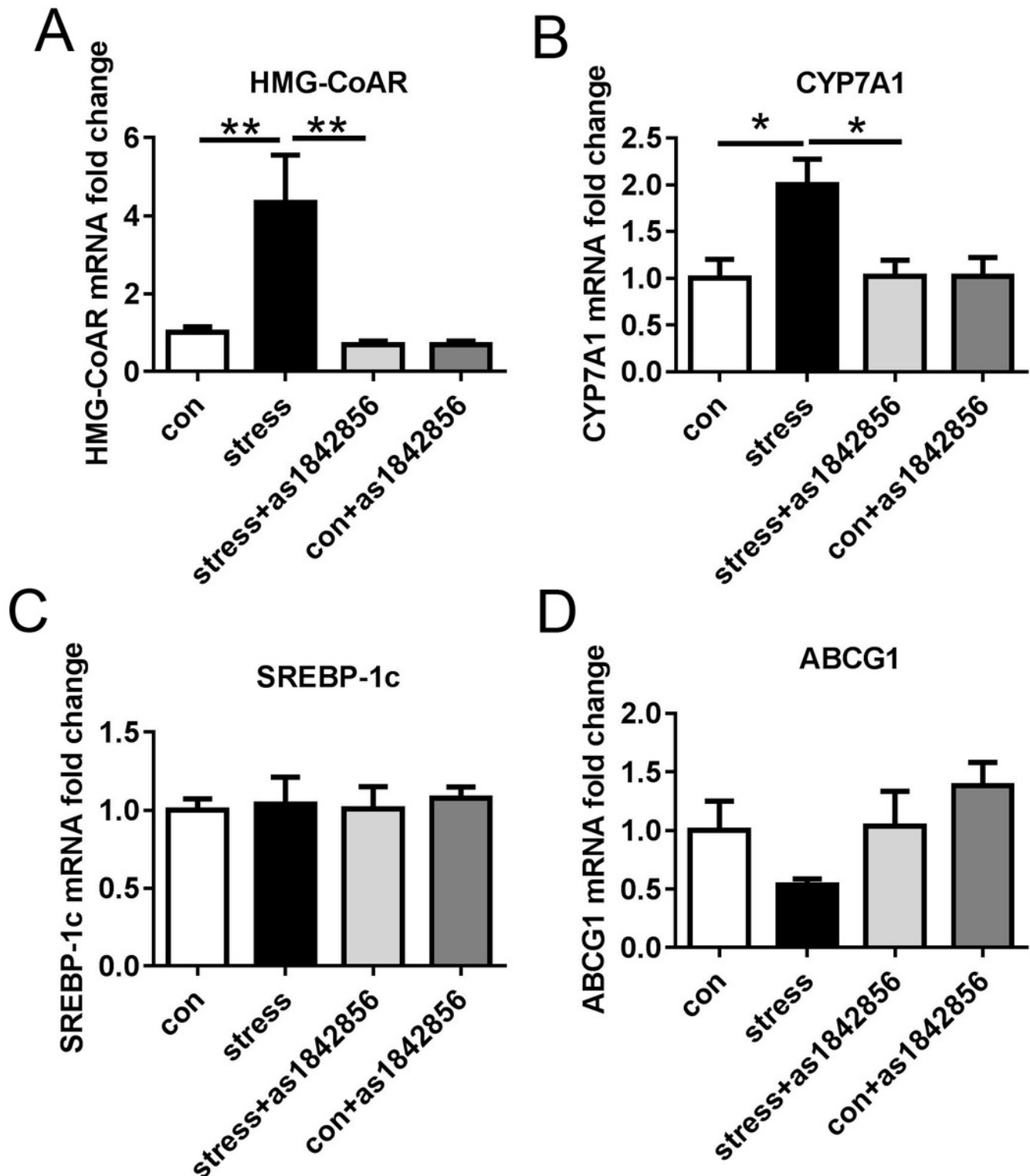
Figure 6

As1842856 protect Hepa1-6 cells against corticosterone-induced liver lipid deposition.

Effects of corticosterone and as1842856 on FoxO1 in Hepa1-6 cells after 48h. Corticosterone (1μM) increased the protein (A and B) and mRNA lever (E) of FoxO1 expression and its downstream gene, PEPCK (F), AND decreased the lever of p-FoxO1 protein (C) and p-FoxO1/FoxO1 (D), while as1842856 (1μM) could reverse this effect except the protein and mRNA lever of FoxO1 expression. *$p<0.05$ **$p<0.01$***p<0.0001. Date presented as mean±SEM, n=3.
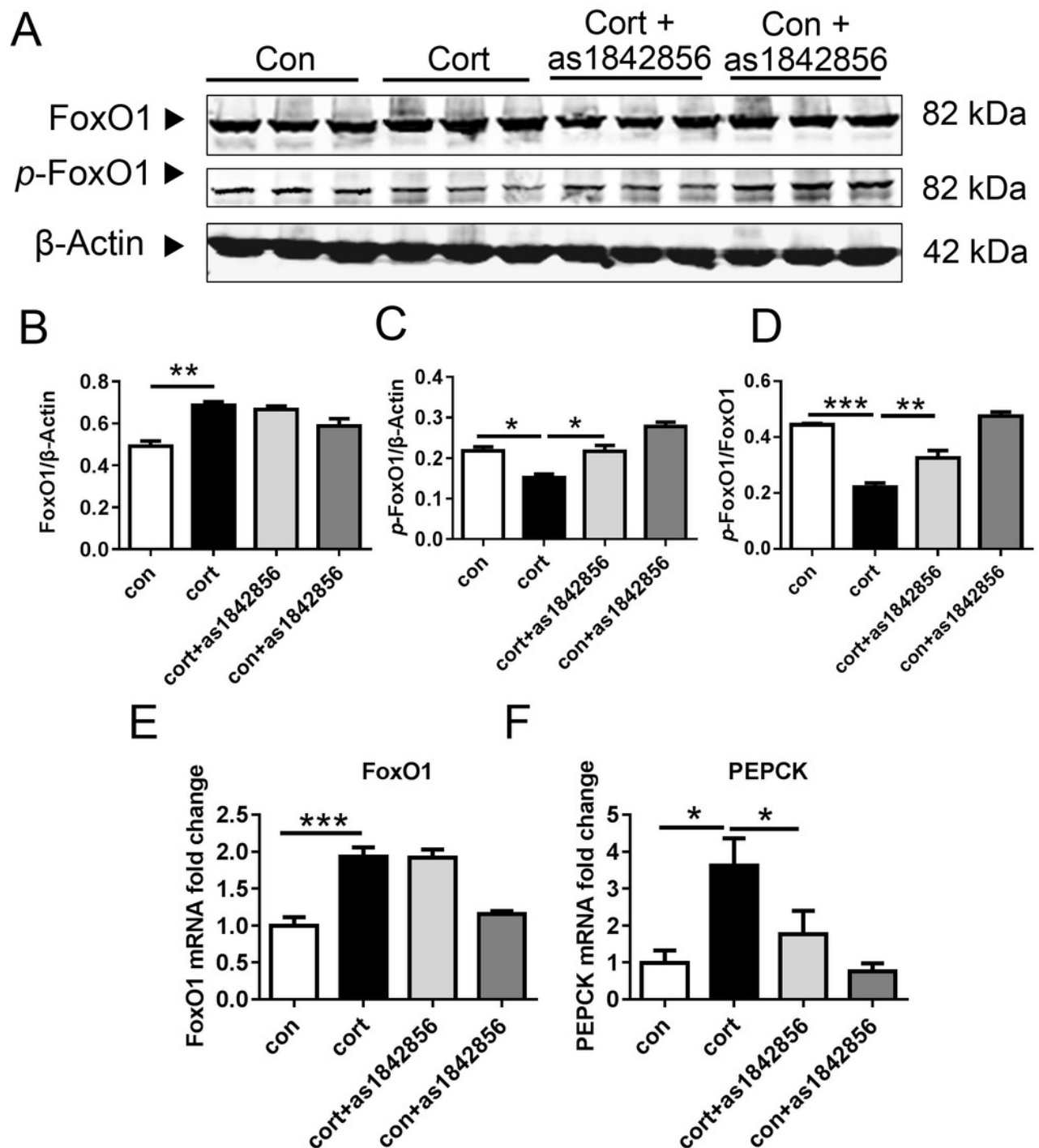
Figure 7

Effects of corticosterone and as1842856 on lipid deposition and TG synthesis genes in Hepa 1-6 cells after 48h.

(A) Representative images showed above from four groups. After treatment with 1μm Corticosterone or/and 1μm as1842856 for 48h, the cells were stained with Oil red O. (B) Corticosterone increased the genes' expression of Fasn, while as1842856 could reverse this effect. (C and D) There was no significant difference among the four groups in the genes' expression of ACC1 and SCD1. ***$p<0.001$. Date presented as mean±SEM, n=3.