

Adenovirus-mediated expression of SIK1 improves hepatic glucose and lipid metabolism in type 2 diabetes mellitus rats

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1 **Abstract**

2 **AIM:** In this study, we investigated the role and mechanism of Salt-induced kinase
3 1 (SIK1) in regulation of hepatic glucose and lipid metabolism in a high-fat food
4 (HFD) and streptozocin (STZ)-induced type 2 diabetes mellitus (T2DM) rat model.

5 **Methods:** A diabetic rat model treated with HFD plus low-dose STZ was
6 developed and was transduced to induce a high expression of SIK1 in vivo via a
7 tail-vein injection of a recombinant adenoviral vector. The effects on hepatic
8 glucogenetic and lipogenic gene expression, systemic metabolism and pathological
9 changes were then determined.

10 **Results:** In T2DM rats, SIK1 expression was reduced in the liver. Overexpression
11 of SIK1 improved hyperglycaemia, hyperlipidaemia and fatty liver, reduced
12 the expression of cAMP-response element binding protein (CREB)-regulated
13 transcription co-activator 2 (CRTC2), phosphoenolpyruvate carboxykinase (PEPCK),
14 glucose-6-phosphatase (G6Pase), pS577 SIK1, sterol regulatory element
15 binding-protein-1c (SREBP-1c) and its target genes, including acetyl-CoA
16 carboxylase (ACC) and fatty acid synthase (FAS), and increased the expression of
17 SIK1, pT182 SIK1 and pS171 CRTC2 in diabetic rat livers with the suppression of
18 gluconeogenesis and lipid deposition.

19 **Conclusion:** SIK1 plays a crucial role in the regulation of glucose and lipid
20 metabolism in the livers of HFD/STZ-induced T2DM rats, where it suppresses hepatic
21 gluconeogenesis and lipogenesis by regulating the SIK1/CRTC2 and SIK1/SREBP-1c
22 signalling pathways. Strategies to activate SIK1 kinase in liver would likely have

23 beneficial effects in patients with T2DM and nonalcoholic fatty liver disease
24 (NAFLD).

25 **Keywords:** adenovirus; Salt-induced kinase 1; gluconeogenesis; lipogenesis; type
26 2 diabetes mellitus; diabetic rats

27 **Introduction**

28 T2DM is characterized by hyperglycemia and insulin resistance (IR) and is the
29 foremost type of diabetes around the world [1]. Diabetes complications such as
30 hyperlipidemia and NAFLD account for an increasing proportion of annual health
31 care costs. Tight glucose control has been associated with a reduced incidence of
32 diabetes complications, underscoring efforts to characterize regulators that function
33 importantly in the pathogenesis of T2DM [2].

34 SIK1, a serine/threonine protein kinase, belongs to the AMP-activated protein
35 kinase (AMPK) [3]. As an energy sensor, AMPK markedly inhibits hepatic
36 glucogenesis and lipogenesis by transcriptional control [4, 5]. In addition, Liver
37 kinase B 1 (LKB1), a major upstream kinase of AMPK, phosphorylates SIK1 at
38 Thr182 in the activation loop (A-loop) of the kinase domain, which is essential for
39 switching on the SIK1 kinase activity, thus resulting in the increase of the kinase
40 activity of SIK1 [6, 7]. Treatment with adrenocorticotropic hormone (ACTH) and the
41 subsequent phosphorylation of the regulatory domain at Ser-577 by protein kinase A
42 (PKA) makes SIK1 translocate to the cytoplasm and lose its repressive properties[3,
43 8]. Knockdown of SIK1 in mice promoted both fasting hyperglycaemia and
44 gluconeogenic gene expression. Conversely, mice treated with adenovirus-expressed

45 SIK1 (Ad-SIK1) exhibited fasting hypoglycaemia and reduce gluconeogenic gene
46 expression [9]. Ad-SIK1 was also effective in reducing blood glucose levels in fasted
47 db/db diabetic mice [9]. These observations demonstrate a key role of SIK1 on
48 glucose metabolism in vivo.

49 The liver is the major organ responsible for glucose production. Hepatic glucose
50 production mainly comes from gluconeogenesis and is critical for maintaining
51 normoglycemia in the fasting state [10]. The cAMP response element binding protein
52 (CREB) and its co-activator, CRTC2, play crucial roles in signal-dependent
53 transcriptional regulation of hepatic gluconeogenesis. CREB transcriptional activity is
54 required for fasting gluconeogenesis [11]. CRTC2 is a key regulator of fasting glucose
55 metabolism that acts through the CREB to modulate glucose output. Phosphorylation
56 of CRTC2 at Ser171 by AMPK results in the inhibition of the nuclear translocation of
57 CRTC2; subsequently, the cytoplasmic localization of CRTC2 prevents its
58 combination with CREB elements [9, 12]. Thus, gluconeogenesis is restrained.
59 Conversely, sequestered in the cytoplasm under feeding conditions, CRTC2 is
60 dephosphorylated and transported to the nucleus where it enhances CREB-dependent
61 transcription in response to fasting stimuli [9]. CRTC2 has been recently found to be a
62 substrate of SIK1 in vivo [9, 12]. SIK1 had been previously identified as a modulator
63 of CREB-dependent transcription in adrenocortical carcinoma cells [13]. Moreover,
64 CREB was found to occupy the SIK1 promoter in chromatin immunoprecipitation
65 assays of primary rat hepatocytes; CRTC2 was recruited to this promoter in response
66 to forskolin treatment [9]. The mRNA levels of CRTC2, PEPCK and G6Pase in

67 SIK1-deficient primary rat hepatocytes were increased, while SIK1 overexpression
68 suppressed the CRTC2 activity [9]. A recent report has shown that the selective
69 salt-induced kinase (SIK) inhibitor HG-9-91-01 promotes dephosphorylation of
70 CRTC2, resulting in enhanced gluconeogenic gene expression and glucose production
71 in hepatocytes, but this effect is abolished when an HG-9-91-01-insensitive mutant
72 SIK is introduced [14]. Interestingly, in primary rat hepatocytes, SIK1 phosphorylated
73 CRTC2 at Ser 171 and in turn promoted its export to the cytoplasm, thereby inhibiting
74 the expression of downstream gluconeogenic genes such as PEPCK and G6Pase [9],
75 suggesting that regulation of CRTC2 activity by SIK1 may be crucial for inhibiting
76 excessive hepatic glucose output. Therefore, the SIK1/CRTC2 signalling pathway will
77 probably represent a novel strategy for suppressing hepatic gluconeogenesis and
78 ameliorating hyperglycaemia. Although SIK1 is implicated in regulation of CRTC2
79 and hepatic glucose output, the glycometabolism of the kinase remains
80 uncharacterized in the HFD/STZ-induced T2DM rat model.

81 In addition, the liver is also one of the major organs regulating lipid metabolism
82 [15]. Hepatic lipogenesis contributes to accumulation of fat in the liver [16].
83 SREBP-1c acts as a master transcriptional regulator for the hepatic lipogenesis by
84 activating its target genes, such as FAS and ACC. Moreover, SREBP-1c is shown to
85 be a direct substrate for SIK1 in vitro [17]. SIK1 blocks lipogenesis by direct
86 phosphorylation of SREBP-1c on multiple serine residues [12]. Ectopic expression of
87 SIK1 in mouse livers reduces lipogenic gene expression and hepatic triglyceride
88 accumulation [12]. This effect was reversed by co-expression of a

89 phosphorylation-deficient Srebp1-c mutant [17]. A previous report has shown that
90 lipogenic genes, such as FAS and ACC, are up-regulated by SIK1 knockdown in
91 mouse liver, whereas overexpression of SIK1 reduces expression levels of SREBP-1c
92 target genes, suggesting that SIK1 could regulate lipogenic gene transcript [17].
93 SIK1-induced phosphorylation of SREBP-1c at Ser329 is thought to be critical for the
94 suppression of SREBP-1c transcription activity [17]. Our previous study
95 demonstrated that overexpression of SIK1 suppressed the expression of SREBP-1c
96 and its target genes in HepG2 cells cultured in a high glucose environment [18]. Thus,
97 modulation of SREBP-1c activity by SIK1 would provide an attractive means for the
98 regulation of hepatic lipogenesis. In the diabetic conditions, normal regulation of
99 gluconeogenesis and lipogenesis is disrupted; hence the SIK1/CRTC2 and
100 SIK1/SREBP-1c pathways may serve as therapeutic targets to modulate metabolic
101 disorders in diabetic patients with NAFLD.

102 To date, the role and mechanism of SIK1 in the liver of the HFD/STZ-induced
103 T2DM rat model remains completely unknown. Because the diabetic rat model treated
104 with HFD plus low-dose STZ replicates the natural history and metabolic
105 characteristics of human T2DM and develops most of the biochemical and
106 pathological symptoms [19-24] associated with T2DM in humans, the diabetic rat
107 model is particularly suitable for pharmaceutical research [25]. Thus, it is of interest
108 to define the effect of SIK1 on hepatic gluconeogenesis and lipogenesis of the
109 HFD/STZ-induced T2DM rat. In the present study, we generates a diabetic rat model
110 treated with HFD plus low-dose STZ and focuses on the role of SIK1 in the hepatic

111 gluconeogenic and lipogenic pathways and their effect on the resulting phenotype of
112 lower fasting glucose levels and ameliorated fatty liver disease. Meanwhile, we use a
113 recombinant SIK1-expressing adenovirus to obtain a high expression of SIK1 in vivo,
114 and then assess its affect on diabetes in the HFD/STZ-induced T2DM rat model. To
115 our knowledge, this is the first study to examine the effects of adenovirus-mediated
116 SIK1 overexpression on hepatic glucose and lipid metabolism in the
117 HFD/STZ-induced T2DM rats.

118 **Materials and Methods**

119 **Recombinant adenovirus production**

120 Ad-Sik1 and negative control adenovirus containing green fluorescent protein
121 (Ad-GFP) were purchased from Gene Chem Co., Ltd. (Shanghai, China). Ad-Sik1
122 and Ad-GFP were obtained with a titre of 1×10^{11} plaque forming units (PFU) /ml. The
123 recombinant adenovirus was stored at -80°C until use. Construction of both vectors
124 was described in S1 Appendix. Sik1-overexpressing rats were established by an
125 injection of Ad-Sik1 or Ad-GFP at an optimized dose of 5×10^9 PFU in $50\mu\text{l}$ (diluted
126 with physiological saline) via tail vein once a week for 8 weeks according to the
127 manufacturer's protocols. Meanwhile, the rats of the control and model groups
128 received physiological saline at the same dosage by tail vein injection.

129 **Animal treatments**

130 Thirty male wistar rats, three to four-weeks-old, weighing approximately 70-100 g,
131 were supplied by BEI JING HFK BIOSCIENCE CO., LTD (Beijing, China). The
132 protocol for using animals was approved by the research Ethics Committee of Tongji

133 Medical College, Huazhong University of Science and Technology (Protocol Number:
134 822). All animals were housed with two rats per cage in an air-conditioned room
135 (22°C ± 3°C, 50%-60% relative humidity) with a 12:12-hour light-dark cycle and
136 were initially fed normal chow and allowed to adapt to their environment for 1 week.
137 After acclimatization, all rats were randomly assigned to 2 groups. The control rats
138 were fed ad libitum with a normal diet and the other rats were fed ad libitum with a
139 HFD to induce diabetes [26]. Four weeks later, rats on HFD were injected with 36
140 mg/kg STZ (dissolved in citrate buffer, pH 4.5) intraperitoneally. Meanwhile, the
141 control rats were injected with the same volume of citrate buffer. Diabetes was
142 defined by fasting serum glucose >11.1 mmol/L 72 h after STZ injection. The diabetic
143 rats were randomly divided into three groups: diabetes mellitus (DM) group (n=6),
144 Ad-Sik1 group (n=8), and Ad-GFP group (n=6). The Ad-Sik1 and Ad-GFP groups
145 received an injection of Ad-Sik1 or Ad-GFP at an optimized dose of 5×10⁹ PFU via
146 tail vein once a week for 8 weeks. The DM and normal control groups were given an
147 equal volume of normal saline. The normal chow (13.68%, 64.44%, and 21.88% of
148 calories derived, respectively, from fat, carbohydrate, and protein) was provided by
149 the Laboratory Animal Center, Huazhong University of Science and Technology
150 (Wuhan, China). The high fat diet (rodent diet with 45% kcal fat) was purchased from
151 WANQIANJIAXING BIOTECHNOLOGY CO., LTD (Wuhan, China). Food intake,
152 water intake and blood glucose were monitored periodically. After an 8-week
153 treatment, the rats were weighed and sacrificed. Fasting blood was collected from the
154 ventral aorta and serum was separated for biochemical analysis. The liver was

155 removed and weighed. Part of the liver was fixed in 4% paraformaldehyde and
156 embedded in paraffin for hematoxylin and eosin (HE) staining and
157 immunohistochemical analysis. The rest of the liver was washed with normal saline
158 and stored at -80°C for RT-PCR and Western blot.

159 **Reagents**

160 STZ was purchased from Sigma (SaintLouis, Missouri, USA). RNAiso Plus was
161 purchased from TaKaRa (Dalian, China). SIK1 antibody was purchased from Novus
162 Biologicals, LLC (Cat #: 82417, Littleton, USA). SREBP-1c antibody, G6Pase and
163 CRTC2 (S171) antibody were purchased from Abcam (Cat #: ab28481, Cat #:
164 ab83690 and Cat #: ab203187, Cambrige, UK). CRTC2 antibody, SIK1 (S577)
165 antibody, SIK1 (T182) antibody, FAS antibody and ACC antibody were purchased
166 from Proteintech Group, Inc. (Cat #:12497-1-AP, Cat #: S4530-2, Cat #: S4529-2, Cat
167 #:10624-2-AP and Cat #:21923-1-AP, Rosemont, USA). PEPCK antibody and β-actin
168 antibody were purchased from Cell Signaling Technology, Inc. (Cat #: 12940 and Cat
169 #: 4967, Danvers, Massachusetts, USA). Goldview DNA dye and DNA Marker I were
170 purchased from TIANGEN BIOTECH (BEIJING) CO., LTD. Horseradish
171 peroxidase-conjugated goat anti-rabbit IgG was purchased from Bioworld Technology,
172 Inc. (Minnesota, USA) as secondary antibody.

173 **Biochemical assay**

174 Serum glucose was measured using enzymatic glucose-oxidase kits (Ruiyuan
175 Biotechnology Co., Ltd, Ningbo, China), TG and total cholesterol (TC) were
176 determined using enzymatic couple colorimetric kits (Huachen Biochemical Co., Ltd,

177 Shanghai, China).

178 **Histological Analysis**

179 Liver tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin,
180 sectioned into 4 μ m sections (Leica, Wetzlar, Germany), and stained with HE for
181 microscopic assessment (Olympus, Tokyo, Japan). The liver cryosections were
182 prepared for oil red O staining.

183 **Immunohistochemistry analysis**

184 The liver tissues were fixed with 4% paraformaldehyde for paraffin embedding.
185 The paraffin-embedded sections were subjected to immunohistochemical staining for
186 SIK1, CRTC2, PEPCK, G6pase, SREBP-1c, FAS and ACC in the liver. The tissue
187 sections were incubated with rabbit anti primary antibody (1:100). After washing with
188 PBST, the sections were incubated with secondary antibody, and the
189 diaminobenzidine method was used. Next, the SIK1, CRTC2, PEPCK, G6pase,
190 SREBP-1c, FAS, ACC and Insulin protein expressions were observed under an
191 optical microscope. All the sections were examined by light microscope. Optical
192 density (OD) was identified as expression intensity of positive staining in the liver
193 tissues, which was semiquantitatively analysed with Image-Pro Plus 6.0 software
194 (Media Cybernetics, Inc., USA).

195 **RT-PCR**

196 Total RNA samples were isolated from the rat liver using RNAiso Plus (D9109;
197 TaKaRa, Dalian, China). RNA samples were converted to cDNA using a RevertAid
198 First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the

199 manufacturer's instructions. Primers were designed using the nucleotide sequence and
200 synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Semi-quantitative
201 PCR conditions were 95°C for 2 minutes, 94°C for 30 seconds, followed by 35 cycles
202 at 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 2 minutes. PCR products
203 were separated by 1.5% agarose gel electrophoresis and visualised under ultraviolet
204 light using a JS-680B Bio-Imaging System camera (PEIQING Technology Limited,
205 Shanghai, China). Relative band intensities of each sample were calculated after being
206 normalized to the band intensity of β-actin. The sequences of the primers used in this
207 study are listed in S1 Table.

208 **Western blot**

209 SIK1, SIK1 (S577), SIK1 (T182), CRTC2, CRTC2 (S171), PEPCK, G6pase,
210 SREBP-1c, FAS and ACC protein expression were determined by Western blotting,
211 which was performed according to standard procedures. The protein concentration in
212 tissue lysates was measured with a BCA protein assay kit (Boster, Wuhan, China)
213 according to the manufacturer's instructions. Protein lysates extracted from the rat
214 liver tissue were electrophoresed using 8-12% sodium dodecyl sulphate-
215 polyacrylamide gel electrophoresis (SDS-PAGE) for separation. Then, samples were
216 transferred onto a nitrocellulose membrane. The membrane was next incubated in 5%
217 milk in a mixture of Tris-buffered saline and Tween 20 (TBST) for 1 h at room
218 temperature to block the membrane. The proteins were incubated with the primary
219 antibody overnight at 4°C (SIK1, 1:1000; SIK1 (S577), 1:2000; SIK1 (T182), 1:2000;
220 CRTC2, 1:500; CRTC2 (S171), 1:500; PEPCK, 1:1000; G6pase, 1:800; SREBP-1c,

221 1:500; FAS, 1:500; ACC, 1:500; β -actin, 1:1000). After washing the membrane 5
222 times in TBST, the membrane was incubated with secondary antibody for 30 min at
223 room temperature. Finally, the protein was detected with electrochemiluminescence
224 (ECL) Western blotting reagents. The optical density (OD) of protein bands was
225 quantified using Image J 1.48 software (National Institutes of Health, USA). The
226 results are expressed as the ratio between the OD value of a target band to the OD
227 value of β -actin.

228 **Statistical analysis**

229 All data are analysed by one-way ANOVA or two-way ANOVA using GraphPad
230 Prism 5.0 software (GraphPad Software, San Diego, CA, USA), and the results are
231 presented as the mean \pm SD. $P<0.05$ was considered statistically significant. In the
232 figures and tables, $*P<0.05$; $**P<0.01$; $***P<0.001$. ns, not significant.

233 **Results**

234 **Effects on body weight, liver weight, FBG, TG and TC**

235 **Figure 1. Effects on body weight, liver weight, FBG, TG and TC in**
236 **HFD/STZ-induced diabetic rats.** (A) Body weight; (B) Liver weight; (C) Liver
237 index; (D) Serum glucose levels ; (E) Serum TG levels ; (F) Serum TC levels . The
238 results are expressed as the mean \pm SD. Significant differences are indicated as
239 $*P<0.05$, $**P<0.01$, $*** P<0.001$. ns, not significant.

240 The HFD/STZ-induced diabetic rats showed classic diabetic symptoms of polyuria,
241 polydipsia and weight loss. These symptoms are related to the presence of
242 hyperglycaemia (blood glucose level fluctuation from 20.09 to 30.61 mmol/L).

243 Neither the Ad-SIK1 group nor the Ad-GFP group showed significant differences in
244 blood glucose or body weight. As shown in Table 1, serum glucose, TG and TC were
245 significantly higher in the DM group compared to the control group. Intriguingly,
246 serum TG was remarkably decreased ($P<0.05$), but serum TC slightly reduced in the
247 Ad-SIK1 group compared to the DM group. Although Ad-SIK1 administration
248 attenuated the HFD/STZ-induced increase in the serum TC level, no significant
249 difference was observed.

250 **Histological examination of liver**

251 **Fig 2. Effects on histology of liver of HFD/STZ-induced diabetic rats.** (A) Liver
252 tissue sections were stained with HE ($\times 200$); (B) oil red O to observe liver lipid
253 content. Scale bar is 50 μ m.

254 The typical HE and oil red O staining results obtained upon histological
255 examination are shown in [Fig 1](#). In consonance with the biochemical data, the staining
256 of liver tissues with HE and oil red O revealed an accumulation of lipid droplets in the
257 liver of the DM group, whereas lipid droplets were rare in the liver of the Ad-SIK1
258 group. Thus, Ad-SIK1 treatment significantly reduced fat deposition compared with
259 the DM group, indicating that Ad-SIK1 administration could markedly improve
260 steatosis. Therefore, these results confirmed the protective effect of SIK1
261 overexpression against histological changes in the liver of HFD/STZ-induced diabetic
262 rats.

263 **Immunohistochemical staining of genes related to hepatic
264 glucose and lipid metabolism.**

265 **Fig.3 Effects on immunohistochemical staining of SIK1, CRTC2, PEPCK,**
266 **G6pase, SREBP-1c, FAS and ACC in liver.** (A) immunohistochemical staining of
267 **SIK1, CRTC2, PEPCK and G6pase; (B)** immunohistochemical staining of
268 **SREBP-1c, FAS and ACC.** Immunohistochemical staining images are 200 times
269 larger under light microscopy Data are presented as the mean \pm SD. Significant
270 differences are indicated as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ns, not significant.
271 Scale bar is 50 μ m.

272 Fig 3 illustrates the immunohistochemical photomicrographs of SIK1, CRTC2,
273 PEPCK, G6pase, SREBP-1c, FAS and ACC in the liver of rats. In the DM and
274 Ad-GFP groups, SIK1-positive staining was much weaker than that in the Ad-SIK1
275 ($p<0.001$). Notably, SIK1 overexpression significantly increased the reduced
276 SIK1-positive staining in the liver of diabetic rats. In contrast, CRTC2, PEPCK,
277 G6pase, SREBP-1c, FAS and ACC stainings were much stronger in the DM and
278 Ad-GFP groups than those in the Ad-SIK1 group ($p<0.001$). Obviously, Ad-SIK1
279 treatment inhibited this enhanced positive staining. In addition, we also verified that
280 SIK1 overexpression inhibited CRTC2 nuclear translocation in the liver tissues *via*
281 immunohistochemical staining. The nuclear expression of CRTC2 protein was
282 obviously increased in the DM and Ad-GFP groups compared to the normal control
283 group; however, the treatment with Ad-SIK1 inhibited the nuclear translocation of the
284 CRTC2 protein.

285 **SIK1 overexpression results in the inhibition of the hepatic**
286 **gluconeogenic program in HFD/STZ-induced diabetic rats.**

287 **Fig 4. Effects on mRNA and protein expression of genes related to glucose**
288 **metabolism in diabetic rats.** (A) Protein levels of SIK1, CRTC2, PEPCK and
289 G6pase in liver; (B) mRNA (relative fold change) levels of SIK1, CRTC2, PEPCK
290 and G6pase in liver. Fold expression levels were measured relative to the expression
291 of β -actin (internal control). Data are presented as the mean \pm SD. Significant
292 differences are indicated as * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ns, not significant. n=6
293 in the control group; n=5 in the DM group; n=6 in the Ad-SIK1 group.

294 To determine whether SIK1 overexpression ameliorates hyperglycaemia by
295 decreasing endogenous glucose production in the liver, we measured the mRNA and
296 protein of SIK1, CRTC2, PEPCK and G6pase in the liver. RT-PCR analysis showed
297 that SIK1 was significantly elevated in the Ad-SIK1 group compared to the DM group,
298 while CRTC2, PEPCK and G6pase were significantly reduced, indicating an
299 inhibitory effect of SIK1 in liver gluconeogenesis (Fig 3B). Indeed, the Western blot
300 results showed that SIK1 was significantly decreased in the DM group compared to
301 the control group, whereas CRTC2, PEPCK and G6pase were significantly elevated in
302 the DM group. SIK1 overexpression significantly increased the protein level of SIK1,
303 but decreased the protein levels of CRTC2, PEPCK and G6pase in liver compared
304 with the DM group (Fig 3A). Meanwhile, the phosphorylation level of SIK1 at Ser577
305 was drastically reduced, whereas the level of pT182 SIK1 and pS171 CRTC2 was
306 significantly increased in the Ad-SIK1 group compared with the DM and Ad-GFP
307 groups. These results suggest that SIK1 could inhibit the hepatic gluconeogenic
308 program in HFD/STZ-induced diabetic rats by regulating the SIK1/CRTC2 signalling

309 pathway.

310 **SIK1 inhibits the hepatic lipogenic program in**
311 **HFD/STZ-induced diabetic rats.**

312 **Fig 5. Effects on mRNA and protein expression of genes related to lipid**

313 **metabolism in diabetic rats.** (A) Protein levels of SREBP-1c, FAS and ACC in liver;

314 (B) mRNA (relative fold change) levels of SREBP-1c, FAS and ACC in liver. Fold

315 expression levels were measured relative to the expression of β -actin (internal control).

316 Data are presented as the mean \pm SD. Significant differences are indicated as * $p<0.05$,

317 ** $p<0.01$, *** $p<0.001$. ns, not significant. n=6 in the control group; n=5 in the DM

318 group; n=6 in the Ad-SIK1 group.

319 To investigate the underlying molecular mechanism of the hypolipidaemic effect

320 of SIK1 overexpression on the diabetic rats, we measured the mRNA and protein of

321 SREBP-1c, FAS and ACC in the liver. Notably, the mRNA expression of SREBP-1c,

322 FAS and ACC in the liver of the DM group increased significantly compared to the

323 control group (Fig 4B). However, Ad-SIK1 treatment significantly reduced the

324 mRNA expression of SREBP-1c, FAS and ACC in the liver compared with the DM

325 group, suggesting the mitigative role of SIK1 on fatty liver. Meanwhile, Western blot

326 analysis revealed that SREBP-1c, FAS and ACC were markedly downregulated in the

327 Ad-SIK1 group compared with the DM group (Fig 4A). Taken together, these

328 findings indicate that the relieving effect of SIK1 overexpression on fatty liver was

329 associated with a significant reduction in the expression of lipogenetic genes such as

330 SREBP-1c, FAS and ACC.

331 **Discussion**

332 Since the discovery of the SIK family, the roles of SIK isoforms (SIK1/2/3) in
333 glucose and lipid metabolism have been extensively investigated [6-9, 12, 14, 17, 18].
334 However, the biological function of SIK1 remains poorly understood in
335 HFD/STZ-induced T2DM rats. In this study, we found that the expression of hepatic
336 SIK1 was markedly decreased in the HFD/STZ-induced T2DM rat model and that
337 administration of Ad-SIK1 lowered fasting blood glucose and ameliorated fatty liver
338 disease, suggesting that a reduction of SIK1 may contribute to the glucose and lipid
339 metabolism disorder in diabetes. Metformin, a widely used hypoglycemic drug, which
340 attenuated hyperglycaemia and NAFLD in HFD/STZ-induced diabetic rats [27],
341 increased SIK1 expression levels in HepG2 cells cultured in high glucose conditions
342 [18]. These findings indicate that SIK1 may be associated with the pathogenesis of
343 T2DM and NAFLD.

344 This study established a model of T2DM rats treated with HFD plus low-dose
345 STZ. The HFD/STZ-induced diabetic rats exhibited classic diabetic symptoms of
346 polyuria, polydipsia and weight loss. The T2DM rats also showed high blood glucose,
347 high TG and TC than normal control rats, which was consistent with previous report
348 [26]. We further determined the effect of Ad-SIK1 on hepatic glucose metabolism in
349 HFD/STZ-induced diabetic rats. Although the function of SIK1 in the
350 HFD/STZ-induced diabetic rat model has not been reported, but RNAi-based
351 knockdown strategies and genetic diabetic mouse models have revealed its role in the
352 regulation of glucose metabolism. First, overexpression of SIK1 reduced fasting blood

353 glucose and gluconeogenic gene expression in db/db mice, while Ad-SIK1 RNAi
354 promoted them [9]. Second, SIK1 activity was reduced in the livers of db/db diabetic
355 mice[12]. In the present study, the administration of Ad-SIK1 resulted in amelioration
356 of hyperglycaemia in HFD/STZ-induced diabetic rats, suggesting that exogenous
357 SIK1 might have a protective effect on T2DM. This is obviously the first report that
358 suggests the potential of adenovirus-mediated SIK1 gene transfer in the management
359 of hyperglycaemia in the HFD/STZ-induced T2DM rat model. More importantly, we
360 for the first time demonstrated that SIK1 mRNA and protein expression were
361 significantly reduced in the livers of HFD/STZ-induced diabetic rats, which was
362 consistent with our previous in vitro studies [8, 18], suggesting that the expression of
363 SIK1 is inhibited in diabetic states.

364 Phosphorylation at Thr182 by LKB1 is essential for switching on the SIK1
365 kinase activity [7, 28]. The Thr182 of SIK1 is phosphorylated by LKB1, resulting in
366 conversion from inactive SIK1 to the active form [6]. Consistent with previous in
367 vitro observations [8, 18], this in vivo study indicated that the level of Thr-182
368 phosphorylation, as well as the expression of SIK1 mRNA and protein, was
369 downregulated in the livers of HFD/STZ-induced diabetic rats, suggesting that the
370 SIK1 kinase activity may be suppressed in diabetic states. As expected, Ad-SIK1
371 treatment significantly elevated the level of pT182 SIK1 compared to the DM group.
372 In addition, the intracellular distribution of SIK1 is closely associated with its
373 functional activity. Ser-577 is a determinant of the intracellular distribution of SIK1.
374 Moreover, inactive SIK1 as well as CREB-repressing active SIK1 are present as

375 Ser577-dephosphorylated forms and are localized in the nucleus [3, 6, 8].
376 Phosphorylation of SIK1 at Ser577, which causes the nucleus export of SIK1, leads to
377 a reduction of the transcriptional modulating activity of SIK1 [6]. On the basis of the
378 ability of Ser 577 phosphorylation to decrease the transcriptional modulating activity
379 of SIK1, we reasoned that phosphorylation level of Ser577 indicated the ability of
380 SIK1 to suppress CREB. Thus, we examined the phosphorylation level of SIK1 at
381 Ser577 in the livers of HFD/STZ-induced diabetic rats. *In vivo*, in the DM and
382 Ad-GFP groups, the phosphorylation of SIK1 at Ser577 was elevated, whereas the
383 expression of SIK1 was reduced, which were reversed by Ad-SIK1 administration,
384 suggesting the possibility that SIK1 acts as a modulator of CREB-dependent
385 transcription in the livers of HFD/STZ-induced diabetic rats.

386 The remarkable feature of T2DM is elevated fasting blood glucose. Dysregulated
387 gluconeogenesis contributes to hyperglycaemia in diabetic rodents and humans [29,
388 30]. SIK1 was shown to inhibit CREB activity by phosphorylating CREB-specific
389 coactivators, CRTC2, at Ser171 to suppress hepatic gluconeogenesis [9, 10]. Serine
390 171 is the primary phosphorylation site that mediates CRTC2 activity [9]. To confirm
391 the importance of Ser 171 for inhibition of the gluconeogenic programme by SIK1,
392 we evaluated the expression of hepatic SIK1, CRTC2 and pS171 CRTC2 in diabetic
393 rats. Our results showed that the mRNA and protein expression of CRTC2 in the DM
394 and Ad-GFP groups was significantly elevated, whereas pS171 CRTC2 was
395 downregulated compared to the control group. Moreover, relative to control Ad-GFP
396 diabetic rats, Ad-SIK1 administration decreased fasting blood glucose, increased

397 pS171 CRTC2 and reduced CRTC2 and gluconeogenic genes, such as PEPCK and
398 G6Pase. The changes in expression of SIK1, CRTC2, PEPCK and G6Pase were also
399 confirmed by immunohistochemistry analysis. Interestingly, the CRTC2 nuclear
400 accumulation observed in the Ad-SIK1 group was lower than that seen in the DM
401 group, suggesting that Ad-SIK1 treatment might deter the translocation of CRTC2
402 into the cell nucleus, thus reducing the transcription of gluconeogenic genes and
403 hepatic glucose output. Consequently, we observed lower blood glucose levels in the
404 Ad-SIK1 group than in the DM group. Taken together, these findings suggest that
405 recombinant SIK1 directly inhibited the hepatic gluconeogenic program in the
406 HFD/STZ-induced diabetic rats by the SIK1/CRTC2 pathway.

407 Lipid metabolic disorder is one of the most common pathophysiological
408 changes in T2DM. Liver plays a vital role in the regulation of systemic lipid
409 metabolism. As a key regulator of hepatic lipogenesis, SREBP-1c was suggested to be
410 involved in the development of NAFLD by contributing to the onset of fatty liver
411 phenotypes [17]. SIK1 regulates hepatic lipogenesis by modulating SREBP-1c
412 activity [17]. To evaluate the effect of adenovirus-mediated SIK1 overexpression on
413 lipogenic gene expression in the livers of HFD/STZ-induced T2DM rats, we
414 transduced diabetic rats with Ad-SIK1 adenovirus or Ad-GFP control viruses. In this
415 study, the HFD/STZ-induced diabetic rats showed characteristics of NAFLD,
416 including elevation of hepatic enzyme levels, significantly increased relative liver
417 weights (liver index), hyperlipidaemia and histological changes such as steatosis and
418 hepatocyte injury. In concert with the histological and immunohistochemistry analysis,

419 Ad-SIK1 administration reduced serum TC and TG levels, and decreases the elevated
420 hepatic mRNA and protein levels of SREBP-1c, FAS and ACC caused by
421 HFD/STZ-induced T2DM, suggesting that overexpression of SIK1 could suppress
422 hepatic lipogenesis by downregulating SREBP-1c and its downstream gene
423 expression. This effect was consistent with previous reports [12, 17].
424 In summary, the present study demonstrates that SIK1 mRNA and protein expression
425 are significantly reduced in the livers of HFD/STZ-induced diabetic rats.
426 Overexpression of SIK1 ameliorates hyperglycaemia and fatty liver by suppressing
427 hepatic gluconeogenesis and lipogenesis in HFD/STZ-induced T2DM rats. This
428 protective effect of SIK1 may be derived from its interference with the SIK1/CRTC2
429 and SIK1/SREBP-1c pathways. Up-regulating hepatic SIK1 expression may represent
430 an attractive means for the treatment of T2DM and NAFLD. Figure 6 illustrates the
431 possible mechanisms of SIK1 in attenuating T2DM with NAFLD.

432 **Figure 6. Proposed mechanisms of the hypoglycemic and hypolipidemic effect of**
433 **SIK1.** Schematic representation of the role of SIK1 in amelioration of T2DM with
434 NAFLD. Overexpression of SIK1 can contribute to preventing hyperglycemia and
435 hepatic lipid accumulation through suppressing gluconeogenesis and lipogenesis with
436 a decrease in the expressions of CRTC2, PEPCK, G6Pase, SREBP-1c, FAS and ACC
437 in liver, thus reducing fasting blood glucose, serum TC, serum TG, hepatic steatosis
438 and liver weight. SIK1, salt-induced kinase 1; CRTC2, CREB-regulated transcription
439 co-activator 2; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase,
440 glucose-6-phosphatase; SREBP-1c, sterol regulatory element binding-protein-1c;

441 ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; TC, total cholesterol; TG
442 triglycerides.

443

444 **Supporting Information**

445 **S1 Appendix.** Method of construction of recombinant adenovirus vectors.

446 **S1 Table.** List of primer sequences for RT-PCR.

447 **S2 Table.** Changes in body weight, liver weight, FBG, TG and TC.

448

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450 DaoFei Song and Chang Wang; Formal analysis, DaoFei Song and Lei Yin; Funding
451 acquisition, XiuYing Wen; Investigation, DaoFei Song; Methodology, XiuYing Wen;
452 Project administration, DaoFei Song; Supervision, XiuYing Wen; Validation, DaoFei
453 Song; Writing – original draft, DaoFei Song; Writing – review & editing, XiuYing
454 Wen.

455 **Conflict of interest:** The authors declare that there are no conflicts of
456 interest.

457

458 **Abbreviations**

459 ACC acetyl-CoA carboxylase

460 ACTH adrenocorticotropic hormone

461 Ad-GFP adenovirus-green fluorescent protein

462 Ad-SIK1 adenovirus-Salt induced kinase 1

463 AMPK AMP-activated protein kinase

464	CREB	cAMP response element binding protein
465	CRTC2	CREB-regulated transcription co-activator 2
466	DM	diabetes mellitus
467	FAS	fatty acid synthase
468	G6Pase	glucose-6-phosphatase
469	HFD	high-fat diet
470	LKB1	serine/threonine kinase 11
471	NAFLD	nonalcoholic fatty liver disease
472	OD	optical density
473	PGC-1 α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
474	PKA	protein kinase A
475	PEPCK	phosphoenolpyruvate carboxykinase
476	PFU	plaque forming units
477	RT-PCR	reverse transcription-polymerase chain reaction
478	SIK1	Salt-induced kinase 1
479	STZ	streptozotocin
480	SREBP-1c	regulatory element binding-protein-1c
481	T2DM	type 2 diabetes mellitus
482	TC	total cholesterol
483	TG	triglycerides
484		

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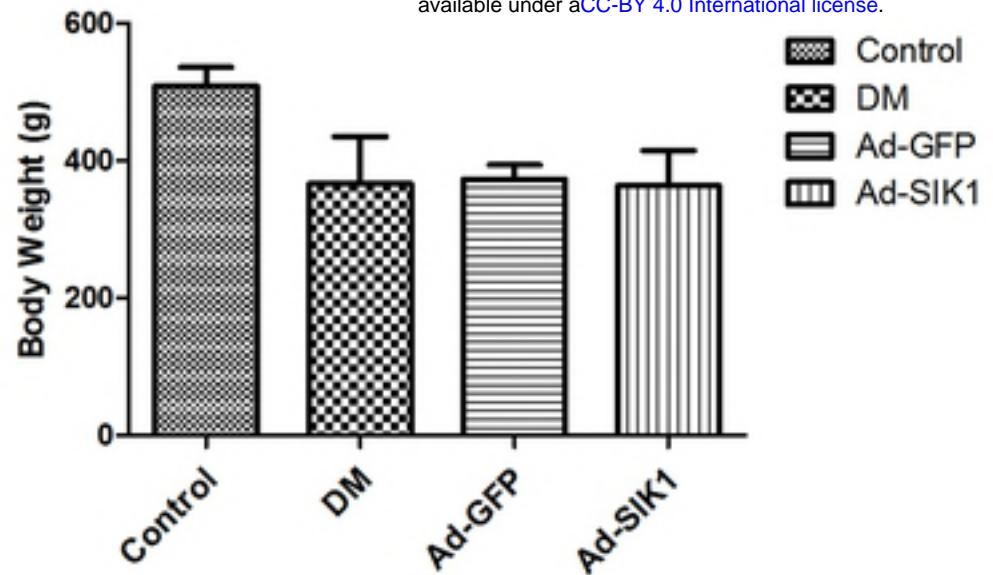
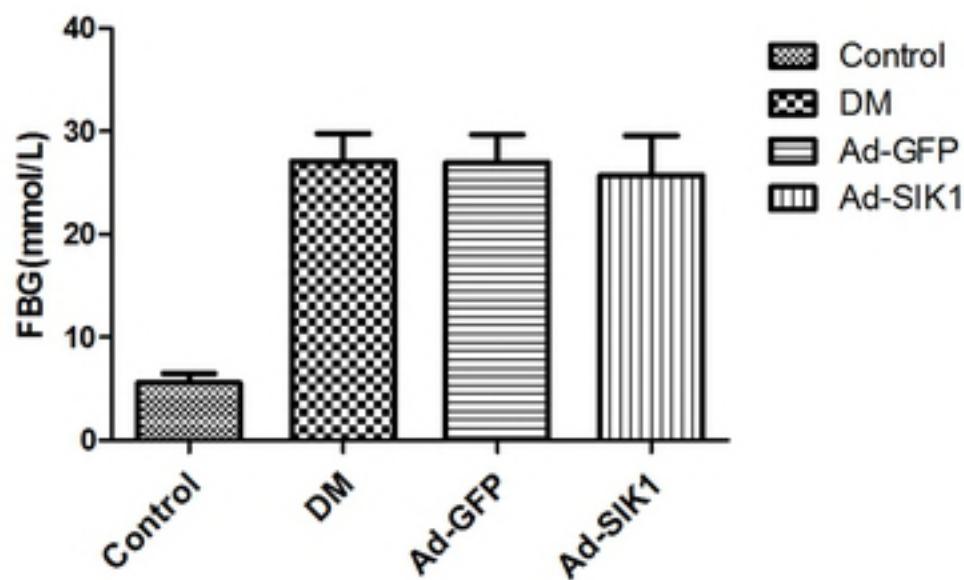
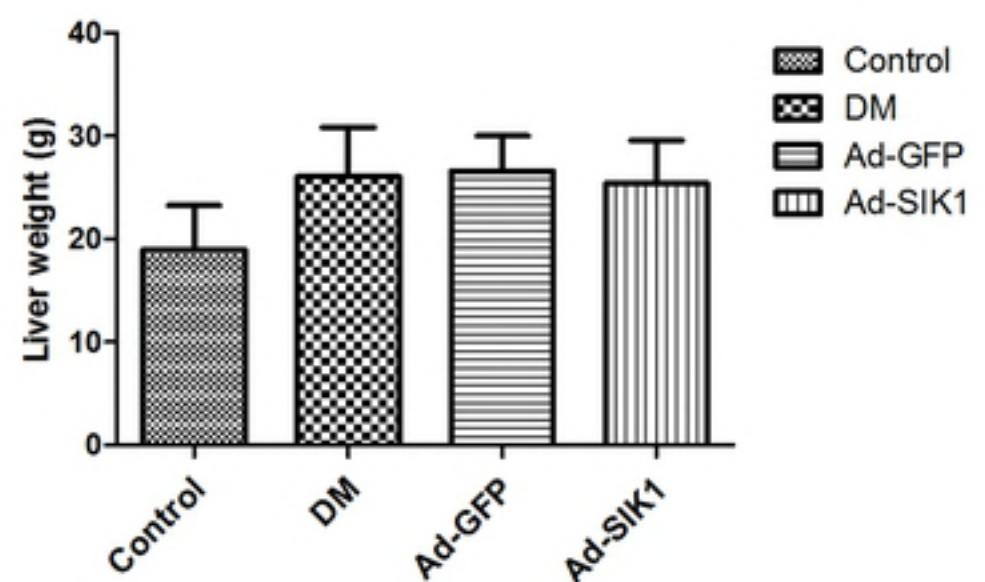
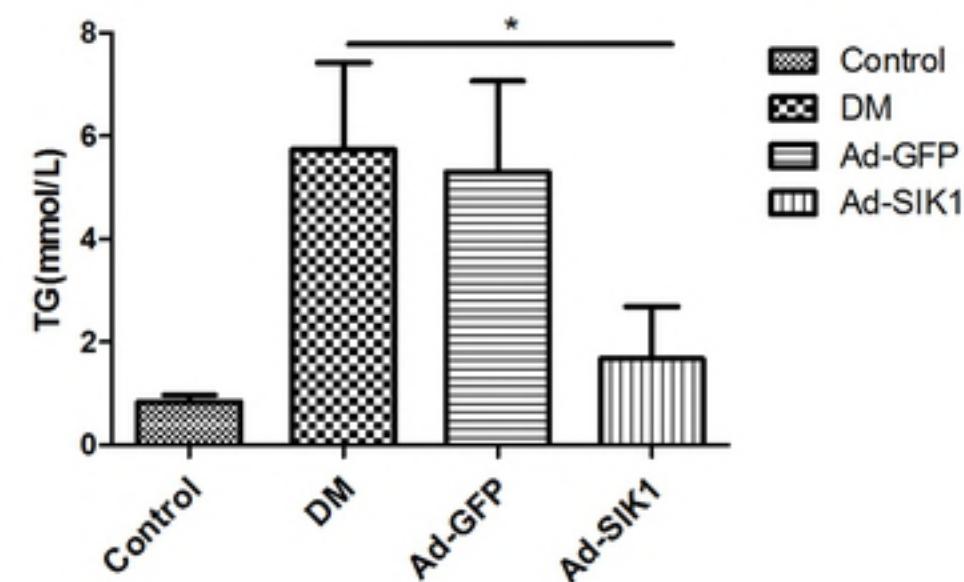
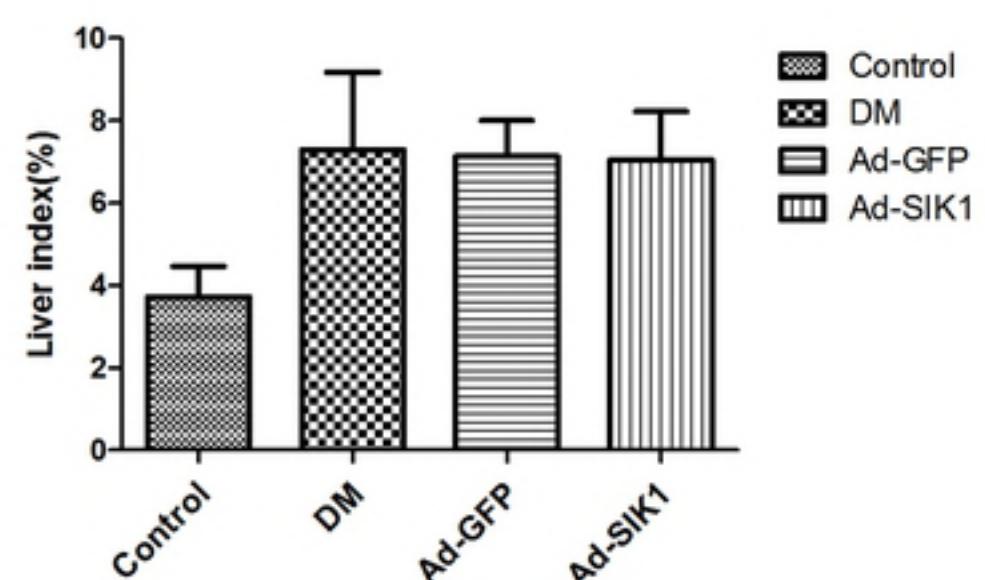
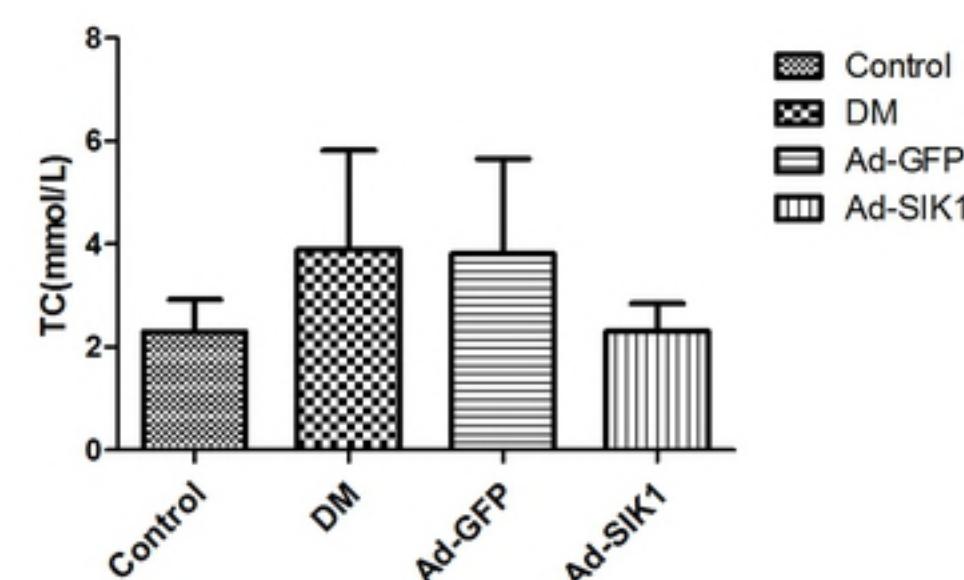
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**D****B****E****C****F****Figure1**

Control

DM

Ad-GFP

Ad-SIK1

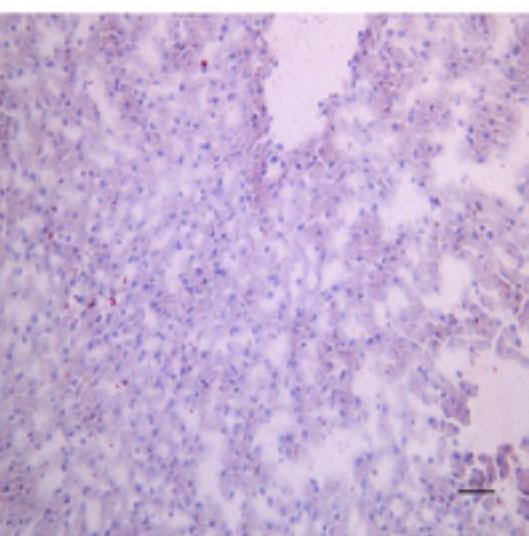
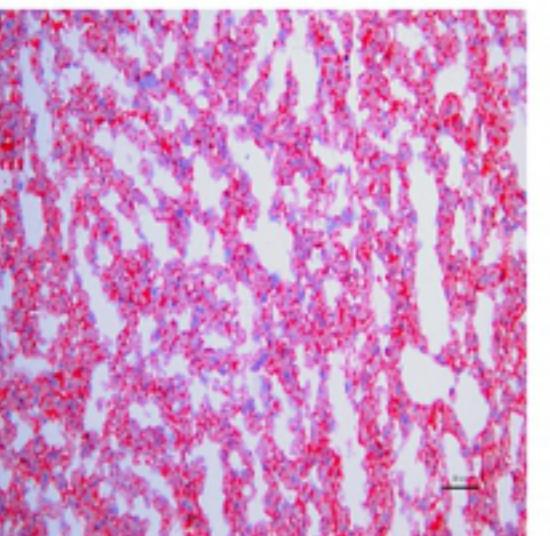
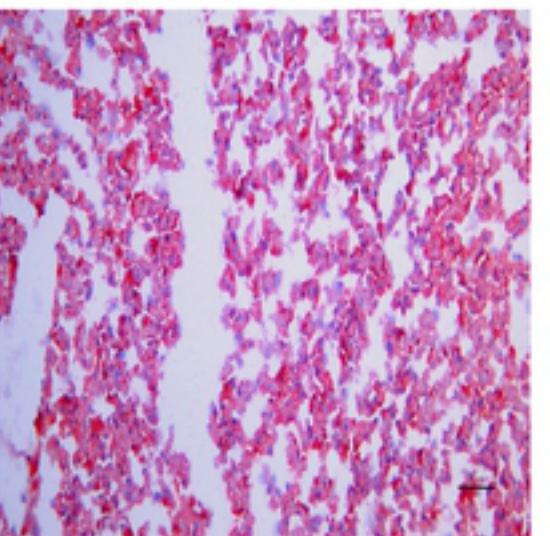
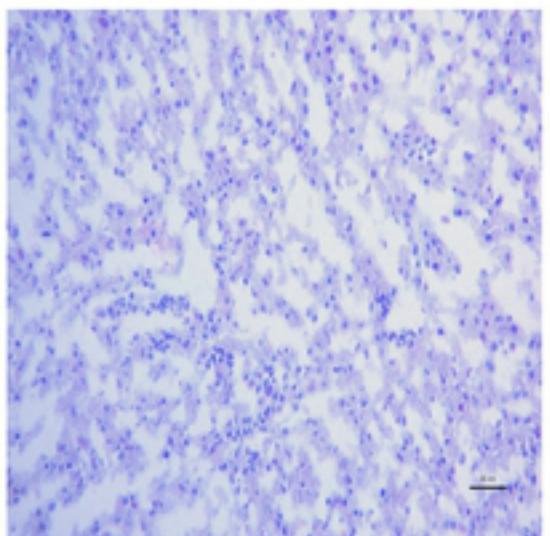
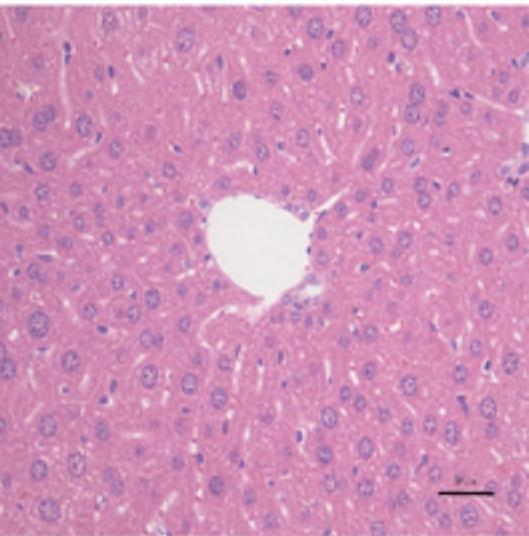
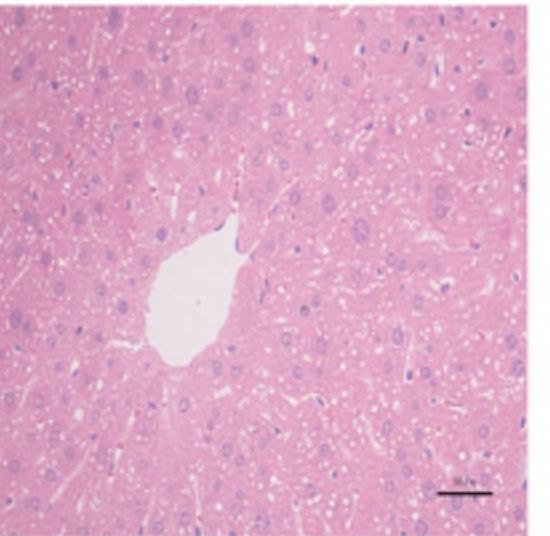
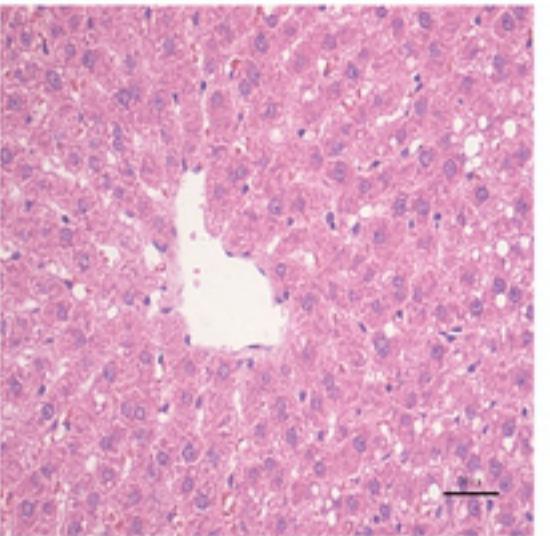
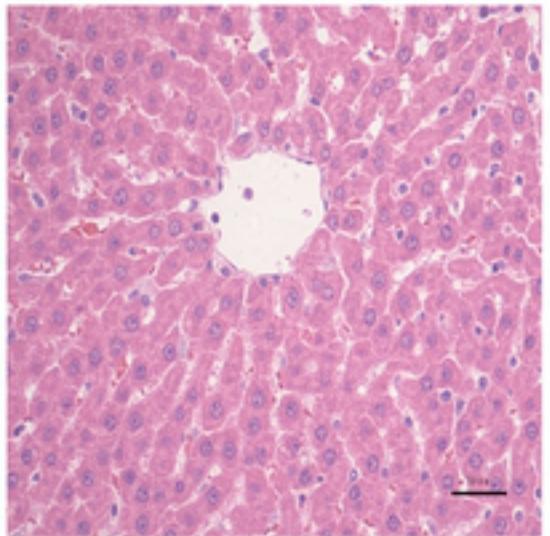


Figure2

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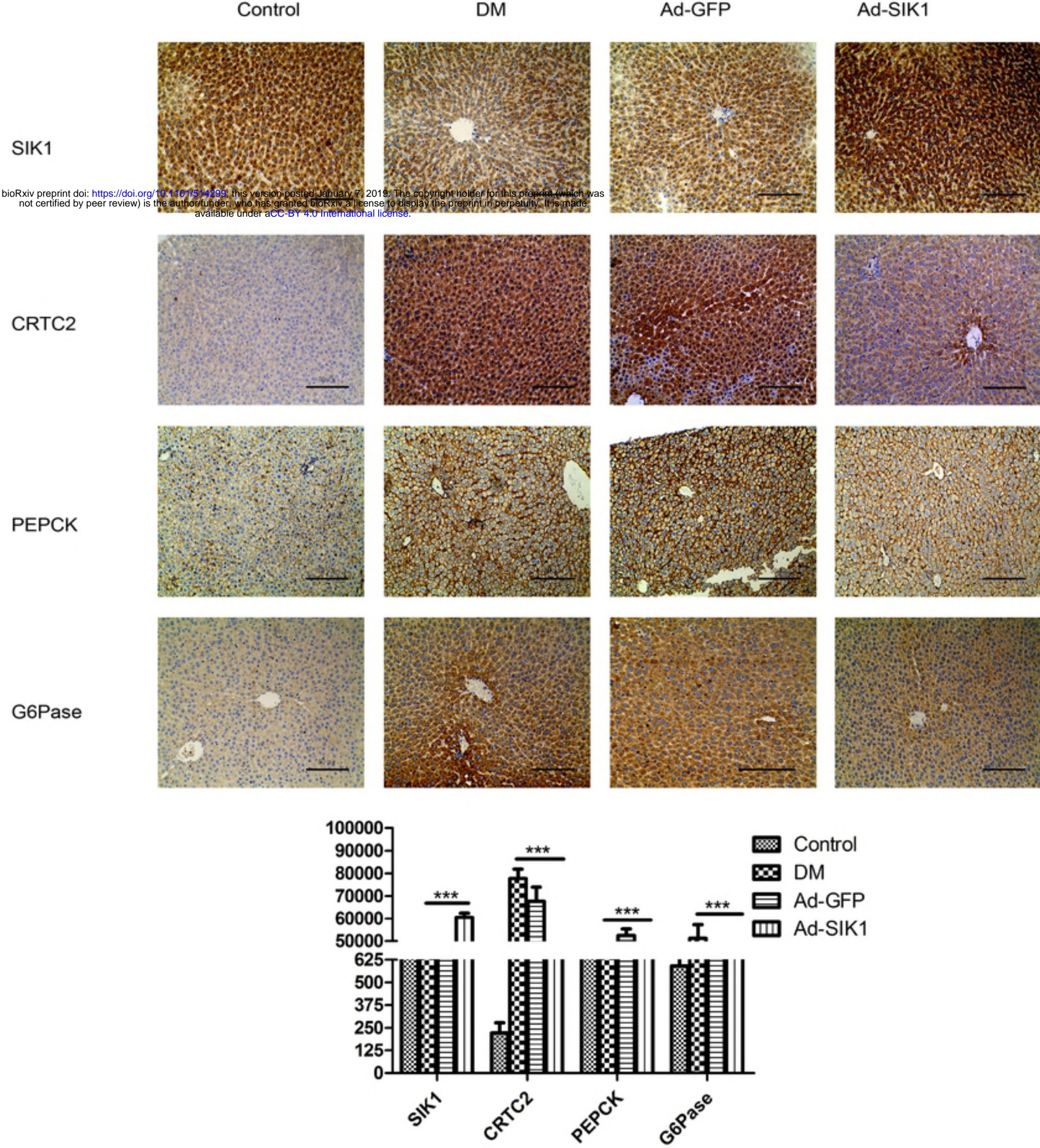


Figure3A

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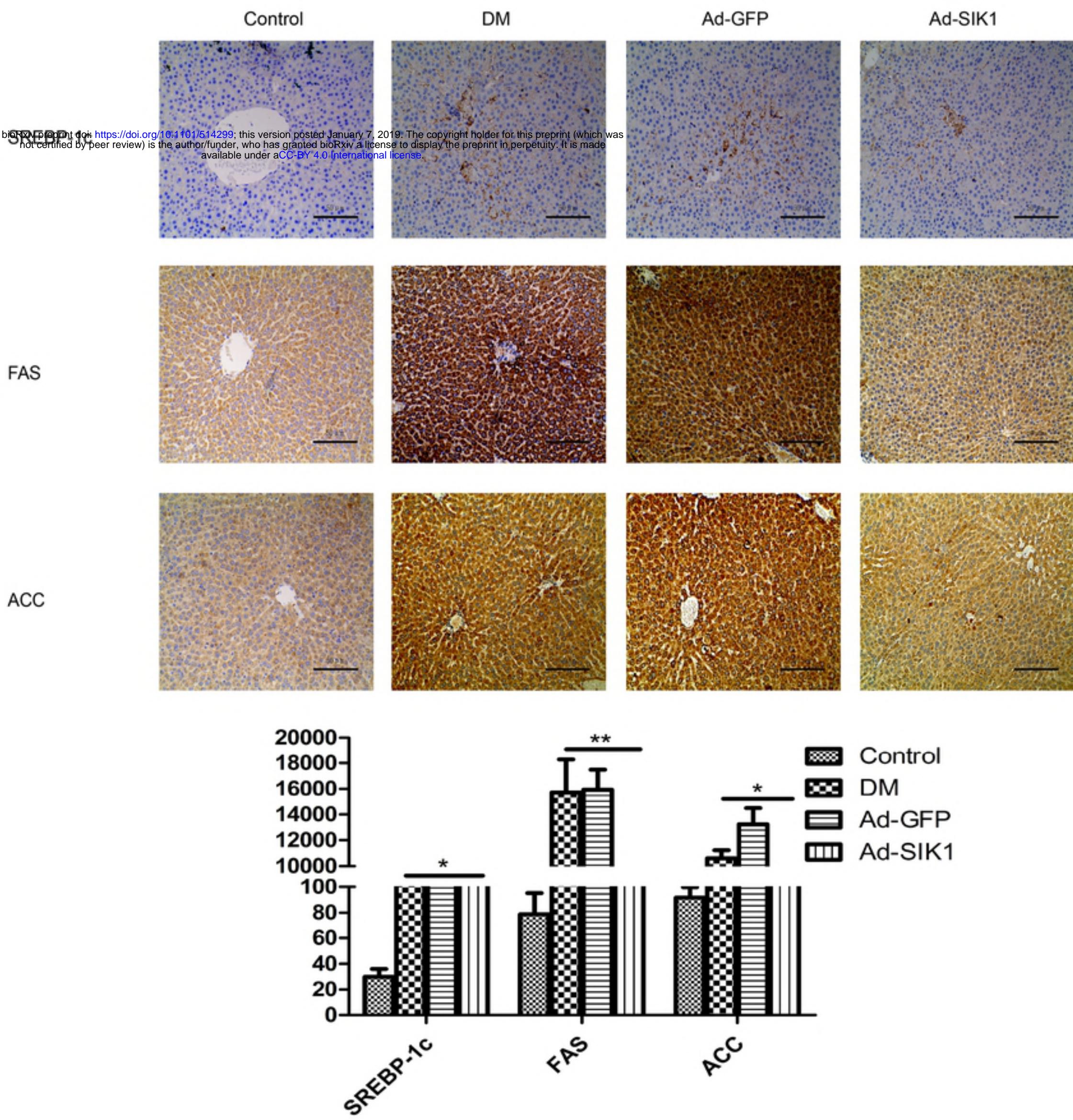


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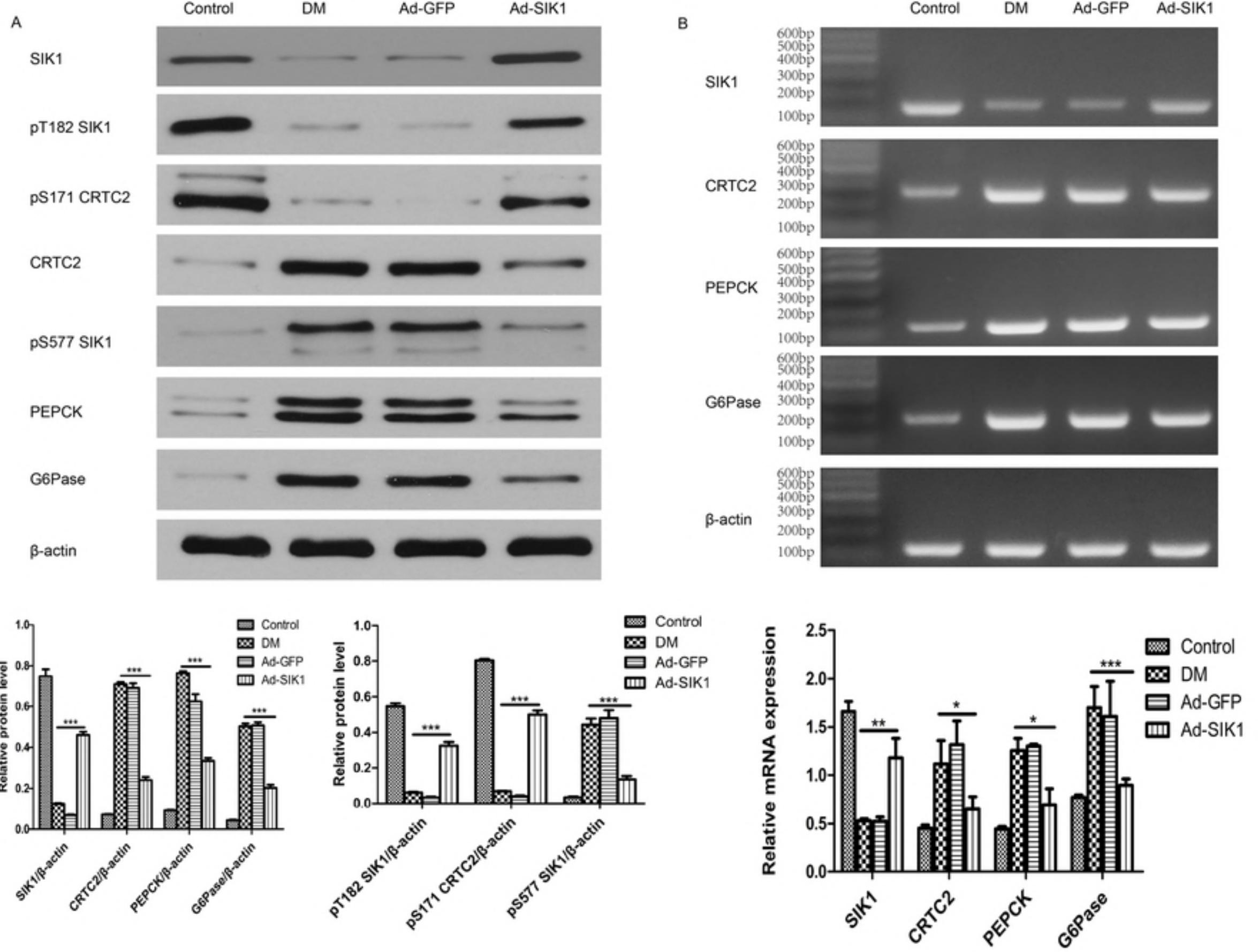


Figure 4

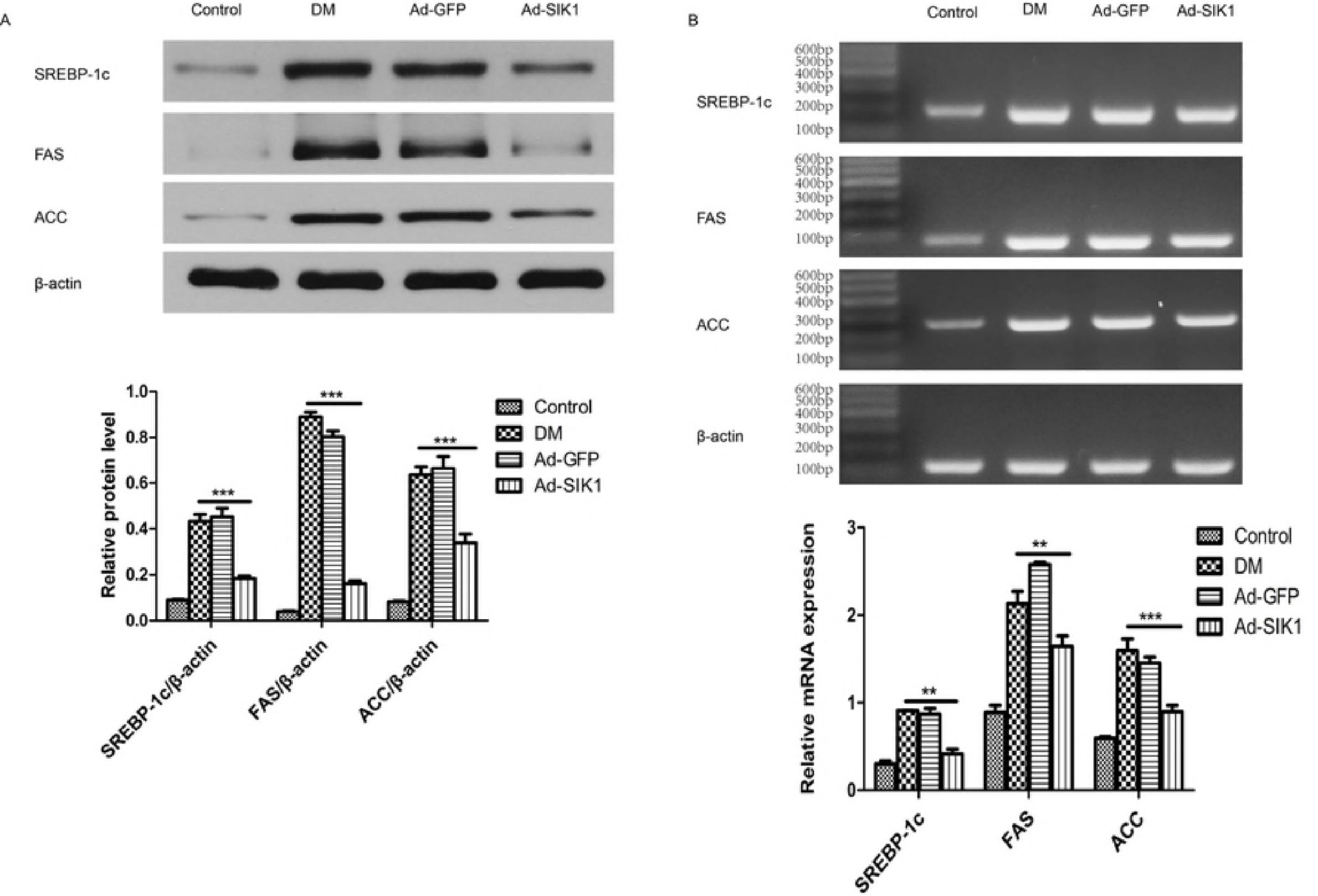


Figure 5

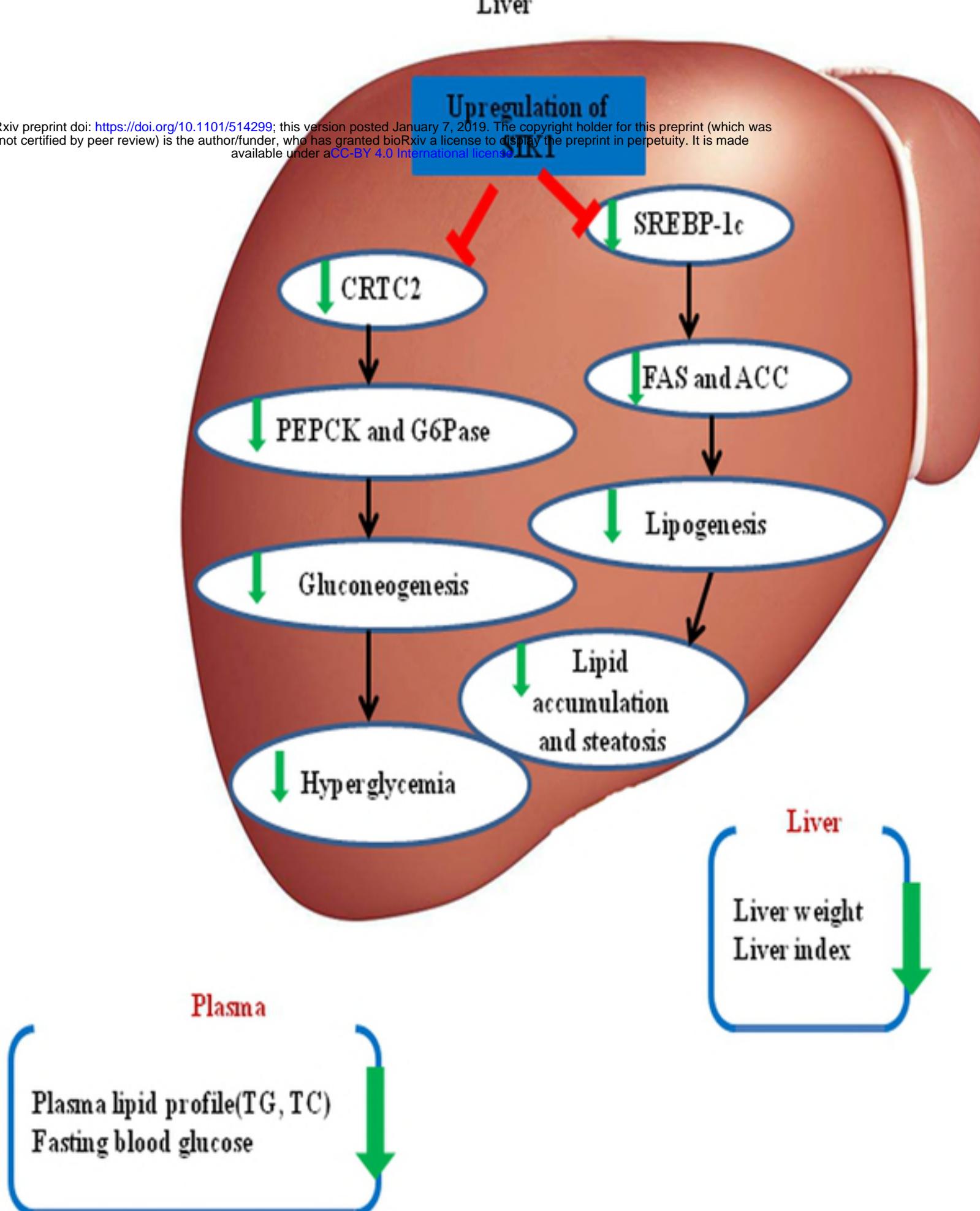


Figure6