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HCBP6 upregulates human *SREBP1c* expression by binding to C/EBPβ-binding site in the *SREBP1c* promoter

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Sterol regulatory element-binding protein-1c (SREBP1c) plays an important role in triglyceride (TG) homeostasis. Although our previous study showed that hepatitis C virus core-binding protein 6 (HCBP6) regulates SREBP1c expression to maintain intracellular TG homeostasis, the mechanism underlying this regulation is unclear. In the present study, we found that HCBP6 increased intracellular TG levels by upregulating *SREBP1c* expression. HCBP6 increased *SREBP1c* transcription by directly binding to the *SREBP1c* promoter (at the −139- to +359-bp region). Moreover, we observed that HCBP6 interacted with C/EBPβ-binding site in the *SREBP1c* promoter both *in vitro* and *in vivo*. These results indicate that HCBP6 upregulates human *SREBP1c* expression by binding to the C/EBPβ-binding site in the SREBP1c promoter. [BMB Reports 2018; 51(1): 33-38]

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver disorders worldwide and includes a series of disorders from liver steatosis to non-alcoholic steatohepatitis that eventually lead to liver cirrhosis and in some cases hepatocellular carcinoma (HCC) (1). In liver steatosis, triglycerides (TGs) accumulate in lipid droplets formed in the cytoplasm of hepatocytes, which leads to NAFLD (2). Thus, serum TG levels increase during NAFLD development (3).

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Sterol regulatory element-binding proteins (SREBPs) contain approximately 1150 amino acids. SREBP precursors are bound to the endoplasmic reticulum (ER) (4). Upon activation, they release their active domain and translocate into the nucleus (5, 6). After entering the nucleus, SREBPs promote the transcription of various genes involved lipogenesis and cholesterogenesis. Mammalian SREBP family contains three proteins, namely, SREBP1a, SREBP1c, and SREBP2, that function as important transcription factors. SREBP2 regulates cholesterol synthesis, SREBP1c regulates TG synthesis, and SREBP1a regulates constant cholesterol and TG synthesis (7-9). SREBPs are regulated through three major processes, *i.e.*, (1) transcription, (2) proteolytic cleavage of SREBP precursors, and (3) post-translational modification. Various evidences suggest that SREBP1c is mainly regulated at the transcriptional level. Moreover, some studies have shown that NF-Y, LXR, Sp1, C/EBPβ, and E-box induce *SREBP1c* transcription (10-12).

We previously identified hepatitis C virus (HCV) core-binding protein 6 (HCBP6) (also called FUNDC2, HCC3, DC44, or PD03104) by performing yeast two-hybrid assay (13). Although our previous study showed that HCBP6 participates in SREBP1c-FASN-mediated TG accumulation, it is unclear whether HCBP6 interacts with the *SREBP1c* promoter (14).

The present study is the first to provide information on the mechanism underlying SREBP1c-mediated TG accumulation In the present study, we analyzed HCBP6-induced activation of the *SREBP1c* promoter in HepG2 cells. Thus, our findings provide new insights on the regulation of *SREBP1c* expression by HCBP6.

RESULTS

HCBP6 upregulates *SREBP1c* expression and promotes TG synthesis in HepG2 cells

To investigate whether HCBP6 promoted SREBP1c expression, HepG2 cells were transfected with pcDNA-*HCBP6* expression plasmid. Results of qRT-PCR and western blotting showed that pcDNA-*HCBP6*-transfected cells showed significant HCBP6 overexpression ($P < 0.01$; Fig. 1A, C). Furthermore, results of qRT-PCR showed that HCBP6 overexpression increased the

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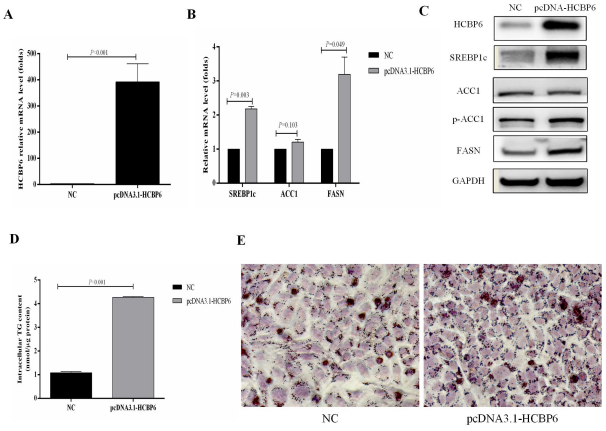

Fig. 1. HCBP6 upregulates *SREBP1c* expression and promotes TG synthesis in HepG2 cells. HepG2 cells were transfected with pcDNA-*HCBP6*. (A) *HCBP6* mRNA expression was analyzed by performing qRT-PCR, which showed successful HCBP6 overexpression. (B) Next, qRT-PCR was performed to determine the expression of all the key genes involved in TG synthesis, including *SREBP1c*, *ACC1*, and *FASN* at 24 h after transfection. Results of qRT-PCR showed that HCBP6 overexpression increased the expression of the genes associated with TG synthesis. (C) Western blotting was performed to determine the protein expression of HCBP6, SREBP1c, ACC1, p-ACC1, and FASN at 48 h after transfection, with GAPDH as a loading control. Results of western blotting showed that HCBP6 overexpression upregulated the protein expression of SREBP1c, p-ACC1, and FASN. (D) Intracellular TG content was determined using the Adipogenesis Assay Kit at 48 h after transfection according to the manufacturer's instructions, and was normalized to the total protein content. HCBP6 overexpression increased intracellular TG content. (E) After 24 h of pcDNA-*HCBP6* transfection, HepG2 cells were treated with oleic acid (0.25 mmol/L) for 24 h and were stained with oil red O. HCBP6 overexpression increased intracellular oil red O staining. Data are expressed as mean ± SEM; Statistically significant difference compared with relative mRNA levels in absence of pcDNA-*HCBP6* (Student's *t*-test; n = 3).

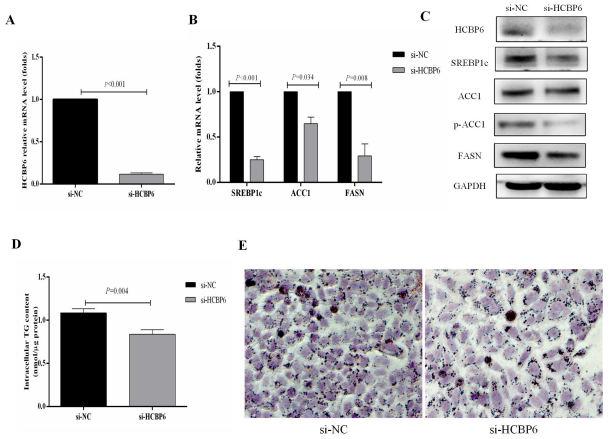

Fig. 2. *HCBP6* silencing decreases SREBP1c expression and suppresses TG synthesis in HepG2 cells. HepG2 cells were transfected with si-*HCBP6* or si-NC. (A) *HCBP6* mRNA expression was analyzed by performing qRT-PCR, which showed successful *HCBP6* silencing. (B) qRT-PCR was performed to analyze the expression of all the key genes involved in TG synthesis, including *SREBP1c*, *ACC1*, and *FASN* at 24 h after transfection. Results of qRT-PCR showed that *HCBP6* silencing decreased the expression of all the genes involved in TG synthesis. (C) Western blotting was performed to determine the protein expression of HCBP6, SREBP1c, ACC1, p-ACC1, and FASN at 48 h after transfection, with GAPDH as the loading control. Results of western blotting analysis showed that *HCBP6* silencing downregulated the protein expression of SREBP1c, p-ACC1, and FASN. (D) Intracellular TG content was determined using the Adipogenesis Assay Kit at 48 h after transfection according to the manufacturer's instructions, and was normalized to the total protein content. *HCBP6* silencing decreased the intracellular TG content. (E) After 24 h of si-*HCBP6* transfection, HepG2 cells were treated with oleic acid (0.25 mmol/L) for 24 h and were stained with oil red O. *HCBP6* silencing decreased intracellular oil red O staining. Data are expressed as mean ± SEM; Statistically significant difference compared with relative mRNA levels in absence of si-*HCBP6* (Student's *t*-test; n = 3).

expression of SREBP1c and FASN ($P < 0.05$; Fig. 1B), and results of western blotting showed that HCBP6 overexpression significantly promoted expression of SREBP1c, p-ACC1, and FASN ($P < 0.01$; Fig. 1C). We next examined whether HCBP6 overexpression induced TG synthesis in HepG2 cells. As expected, HCBP6-overexpressing HepG2 cells showed TG synthesis (Fig. 1D, E), as indicated by increased intracellular TG content and results of oil red O staining.

HCBP6 silencing decreases *SREBP1c* expression and suppresses TG synthesis in HepG2 cells

To examine whether *HCBP6* knockdown affected SREBP1c expression, we transfected HepG2 cells with a specific siRNA against *HCBP6* (si-*HCBP6*) to silence *HCBP6* expression. The efficacy of siRNA-induced *HCBP6* silencing is shown in Fig. 2. *HCBP6* mRNA and protein expression levels significantly decreased in si-*HCBP6*-transfected cells (Fig. 2A, C). Moreover, results of qRT-PCR and western blotting showed that SREBP1c, ACC1, p-ACC1, and FASN expression decreased significantly in si-*HCBP6*-transfected cells, which was consistent with the decrease in HCBP6 expression ($P < 0.05$; Fig. 2B, C). Moreover, intracellular TG levels decreased significantly in si-*HCBP6*-transfected cells compared with that in negative control siRNA (si-NC)-transfected cells (Fig. 2D). Results of oil red O staining showed that si-*HCBP6* transfection significantly decreased lipid droplet accumulation (Fig. 2E) after 48 h. These results indicate that *HCBP6* silencing decreases *SREBP1c* expression and suppresses lipid synthesis.

HCBP6 upregulates the transcriptional activity of the *SREBP1c* promoter in HepG2 cells

Deletion derivatives of the *SREBP1c* promoter named P-P6 (Fig. 3A, left) and these derivatives were respectively transiently transfected in HepG2 cells. The luciferase activity

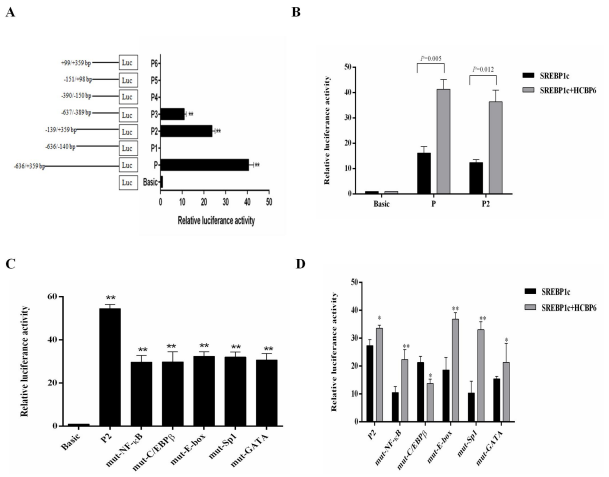

Fig. 3. HCBP6 upregulates transcriptional activity of the *SREBP1c* promoter in HepG2 cells. (A) Deletion fragments of the *SREBP1c* promoter were cloned into luciferase reporter vector pGL4.10-Basic based on the findings of Promoter 2.0 and Promoter Scan prediction (left). HepG2 cells were cotransfected with containing the different deletion fragments of the *SREBP1c* promoter or the pGL4.10-Basic vector and *Renilla* luciferase vector (pRL-TK), which served as an internal control. Luciferase activity was measured after 24 h of transfection. Results of the luciferase reporter assay showed that the P, P2, and P3 promoter fragments were active, with the P2 fragment showing the highest transcriptional activity (right). (B) HepG2 cells were cotransfected with 60 ng vectors containing the different *SREBP1c* promoter fragments or the pGL4.10-Basic vector with or without 180 ng pcDNA-*HCBP6* plasmid and pRL-TK. Luciferase activity was measured after 24 h of transfection. HCBP6 overexpression enhanced the activity of the P, P2 and P3 promoter fragments. (C) Site-directed mutagenesis of NF-κB-, C/EBPβ-, E-box-, Sp1-, and GATA-binding sites in the P2 promoter fragment. The basal activity of the mutation P2 promoter fragment was measured as described in panel A (right). We observed that mutant P2 *SREBP1c* promoter fragments were active; however, their activity was significantly lower than that of the wild-type P2 promoter fragment (D) Luciferase activity of the mutant promoter fragments was measured by cotransfecting HepG2 cells with vectors containing the different *SREBP1c* promoter fragments or the pGL4.10-Basic vector with or without pcDNA-*HCBP6* plasmid and pRL-TK. Luciferase activity of all the mutant P2 *SREBP1c* promoter fragments, except the mut-C/EBPβ promoter fragment, increased for 24 h in presence of HCBP6 overexpression. Data are expressed as the ratio of relative luciferase activity normalized to that of the pGL4.10-Basic vector. *P $<$ 0.05 and **P $<$ 0.01 indicate statistically significant difference compared with promoter activity in the absence of pcDNA-*HCBP6*, as determined using Student's *t*-test. ANOVA was used to compare the activity of different *SREBP1c* promoter fragments (n = 3).

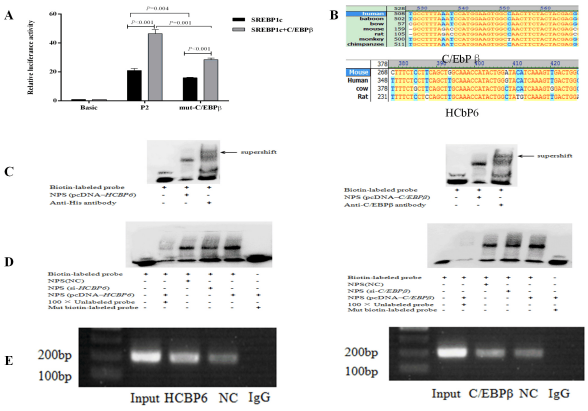

Fig. 4. HCBP6 directly binds to the C/EBPβ-binding site in the *SREBP1c* promoter. (A) Activities of the wild-type P2 promoter (P2) and P2 promoter with a mutated C/EBPβ-binding site (mut-C/EBPβ) were measured for 24 h in the absence or presence of pcDNA-*HCBP6*. Luciferase activity was measured after 24 h of transfection. C/EBPβ enhanced the activity of the P2 and mut-C/EBPβ promoter fragments; however, the activity of the mut-C/EBPβ promoter fragment was significantly lower than that of the wild-type P2 promoter fragment in presence of pcDNA-*C/EBPβ*. (B) C/EBPβ and HCBP6 is evolutionarily conserved in different mammals. (C) The presence of supershifted bands when an anti-His or anti-C/EBPβ antibody was added confirmed the specificity of interaction. (D) EMSA was performed using the LightShift chemiluminescence EMSA kit. Next, 6 μg nuclear protein extracts showing HCBP6 or C/EBPβ overexpression in the absence or presence of 100-fold excess of the unlabeled probe or mutant biotin-labeled probe (left and right, respectively) were incubated with biotin-labeled probes. Results indicate that HCBP6 and C/EBPβ interacted with the C/EBPβ-binding site in the *SREBP1c* promoter *in vitro*. (E) ChIP assay was performed using the EZ-Zyme Chromatin prep kit and EZ-ChIP kit. Chromatin solution was immunoprecipitated using 5 μg anti-His antibody, anti-C/EBPβ antibody or 5 μg normal anti-IgG antibody, followed by overnight incubation with 20 μl protein A agarose beads at 4°C. Next, the immunoprecipitated DNA was amplified by performing qRT-PCR targeting −236- to −37-bp region of the *SREBP1c* promoter. Total chromatin was used as a positive control, and normal rabbit IgG was used as a negative control. Results of the ChIP assay showed that both C/EBPβ and HCBP6 interacted with the *SREBP1c* promoter in HepG2 cells. Data are expressed as mean ± SEM. Statistically significant difference between the wild-type P2 and mut-C/EBPβ promoter fragments in absence or presence of pcDNA-*C/EBPβ*, as determined by performing Student's *t*-test (n = 3).

of P was 40.36-fold higher than that of the pGL4.10-Basic vector (P = 0.015), and the luciferase activity of P2 was 23.80-fold higher than that of the pGL4.10-Basic vector (P = 0.042). Analysis of the luciferase activity showed that the *SREBP1c* promoter fragment containing −139- to +359-bp region had core transcriptional activity (Fig. 3A, right; nucleotides are numbered from the transcription start site designated as +1).

Cotransfection with pcDNA-*HCBP6* significantly increased the transcriptional activity of the P and P2 derivatives of the *SREBP1c* promoter (Fig. 3B). Analysis by using Genomatix software predicted the presence of several putative cis-acting elements in the P2 fragment of the *SREBP1c* promoter containing NF-κB-, C/EBPβ-, E-box-, Sp1-, and GATA-binding sites. We constructed a series of *SREBP1c* promoter fragments with mutations in these binding sites by performing site-directed mutagenesis and transfected HepG2 cells with vectors containing these mutant promoter fragments. The basal activity

of the mutant promoter fragments decreased to different degrees (Fig. 3C). However, cotransfection with pcDNA-*HCBP6* increased the activity of all the mutant promoter fragments, except that of mut-C/EBPβ promoter fragment (Fig. 3D). These results indicate that HCBP6 overexpression increased the activity of the *SREBP1c* promoter by binding to the region from −109 to −106 bp, which is almost similar to the binding region for C/EBPβ.

HCBP6 directly binds to the C/EBPβ-binding site in the *SREBP1c* promoter

C/EBPβ enhanced the activity of the *SREBP1c* promoter. Moreover, introduction of specific mutations in the C/EBPβ-binding site inhibited the ability of C/EBPβ to stimulate the transcriptional activity of the *SREBP1c* promoter (Fig. 4A).

Furthermore, Both C/EbPβ and HCbP6 were conserved among mammals (Fig. 4B). Next, we performed electrophoretic mobility shift assay (EMSA) by using nuclear protein extracts (NPS) of HepG2 cells to determine whether HCBP6 interacted with the −109- to −106-bp region of the *SREBP1c* promoter *in vitro*. A biotin-labeled probe led to the formation of a DNA-protein complex. The presence of supershifted bands when an anti-His or anti-C/EBPβ antibody was added confirmed the specificity of interaction (Fig. 4C). The over-expression HCBP6 protein group produced the largest amount of complexes (Fig. 4D, left, lane 5), and non-transfected protein group came second (Fig. 4D, left, lane 3), and si-*HCBP6* protein group came the least complex (Fig. 4D, left, lane 4). To assess the specificity of this protein-DNA interaction, cross-competition using a 100-fold molar excess of unlabeled probe (Fig. 4D, left, lane 2) and mutant biotin-labeled probe (Fig. 4D, left, lane 6) containing the C/EBPβ-binding site from SREBP1c promoter was performed, resulting in reduction amounts of complex formation. Importantly, these results are consistent with C/EBPβ-binding site from SREBP1c promoter spanning −109- to −106-bp (Fig. 4D, right).

Chromatin immunoprecipitation (ChIP) assay to determine whether HCBP6 interacted with the −109- to −106-bp region of the *SREBP1c* promoter *in vivo* in HepG2 cells. Chromatin was immunoprecipitated using anti-His antibody, anti-C/EBPβ antibody or 5 μg normal anti-IgG antibody, and DNA fragments of expected size were used as templates for performing qRT-PCR (Supplementary Table 1). Results of the ChIP assay showed that both HCBP6 and C/EBPβ interacted with the −109- to −106-bp region of the *SREBP1c* promoter (Fig. 4E).

DISCUSSION

The HCV core protein performs different functions. To elucidate its biological role, we previously screened cellular proteins that directly interacted with the HCV core protein by using a human liver cDNA library (13). We found that many proteins interacted with the HCV core protein, including apolipoprotein A1, apolipoprotein A2, and translin (15). Based on these findings, we focused on the role of HCV infection in the development of liver steatosis (16, 17). We found that the HCV core protein modulated cholesterol homeostasis through SREBP2 pathway (18). Moreover, several studies have shown that the HCV core protein plays an important role in the development of HCV-induced liver steatosis (19-21). In our previous study, we performed the yeast two-hybrid assay to determine the role of new genes, particularly *HCBP6*, in the development of liver steatosis; however, biological functions of proteins encoded by these genes are unclear. Our previous study showed that HCBP6 participated in cell proliferation, signal transduction, growth, and differentiation (22). Therefore, we have validated that HCBP6, an HCV core-binding protein, affected lipid metabolism.

In the present study, we overexpressed and knocked down *HCBP6* in HepG2 cells to determine whether HCBP6 promoted intracellular lipogenesis through SREBP1c pathway (Fig. 1 and 2). We found that HCBP6 promotes lipogenesis and that *SREBP1c* is a potential target and is positively regulated by HCBP6. Next, we identified HCBP6-binding site in the *SREBP1c* promoter. Bioinformatics analysis showed that HCBP6 interacted with the C/EBPβ-binding site in the *SREBP1c* promoter. (Fig. 3C). We confirmed the interaction between HCBP6 and the C/EBPβ-binding site in the *SREBP1c* promoter by isolating the 5′-flanking region of *SREBP1c* from the DNA of HepG2 cells and by performing a series of experiments, including the luciferase reporter assay, site-directed mutagenesis, EMSA, and ChIP assay (Fig. 3 and 4).

SREBP1c plays a key role in energy homeostasis by regulating lipogenesis in the liver (23, 24). SREBP1c regulation in the liver has been extensively studied, and factors such as LXR, Sp1, NF-Y, and E-box are known to induce SREBP1c in hepatocytes (11, 25-27). In the present study, we performed site-directed mutagenesis to confirm that C/EBPβ interacted with the −109- to −106-bp region of the *SREBP1c* promoter to modulate *SREBP1c* transcription (Fig. 4A). Results of EMSA and ChIP assay performed in the present study indicate that HCBP6-related factors perform a direct key role in the regulation of *SREBP1c* expression by binding to the C/EBPβ-binding site in the *SREBP1c* promoter (Fig. 4D, E). Previous studies have shown that HCBP6 is located in the nucleus (28, 29). Thus, the results of the present study suggest that HCBP6 functions as a transcription factor to regulate SREBP1c expression both *in vitro* and *in vivo*. Moreover, we found that both C/EBPβ and HCBP6 are a part of a protein complex that binds to the *SREBP1c* promoter.

C/EBPβ belongs to CCAAT/enhancer-binding protein family that includes basic leucine zipper transcription factors that regulate lipid metabolism in the liver by regulating transcription and translation (30, 31). In addition, C/EBPβ is a pioneer transcription factor that binds to the *PPARγ* promoter and induces PPARγ expression to activate liver steatosis and inflammation (32, 33). In addition to its role in inducing the *PPARγ* promoter to activate lipogenesis in the liver, C/EBPβ

binds upstream of 368 nucleotides in the *SREBP1c* promoter (34). We also found that C/EBPβ interacted with the *SREBP1c* promoter to induce TG accumulation. Particularly, we observed that mutation of the C/EBPβ-binding site in the *SREBP1c* promoter, overexpression C/EBPβ can still enhance SREBP1c promoter activity, but the ability to enhance SREBP1c promoter activity has been decreased (Fig. 4A). Thus, our results suggest that C/EBPβ is important for regulating *SREBP1c* transcription and there is more than one of C/EBPβ-binding-sites in the SREBP1c promoter.

While our previous study showed that HCBP6 inhibited the SREBP1c pathway to reduce TG level, results of the present study indicate that HCBP6 promotes *SREBP1c* transcription to increase lipid accumulation (14). This finding indicates that HCBP6 and SREBP1c are a part of a complex inter-regulatory network and not a fixed canonical cascade and are activated by factors such as extracellular signals and differentiation status of involved cells (30).

In summary, the present study is the first to show that HCBP6 induces *SREBP1c* expression to increase TG levels in HepG2 cells by directly interacting with the C/EBPβ-binding site in the *SREBP1c* promoter. These results also suggest that the effect of HCBP6 on lipogenesis in the liver is partially mediated by the increase in *SREBP1c* transcription. However, further studies are required to explore a systematic and comprehensive study that aims to demonstrate the regulatory mechansim by C/EBPβ and HCBP6 on same region of *SREBP1c* promoter.

MATERIALS AND METHODS

Detailed informition is in the Supplementary Material.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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