Canonical Notch Pathway Protects Hepatocytes from Ischemia/Reperfusion Injury in Mice by Repressing Reactive Oxygen Species Production Through JAK2/STAT3 Signaling

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Hepatic ischemia/reperfusion (I/R) injury is initiated by reactive oxygen species (ROS) accumulated during the early reperfusion phase after ischemia, but cellular mechanisms controlling ROS production and scavenging have not been fully understood. In this study, we show that blocking Notch signal by knockout of the transcription factor RBP-J or a pharmacological inhibitor led to aggravated hepatic I/R injury, as manifested by deteriorated liver function and increased apoptosis, necrosis, and inflammation, both *in vitro* and *in vivo*. Interruption of Notch signaling resulted in increased intracellular ROS in hepatocytes, and a ROS scavenger cured exacerbated hepatic I/R injury after Notch signaling blockade, suggesting that Notch signal deficiency aggravated I/R injury through increased ROS levels. Notch signal blockade resulted in down-regulation of Hes5, leading to reduced formation of the Hes5-STAT3 complex and hypophosphorylation of STAT3, which further attenuated manganese superoxide dismutase (MnSOD) expression and increased ROS and apoptosis. Indeed, overexpression of a constitutively active STAT3 rescued MnSOD expression and I/R injury–induced apoptosis in the absence of Notch signaling. Finally, forced Notch activation by ligand stimulation or Hes5 overexpression reduced intracellular ROS and protected hepatocytes from apoptosis after I/R injury through the activation of STAT3 and MnSOD expression. Notch signal protects hepatocytes from I/R injury by Hes5-dependent activation of STAT3, which activates the expression of MnSOD, leading to the scavenging of ROS. (HEPATOLOGY 2011;54:979-988)

Hepatic ischemia/reperfusion (I/R) injury is initiated by the accumulation of reactive oxygen species (ROS). The depletion of intracellular adenosine triphosphate by anoxia followed by reoxygenation results in massive production of ROS in mitochondria,[1-3] in addition to other sources.[4] ROS accumulates in cells when its production exceeds the scavenging capacity of the major scavenger manganese superoxide dismutase (MnSOD) and other enzymes.[5,6] ROS impairs cells directly through lipid peroxidation,

Abbreviations: ALT, alanine aminotransferase; APC, allophycocyanin; AST, aspartate aminotransferase; BM, bone marrow; DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell sorting; GSI, γ-secretase inhibitor; iNOS, inducible nitric oxide synthase; I/R, ischemia/reperfusion; KO, knockout; MnSOD, manganese superoxide dismutase; mRNA, messenger RNA; PCR, polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

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protein oxidation, and DNA damage, which together finally induce cell death. Moreover, ROS and oxidized molecules act as signaling molecules to activate nuclear factor κB and activator protein 1 followed by inflammatory responses.[6-8] I/R injury also activates stress signaling and signaling through Toll-like receptors (TLRs), leading to cell damage through signaling mediated by mitogen-activated protein kinase, Akt, and other pathways.[9,10] However, molecular mechanisms controlling cellular I/R responses have not been fully elucidated.

The RBP-J–mediated Notch signaling regulates both development and cell responses to extracellular insults.[11-13] Recent results have suggested that Notch signaling plays a role in I/R and ROS accumulation,[14,15] but the molecular mechanisms have not been established. In the present study, we show that the Notch–RBP-J pathway protects hepatocytes from I/R injury by repressing the production of ROS through JAK2/STAT3 signaling.

Materials and Methods

Mice. *RBP-J*–floxed (*RBP-J*^{f}) mice[16] and *Mx-Cre* transgenic mice (provided by K. Rajewsky) were maintained on a C57BL/6 background and were genotyped by way of polymerase chain reaction (PCR).[16] The Cre-mediated deletion of *RBP-J* was induced in 1-month-old RBP-J^{f}-MxCre mice by using poly(I)-poly(C) (Sigma, St. Louis, MO) exactly as described.[11,16] Partial hepatic warm ischemia was induced as described.[17] Briefly, Carprofen (Pfizer Animal Health) was administered subcutaneously (5 mg/kg) 2 hours before surgery for analgesia. Mice were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally) and were injected with heparin (100 U/kg) just before the surgical procedure. A midline laparotomy was performed, and an atraumatic clip was used to interrupt blood supply to the left lateral and median lobes of liver. After hepatic ischemia for 90 minutes, the clip was removed to initiate reperfusion. Sham controls underwent the same surgical procedure but without hepatic ischemia. Sometimes, mice were pretreated with Mn(III)-TBAP (Cayman Chemical, Ann Arbor, MI) (500 μM, 10 mL/kg body weight intraperitoneally) to scavenge ROS immediately before the procedure.[18] Bone marrow (BM) transplantation was performed as described,[16] and recipient mice were maintained with water containing antibiotics for 8 weeks prior to hepatic I/R injury. All surgical procedures were accomplished in a clean surgery room with sterilized instruments. Mice were fed with antibiotic-containing water after surgery and were euthanized by venesection at the end of experiments. All animal experiments were performed following institutional Animal Experiment Administration Committee guidelines. In addition, all animals received human care referring to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

Cell Culture and Transfection. For the isolation of hepatocytes, mice were perfused with 15 mL of prewarmed collagenase D (0.05%, Sigma-Aldrich) through the portal vein for 15 minutes. Livers were then removed and minced, and hepatocytes were pelleted by centrifugation at 50*g* for 3 minutes three times. The purity of hepatocytes exceeded 90%. Hepatocytes were cultured in Williams' E medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal bovine serum, 0.5 IU/mL insulin, and 10 μg/mL dexamethasone. *In vitro* I/R of hepatocytes was performed as described.[19] A γ-secretase inhibitor (GSI IX; Calbiochem, La Jolla, CA) was used at the concentration of 75 μM, with dimethyl sulfoxide (DMSO) as a control. Mn(III)-TBAP was added at a concentration of 100 μM.

The human hepatocyte line HL7702 and mouse macrophage line RAW264.7 were cultured with RPMI1640 supplemented with 20% and 10% fetal bovine serum, respectively. Transfection of HL7702 cells was performed with Lipofectamine 2000 (Invitrogen) according to the recommended protocol. The Hes5 overexpression vector was constructed by inserting rat *Hes5* complementary DNA[20] into pcDNA3.1. The plasmids pcDNA3-6 × Myc-mSTAT3 and pcDNA3-6 × Myc-mSTAT3-Y705F, which are vectors for expressing myc-tagged constitutively active STAT3 (STAT3^{C}) and the control STAT3, respectively, were provided by Yongzhan Nie.[21]

For coculture of hepatocytes and OP9 cells, OP9-Dll1 or OP9-GFP cells[22] (1×10^5) were seeded in 12-well plates. After cell adherence, HL7702 cells (5×10^5) were seeded and cultured for 12 hours before being subjected to I/R injury *in vitro*.

Histology. Hematoxylin and eosin staining and immunohistochemistry of myeloperoxidase were performed using standard procedures. Rabbit anti-mouse myeloperoxidase and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G were purchased from Thermo (Astmoor Runcorn, UK) and Boster BioTec (Wuhan, China), respectively. Terminal deoxynucleotidyl transferase–mediated deoxyuridine

triphosphate nick-end labeling (TUNEL) was performed using a kit (Promega, Madison, WI) according to the manufacturer's protocol. To quantify histological images, at least five random fields of each section were counted.

Biochemistry. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using a Chemistry Analyzer (AU400, Olympus, Tokyo, Japan). Nitric oxide was measured as described.[23]

Fluorescence-Activated Cell Sorting. Mice were perfused through the portal vein with ice-cold phosphate-buffered saline. Liver fragments (300-500 mg) were dissected, rinsed with medium, and treated with collagenase D (0.1%) at 37°C for 45 minutes. Hepatocytes were pelleted by centrifugation at 50*g* for 3 minutes three times, followed by centrifugation at 300*g* for 5 minutes to acquire nonparenchymal cells. Fluorescence-activated cell sorting (FACS) was performed with routine protocols using a FACSCalibur flow cytometer (BD Immunocytometry Systems). Cells were stained with fluorescein isothiocyanate–Ly6G (1A8), allophycocyanin (APC)-CD3 (145-2C11), and APC-CD4 (RM4-5) antibodies (BD Bioscience Pharmingen) or biotinylated F4/80 (BM8, eBioscience) and biotinylated Flk1 (Avas12a1, eBioscience) with phycoerythrin-streptavidin and APC-streptavidin (Biolegend, San Diego, CA). To measure intracellular ROS, cells were stained with 2′,7′-dichlorofluorescin (Beyotime, Haimen, China) following the recommended protocols and were analyzed by way of FACS. ROS was quantified using mean fluorescence intensity.[15] To detect apoptosis of HL7702 cocultured with OP9 cells, cells were stained with APC-Annexin V (eBioscience) following the recommended protocols and were analyzed by way of FACS with hepatocytes gated through forward scatter and side scatter plots.

Immunoprecipitation and Western Blot Analysis. Cell extracts were mixed with anti-Hes5 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-STAT3 (EPR787Y, Epitomics, Burlingame, CA) and were immunoprecipitated with protein A beads (Amersham Bioscience, Uppsala, Sweden) followed by intensive washing.

Western blot analysis was performed routinely, with primary antibodies including anti-Hes5, anti-MnSOD (Santa Cruz Biotechnology), anti–phospho-STAT3, anti-STAT3, anti-Akt, anti–phospho-Akt (Signalway Antibody, Pearland, TX), anti-SOCS3 (Cell Signaling Technology, Boston, MA), or anti–β-actin (Sigma-Aldrich). As secondary antibodies, anti-rabbit immunoglobulin G or anti-mouse immunoglobulin G (Boster BioTec) were used.

Real-Time Reverse-Transcription PCR. Real-time reverse-transcription PCR (RT-PCR) was performed essentially as described.[11] Primers are listed in Supporting Table S1.

Statistical Analysis. Data were analyzed with SPSS version 12.0 software. Results are expressed as the mean ± SD. Comparisons between groups were performed using an unpaired Student *t* test. $P < 0.05$ was considered statistically significant.

Results

Notch Pathway Responds to Hepatic I/R Injury. Normal mice were subjected to hepatic I/R injury, and messenger RNA (mRNA) expression of Notch1, 2, and 3; Dll1 and 4; Jag1 and 2; and Hes1 and 5 in liver was examined 6 hours after reperfusion. Among them, the mRNA level of Notch1, Notch2, Dll4, Jag2, and Hes5 was significantly up-regulated (Fig. 1A). Notch1 intracellular domain increased in the livers of mice suffering I/R injury (Fig. 1B; Supporting Fig. 1), suggesting Notch signal activation during I/R injury.

Notch Signal Blockade in Hepatocytes Leads to Increased Apoptosis During I/R In Vitro. The human hepatocyte line HL7702 was subjected to *in vitro* I/R.[19] TUNEL staining revealed significantly increased apoptosis in cells suffering I/R injury (Fig. 1C,D), and the number of viable cells decreased concomitantly (Fig. 1E). Notably, when Notch signaling was blocked by GSI, I/R induced remarkably increased apoptosis and decreased cell viability (Fig. 1C-E). The culture supernatants of I/R-injured HL7702 cells in the absence of Notch signaling had stronger ability to stimulate macrophages for tumor necrosis factor α (TNFα) production, suggesting that these hepatocytes produced more endogenous damage-associated molecular pattern (Fig. 1F).[24] These data suggest that blocking Notch signal in hepatocytes resulted in aggravated I/R injury.

Notch Signal Deficiency Exacerbates Hepatic I/R Injury In Vivo. In poly(I)-poly(C)–induced $RBP^{f/f}$-MxCre (RBP-J knockout [KO]) and $RBP^{f/+}$-MxCre (control) mice, ≈90% of the floxed *RBP-J* allele was deleted in liver.[16] When the *RBP-J* KO and control mice were subjected to hepatic I/R injury, significantly higher levels of serum ALT and AST were detected 6 hours and 24 hours after reperfusion (Fig. 2A,B). Histological examination of liver showed that in *RBP-J* KO mice, I/R induced more intensified tissue degeneration and focal necrosis than in control mice (Fig. 2C). TUNEL staining detected significantly more apoptotic cells in the liver sections from *RBP-J* KO mice

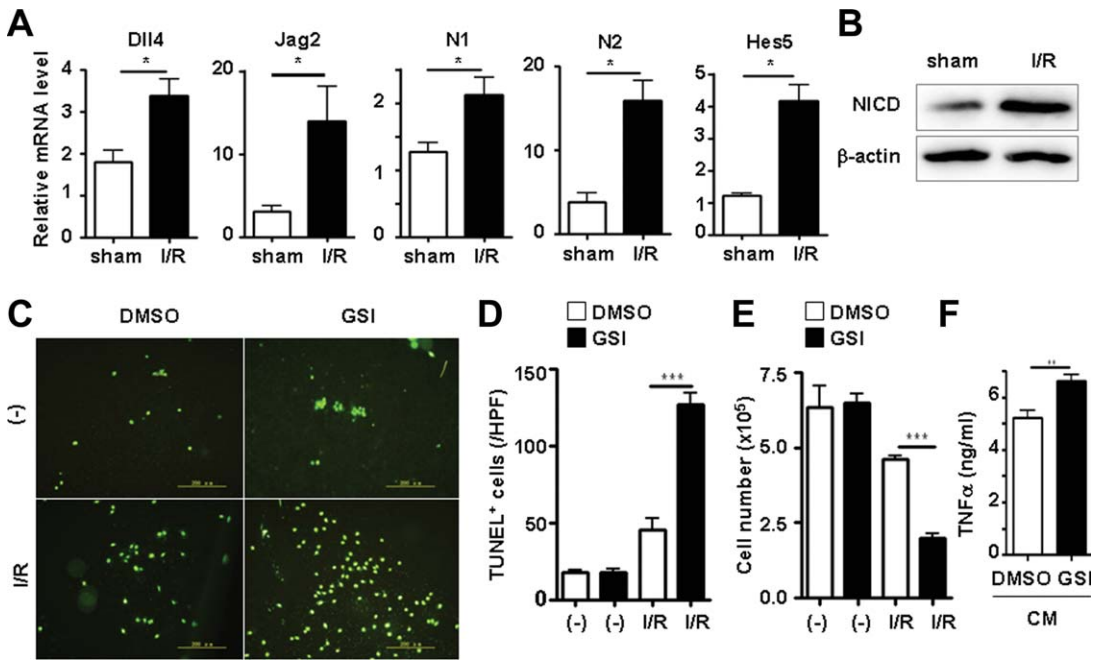

Fig. 1. Blocking Notch signaling exacerbates hepatic I/R injury *in vitro*. (A, B) mRNA expression of Dll4, Jag2, Notch 1, Notch2, and Hes5 (A) and the level of Notch1 intracellular domain (NICD) (B) in mouse liver after hepatic I/R (6 hours after reperfusion). (C, D) HL7702 cells were subjected to I/R *in vitro* in the presence of DMSO or GSI. Apoptotic cells were stained by TUNEL 6 hours after reperfusion (C, magnification $\times 200$) and were quantified (D). (E) Number of viable HL7702 cells in (C). (F) Culture supernatants (CM) in (C) were collected and were used to treat RAW264.7 cells. TNFα production by RAW264.7 was assessed 12 hours later. Bars = mean $\pm$ SD (n = 4). $^*P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

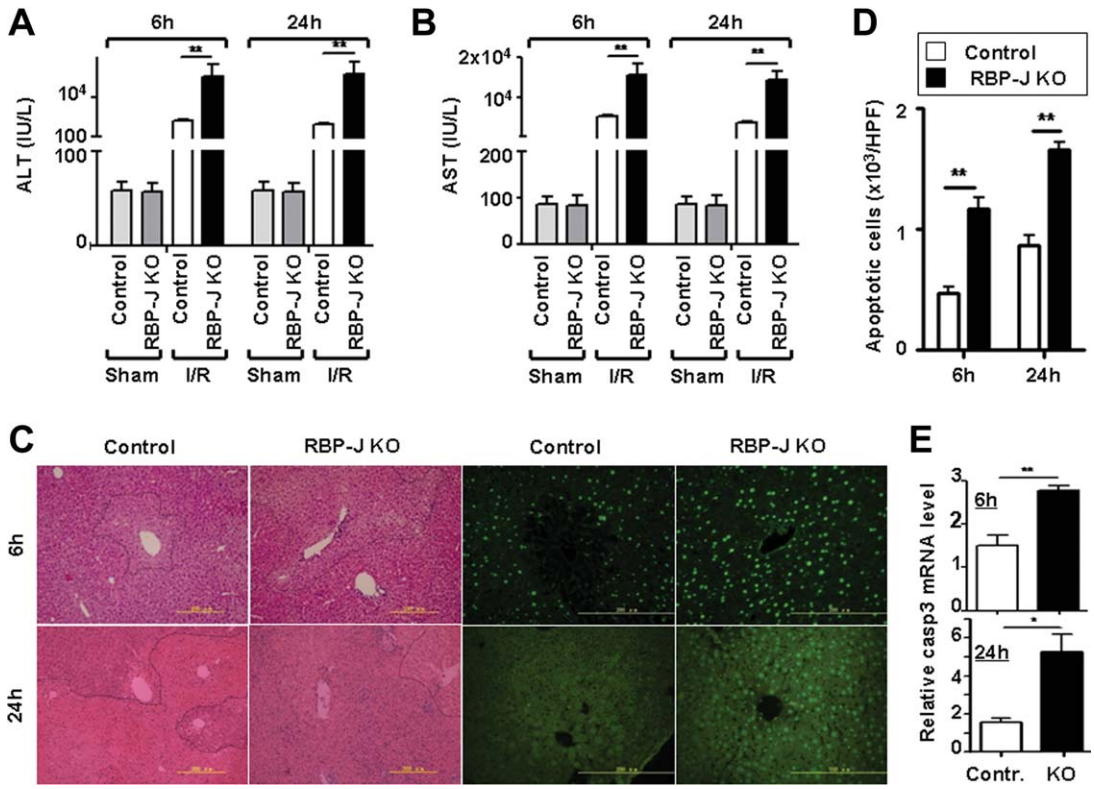

Fig. 2. *RBP-J* disruption leads to aggravated hepatic I/R *in vivo*. *RBP-J* KO or control mice were subjected to hepatic ischemia and were examined 6 hours or 24 hours after reperfusion. (A, B) Serum ALT (A) and AST (B). (C) Liver sections were stained with hematoxylin and eosin (left, magnification $\times 400$), with the outlined areas showing hepatic necrosis, or TUNEL (right). (D) Quantitative comparison of apoptotic cells upon TUNEL in (C). (E) The mRNA level of caspase-3 was analyzed by way of real-time RT-PCR, with β-actin as a reference control. Bars = mean $\pm$ SD (n = 4). $^*P < 0.05$. $^{**}P < 0.01$.

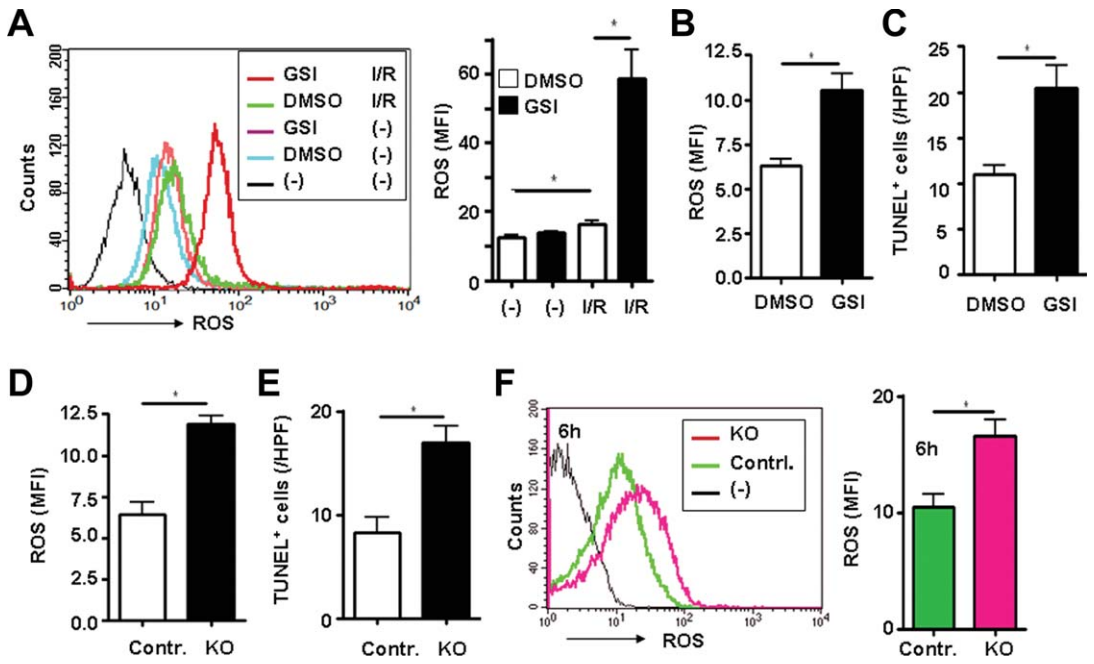

Fig. 3. Notch signal blockade results in increased ROS in hepatocytes. (A) HL7702 cells were treated by I/R *in vitro* in the presence of DMSO or GSI. ROS were examined by way of FACS (left) and were quantified by way of mean fluorescence intensity (right). (B-E) I/R *in vitro* was performed with normal primary hepatocytes in the presence of DMSO and GSI (B,C) or with *RBP-J* KO and control primary hepatocytes (D,E). ROS and apoptosis were assessed by way of FACS (B,D) and TUNEL (C,E), respectively. (F) *RBP-J* KO and control mice were subjected to hepatic I/R injury. Hepatocytes were isolated and examined for ROS by way of FACS (left) and quantified (right). Bars = mean ± SD (n = 4). *$P < 0.05$.

(Fig. 2C,D), and the mRNA levels of caspase-3 increased in the liver of *RBP-J* KO mice after reperfusion (Fig. 2E). Moreover, reperfusion resulted in strengthened inflammatory responses in *RBP-J* KO mice, as shown by increased infiltration of inflammatory cells, including neutrophils, macrophages, and T cells (Supporting Fig. 2A,B), and production of the inflammatory cytokines TNFα, interleukin-6, interleukin-1β, and interferon-γ; chemokine ligand 3; and intercellular cell adhesion molecule 1 (Supporting Fig. 2C). Therefore, disruption of Notch signaling resulted in aggravated I/R injury in mice.

It is noteworthy that in *RBP-J* KO mice, *RBP-J* deletion also occurs with high efficiency in hematopoietic cells,[16] which participate in hepatic I/R injury.[8] However, mice accepting *RBP-J* KO BM cells and control BM cells exhibited similar tissue necrosis and cell apoptosis, as well as serum ALT and AST levels, after I/R injury (Supporting Fig. 3A-D). These data suggest that aggravation of I/R injury upon Notch signal blockade might be attributed to hepatic but not BM-derived cells.

Notch Blockade Leads to Increased ROS After Hepatic I/R Injury. We examined ROS by way of FACS in hepatocytes suffering I/R in the absence of Notch signaling using several systems. As shown in Fig. 3A, whereas I/R injury of HL7702 cells led to mildly increased ROS levels, blocking Notch signaling by GSI resulted in remarkably higher levels of ROS after reperfusion. Meanwhile, GSI treatment significantly up-regulated inducible nitric oxide synthase (iNOS) expression and down-regulated Bcl-xL (Supporting Fig. 4A), which might be due to increased ROS levels.[15,25,26] In normal primary hepatocytes, I/R *in vitro* in the presence of GSI induced higher levels of ROS after reperfusion, accompanied by increased apoptosis (Fig. 3B,C). The same phenomena were detected in *RBP-J*–deficient hepatocytes (Fig. 3D,E). I/R-injured *RBP-J* KO hepatocytes also expressed higher level of iNOS and produced more nitric oxide than control (Supporting Fig. 4B-E). Finally, hepatocytes from *RBP-J* KO mice had higher levels of ROS (Fig. 3F) and iNOS mRNA (Supporting Fig. 4F) than control mice upon I/R injury. These data collectively indicate that Notch blockade led to increased ROS levels during I/R injury. In sinusoidal endothelial cells, Notch interruption also resulted in increased ROS and cell death (Supporting Fig. 5), suggesting that the role of Notch signaling in ROS production was not limited to hepatocytes.

Scavenging ROS Cancels Aggravated Hepatic I/R Injury Caused by Notch Signal Deficiency. In HL7702 cells subjected to I/R injury, Mn(III)-TBAP[18] effectively decreased ROS in both the GSI-treated

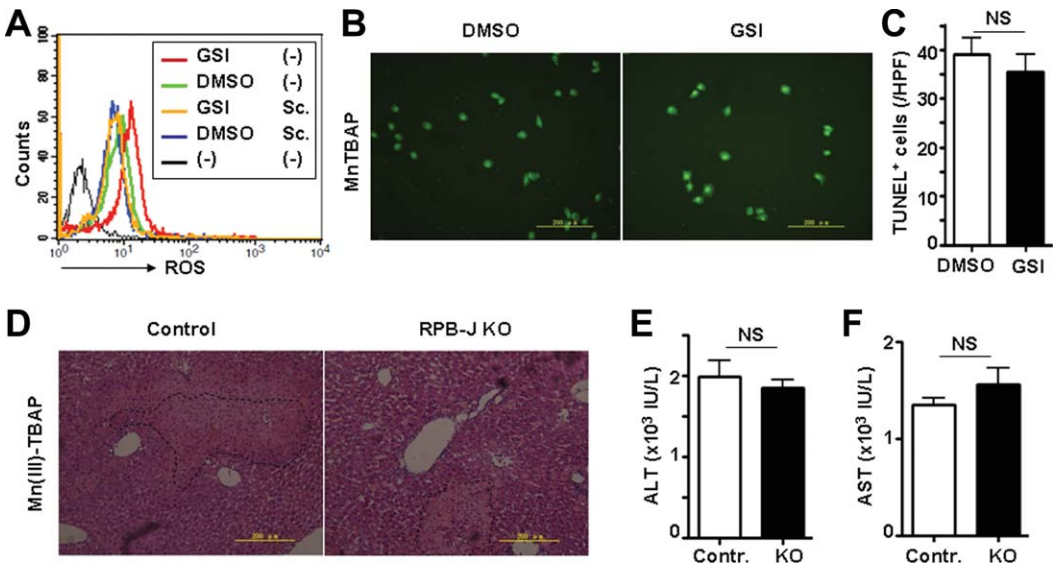

Fig. 4. ROS scavenger alleviates hepatic I/R injury aggravated by Notch signal deficiency. (A-C) HL7702 cells were subjected to I/R *in vitro* in the presence of DMSO or GSI, with or without the ROS scavenger Mn(III)-TBAP (Sc.). ROS were assessed by way of FACS 6 hours after reperfusion (A). Apoptotic cells were stained by TUNEL (B, magnification ×200), and were quantified (C). (D) *RBP-J* KO or control mice were injected intraperitoneally with Mn(III)-TBAP before hepatic I/R. Liver sections were stained with hematoxylin and eosin, with the outlined areas showing hepatic necrosis. (E, F) Serum ALT (E) and AST (F) of mice in (D). Bars = mean ± SD (n = 4), NS, not significant.

group and the control group (Fig. 4A). The aggravated apoptosis after I/R in the presence of GSI was also cancelled (Fig. 4B,C). We treated *RBP-J* KO and control mice with Mn(III)-TBAP before hepatic I/R injury. Histological staining indicated that upon Mn(III)-TBAP administration, *RBP-J* KO and control mice showed a similar degree of liver cell necrosis after hepatic I/R (Fig. 4D) and similar serum ALT and AST levels (Fig. 4E,F). These findings suggest that blocked Notch signaling aggravated hepatic I/R injury through increased ROS production.

Disruption of Notch Signal Leads to Down-regulation of MnSOD During Hepatic I/R Injury. Using RT-PCR, we found that although the expression of xanthine oxidase increased after I/R in the presence of GSI, the expression of monoamine oxidase A, monoamine oxidase B, or $p66^{Shc}$ did not change significantly (Supporting Fig. 6). Mitochondrial respiration provided more than 90% of intracellular ROS, which is scavenged by MnSOD.[27] In HL7702 suffering from I/R in the presence of GSI, the expression of MnSOD was down-regulated significantly at both the mRNA (Fig. 5A) and protein (Fig. 5B; Supporting Fig. 7A) levels. Consistently, in *RBP-J* KO mice subjected to hepatic I/R injury, MnSOD expression in liver was also down-regulated significantly (Fig. 5C; Supporting Fig. 7B). These data suggest that blocking Notch signaling down-regulated MnSOD expression, leading to decreased scavenging of ROS and aggravated hepatic I/R injury.

Notch Signal Blockade Attenuates STAT3 Activation in Hepatocytes During Hepatic I/R Injury. There was no obvious difference in Akt phosphorylation, which regulates ROS,[28] between GSI-treated and control HL7702 hepatocytes after I/R injury (Supporting Fig. 8A). However, decreased phosphorylation of STAT3 was observed in both GSI-treated HL7702 cells and liver extracts from *RBP-J* KO mice after I/R injury (Fig. 5D; Supporting Fig. 7C,D). Because STAT3 transactivates MnSOD,[19,29] blocking Notch signaling might down-regulate the transcription of MnSOD through decreased STAT3 activation, leading to increased ROS and aggravated I/R injury.

SOCS3, an inhibitor of STAT3 activation, was slightly up-regulated in GSI-treated HL7702 hepatocytes (Supporting Fig. 8B) during I/R injury, in contrast to Notch-deficient macrophages.[23] Hes proteins bind to STAT3 and facilitate phosphorylation of STAT3 by JAK2 (data not shown).[30] Using immunoprecipitation, we found that during I/R injury in both mice and HL7702 cells, the absence of Notch signaling resulted in decreased Hes5-STAT3 complex (Fig. 5E,F; Supporting Fig. 9). This finding indicated that disruption of Notch signaling reduced STAT3 activation by decreasing the expression of Hes5 under I/R.

Constitutive STAT3 Activation Abrogates Notch Blockade-Induced Aggravation of Hepatic I/R Injury. HL7702 hepatocytes were transfected with constitutively active STAT3 ($STAT3^{C}$) (Supporting Fig. 10A). TUNEL staining indicated that overexpression of constitutively

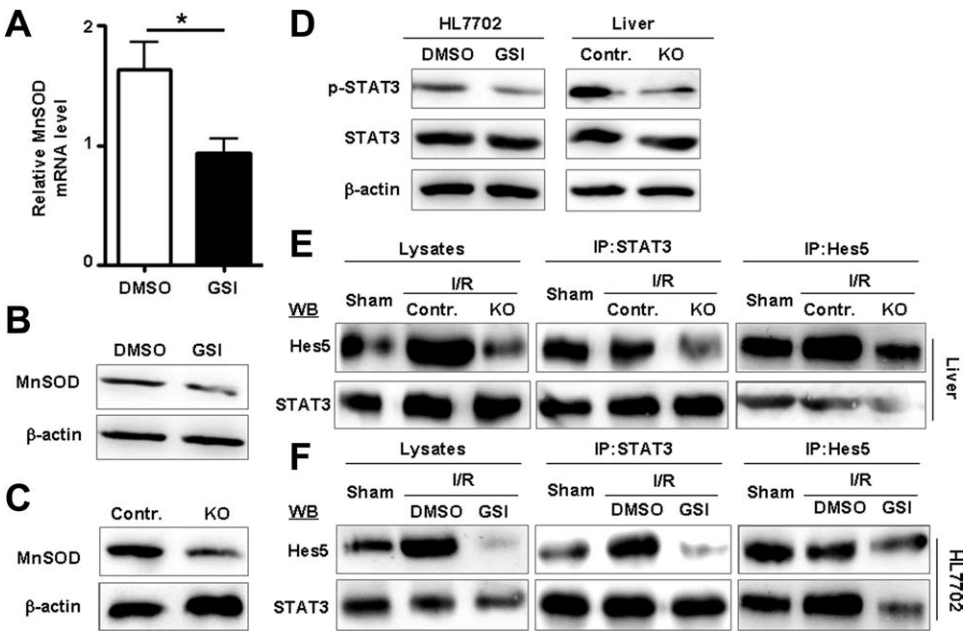

Fig. 5. Blocking Notch signal leads to attenuated STAT3 phosphorylation and down-regulation of MnSOD. (A,B) HL7702 cells were subjected to I/R *in vitro* in the presence of DMSO or GSI, and MnSOD expression was determined by way of real-time RT-PCR (A) and western blot analysis (B). (C) MnSOD expression in livers of *RBP-J* KO and control mice subjected to hepatic I/R injury. (D) Detection of phosphorylated (p-STAT) or total STAT3 by western blotting using protein extracts from I/R-injured HL7702 cells or livers of *RBP-J* KO and control mice subjected to hepatic I/R injury. (E,F) Immunoprecipitation. Cell lysates were prepared from I/R-injured livers of *RBP-J* KO or control mice (E) or HL7702 cells (F) subjected to hepatic I/R injury and were used for immunoprecipitation as shown.

active STAT3 abrogated GSI-induced increase of apoptosis during I/R *in vitro* (Fig. 6A,B). Constitutively active STAT3 also reduced the level of ROS, which increased upon Notch signal deficiency during I/R (Fig. 6C,D). Using Western blot analysis, we found that constitutively active STAT3 up-regulated MnSOD, which was repressed by GSI during I/R injury (Fig. 6E; Supporting Fig. 11A).

Notch Activation Protects Hepatocytes from I/R Injury by Reducing ROS. HL7702 cells were triggered by coculturing with OP9-Dll1[22] and were subjected to I/R injury *in vitro*. Compared with HL7702

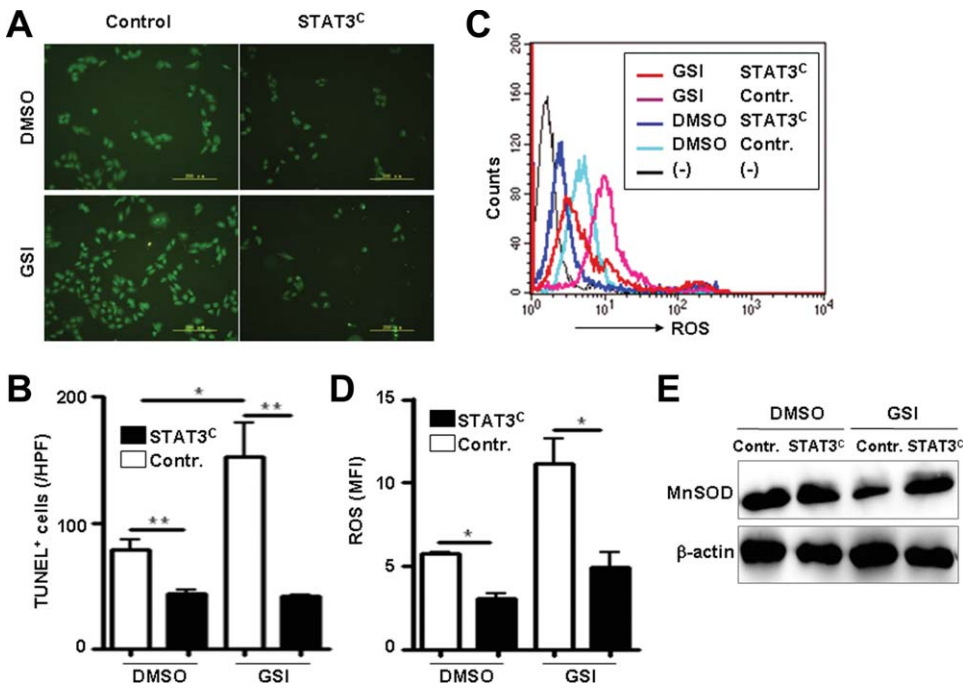

Fig. 6. Overexpressing constitutively active STAT3 rescues aggravated I/R injury caused by Notch signal deficiency. HL7702 cells stably transfected with constitutively active STAT3 ($STAT3^C$)-expressing or control plasmids were subjected to I/R injury *in vitro* in the presence of DMSO or GSI and were examined 6 hours after reperfusion. (A) TUNEL staining (magnification ×200). (B) Quantification of $TUNEL^+$ cells. (C) ROS levels analyzed by way of FACS. (D) ROS levels quantified. (E) MnSOD expression determined by western blot analysis. Bars = mean ± SD (n = 5). *$P < 0.05$. **$P < 0.01$.

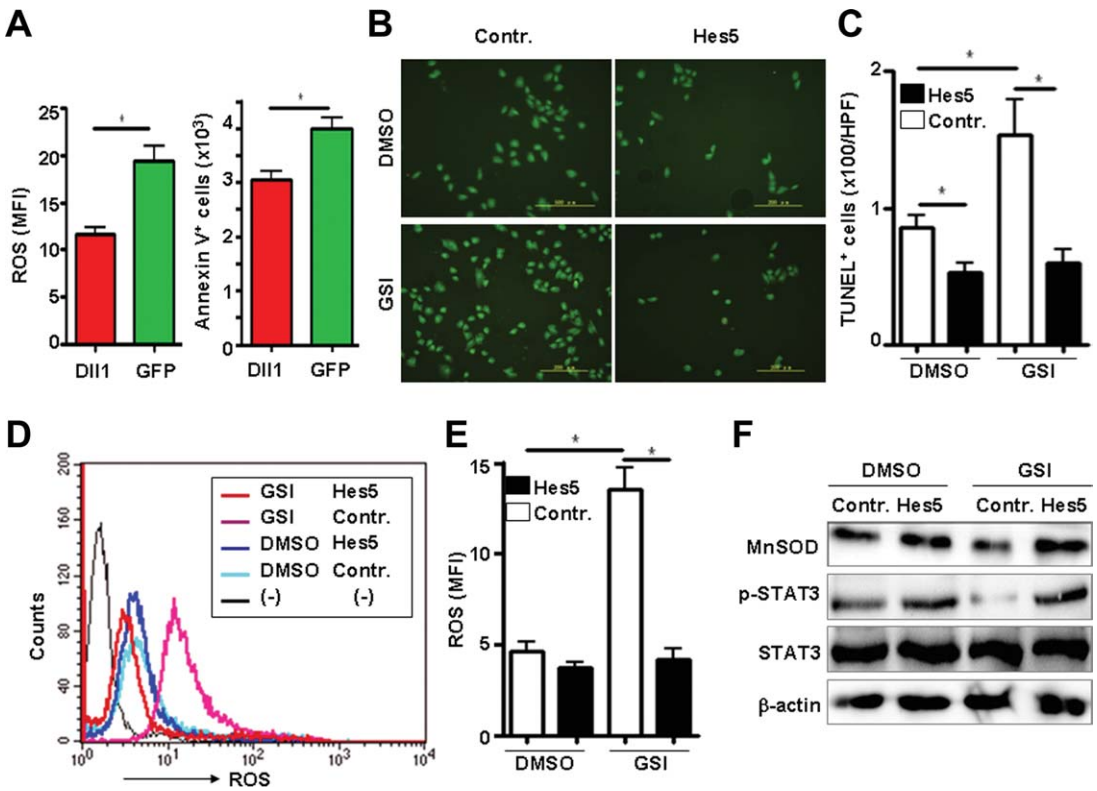

Fig. 7. Forced Notch activation protects hepatocytes from I/R injury. (A) HL7702 cells were cocultured with OP9-Dll1 or OP9-GFP cells and were treated with I/R injury *in vitro*. ROS (left) and apoptotic cells (right) were analyzed by way of FACS and were quantified. (B-E) HL7702 cells stably transfected with Hes5-expressing or control plasmids were subjected to I/R *in vitro* in the presence of DMSO or GSI and were analyzed 6 hours after reperfusion. (B,C) TUNEL staining (magnification ×200) and quantification (C). (D,E) ROS analyzed by way of FACS and quantification (E). (F) Western blot analysis of MnSOD, p-STAT3, and STAT3. Bars = mean ± SD (n = 5). *$P < 0.05$.

cells cocultured with OP9-GFP, forced activation of Notch reduced ROS and apoptosis (Fig. 7A) after reperfusion, suggesting that Notch activation protected hepatocytes from I/R injury by reducing ROS.

Hes5 is the major effector of Notch signal in hepatocytes during I/R injury (Fig. 1A; Fig. 5E,F). HL7702 hepatocytes were stably transfected with pcDNA3.1-Hes5 or pcDNA3.1 (Supporting Fig. 10B) and were subjected to I/R *in vitro* in the presence of DMSO or GSI. Overexpression of Hes5 ameliorated apoptosis during I/R injury, even in the presence of GSI (Fig. 7B,C). Concomitantly, increased ROS (Fig. 7D,E), decreased MnSOD, and STAT3 phosphorylation were also reversed by overexpression of Hes5 (Fig. 7F; Supporting Fig. 11B). These data further suggest that Notch signaling protected hepatocytes through the Hes5-STAT3-MnSOD-ROS pathway during I/R injury.

Discussion

Our results demonstrated *in vitro* and *in vivo* that Notch signaling regulates I/R injury by modulating ROS. The homeostasis of ROS is maintained by its production and scavenge.[31] The results reported here indicate that MnSOD, a critical ROS scavenger in mitochondria, is the major target molecule of Notch signaling in regulating ROS. It has been proved that the transcription of MnSOD is regulated by STAT3[19,29] depending on the JAK2/STAT3 protein complex that is formed by the interaction of Hes proteins with STAT3.[30] We found that Hes5 was the major downstream effector of Notch signaling in hepatocytes during I/R injury. Thus, disruption of Notch signaling resulted in decreased Hes5-STAT3 complex, and overexpressing constitutively active STAT3 or Hes5 rescued MnSOD expression, leading to reduced ROS levels and hepatocyte apoptosis subjected to I/R in the absence of Notch signaling. In summary, the data presented in this study establish a signal axis by which canonical Notch signaling regulates hepatic I/R injury: the activated Notch receptors up-regulate Hes5 through transcription factor RBP-J, and Hes5 facilitates STAT3 activation through the formation of a Hes5-JAK2/STAT3 complex,[30] which in turn activates the transcription of *MnSOD* gene to scavenge ROS and restricts I/R injury (Supporting Fig. 12, left).

The JAK/STAT pathway mediates cytokine signaling and participates in the initiation, propagation, and resolution of inflammation.[32] The basic players in this pathway include four JAK kinases and seven STAT members, with other modifiers such as SOCSs. The JAK2/STAT3-SOCS3 module mediates proinflammation or anti-inflammation signaling, depending on cell types and other environmental cues, and is involved in both hepatic and myocardial I/R injury.[33,34] We have shown recently that Notch signaling down-regulates JAK2/STAT3 signaling through the up-regulation of SOCS3 in macrophages.[23] This signal could be enhanced through the auto-amplification of Notch signaling by TLR-induced and RBP-J–dependent induction of Notch ligands,[35] likely to result in down-regulation of MnSOD and increased ROS levels, to facilitate the destruction of ingested pathogens in macrophages (Supporting Fig. 12, right). In tissue parenchymal cells such as hepatocytes, as shown in the current study, I/R also up-regulates Notch signal activation, but it assists JAK2/STAT3 signaling without the activation of SOCS3 expression, resulting in the up-regulation of MnSOD and increased scavenging of ROS, restricting the extent of tissue damage. Therefore, it appears that, as one of the early response signals, the Notch pathway plays differential roles through JAK2/STAT3-MnSOD in macrophages and hepatocytes—namely, increasing ROS in macrophages to destroy pathogens but reducing ROS in hepatocytes to protect cells. This scenario might also be an explanation of the contradictory observations about the roles of Notch signaling in myocardial and brain I/R injuries.[12,14] However, mechanisms such as epigenetic elements by which Notch signal differentially regulates SOCS3 expression between macrophages and hepatocytes should be determined by further studies.

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