Materials and methods

Animals and surgery. A total of 24 Male Sprague-Dawley rats (6 weeks old) that weighed 200-220 g were purchased from Sino-British SIPPR/BK Lab Animal Ltd. Rats were randomized into four groups: Sham, hepatic I/R, ischemia preconditioning-I/R pretreatment (IPC) and COX2 inhibitor (NS-398) pretreatment-I/R (I/R + NS-398) groups (n=6). All animals were fasted for 12 h before surgery and anesthetized by intraperitoneal (i.p.) injection of 40 mg/kg pentobarbital. A model for partial warm I/R in the liver was generated as described previously (1). Sham-operated rats underwent the same surgical procedures but without vascular occlusion, and I/R rats were subjected to 60 min of ischemia. IPC rats were subjected to 10 min of ischemia and 10 min of reperfusion before the 60 min ischemic insult. To inhibit COX-2 activity, a single dose of the COX-2 inhibitor NS-398 (30 mg/kg, Santa Cruz Biotechnology, Inc.) was dissolved in DMSO and then administered intraperitoneally 10 min prior to the onset of liver ischemia. During surgery, the animals' core body temperature was maintained at 37˚C using heat pads and lamps. The animals were sacrificed by an intraperitoneal injection of sodium pentobarbital (100 mg/kg) at 2 h after reperfusion. Death of the rats was verified by a combination of criteria, including lack of pulse, breathing, corneal reflex, response to a firm toe pinch and graying of the mucous membranes. liver samples were collected for further analysis.

Total RNA extraction and reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted using Trizol (Thermo Fisher Scientific, Inc.) from liver tissue samples from each group. RNA was reverse transcribed using an RT kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The extracted RNA was heated for 15 min at 70˚C and cooled on ice to 4˚C. In total, 1 μg RNA was diluted in RNase-free water to a volume of 14 μl. Then, 4 μl 5x reaction buffer, 0.5 μl Oligo-dT primers, 0.5 μl dNTPs and 1 μl reverse transcriptase were mixed together (total volume, 20 μl). The mixture was incubated for 60 min at 42˚C and heated to 75˚C for 5 min to terminate the reaction. Then, 1 μl cDNA was amplified in a 15-μl reaction volume containing 1.5 μl 10x Taq buffer, 0.3 μl sense primer (10 μmol/l), 0.3 μl antisense primer (10 μmol/l), 0.5 μl Takara Taq (Takara Bio, Inc) and 2 μl dNTPs mixture. Amplification was initiated with 5 min of denaturation at 94˚C. The PCR conditions were as follow; denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and polymerization at 72˚C for 30 sec. To ensure amplification was in the linear range the optimal number of cycles was identified. After the last amplification cycle, the samples were incubated at 72˚C for 10 min and then maintained at 4˚C. The following primer sequences were used: EP1 forward, 5'-AACAGGCGGTAACGGCACATC-3' and reverse, 5'-TGGCGAACAACAGGAAGG-3' (152 bp); EP2 forward, 5'-TCGCCATCGGACACCCTTAC-3' and reverse, 5'-GTTGAGCAGCGGCAGAGAAC-3' (116 bp); EP3 forward, 5'-CTTCAATCAGATGTCAGTAG-3' and reverse 5'-TTCTTAGCAGATAAACC-3' (134 bp); and EP4 forward, 5'-ACAGCCCAGTGACCATTCCC-3' and reverse, 5'-ACAGCCAGCCCACATACCAG-3' (136 bp); GAPDH forward, 5'-GTCGGTGTGAACGGATTTG-3' and reverse, 5'-TCCCATTCTCAGCCTTGAC-3' (181 bp). In total, 10 μl of each PCR reaction was electrophoresed on a 2% agarose gel in TBE buffer containing ethidium bromide, scanned with a NucleoVision imaging workstation (NucleoTech, Inc.), and quantified with the use of GelExpert version 3.5 (NucleoTech, Inc.); the results were determined by using ratios that compared the expression of the gene of interest to that of GAPDH, as described previously (2).

Information and histology of human liver biopsy specimens. Three normal human liver and 3 I/R liver (transplant allograft) tissue samples were obtained from patients (male; age, 38-66 years; median age, 43 years) who had undergone surgery (between July and September, 2012) at Changzheng Hospital (Shangai, China). Hepatic steatosis and cirrhosis were excluded by visual inspection from the surgeon at the time of tissue specimen collection during surgery. Liver samples from the transplant allografts underwent ~20 min of warm ischemia, 6 h of cold ischemia and 60 min of reperfusion. Each subject provided informed written consent. The present study was approved by the Chinese Ethics Committee of Registering Clinical Trials (approval file no. ChiECRCT-2011001). The samples were fixed using formalin overnight at 4˚C and embedded in paraffin. Human liver tissue sections (5 μm) were then immunostained using the EnVision method (DakoCytomation; Agilent Technologies, Inc.) according to the manufacturer's instructions. An anti-EP4 antibody (Santa Cruz Biotechnology, Inc.) was applied, and 3,3'-diaminobenzidine (Sigma-Aldrich; Merck KGaA) was used for color development, followed by hematoxylin counterstaining, as described previously (2).

References

1. Selzner N, Selzner M, Jochum W and Clavien PA: Ischemic preconditioning protects the steatotic mouse liver against reperfusion injury: An ATP dependent mechanism. J Hepatol 39: 55-61, 2003.
2. Kuzumoto Y, Sho M, Ikeda N, Hamada K, Mizuno T, Akashi S, Tsurui Y, Kashizuka H, Nomi T, Kubo A, *et al*: Significance and therapeutic potential of prostaglandin E2 receptor in hepatic ischemia/reperfusion injury in mice. Hepatology 42: 608-617, 2005.

Figure S1. A COX2 inhibitor markedly suppressed EP mRNA expression in the liver at 2 h of reperfusion. The mRNA expression of EP1, EP2, EP3 and EP4 was examined in rodent livers at 2 h of reperfusion. Higher mRNA expression of EP1, EP2, EP3 and EP4 was found in the animals that underwent I/R insult compared with the sham-treated animals. Similar to ischemia preconditioning, COX2 inhibitor NS-398 pretreatment suppressed the expression of EP1, EP2, EP3 and EP4 mRNA induced by I/R. $^{*}P<0.05$ vs. sham. $^{\#}P<0.05$ vs. I/R. EP, prostaglandin E receptor; I/R, I/R, ischemia/reperfusion; COX2, cyclooxygenase-2.

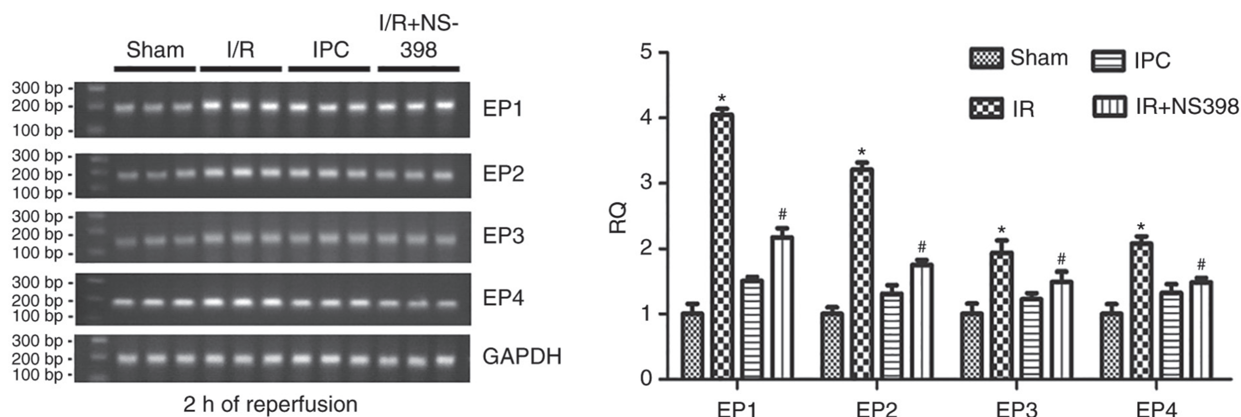

Figure S2. Difference in EP4 expression between human normal liver and transplanted liver samples. Human liver tissue samples were obtained by liver biopsy during surgery, and detailed information is available in the Supplementary Materials and methods. Immunohistochemical analysis of EP4 protein expression was performed with normal liver tissue (No. 1-3) and transplanted tissue (No. 4-6). magnification, x100. Staining intensity is indicated as follows: No signal (-), weak signal (+) and strong signal (++). Higher EP4 expression was found in the human allograft biopsy samples compared with the normal tissue samples. No., Number; EP4, prostaglandin E receptor subtype 4.