

## Interplay of neuropilin-1 and semaphorin 3A after partial hepatectomy in rats

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### Abstract

**AIM:** To elucidate the role of neuropilin-1 (Nrp-1) and semaphorin 3A (Sema3A) in sinusoidal remodeling during liver regeneration in rats.

**METHODS:** Male Wistar/ST rats at 7 wk of age, weighing about 200 g, were used for all animal experiments. *In vivo*, at 24, 48, 72, 96, 144 and 192 h after two-thirds partial hepatectomy (PHx), the remnant livers were removed. Liver tissues were immunohistochemically stained for Nrp-1, Sema3A and SE-1, a liver sinusoidal endothelial cell (SEC) marker. Total RNA of the

liver tissue was extracted and reversely transcribed into cDNA. The mRNA expression of Sema3A was analyzed by quantitative real-time polymerase chain reaction and normalized to that of ribosomal protein S18. *In vitro*, SECs were isolated from rat liver and cultured in endothelial growth medium containing 20 ng/mL vascular endothelial cell growth factor. Migration of SECs in primary culture was assessed by cell transwell assay with or without recombinant Sema3A. Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method.

**RESULTS:** *In vitro*, immunohistochemistry study revealed that Sema3A and Nrp-1 were constitutively expressed in hepatocytes and SECs, respectively, in normal rat liver tissues. Nrp-1 expression in SECs was quantified by the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1. Between 24 h and 96 h following resection of liver, Nrp-1 expression in SECs was transiently increased. Compared with the baseline ( $5.2\% \pm 0.1\%$ ), Nrp-1 expression in SECs significantly increased at 24 h ( $17.3\% \pm 0.7\%$ ,  $P < 0.05$ ), 48 h ( $39.1\% \pm 0.6\%$ ,  $P < 0.01$ ), 72 h ( $46.9\% \pm 4.5\%$ ,  $P < 0.01$ ) and 96 h ( $29.9\% \pm 3.8\%$ ,  $P < 0.01$ ) after PHx, then returned to the basal level at termination of liver regeneration. Interestingly, the expression of Sema3A was inversely associated with that of Nrp-1 in liver after PHx. Sema3A mRNA expression was significantly reduced by about 75% over the period 24-144 h after PHx ( $P < 0.05$ ), and returned to basal levels at 192 h after PHx. *In vitro*, SECs isolated from rats after PHx (PHx-SECs) were observed to migrate to the lower chamber of the cell transwell system after incubation for 24 h, but not cells from normal rats (CONT-SECs), indicating that mobility of PHx-SECs increases as compared with that of CONT-SECs. Moreover, recombinant Sema3A significantly attenuated migration in PHx-SECs in primary culture (vehicle-treated

100%  $\pm$  7.9% *vs* Sema3A-treated 42.6%  $\pm$  5.4%,  $P < 0.01$ ), but not in CONT-SECs. Compared with CONT-SECs, the apoptotic rate of PHx-SECs decreased by 78.3% ( $P < 0.05$ ). There was no difference in apoptosis between CONT-SECs that were treated with vehicle and Sema3A. However, in PHx-SECs, apoptosis was induced by the presence of 5 nmol Sema3A for 24 h (vehicle-treated 21.7%  $\pm$  7.6% *vs* Sema3A-treated 104.3%  $\pm$  8.9%,  $P < 0.05$ ). In addition, immunohistochemistry confirmed the increased expression of Nrp-1 in PHx-SECs, while it was noted to a lesser extent in CONT-SECs.

**CONCLUSION:** The interplay of Nrp-1 and Sema3A shown in our results may lead to a better understanding of interaction between sinusoidal remodeling and SECs during liver regeneration.

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**Key words:** Neuropilin-1; Semaphorin 3A; Sinusoidal remodeling; Liver regeneration; Two-thirds partial hepatectomy; Angiogenesis; Liver sinusoidal endothelial cells

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## INTRODUCTION

In liver, adult hepatocytes are long-lived and normally do not undergo cell division, but they maintain the ability to proliferate in response to toxic injury or partial resection of liver<sup>[1,2]</sup>. The process of liver regeneration (LR) is complex and involves a finely orchestrated balance of cell proliferation to reconstruct the normal liver architecture. Although hepatocyte proliferation is a major feature of the regenerating liver, it involves the interactions of other hepatic cell types (non-parenchymal cells), including Kupffer cells, stellate cells, sinusoidal endothelial cells (SECs) and biliary epithelial cells<sup>[3-5]</sup>.

In the rat liver after two-thirds hepatectomy, the rate of DNA synthesis in hepatocytes begins to increase after about 12 h and peaks around 24 h. However, the induction of DNA synthesis occurs later in the non-parenchymal cells (at approximately 48 h for Kupffer and biliary epithelial cells, and at approximately 96 h for SECs), and subsequently, LR terminates at approximately 192 h<sup>[6,7]</sup>. In the late stage of this process, angiogenesis and/or vasculogenesis is essential to supply blood flow to newly

replicating avascular islands of hepatocytes, in which SECs migrate and proliferate, leading to the formation of new functional sinusoids<sup>[8,9]</sup>.

Indeed, many of the growth factors have been reported to be upregulated in a regenerating liver. Vascular endothelial growth factor (VEGF) is a major proangiogenic factor and is thought to improve sinusoid reconstruction during the LR process<sup>[10,11]</sup>. Hepatocyte growth factor (HGF) levels are dramatically increased after partial hepatectomy, but HGF is also a potent proangiogenic factor *in vivo* and stimulates endothelial cell protease production, motility, proliferation and differentiation *in vitro*.

Recently, the importance of neuropilins (Nrps) as proangiogenic factors has been highlighted<sup>[12]</sup>. Nrps are transmembrane glycoproteins with large extracellular domains that interact with both class 3 semaphorins and VEGF, and they are involved in the regulation of many physiological pathways including angiogenesis<sup>[13,14]</sup>. The semaphorins were initially described as axon guidance factors that affect the development of the central nervous system<sup>[15]</sup>. However, semaphorin receptors were subsequently found to be expressed by multiple types of cells including endothelial cells and cancer cells. These observations were followed by studies that indicate that the semaphorins can modulate the behavior of cancer cells and endothelial cells<sup>[16,17]</sup>.

In this study, we report the inverse expression of Nrp-1 and semaphorin 3A (Sema3A) in liver tissues during liver regeneration in rats. We further show that Sema3A significantly attenuated migration and induced apoptosis in SECs isolated from rats after partial hepatectomy, but not in cells from normal rats.

## MATERIALS AND METHODS

### Animals and treatment

Male Wistar/ST rats, 7 wk of age, weighing about 200 g (Sankyo lab, Tokyo, Japan), were used for all animal experiments. Two-thirds partial hepatectomy (PHx) was performed under sevoflurane inhalant anesthesia according to the method of Higgins *et al*<sup>[18]</sup>. The left lateral and median lobes of the liver were excised. At 24, 48, 72, 96, 144 and 192 h after PHx, the rats were anesthetized and the remnant livers were removed. Control livers were obtained from sevoflurane-anesthetized non-manipulated animals.

Liver tissues were obtained from the right lateral lobe, embedded in Tissue Tek OCT Compound (Electron Microscopy Sciences, Hatfield, PA), frozen and stored at -80 °C until use. All procedures involving animals were approved by the Animal Care and Use Committee of Juntendo University, which conforms to the National Institutes of Health Guideline.

### Immunofluorescent staining

Double staining of Nrp-1 and SE-1 was performed as follows: tissue fixed with 4% paraformaldehyde for 10 min at

room temperature, incubated with goat polyclonal anti-Nrp-1 antibody (R and D Systems, Minneapolis, MN) (diluted 1:50) and mouse monoclonal SE-1 (Immuno-Biological Laboratories, Gunma, Japan) (diluted 1:20) at 4 °C overnight prior to incubation with Alexa-488 rabbit anti-goat (diluted 1:300) and Alexa-594 donkey anti-mouse antibody (diluted 1:300) (Invitrogen, Carlsbad, CA) for 1 h, then mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories, Burlingame, CA). The immunostained samples were observed and analyzed with a BIOREVO BZ-9000 microscope system (KEYENCE, Osaka, Japan).

For Sema3A staining, frozen sections of liver tissues were fixed with -20 °C acetone for 5 min, incubated with rabbit polyclonal anti-Sema3A antibody (diluted 1:200) (Abcam, Cambridge, United Kingdom) at 4 °C overnight prior to incubation with Alexa-488 goat anti-rabbit antibody (diluted 1:300) for 1 h.

#### Quantitative real-time polymerase chain reaction

Total RNA of the liver tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and reversely transcribed into cDNA using the RT reagent kit (TaKaRa Bio Inc., Shiga, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using ABI PRISM 7900HT Sequence Detector System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The following primers were used: Sema3A (accession number: NM\_017310.1) CTG CTC CGA CTT GCA GCA TC (sense) and CGC CTC TGA AAT TGC CAA TAT ACC (antisense); ribosomal protein S18 (RPS18) (accession number: NM\_213557.1) AAG TTT CAG CAC ATC CTG CGA GTA (sense) and TTG GTG AGG TCA ATG TCT GCT TTC (antisense). The Sema3A mRNA expression was normalized to that of RPS18.

#### Preparation of SECs in primary culture

SECs were isolated from rat liver based on a modification of the method of Braet *et al.*<sup>[19]</sup>, as we previously reported<sup>[20]</sup>. Briefly, liver was perfused with 0.05% collagenase A and 0.001% DNase. SECs were then purified by isopycnic sedimentation in a two-step Percoll gradient (25% and 50%). SECs were cultured in EBM-2 medium containing 20 ng/mL VEGF. These cells displayed a number of fenestrae in sieve plates, the characteristic of SECs *in vivo*. In separate experiments, SECs were isolated from rats 4 d after PHx (PHx-SECs).

#### Cell transwell migration assay

SEC transwell migration assay was performed using 24-well culture inserts (BD Biosciences, San Jose, CA). Polyethylene terephthalate membranes with a pore size of 8 µm were coated with fibronectin. SECs were loaded to the upper chamber. Recombinant Sema3A (R and D Systems, Minneapolis, MN) or vehicle was added to the

lower chamber. After incubation for 24 h, non-migrated cells on the upper surface of the filter were removed with a cotton swab and the cells which traversed and spread on the lower surface of the membrane were fixed with 2.5% glutaraldehyde and stained with hematoxylin. The average number of the cells per field was counted  $\times 10$  objective magnification from five microscopic fields. Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated control.

#### Apoptosis assay

After incubation with 5 nmol recombinant Sema3A for 24 h, SECs were fixed with 4% paraformaldehyde for 10 min at room temperature. Apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method with a fluorescent apoptosis detection system kit (Promega, Madison, WI).

#### Statistical analysis

Results are expressed as the mean  $\pm$  SE. Statistical analysis were performed using GraphPad Prism v5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was determined by one-way analysis of variance.

## RESULTS

#### Nrp-1 expression in SECs increases in regenerating liver after PHx

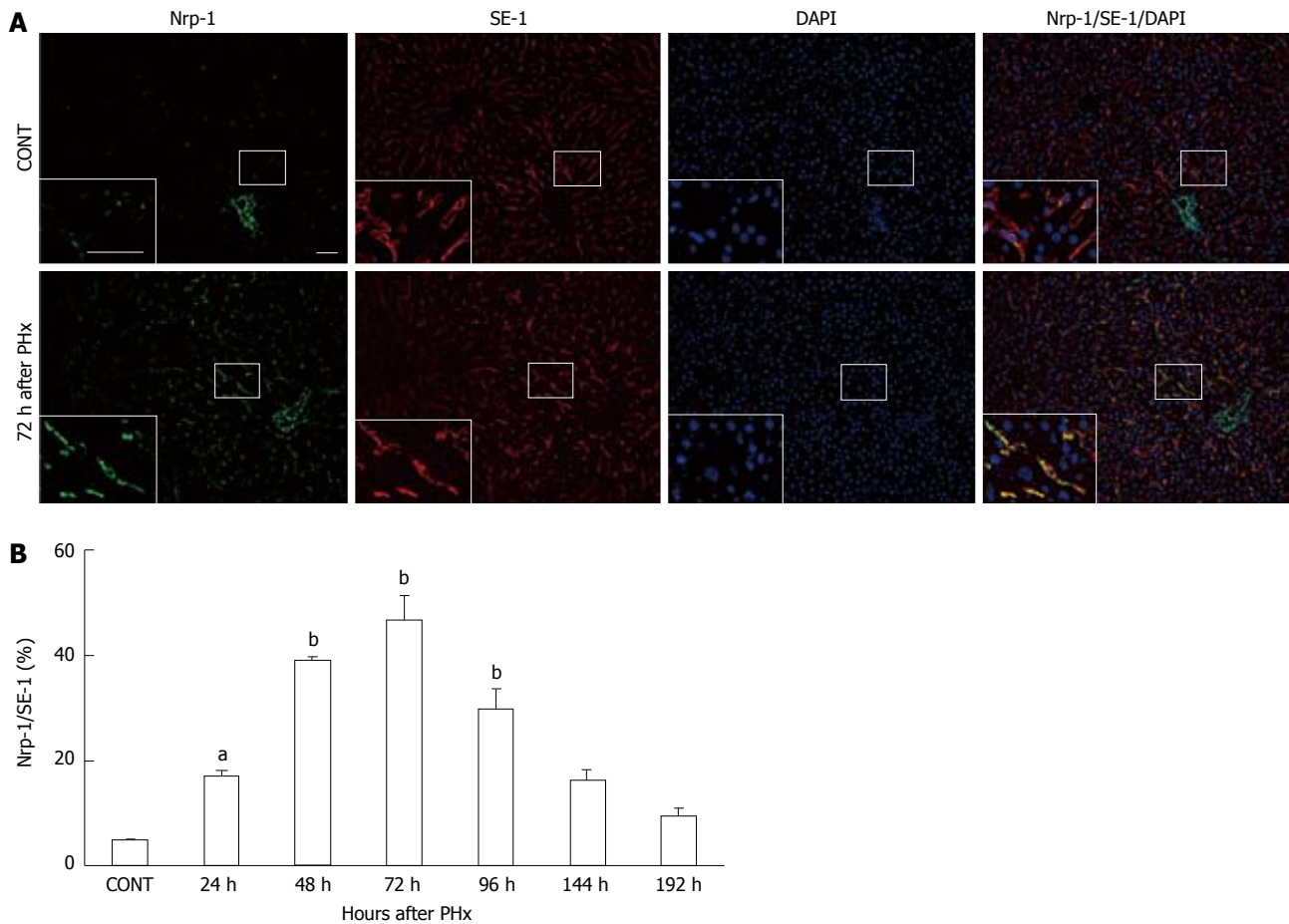
To investigate Nrp-1 expression in SECs during LR, liver tissues after PHx were analyzed by immunofluorescent staining with anti-Nrp-1 antibody and SE-1, a monoclonal antibody that specifically reacts with rat liver SECs<sup>[21,22]</sup>. As shown in Figure 1A, SECs stained with SE-1 were positive for Nrp-1 in control liver, and Nrp-1 expression in SECs was enhanced in regenerating liver at 72 h after PHx.

Nrp-1 expression in SECs was quantified by the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1. Nrp-1 expression in SECs significantly increased at 24 h ( $P < 0.05$ ), peaked at 72 h ( $P < 0.01$ ), and recovered to the basal level at 144 h after PHx (Figure 1B).

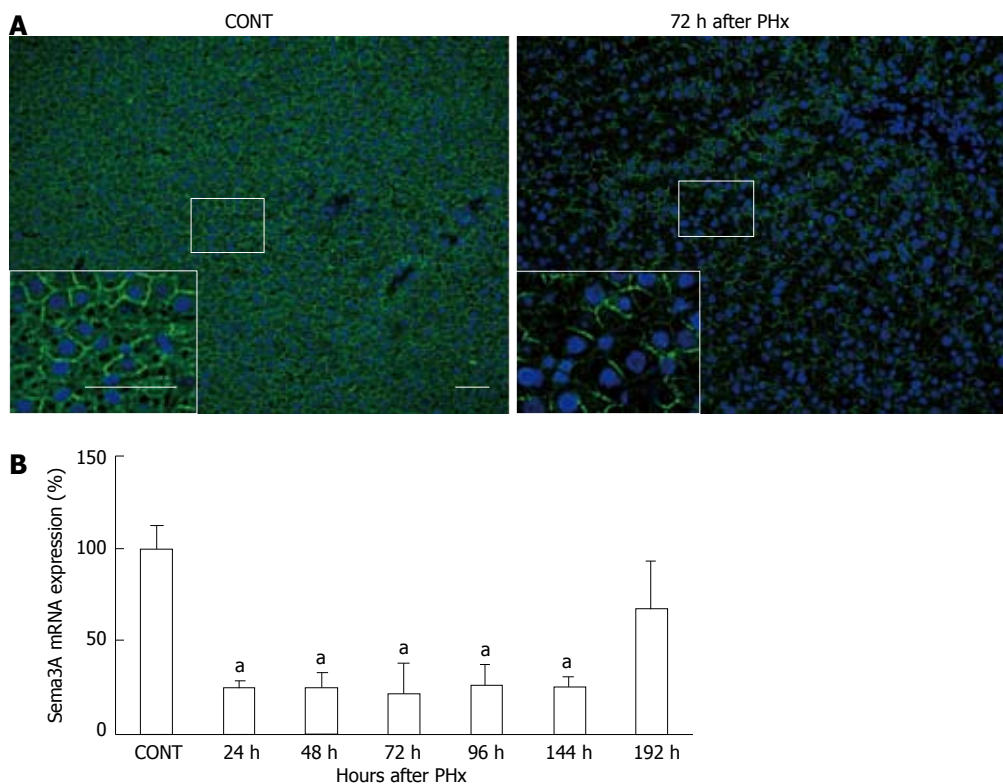
#### Sema3A expression decreases in regenerating liver after PHx

To test the expression of Sema3A during LR, liver tissues were examined by immunofluorescence with anti-Sema3A antibody in control rats and rats at 72 h after PHx. Sema3A was strongly stained in hepatocytes, which display round and big nuclei, in control liver. However, Sema3A expression was attenuated at 72 h after PHx (Figure 2A). Furthermore, real-time PCR analysis confirmed the decline of Sema3A expression during the process of LR. As shown in Figure 2B, Sema3A mRNA expression was significantly reduced by about 75% over the period 24-144 h after PHx ( $P < 0.05$ ), and returned

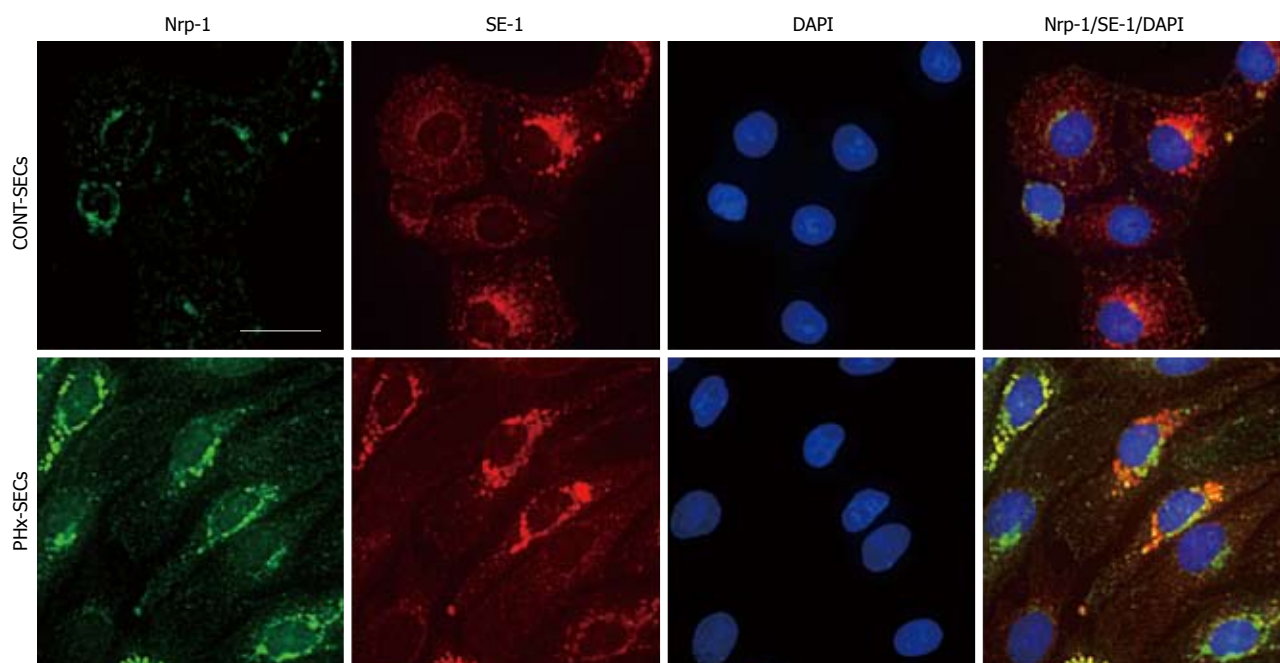




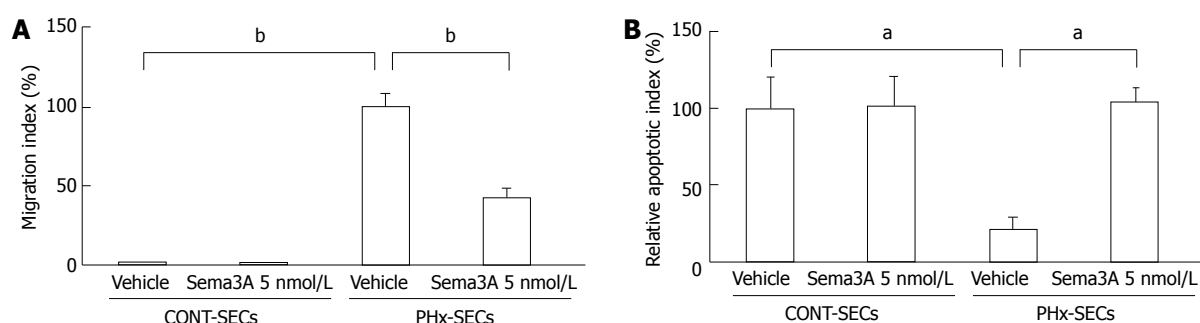
**Figure 1 Neuropilin-1 expression in sinusoidal endothelial cells in regenerating liver.** A: Immunofluorescent examination of neuropilin-1 (Nrp-1) in control liver and regenerating liver 72 h after partial hepatectomy. Green: Nrp-1; Red: Sinusoidal endothelial cells stained with SE-1; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 50  $\mu$ m; B: Graphic representation of the percentage of immunostained area with anti-Nrp-1 antibody in relation to the area stained with SE-1 (%). Results are expressed as the mean  $\pm$  SE. <sup>a</sup> $P$  < 0.05, <sup>b</sup> $P$  < 0.01 vs control liver ( $n$  = 3). PHx: Partial hepatectomy.



**Figure 2 Semaphorin 3A expression in liver tissues after partial hepatectomy.** A: Immunofluorescent examination of semaphorin 3A (Sema3A) in control liver and regenerating liver 72 h after partial hepatectomy (PHx). Green: Sema3A; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride. Scale bar, 50  $\mu$ m; B: Sema3A mRNA expression in liver tissues after PHx. Sema3A mRNA expression was quantified by real-time polymerase chain reaction and normalized to ribosomal protein S18 mRNA level. Results are expressed as the mean  $\pm$  SE. <sup>a</sup> $P$  < 0.05 vs control liver ( $n$  = 3).



**Figure 3** Immunofluorescent examination of neuropilin-1 and SE-1 in sinusoidal endothelial cells in primary culture. A: Neuropilin-1 (Nrp-1) expression in sinusoidal endothelial cells (SECs) isolated from normal rats (CONT-SECs); B: Nrp-1 expression in SECs isolated from rats after partial hepatectomy (PHx-SECs). Green: Nrp-1; Red: SE-1; Blue: Nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 20  $\mu$ m.



**Figure 4** Role of semaphorin 3A in sinusoidal endothelial cell migration and apoptosis. A: Effect of semaphorin 3A (Sema3A) on migration of sinusoidal endothelial cells (SECs) isolated from normal rats (CONT-SECs) and rats after partial hepatectomy (PHx-SECs). SEC migration was assessed by cell transwell assay in the presence or absence of 5 nmol Sema3A for 24 h. Migration index represents the number of migratory cells/number of migratory cells in vehicle-treated PHx-SECs; B: Effect of Sema3A on apoptosis of CONT-SECs and PHx-SECs. Following incubation of SECs with 5 nmol Sema3A or vehicle for 24 h, apoptotic cells were determined by a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling method. Results are expressed as the mean  $\pm$  SE. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs vehicle-treated PHx-SECs ( $n = 4$ ).

to baseline at 192 h after PHx.

#### **Nrp-1 expression increases in PHx-SECs as compared with CONT-SECs in primary culture**

To confirm that Nrp-1 is expressed in SECs, immunofluorescent analysis was performed in SECs isolated from rat liver. Both CONT-SECs and PHx-SECs at 24 h in culture were stained positive for Nrp-1 and SE-1, and Nrp-1 expression was increased in PHx-SECs as compared with CONT-SECs (Figure 3A and B). The up-regulated Nrp-1 expression in PHx-SECs was consistent with *in vivo* data shown in Figure 1. Although fluorescence is observed in the perinuclear region as well as the plasma membrane, this may suggest that Nrp-1 presents intracellular trafficking between plasma membrane and

intracellular compartments, such as endoplasmic reticulum, Golgi, endosomes and lysosomes.

#### **Sema3A attenuates migration and elicits apoptosis of PHx-SECs**

To investigate the effect of Sema3A on SEC mobility, migration of cells was quantified by cell transwell assay in SECs isolated from rats after PHx (PHx-SECs) and normal rats (CONT-SECs). As a result, PHx-SECs were observed to migrate to the lower chamber after incubation for 24 h, but not CONT-SECs, indicating that mobility of PHx-SECs increases as compared with that of CONT-SECs. Moreover, 5 nmol Sema3A significantly attenuated migration of PHx-SECs by 57% ( $P < 0.01$ , Figure 4A), while it did not affect proliferation of PHx-SECs (data

not shown).

Based on previous reports showing that Sema3A induces apoptosis<sup>[23-25]</sup>, we explored the effect of Sema3A on apoptotic rate in PHx-SECs and CONT-SECs with TUNEL assay. Compared with CONT-SECs, apoptotic rate in PHx-SECs decreased by 78% ( $P < 0.05$ , Figure 4B). There was no difference in apoptosis between CONT-SECs that were treated with vehicle and Sema3A. However, in PHx-SECs, apoptosis was induced by the presence of 5 nmol Sema3A for 24 h ( $P < 0.05$ , Figure 4B).

## DISCUSSION

The adult hepatocyte is normally a quiescent, highly differentiated cell, but almost immediately it can begin the process of replication in response to conditions that induce cell loss by physical, infectious, or toxic injury. The events involved in the regenerative response are precise, carefully orchestrated, and highly regulated. Indeed, the hepatocyte is a key player in the process of LR, but other cell types, including non-parenchymal cells, also play important roles in reformation of the normal liver architecture.

Among non-parenchymal cells, SECs line the intrahepatic circulatory vessels, named sinusoids, and provide a large surface area for nutrient absorption. Thus, in regenerating liver, SECs play a central role in reconstruction of sinusoids through which blood flow is supplied into the hepatocytes<sup>[8,9,26]</sup>. Previous reports using electron microscopy have shown that SECs proliferate and migrate into initially formed avascular clusters of proliferating hepatocytes, leading to the formation of new functional sinusoids<sup>[9]</sup>.

In the process of sinusoidal remodeling, various proangiogenic factors are produced in response to up-regulation of hypoxia-inducible factor, which is generated within the avascular clusters of hepatocytes. Among these, vascular endothelial growth factor (VEGF) has been shown to be a key regulator of hepatic neovascularization, and is involved in three tyrosine kinase receptors (VEGFR1, R2, and R3), and two non-tyrosine kinase receptors (Nrp-1 and Nrp-2) in inducing signal transduction<sup>[27]</sup>.

Neuropilins were first identified as receptors for class 3 semaphorins, a family of soluble molecules with neuronal guidance functions<sup>[28,29]</sup>. During development, Nrp-1 is preferentially expressed in arteries, and Nrp-2 is expressed in veins and lymphatics<sup>[30]</sup>. There is a degree of specificity in semaphorin/neuropilin binding and activity, with semaphorin 3A (Sema3A) binding to Nrp-1, Sema3F binding to Nrp-2 and Sema3B binding to both receptors on endothelial cells<sup>[13]</sup>. Recently it has been suggested that these semaphorins may interact with the vasculature in an inhibitory manner. Indeed, *in vitro* studies suggest that class 3 semaphorins compete with VEGF for Nrp-1/Nrp-2 binding, thereby inhibiting mitogenic effects of VEGF in endothelial cells, and leading

to apoptosis and inhibition of migration in breast cancer cell lines<sup>[31,32]</sup>.

In this study, to investigate interplay of Nrp-1 and Sema3A in sinusoidal remodeling during LR, we examined the expression of Nrp-1 in liver tissues after PHx in rats, and found that Nrp-1 is constitutively expressed in SECs in normal liver, but its expression is increased following resection of liver, returning to the basal level at termination of liver regeneration. This is consistent with well-documented findings that, following hepatocyte proliferation, DNA synthesis of SECs begins in the late stage of LR<sup>[2,7]</sup>. Increased expression of Nrp-1 was further confirmed by *in vitro* study with SECs isolated from rats after PHx, while it was noted to a lesser extent in cells from normal liver.

Interestingly, the inverse expression of Sema3A and Nrp-1 was observed in liver during the process of LR. Immunohistochemistry also indicated the expression of Sema3A in hepatocytes, suggesting the possibility that Sema3A may interact with Nrp-1 that is expressed in SECs. Indeed, our *in vitro* results show that Sema3A significantly attenuated migration and induced apoptosis in SECs isolated from rats after PHx, but not in cells from normal rats.

Recently, many studies have illustrated the crucial role of Nrp-1 in cell proliferation in development and cancer. The absence of a functional Nrp-1 receptor results in embryonic lethality as a result of impaired heart and blood vessel development, indicating that this receptor plays a central regulatory role during developmental angiogenesis<sup>[33]</sup>. Furthermore, in endothelial-specific Nrp-1 knockouts, certain arterial markers are missing from arterioles and arteries<sup>[34,35]</sup>. Alternatively, a selective Sema3A inhibitor has been shown to enhance regenerative responses and functional recovery of the injured spinal cord<sup>[36]</sup>.

Although further studies are required to reveal the underlying mechanism, our results showing inverse expression of Nrp-1 and Sema3A in liver after PHx may provide a better understanding of sinusoidal remodeling in LR.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The process of liver regeneration (LR) is complex and involves a finely orchestrated balance of cell proliferation to reconstruct the normal liver architecture. Although hepatocyte proliferation is a major feature of the regenerating liver, it involves the interactions of other hepatic cell types (non-parenchymal cells), including Kupffer cells, stellate cells, sinusoidal endothelial cells and biliary epithelial cells.

### Research frontiers

Many of the growth factors have been reported to be upregulated in a regenerating liver. Vascular endothelial growth factor (VEGF) is a major proangiogenic



factor and is thought to improve sinusoid reconstruction during the LR process.

### Innovations and breakthroughs

Recently, the importance of neuropilins (Nrp) as proangiogenic factors has been highlighted. Nrps are transmembrane glycoproteins with large extracellular domains that interact with both class 3 semaphorins and VEGF, and are involved in the regulation of many physiological pathways including angiogenesis. Semaphorin receptors were subsequently found to be expressed by multiple types of cells including endothelial cells.

### Applications

The findings of this study may offer new insights into the role of Nrp-1 and semaphorin 3A (Sema3A) as a target for novel therapeutic strategies in impaired liver regeneration.

### Terminology

Nrp-1 was originally characterized as a Sema3A receptor regulating axon guidance, and it was later demonstrated that Nrp-1 is also important for vascular morphogenesis. Nrp-1 acts as receptor for VEGF, whereby it serves to promote VEGF-induced angiogenesis. Because Sema3A inhibits angiogenesis through Nrp-1, it has been suggested that it acts by merely competing with VEGF and thus inhibiting its proangiogenic effects.

### Peer review

The authors present interesting results on the interaction of neuropilin and semaphorin in control of angiogenesis during liver regeneration. Overall, the results presented support the conclusions.

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