Methylation of Septin9 Mediated by DNMT3a Enhances Hepatic Stellate Cells

Activation and Liver Fibrogenesis

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Abbreviations: Sept9, septin9; ECM, extracellular matrix; HSCs, hepatic stellate cells; α -SMA, α -smooth muscle actin; Col1a1, type I collagen; TGF- β 1, transforming growth factor- β 1; HCC, hepatocellular cancer; 5-azadC, 5-aza-2'-deoxycytidine; CRC, colorectal cancer; DNMTs, DNA methyltransferase enzymes; RRBS, reduced representation bisulfite sequencing; BSP, bisulfite pyrosequencing PCR; MSP, methylation-specific PCR.

Abstract

Liver fibrosis, resulting from chronic and persistent injury to the liver, is a worldwide health problem. Advanced liver fibrosis results in cirrhosis, liver failure and even hepatocellular cancer (HCC), often eventually requiring liver transplantation, poses a huge health burden on the global community. However, the specific pathogenesis of liver fibrosis remains not fully understood. Numerous basic and clinical studies have provided evidence that epigenetic modifications, especially DNA methylation, might contribute to the activation of hepatic stellate cells (HSCs), the pivotal cell type responsible for the fibrous scar in liver. Here, reduced representation bisulfite sequencing (RRBS) and bisulfite pyrosequencing PCR (BSP) analysis identified hypermethylation status of Septin9 (Sept9) gene in liver fibrogenesis. Sept9 protein was dramatically decreased in livers of CCl4-treated mice and immortalized HSC-T6 cells exposed to TGF- β 1. Nevertheless, the suppression of Sept9 could be blocked by DNMT3a-siRNA and DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-azadC). Overexpressed Sept9 attenuated TGF- β 1-induced expression of myofibroblast markers α -SMA and Col1a1, accompanied by up-regulation of cell apoptosis-related proteins. Conversely, RNAi-mediated silencing of Sept9 enhanced accumulation of extracellular matrix. These observations suggested that Sept9 contributed to alleviate liver fibrosis might partially through promoting activated HSCs apoptosis and this anti-fibrogenesis effect might be blocked by DNMT-3a mediated methylation of Sept9. Therefore, pharmacological agents that inhibit Sept9 methylation and increase its expression could be considered as valuable treatments for liver fibrosis.

Keywords: Septin9; liver fibrosis; DNA methylation; apoptosis; DNMT3a

45 1. Introduction

46 Liver fibrosis is one of the serious public health problems and is associated with significantly
47 increased risk of terminal liver cirrhosis and hepatocellular carcinoma (HCC). Liver fibrogenesis is
48 characterized by progressive accumulation of extracellular matrix (ECM) and can be triggered by
49 many liver diseases, such as hepatitis, non-alcoholic fatty liver disease(NAFLD) and alcoholic liver
50 disease(ALD) (Bataller and Brenner, 2005; Hernandez-Gea and Friedman, 2011). Activation of the
51 liver resident perisinusoidal cell type, hepatic stellate cell (HSC), plays a key role in the pathogenesis
52 of liver fibrosis and constantly influences the disease progression. (Moreira, 2007). Upon liver
53 insults, the quiescent HSCs trans-differentiate into fibrogenic myofibroblasts-like cells, identified by
54 increased expression of α -smooth muscle actin (α -SMA), and secrete profibrogenic mediators to
55 promote ECM proteins enriched in type I collagen (Colla1), resulting in the progression of hepatic
56 fibrosis(Henderson and Iredale, 2007). Hence, blocking of HCSs activation and promoting of
57 collagen degradation are thus the most effective therapeutic strategies for human hepatic fibrosis. It
58 has been demonstrated that transforming growth factor- β 1 (TGF- β 1), an inflammatory cytokine
59 secreted by different types of hepatic cells (HSCs, sinusoidal endothelial cells, Kupffer cells and
60 hepatocytes), is the main fibrogenic cytokine among HSCs activation(Friedman, 1999). Data
61 emerging from *in vivo* and *in vitro* have identified apoptosis of activated HSCs might be one of the
62 resolution of liver fibrosis. (Iredale, 2007; Wu *et al.*, 2015). Although experimental studies have
63 indicated revolution to prevent fibrosis progression, there is still a long way to characterize the
64 underlying molecular mechanisms and find the most effective inducers of activated HSCs apoptosis
65 to provide standard treatment for hepatic fibrosis in human.

66 Genomic methylation is a key epigenetic modification that influences gene expression. During DNA

67 methylation procedure, a methyl group is integrated to the carbon 5'-end of the cytosine ring in the
68 CpG dinucleotide, yielding a conversion to methylcytosine(Jones, 2012). Previous studies have
69 concluded that the transcriptional activity of genes were impaired when they were methylated in
70 CpG island (Ramirez-Carrozzi *et al.*, 2009) (Deaton and Bird, 2011; Lokk *et al.*, 2014). Moreover,
71 DNA methylation in mammals is generally catalyzed by a family of DNA methyltransferase
72 (DNMTs) enzymes, including DNMT1, DNMT3a and DNMT3b (Valente *et al.*, 2014). DNMT1, the
73 most abundant methyltransferase in mammals, is involved in maintaining established DNA
74 methylation which was triggered by DNMT3a and DNMT3b (Baylin and Herman, 2000; Eng *et al.*,
75 2000). 5-aza-2'-deoxycytidine (5-AzaC) is an inhibitor of DNA methylation, it blocks enzymatic
76 activity of the three methyltransferase(Lindner *et al.*, 2013; Wang *et al.*, 2016a). Accumulating
77 evidence have implied that DNA methylation occurs in genes involved in fibroblast activation and
78 fibrogenesis(Bian *et al.*, 2014; Tao *et al.*, 2014), and up-regulation of DNMTs have also confirmed
79 in fibrotic diseases. Hence, the studies of genome-wide and loci-specific DNA methylation in
80 fibrotic organs (e.g., liver, kidney, lung and heart) will identify the novel DNA methylation
81 biomarkers for fibrotic diseases of different organs.

82 Septin9 (Sept9) is a member of highly conserved septin family, which coding for GTPase
83 proteins(Estey *et al.*, 2011). These GTPase proteins integrate into polyplidity and constitute
84 filamentous structures which are crucial for cytoskeletal organization, cell division, cell cycle control
85 and membrane-remodeling events(Estey *et al.*, 2011) (Sandrock *et al.*, 2011). Sept9 is distinguished
86 by its numerous genomic organization that contains 219 kb and alternative splicing at both 3' and 5'
87 ends of Sept9 pre-mRNAs yields 18 individual transcripts to encode 15 isoforms(McIlhatton *et al.*,
88 2001; Peterson and Petty, 2010). Previous studies have been conducted on the different expression

89 patterns of the Sept9 transcripts. For instance, the transcript Sept9_v1 was found to be overexpressed
90 in many tumors, including ovarian, breast and prostate cancer while the expression of Sept9_v1 was
91 almost undetectable in the normal tissue of these organs(Scott *et al.*, 2006; Gonzalez *et al.*, 2007).
92 And Sept9_v4 and Sept9_v4* encoded for the same polypeptide fragment, but only the Sept9_v4*
93 polypeptide chain was confirmed to be involved in tumorigenesis (McDade *et al.*, 2007; Connolly *et al.*, 2011b). However, controversial to conventional septin genes effect on promoting tumor in
94 cancers, expression levels of Sept9_v3 were diversiform in different tumor cells. Subsequent studies
95 proved the diversity of Sept9_v3 expression was due to its different DNA methylation status in
96 cancers (Lofton-Day *et al.*, 2008). Nowadays, methylation of Sept9 in human disease has aroused
97 people's concern. In recent years, a number of basic and clinical studies have provided evidence of
98 Sept9 hypermethylation status in human diseases, such as malignant pleural effusions(Dietrich *et al.*,
99 2013), noninvasive lung cancer(Powrozek *et al.*, 2014), esophageal squamous cell carcinoma(Kuo *et al.*, 2014), cholangiocarcinoma(Andresen *et al.*, 2015) and even diabetes(Dayeh *et al.*, 2014). More
100 importantly, Sept9 DNA methylation assay has also been developed for plasma and methylated
101 Sept9 in peripheral blood was validated as a sensitive biomarker of colorectal cancer
102 (CRC)(Grutzmann *et al.*, 2008; deVos *et al.*, 2009), which is now available for clinical diagnosis in
103 blood-based screening test (Epi proColon®) for CRC in Europe(Payne, 2010).
104
105 In this study, we analyzed methylome of primary HSCs from carbon tetrachloride-induced liver
106 fibrotic mice via the RRBS method, and we found Sept9 was also hypermethylated in liver fibrosis.
107 To advance our understanding of fibrosis-derived epigenetic changes in Sept9, we verified the
108 hypermethylation profile of Sept9 in HSCs *in vivo* and *in vitro* by using bisulfite pyrosequencing
109 PCR (BSP) and methylation-specific PCR (MSP) method. Furthermore, our findings revealed the
110

methylation of Sept9 might be mediated by DNMT3a and forced expression of Sept9 could alleviate liver fibrosis *in vitro*.

2. Materials and methods

2.1 Animals, mouse models of liver fibrosis

All of the animal experimental protocols were reviewed and approved by the Ethics Committees of Anhui Medical University. 8-week-old male C57BL/6J mice (18-20g weight), obtained from the Experimental Animal Center of Anhui Medical University, was subcutaneously injected of carbon tetrachloride (CCl₄) for advanced liver fibrosis model. Mice were randomly divided into two groups (eight mice pergroup), including vehicle group and model group. Liver fibrosis was set up using the procedure described previously (Miao *et al.*, 2016) with some modification. Briefly, liver fibrosis was induced by biweekly subcutaneous injection of a 20% solution of CCl₄ in olive oil at a dose of 0.01ml/g/mouse for 4 weeks. Vehicle control mice were injected subcutaneously with the same volume of 100% olive oil at the same time intervals. 24 h after the last injection, mice were anesthetized by 10% chloral hydrate and hepatic tissues were harvested for the further analysis. The hepatic lobes were used for hematoxylin and eosin (H&E) staining, Masson staining, Sirius-Red staining and immunofluorescent staining by fixation with 4% formalin.

2.2 Cell culture and treatments

Primary mouse HSCs were isolated from the liver tissues of vehicle control mice and CCl₄ treated mice according to a previous study presented by Si *et al.* (Si *et al.*, 2008). All the primary cells were analyzed by Reduced Representation Bisulfite Sequencing (RRBS) to reveal methylation status of

133 **genes** in quiescent and activated **mouse** HSCs.

134 HSC-T6 cell line was purchased from Shanghai FuMeng Gene Bio-technology Co., LTD. (Shanghai,
135 China). HSC-T6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Keygen,
136 China) supplemented with 10% fetal bovine serum(FBS, Beyotime, China), 100 U/ml penicillin and
137 100 mg/ml streptomycin and incubated at 37 °C with 5% **CO₂**. When cell density approximately
138 **cultured** to 80%, the HSC-T6 cells were cultured in serum-free DMEM in the presence of 10 ng/mL
139 TGF-β1 (Peprotech, USA) or 1 μM 5-azadC(Sigma-Aldrich, St. Louis, MO).

140

141 *2.3 Reduced representation bisulfite sequencing (RRBS)*

142 High-molecular-weight genomic DNA was extracted from primary mouse HSCs using the Genomic
143 DNA extraction kit (Genaray, China) according to the manufacturer's protocol. 100 ng of pooled
144 genomic DNA with an equal amount from three mice was used for the construction of each RRBS
145 library. Genomic DNA was digested overnight with *MspI* restriction enzyme ((recognition site
146 C⁺CGG, NEB, Ipswich, MA, USA) at 37 °C. After purification, single A nucleotides were added to
147 the 3'-end UTR, followed by ligation to methylated adapter with T overhang. Bisulfite conversion
148 was performed with a ZYMO EZ DNA Methylation-Gold™ Kit (ZYMO, Irvine, CA, USA). The
149 final libraries were generated by PCR amplification of 25 cycles. RRBS libraries were analysed by
150 an Agilent 2200 Bioanalyzer (Agilent Technologies). Sequencing data were performed on an
151 Illumina 2500 for 100 bp bidirectional reads, yielding ~5G clean data. Finally, the identified
152 methylated/hydroxymethylated genes from *in vivo* quiescent **and** activated HSCs were compared by
153 two tailed unpaired Student's t test and minimal significance set at $p < 0.01$.

154

2.4 Bisulfite pyrosequencing and Methylation-specific PCR

To clarify the methylation status of septin9 gene, 6 CpG sites in the CpG island of the septin9 gene region were amplified using the bisulfite sequencing PCR (BSP) of the bisulfite-treated DNA. The primary mouse HSCs derived from the liver tissues of CCl_4 treated mouse were sequenced. The primers used for BSP : Forward, GTGAGTTAGTTTTGAAGGTTTTTTG; Reverse, TTTTCTTTTAATTTATTAATTTTATTTAA. Methylation-specific PCR (MSP) was then performed with methylated and unmethylated Sept9 PCR primers to determine the methylation status of Sept9. The primers used for MSP: Sept9-M, forward: 5'-CGGGCGTAATAGTTAGTGTC-3', reverse: 5'-CTCCCGATAACCGAACTAAC-3'; Sept9-U, forward: 5'-AGGTGGGTGTAATAGTTA GTGTT-3', reverse: 5'-CTCCCAATACCAAATAACC-3'.

2.5 Total RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from mouse liver tissues and HSC-T6 cells using TRIzol reagents (Invitrogen, USA) and uniformly quantified to 1000ng/ μl by the Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). And then the first-strand cDNA was synthesized via PrimeScript[®] RT reagent kit (Takara, JAP) according to the manufacturer's instructions. SYBR-Green real-time quantitative PCR analyses for mRNAs of Sept9, α -SMA, Colla1, DNMT3a and internal control (GAPDH and β -actin) were performed by using QuantiNova SYBR Green PCR Kit (Qiagen, Germany) in the Pikoreal 96 real-time PCR system (Thermo Scientific, USA). And all experiments were done in triple replication. Primer sequences were listed in Table 1.

2.6 Western blot analysis

Mouse liver tissues and rat HSC-T6 cells were lysed with RIPA lysis buffer (contained 1%PMSF)

178 (Beyotime, China). Whole extracts were prepared, and protein concentration was measured by a
179 BCA protein assay kit (Beyotime, China). Total protein of each sample (30 - 50 μ g) was separated
180 by SDS-PAGE gel and then transferred onto PVDF membranes (Millipore Corp, Billerica, MA,
181 USA). The transferred membranes were blocked in TBS/Tween-20 (0.05%) containing 5 % skim
182 milk at 37°C. After 3 h, transferred the membranes to 4°C with specific primary antibodies overnight.
183 Rabbit polyclonal recognizing α -SMA, Colla1, DNMT1, DNMT3a, DNMT3b and β -actin (Bioss,
184 China) was diluted 1:500, Bax and Bcl-2 (Boster, China) were diluted 1:200, rabbit monoclonal
185 antibodies recognizing cleaved-caspase3 (cell Signaling, USA) were diluted 1:500, rat monoclonal
186 antibody recognizing Sept9_v3 (Sigma, USA) was used at 1:500. HRP-conjugated IgG anti-rabbit
187 and anti-rat antibodies were used as secondary antibodies (1:10000) at 37°C for 1 h. After
188 thoroughly washed in TBST and soaked in distilled water, blotting was detected by the enhanced
189 chemiluminescence system with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific). All
190 experiments were repeated for three times.

191

192 2.7 Plasmid construction and transfection of HSC-T6

193 Ectopic expression of Sept9 was achieved by using the GV141-Sept9 plasmid (GeneChem, China)
194 transfection and empty GV141 vector (GeneChem, China) was used as a control. The constructed
195 plasmid was transfected into HSC-T6 cells using Lipofectamine 2000 (Invitrogen, USA) in
196 Opti-MEM (Gibco, USA) according to the manufacturer's instructions. 6 h after transfection, the
197 culture medium was replaced by DMEM with 10ng/ml TGF- β 1 (Peprotech, USA), 24h later cells
198 were collected for RT-qPCR and 48h for Western blot and Flow cytometry analyses.

199

2.8 RNA interference (RNAi) analysis

RNAi experiments in HSC-T6 cells were performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. For DNMT1, DNMT3a, DNMT3b and Sept9 silencing, HSC-T6 cells were transfected with DNMT1-siRNA, DNMT3a-siRNA, DNMT3b-siRNA, Sept9-siRNA or negative control siRNA (GenaPharma, China) and the culture medium was replaced by DMEM with 10ng/ml TGF- β 1 at 6 h after transfection. The following siRNA sequences were used: DNMT1-siRNA (sense:5'-CCCAGAGUAUGCACCAAUATT-3' and antisense: 5'-UAUUG-GUGCAUACUCUGGGTT-3'); DNMT3a-siRNA (sense:5'-GCGUCACACAGAAGCAUAUTT-3' and antisense: 5'-AUAUGCUUCUGUGUGACGCTT-3'); DNMT3b-siRNA (sense:5'-AGAUGA-CAGGUGCCCAGAGUU-3' and antisense: 5'-CUCUGGGCACCUGUCAUGUUU-3'); siRNA-control with scrambled sequence: (sense:5'-UUCUCCGAACGUGUCACGUTT -3'and anti-sense:5'-ACGUGACACGUUCGGAGAATT-3'); Sept9-siRNA (sense:5'-GAAGUCAUAU-CAACCGGATT-3' and antisense:5'-UUCCGGUUGAUUAUUGACUUCTT-3');

2.9 Cell cycle analysis

Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) was performed for cell cycle analysis. After 48 h of transfection, cells were trypsinized and washed for three times by cold PBS, and then fixed in 70% ethanol in PBS at 4 °C. After overnight fixation, cells were washed again with cold PBS for one time and incubated with propidium iodide (PI) staining buffer at 37 °C for 30min in the dark. The cell cycle was analyzed on BD FACSVerse (BD Biosciences) and cell populations were quantified by the ModFit data analysis software package (Verity Software House, USA).

2.10 Cell apoptosis analysis

Annexin-V-FITC Apoptosis Detection Kit (Bestbio, China) was used to quantify apoptotic cells on BD FACSVerse (BD Biosciences). Cells were distinguished into viable cells, early apoptotic cells, advanced apoptotic cells and dead cells. Data of apoptosis was analyzed by FlowJo software (TreeStar, USA). The percentage of apoptotic cells were compared between different groups.

2.11 MTT cell viability assay

The proliferation of HSC-T6 cells was determined by standard 3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assay. Cells were seeded in 96-well culture plates at a density of 5×10^3 cells per well, after cells adherence they were transfected with mock-vehicle GV141 or GV141-Sept9 as described above. 24 h later, 20 μ L of 5 mg/mL MTT (Sigma, USA) was added to each well and incubated at 37 °C for another 4 h; after that, cell medium was replaced by 150 μ L dimethyl sulfoxide (DMSO). And then, the absorbance was determined via a Thermomax microplate reader (Bio-Tek EL, USA) at 490 nm wavelength.

2.12 Immunohistochemistry

Liver tissues were fixed in 4% neutral buffered formalin solution. After 24-48 h, tissues were embedded in paraffin and sliced for routine histology. Tissue sections were dewaxed in xylene and dehydrated in alcohol, and then antigen retrieval was achieved by incubating in citric saline for 15 min. After blocking of 0.3% hydrogen and 2% bovine serum albumin, thin sections were incubated with primary antibody against Sept9 (1:200) and α -SMA (1:200) at 4°C overnight. After rinsing of PBS for three times, the slides were incubated with HRP-conjugated IgG secondary antibody for 60

244 min at 37°C. α -SMA and Sept9 expression was visualized by 3, 3'-diaminobenzidine
245 tetrahydrochloride (DAB) staining. Subsequently, the sections were counterstained with hematoxylin
246 for 30 s and dehydrated, α -SMA and Sept9 positive areas within the fibrotic region were then
247 observed.

248

249 2.13 Double Immunofluorescent Staining

250 To determine the colocalization of α -SMA or DNMT3a and Sept9 *in vivo*, double
251 immunofluorescent staining was performed. Frozen tissue sections were firstly incubated with 10%
252 bovine serum albumin for one hour at room temperature to block unspecific staining. After rinsing,
253 TRITC-conjugated anti- α -SMA/DNMT3a antibody (1:200) in combination with FITC-conjugated
254 anti-Sept9 antibody (1:200) were used to incubate. Then the stained sections were examined with
255 inversion fluorescence microscopy. α -SMA and DNMT3a were shown as redfluorescence, and Sept9
256 as greenfluorescence, respectively.

257

258 2.14 Statistical analysis

259 All results were presented as mean $\pm$ SD and were analyzed using the GraphPad Software. Statistical
260 significance was determined by either the Student's t-test or One-Way ANOVA (LSD), and
261 significance was defined as $p < 0.05$.

262

263 3. Results

264 3.1 Expression of Sept9 protein was down-regulated in *CCL₄*-induced mouse advanced liver fibrosis
265 model

266 To determine the expression of Sept9 in hepatic fibrosis, we firstly performed immunohistochemistry
267 assays on the liver tissues from CCl₄-induced murine liver fibrosis model. As shown in Fig.1A, all
268 liver tissues from CCl₄-treated mice presented with fibrotic lesions and marked hepatomegaly. The
269 degree of liver fibrosis was investigated through hematoxylin eosin (H&E), Masson Trichrom and
270 Sirius-Red staining. Histologically, H&E, Masson and Sirius-Red staining showed the CCl₄ injection
271 resulted in prominent liver inflammatory infiltration, necrosis and formation of regenerative nodules
272 and fibrotic septa in the main block of mouse livers when compared with liver tissues from
273 vehicle-treated groups (Fig.1B and C). Furthermore, immunostaining from CCl₄-treated groups
274 showed myofibroblast marker α -SMA was extensively stained, whereas the liver tissue sections from
275 vehicle treated mice were scarcely stained (Fig.1D). These data indicated us CCl₄-induced murine
276 liver fibrosis model was successfully established for us to study the liver fibrogenesis. Next, as
277 shown in Fig.1D, Sept9 immunostaining signal was decreased in fibrotic liver tissue sections from
278 CCl₄ treated mice, compared to control group. More importantly, the results of double
279 immunofluorescence staining indicated that Sept9 (green) was primarily co-localized with α -SMA
280 (red) in the fibrotic areas, indicating hepatic stellate cells may be one of the main cell types which
281 express Sept9 (Fig.1E). Consistently, the aberrant expression of α -SMA, Colla1 and Sept9 were
282 further demonstrated by Western blot analysis. As compared to the vehicle-treated groups,
283 expression levels of α -SMA and Colla1 protein were gradually increased along with the degree of
284 liver fibrosis progressed in mice while Sept9 protein expression was decreased in those livers
285 (Fig.1F). In addition, decreased expression of Sept9 mRNA was also observed in CCl₄-treated mouse
286 livers via Real-time quantitative PCR assay (Fig.1G).

287

3.2 Decreased expression of Sept9 in *mouse* primary HSCs and TGF- β 1 induced HSC-T6 cell lines

In response to stimulating factors, the trans-differentiation from quiescent stellate cells into proliferative myofibroblast-like cells, is the pivotal step for the progression of hepatic fibrosis(Henderson and Iredale, 2007). Western blot analysis further confirmed the abnormal expression of Sept9 protein in primary HSCs, which were perfused from livers of vehicle-treated and CCl₄-treated mice (Fig. 2A). And cytokine TGF- β 1 is believed to be one of the most potent inducer of HSCs activation, increasing of myofibroblast markers, α -SMA and collagens, can be dramatically triggered in TGF- β 1 treated HSCs (Friedman, 1999). To reveal the association of Sept9 in the activation of HSCs *in vitro*, we next measured the expression of Sept9 protein in cultured rat HSC-T6 cells treated with TGF- β 1. Here, as illustrated in Fig. 2B, treatment of TGF- β 1 could induced the up-regulation of α -SMA and Colla1 in HSC-T6 cells, and we could find the cells were activated most significantly at the concentration of 10 ng/ml TGF- β 1. Subsequently, the decreased expression of Sept9 protein was confirmed in HSC-T6 cells incubated with TGF- β 1 (10 ng/ml) for 48 h (Fig. 2C). And RT-qPCR analysis also suggested the down-regulation of Sept9 mRNA in TGF- β 1 activated HSCs (Fig. 2D). All these data were consistent with the results from experiments *in vivo*.

3.3 Ectopic expression of Sept9 alleviated TGF- β 1-induced up-regulation of α -SMA and Colla1 in HSC-T6 cells and promotes activated stellate cells apoptosis

The aberrant alteration of Sept9 mRNA and protein expression levels *in vivo* and *in vitro* urged us to elucidate the functional role of Sept9 in stellate cells activation and liver fibrogenesis. RT-qPCR analysis was performed 24 h after transfection and showed that Sept9 mRNA expression was

310 remarkably increased in HSC-T6 cells transfected with Sept9 plasmid (GV141-Sept9), compared
311 with those transfected with control plasmid (Fig.3A). Meanwhile, surface expression levels of
312 α -SMA and Colla1 were significantly repressed in GV141-Sept9 transfected cells as compared to
313 mock-vehicle groups (Fig.3A). These results suggested that forced expression of Sept9 could block
314 the activation of stellate cells even at baseline in culture. Subsequently, as illustrate in Fig.3B,
315 Western blot analysis demonstrated that TGF- β 1-induced increase of α -SMA and Colla1 could be
316 also suppressed by overexpression of Sept9, indicating that Sept9 might alleviate the activation of
317 HSC-T6 cells *in vitro*. Next, we further explored the underlying mechanism by which exotic
318 expression of Sept9 remitted TGF- β 1-induced HSC-T6 cells activation. Given experimental models
319 of liver fibrosis consistently reported that eradication of activated HSCs by apoptosis was a key step
320 in the onset of fibrosis regression (Iredale *et al.*, 1998), we evaluated effects of Sept9 overexpression
321 on the apoptosis of activated HSC-T6 cells. As compared to the vehicle groups (1.29%), high rate of
322 early apoptotic cells (46.5%) could be observed in GV141-Sept9 transfected HSCs via the flow
323 cytometric analyses (Fig.3C). Similar with previous observations (Mao *et al.*, 2013), Sept9
324 overexpression was commonly involved in cell apoptotic response. To further confirm Sept9 induced
325 activated stellate cells apoptosis, we performed Western blot analysis to investigate whether Sept9
326 could influence expression levels of cell apoptosis-related proteins, Bax, Bcl-2 and cleaved-caspase3.
327 As illustrated in Fig.3D, overexpression of Sept9 notably increased the Bax/Bcl-2 ratio, and active
328 forms of caspase3 was also obviously elevated in activated HSC-T6 cells transfected with
329 GV141-Sept9, conceivably suggesting that apoptosis of activated HSC-T6 cells was promoted by
330 Sept9. Consistently, paralleled expression patterns and co-localization of Sept9 and cleaved
331 caspase-3 was also examined in the ipsilateral cortex by Mao and colleagues (Mao *et al.*, 2013),

implying Sept9-induced apoptosis might be partly mediated by caspase3-regulated apoptosis pathway.

Moreover, by cell cycle analysis, we found that induction of Sept9 increased the percentage of activated HSCs in G0/G1 phase while a remarkable decrease of cell numbers in the S phases could be observed (Fig.3E). Additionally, the MTT assay indicated that the viability of HSC-T6 cells was significantly suppressed (~35% loss) by GV141-Sept9 as compared to the mock-vehicle groups (Fig.3F). Hence, Sept9 could also inhibit the activated HSC-T6 cells proliferation via perturbing G1 to S phase progression in cell cycle. Taken together, Sept9 might suppress the activation of HSCs induced by TGF- β 1, at least in part, via enhancing cell cycle arrest and cell apoptosis in liver fibrogenesis.

3.4 Depletion of Sept9 promotes TGF- β 1-induced expression of α -SMA and Colla1 in HSC-T6 cells

To further establish the important role of Sept9 during HSCs activation, siRNA specific for rat Sept9 (Sept9-siRNA) was used to knockdown Sept9 expression. As compared with scrambled-siRNA, Sept9-siRNA induced a significant decrease of Sept9 mRNA in non-treated HSC-T6 cells after 24 h transfection, accompanied by increased α -SMA and Colla1 mRNA expression (Fig.4A). Western blotting also showed that in comparison to cells transfected with scrambled-siRNA, Sept9-siRNA induced a significant decrease of Sept9 protein in TGF- β 1-treated HSC-T6 cells after 48 h transfection (Fig.4B). Consistently, both of α -SMA and Colla1 were up-regulated in response to Sept9-siRNA as compared to the scrambled-siRNA (Fig.4B), revealed that deficiency of Sept9 promoted the activation of HSC-T6 cells at baseline and aggravated phenotype changes of activated HSC-T6 cells. Accordingly, we also performed flow cytometric analyses to assess whether apoptosis and cycle of cells could be influenced by Sept9-siRNA. Slightly decrease of apoptotic cell proportion

could be observed in HSC-T6 cells transfected with Sept9-siRNA, even though only a very small number of cell apoptosis was triggered by scrambled-siRNA. Thus, the extraneous depletion of Sept9 could aggravate the liver fibrogenesis (Fig.4C).

357

358 3.5 Hypermethylation status of Sept9 in primary mouse HSCs from *CCl₄*-induced murine liver 359 fibrosis model

Emerging evidence have suggested that epigenetic modifications, especially DNA methylation may contribute to the activation of hepatic stellate cells. Generally, transcriptional activity of gene is impaired when it's methylated. Given the loss of Sept9 gene and its particular function in fibrogenesis, we performed RRBS (reduced representation bisulfite sequencing) analysis to clarify the potentially upstream regulatory mechanism on Sept9. According to the result of RRBS, 10 CpG sites of Sept9 gene were hypermethylated in *CCl₄* treated mice (Fig. 5A). We next used Bisulfite pyrosequencing PCR (BSP) on bisulfite-treated DNA from primary mouse HSCs to confirm the methylated patterns of Sept9. The results showed us all the six sites we randomly selected from RRBS results were in high methylation ratio (Fig. 5B). In mammals, DNA methylation is catalyzed by three different DNA methyltransferases, DNMT1, DNMT3a and DNMT3b (Valente *et al.*, 2014). Consistently with previous studies, up-regulation of DNMTs (DNMT1, DNMT3a and DNMT3b) were observed in liver tissues from *CCl₄*-treated mice (Fig.5C). Subsequently, TGF- β 1 induced up-regulation of DNMTs *in vitro* were also showed by Western blot analysis (Fig.5D). Next, we treated activated HSC-T6 cells with DNMTs inhibitor 5-AzadC to explore the methylation effect of DNMTs on Sept9 in the progression of hepatic fibrosis. As illustrated in Fig. 5E, expression levels of Sept9 protein were elevated in cells exposed to 5-AzadC, suggesting that an epigenetic

376 mechanism was operated to prevent Sept9 expression in activated HSCs, which further verified the
377 information of RRBS. Whereas, reduced expression of α -SMA and Colla1 were observed in
378 5-AzadC treated cells. We also used MSP to detect the extent of methylation at the CpG island,
379 5-AzadC treatment reversed TGF- β 1-induced aberrant hypermethylation of Sept9 gene (Fig.5F).
380 Taken together, these findings convincingly indicated that inhibition of Sept9 expression in the
381 progression of HSC-T6 cell activation might be mediated by DNA methylation, which was
382 significantly correlated with the fibrogenesis.

383

384 3.6 Silencing of DNMT-3a blocked TGF- β 1 induced down-regulation of Sept9

385 Next, we investigate which DNMT might be responsible for the inhibition of Sept9 in liver fibrosis.
386 SiRNAs specific for rat DNMT1, DNMT3a and DNMT3b (DNMT1-siRNA, DNMT3a-siRNA and
387 DNMT3b-siRNA) were used to knockdown the three DNMTs respectively. As shown in Fig.6A, all
388 three siRNAs induced significant decrease of corresponding DNMTs in TGF- β 1-induced HSC-T6
389 cells after 48 h transfection, in comparison to those cells transfected with scrambled-siRNA.
390 However, elevated Sept9 expression was only observed in the cells transfected with
391 DNMT3a-siRNA, relative to cells transfected with scrambled-siRNA (Fig.6A). There was no
392 significant difference of Sept9 expression levels between scrambled-siRNA and DNMT1-siRNA or
393 DNMT3b-siRNA transfected groups. Meanwhile, relief of Sept9 methylation was observed in
394 DNMT3a-siRNA transfected HSC-T6 cells via MSP analysis (Fig.6B). In order to verify whether
395 DNMT3a specific for Sept9 methylation, double immunofluorescence stain of Sept9 and DNMT3a
396 was performed in mouse liver tissue slices (frozen), and co-localization of these two protein could be
397 observed (Fig.6C). Furthermore, DNMT3a-siRNA induced similar effect with Sept9 plasmid on

398 α -SMA expression, echoed the negative relation of DNMT3a and Sept9 biological function during
399 HSCs activation (Fig.6D). These results indicated that DNMT3a might contribute to the
400 down-regulation of Sept9 during HSC activation through DNA methylation.

401

402 4. Discussion

403 Liver fibrosis can be caused by almost all chronic liver diseases, including chronic HBV and HCV
404 infection, alcohol or nonalcoholic liver disease. It is characterized by excessive accumulation of
405 extracellular matrix (ECM) and presents in intrahepatic connective tissue abnormal hyperplasia at the
406 macro-level (Jiao *et al.*, 2009; Troeger *et al.*, 2012). More and more studies paid attention to liver
407 fibrosis not only because there is no effective anti-fibrotic treatment strategy has yet been built in
408 humans, continuous progresses of liver fibrosis ultimately results in cirrhosis or even HCC(Bataller
409 and Brenner, 2005). Differentiation of quiescent hepatic stellate cells into activated and proliferated
410 myofibroblasts, the principal source of ECM, act as a pivotal role in the pathogenesis of liver fibrosis
411 (Hernandez-Gea and Friedman, 2011). In the past, liver fibrosis was regarded as an irreparable
412 pathological process because of the distortion of liver parenchyma and vascular architecture (Popper
413 and Uenfriend, 1970), but increasingly clinical and experimental researches have persuasively
414 revealed that fibrosis alleviation may occur upon the elimination of etiological agents and apoptosis
415 of activated HSCs.(Ellis and Mann, 2012). Therefore, suppression of HSCs activation and blocking
416 of ECM deposition, finally remodeling the liver's architecture is the crucial therapeutic strategy for
417 liver fibrosis. However, the molecular mechanisms underlying regulation of HSCs activation and
418 apoptosis are still poorly understood. Here, our study provided the initial evidence that Sept9 gene
419 was methylated in liver fibrosis, caused loss of Sept9 expression *in vivo* and *in vitro*. Moreover,

420 protein and mRNA expression levels of Sept9 were closely correlated with the progresses of HSCs
421 activation as measured by the expression of myofibroblast markers α -SMA and Colla1.

422 Human septins are an evolutionarily conserved family of GTP-binding proteins comprising 13
423 isoforms that are differentially expressed in a cell- and tissue-specific manner. Septins firstly
424 characterized in *Saccharomyces cerevisiae* by mutations that regulate budding morphology and cell
425 cycle progression(Hartwell, 1971). According to sequence similarity, all isoforms have been
426 classified into the four subgroups: Sept2, Sept3, Sept6 and Sept7(Kinoshita, 2003a). Nowadays,
427 septins have been identified in many other eukaryotes, ranging from fungi to humans(Kinoshita,
428 2003b; Sandroek *et al.*, 2011; Mostowy *et al.*, 2014), except for plants(Oh and Bi, 2011). When
429 purified from cells and tissues, septins assembled into the Sept2–Sept6–Sept7 apolar hexamer
430 complex (Sellin *et al.*, 2011). However, in dividing non-neuronal cells, septins complex additionally
431 contain Sept9, which occupies the terminal positions, to form octamers(Kim *et al.*, 2011). Almost all
432 human septins, including Sept9, exhibit extensive alternative splicing, yielding diversity at the 5' and
433 3' ends of the transcripts. Seven transcripts of Sept9 have been identified to encod five distinct
434 isoforms (Sept9_v1-Sept9_v5)(Scott *et al.*, 2005). Altered expression of several septin isoforms have
435 been revealed in human carcinoma (Connolly *et al.*, 2011a), and aberrant levels of septins closely
436 correlate with tumorigenic phenotypes, such as increased cell proliferation, invasiveness, and
437 chemotherapy resistance(Amir and Mabjeesh, 2007; Chacko *et al.*, 2012). However, a growing
438 body of research is focused on the methylation of another variant Sept9_v3. Diana Connolly and
439 colleague clarified Sept9 isoforms differ in their respective cellular distribution and promigratory
440 potential (Connolly *et al.*, 2011b). They performed a comprehensive analysis of the Sept9 isoforms at
441 the levels of mRNA and protein expression and methylation status in carcinoma cell lines and tumor

442 tissues. As compared to Sept9_v1, hypermethylated Sept9_v3 was more likely to be observed, with
443 repression of mRNA and protein level. Subsequently, hypermethylation status of Sept9 was
444 gradually identified in malignant pleural effusions, noninvasive lung cancer, esophageal squamous
445 cell carcinoma, cholangiocarcinoma and even diabetes(Dietrich *et al.*, 2013; Dayeh *et al.*, 2014; Kuo
446 *et al.*, 2014; Powrozek *et al.*, 2014; Andresen *et al.*, 2015). More specifically, assessment of
447 methylated Sept9 in peripheral blood has been practically applied for clinical detection of
448 asymptomatic colorectal cancer (Church *et al.*, 2014). Moreover, CheeKian Tham *et al.* stated
449 postoperative plasma methylation level of Sept9 was an independent predictor of recurrence and
450 survival of CRC patients (Tham *et al.*, 2014). And RT-qPCR based qualitative assay for detection of
451 methylated Sept9 DNA in human plasma was also validated (Potter *et al.*, 2014). Subsequent data
452 from cycle threshold value showed the methylated Sept9 could be also detected in CRC tissue
453 samples (Toth *et al.*, 2014).

454 Epigenetic regulation of gene expression allows for the hereditary feature and environment factors
455 change phenotype in a manner independent of the DNA sequence(Bird, 2007). Gene silencing
456 associated with DNA methylation, a crucial epigenetic control mechanism, has been identified to
457 occur through the course of human development, maturation and aging (Bird, 2002; Burzynski,
458 2003). Even the DNA methylation patterns of fetal reflects tissue origin and developmental age
459 (Sliker *et al.*, 2015). Human lifestyle and nutritional status were also believed to affect DNA
460 methylation levels(Niculescu, 2012). High fructose or fat intake could induce DNA methylation,
461 suggesting DNA methylation is involved in the metabolism of human body (Ohashi *et al.*, 2015;
462 Wang *et al.*, 2015). According to the liver is the human body's largest metabolism organ, there is
463 increasing evidence that DNA methylation also participated in pathological process of various liver

diseases. Both of alcohol abuse and tobacco exposure, two major risk factor for the development of liver related disorders, induced alter in gene methylation status(Mansoori and Jain, 2015; Shen *et al.*, 2015). Moreover, both of *in vitro* and *in vivo* studies have demonstrated that some anti-inflammatory factors, such as Apolipoprotein A1 (ApoA1) and PPAR- γ , could be suppressed by Hepatitis B/C virus core protein through promoter methylation, resulting in deterioration of hepatitis or eventually progress to cancer (Zhao *et al.*, 2015; Wang *et al.*, 2016b). Genome-wide DNA methylation analysis by Nobuhisa Yamada *et al.* revealed that there were 2,670 CpG sites that significantly differed in regards to the methylation levels between tumor and non-tumor liver tissues in hepatocellular carcinoma (HCC)(Yamada *et al.*, 2016). And further methylation-specific PCR (MSP) combined with expression analysis showed that, many tumors suppressor genes (such as APC, CDKN2A, and GSTP1) were significantly hypermethylated and inhibited in HCC compared to the non-tumor liver tissues(Yamada *et al.*, 2016). Besides, methylated CpG tandems amplification and sequencing (MCTA-Seq) method also detected hypermethylated CpG islands in circulating cell-free DNA of HCC patients, contributed to the improvement of HCC diagnosis in a clinical setting(Wen *et al.*, 2015). Nowadays, data emerging from methylation screening showed that DNA methylation is a crucial step in the transdifferentiation of HSCs (Page *et al.*, 2016), methylation status change from hypo- to hypermethylation might contribute to the progression of liver fibrosis (Komatsu *et al.*, 2012). More importantly, 5-azadC, an inhibitor of DNA methyltransferase, reportedly alleviated the pathological process of renal, lung and liver fibrosis *in vivo* and *in vitro* (Huan *et al.*, 2015) (Mann *et al.*, 2007; Bechtel *et al.*, 2010). These identifications indicated the importance role of DNA methylation in the progression of fibrotic diseases.

485 In this study, reduced representation bisulfite sequencing (RRBS) techniques and BSP assay
486 identified the hypermethylation status of Sept9 in primary HSCs from fibrotic mice. Then DNA
487 methylation associated Sept9 gene silencing was observed in the fibrotic liver tissues from
488 CCl₄-treated mice *in vivo* by Western blot and RT-qPCR analysis. And its decrease strongly
489 correlated with the HSCs activation process as measured by the induction of α -SMA and Colla1,
490 two biomarkers of stellate cells activation and liver fibrogenesis. Abnormal expression of Sept9
491 during HSCs activation also occurred *in vitro* and loss-of-function analyses revealed DNMT3a, a
492 member of DNA methyltransferase enzymes family, might contribute to Sept9 reduction.
493 Overexpression of Sept9 blocked the expression of α -SMA and Colla1 at both mRNA and protein
494 levels, indicating that ectopic expression of Sept9 could prevent the activation of HSCs and alleviate
495 the excessive collagen deposition. Furthermore, in combination with the results of flow cytometric
496 analysis, we suggested that Sept9 suppressed collagen production might through promoting activated
497 HSCs apoptosis, which was widely recognized as an important approach of liver fibrosis relief. And
498 the pro-apoptotic effect of Sept9 on activated HSCs was simultaneously confirmed by the
499 up-regulating of protein Bax/Bcl-2 ratio and activated-capase3 expression in Sept9 overexpressed
500 HSCs. Meanwhile, by cell cycle analysis, we further found that forced expression of Sept9 increased
501 the percentage of activated HSCs in G0/G1 phase, along with an apparent reduction of cell numbers
502 in the S phase. Additionally, therapeutic Sept9-siRNA enhanced effects of TGF- β 1 on α -SMA and
503 Colla1 expression and slightly reduced normal programmed cell apoptosis. According to these
504 results we suggest that DNA methylation associated Sept9 gene silencing is required for HSCs
505 activation, and ectopic expression of Sept9 may block liver fibrogenesis (Fig.7)(Friedman, 2012).

506 In summary, our findings in the present study suggested that methylation of Sept9 plays a pivotal
507 role during hepatic stellate cell activation and liver fibrogenesis. Forced expression of Sept9 offsets
508 TGF- β 1-induced HSC-T6 cells activation and even evokes cells apoptosis while silencing of Sept9
509 promote the process of fibrosis. These results indicate the therapeutic potential of Sept9 for treatment
510 of liver fibrosis. Nevertheless, the underlying mechanism of Sept9 in the setting of liver fibrosis has
511 not been fully investigated. Hence, more basic and clinical studies will be conducted urgently to
512 explore the molecular mechanism, especially the substrates and downstream signaling pathways that
513 are regulated by Sept9. To our knowledge, this is the first report of DNA methylation associated
514 Sept9 gene silencing during liver fibrosis *in vivo* and *in vitro*. This study gives us novel insights into
515 the potential roles of Sept9 in the development of stellate cell activation and encourages future
516 studies to determine the value of methylated Sept9 as a novel biomarker and therapeutic target in
517 hepatic fibrosis.

518

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523 **Conflict of interest**

524 The authors declare that there are no conflicts of interest.

525

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705

706 **Figure Legends**

707 **Fig. 1. Down-regulation of Sept9 in the CCl4-induced mouse liver fibrosis models, n=8 mice per**
708 **group.** (A) Gross pathology of livers from vehicle control and fibrotic mice, representative of all
709 animals studied. (B-C) Pathology observation of the representative hematoxylin and eosin (H&E),
710 Massion and Sirius-Red staining of mouse liver sections. (original magnification, $\times 200$ and $\times 400$).
711 (D) The expression of α -SMA and Sept9 were assessed by immunohistochemistry staining analysis.
712 Representative views from each group are presented (original magnification, $\times 400$). (E) Mouse liver
713 tissues were double stained with α -SMA and Sept9 antibodies. Representative photomicrographs are
714 shown (original magnification, $\times 200$) (F) Protein expression levels of α -SMA, Colla1, Sept9 and
715 β -actin control were analyzed by Western blot in mouse liver tissues. Representative blots of three
716 independent experiments are shown. * $p < 0.05$, ** $p < 0.01$ vs. vehicle control. (G) Total RNAs extracts
717 were made from the mouse liver tissues. The expression of α -SMA, Colla1 and Sept9 mRNA were
718 assessed by RT-qPCR. The results are expressed as relative expression against vehicle control.
719 ** $p < 0.01$ vs. vehicle control.

720

721 **Fig. 2. Decreased expression of Sept9 in mouse primary HSCs and TGF- β 1 induced HSC-T6**
722 **cell lines.** (A) Expression levels of myofibroblast markers (α -SMA and COL1a1), Sept9 and β -actin
723 control protein were assayed by Western blot in primary mouse HSCs derived from the liver tissues

724 of CCl4 treated mice, and vehicle control mice. * $p < 0.05$, ** $p < 0.01$ vs vehicle control. (B) HSC-T6
725 cells were stimulated with different concentrations of TGF- β 1 (0, 2, 5, 10 ng/ml) for 48 h. The
726 protein level of myofibroblast markers (α -SMA and Colla1) were assessed by Western blot. The
727 results are shown as relative expression against non-treated cells. ** $p < 0.01$ vs. non-treated cells. (C)
728 HSC-T6 cells were stimulated with TGF- β 1 (10 ng/ml) for 48h. The protein level of α -SMA and
729 Sept9 were measured by Western blot. Representative blots of three independent experiments are
730 shown. ** $p < 0.01$ vs. non-treated cells. (D)HSC-T6 cells were stimulated with TGF- β 1 (10 ng/ml) for
731 24h. The mRNA level of α -SMA, Colla1 and Sept9 were examined by RT-qPCR. The results are
732 expressed as relative expression against non-treated cells. * $p < 0.05$, ** $p < 0.01$ vs. non-treated cells.

733

734 **Fig. 3. Effect of Sept9 on activated HSCs.** (A) Total RNAs were isolated 24h after transfection of
735 plasmids in non-treated HSCs. The expression of Sept9, α -SMA and Colla1 mRNA was tested by
736 RT-qPCR. The results are expressed as relative expression against control expression without
737 transfection. * $p < 0.05$, ** $p < 0.01$ vs GV141-control. (B) The expression levels of Sept9, α -SMA and
738 Colla1 protein were determined by western blot in plasmids transfected TGF- β 1-induced HSC-T6
739 cells. Representative images of three independent experiments are shown. ** $p < 0.01$ vs non-treated
740 cells, # $p < 0.05$, ## $p < 0.01$ vs GV141-control. (C) Effect of Sept9 on apoptosis of TGF- β 1-treated
741 HSC-T6 cells. One representative experiment of the three independent experiments is demonstrated.
742 (D)The expression levels of apoptosis-associated Bax, Bcl-2 and proteolytic cleavage of caspase-3
743 protein were analyzed by Western blot in Sept9-transfected TGF- β 1-treated HSCs. Representative
744 blots of three independent experiments are shown. ** $p < 0.01$ vs GV141-control. (E) Effect of Sept9
745 on cell cycle in TGF- β 1-activated HSC-T6 cells. Representative images of three independent

746 experiments are shown. (F) Sept9 significantly decreased cell proliferation of TGF- β 1-treated
747 HSC-T6 cells. Proliferation of HSC-T6 cells was tested by MTT assay. Data represent the mean $\pm$
748 SD from three independent experiments. **p<0.01 vs GV141-controls.

749

750 **Fig. 4. Effect of Sept9 silencing on activated HSCs.** (A) HSC-T6 cells without TGF- β 1 treatment
751 were transfected with Sept9-siRNA or scrambled-siRNA respectively. The expression of Sept9,
752 α -SMA and Colla1 mRNA was determined by RT-qPCR. The results are expressed as relative
753 expression against control expression without transfection. *p<0.05, **p<0.01 vs scrambled-siRNA.
754 (B) The expression levels of Sept9, α -SMA and Colla1 protein were analyzed by Western blot in
755 Sept9-siRNA transfected TGF- β 1-induced HSC-T6 cells. Representative images of three
756 independent experiments are shown. *p<0.05, **p<0.01 vs non-treated cells, #p<0.05, ##p<0.01 vs
757 scrambled-siRNA. (C) Effect of Sept9-siRNA on apoptosis of TGF- β 1-activated HSCs. One
758 representative experiment of the three independent experiments is demonstrated.

759

760 **Fig. 5. Methylation status of Sept9 and aberrant expression of DNMTs in liver fibrosis.**
761 (A) Result of RRBS analysis identified methylated region of Sept9 gene in primary mouse HSCs
762 derived from the liver tissues of CCl4 treated mice (B) Status of methylation at the CpG site of Sept9
763 gene: Bisulfite sequencing revealed a methylated CpG island in the Sept9 gene of **mouse** primary
764 HSCs. (C) Expression levels of DNMTs (DNMT1, DNMT3a, DNMT3b) and β -actin control protein
765 were assayed by Western blot in the liver tissues of CCl4 treated mice, and vehicle control mice.
766 **p<0.01 vs vehicle control. (D) Whole-cell extracts were isolated from different concentrations of
767 TGF- β 1 (0, 2, 5, 10 ng/ml) treated HSC-T6 cells. The protein level of DNMTs (DNMT1, DNMT3a,

DNMT3b) and β -actin control protein were assessed by Western blot. The results are expressed as relative expression against control expression without treatment. * $p < 0.05$, ** $p < 0.01$ vs. vehicle control. (E). Activated HSCs were cultured in the presence of 1 mM 5-AzaC for 48 h. Whole-cell protein extracts were made from the cells. α -SMA, Col1a1, and Sept9 protein were analyzed by western blotting. Representative blots of three independent experiments are shown. ** $p < 0.01$ vs. non-treated cells, ## $p < 0.01$ vs. 5-AzaC treated cells. (F). MSP analysis of Sept9 in HSC-T6 cells challenged with or without 5-azaC. All the 3 groups show the presence of both unmethylated and methylated DNA respectively. The bands detected by the methylated primer represent methylated Sept9 (M), and the bands detected by the unmethylated primer represent unmethylated Sept9 (U).

Fig. 6. siRNAs directed against DNMTs respectively and DNMT3a-siRNA was able to up-regulate Sept9 expression level. (A) HSC cells were transfected with DNMT1-siRNA, DNMT3a-siRNA, DNMT-3b or scrambled-siRNA respectively. Whole-cell extracts were isolated 48 h after transfection. Protein levels of DNMT1, DNMT3a, DNMT3b, Sept9 and β -actin were measured by western blotting. Representative images of three independent experiments are shown. ** $p < 0.01$ vs scrambled-siRNA. (B) MSP analysis of Sept9 in TGF- β 1-treated HSCs-T6 cells with RNAi transfection. Both groups show the presence of both unmethylated and methylated DNA respectively. The bands detected by the methylated primer represent methylated Sept9 (M), and the bands detected by the unmethylated primer represent unmethylated Sept9 (U). (C) Mouse liver tissues were double stained with Sept9 and DNMT3a antibodies. Representative photomicrographs are shown (original magnification, $\times 200$) (D) Expression levels of α -SMA protein were measured by Western blot in DNMT3a-siRNA transfected TGF- β 1-treated HSCs. Representative blots of three

790 independent experiments are shown. **p<0.01 vs scrambled-siRNA.

791

792 **Fig.7. A schematic model of Sept9 function in liver fibrosis.** Liver injury induces the expression of
793 DNA methyltransferase enzyme, DNMT3a, and promotes the methylation effect of DNMT3a on
794 Sept9. Then quiescent hepatic stellate cells are activated due to the suppression of Sept9, leading to
795 excessive ECM deposition and fibrosis progression. However, ectopic Sept9 causes apoptosis of
796 activated stellate cells and collagen degradation, resulting in fibrosis remission.

797

798

Table1. Primer sequences for Real-time qPCR.

Gene	Forward Primer	Reverse Primer
α -SMA(rat)	5'-CGAAGCGCAGAGCAAGAGA-3'	5'-CATGTCGTCCCAGTTGGTGAT-3'
Colla1(rat)	5'-GATCCTGCCGATGTCGCTAT-3'	5'-TGTAGGCTACGCTGTTCTTGCA-3'
Sept9_v3(rat)	5'-ATATCAACCGGAAGAAACGC-3'	5'-GCGCTTCATGAACTCAATGT-3'
DNMT3a(rat)	5'-GGCCCAT TCGATCTGGTGA-3'	5'-CTTGGCTATTCTGCCGTGTTC-3'
β -actin(rat)	5'-CACCCGCGATACAACCTTC-3'	5'-CCCATACCCACCATCACACC-3'
α -SMA(mouse)	5'-CGGGAGAAAATGACCCAGATT-3'	5'-AGGGACAGCACAGCCTGAATAG-3'
Colla1(mouse)	5'-GGAGAGTACTGGATCGACCCTAAC-3'	5'-ACACAGGTCTGACCTGTCTCCAT-3'
Sept9_v3(mouse)	5'-CGATCACCCACGATATTGAA-3'	5'-CTGCCAGCAGTTCTCATTGT3'
GAPDH(mouse)	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

Figure*Fig.1 Wu et al.***A.** **B.** **C.****Masson****Sirius** **D.** α -SMA

Sept9

E.**Dapi** α -SMA (Red)

Sept9 (Green)

Merge **F.** **G.**

A.

B.

C.

D.

A.

B.

C.

D.

E.

F.

A.

B.

C.

A.

Chromosome	Start	End	Gene	DMR start	DMR end	Numbers of DMR
chr11	117359470	117360422	Septin9	117359502	117359601	10

B.

Methylation of Sept9

Legend: ■ unmethylated ■ methylated ■ not present

C.

D.

E.

F.

A.

B.

C.

D.