RESEARCH ARTICLE

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Type XVIII collagen is essential for survival during acute liver injury in mice

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SUMMARY

The regenerative response to drug- and toxin-induced liver injury induces changes to the hepatic stroma, including the extracellular matrix. Although the extracellular matrix is known to undergo changes during the injury response, its impact on maintaining hepatocyte function and viability in this process remains largely unknown. We demonstrate that recovery from toxin-mediated injury is impaired in mice deficient in a key liver extracellular matrix molecule, type XVIII collagen, and results in rapid death. The type-XVIII-collagen-dependent response to liver injury is mediated by survival signals induced by α1β1 integrin, integrin linked kinase and the Akt pathway, and mice deficient in either α1β1 integrin or hepatocyte integrin linked kinase also succumb to toxic liver injury. These findings demonstrate that type XVIII collagen is an important functional component of the liver matrix microenvironment and is crucial for hepatocyte survival during injury and stress.

INTRODUCTION

A central feature of liver function is the ability to metabolize foreign substances and sense pathogens in the circulating blood stream. In order to maintain tissue homeostasis, the liver has the capacity to mount a rapid response to toxin-mediated injury or pathogen infection and regenerate damaged parenchymal tissue. Various cytokines and growth factors and their requisite signaling pathways have been implicated in this process. A recent modeling study suggests that the hepatocyte-sinusoid interface plays a crucial role in the repopulation of hepatocytes after acute toxic injury (Michalopoulos, 2007; Ding et al., 2010). The role of liver extracellular matrix (ECM) in toxic liver injury is largely unknown, but the ECM is speculated to be important for hepatocyte function and viability (Bissell et al., 1987; Dunn et al., 1989; Flaim et al., 2005). Key metabolic and homeostatic features *in vitro* such as cytochrome P450 gene expression and serum protein production are influenced by interactions between ECM molecules and hepatocytes via cell surface integrin receptors (Hamilton et al., 2001; Oda et al., 2008; Page et al., 2007). Additionally, studies have demonstrated that the ECM composition used in hepatocyte cultures might impact cellular architecture, morphology and polarity (Berthiaume et al., 1996; LeCluyse et al., 1994). Liver ECM molecules have different distribution patterns in normal and pathological states, but *in vivo* studies of the impact of these changes are lacking. Understanding the functional role of ECM molecules in toxic liver injury could have important therapeutic implications and considerable impact on hepatic bioengineering applications and *in vitro* modeling of xenobiotic metabolism.

Type XVIII collagen is a prominent ECM component in the liver. This member of the multiplexin family of collagens is highly expressed in liver, and levels have been shown to increase further during pro-fibrotic stages of fibrosis, cirrhosis and various cancers of the liver (Jia et al., 2001; Musso et al., 1998; Musso et al., 2001). Genetic deletion of type XVIII collagen has been reported to result in structural defects in ECM structure, including the expansion and disorganization of several tissue basement membranes (Fukai et al., 2002; Utriainen et al., 2004), including the brain and retina, heart, kidney, and choroid plexus. No overt liver phenotype during development and postnatal homeostasis has been reported in mice lacking type XVIII collagen (Marneros and Olsen, 2005; Seppinen and Pihlajaniemi, 2011).

RESULTS

Because type XVIII collagen is highly expressed in the liver and plays an important role in ECM architecture, we explored its role in adaptive responses and regeneration after acute toxic liver injury in mice. Toxin-induced liver injury using carbon tetrachloride (CCl_4) provides a well-defined model in which acute injury and the subsequent regenerative response can be assessed. A survey of ECM molecules demonstrated variable expression in and around the normal central vein region, a region most susceptible to toxin-induced injury, with high expression observed for type XVIII collagen (supplementary material Fig. S1A-D). A single injection of CCl_4 results in centrilobular necrosis and apparent degradation of type XVIII collagen (Fig. 1) after 48 hours. We also observed an increase in expression of the basement-membrane-associated

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TRANSLATIONAL IMPACT

Clinical issue

Drug and xenobiotic metabolism occurs primarily in the liver and drug- or toxin-induced hepatic injury is a principal cause of acute liver failure, for which there are limited therapeutic options. From a drug development perspective, toxic liver injury can lead to drug withdrawal from the market and represents a large obstacle in the development of pharmaceuticals. The liver's regenerative response to drug- or toxin-induced injury involves the expression of various cytokines and growth factors as well as changes to the hepatic stroma, including the extracellular matrix, but is poorly understood. A complete understanding of the mechanisms underlying liver injury and of the tissue response to injury is central to the development of new treatment options for acute liver failure.

Results

In this study, the authors investigate the functional role of the liver extracellular matrix in toxic liver injury. Using a mouse model of acute toxin-induced liver injury, they identify an interaction between the extracellular matrix and hepatocytes that promotes cell survival. They show that a newly identified interaction between the key extracellular matrix molecule type XVIII collagen and its receptor α1β1 integrin is involved in this survival response. Type XVIII collagen also plays a crucial role in maintaining the provisional matrix during the injury response. Moreover, its expression is regulated by the pleiotrophic cytokine TGFβ, which is induced after acute liver injury.

Implications and future directions

This work identifies type XVIII collagen and α1β1 integrin as key determinants of resistance to drug- or toxin-induced liver injury and suggests that the interaction of hepatocytes with the extracellular matrix is an essential element of the liver injury response. Further studies are now needed to determine exactly how integrin binding to the extracellular matrix promotes the survival of hepatocytes during liver injury. The development of therapeutic strategies that focus on promoting these interactions could facilitate the repair of acute liver injury. In addition, these findings might provide the basis for the identification of therapeutic strategies designed to prevent drug- or toxin-induced liver injury.

short-chain variant of type XVIII collagen in livers in the early phase (48 hours) of CCl_4 injury (Fig. 1Q). Immunofluorescence analysis indicates that type XVIII collagen protein levels in the centrilobular region begin to return at 144 hours post-CCl_4 injection and are fully covered at 192 hours (Fig. 1N,O), and *Col18a1* gene expression levels returned to normal at 192 hours (Fig. 1Q).

In order to examine the functional role of type XVIII collagen during liver injury, we utilized the CCl_4 model of chronic liver injury. Liver metabolism was first primed by administering phenobarbital and mice were subsequently challenged with a single injection of CCl_4 every 3-4 days. Mice deficient in type XVIII collagen (*Col18a1*$^{-/-}$) were moribund within the first few days of CCl_4 challenge (Fig. 2A). Histological analysis of wild-type mice revealed low to moderate levels of damage around the centrilobular venules (Fig. 2B,C). In contrast, *Col18a1*$^{-/-}$ mice exhibited substantial levels of lipid accumulation with signs of degeneration extending into the mid and periportal zones (Fig. 2D,E). Masson's trichrome staining did not reveal any overall difference in ECM deposition between wild-type and *Col18a1*$^{-/-}$ mice.

Endostatin, a C-terminal cleavage product of type XVIII collagen, is known to function as an endogenous angiogenesis inhibitor and has been shown to potentially possess additional biological activity (O'Reilly et al., 1997; Rehn et al., 2001; Su et al., 2012; Sudhakar et al., 2003). Circulating levels of this type XVIII collagen fragment have been shown to increase in animal models of liver regeneration (Colakoglu et al., 2007) and the role of this circulating endostatin has not been established to date. We speculated that type XVIII collagen, via its endostatin domain, might mediate the survival of hepatocytes during liver injury. Biologically active recombinant mouse endostatin was administered twice daily (the half-life of endostatin is estimated at 12-14 hours) to *Col18a1*$^{-/-}$ mice with CCl_4-induced toxic liver injury. Supplementation with exogenous endostatin did not affect the progression of CCl_4-induced liver disease in *Col18a1*$^{-/-}$ mice. None of the *Col18a1*$^{-/-}$ mice survived past day 5 despite endostatin administration (Fig. 2A). These results suggest that soluble endostatin, which is derived from type XVIII collagen, does not mediate hepatocyte survival action in this setting.

TUNEL staining of liver sections of *Col18a1*$^{-/-}$ mice revealed significant cell death when compared with control mice 48 hours

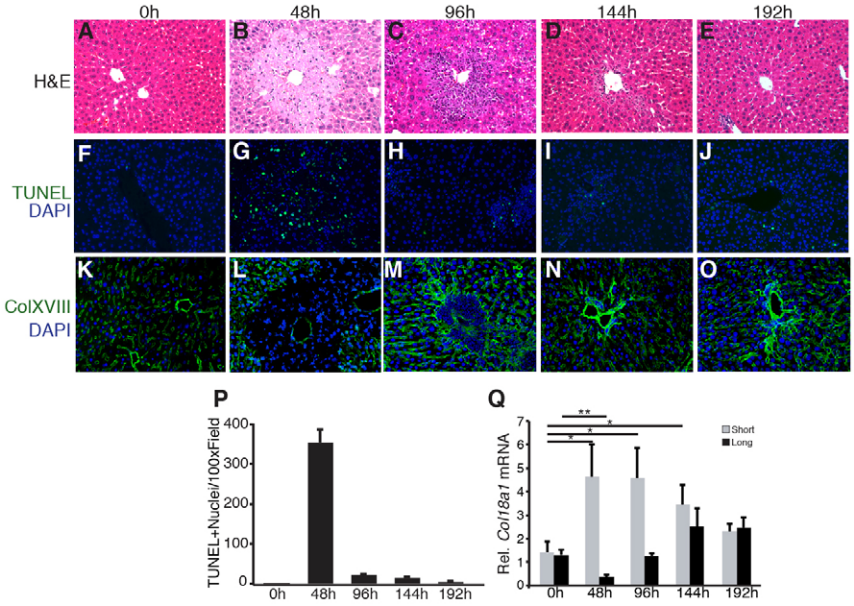

Fig. 1. Type XVIII collagen expression during the acute injury response in the liver. Acute injury was induced with a single dose of the hepatotoxin carbon tetrachloride (CCl_4) in order to examine the kinetics of the liver injury response and changes in the liver extracellular matrix. The single dose of CCl_4 was administered and mice were sacrificed at the 0, 48, 96, 144 or 192 hour time points. (A-E) H&E stain indicates damage and subsequent repair to the centrilobular zone after CCl_4 administration. (F-J) TUNEL staining and (P) quantification indicates the kinetics of cell death during the acute injury response. (K-O) Immunofluorescence of type XVIII collagen (ColXVIII) and (Q) gene expression levels of the long and short variant of type XVIII collagen in wild-type mice treated with CCl_4. *P<0.05; **P<0.02.

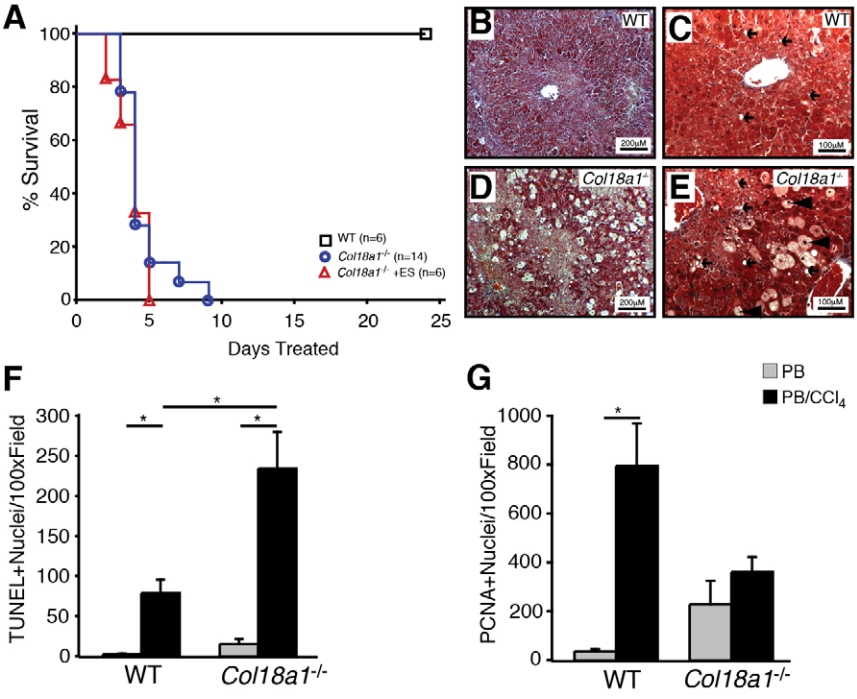

Fig. 2. Type XVIII collagen plays a crucial role in the response of liver to acute injury. (A) Wild-type (n=6) and *Col18a1*$^{-/-}$ (n=14) mice were subjected to a 24-day CCl_4 challenge. Recombinant endostatin (ES) was administered to an additional *Col18a1*$^{-/-}$ cohort (n=6) treated with CCl_4. The Kaplan-Meier curve shows that *Col18a1*$^{-/-}$ mice had a severe acute response to the hepatotoxin and were moribund within the first few days of treatment. Endostatin administration did not improve survival in these mice. (B-E) Masson's trichrome staining of (B,C) wild-type and (D,E) *Col18a1*$^{-/-}$ mice after CCl_4 induction reveals extensive damage within the centrilobular zone of *Col18a1*$^{-/-}$ mice, including lipid accumulation (arrows) and ballooning degeneration (arrowheads). Less-extensive damage was observed in wild-type mice. (F) TUNEL staining after 48 hours in sham- and CCl_4-treated wild-type and *Col18a1*$^{-/-}$ mice reveals an increase in cell death in the *Col18a1*$^{-/-}$ (n=4) mice as represented in the bar graph. (G) Centrilobular proliferation was observed in wild-type mice 48 hours after CCl_4 injections, whereas *Col18a1*$^{-/-}$ mice displayed a mild proliferative response (n=4) at the same time point, as represented in the bar graph. *P<0.05.

after the CCl_4 challenge (Fig. 2F). Proliferation as assayed by staining for proliferating cell nuclear antigen (PCNA) status was similar in CCl_4-challenged *Col18a1*$^{-/-}$ and wild-type mice at the 48-hour time point (Fig. 2G). To further confirm this observation, we performed liver regeneration (a process highly dependent on the proliferative capacity of the hepatocytes) studies using *Col18a1*$^{-/-}$ mice. We did not observe a difference in liver regeneration rates when compared with control mice (supplementary material Fig. S2) 7 days post-hepatectomy. Additionally, we noted an increase in cell death (Fig. 2F,G) in *Col18a1*$^{-/-}$ mice primed with phenobarbital prior to CCl_4 administration. Histology also revealed expanded areas of degeneration in *Col18a1*$^{-/-}$ mice treated with phenobarbital (supplementary material Fig. S3). This suggests that type XVIII collagen has a crucial role in preserving the integrity of the liver in response to xenobiotics and the survival and/or viability of hepatocytes during toxic liver injury.

The cytochrome P450 enzyme CYP2E1 is predominately responsible for metabolizing CCl_4, generating reactive oxygen species that induce centrilobular hepatic necrosis (Wong et al., 1998). Hepatocyte-ECM interactions might modulate cytochrome P450 gene expression *in vitro*; therefore, we explored whether the loss of type XVIII collagen results in altered expression of the *Cyp2e1* gene. When wild-type and *Col18a1*$^{-/-}$ mice were treated with phenobarbital alone or together with CCl_4, no significant differences in *Cyp2e1* gene expression were observed (Fig. 3A). Albumin gene expression was reduced in both wild-type and *Col18a1*$^{-/-}$ groups treated with CCl_4 when compared with control mice, but treatment with CCl_4 had a significantly stronger effect in *Col18a1*$^{-/-}$ mice than in wild-type mice (Fig. 3B). Furthermore, primary mouse hepatocytes cultured on Matrigel supplemented with purified recombinant type XVIII collagen revealed significantly enhanced albumin production and viability when compared with hepatocytes cultured on Matrigel alone (Fig. 3C,D). These results support the notion that type XVIII collagen is important for hepatocyte survival and function and thus might contribute to the responses that are responsible for protection of the liver during toxic injury.

Our results suggest that hepatocyte interaction with type XVIII collagen is important for maintaining hepatocyte survival and function in the liver injury setting. Because type XVIII collagen can bind to integrins (Faye et al., 2009), we considered the possibility that survival cues mediated via integrin signaling might be deficient in the absence of type XVIII collagen. In order to address the capacity of hepatocytes to bind to type XVIII collagen in an integrin-dependent manner, cell adhesion assays with AML12 hepatocytes were performed in the presence of magnesium or EDTA. The AML12 hepatocytes adhered to purified recombinant type XVIII collagen, but EDTA significantly diminished this binding, suggestive of integrin-dependent adhesion to type XVIII collagen (Fig. 4A).

To confirm integrin-dependent cell adhesion of hepatocytes to collagen XVIII, we performed antibody-blocking assays for α1β1 and α5β1 integrins. Our analysis and previous literature suggests that these two integrins are highly expressed in the liver and particularly in hepatocytes (supplementary material Fig. S4) (Binamé et al., 2008; Pinkse et al., 2004). These integrins are also implicated in mediating survival signaling in epithelial cells (Howlett et al., 1995; Liu Tsang et al., 2007). A significant decrease in adhesion was observed when cells were pre-treated with α1-blocking antibody (Fig. 4B). We also noted a strong correlation between type XVIII collagen expression and expression of α1β1 integrin in the central vein region and the adjacent sinusoids of the liver (Fig. 4C-E). Such strong colocalization was not observed between type XVIII collagen and α5β1 integrin in the central vein region. However, we did observe colocalization between type XVIII collagen and α5β1 integrin in the liver sinusoids outside of the centrilobular zone. Additionally, AML12 cell adhesion was not

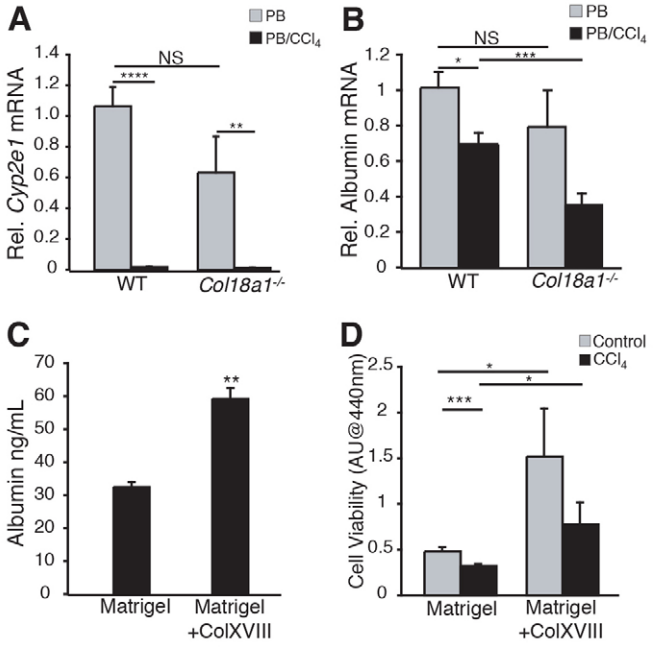

Fig. 3. Altered hepatocyte function and cell turnover in the absence of type XVIII collagen. (A) Levels of *Cyp2e1* gene expression assessed by quantitative real-time PCR were similar between wild-type (WT) and *Col18a1*$^{-/-}$ mice after induction with phenobarbital (PB; *n*=4). These levels were significantly decreased upon treatment with CCl_4. (B) Albumin gene expression in WT and *Col18a1*$^{-/-}$ mice treated with CCl_4 was decreased when compared with phenobarbital-treated mice as determined by quantitative real-time PCR (*n*=4). The decrease in albumin gene expression between WT and *Col18a1*$^{-/-}$ mice treated with CCl_4 was significant. (C,D) Isolated primary hepatocytes cultured on Matrigel™ supplemented with recombinant chicken type XVIII collagen (ColXVIII) exhibited (C) increased albumin production compared with Matrigel alone and (D) improved viability, as determined by WST-1 assay, upon treatment with CCl_4 when compared with Matrigel alone (performed in triplicate). AU, absorbance units. *P<0.05, **P<0.02, ***P<0.005, ****P<0.001; NS, not significant.

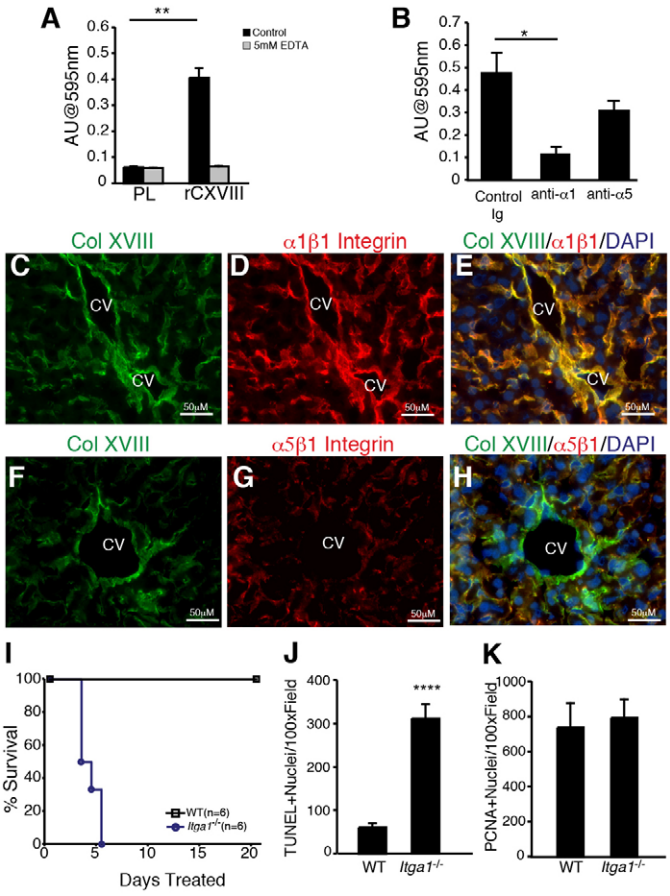

Fig. 4. Type XVIII collagen binds the α1β1 integrin on hepatocytes. (A) Non-tissue-culture-treated 96-well plates were coated with recombinant type XVIII collagen (rCXVIII) at a concentration of 25 μg/ml and AML12 cells were allowed to adhere in 1:1 F12/DMEM supplemented with 1% BSA supplemented with 1 mM $MgCl_2$ (control) or 5 mM EDTA. AU, absorbance units; PL, plastic. (B) AML12 cells were treated with azide-free/low-endotoxin antibodies and subsequently allowed to adhere to plates coated with type XVIII collagen to determine the integrins that promote binding to this ECM constituent. (C-E) Type XVIII collagen and the α1β1 integrin receptor colocalize in the central vein (CV). (F-H) The α5β1 integrin receptor colocalizes with type XVIII collagen to a lesser extent in the central vein and centrilobular zone than does the α1β1 integrin receptor. (I) Kaplan-Meier survival curve of *Itga1*$^{-/-}$ mice subjected to 1 week of phenobarbital and CCl_4. (J) TUNEL staining in sham- and CCl_4-treated (48 hour) wild-type (*n*=4) and *Itga1*$^{-/-}$ (*n*=3) mice reveals an increase in cell necrosis in the *Itga1*$^{-/-}$ mice. (K) *Itga1*$^{-/-}$ (*n*=3) mice maintained a similar proliferation rate compared with wild type (*n*=4) when treated with CCl_4, as determined by PCNA staining. *P<0.05, **P<0.02, ****P<0.001.

blocked by the α5β1 integrin blocking antibody, suggesting that this integrin does not bind to type XVIII collagen (Fig. 4B).

Because the α1β1 integrin heterodimer is expressed at the ECM-cell interface of cells in the centrilobular zone and our antibody blocking studies suggests that it is a receptor for type XVIII collagen, we utilized mice deficient in the α1 integrin subunit (*Itga1*$^{-/-}$ mice) to test whether α1β1 integrin plays a role in hepatocyte survival during CCl_4 injury. We induced liver injury in *Itga1*$^{-/-}$ mice with CCl_4 and compared disease progression to that of age-matched littermate control mice. Whereas the control mice survived throughout the 21-day treatment period, the *Itga1*$^{-/-}$ mice were moribund within 5 days post CCl_4 injection, similarly to what was observed with the *Col18a1*$^{-/-}$ mice (Fig. 4I). Apoptosis was significantly increased in *Itga1*$^{-/-}$ mice treated for 48 hours with CCl_4 (Fig. 4J), whereas proliferation rates remained similar to wild-type mice (Fig. 4K). In an effort to begin defining signaling pathways that might play a role in α1β1-integrin-mediated hepatocyte survival, we investigated a potential role for integrin linked kinase (ILK) signaling. Recent reports have implicated ILK signaling in hepatocytes during their response to injury (Gkretsi et al., 2008; Gkretsi et al., 2007). As shown in Fig. 5A-C, type XVIII collagen and α1β1 integrin colocalize on the surface of AML12 hepatocytes during the early phase of cell adhesion. Blockade of α1β1 integrin restricts cell spreading over time and limits ILK concentration at focal adhesions (Fig. 5D-G). In order to assess the significance of ILK in liver injury, we generated mice that had a specific deletion of ILK in hepatocytes by crossing albumin-cre mice to mice carrying floxed alleles of *Ilk* (*Alb*$^{Cre+}$*Ilk*$^{flox/flox}$, *Ilk*^{HepKO}). These mice were sensitive to CCl_4-induced liver injury and displayed increased levels of hepatic

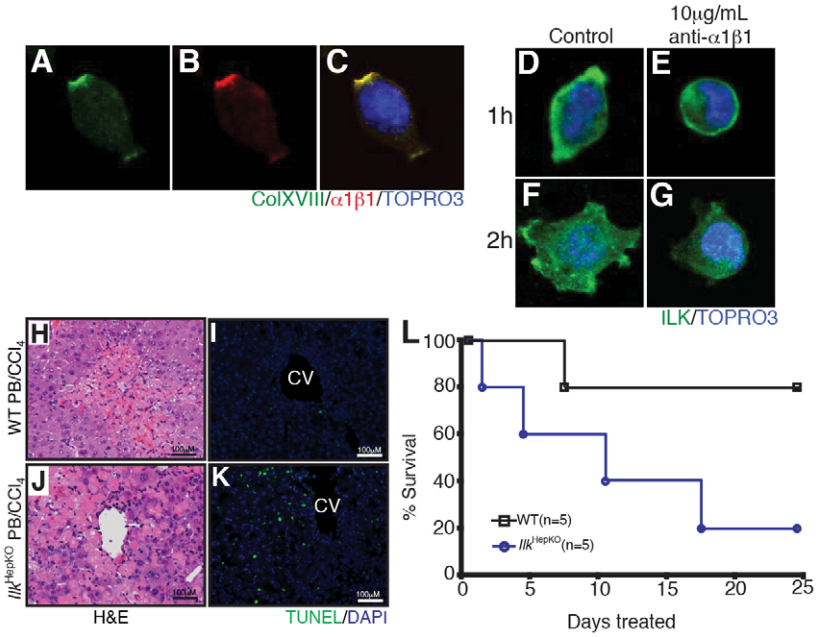

Fig. 5. Histology and cell death of CCl_4-injured *Ilk*^{HepKO} and *Ilk*^{HepWT} mice. (A-C) Type XVIII collagen and α1β1 integrin colocalize on AML12 hepatocytes during early adhesion events. (D-G) Antibody blockade of α1β1 integrin restricts cell spreading and ILK concentration at focal adhesions when AML12 cells are allowed to adhere for (D,E) 1 hour and (F,G) 2 hours. (H-K) *Ilk*^{HepKO} mice had extensive cellular necrosis compared with *Ilk*^{HepWT} mice as determined by H&E and TUNEL staining. (L) Genetic ablation of *Ilk* in hepatocytes (*Ilk*^{HepKO} mice; *n*=5) leads to an increase in the number of moribund mice when compared with wild-type (*n*=5) mice in a 25-day CCl_4 survival time course. PB, phenobarbital.

necrosis (Fig. 5H-K) with significant morbidity occurring within 2 weeks (Fig. 5L). We also examined Akt phosphorylation in the livers of *Col18a1*$^{-/-}$ and wild-type mice. Levels of nuclear phosphorylated Akt were substantially lower in *Col18a1*$^{-/-}$ mice (supplementary material Fig. S5) and correlated with an overall decrease in Akt phosphorylation (supplementary material Fig. S5).

The formation of a provisional matrix is an essential component of the tissue injury response (Bezerra et al., 1999). Fibrinogen is converted to fibrin and deposited in the centrilobular zone of the murine liver after injury. Previous studies have established an interaction between type XVIII collagen and fibrin (Tang et al., 2009), prompting us to explore the nature of fibrin deposition in *Col18a1*$^{-/-}$ mice after injury. Immunohistochemical analysis of *Col18a1*$^{-/-}$ mice revealed that only small amounts of fibrin are deposited within the centrilobular zone, in a patchy manner, after 48 hours exposure to CCl_4, whereas distribution is more uniform within the centrilobular zone of wild-type mice (Fig. 6A-E). This altered deposition was independent of fibrinogen gene expression: as levels of α, β and γ chains were comparable in CCl_4-challenged wild-type and *Col18a1*$^{-/-}$ mice (Fig. 6F-H). These results support the notion that the impaired repair capacity of the liver in *Col18a1*$^{-/-}$ mice might also be due to defective fibrin synthesis and/or deposition.

Next, we explored the mechanisms that might control type XVIII collagen gene expression during liver injury and the subsequent regenerative process. FoxA2 is a transcription factor that is highly expressed in liver and is responsible for liver-specific gene expression. It has been established that gene expression of the long-chain variant of type XVIII collagen is highly dependent on FoxA2 activity (Liétard et al., 2000). Additionally, recent studies indicate that TGFβ signaling via Smad2/3 activation can directly impact FoxA2 expression and activity levels (Zhang et al., 2011). Because TGFβ is expressed during CCl_4-mediated liver injury, we examined Smad2/3 responsiveness in the injured centrilobular zone during injury and regeneration. We observed nuclear localization of Smad2 in the hepatocytes in and adjacent to the injured centrilobular zones at 48 hours post CCl_4 intoxication (Fig. 7A-E). To explore the direct relationship between TGFβ signaling and type XVIII collagen expression, we treated AML12 cells with recombinant TGFβ1. We observed that expression level of the long-chain variant of type XVIII collagen was decreased by tenfold and short-chain levels were increased fourfold (Fig. 7F). Immunocytochemical staining for type XVIII collagen revealed increased density on the cell surface when cells were treated with TGFβ1 (Fig. 7G,H).

DISCUSSION

We demonstrate that type XVIII collagen plays a crucial role in maintaining the liver microenvironment during acute hepatotoxic injury. Numerous reports suggest that hepatocyte-matrix interactions play an important role in the metabolic status of cultured primary hepatocytes. In our *Col18a1*$^{-/-}$ mice, we did not observe alterations in the levels of *Cyp2e1*, the primary cytochrome P450 enzyme responsible for metabolizing CCl_4. Despite normal levels of *Cyp2e1* expression, the absence of type XVIII collagen led to a high degree of hepatic necrosis and reduced hepatocyte functionality.

Mice deficient in type XVIII collagen develop a normal liver with regenerative capacity following partial hepatectomy. We observed that proliferative rates during the CCl_4-induced injury response lagged slightly but not to a significant degree. These data suggest that the genetic deletion of type XVIII collagen does not significantly impair hepatocyte proliferation under normal or injury conditions. However, we did observe significant differences in cell death, suggesting that type XVIII collagen plays a key role in cell survival. We identified α1β1 integrin as a newly identified receptor for type XVIII collagen and observed similar defects in the cell survival response in mice deficient in α1β1 integrin and hepatocyte integrin linked kinase (*Ilk*^{HepKO}), a potential downstream mediator of integrin activation. Although we identified a potential pathway by which type

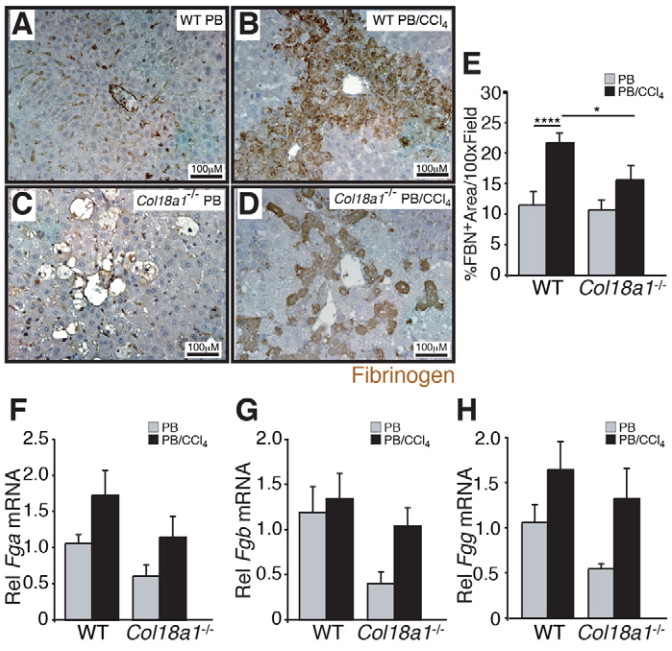

Fig. 6. Altered matrix deposition in *Col18a1*$^{-/-}$ mice with acute liver injury. (A-D) 48 hours after a single dose of CCl_4, fibrin deposition within the damaged centrilobular zone of *Col18a1*$^{-/-}$ mice was patchy compared with wild type (WT) and (E) decreased significantly as determined by immunohistochemistry. (F-H) α, β and γ fibrinogen chain mRNA levels as determined by quantitative real-time PCR did not reveal significant changes in gene expression in 48-hour CCl_4-treated WT and *Col18a1*$^{-/-}$ mice (n=4). *P<0.05, ****P<0.001.

XVIII collagen exerts its pro-survival effects, it is important to point out that the molecules we focused on also probably function independent of type XVIII collagen and therefore the phenotypes we observed through genetic deletion of these molecules are complex and will require further analysis.

Much of the functional biological activity of type XVIII collagen has been attributed to its C-terminal domain, commonly referred to as endostatin. This matricryptin (fragment derived from proteolytic cleavage of ECM) plays a well-established role in tumor angiogenesis and newly identified roles in neuronal development are beginning to emerge (O'Reilly et al., 1997; Seppinen and Pihlajaniemi, 2011; Su et al., 2012). Soluble endostatin levels have been shown to increase in various types of liver injury and disease. We sought to determine whether the endostatin domain of type XVIII collagen could play a beneficial role during the injury response. We did not observe a beneficial effect of providing physiological levels of endostatin to type-XVIII-collagen-deficient mice exposed to CCl_4. This suggests that the protective role of type XVIII collagen is likely to be derived from activity that is independent of the endostatin domain. Because we administered non-native endostatin in this trial, we cannot rule out that the endostatin domain still intact with the rest of the type XVIII collagen could elicit the protective effects during acute toxin-induced liver injury.

It is also interesting to note the shift in isoform expression during the liver injury response. We observed a significant decrease in expression of the long isoform of *Col18a1*, whereas that of the short isoform was significantly increased. The long isoform is controlled by a proximal promoter containing hepatocyte-specific response elements, whereas expression of the short isoform is controlled by a more distal upstream promoter region with response elements that are less understood (Elamaa et al., 2003; Muragaki et al., 1995; Rehn et al., 1996). Our data suggests that TGFβ signaling, a key pathway involved in the acute and chronic liver injury response, might play and important role in controlling *Col18a1* isoform expression.

Collectively, our findings demonstrate a crucial contribution of ECM-hepatocyte interaction to the protection against acute damage in hepatocytes. We provide evidence that type XVIII collagen binds to α1β1 integrin and this interaction might serve to induce hepatocyte survival signals via ILK. Previous studies have shown that ILK plays a key role in liver regeneration and the injury response (Apte et al., 2009; Gkretsi et al., 2008; Gkretsi et al., 2007). The studies indicate that outside-in signaling derived from the ECM is important in the setting of injury and such signaling re-enforces processes that protect hepatocytes from rapid damage. We believe that type XVIII collagen and its receptor, α1β1 integrin, are key determinants of resistance to drug- or toxin-induced liver injury. The results provide a basis for identification of new therapeutic targets aimed at preventing or repairing liver injury. Additionally, we demonstrate successful production of type XVIII collagen and the use of recombinant type XVIII collagen in standard cell culture experiments. Exposure of cells to type XVIII collagen leads to an enhanced production of albumin, which is associated with increased hepatocyte viability. These studies offer novel opportunities to evaluate gene expression profiles and secretion of liver-specific products when hepatocytes are cultured in a 3D Matrigel containing type XVIII collagen.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma (St Louis, MO) or Fisher Scientific (Waltham, MA) unless otherwise noted. Secondary antibodies for immunofluorescence were purchased from Jackson ImmunoResearch Laboratories (Westgrove, PA) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma.

Animal care and use

All mice were housed under standard conditions in the Beth Israel Deaconess Medical Center Animal Research Facility (BIDMC; Boston, MA). All experiments were conducted with the ethical approval of the Institutional Animal Care and Use Committee of the BIDMC. *Col18a1*$^{-/-}$ mice were generated in Dr Bjorn Olsen's laboratory at Harvard Medical School (Fukai et al., 2002). *Itga1*$^{-/-}$ mice were a kind gift from Dr Humphrey Gardner (Gardner et al., 1996). *Ilk* floxed mice were a gift from Dr Shoukat Dedhar (British Columbia Cancer Research Centre) (Terpstra et al., 2003). Albumin-Cre transgenic mice were purchased from The Jackson Laboratory. *Ilk* floxed mice and albumin-Cre mice were interbred to generate *Ilk*^{HepKO} (*Alb*$^{Cre+}$*Ilk*$^{flox/flox}$) and *Ilk*^{HepWT} (*Alb*$^{Cre-}$*Ilk*$^{flox/flox}$) littermates.

Carbon-tetrachloride-induced liver injury model

The CCl_4 model of hepatotoxicity was performed as described (Zeisberg et al., 2007). Mice were given phenobarbital in their

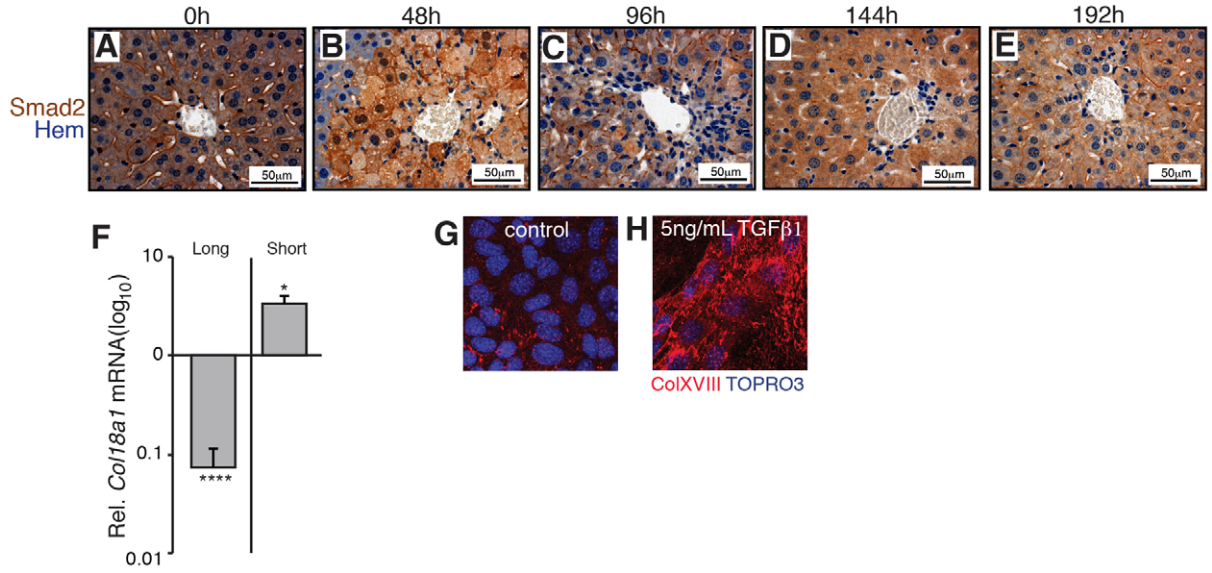

Fig. 7. TGFβ signaling controls type XVIII collagen gene expression. A single dose of CCl_4 was administered and mice were sacrificed at 0, 48, 96, 144 and 192 hour time points. (A-E) DAB stain indicates Smad2 localization during the injury response. Hem, hematoxylin. (F) Gene expression levels for long- and short-chain Col18a1 in mice treated with recombinant TGFβ1 as determined by quantitative real-time PCR. Three independent experiments were performed for this analysis. (G,H) AML12 cells treated with 5 ng/ml recombinant TGFβ1 and stained for type XVIII collagen with TOPRO3 counterstain. *P<0.05, ****P<0.001.

drinking water (0.1%) for 24 hours prior to CCl_4 administration. Two types of CCl_4 trials were performed in the study. Mice either received a single dose of CCl_4 and were allowed to recover (recovery time course) or received CCl_4 injections every 3-4 days (survival time course). Moribund mice were euthanized for the Kaplan-Meier curve. For the endostatin therapeutic trial, mice received twice-daily injections intravenously (i.v.; 500 μl of 140 ng/ml) of recombinant endostatin. Sex- and age-matched animals were used throughout the study.

Cell adhesion assay

Recombinant chicken type XVIII collagen was overexpressed as described previously (Dong et al., 2003) and purified by anion exchange chromatography (see below for details). AML12 hepatocytes maintained in standard media of 10% fetal bovine serum (FBS), 1× insulin/transferrin/selenium (GIBCO), 1× penicillin/streptomycin, 1:1 DMEM:F12, were utilized for cell adhesion assays. Cells were passaged the day before the assay was performed. Non-tissue 96-well plates were coated with ECM molecules at the indicated concentrations overnight at 4°C. The next day wells were washed with phosphate buffered saline (PBS) three times and then incubated with blocking buffer containing 1% BSA in PBS for approximately 1 hour at room temperature. Cells were trypsinized, counted and resuspended in 1:1 DMEM/F12 containing 0.1% BSA. The cells were allowed to adhere for 1 hour at 37°C, 5% CO_2. For the integrin-dependent adhesion assay, cells were premixed with 1 mM $MgCl_2$ or 10 mM EDTA. For integrin blocking studies, cells were premixed with azide free/low endotoxin (BD Biosciences, San Jose, CA) antibodies (Ha31/8 for α1 and 5H10-27 for α5) or control IgM at 10 μg/ml and incubated for 30 minutes at room temperature prior to adhesion. The plate was then washed three times with PBS. Adherent cells were fixed in 95% ethanol for 10 minutes. Cells were stained with 0.1% crystal violet, in 5% ethanol for 30 minutes at room temperature. The crystal violet solution was washed away with lukewarm tap water. Cells were then lysed with 75 μl of 10% acetic acid. The cell lysate was pipetted into a new 96-well plate and measured at 595 nm in a microplate spectrophotometer.

Liver regeneration

Partial hepatectomy in *Col18a1*$^{-/-}$ mice and wild-type control mice was performed. Anesthesia was induced by peritoneal injection of ketamine (50 mg/kg of body weight). The large median lobe (including right central lobe and left central lobe) and left lateral lobe, which constitute approximately 70% of the total liver, were separately ligated and resected. For sham surgery, the abdominal wall was opened and the xyphoid process was removed before the abdominal wall was closed. The mass of the resected liver tissue was measured after surgery, and that of the remnant liver was determined after sacrificing of the animals 7 days after surgery. When mice were sacrificed at the indicated times after the partial hepatectomy, the remnant liver was excised and weighed, and the liver regeneration rate (%) was expressed as follows: remnant liver weight/(original whole liver weight)×100.

Type XVIII collagen expression and purification

Type XVIII collagen from chicken was overexpressed in EBNA-293 cells (a gift from Dr Willi Halfter, University of Pittsburgh, PA). Cell culture supernatants were collected and frozen at –80°C until purification. The frozen supernatant was thawed and diluted 1:1 in ice-cold ammonium sulfate. The mixture was allowed to precipitate at 4°C overnight with gentle stirring. The next day the precipitate was collected by centrifugation at 2000 ***g*** for 10 minutes. The pellet was washed twice with ice-cold 25 mM Tris, 150 mM NaCl, pH 7.4 (TBS) and then re-suspended in TBS. Next, the material was loaded onto a monoQ column pre-equilibrated with

TBS. The column was washed with 10 column volumes (CV) of TBS followed by 20 CV of 25 mM Tris, 500 mM NaCl, pH 7. Subsequently, the column was eluted with 25 mM Tris, 1500 mM NaCl, pH 7. Elution fractions were collected every minute. The flow rate for the load, wash and elution steps was 1 ml/minute. All steps were carried out at 4°C. Protein-containing fractions were determined by the BCA assay. All protein-containing fractions were assessed for type XVIII collagen by immunoblot (see below) using anti-Myc antibody and an anti-chicken type XVIII collagen hybridoma supernatant (6C4). Type-XVIII-collagen-containing fractions were pooled and dialyzed (MWCO 5000 kDa) overnight against cold TBS at 4°C. Chicken type XVIII collagen and mouse type XVIII collagen share about 75% similarity at the amino acids level.

Protein electrophoresis and immunoblot

Tissue lysates were homogenized in RIPA buffer and diluted 1:1 in a Lammli buffer containing 10% β-mercaptoethanol and heated at 95°C for 10 minutes. Samples were centrifuged briefly, loaded and run on 8% Tris-HCl SDS-PAGE gel. The protein was then transferred to a nitrocellulose membrane. Transfer efficiency was determined by Ponceau staining of the nitrocellulose membrane. Membranes were washed in TBS containing 0.1% Tween 20 (TBST). Blots were blocked in 5% non-fat milk/TBST for 1 hour at room temperature. 6C4 anti-chicken type XVIII collagen hybridoma supernatant was diluted 1:1 in 5% BSA/TBST and membranes were incubated for 16 hours at 4°C. Antibodies against pAkt1/2/3(Ser473) (1:2000, clone D9E, Cell Signaling) was diluted in 5%BSA/TBST, whereas Akt1/2/3 (1:2000, H-136, Santa Cruz) and GAPDH (1:2000, clone 6C5, Chemicon) were diluted in 5% milk/TBST. All blots were incubated with primary antibodies overnight at 4°C. Subsequently, membranes were washed extensively and incubated with a HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Sigma, diluted 1:10,000 in 5% milk/TBST) for 1 hour at room temperature. Blots were washed extensively and probed with HyGLO chemiluminescence reagent (Denville).

WST-1 cell viability assay

Primary hepatocytes were isolated as previously described (Zeisberg et al., 2007) and plated on Matrigel with or without exogenous recombinant chicken type XVIII collagen (3 μg/ml) in normal growth media containing 10% FBS, 2 μg/ml insulin, 1× penicillin/streptomycin, DMEM. Cells were allowed to recover for 48 hours and then cultured in CCl_4-containing media (1.8 ml CCl_4/l normal growth media) for 24 hours. The CCl_4-containing media was replaced with normal growth media and cell viability was assessed with the WST-1 reagent (Roche Applied Science, Branford, CT) as per the manufacturer's recommendations.

Immunofluorescence

Liver tissue was embedded in OCT compound and snap frozen in liquid nitrogen. Sections were cut to 5-8 μm thickness from OCT blocks. Sections were fixed in ice-cold acetone at −20°C for ~30 minutes and allowed to air dry. Tissue specimens were blocked in TBS containing 1% BSA and 1% donkey serum. Specimens were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The next day slides were washed with TBS for 5 minutes a total of three times. The slides were then incubated with the appropriate FITC- or TRITC-conjugated secondary antibodies diluted in blocking buffer (1:200 dilution). Primary antibodies against the following were used at the indicated dilutions: type XVIII collagen/endostatin (AF570, 1:100, R&D Systems, Minneapolis, MN), α1 integrin (Ha31/8, 1:100, BD Biosciences, San Jose, CA), collagen III (1330-01, 1:200, Southern Biotech, Birmingham, AL), laminin (L9393 1:500, Sigma, St Louis, MO), collagen IV (125I, 1:100, MP Biomedical, Santa Ana, MA), perlecan (A7L6, 1:500, Chemicon, Billerica, MA) and FoxA2 (1:200, Cell Signaling, Danvers, MA). Images were acquired on a Zeiss (San Diego, CA) Axiophot or LSM 510 inverted confocal microscope and processed using ImageJ NIH software.

TUNEL assay

Formalin-fixed paraffin-embedded liver tissue was sectioned and deparaffinized by a xylene (100%)/graded alcohol procedure (100%, 95%, 90%, 70%, distilled water). Sections were permeabilized by microwave irradiation in a 10 mM citrate, 0.1% Tween 20 buffer. TUNEL staining was carried out per the manufacturer's recommendation (Roche Applied Science, Branford, CT) and total nuclei were stained with propidium iodide (1 μg/ml). This analysis was performed 48 hours after a single CCl_4 injection.

PCNA proliferation assay

Liver sections were prepared for immunofluorescence as described for the TUNEL assay. After permeabilization/antigen retrieval, slides were incubated in a blocking buffer from the Mouse on Mouse (M.O.M.) fluorescein kit (Vector Labs, Burlingame, CA) for 30 minutes. Monoclonal anti-mouse PCNA (1:200, Dako, Carpinteria, CA) was diluted 1:200 in the M.O.M. diluent buffer and incubated with sections overnight at 4°C. The subsequent staining procedure was carried out according to the manufacturer's recommendations.

Immunohistochemistry of paraffin-embedded tissue

Sections were prepared for immunohistochemistry as described for the TUNEL assay. Staining was carried out using VECTASTAIN ABC kit (Vector Labs, Burlingame, CA) per the manufacturer's recommendations. Polyclonal rabbit anti-mouse fibrinogen (Dako, Carpinteria, CA) was diluted 1:200 in blocking buffer and sections were incubated overnight for 16 hours at 4°C. DAB reagent was used for detection and sections were counterstained with hematoxylin followed by an acid/ethanol (0.25% 12.1 N HCl, 70% ethanol) wash.

Quantitative PCR

RNA was prepared from snap-frozen tissue stored at −80°C using the Trizol extraction per the manufacturer's recommendations (Life Technologies, Grand Island, NY). RNA yield and quality was assessed by UV spectrophotometry. 2 μg of RNA (final concentration of 100 μg/ml) was converted to cDNA using the cDNA archive kit (Life Technologies, Grand Island, NY) with random hexamers for amplification. cDNA was diluted tenfold in TE buffer and 1 μl was used for subsequent reactions. Quantitative real-time PCR was carried out using SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY) on a 7300 Real Time PCR system (Life Technologies, Grand Island, NY). Primer sequences

were identified from the PrimerBank database, which is freely accessible at http://pga.mgh.harvard.edu/primerbank/index.html or Primer-BLAST. Primer sequences can be found in supplementary material Table S1. PCR results were analyzed using ABI Prism SDS software and Excel.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (s.e.m.). Statistical significance was determined by a paired-sample Student's *t*-test using a one- or two-tailed distribution.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

R.K., M.B.D. and C.Y. designed the study. M.B.D., C.Y., H.T., P.M.B., D.K. and H.S. performed experiments. M.B.D., M.Z. and R.K. analyzed the data. B.R.O. provided critical reagents. B.R.O. and M.Z. provided key conceptual insight. M.B.D. and R.K. wrote the paper.

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SUPPLEMENTARY MATERIAL

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