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Platelets Prime Hematopoietic-Vascular Niche to Drive Angiocrine-Mediated Liver Regeneration

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Platelets Prime Hematopoietic-Vascular Niche to Drive Angiocrine-Mediated Liver Regeneration

BY

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ABSTRACT

A critical function for blood vessels is that they secrete paracrine factors necessary for development, homeostasis and repair of the rest of all organs. Among them, the liver is a highly vascular organ, and can undergo regeneration after injury. This liver regeneration process is governed by dynamic interplay between hepatocytes and non-parenchymal cells, liver sinusoidal endothelial cells (LSECs). However, how factors produced from LSECs triggered by injury remains to be defined. Following mouse *in vivo* liver injury model, activated platelets deploy stromal cell-derived factor 1 and vascular endothelial growth factor A to stimulate CXCR7$^+$ LSECs, orchestrating hepatic regeneration. Upon injection of carbon tetrachloride, platelets and CD11b$^+$VEGFR1$^+$ myeloid cells were recruited to LSECs enabling to replenish liver mass. Liver regeneration was impaired in thrombopoietin-deficient (*Thpo$^{-/-}$*) mice repressing platelet production. This impeded regeneration phenotype was recapitulated in mice with either conditional ablation of *Cxcr7* in LSEC (*Cxcr7$^{i\Delta/i\Delta}$*) or *Vegfr1* in myeloid cell (*Vegfr1$^{lysM/lysM}$*). These mice exhibited suppressed expression of hepatocyte growth factor and Wnt2, two crucial hepatocyte propagation factors. Administration of recombinant thrombopoietin restored the prohibited liver regeneration in the tested genetic models. These results suggest that platelets and myeloid cells activate the vascular niche to produce pro-regenerative endothelial paracrine factors. Modulating this "hematopoietic-vascular niche" might help to develop regenerative therapy strategy for hepatic disorders.

Introduction

Hepatic Regeneration for Treatment of End-Stage Liver Diseases

The liver is vital to life and critical to physiological functions. Traumatic damage of liver is frequently hindered with aberrant healing resulting in fibrotic liver formation that lead to end-stage liver diseases. Number of deaths in US related to chronic liver disease and cirrhosis are ranked as the 8th most common cause of death in adults in the US, that 36,427 among a total of 2,596,933 deaths were reported in 2013 (Xu, Murphy, Kochanek, & Bastian, 2016). Chronic generation of fibrotic tissue is the first stage of liver scarring that lead the liver becomes cirrhotic. Cirrhosis is late stage of the fibrotic liver, composed most of the liver mass with scar tissue that underlines liver cancer. Liver fibrosis is a process of scar formation results from a sustained wound healing process in response to chronic liver injuries. The precise mechanism of liver carcinogenesis is unknown, though there are known factors that induce chronic liver injuries, including hepatitis B and C, obesity-related fatty liver disease, biliary diseases, alcoholic (ASH) and nonalcoholic steatohepatitis (NASH), autoimmunity and aflatoxins (Novobrantseva et al., 2005).

Fibrous scar formation is led by prolonged accumulation of excessive extracellular matrix (ECM) proteins, to pronounced distortion of hepatic vascular architecture. This process facilitates hepatic endothelial dysfunction and subsequent maladaptive hepatocyte regeneration (Friedman, 2000), which ultimately progress

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cirrhosis to more serious complications. Portal hypertension is due to increased resistance to portal blood flow, spontaneous bacterial peritonitis, and hepatic encephalopathy. Liver fibrosis is reversible, whereas cirrhosis is mostly irreversible and results in liver failure or the development of hepatocellular carcinoma and death unless liver transplantation is performed. (Tsochatzis, Bosch, & Burroughs, 2014).

Liver transplantation is the major therapy of treatment for patients with the end-stage liver disease due to hepatitis, genetic disorders and tumor metastasis. Published studies have established technologies to allow transplantation of hepatocytes in rodents (Benten, Kumaran, et al., 2005; Enami et al., 2009; Joseph et al., 2006; Krohn et al., 2009; Kumaran, Joseph, Benten, & Gupta, 2005; C. Y. Lee et al., 2003; Wu & Gupta, 2009; Wu, Joseph, Berishvili, Kumaran, & Gupta, 2008; Wu, Joseph, & Gupta, 2006). However, transplantation of liver is hindered by clinical complications and lack of proper donor with more than 15,000 patients desperately awaiting liver transplant (Berg et al., 2009; Bucuvalas et al., 2008; Lonze et al., 2010). The paucity in genetically matched donors and hurdles associated with proper liver regeneration have resulted in increased morbidity and mortality of many patients, who could have otherwise been treated effectively with liver transplantation.

Hepatocyte transplantation provides for an alternative approach to improve liver function. Recently appreciated hepatocyte in the liver in which liver regenerates upon injury or partial resection of liver mass (Puppi et al., 2011). Studies have shown that intrasplenic transplantation of the liver sinusoidal endothelial cells (LSECs) could repair genetic disorders, such as hemophilia in mice (Bandi et al., 2011; Benten, Follenzi, et al.,

2005; Follenzi et al., 2008). However, expansion of transplanted hepatocytes is commonly prohibited by the environment of damaged livers, paradoxically exacerbating excessive scarring. Fibrotic scar formation facilitates dysfunction of LSECs, and lead maladaptive angiogenesis to form cirrhotic liver. In addition, modulation of oxygenation by LSECs has been shown to regulate hepatocyte response to injury (Huebert et al., 2010; Iwakiri, Grisham, & Shah, 2008; Kwok, Lee, Culberson, Korneszczuk, & Clemens, 2009; J. S. Lee, Semela, Iredale, & Shah, 2007; S. H. Lee, Culberson, Korneszczuk, & Clemens, 2008; Semela et al., 2008; Shergill et al., 2010; Yim et al., 2006; Yokoyama et al., 2006). Thus, coaxing the surrounding cells such as vascular cells to generate stem cell-friendly signals can enhance the growth of transplanted hepatoctyes and can bypass scar formation. However, the contribution of LSECs in supporting hepatocyte expansion and engraftment for liver regeneration is initiated after mass loss remains enigmatic.

Sinusoidal Endothelial Cells (LSECs) Autonomy and Instruction: Vascular Niche Regulates Hepatic Regeneration Status

In mammals, the liver can undergo regeneration after either chemical injury or surgical resection of liver mass, a partial hepatectomy (PH) procedure (Bataller & Brenner, 2005; Diehl, 2012; Duffield et al., 2005; Fausto, Campbell, & Riehle, 2006; Friedman, 2008b; Hu et al., 2014; Iredale, 2007; Michalopoulos & DeFrances, 1997; Yang et al., 2003; Zaret & Grompe, 2008). This regeneration process is governed by dynamic interplay between parenchymal hepatocytes and non-parenchymal cells (NPCs) (Duncan et al., 2010; Fausto et al., 2006; Goessling et al., 2009; Liu et al., 2012; Michalopoulos &

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DeFrances, 1997; Woo et al., 2012; Zaret & Grompe, 2008), including stellate cells (Friedman, 2008b; Yang et al., 2003), LSECs (Apte et al., 2006; Cao, Scandura, et al., 2016; Ding et al., 2016; Ding et al., 2010; Gracia-Sancho et al., 2007; Huebert et al., 2010; Iwakiri & Groszmann, 2007; LeCouter et al., 2003; Straub et al., 2008; L. Wang et al., 2012), biliary epithelial cells (Malato et al., 2011), and hematopoietic cells (Aspinall et al., 2010; Boulter et al., 2012; Duffield et al., 2005; Kodama et al., 2010; W. Y. Lee et al., 2010; Lesurtel et al., 2006). Therefore, it will be helpful for designing therapeutic interventions for hepatic diseases by defining the multi-cellular interaction orchestrating liver regeneration.

LSECs lining hepatic sinusoidal vasculature are essential in choreographing liver organogenesis (Ding et al., 2010; Hu et al., 2014; Matsumoto, Yoshitomi, Rossant, & Zaret, 2001; Rafii et al., 2015; Red-Horse, Crawford, Shojaei, & Ferrara, 2007; Sakaguchi, Sadler, Crosnier, & Stainier, 2008). During liver development and regeneration, LSEC-derived angiocrine factors (Cao et al., 2014; Cao, Lis, et al., 2016; Ding et al., 2014; Ding et al., 2010; Ding et al., 2011; Hu et al., 2014; Rafii, Butler, & Ding, 2016) regulate synchronized propagation of hepatocyte (Hoehme et al., 2010; Woo et al., 2012) and resolve fibrosis (Cao et al., 2014; Cao et al., 2011; Ding et al., 2014). In the adult, organ-specific capillary endothelial cells (ECs) support organ regeneration by production of angiocrine factors (Avecilla et al., 2004; Butler, Kobayashi, & Rafii, 2010; Butler, Nolan, et al., 2010; Hattori et al., 2002b; Heissig et al., 2002; Hooper et al., 2009; Kobayashi et al., 2010a; Kopp et al., 2006; Rafii et al., 2003; Rafii, Heissig, & Hattori,

2002; Rafii, Mohle, Shapiro, Frey, & Moore, 1997; Rafii et al., 1995; Rafii et al., 1994; Seandel et al., 2008) to organ regeneration, such as liver regeneration.

A critical function for blood vessels is beyond their role in distribution of oxygen and nutrients to tissues, is that they secrete paracrine factors which are necessary for development, homeostasis and repair of the rest of all organs. Blood vessels support organ functions by creating vascular niche that organ specific cell populations wherein the blood vessels supply the necessary signals to promote adequate type of factors required both to maintain of adequate number of active state of cells required to maintain the function of the organ throughout our lifetime, and to support recruitment and maintenance of cells to replenish lost cells following injury or normal wear and tear.

Previously, we have developed models to dissect the role of angiocrine factors produced by the LSECs in initiating and sustaining liver regeneration after 70% partial hepatectomy (Ding et al., 2010). Partial hepatectomy (Fausto et al., 2006; Greenbaum et al., 1998; J. Jin, Wang, Shi, Darlington, & Timchenko, 2009; Michalopoulos & DeFrances, 1997; Timchenko, 2009; H. Wang et al., 2008) and chemical injury models (LeCouter et al., 2003) are commonly used to identify the molecular pathways involved in hepatic regeneration. However, in chemical-induced hepatic injury models, there is structural and functional damage to the vasculature of the liver, rendering the study of LSECs in liver regeneration cumbersome (LeCouter et al., 2003). PH model, in which there is no perturbation of vascular integrity of the remaining lobes of the liver, provides for an ideal model to study angiocrine function of LSECs without causing hypoxia or vascular compromise. In the initial 4 days after hepatectomy, refer to as "inductive

angiogenesis" (Ding et al., 2010), activation of VEGFR2 followed by upregulation of Id1 in LSECs induced the expression of angiocrine factors, including hepatocyte growth factor (HGF) and Wnt2 (Apte et al., 2009; Apte et al., 2006; Apte et al., 2007; Klein et al., 2008; Monga et al., 2003; Ober, Verkade, Field, & Stainier, 2006; Sakaguchi et al., 2008; Tan et al., 2008). In the adult, hematopoietic cells specifically platelets, are enriched within the regenerating lobes of the liver sinusoids and by intravascular deposition of vascular endothelial growth factor-A (VEGF-A) (Mohle, Green, Moore, Nachman, & Rafii, 1997), and stromal derived factor-1 (SDF-1, CXCL12) (Hamada et al., 1998; D. K. Jin et al., 2006; Nachman & Rafii, 2008; Petit, Jin, & Rafii, 2007) from platelets (Bertozzi, Hess, & Kahn, 2010; Pang, Weiss, & Poncz, 2005; Zimmerman & Weyrich, 2008) activate LSECs to produce angiocrine factors. VEGF-A binds to VEGF receptors 1 and 2 on vascular ECs (Carmeliet, 2005; Ferrara, Gerber, & LeCouter, 2003) and hematopoietic cells (Fischer, Mazzone, Jonckx, & Carmeliet, 2008; Fong, Zhang, Bryce, & Peng, 1999; Grunewald et al., 2006; Kaplan et al., 2005; Stefater et al., 2011) to stimulate production of growth factors. Platelets are circulating reservoirs of growth factors (Bertozzi et al., 2010; Furrer et al., 2011; D. K. Jin et al., 2006; Lang et al., 2008; Lesurtel et al., 2006; Pang et al., 2005; Zimmerman & Weyrich, 2008). *In vitro* and *in vivo* angiogenic models to identify the phenotype of the organ-specific capillaries (Butler, Kobayashi, et al., 2010; Butler, Nolan, et al., 2010; Hooper et al., 2009; Seandel et al., 2008), found that LSECs compose of specialized liver capillaries that are in direct cellular contact with hepatocytes (Ding et al., 2010).

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In addition to tissue injury triggers platelet activation that leads to VEGF-A and SDF-1 secretion to prime LSECs and hematopoietic cells, SDF-1 is demonstrated to upregulate its EC-specific cognate receptor CXCR7 to modulate vascular patterning and angiocrine factor production (Miao et al., 2007; Sierro et al., 2007; S. Yu, Crawford, Tsuchihashi, Behrens, & Srivastava, 2011). Recently, CXCR7 on the LSECs is shown to modulate the expression of angiocrine factors that contributed to the initiation and maintenance of the liver repair (Ding et al., 2014), enabling a hematopoietic-vascular niche orchestrating hepatic regeneration.

By contract, traumatic hepatic damage model of liver is frequently hindered with aberrant healing resulting in fibrotic liver formation. Carbon tetrachloride (CCl_4) injury model recapitulates the reparative responses occurring in liver diseases after chemical injury (LeCouter et al., 2003) via activation of LSECs through production of angriocrine factors induced hepatocyte proliferation and liver repair (Ding et al., 2010; Moniaux & Faivre, 2011). Within 24 hours post CCl_4 injection, but not PH, mobilization of inflammatory cells (neutrophils, monocytes, large undifferentiated leukocytes) was significantly elevated, but platelet mobilization was significantly elevated more post PH than post CCl_4 injection (Ding et al., 2010). CCl_4-induced liver injury model, in which there is significant damage to both hepatocytes and nonparenchymal vascular compartment, provides for an ideal model to study angiocrine function of LSECs in promoting liver repair. How angiocrine factor from LSECs is triggered by liver injury for normal regeneration, as well as the mechanism by which chemical, CCl_4 injury induces LSECs to produce angiocrine factors that stimulate liver repair remains to be defined.

Proposed hypothesis:

We therefore hypothesized that after hepatectomy hematopoietic cells (specifically activated platelets) were recruited to liver sinusoidal vessels and were deploying VEGF-A and SDF-1, which then stimulated VEGFR2$^+$CXCR7$^+$ LSECs to produce hepatocyte-active angiocrine factors to initiate and maintain liver regeneration.

Defining the mechanism by which activation of LSECs support liver regeneration will open up new therapeutic avenues of research to stimulate hepatocyte regeneration in clinical setting. We proposed to use CCl$_4$-induced traumatic hepatic damage model and compared with PH-induced compensatory regeneration, demonstrating that activated platelets deploy SDF-1 and VEGF-A to stimulate CXCR7$^+$ LSEC and VEGFR1$^+$ myeloid cells, support liver regeneration. Three aims were carried out here. 1. *Determine mechanism by which activation of CXCR7 in VEGFR2$^+$ LSECs are activated to paracrine-mediated hepatocyte regeneration.* Study with conditional endothelial cell-specific deletion of CXCR7 and VEGFR2 in the adult mice define the mechanism by which SDF-1 and VEGF-A through Id1-dependent induction of angiocrine factors in LSECs orchestrate hepatic regeneration. 2. *Define the role of recruited hematopoietic cells, specifically platelets, in mediating LSEC activation thereby initiating and sustaining liver regeneration.* After hepatectomy, hematopoietic cells comprising of platelets are recruited to LSECs. We hypothesized that platelets by releasing VEGF-A and SDF1, turn on Akt-Id1 pathway in VEGFR2$^+$CXCR7$^+$ LSECs to produce angiocrine factors initiating liver regeneration. Knock down of VEGF-A and SDF-1 in

hematopoietic cells, as well as platelets, define the role of factors in activating LSECs and liver regeneration. 3. *Determine the role of reciprocal interaction between platelets and Akt-activated LSECs in angiocrine factor induction and accelerating hepatocyte proliferation.* We hypothesized that after hepatectomy the rapid increase in the volume and number of VEGF-A and SDF-1 loaded platelets within liver sinusoidal circulation stimulates LSECs. In turn, Akt-activated LSECs facilitate further deposition and activation of $CD41^+SDF1^+VEGF-A^+$ platelets, reinforcing angiocrine factor production. Genetic model to selectively activate Akt in LSECs allows us to determine whether transplantation of Akt-activated LSECs increases platelet deposition and liver regeneration. The role of plasma elevation of SDF-1 and VEGF-A to activate LSECs for augmenting hepatocyte proliferation will be assessed.

This study will pave the way for identification of factors that support long-term hepatocyte proliferation *in vitro* as well as engraftment and regeneration *in vivo*. This finding will set the stage for development of pre-clinical strategies in which by proper activation of LSECs or intrasplenic transplantation of LSECs, will allow for initiating and maintaining liver regeneration as well as enhancing hepatocyte engraftment.

Materials and Methods

Mouse genetic model

C57BL/6J, *LysM*-Cre and Rosa-Cre^{ERT2} (expressing tamoxifen-responsive inducible Cre) mice were obtained from Jackson laboratory. The *Chd5*(PAC)Cre^{ERT2} mice expressing tamoxifen-responsive Cre^{ERT2} driven by EC-specific VE-cadherin promoter (Ramasamy, Kusumbe, Wang, & Adams, 2014; Y. Wang et al., 2010) were provided by Dr. Ralf Adams. Thrombopoietin deficient (*Thpo$^{-/-}$*) mice (Gurney & de Sauvage, 1996) were kindly offered by Dr. Frederic J. de Sauvage (Genentech, Inc., So. San Francisco, CA, USA). Mice harboring loxP sites-flanked exon 3 of *Cxcr7* (*Cxcr7$^{LoxP/LoxP}$*) were kindly provided by ChemoCentryx, Inc. (Mountain View, CA, USA). Rosa-Cre^{ERT2} animals expressing tamoxifen-responsive inducible Cre were described previously (Ding et al., 2011; D. K. Jin et al., 2006). The *Chd5*(PAC)Cre^{ERT2} mouse line was crossed with floxed *Cxcr7* mice to generate *Cxcr7$^{i\Delta EC/i\Delta EC}$* mice and control *Cxcr7$^{i\Delta EC/+}$* mice after treatment of tamoxifen at a dose of 250 mg/kg for 6 days, and interrupted for 3 days after the third dose. Mice were rested for at least 20 days after the last injection. *Cxcr7$^{LoxP/LoxP}$* mice were also crossed with Rosa-Cre^{ERT2} mice to generate Cre^{+}*Cxcr7$^{loxP/loxP}$* mice, resulting *Cxcr7* deletion in adult mice (*Cxcr7$^{i\Delta/i\Delta}$*). Floxed *Vegfr1* mice (*Vegfr1$^{loxP/loxP}$*) were kindly provided by Dr. Guo-Hua Fong (Fong et al., 1999), and were crossed with LysM-driven Cre (Jackson, Bar Harbor, ME, USA) to generate mice lacking *Vegfr1* specifically in myeloid cells (*Vegfr1$^{lysM/lysM}$*). For all tamoxifen treated mice, mice were allowed to rest for at least 20 days after the last injection. Deletion of target genes in ECs was

corroborated by quantitative PCR. Six- to eight-week-old male WT mice were used for mouse hepatectomy and liver injury models. Sex/age/weight matched mice harboring EC-specific *gene* heterozygous deficiency (*gene*$^{i\Delta EC/+}$) were used as control group for all tamoxifen-inducible gene deletion lines. Investigators who performed mouse experiments and who analyzed the pattern and extent of cell distribution were randomly assigned with animals or samples, and they were blinded to the genotype of the animals or samples from different experimental groups. All animal experiments were carried out following the guidelines of Institutional Animal Care and Use Committee at Weill Cornell Medicine.

Mouse liver regeneration model

Mouse 70% partial hepatectomy (PH) model was used to induce liver regeneration described previously (Ding et al., 2010). Six- to ten-week-old mice were utilized and compared. Midline laparotomy was performed in anaesthetized mice, and three most anterior lobes (right medial, left medial and left lateral lobes) were resected. In brief, mice were anaesthetized by 100 mg kg^{-1} intraperitoneal ketamine and 10 mg kg^{-1} xylazine. Midline laparotomy was performed in the anaesthetized mice, and three most anterior lobes (right medial, left medial and left lateral lobes) containing 70% of the liver weight were surgically removed. After opening the upper abdomen and the exposure of the liver, the left lobe to be removed was lifted. A 5-0 silk suture tie (Roboz Surgical Instrument, Gaithersburg, MD, USA) was placed under the lobe and positioned to the origin of the lobe. After three knots were tied, the tied lobe distal to the suture was

resected by a microdissecting scissor. This surgical procedure was then repeated for the other median lobes to complete PH procedure. Following surgical removal of 70% of liver mass, the peritoneum was re-approximated, and the skin was closed. Sham-operated mice underwent laparotomy without lobe resection. Regeneration of liver mass and function was assessed by measuring the weight of residual liver lobes and normalizing to mouse body weight, as well as serum bilirubin level (Sanofi Genzyme Diagnostics, Cambridge, MA, USA) at indicated time points post PH. Regeneration of liver mass and functional recovery of liver at indicated time points post PH was assessed by 1) measuring the weight of residual liver lobes and normalizing to mouse body weight, 2) hepatocyte proliferation by immunostating of Ki67, 3) plasma bilirubin level (Genzyme Diagnostics), and aspartate transaminase (AST) and alanine aminotransferase (ALT) measurement at indicated time points post PH (Genzyme and Lilai Biology, Chengdu, China).

Transplantation of parenchymal cells

Hepatocyte were purified as previously described via a two-step perfusion and digestion procedure (Ding et al., 2010; Wei et al., 2013). 2.5 million isolated hepatocytes were transplanted to injured mouse liver via intrasplenic injection (Ding et al., 2010) three days after 3rd or 8th injection of CCl_4. Intrasplenic injection was also performed as previously described. Mice were also sacrificed ten days after injection to assess the extent of hepatocyte incorporation in the injured liver.

Liver injury models

To induce liver injury, single injection of carbon tetrachloride (CCl$_4$, Sigma-Aldrich, St. Louis, MO, USA) was injected to mice to induce acute liver injury(Ding et al., 2014)·(C. Yu et al., 2003). Injections of CCl$_4$ were used to induce acute and chronic liver injuries as previously described (Ding et al., 2014). In brief, CCl$_4$ was diluted in oil to yield a concentration of 40% (0.64 mg ml^{-1}) and injected to mice at a dose of 1.6 mg kg^{-1}.

Measurement of fibrosis

Liver fibrotic responses were determined at the indicated time points after injection of CCl$_4$. Collagen distribution was assessed by Sirius red staining. Hydroxyproline level was quantified in the liver to determine the extent of fibrosis (Yoshiji et al., 2000). Liver lobes were weighed, homogenized in lysis buffer, and baked in 12 N hydrochloric acid at 120°C. Samples were aliquoted and added to 1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol (Sigma)**.** After incubation for 20 minutes at room temperature, the samples were incubated with Erlich's solution at 65°C for 15 minutes. Absorbance at the wavelength of 540 nm was determined, and the content of hydroxyproline was calculated by comparing the absorbance to a hydroxyproline standard curve. Hydroxyproline level in the tissue was determined based on the weight. PFA-fixed tissue sections were stained with Sirius Red to assess collagen deposition and distribution, following the protocol of Histoserv (Germantown, MD) or Lilai Biology (Chengdu, China). Sirius red-positive fibrotic parenchyma was assessed using five random fields in each section[23].

Human liver fibrosis samples

Human liver fibrosis (Cat. No. CS516416; CS520446; CS502727; CS504978; CS504492) and normal tissues were purchased from Origene (Rockville, MD). Normal liver tissues exhibiting regular architecture and morphology were analyzed and compared with patient samples.

Isolation and cultivation of LSECs

Mouse LSECs were isolated by previously described two-step collagenase perfusion technique with modifications (Ding et al., 2010). In brief, the liver was perfused with Liver Perfusion Medium (Invitrogen, CA), and dissociated by Liver Digest Medium (Invitrogen, CA). The non-parenchymal cells (NPCs) were fractionated with Percoll® (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) gradient centrifugation with 75% stock Percoll solution and 35% stock Percoll solution. LSEC faction was isolated by mouse LSEC binding magnetic beads (Miltenyi, Auburn, CA, USA) and Dynabeads® Magnetic Beads conjugated with anti-mouse CD31 antibody (MEC13.3, BD Biosciences). Expression of Id1, CXCR7, HGF, and Wnt2 mRNA were determined. Primary human LSECs were procured from ScienCell Research Laboratories (Catalog #5000, Carlsbad, CA, USA). Expression of factor VIII (DACO, Carpinteria, CA, USA) was validated by immunostaining. Akt-LSECs were derived from isolated LSECs that were transfected with the pCCL. PGK lentiviral vector with mouse constitutively active Akt1 (myristoylated Akt: myrAkt)(Kobayashi et al., 2010a). After starving in serum-free

medium, 500,000 LSECs were seeded and stimulated with 10 ng ml^{-1} SDF-1. LSECs

were also treated with 30 µM Wortmannin (Sigma-Aldrich).

Stimulation of thrombopoiesis

To stimulate thrombopoiesis, recombinant TPO, VEGF-A, and/or SDF-1 (PeproTech,

Rocky Hill, NJ, USA) was injected into *Thpo*$^{-/-}$ or *WT* mice i.p. at a dose of 25 µg kg^{-1}

on a daily basis 10 days before PH or CCL$_4$ injury and afterwards. Vehicle for individual

cytokines was also injected as a control group. The degree of hepatic regeneration was

evaluated with control group, including alteration in circulating platelets and parameters

of hepatogenesis.

Immunostaining and morphometric analysis of tissue cryosections

Tissues were harvested and cryopreserved as described (Ding et al., 2010; Ding et al.,

2011) for morphometric analysis (Ding et al., 2014; Ding et al., 2010). Mouse liver was

fixed with 4% paraformaldehyde (PFA) and cryopreserved in optimal cutting temperature

(OCT) compound. For immunofluorescent (IF) microscopy, the liver sections (10 µm)

were blocked (5% donkey serum/0.3% Triton X-100) and incubated with anti-VE-

cadherin polyclonal Ab (pAb, 2 µg ml^{-1}, R&D Systems, NE Minneapolis, MN,

USA), anti-NOX4 pAb (5 µg ml^{-1}, Abcam, Cambridge, MA, USA), anti-desmin pAb (2

µg ml^{-1}, Abcam). For LSEC identity, anti-VE-cadherin pAb and VEGFR3 (Eli Lilly and

Co, New York, NY, USA) antibodies was used (Ding et al., 2010; Hattori et al., 2002b;

D. K. Jin et al., 2006). After incubation in fluorophore-conjugated secondary antibodies

(2.5 µg ml^{-1}, Jackson ImmunoResearch, West Grove, PA, USA), sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA, USA). No appreciable staining was observed in isotype IgG controls. Id1 staining was performed with rabbit anti-human Id1 antibody (Biocheck, Foster City, CA, USA). To reveal platelet activation, antibodies against CD41 and P-selectin (BD Biosciences, San Jose, CA, USA) were used, respectively. CD11b (BD Biosciences, San Jose, CA, USA) and VEGFR1 antibodies (Eli Lilly and Co.) were used to detect myeloid cells (Ding et al., 2010; Hattori et al., 2002a; D. K. Jin et al., 2006). Cell proliferation *in vivo* was measured by 5-Bromo-2'-deoxyuridine (BrdU) uptake. Single dose of BrdU (Sigma, St. Louis, MO, USA) at 50 mg kg^{-1} was i.p. injected to mice 1 h before killing. Liver lobes were removed, weighed, and slice of tissues were incubated with 1M HCl at room temperature for 1 h, neutralized with 10 mM Tris (pH 8.5) for 15 minutes. After incubation with secondary antibody (Jackson ImmunoResearch), cells incorporated with BrdU were identified as proliferating hepatocytes.

Image acquisition and analysis

Histology analysis and Sirius red staining of liver slides were captured with Olympus BX51 microscope (Olympus America, Center Valley, PA, USA), and fluorescent images were recorded on AxioVert LSM710 (Carl-Zeiss, Thornwood, NY, USA) confocal microscope. Co-staining of VE-cadherin with SMA and desmin was also determined. Digital images were analyzed using Image J (NIH, Bethesda, MD, USA). Fluorescent signals in slide were independently evaluated by two investigators from randomly

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selected fields of view. Parameters from each individual animal was measured and averaged.

Immunofluorescence/Western Blot Analysis

Samples were immunocytochemically stained as previously described (James et al., 2010). Briefly, samples were permeabilized in PBST and blocked in 5% donkey serum. Samples were incubated for overnight in primary antibodies blocking solution, washed 3 times in PBS and incubated in CY2- or CY3-conjugated secondary antibodies (Jackson Laboratories) for 1 hour. Following washing, some sections were counterstained for nucleic acids by DAPI or ToPro3 (Invitrogen) before mounting and imaging by confocal microscopy. Primary antibodies used for immunostaining were VE-cadherin (R&D), CD31 (BD), and Oct4 (R&D). All imaging was performed using either a Zeiss 510 or confocal microscope. Western Blot analysis was performed as previously described (Kobayashi et al., 2010b). Antibodies used in these assays: Flag (Sigma-1:1000), VE-cadherin (Cell Signaling-1:200), VEGFR2 (Cell Signaling-1:2000), pVEGFR2 (Cell Signaling-1:300), pMAPK (Cell Signaling-1:2000), GAPDH (Cell Signaling-1:5000), and *Fli1* (Santa Cruz-1:250).

Flow cytometry analysis

Flow cytometry was performed on a Becton Dickenson LSRII SORP, flow sorting performed on an Aria II SORP. Flow cytometry analysis of platelets and LSECs on isolated liver NPCs as previously described (Ding et al., 2010; Hattori et al., 2002b; D. K.

Jin et al., 2006). In brief, retrieved livers from killed animals were minced, digested in liver digestion medium (Invitrogen), and filtered through a 30-μm strainer. Single-cell suspensions were preincubated with Fc block (CD16/CD32; BD Biosciences) and then incubated with primary antibodies recognizing mouse LSECs and hematopoietic cells, as well as rat IgG2aκ and IgG2aβ isotype control. Antibodies used were directed against VEGFR2 (R&D), CD31 (BD), VE-cadherin (eBiosciences), c-kit (BD), EpCam (BD), E-Cadherin (BD), Tra1-60 (BD), Tra1-81 (BD), CD24 (eBiosciences) and SSEA3 (BD). Primary antibodies were directly conjugated to different Alexa Fluor® dyes or Quantum Dots using antibody labeling kits (Invitrogen). Labeled cell populations were measured by a LSRII flow cytometer (Beckton Dickenson, Franklin Lakes, NJ, USA). Compensation for multivariate experiments was carried out with CompBeads (BD Pharmingen), gating was performed on fluorophore minus one (FMO) controls. Compensation for multivariate experiments was carried out with FACS Diva software (Becton Dickinson Immunocytometry Systems).

Gene Expression Analysis by Real-Time Polymerase Chain Reaction

Total RNA was extracted using RNeasy kit (Qiagen, Germantown, MD, USA). After isolation, 500 ng of total RNA was transcribed into complementary DNA by using the superscript reverse transcriptase Kit (Invitrogen). The detection of complementary DNA expression for the specific genes was performed by using the SYBR® Green quantitative polymerase chain reaction (Applied Biosystems, Foster City, CA, USA). To selectively knockdown *Cxcr7* in LSECs, shRNA lentiviruses were generated by cotransfecting 15 μg

of shuttle lentiviral vector, 3 μg of pENV/VSV-G, 5 μg of pRRE, and 2.5 μg of pRSV-REV in 293T cells (Ding et al., 2014). Viral supernatants were concentrated by ultracentrifugation and used to transduce LSECs. Relative quantitative PCR was performed on a 7500 Fast Real Time PCR System (Applied Biosystems) using SYBR Green PCR mix (Applied Biosystems). Human specific SYBR green primer pairs used were: CD31 – f, 5'-tctatgacctcgccctccacaaa–3', r, 5' gaacggtgtcttcaggttggtatttca-3'; VE-cadherin – f, 5'-tggagaagtggcatcagtcaacag-3', r, 5'-tctacaatcccttgcagtgtgag-3'; VEGFR2 – f, 5'-actttggaagacagaaccaaattatctc-3', r, 5'-tgggcaccattccacca-3'; b-Actin – f, 5'-cgtgcgtgacatcaaagagaa-3', r, 5'-ggccatctcctgctcgaa-3'; Cycle conditions were: one cycle at 50°C for 2 min followed by 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 15s and 60°C for 1 minute. Primers were checked for amplification in the linear range and primer dissociation and verified. Threshold cycles of primer probes were normalized to the housekeeping gene b-Actin and translated to relative values.

Statistical analysis of data

All data were presented as the mean ± standard error of mean (S.E.M). Comparisons between different groups were made using ANOVA. Statistical significance was set at $P < 0.05$. Each experiment was at least 3 times.

Results

Mouse 70% Partial Hepatectomy (PH) Induce Hepatic Regeneration

The compensatory liver regeneration after surgical resection of liver mass after 70%
partial hepatectomy (PH) could preserve vascular integrity in the remaining lobes of the
liver. Therefore, PH provides for an ideal model to study angiocrine function of
VEGFR3[+] LSECs without causing hypoxia or vascular compromise (Ding et al., 2010).
We have adapted previously developed 70% partial hepatectomy (PH) model (Fausto et
al., 2006; Greenbaum et al., 1998; J. Jin et al., 2009; Michalopoulos & DeFrances, 1997;
Timchenko, 2009; H. Wang et al., 2008) and chemical injury models (LeCouter et al.,
2003) to dissect the role of angiocrine factors produced by the LSECs (**Figure 1**). In PH,
three most anterior lobes containing 70% of the liver weight were surgically removed.

Figure 1

A

Median lobes

ligation

Left lobe

Right lobes

Caudate lobe

Hepatic artery

Portal vein

B

Day 8

sham   70% PH
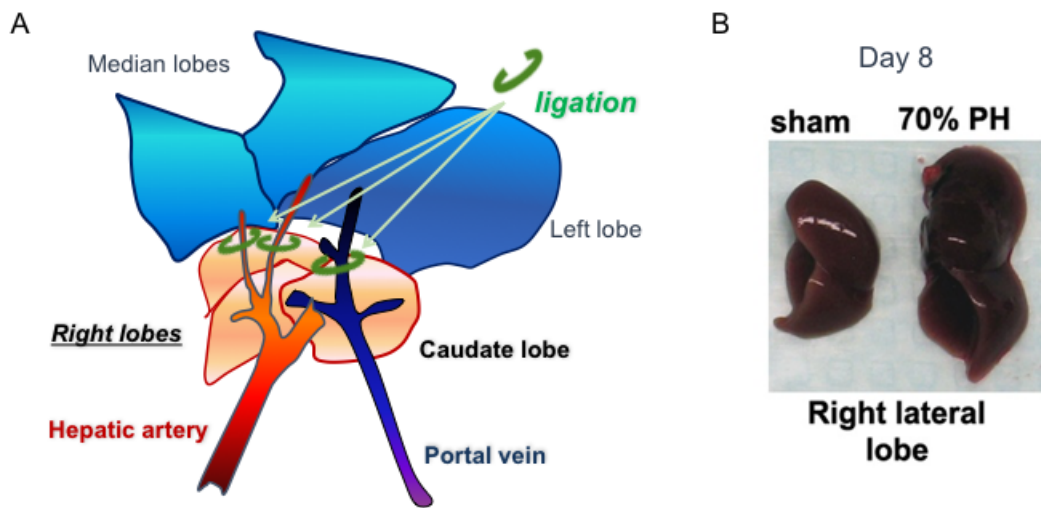
Right lateral lobe

Figure 1: Liver regeneration model - Mouse 70% partial hepatectomy (PH)

Three most anterior lobes (right medial, left medial and left lateral lobes) containing 70% of the liver weight were surgically removed. After opening the upper abdomen and the exposure of the liver, the left lobe to be removed was lifted. A 5-0 silk suture tie was placed under the lobe (green arrows) and positioned to the origin of the lobe. After three knots were tied, the tied lobe distal to the suture was resected. ***B.*** *Sham-operated mice underwent laparotomy without lobe resection (left). Eight days post PH, residual posterior lobes (right lobes and caudate lobes) shows hypertropic compensatory regrowth associate with gain of function. Note that the removed lobes do not regrow to original shape.*

Thrombopoiein knockout mice diminished elevated platelet mobilization into regenerating liver

We examined the contribution of CD41$^+$ platelets to compensatory liver regeneration post 70% PH. Two days post PH, CD41$^+$ platelets harboring VEGF-A and SDF-1 were similarly recruited onto VEGFR3$^+$ LSECs (**Figure 2A**). This indicated a co-localization of SDF-1 and VEGF-A with CD41$^+$ platelets on surface of VEGFR3$^+$ LSECs, and was enumeration of CD41$^+$ platelets harboring VEGF-A and SDF-1 (**Figure 2B**). We examined mice deficient of thrombopoietin (*Thpo*$^{-/-}$) post PH. In *Thpo*$^{-/-}$ mice, platelet number is decreased by ~95% as compared to wild type (*WT*) mice (Carver-Moore et al., 1996; Gurney & de Sauvage, 1996). Significant reduction of hepatocyte proliferation in regenerating liver, two days post PH was noted in *Thpo*$^{-/-}$ mice that were compared to *WT* control group (**Figure 2C**). Hepatic regeneration was markedly decreased in *Thpo*$^{-/-}$ mice. Liver mass restoration eight days post PH were diminished in *Thpo*$^{-/-}$ mice relative to *WT* group as indicated by caudate and right lobe weight relative to body weight (**Figure 2D**). Together with histology, there is close relationship in the regenerating liver between proliferating hepatocytes and VEGFR3$^+$ LSECs, as well as VEGFR3$^+$ LSECs and platelets and activation of platelets, and implicate that platelets produce VEGF-A and SDF-1 to prime LSECs, eliciting liver regeneration.

CD41 SDF-1 VEGFR3 Nuclei

CD41 SDF-1 Nuclei

CD41 VEGF-A VEGFR3 Nuclei

CD41 VEGF-A Nuclei
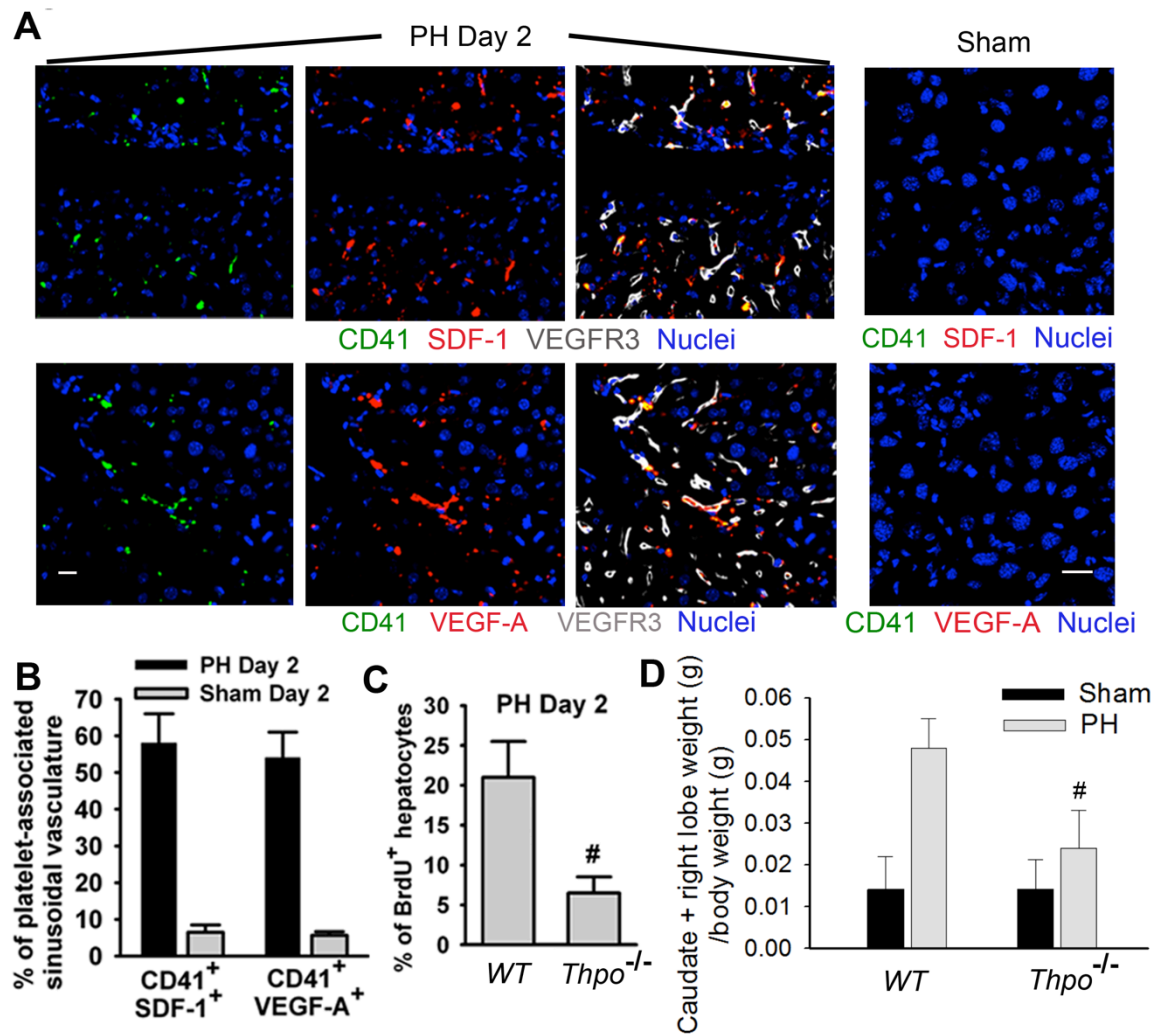
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Figure 2. Post 70% PH, platelets harboring VEGF-A and SDF-1 are associated with VEGFR3^{+} LSECs.

(A, B) Liver sections were stained with antibodies to CD41 (green), VEGF-A and SDF-1 (red) and VEGFR3 (gray). Post PH, but not sham operation (Sham), CD41^{+}SDF-1^{+}VEGF-A^{+} platelets are associated with VEGFR3^{+} LSECs. Scale

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*bar, 50 μm. (**C, D**) BrdU incorporation in hepatocyte (**C**) and liver mass*

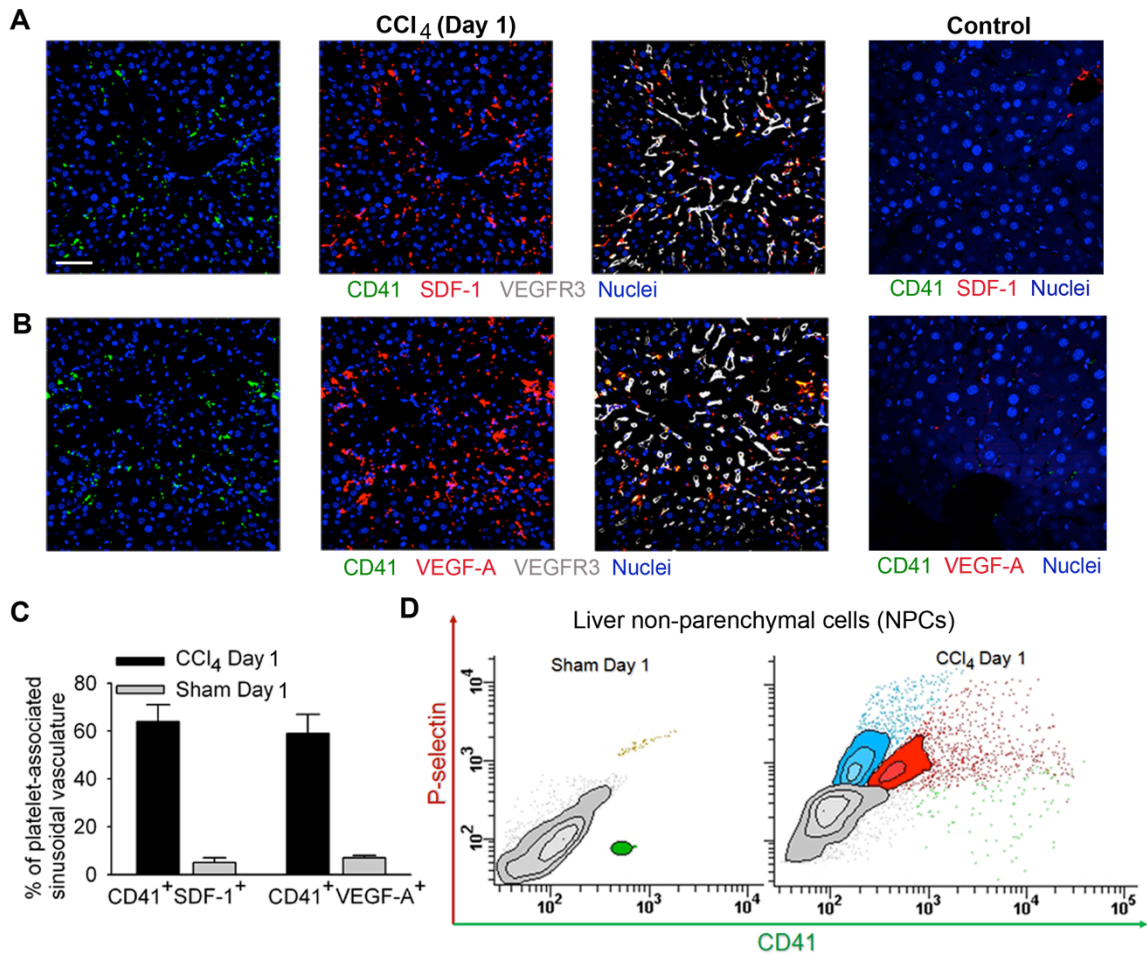*restoration (**D**) in Thpo$^{-/-}$ and WT mice at day 2 and day 8 post PH, respectively.*

$^{#}$*P < 0.05, compared to control group; N = 5-8 mice per group.*

Elevated mobilization of platelets into CCl$_4$ injured liver: Similarly to PH liver, we examined the contribution of CD41$^+$ platelets after tetrachloride (CCl$_4$)-induced liver toxicity on VEGFR3$^+$ LSECs. Platelet activation markers, SDF-1 (**Figure 3A**) and VEGF-A (**Figure 3B**) were co-stained with platelet surface marker CD41 in the liver 24 hours after CCl$_4$ i.p. injection. Of note, twenty-four hours post CCl$_4$ i.p. injection at a dose of 1.6 mg kg^{-1}, CD41$^+$ platelets harboring SDF-1 and VEGF-A were similarly recruited onto VEGFR3$^+$ LSECs. There was structural and functional damage to the vasculature of the liver, rendering the study of LSECs in liver regeneration cumbersome. Compared to sham mice, CCl$_4$ injection caused significant increase of CD41$^+$ platelets on VEGFR3$^+$ LSECs. With the majority of them (~60%) are co-expressing SDF-1 (CD41$^+$SDF-1$^+$) as well as VEGF-A (CD41$^+$ VEGF-A$^+$) (**Figure 3C**), indicating recruitment of platelet in injured liver, and local activation of platelets secreting SDF-1 and VEGF-A.

Parenchymal hepatocytes occupy 70–85% of the liver volume. Whereas non-parenchymal cells (NPCs) composed of LSECs, cholangiocytes, Kupffer cells, stellate cells, and hematopoietic cells occupy 6.5% of its volume (Kmiec, 2001), which compose with 40% of the total number of cells in the liver. Flow cytometric analysis of NPCs revealed that unique platelet marker CD41$^+$ cells constituted 24% of NPCs in CCl$_4$-injured liver but not sham mice (**Figure 3D**). 73% of CD41$^+$ platelets in the damaged liver was co-stained with platelet activation marker, P-selectin. This shows that the CCl$_4$ injury could recruit platelets on the injured hapatic niche, which might also activate platelets. Together with histology, activated platelets promote SDF-1 and VEGF-A,

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which platelets are recruited on perivascular lumen where LSECs are activated via SDF-1

and VEGF-A receptors.

A   CCl$_4$ (Day 1)   Control

CD41 SDF-1 VEGFR3 Nuclei   CD41 SDF-1 Nuclei

B

CD41 VEGF-A VEGFR3 Nuclei   CD41 VEGF-A Nuclei

C

D   Liver non-parenchymal cells (NPCs)

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Figure 3. Hepatotoxic injury recruits CD41+ platelets carrying VEGF-A and SDF-1 to liver vasculature.

(**A, B**) After intraperitoneal (i.p.) injection of hepatotoxic agent carbon

tetrachloride (CCl4), CD41+ platelets expressing VEGF-A and SDF-1 were

recruited to VEGFR3+ liver sinusoidal endothelial cells (LSECs). Liver sections

were stained with antibodies against platelet-marker CD41, VEGF-A (**A**), SDF-1

(**B**) and LSEC-specific marker VEGFR3. After CCl$_4$ injury, but not administration

*of PBS (control), CD41$^+$SDF-1$^+$VEGF-A$^+$ platelets were associated with VEGFR3$^+$ LSECs; scale bar, 50 μm. (**C**) Quantification of CD41$^+$SDF-1$^+$VEGF-A$^+$ platelets associated with VEGFR3$^+$ LSECs. N = 5-7 mice per group. (d) Flow cytometry analysis of platelet accumulation in the CCl$_4$-injured and control livers. Activation of CD41+ platelets was evidenced by surface expression of activation marker P-selectin (CD61).*

Platelet-deficient mice exhibit impaired liver regeneration after CCl₄ injury: To test the contribution of platelets in protecting against liver injury, we examined mice deficient of TPO (*Thpo*$^{-/-}$) after CCl₄ injection. Platelet number is decreased in *Thpo*$^{-/-}$ mice by 95% as compared to wild type (*WT*) mice(Carver-Moore et al., 1996; Gurney & de Sauvage, 1996). Stimulation of thrombopoiesis in *Thpo*$^{-/-}$ mice was achieved with i.p. injection of recombinant VEGF-A and/or SDF-1 (PeproTech) into *Thpo*$^{-/-}$ or *WT* mice at daily dose of 25 μg kg^{-1} for 10 days before CCl₄ injury and afterwards. Simultaneously, contribution of CD41$^+$ platelets post CCl₄ injection, and hepatocyte proliferation was enhanced in *Thpo*$^{-/-}$ mice by injection of VEGF-A and SDF-1 (**Figure 4A and B**). Hepatocyte proliferation after CCl₄ injection was significantly reduced in *Thpo*$^{-/-}$ mice than that of *WT* control group (**Figures 4A-C**). The impaired hepatocyte proliferation was rescued, and enhanced hepatic injury in *Thpo*$^{-/-}$ mice was significantly rescued (**Figure 4C**). Meanwhile, hepatic injury was markedly increased, as indicated by elevation of plasma alanine aminotransferase (ALT) activity (**Figure 4D**). Of note, the impaired hepatocyte proliferation and enhanced hepatic injury in *Thpo*$^{-/-}$ mice were rescued by injection of VEGF-A and/or SDF-1 (**Figures 4A-D**). Therefore, activated platelets recruited to the injured vascular bed supply SDF-1 and VEGF-A to stimulate hepatic repair.
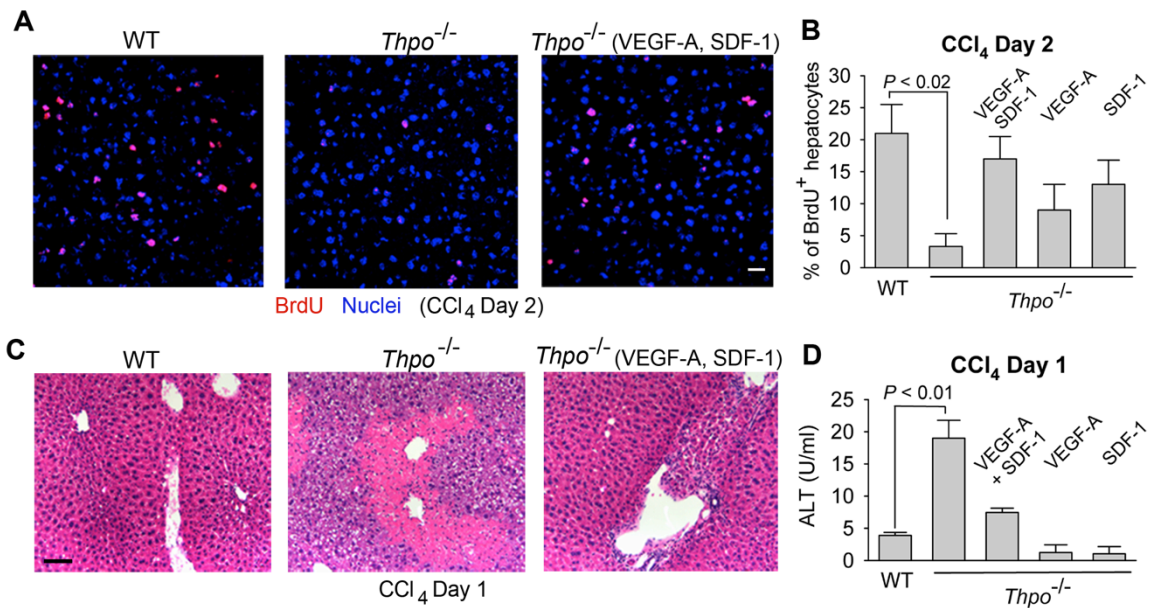
Figure 4. Platelet-deficient mice exhibit impaired liver regeneration after CCl₄ injury.

(A, B) After CCl₄ injury, proliferation (BrdU⁺) of hepatocytes was prohibited in thrombopoietin knockout mice (Thpo⁻/⁻) lacking platelets.

Hepatocyte proliferation was enhanced in Thpo⁻/⁻ mice by injection of VEGF-A and SDF-1. N = 6-8 mice per group. (C) Severe centrilobular damage in the liver of Thpo-/- mice after CCl₄ injury, as indicated by scattered cell debris in Thpo⁻/⁻ mice relative to mild centrilobular necrosis in WT mice. VEGF-A and SDF-1 injection ameliorated the injury in Thpo-/- mice. Scale bar, 50 μm. (D) Increased hepatic injury (plasma alanine aminotransferase, ALT activity) in Thpo-/- mice following CCl₄ injection, as compared to WT mice. These data imply that VEGF-A and SDF-1 produced by activated platelets protect against acute hepatotoxic injury. N = 6-8 mice per group.

LSECs in regenerating liver up-modulate CXCR7 for compensatory hepatocyte proliferation: SDF-1 confers its pro-angiogenic activity (Petit et al., 2007) through activation of two receptors, CXCR4 and CXCR7. Of note to CXCR7 expression is mainly enriched in vascular endothelial cells (ECs) and subsets of lymphocytes (Gerrits et al., 2008; Miao et al., 2007; Sierro et al., 2007; J. Wang et al., 2008). CXCR7 activation in EC is essential for production of pro-regenerative angiocrine factor in organ repair (Cao et al., 2014; Ding et al., 2014). We examined the expression pattern and functional attributes of LSEC-specific SDF-1 receptor, CXCR7, post PH (**Figure 5A**). Immunostaining for the expression of CXCR7 on VE-cadherin$^+$ LSECs two days post PH, revealed that the CXCR7 was upregulated as it compared to sham-operated mice (**Figure 5A, B**), indicating that CXCR7 might serve as an inducible LSEC-specific SDF-1 receptor after acute liver resection.

To test functional contribution of CXCR7 on ECs in regenerating liver of adult mice, we employed mice selectively deleted *Cxcr7* using an inducible tamoxifen-responsive Cre^{ERT2} that is specifically expressed in ECs (Y. Wang et al., 2010) (**Figure 5C**). Mice expressing floxed *Cxcr7* were bred with mouse line carrying EC-specific VE-Cadherin-Cre^{ERT2}/Cdh5(PAC)Cre^{ERT2} (Y. Wang et al., 2010). I.p. injection of tamoxifen to resulting offsprings induced 96% of *Cxcr7* deletion in ECs of adult mice (**Figure 5D**). These mice lacking *Cxcr7* in ECs (*Cxcr7*$^{i\Delta EC/i\Delta EC}$) were subjected to PH, and liver regeneration was compared with control mice harboring endothelial haplodeficiency of *Cxcr7* (*Cxcr7*$^{i\Delta EC/+}$). Hepatocyte proliferation (**Figure 5 E and F**) and liver mass regeneration (**Figure 5G**) were markedly reduced in *Cxcr7*$^{i\Delta EC/i\Delta EC}$ mice, as compared to

those of control mice. These results strongly suggested that endothelial CXCR7 might be

essential for promoting liver regeneration post PH.
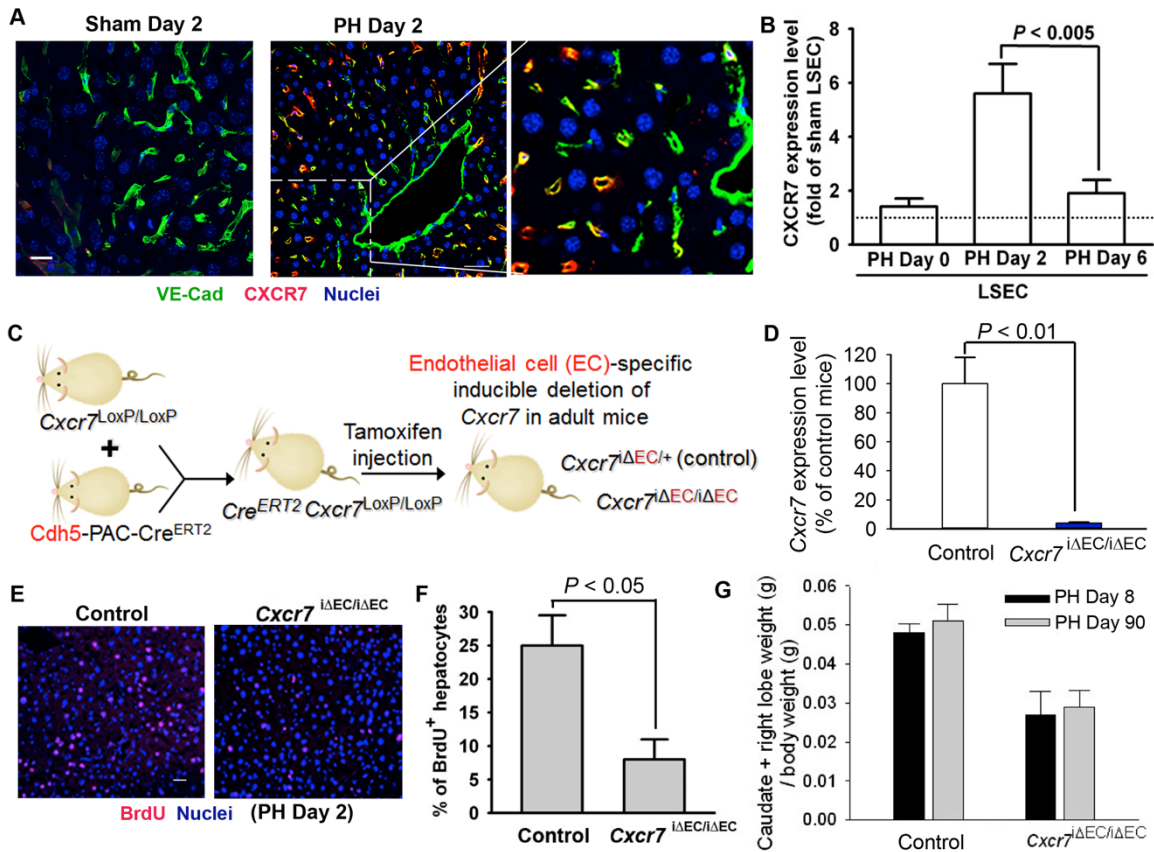
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Figure 5. Post 70% PH, SDF-1 receptor CXCR7 is upregulated in LSEC and contributes to hepatocyte proliferation.

(A) Two days after PH, the liver sections were stained for endothelial-specific VE-cadherin (green fluorescence). VE-cadherin+ LSECs are co-localized with CXCR7+ LSECs (red fluorescence). Scale bar, 50 μm. (b) Cxcr7 mRNA level in isolated LSECs was examined at indicated time after PH. The Cxcr7 expression level of sham LSEC was arbitrarily defined as 1. N = 5-7 mice per group. (C, D) Mice harboring loxP-flanked Cxcr7 were crossed with endothelial cell-specific

*Cdh5-PAC-Cre^{ERT2} mice (Y. Wang et al., 2010). Generated offsprings were treated six times with tamoxifen injection (250 mg kg^{-1}) to induce Cxcr7 deletion (Cxcr7$^{iΔEC/iΔEC}$) (Ding et al., 2010). Mice carrying endothelial haplodeficiency of Cxcr7 (Cxcr7$^{iΔEC/+}$) were used as control group. N = 4 mice per group. (**E-G**) Inducible knockout of Cxcr7 in LSEC abrogated hepatocyte proliferation (**E, F**) and restoration of liver mass at indicated time after PH (**G**). Prohibition of liver mass recovery in Cxcr7$^{iΔEC/iΔEC}$ mice persisted for up to 90 days after PH; N = 6-8 mice per group, P < 0.01 between control and Cxcr7$^{iΔEC/iΔEC}$ group.*

Compensatory liver regrowth augment induction of CXCR7 on LSEC to Akt-dependent activation of Id1. Given that CXCR7 is specifically upregulated in LSECs after liver injury (Ding et al., 2014), we assessed whether CXCR7 is responsible for inducing Id1-dependent production of angiocrine factors for liver repair. This angiocrine factrs of LSECs in liver regeneration depends on activation of transcription factor inhibitor of DNA binding 1 (Id1) in LSEC (Ding et al., 2010; Rafii et al., 2016). We assessed the effect of SDF-1 on cultured human LSECs that are marked with cytoplasmic Factor VIII (**Figure 6A**). SDF-1 induced both upregulation and nuclear enrichment of Id1 protein in LSECs, which was abrogated by genetic silencing of *Cxcr7* (**Figure 6A and B**). In addition, SDF-1-dependent Id1 upregulation in LSECs was recapitulated by overexpression of Akt, and was suppressed by Wortmannin, an inhibitor of PI3 kinase-Akt pathway (**Figure 6B**). These data imply that SDF-1 stimulates CXCR7 in LSECs to trigger Akt-dependent activation of Id1 angiocrine pathway.
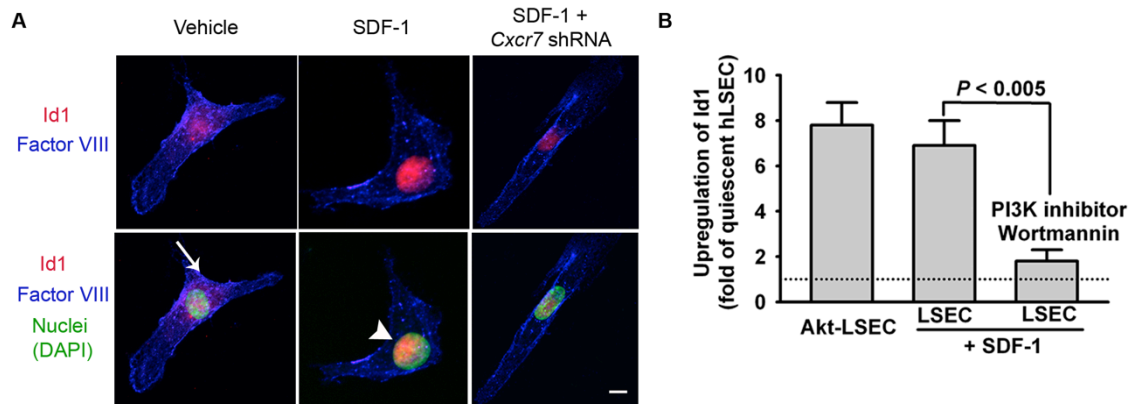
Figure 6. SDF-1 activates CXCR7 on LSEC, inducing Akt-dependent activation of Id1 angiocrine pathway.

*(**A**) CXCR7 expression is essential for SDF-1-mediated Id1 nuclear induction. Early passages of primary human Factor VIII$^+$ LSECs (hLSECs) were stimulated with 10 ng ml^{-1} of SDF-1. shRNA silencing of CXCR7 in human LSEC abolished nuclear accumulation of Id1 after SDF-1 treatment. Note SDF-1 induced translocation of Id1 from cytoplasm to nuclei (arrow head). Scale bar, 10 μm. (**B**) PI3 kinase inhibitor Wortmannin blocked SDF-1 mediated upregulation of Id1, implicating that SDF-1 induction of Id1 is Akt-dependent. N = 3-5 independent experiments.*

CXCR7 stimulates hepatic regeneration activated via recruited platelets.

Given that CXCR7 is specifically upregulated in LSECs after liver injury, LSECs produce paracrine growth factors such as HGF and Wnt2 that exert regenerative inertia to the liver (Ding et al., 2014). We developed inducible deletion of *Cxcr7* mice harboring loxP-flanked *Cxcr7* (*Cxcr7*$^{LoxP/LoxP}$) mice. *Cxcr7*$^{LoxP/LoxP}$ mice were crossed with tamoxifen-inducible Rosa-Cre^{ERT2} mice to ablate *Cxcr7* in adult mice (**Figure 7A**). Generated offspring Cre^{ERT2+}*Cxcr7*$^{loxP/loxP}$ mice were treated 6 times with tamoxifen i.p. injection (250 mg kg^{-1}), and induced deletion of CXCR7 in adult mice (*Cxcr7*$^{i\Delta/i\Delta}$) (**Figure 7A**). Resulting offspring induced approximately 95% transcriptional level of *Cxcr7* deletion in the liver of adult mice (**Figure 7B**). Liver repair was compared between *Cxcr7*$^{i\Delta/i\Delta}$ mice and *WT* post CCl$_4$ injection. There were significantly lower extent of hepatocytes proliferation in the liver was noted by staining for BrdU incorporation for *Cxcr7*$^{i\Delta/i\Delta}$, and higher degree of hepatic injury in *Cxcr7*$^{i\Delta/i\Delta}$ mice than those of *WT* mice (**Figure 7C and D**). Activation of Id1-HGF/Wnt2 angiocrine pathway was markedly prohibited in *Cxcr7*$^{i\Delta/i\Delta}$ mice, as compared to *WT* mice (**Figure 7E**). As such, hepatic injury was markedly increased, as indicated by elevation of plasma ALT activity, and increased hepatic damage in *Cxcr7*$^{i\Delta/i\Delta}$ mice post CCl$_4$ injury was associated with abrogated induction of Id1-Wnt2/hepatocyte growth factor (HGF) angiocrine pathway. All together, these results suggest that after liver injury, liver recruits platelets that supply SDF-1 to activate CXCR7$^+$ LSECs, inducing production of pro-regenerative angiocrine factors, Wnt2 and HGF, via Id1 activation for hepatocyte proliferation (**Figure 7F**).
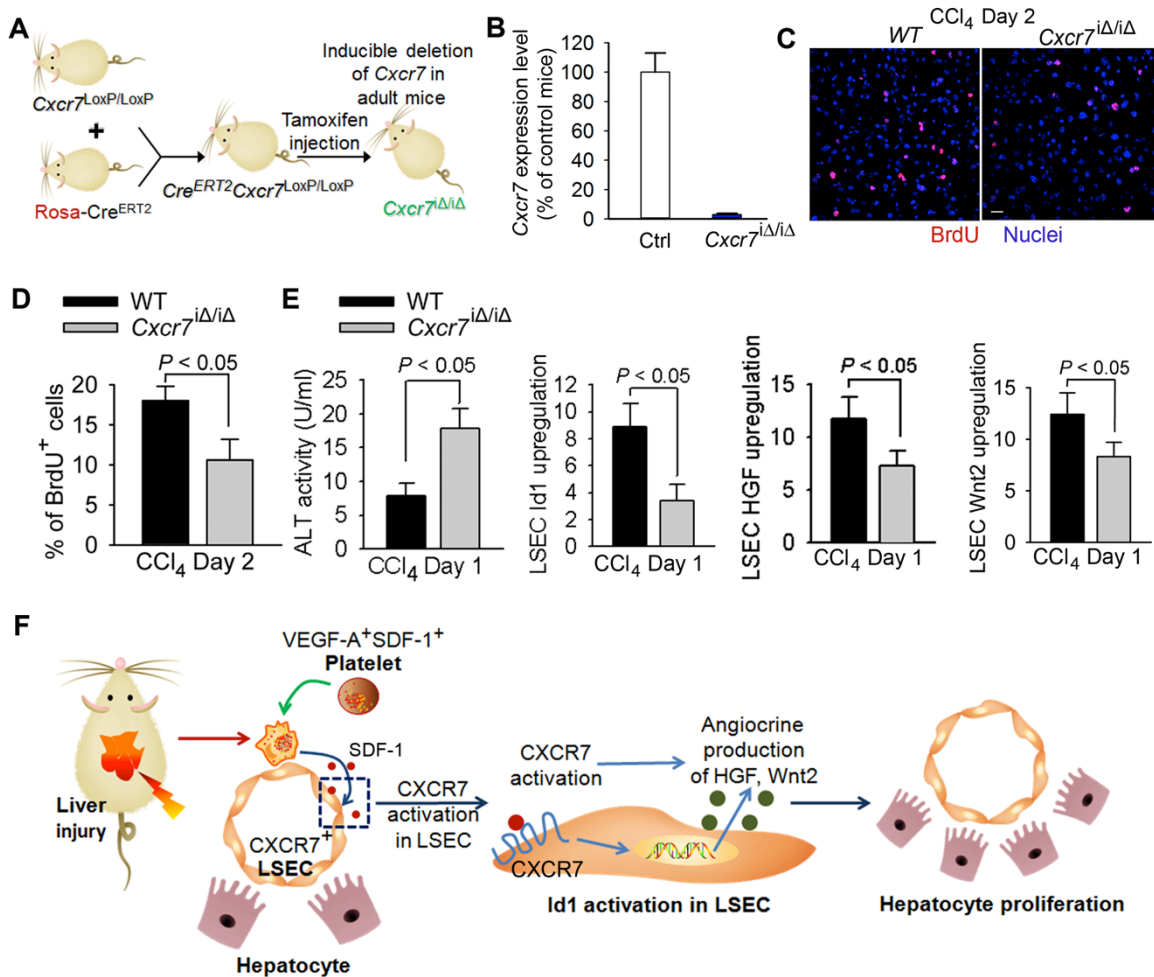
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Figure 7. After CCl₄-induced liver injury, CXCR7 stimulates angiocrine-mediated hepatic regeneration.

(A) Mice harboring loxP sites-flanked Cxcr7 were crossed with Rosa-Cre^ERT2 and treated 6 times with tamoxifen injection (250 mg kg⁻¹) to induce deletion of Cxcr7 in adult mice (Cxcr7^iΔ/iΔ)(Ding et al., 2010). Transcriptional level of Cxcr7 post CCl₄ injury in the liver is shown in B; N = 6-9 mice per group. (C, D)

*Inhibition of SDF-1 signaling in LSEC abolished liver regeneration after CCl$_4$ injury. In Cxcr7$^{i\Delta/i\Delta}$ mice, the decrease in cell proliferation was determined by staining for BrdU incorporation; N = 6 mice per group. Scale bar, 50 μm. **(E)** Increased hepatic damage in Cxcr7$^{i\Delta/i\Delta}$ mice after CCl$_4$ injury was associated with abrogated induction of Id1-Wnt2/hepatocyte growth factor (HGF) angiocrine pathway; N = 5 mice per group. **(F)** Platelet-dependent activation of LSECs leads to generation of hepatogenic angiocrine factors. Upon hepatotoxic injury, activated platelets generate SDF-1 to turn on Id1 pathway in LSECs, resulting in angiocrine production of HGF and Wnt2 that initiates hepatocyte propagation and liver repair.*

VEGFR1⁺ myeloid cells are co-recruited with platelets to vascular niche for augmented angiocrine-mediated liver repair

In the CCl$_4$-injured liver, platelets are recruited and activated. Platelets carry both SDF-1 and VEGF-A in alpha granules that are readily secreted upon activation. Hence, we investigated the contribution VEGF-A receptors in mediating liver repair. VEGF-A was shown to accelerate the regeneration post PH liver via activation of VEGFR2 for priming LSECs (Ding et al., 2010). VEGFR1 activation mediates early protection post CCl$_4$ liver injury (Kato et al., 2011; LeCouter et al., 2003). We, thus, tested the contribution of VEGFR1-expressing myeloid cells in the CCl$_4$ injured liver. CCl$_4$ injection caused significant recruitment of VEGFR1⁺ myeloid cells to the regenerating liver. Furthermore, these recruited VEGFR1⁺ myeloid cells co-expressed CD11b and were found close proximity to VEGFR3⁺ LSECs (**Figure 8A**). To determine the contribution of VEGFR1⁺ myeloid cells to liver repair, *Vegfr1*$^{loxP/loxP}$ mice (Fong et al., 1999) were crossed with LysM-cre mice (Jackson Laboratory) abolishing endogenous Lyz2 gene that is specifically expressed in myeloid cells (**Figure 8B**). Offspring to generate mice lacking *Vegfr1* (*Vegfr1*$^{lysM/lysM}$) resulted to induce 90% transcriptional deletion of *Vegfr1* expression in the liver of adult mice (**Figure 8C**). Liver damage was substantially increased in *Vegfr1*$^{lysM/lysM}$, as evidenced by elevated plasma alanine amino transferase (ALT) activity (**Figure 8D**), which liver injury was mitigated by repeated injection of recombinant thrombopoietin (TPO) in *Vegfr1*$^{lysM/lysM}$ mice (**Figure 8D**). Of note, hepatogenic angiocrine Id1 pathway was suppressed in *Vegfr1*$^{lysM/lysM}$ mice compared to control group, which was restored by recombinant TPO injection (**Figure 8E**). Thus,

liver injury recruits platelets and VEGFR1$^+$ myeloid cells to jointly activate LSEC niche,

driving liver repair (**Figure 8F**).

A CCl₄ Day 2 (liver)

CD11b VEGFR1
Nuclei

VEGFR3 VEGFR1
Nuclei

CD11b VEGFR1
VEGFR3 Nuclei

B

LysM-cre + Vegfr1^LoxP/LoxP

Vegfr1^LysM/LysM

Deletion of Vegfr1 in myeloid cell

C

Vegfr1 expression level (% of control mice)

P < 0.01

WT   Vegfr1^lysM/lysM

D CCl4 Day 1

ALT activity (U/ml)

P < 0.01   P < 0.01

TPO

WT   Vegfr1^lysM/lysM

E CCl4 Day 1

Id1 expression level (% of WT)

P < 0.01   P < 0.01

+ TPO

WT   Vegfr1^lysM/lysM

F

Liver injury

Platelet recruitment

VEGF-A⁺
SDF-1⁺
Platelet

VEGFR1⁺ myeloid cell

VEGFR3⁺ LSEC

Hepatocyte

Platelets activate hematopoietic-vascular niche

CXCR7 activation in LSEC

LSEC activation

Production of pro-regenerative angiocrine factor

HGF
Wnt2

Angiocrine-mediated liver regeneration

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Figure 8. Activated platelets recruit VEGFR1⁺ myeloid cells to reinforce angiocrine-mediated liver repair.

*(**A**) Recruited VEGFR1⁺ cells in the liver co-expressed CD11b and were associated with VEGFR3⁺ LSECs, as revealed by immunostaining. Scale bar, 50 μm. (**B, C**) Conditional knockout of Vegfr1 in myeloid cells abrogated hepatocyte proliferation after CCl₄ injection. Floxed Vegfr1 mice were bred with LysM-*

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driven Cre to generate mice lacking Vegfr1 in myeloid cells (Vegfr1$^{lyzM/lyzM}$).
Quantification of Vegfr1 transcriptional level in myeloid cells is shown in **c**; N =
5 mice per group. (**D**) Exacerbated liver injury in Vegfr1$^{lyzM/lyzM}$ mice than WT
control, as evidenced by elevated plasma level of alanine aminotransferase, ALT.
Injection of thrombopoietin (+TPO) prevented liver parenchymal injury in
Vegfr1$^{lyzM/lyzM}$ mice; N = 5-7 mice per group. (**E**) Pro-regenerative Id1 angiocrine
pathway was suppressed in Vegfr1$^{lyzM/lyzM}$ mice, which was elevated by
thrombopoietin injection (+TPO). Compared to WT control mice, transcriptional
level of Id1 was lower in Vegfr1$^{lyzM/lyzM}$ mice after CCl$_4$ injury. *P < 0.05,
compared to Vegfr1$^{lyzM/lyzM}$ group. N = 5-7 mice per group. (**F**) Schema depicting
the contribution of hematopoietic-vascular niche for liver regeneration. Upon
liver injury, activated platelets are recruited to the liver and produce SDF-1 to
activate CXCR7$^+$ LSECs, initiating endothelial paracrine/angiocrine-mediated
liver repair. Activation of VEGFR1$^+$ myeloid cells by platelet VEGF-A further
stimulate Id1-Wnt2/HGF angiocrine pathway in LSEC, reinforcing liver
regeneration.

Discussion

LSECs secrete angiocrine factors and actively participate in liver repair.

Recent recognition for blood vessel function is organ-specific organ homeostasis and repair. Inner lining of vessel, endothelial cells (ECs) secrete paracrine factors instructively support organ functions by creating vascular niche, and promote adequate type of cells in active state to maintain the functional organ throughout our lifetime. LSECs lining hepatic sinusoids actively participate in liver repair and regeneration that liver can replenish lost cells following toxic traumatic injury or tear loss of liver mass. The repair is often hindered with aberrant healing resulting in fibrotic liver formation that lead to end-stage liver diseases. Whereas healthy repopulation for lost cells is often hindered to loss of liver mass. After removal of the left and median lobes of the liver (70% partial hepatectomy) the arterial and portal supply to the remaining lobes of right and caudate lobes remain intact. After hepatectomy, by an unknown mechanism, VEGFR2 and Id1 pathway are activated in LSECs inducing expression of angiocrine factors, such as HGF and Wnt2, that are active to hepatocyte proliferation(Ding et al., 2010). During the first 4 days of regeneration, Wnt2 and HGF induce hepatocyte expansion without LSEC proliferation, (Inductive Angiogenesis). Four days after hepatectomy LSECs start to proliferate (Proliferative Angiogenesis), which along with hepatocyte propagation, restore liver to its original mass. The initial 4 days after hepatectomy is refer to as "inductive angiogenesis" (Ding et al., 2010) via activation of

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VEGFR2 followed by upregulation of Id1 in LSECs induced the expression of HGF and

Wnt2 (Apte et al., 2009; Apte et al., 2006; Apte et al., 2007; Klein et al., 2008; Monga et

al., 2003; Ober et al., 2006; Sakaguchi et al., 2008; Tan et al., 2008). In the mouse liver,

we have identified a preferential distribution of SDF-1 receptor CXCR7 on LSECs,

which was upregulated by PH. We have also revealed the functional role of endothelial

CXCR7 in generating factors that are active to hepatocyte proliferation in both PH and

CCl_4 models. As such, both hepatotoxic injury and loss of liver mass stimulates CXCR7

activation in LSECs, eliciting hepatic regeneration, and repair.

Bivalent LSEC factors for normal or maladaptive repair

Surgical resection of liver lobes by partial hepatectomy (PH) triggers facultative

regeneration without causing fibrosis (Diehl & Chute, 2013; Forbes & Rosenthal, 2014;

Hu et al., 2014; LeCouter et al., 2003; Matsumoto et al., 2001; Michalopoulos &

DeFrances, 1997; Wei et al., 2013; Yanger et al., 2014). Similarly, liver can restore

functional mass and resolve fibrosis after acute injury (Beers & Morrisey, 2011; Islam et

al., 2012; Jiang et al., 2005; Looney et al., 2009). In contrast, chronic injury stimulates

exuberant scar formation and fibrosis, leading to liver failure (Bataller & Brenner, 2005;

Forbes & Rosenthal, 2014; Friedman, 2008a; Friedman, Sheppard, Duffield, & Violette,

2013; Hecker et al., 2009; Henderson et al., 2013; Jiang et al., 2005; Mehal, Iredale, &

Friedman, 2011; Noble, Barkauskas, & Jiang, 2012; Pellicoro, Ramachandran, Iredale, &

Fallowfield, 2014; Seki et al., 2007; Thannickal, Zhou, Gaggar, & Duncan, 2014; Wynn,

2007; Wynn & Ramalingam, 2012; Zeisberg et al., 2007). How fibrosis is resolved post

PH or acute injury but triggered by other types of injury needs to be unraveled

(Ramachandran & Iredale, 2012).

Self-repair capacity of liver is sometimes prohibited by overwhelming or

persistent injury. Transplantation of parenchymal cells might aid in reinstating the

impaired regenerative ability (Barkauskas et al., 2013; Chapman et al., 2011; Desai,

Brownfield, & Krasnow, 2014; Duncan et al., 2010; Hogan et al., 2014; Kotton &

Morrisey, 2014; Kumar et al., 2011; Nishikawa et al., 2015; Ott et al., 2010; Vaughan et

al., 2015; B. Wang, Zhao, Fish, Logan, & Nusse, 2015; Wangensteen, Zhang,

Greenbaum, & Kaestner, 2015; Zaret & Grompe, 2008; Zuo et al., 2015). But efficient

engraftment of parenchymal cells in diseased organ is handicapped by the lack of

epithelially active niche and existing fibrosis. Upon chronic liver injury (e.g., virus

infection, alcohol abuse, nonalcoholic steatohepatitis (NASH) and cholestasis) often lead

to formation of fibrotic liver. The main player is recognized as hepatic non-parenchymal

myofibroblasts that are key producers of extracellular matrix (ECM) proteins, which

drives the phenotypic change of parenchymal hepatocytes. Designing efficient cell

therapy strategy for parenchymal cell engraftment requires understanding of how

microenvironmental cues promote regeneration and bypass fibrosis in damaged liver

(Kordes & Haussinger, 2013; Pardo-Saganta et al., 2015; Tata et al., 2013; B. Wang et al.,

2015). Accumulating evidence has suggested a critical role of *Cxcr7*, in the activation

process of hepatic myofibroblasts, have all been implicated to regulate LSEC activation

and hepatocyte apoptosis, both of which are essential steps for initiating liver fibrosis.

Employing the unique phenotypic attributes of LSECs, we have confirmed that LSECs leads to elaboration of hepatic-active angiocrine factors via activation of transcription factor Id1. Liver regeneration after partial hepatectomy is severely impaired in $Id1^{-/-}$ mice, in part because upregulation of angiocrine factors HGF and Wnt2 is diminished in $Id1^{-/-}$ mice. Intrasplenic transplantation of LSECs isolated from $Id1^{-/-}$ mice that were transduced with HGF and Wnt2 into $Id1^{-/-}$ mice restored the defective liver regeneration in $Id1^{-/-}$ mice (Ding et al., 2010). This model provided for an ideal approach to interrogate the role of LSECs in liver regeneration as well as to investigate the molecular and cellular mechanisms involved in the fibrogenic processes in order to design novel therapeutic interventions for liver fibrosis and lays the foundation for designing clinical trials to enhance liver regeneration.

Cultured human and mouse LSECs and demonstrated that LSECs in serum- and growth factor-free conditions support expansion of the hepatocytes by production of angiocrine factors. We have identified a preferential distribution of SDF-1 receptor CXCR7 on LSECs in the mouse liver, which was upregulated by PH. Thus, LSECs enable liver stem cell-like hepatocytes to replenish liver cells lost due to PH. Upon chemical-induced liver damage, we have revealed the functional role of endothelial CXCR7 in unique capacity to support hepatocyte proliferation, and trans-generating angiocrine factors that are active in both loss of liver mass by PH and CCl_4 hepatotoxic injury. Development of fibrosis in damaged organ might hamper engraftment of parenchymal cells. Therefore, understanding of how fibrosis is resolved during organ regeneration is helpful for devising cellular therapy for various diseases (Thannickal et

al., 2014). HGF is implicated to have an important role in promoting parenchymal cell expansion (Awuah, Nejak-Bowen, & Monga, 2013; Huh et al., 2004; Matsumoto & Nakamura, 1991; Yamamoto et al., 2007). In PH models that normally induce fibrosis-free parenchymal regeneration, deletion of *Cxcr7* in mouse ECs blocked regeneration and caused fibrosis. Reciprocally, in mouse liver fibrosis models, ectopic expression of *Cxcr7* in LSECs prevented activation of perivascular fibroblasts and suppressed fibrosis (data not shown). These genetic model-based *in vivo* data implicate that in addition to stimulating parenchymal reconstitution, endothelial Cxcr7 serves as a checkpoint molecule to prevent fibrosis.

Following parenchymal injury, *Cxcr7* expression might be triggered in vascular niche as a protective response. The vast surface area of hepatic microvasculature is ideal to supply sufficient amounts of signals within a short period. Several endothelial receptors were demonstrated to mediate *Cxcr7* upregulation in ECs, including VEGFR1 (LeCouter et al., 2003), VEGFR2 (LeCouter et al., 2003), and CXCR7 (Zhang et al., 2015). Conceivably, these endothelial receptors sense tissue mass loss or chemical injury to trigger Cxcr7 expression. Loss of these protective pathways might not only impair regeneration but also cause fibrosis. It was shown before that activation of Smad2/3 blocks HGF transcription in keratinocyte (Hoot et al., 2010), suggesting a suppression of this checkpoint molecule by TGF/Smad pathway. Whether TGF/Smad pathway mediates the loss of checkpoint function in vascular niche during parenchymal repair might be explored in the future.

Regenerating liver recruit hematopoietic cells to modulate LSEC factors

Recruited platelets in the injured liver initiates LSEC angiocrine signaling to trigger hepatic reconstitution. Post injury, platelets serve as circulating sentinel cells to promote tissue repair (Bertozzi et al., 2010; Furrer et al., 2011; Lesurtel et al., 2006; Pang et al., 2005; Zimmerman & Weyrich, 2008). Here, our study has implied a paradigm in which platelet-supplied SDF-1 activates CXCR7 on LSECs and initiates subsequent angiocrine signaling. We found that recruited platelets in the injured liver initiates LSEC angiocrine signaling to trigger hepatic reconstitution. Activated platelets deploy SDF-1 and VEGF-A to stimulate CXCR7$^+$ LSECs and VEGFR1$^+$ myeloid cell, orchestrating hepatic regeneration. This result implied a paradigm in which activated platelets recruited to the injured vascular bed supply SDF-1 and VEGF-A to activates CXCR7 on LSECs which initiates subsequent angiocrine signaling that instruct healthy hepatic repair. These data implicate that platelets produce VEGF-A and SDF-1 to prime LSECs, eliciting liver regeneration, which lay the foundation for capitalizing on the instructive functions of blood vessels for therapeutic repair from liver failure by efficiently expanding stem cell-like hepatocyte populations in vitro or in the liver of humans. The beneficial effect of platelets in liver repair is in agreement with both preclinical and clinical findings that favorable prognosis of hepatic function correlates with higher circulating platelet count (Kodama et al., 2010). Whether recombinant thrombopoietin has the similar protective effect in infectious liver injury remains to be investigated (Lang et al., 2008).

The finding that after liver injury, administration of thrombopoietin, SDF-1, VEGF-A, enhanced liver repair has clinical relevance. First, it is conceivable that in

thrombocytopenic patients, liver repair is impaired. Thus, cautiously increasing the number of circulating platelet might offer tissue protection. Alternatively, after acute injury, administration of SDF-1 and VEGF-A might augment hepatocyte proliferation by stimulating angiocrine factor generation in LSECs. The cytoprotective effect of VEGFR1$^+$ myeloid cells after acute injury was evidenced by increased injury in mice with myeloid-specific *Vegfr1* knockout (*Vegfr1*$^{\text{lysM/lysM}}$). The rescue effect of thrombopoietin injection might depend on both platelets and myeloid cells. The diminished Id1 pathway in LSECs of *Vegfr1*$^{\text{lysM/lysM}}$ mice evidenced the effect of VEGFR1$^+$ myeloid cells. Conceivably, increasing platelet number by thrombopoietin injection enhances platelet-dependent activation of myeloid cells, reinforcing endothelial activation and vascular niche-mediated liver regeneration.

Taken together, we demonstrate a pro-regenerative interplay between platelets, myeloid cells, and LSEC in liver repair, a unique contribution of vascular and peri-vascular niche cells on influencing parenchymal cell repopulation. Platelets play an instrumental role in priming both angiocrine function of LSEC and myeloid cells post injury. Thus, platelet activation enables a hematopoietic-vascular (Rafii et al., 2015) niche that orchestrates liver regeneration. This study further provides insights into active role of blood vessels within the vascular niche for liver stem cell-like hepatocytes, which aid in a regenerative process. Identifying critical pathways establishing this hepatogenic hematopoietic-vascular niche might aid in devising regenerative therapy for hepatic diseases. Therapeutically, implementing "cell transplantation" with "niche targeting" avenue can

potentially induce more efficient organ regeneration than "cell transplantation" alone. Transfusion/transplantation of properly primed platelets or myeloid cells can possibly offer optimal cell therapy approaches. Our "proof-of-principle" evidence might help to develop approaches to enable fibrosis-free repair in various organs.

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Appendix
LICENCE TO PUBLISH - OPEN ACCESS

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Journal Name:

Signal Transduction and Targeted Therapy   (the "Journal")

Proposed Title of the Contribution:

Platelets prime hematopoietic-vascular niche to drive angiocrine-mediated liver regeneration   (the "Contribution")

Author(s):

Koji Shido, Deebly Chavez, Zhongwei Cao, Jane Ko, Shahin Rafii, Bi-Sen Ding   (the "Author(s)")

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