

Fibrinolysis-independent role of plasmin and its activators in the haematopoietic recovery after myeloablation

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Abstract

Proteinases have been implicated in the mobilization of haematopoietic progenitor cells (HPCs) from the bone marrow (BM). Here, we report the involvement of the plasminogen (Plg) system in the haematopoietic recovery following chemotherapy. By using gene-deficient mice, we found that plasmin and its activators tPA and uPA play a role in the haematopoietic recovery upon delivery of the cytotoxic agent 5-fluoro-uracil (5-FU). The impaired haematopoietic recovery of Plg-deficient (Plg^{-/-}) mice after 5-FU was not rescued by depletion of fibrinogen, indicating that it was not due to defective fibrinolysis. Instead, loss of Plg impaired breakdown of fibronectin, VCAM-1 and laminin-BM matrix proteins involved in adhesion of HPCs to their BM microenvironment and in transendothelial migration of HPCs. These findings provide novel insights in how plasmin regulates haematopoietic recovery upon cytotoxic myeloablation.

Keywords: plasmin • fibrinolysis • extracellular matrix remodelling • bone marrow • progenitors

Introduction

Recent studies reveal that haematopoietic progenitor cells (HPCs) sense signals from the bone marrow (BM) microenvironment [1]. In response to cytotoxic stress, cells in the BM secrete proteinases such as MMP-9 [2, 3]. These enzymes proteolytically inactivate adhesive signals including membrane-bound KitL (mKitL), cKit, SDF-1 and CXCR-4 that anchor HPCs in the BM microenvironment; their inactivation allows HPCs to proliferate and become released in the peripheral blood [2, 3].

Plasminogen (Plg) is activated to plasmin *via* proteolytic cleavage by two plasminogen activators (PA), i.e. tissue-type PA (tPA) and urokinase-type PA (uPA); the latter binds to membrane-anchored uPAR (uPAR). We recently found that uPAR is a novel adhesive signal for HPCs, and that plasmin inactivates uPAR,

thereby enabling mobilization of HPCs from the BM [4]. Besides, plasmin also inactivates another adhesive signal for HPCs, namely mKitL, which could also contribute to the impaired haematopoietic recovery upon loss of Plg [4].

However, we explored in this study whether plasmin might regulate haematopoietic recovery *via* (yet) additional mechanisms. Indeed, plasmin is capable of cleaving a wide spectrum of substrates, including cell surface receptors, extracellular matrix (ECM) proteins and fibrin (reviewed in [5]). Several of these plasmin targets are present in the BM and regulate haematopoietic recovery. For instance, VCAM-1 and the ECM proteins fibronectin and laminin ensure adhesion of HPCs in the BM microenvironment, whereas their breakdown facilitates HPC migration to the peripheral blood and repopulation of blood cells [2, 6–12]. In addition, fibrinogen supports the proliferation and differentiation of HPCs *in vitro* [13–15]. In the BM, fibrinogen is localized at the luminal side of sinusoidal vessels, where it might promote maturation of megakaryocytes [16].

It remains unknown whether, in response to cytotoxic stress, plasmin affects haematopoiesis *via* mechanisms other than cleavage of uPAR and mKitL, for instance *via* fibrinolysis. This is a relevant question, as health and survival of Plg^{-/-} mice are

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improved after inter-crossing with mice lacking fibrinogen (Fbg^{-/-}), indicating a critical role of the fibrinolytic system [17]. In addition, based on fibrin(ogen) immunostaining results, a recent paper suggested that fibrin(ogen) might play a role in haematopoietic regeneration following chemo-myeloablation, however, without providing functional evidence for a role of fibrinolysis [18]. It also remains outstanding whether plasmin cleaves any of the abovementioned ECM proteins, onto which HPCs adhere and are retained.

Even though administration of exogenous tPA improved haematopoietic recovery [18], it still remains unknown whether the endogenous PA are important in this process and, if so, whether tPA or uPA are relevant. We therefore analysed the role of the PA in the response to cytotoxic stress in further detail. Since initial experiments revealed that loss of tPA or uPA did impair haematopoietic recovery (unlike what has been reported recently [18]), we analysed haematopoietic recovery in mice lacking tPA and/or uPA, and, for reasons of comparison and in order to study the role of fibrinolysis, also in mice lacking Plg, as such and after depletion of fibrinogen.

Experimental methods

Animal studies

Wild-type (WT) mice and mice lacking Plg, uPA, tPA, and both uPA and tPA (all in mixed 75% C57Bl6 25% 129Sv background) were used. For all experiments, age-, gender- and strain-matched mice were used. Mice were maintained in HEPA-filtered IVC units. All experiments were performed according to the guidelines for care and use of laboratory animals approved by the institutional ethical animal care committee. Mice were injected with a bolus i.v. of 5-FU (150 or 200 mg/kg, Fluroblastin®, Pharmacia, Brussels, Belgium). Peripheral blood was repetitively sampled by retro-orbital puncture under light anaesthesia, and full blood counts (EDTA buffered) were determined on a haemocytometer (Cell-Dyn 1300, Abbott, Louvain-la-Neuve, Belgium). Tranexamic acid (Exacyl®, Bournonville, Brussels, Belgium) was administered *via* osmotic minipumps (1.8 mg/day; Alzet 2001, Charles River, Brussels, Belgium) and *via* drinking water (20 mg/ml), as described [19]. Ancrod (1 IU; NIBSC, Hertfordshire, UK) was administered *via* daily i.p. injections, as described [20, 36].

FACS analysis

Murine BMCs were filtered through a 40 or 70 µm nylon mesh (Falcon, VWR, Haasrode, Belgium). To quantify Lin⁻Sca1⁺ BMCs, we combined magnetic bead depletion (EasySep, Stem Cell Technologies, Grenoble, France) with flow cytometry. Staining was performed with PE- or FITC-labelled anti-mouse antibodies against the following antigens: Sca-1, BrdU (all from BD Biosciences, Erembodegem, Belgium), and cKit (eBioscience, Halle-Zoersel, Belgium). Control stainings included appropriate isotype control antibodies. Non-specific binding was prevented by addition of mouse serum (DakoCytomation, Heverlee, Belgium), as alternative for Fc-receptor block. Apoptosis was analysed using TUNEL staining (fluorescein *in situ* cell death detection kit, Roche, Brussels, Belgium), as described previously [21]. For cell cycle analysis, BM cells were stained with primary antibodies, fixed in 70% ethanol and treated with propidium iodide (PI/RNase, BD Biosciences), as previously

described [2]. To determine HSC proliferation, mice were injected i.p. with BrdU (1 mg) every 8 hrs and the number of BrdU⁺ Lin⁻Sca1⁺ BMCs were quantified by flow cytometry, as described previously [22].

Immunohistochemistry

Mice were killed by cervical dislocation, the femurs were removed, fixed in 2% paraformaldehyde in phosphate buffer solution (PBS) for 24 hrs, and decalcified in 0.5 M EDTA solution for 8 days. After dehydration and paraffin embedding, 10 µm longitudinal sections were prepared on Superfrost Plus slides. Immunohistochemistry was performed using antibodies against tPA, uPA (both from Santa Cruz, Boechout, Belgium), laminin and fibronectin (both from Sigma, Bornem, Belgium), and fibrin (ogen) (Nordic, Tilburg, Netherlands). Specificity for PA staining was performed with the use of deficient mice. Corresponding secondary antibodies labelled with HRP or biotin (for signal amplification *via* TSA (Perkin Elmer, Zaventem, Belgium) or *via* Vectastain ABC kit (Vector Laboratories, Brussels, Belgium) were used. For light microscopy, sections were developed with 3,3'-diaminobenzidine (DAB, Sigma) as a chromogen substrate and counterstained with Harris Haematoxylin. For fluorescence imaging, sections were counterstained with DAPI and mounted with Vectashield (Vector Laboratories). Analysis was performed on a Zeiss Axioplan2 connected to a 3CCD video camera (DXC-930P, Sony), and KS300 software (Zeiss, Zaventem, Belgium). Laminin or fibronectin immunopositivity was quantified by integration of the stained area and expressed as the ratio of the immunopositive area over the BM matrix and near the endosteum, respectively.

ELISA, reagents, protease activity and Western blotting

BM extracellular fluid (BM plasma) from mice was obtained as described previously [23]. Briefly, femurs were flushed with PBS; after centrifugation, the supernatant was collected and frozen for analysis. Total protein amounts on BM plasma samples were determined with the BCA protein analysis kit (Perbio, Erembodegem, Belgium), to control for loading. Murine sVCAM-1 protein levels were quantified in BM plasma samples using a commercially available ELISA (R&D Systems, Abingdon, UK), which uses antibodies specifically raised against sVCAM-1 according to manufacturer. Western blotting was performed on reduced BM plasma samples, which were neutralized with a cocktail of protease inhibitors (Complete Inhibitor + EDTA; Roche). ECM components were detected with rabbit antibodies against fibronectin and laminin (both from Sigma), appropriate secondary HRP-labelled antibodies (DakoCytomation) and the ECL detection system (Amersham Biosciences, Diegem, Belgium). Notably, we used an affinity purified rabbit anti-laminin polyclonal antibody from Sigma, which specifically recognizes (vascular) basement membrane laminins including the beta1 and gamma1 chain, and its plasmin cleavage products ([24, 25] and instructions of the manufacturer). We used an affinity purified rabbit anti-fibronectin polyclonal antibody from Sigma, which specifically recognizes fibronectin and its plasmin cleavage products ([25] and instructions of the manufacturer).

In vitro cleavage experiments

For studying *in vitro* cleavage of membrane-bound VCAM-1, we used the mouse BM cell line OP9, which expressed VCAM-1 as determined on flow cytometry (not shown). Cells were seeded in 48-well plate (2.5 × 10⁵ cells per well), allowed to adhere, and starved overnight in serum-free medium.

Thereafter, cells were stimulated with human active plasmin (1 nM), diluted in serum-free medium, and incubated at 37°C for 5 hrs. This protocol yielded reproducible plasmin activity (assayed by S-2403) without affecting cellular viability (not shown). Levels of VCAM-1 in cell lysates and conditioned medium were quantified using commercially available ELISAs (R&D Systems).

Statistics

We used SPSS v. 11.0 for statistical calculations. Unless stated otherwise, data (mean ± S.E.M.) were statistically analysed by an unpaired Student's t-test. To determine the genotypic differences in white blood cell (WBC) counts after 5-FU, an ANOVA for repeated measurements was used, complemented with Dunnett's multiple comparison t-test (correction for multiple testing using a single control group) to identify statistically significant genotypic differences at each individual time point. Cox regression was used to analyse the genotypic differences in survival after 5-FU. $P < 0.05$ was considered statistically significant.

Results

Expression of PA in the BM

Proteinases have been implicated in the haematopoietic recovery of 5-fluorouracil (5-FU)-induced myeloablation [2]. To evaluate a possible role of the Plg system, we determined the expression pattern and levels of its components in the BM of WT mice. Integrin expression in stem cells from bone marrow and adipose tissue during chondrogenic differentiation Initial immuno-histochemical experiments revealed that uPA was expressed in stromal and haematopoietic cells in steady-state conditions and, even stronger so after 200 mg/kg 5-FU (Fig. 1A). Expression of tPA was largely, though not exclusively, confined to endothelial sinusoids in steady-state conditions (Fig. 1A). After 200 mg/kg 5-FU, a weaker tPA signal was detected in these cells, presumably because endothelial cells were destroyed by 5-FU (not shown) [26]; interestingly, however, tPA staining was stronger in cells lining the endosteum (putatively identified as osteoblasts) after 5-FU (Fig. 1A). We next analysed, by casein zymography, whether activity levels of tPA and uPA in the BM plasma of WT mice changed during haematopoietic recovery in response to 5-FU. As a result of the BM damage, tPA and uPA levels were reduced at 7 days after 200 mg/kg 5-FU (Fig. 1B). Indeed, compared with day 0, both tPA and uPA activity were reduced at 7 days after 5-FU (in artificial units: tPA: 90 ± 10 at 0 days *versus* 30 ± 20 at 7 days; uPA: 300 ± 10 at 0 days *versus* 160 ± 60 at 7 days; $n = 4$; $P \leq 0.05$). However, during the recovery period from 10–14 days onwards, tPA and, especially, uPA levels were elevated above steady-state (Fig. 1B).

Loss of Plg or its activators impairs haematopoietic recovery

We first characterized the haematopoietic response to 5-FU in WT mice and in mice lacking plasminogen (Plg^{-/-}), which are unable

to generate plasmin [27]. To avoid any confounding interpretation due to the progressive development of morbidity in Plg^{-/-} mice beyond 16 weeks of age, we consistently used 9- to 12-week-old healthy Plg^{-/-} mice without overt signs of morbidity. No major haematopoietic defects were found in these mice in steady-state conditions (not shown). Administration of 200 mg/kg 5-FU i.v. to WT mice resulted in an initial reduction in WBC counts on day 7, followed by a rebound leukocytosis by day 14, which normalized at day 17 (Fig. 1C). By contrast, 5-FU induced a much more profound leukopenia in Plg^{-/-} mice on day 7 and 11, without rebound leukocytosis at all on day 14 (Fig. 1C), indicating that plasmin participates in myeloid recovery after 5-FU. Similar data were obtained when WT mice were treated with the plasmin inhibitor tranexamic acid (Fig. S1). Microscopic analysis revealed a more severe BM aplasia in Plg^{-/-} than WT mice, 7 days after 5-FU (Fig. 1D). Also, FACS analysis for Sca-1⁺, Sca-1⁺cKit⁺, Lin⁻Sca-1⁺ or Lin⁻Sca-1⁺cKit⁺ cells revealed that the number of HPCs was significantly reduced in the BM of Plg^{-/-} mice after 5-FU (Fig. 1E). This deficit was, however, not caused by genotypic differences of HPC counts in the BM in steady-state conditions (Fig. 1E). Additional experiments revealed that the reduced HPC counts in the BM of Plg^{-/-} mice after 5-FU was at least in part explained by impaired HPC proliferation (Fig. S2). Also, up to 75% of Plg^{-/-} mice died already from day 4 after 5-FU onwards, whereas only 10% of WT mice succumbed, and only at later times (Fig. 1F). As found previously with this mouse model [2], the WBC counts normalized in the few surviving Plg^{-/-} mice (and tPA^{-/-}:uPA^{-/-} mice; see below) at 17 days after 5-FU (Fig. 1C). Thus, plasmin proteolysis plays a role in the haematopoietic recovery in response to 5-FU.

We also analysed the response to 5-FU in mice lacking uPA (uPA^{-/-}), tPA (tPA^{-/-}), or both activators (tPA^{-/-}uPA^{-/-}). No major haematopoietic defects were found in these mice in steady-state conditions (not shown). Compared with WT mice, WBC counts in tPA^{-/-}uPA^{-/-} mice were significantly lower at day 7 and 11 after administration of 200 mg/kg 5-FU, and failed to become compensatorily up-regulated by day 14 (Fig. 1C). WBC counts were also lower in uPA^{-/-} and tPA^{-/-} mice at 7 days after 5-FU (Fig. 1C) and WBC recovery remained impaired in uPA^{-/-} mice (Fig. 1C). In contrast to WT mice, up to 75% of tPA^{-/-}:uPA^{-/-} mice died already from day 4 after 5-FU onwards, whereas a large fraction (60%) of uPA^{-/-} mice died at 10 days after 5-FU (Fig. 1F). tPA^{-/-} mice survived (Fig. 1F), presumably because loss of tPA promotes megakaryopoiesis after 5-FU (Note S1). Thus, uPA and tPA play a role in haematopoietic recovery after cytotoxic therapy.

Plasmin stimulates haematopoietic recovery, independently of fibrinolysis

To investigate whether plasmin stimulates haematopoietic recovery by promoting fibrinolysis (which is defective in Plg^{-/-} mice [5, 17, 27]), we performed the following experiments. We first analysed by immunostaining the expression pattern of fibrin(ogen)

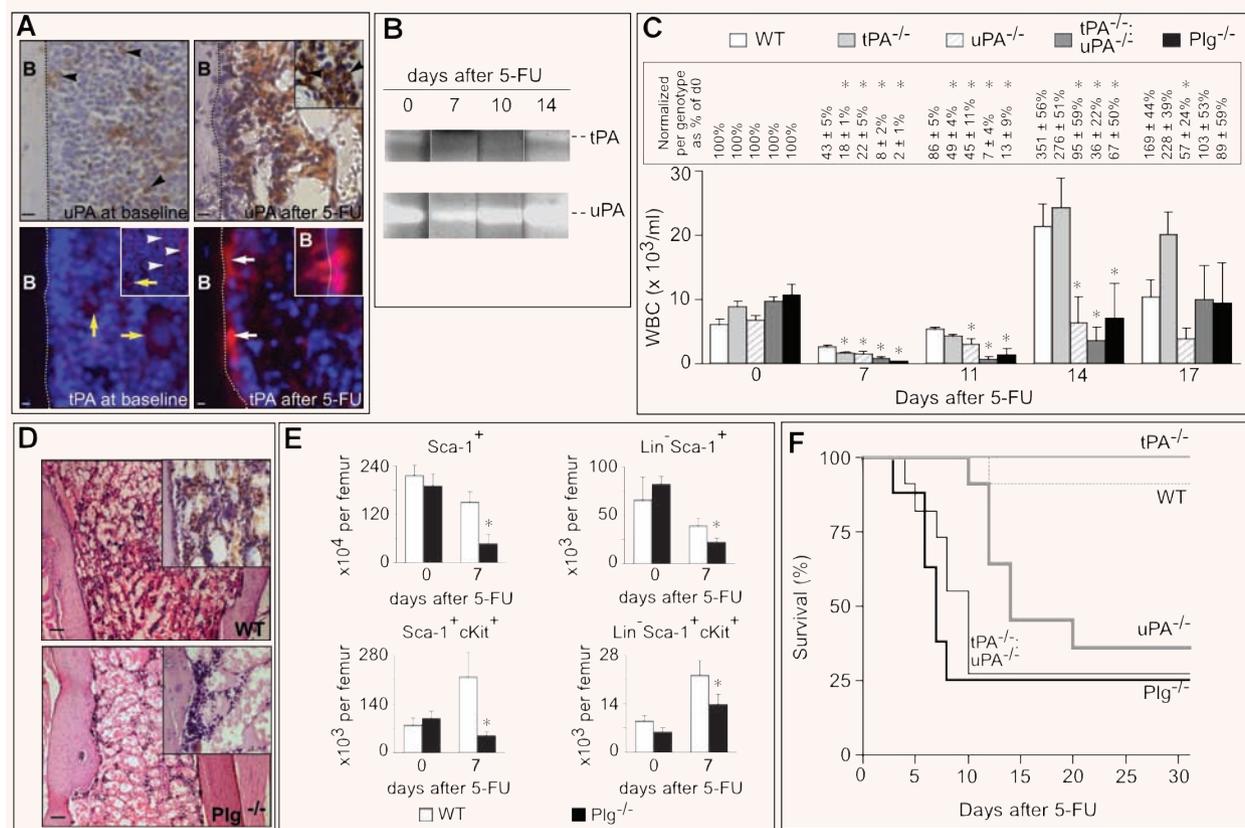


Fig. 1 Impaired haematopoietic recovery after 5-FU in $Plg^{-/-}$ mice. **(A)** Immunostaining for uPA and tPA on longitudinal sections through the femur of WT mice. uPA immunostaining (brown) in steady-state conditions (*upper left*) and after 5-FU (*upper right*) revealed that uPA was expressed by stromal and haematopoietic cells (black arrowheads, and *inset*). Note the enhanced expression of uPA in BM stroma after 5-FU. Immunostaining for tPA (red) in steady-state conditions (*lower left*) revealed expression of tPA predominantly in sinusoids (white arrowheads; *inset*) and in megakaryocytes (yellow arrows). In contrast, 5-FU (*lower right*) increased tPA expression in cells lining the endosteum (white arrows); high magnification imaging showed that cells lining the endosteum (DAPI nuclear counterstaining; blue) indeed expressed tPA (*inset*). The dotted line demarcates the border between the cortical bone (left) and BM (right). Magnification bars: 50 μ m. **(B)** Casein zymography, revealing an initial reduction (7 days) and subsequent increase (14 days) in tPA and uPA activity (evidenced by the lysis and clearing of the gel) in BM plasma of WT mice after 5-FU. Each BM plasma sample loaded on the gel contains an equal amount of protein. Representative example of 4 experiments. **(C)** White blood cell (WBC) counts in WT, $uPA^{-/-}$, $tPA^{-/-}$, $tPA^{-/-}uPA^{-/-}$ and $Plg^{-/-}$ mice at the indicated days after 5-FU ($n = 8-12$). $P < 0.05$ for all genotypes versus WT (ANOVA). *: $P < 0.05$ versus WT per time point (Dunnett). The values (%) above the bars *in the text box* indicate the percentage reduction as compared to the respective steady-state value for each genotype. **(D)** Representative pictures from BM sections (H&E) of WT (*upper*) and $Plg^{-/-}$ (*lower*) mice at day 7 after 5-FU (200 mg/kg i.v.), revealing impaired repopulation of the bone marrow in $Plg^{-/-}$ mice. Note the hypocellular BM in $Plg^{-/-}$ mice. Magnification bars: 100 μ m. **(E)** The number of HPCs in the BM of WT and $Plg^{-/-}$ mice in steady-state conditions and at 7 days after 5-FU (200 mg/kg i.v.). *Upper*: Sca-1⁺ and Lin⁻Sca-1⁺ HPCs. *Lower*: Sca-1⁺cKit⁺, Lin⁻Sca-1⁺cKit⁺ HPCs. *: $P < 0.05$ versus WT ($n = 3-8$). **(F)** Survival of WT, $uPA^{-/-}$, $tPA^{-/-}$, $tPA^{-/-}uPA^{-/-}$ and $Plg^{-/-}$ mice after 200 mg/kg 5-FU ($n = 8-12$). $P < 0.05$ for $Plg^{-/-}$, $tPA^{-/-}uPA^{-/-}$ and $uPA^{-/-}$ versus WT (Cox regression).

in the BM in steady-state conditions and after 5-FU. When using an antibody that recognizes both fibrinogen and fibrin, a fibrin(ogen) immunoreactive signal was detected mainly at the luminal side of sinusoidal BM vessels in steady-state conditions (Fig. 2A), consistent with previous reports [16]. After 5-FU, fibrin(ogen) immunoreactivity was also detectable in the BM matrix (Fig. 2A), likely caused by extravasation of plasma proteins through

damaged vessels following cytotoxin exposure [26]. A similar pattern of fibrin(ogen) staining was observed in $Plg^{-/-}$ mice (Fig. 2A). Consistent with previous findings [27], we did not detect intravascular fibrin clots nor excessive deposition of fibrin(ogen) in the BM of $Plg^{-/-}$ mice (Fig. 2A, Fig. S3).

We then analysed whether depletion of fibrinogen would rescue the haematopoietic recovery defect in $Plg^{-/-}$ mice (as one would

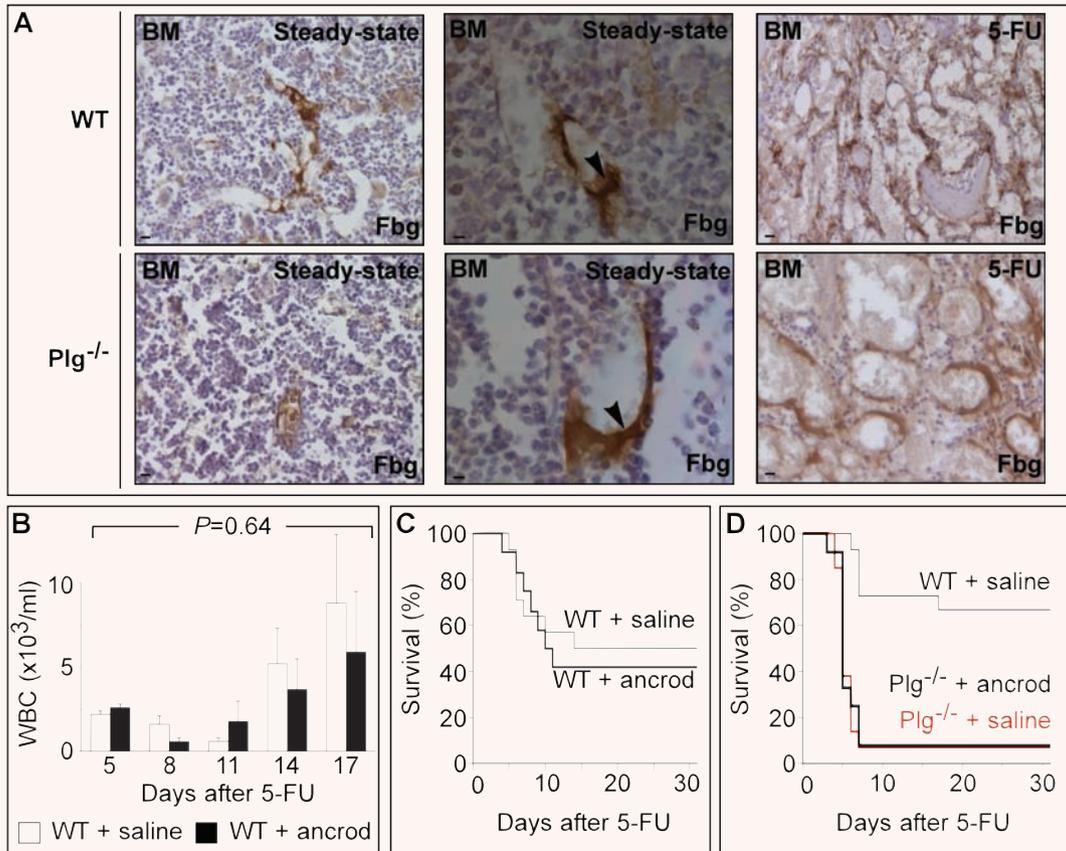


Fig. 2 Role of fibrin(ogen) and fibrinolysis in haematopoietic recovery after 5-FU. **(A)** Immunostaining for fibrin(ogen) (brown) on longitudinal sections through the femur of WT (upper) and $Plg^{-/-}$ (lower) mice. Note that no genotypic differences were found. *Left column:* in steady-state conditions, fibrin(ogen) was predominantly expressed at the luminal side of sinusoidal BM vessels. *Middle column:* in steady-state conditions, we did not observe intravascular thrombi. *Right column:* after 5-FU, expression of fibrin(ogen) increased in the BM matrix, likely because of extravasation of plasma fibrinogen through the dilated leaky sinusoids. Magnification bars: 50 μm in outer panels and 10 μm in middle panels. **(B)** WBC counts in WT mice, treated with daily i.p. injections of vehicle or ancrod, at the indicated days after 5-FU (200 mg/kg i.v.). $P = NS$ versus vehicle (ANOVA); $P = NS$ versus vehicle per time point ($n = 10-13$). **(C)** Compared with vehicle, survival of ancrod-treated WT mice after 5-FU (200 mg/kg i.v.) was similar. Please note that, compared to Fig. 1F, the survival of control mice was reduced, likely because of the daily i.p. injections. $P = NS$ versus vehicle (Cox regression; $n = 10-13$). **(D)** Compared with WT mice, survival of vehicle- or ancrod-treated $Plg^{-/-}$ mice after 5-FU (150 mg/kg i.v.) was reduced. Please note the similar survival curves for vehicle- and ancrod-treated $Plg^{-/-}$ mice. $P < 0.05$ versus WT (Cox regression; $n = 10-12$).

expect in case impaired fibrinolysis contributes to the impaired haematopoietic recovery in $Plg^{-/-}$ mice), but first assessed whether depletion of fibrinogen itself would alter haematopoietic recovery in response to 5-FU. We therefore treated WT mice with ancrod – a snake venom that depletes fibrinogen in blood and tissues by more than 90% [20, 36]. Initial studies revealed that, consistent with previous studies [20, 36], daily intraperitoneal injection of 1 IU ancrod effectively reduced the levels of fibrinogen in blood and BM plasma below the detection limit, but did not affect peripheral blood parameters including WBC and platelet counts (not

shown). Overall, depletion of fibrinogen did not alter the haematopoietic recovery of WT mice in response to 5-FU (200 mg/kg) ($n = 10$; $P = 0.64$ by ANOVA; Fig. 2B). Ancrod did also not affect the survival of WT mice after 5-FU (Fig. 2C). We then treated $Plg^{-/-}$ mice with ancrod and analysed their response to 5-FU. Ancrod did not alter the impaired haematopoietic recovery and survival after 5-FU (150 mg/kg) in $Plg^{-/-}$ mice (Fig. 2D and not shown). Hence, the haematopoietic recovery defect in $Plg^{-/-}$ mice was likely not attributable to excessive fibrin depositions, and fibrinolysis appears not to be required for haematopoietic recovery.

Plasmin remodels the BM microenvironment after 5-FU

Since plasmin did not regulate haematopoietic recovery through fibrinolysis, we explored alternative mechanisms. Haematopoietic recovery after 5-FU requires breakdown of ECM proteins such as fibronectin and VCAM-1, onto which HPCs adhere [7], or laminin around BM sinusoidal vessels [28, 29], which impedes transendothelial migration and mobilization of HPCs [8–10]. Degradation of these ECM proteins facilitates the release and migration of HPCs, required for repopulating the BM and peripheral blood [2, 6–12]. Plasmin-mediated matrix breakdown facilitates cell migration in diverse conditions [30, 31], whereas plasmin is capable of cleaving fibronectin, laminin and VCAM-1 *in vitro* ([32]; Note S2). Since reduced degradation of these ECM proteins in $\text{Plg}^{-/-}$ mice might therefore contribute to their impaired haematopoietic recovery after 5-FU, we analysed whether plasmin was involved in the degradation of fibronectin, laminin and VCAM-1 in the BM after 5-FU *in vivo*.

Since it is unknown whether 5-FU alters the amount, deposition and localization of fibronectin, laminin and VCAM-1 in the BM microenvironment, we first performed immunostaining for fibronectin and laminin on BM sections, and measured by ELISA sVCAM-1 levels in BM plasma of WT mice after 200 mg/kg 5-FU. In steady-state conditions, fibronectin was detected near the endosteum, whereas laminin was predominantly found around sinusoidal vessels, as reported previously [28] (Fig. 3A). At day 7 after 5-FU, the fibronectin-immunoreactive signal near the endosteum and the laminin-positive signal around the sinusoidal vessels were much weaker (Fig. 3A), but became nearly normal again by 14 days after 5-FU (Fig. 3A). sVCAM-1 levels in the BM plasma became transiently increased by ~1.6-fold on day 7 after 5-FU (% of baseline levels: $157 \pm 18\%$ at 7 days and $85 \pm 14\%$ at 10 days after 5-FU; $n = 4$; $P < 0.05$ on day 7). Hence, administration of 5-FU appears to result in the degradation of fibronectin, laminin and VCAM-1 in the BM microenvironment.

We then morphometrically quantified the amount of immunoreactive fibronectin and laminin in the BM of $\text{Plg}^{-/-}$ mice at day 7 after 200 mg/kg 5-FU. No genotypic differences were detected in baseline conditions (Fig. 3B; not shown). Substantially larger deposits of fibronectin were detected in $\text{Plg}^{-/-}$ mice at day 7 after 5-FU (% fibronectin⁺ area: $24 \pm 7\%$ in $\text{Plg}^{-/-}$ mice *versus* $7 \pm 2\%$ in WT mice; $n = 4$; $P < 0.05$). Laminin was also more abundant in 5-FU-treated $\text{Plg}^{-/-}$ mice (laminin⁺ area: $11.8 \pm 3.8\%$ in $\text{Plg}^{-/-}$ mice *versus* $4.5 \pm 1.8\%$ in WT mice; $n = 4$; $P < 0.05$; Fig. 3B), suggesting that loss of plasmin impaired degradation of these ECM proteins. Immunoblotting confirmed that fibronectin and laminin in BM plasma samples were degraded less in $\text{Plg}^{-/-}$ mice at day 7 after 5-FU (Fig. 3C). ELISA measurements further demonstrated that, in contrast to WT mice, sVCAM-1 levels failed to increase in the BM plasma of $\text{Plg}^{-/-}$ mice on day 7 after 5-FU (% of baseline levels: $97 \pm 18\%$ in $\text{Plg}^{-/-}$ mice *versus* $157 \pm 18\%$ in WT mice; $n = 4$; $P < 0.05$). Thus, during 5-FU recovery, plasmin breaks down various ECM proteins, which anchor HPCs in the BM microenvironment.

Discussion

This study highlights a role for the Plg system in the haematopoietic recovery after myeloablation. In response to 5-FU, plasmin and the PA *play a role* in the restoration of WBC counts. Plasmin regulates haematopoietic recovery after 5-FU *via* several mechanisms, *i.e.* by stimulating the proliferation of chemo-resistant HPCs, and by promoting proteolytic breakdown of adhesive ECM proteins in the BM microenvironment. Notably, however, the haematopoietic effect of plasmin was independent of fibrinolysis.

Administration of 5-FU causes several changes in the BM microenvironment that affect proliferation, migration and mobilization of HPCs, and the repopulation of the BM and blood [2, 11]. In the early phase after 5-FU, HPCs are released from their BM microenvironment by inactivation of adhesive signals, resulting in the proliferation and differentiation of HPCs [2, 11]. Our data indicate that plasmin participates in the proliferation of HPCs in response to 5-FU; we presume that this mitogenic effect is, at least in part, secondary to the fact that plasmin inactivates adhesive signals for HPCs (though we obviously cannot exclude additional mechanisms). Apart from cleaving the membrane-associated form of uPAR (a novel adhesive signal for HPCs [4]), plasmin also regulates the cleavage of the adhesive signal mKitL into sKitL [18] (and unpublished findings), which is known to promote HPC proliferation and migration [2, 11]. The present findings now reveal that plasmin also stimulates the breakdown of VCAM-1 and fibronectin, onto which HPCs adhere in the BM microenvironment [7], and which would facilitate de-adhesion and migration of HPCs [6, 7]. Moreover, plasmin promotes the degradation of laminins, which are present around BM sinusoidal vessels [28, 29]; since these ECM proteins impede transendothelial migration and mobilization of HPCs [8–10], their breakdown by plasmin would also enhance haematopoietic recovery. Hence, plasmin promotes haematopoietic recovery after myeloablation, at least in part, *via* breakdown of adhesive signals and migration barriers for HPCs.

We also explored whether the role of plasmin in haematopoietic recovery relied on fibrinolysis. Indeed, $\text{Plg}^{-/-}$ mice suffer considerable morbidity and mortality, associated with excessive intra- and extravascular deposition of fibrin [5, 17, 27]. Notably, health and survival of $\text{Plg}^{-/-}$ mice were substantially improved on a fibrinogen-deficient background, illustrating the importance of the fibrinolytic system in health and disease [17]. A recent paper suggested that plasmin-mediated fibrinolysis might play a role in haematopoietic regeneration following myeloablation, but did not provide any functional evidence for this hypothesis [18]. However, our study indicates that plasmin does not regulate haematopoietic recovery after 5-FU *via* fibrinolysis. A role of plasmin, independently of fibrinolysis, has been previously documented in other conditions [31, 33].

Our data are consistent with a recent study by Heissig *et al.*, documenting an impaired recovery and survival of $\text{Plg}^{-/-}$ mice after 5-FU [18]. Our results differ, however, from their findings in other aspects. For instance, Heissig *et al.* show that administration

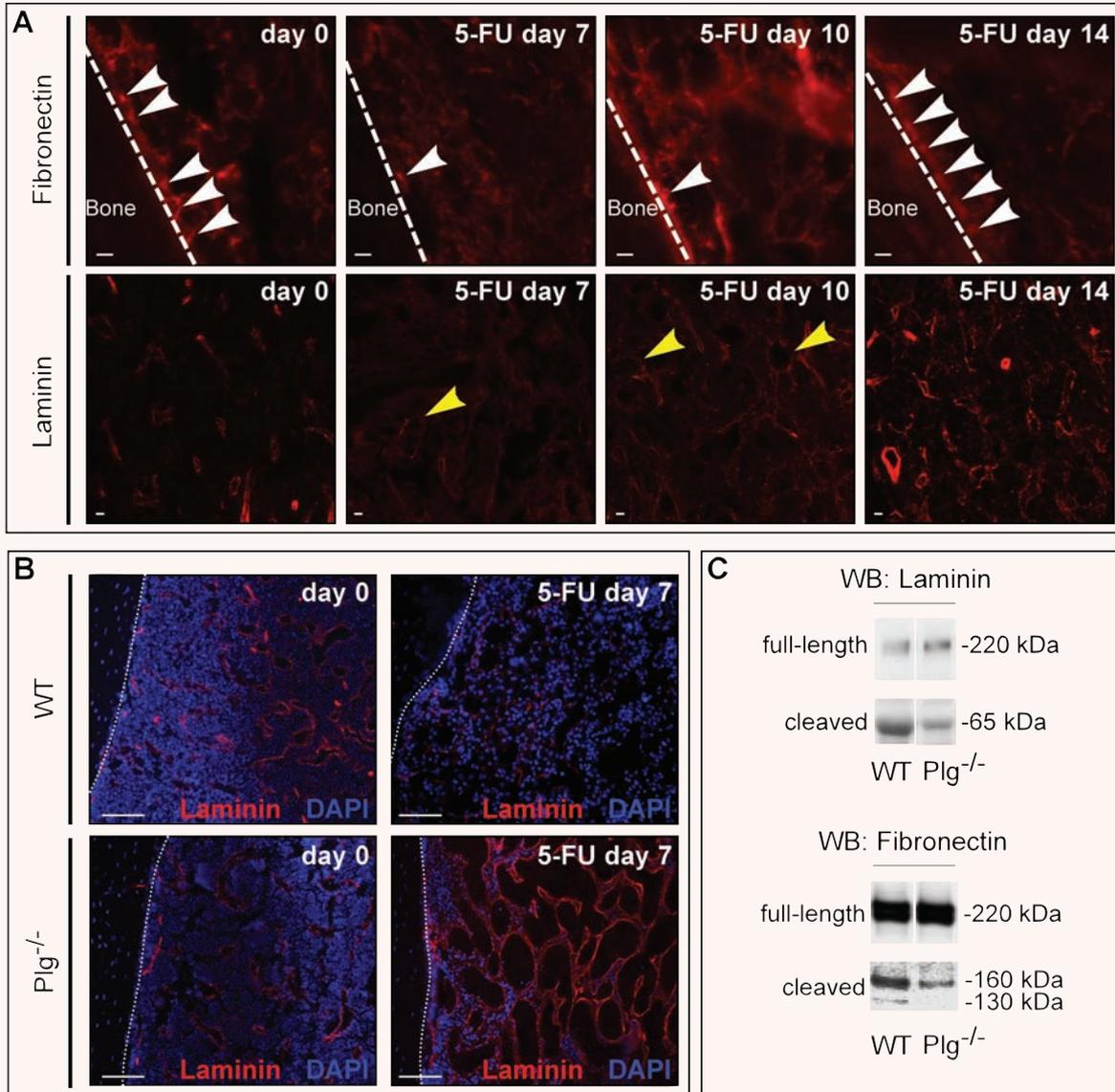


Fig. 3 Impaired ECM remodelling after 5-FU in Plg^{-/-} mice. **(A)** Immunostaining (red) of fibronectin (*upper*) and laminin (*lower*) on longitudinal sections through the femur of WT mice at different time points after 5-FU (200 mg/kg i.v.). *Upper*: Note the loss of fibronectin expression near the endosteum (white arrow heads) at 7 and 10 days after 5-FU. The dotted line demarcates the border between the cortical bone (left) and BM (right). *Lower*: Note the loss of laminin expression around the sinusoids (yellow arrow heads) at 7 and 10 days after 5-FU. Magnification bars: 10 μ m. **(B)** Laminin immunostaining (red) and nuclear DAPI staining (blue) of longitudinal sections through the femur, revealing a comparable pattern of laminin immunoreactivity around sinusoids in WT (*upper left*) and Plg^{-/-} (*lower left*) mice in steady-state conditions. At 7 days after 5-FU, laminin is more abundant around and in the interstitial space between the vascular sinusoids in Plg^{-/-} (*lower right*) than WT (*upper right*) mice. The dotted line demarcates the border between the cortical bone (left) and BM (right). Magnification bars: 100 μ m. **(C)** Immunoblots, showing less degradation of fibronectin and laminin in BM plasma samples of Plg^{-/-} than WT mice after 5-FU. The apparent molecular weights of the degradation products of fibronectin and laminin, cleaved by plasmin, are consistent with previous reports [25, 32]. Laminin: intact beta/gamma form (220 kD), plasmin cleavage product (65 kD); fibronectin: intact monomer (220 kD); plasmin cleavage product (160 and 130 kD).

of exogenous tPA, *via* conversion of mKitL into sKitL (in part independently of plasmin generation), stimulates haematopoietic regeneration, but also mention (as data not shown) that endogenous tPA and uPA are redundant [18]. In contrast, our data clearly show that single loss of uPA as well as loss of tPA alone each impairs haematopoietic recovery. We do not have a precise explanation for the differences between both studies, and can only speculate that differences in experimental conditions may contribute. Whatever the explanation, a role for uPA might not be unexpected, given its up-regulated expression in the BM after myeloablation and well-known prime role in tissue remodelling in various pathological conditions [20, 31].

In conclusion, plasmin and both plasminogen activators play a role in haematopoietic recovery following cytotoxic therapy, in part, through remodelling of the BM matrix but independent of fibrinolysis.

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Disclosures

The authors declare that they have nothing to disclose.

Supporting Information

The following supporting information is available for this article:

Note S1: Compared with WT mice, tPA^{-/-} mice exhibited an impaired recovery of WBCs at day 7 but normal survival after 200 mg/kg 5-FU. We observed that, at 7 days after 5-FU, the BM of tPA^{-/-} mice contained increased numbers of (activated) megakaryocytes, and that recovery of platelets in the peripheral blood was accelerated (data not shown). Further evidence that megakaryocytes might modulate the course of haematopoietic restoration in tPA^{-/-} mice following 5-FU is suggested by the observation that inhibition of megakaryocyte recovery by anti-CD144 antibodies, as used previously [12], impaired the recovery of WBCs and platelets after 5-FU in tPA^{-/-} mice, and reduced their survival (data not shown). These data will be presented in detail elsewhere.

Note S2: As VCAM-1 is sensitive to cleavage by serine proteases such as thrombin and neutrophil elastase [34, 35], we investigated whether plasmin might cleave membrane-bound VCAM-1 *in vitro*. To this end, we tested the effect of plasmin on cultured OP9 stromal cells, which expressed VCAM-1 as examined by flow cytometry (not shown). Compared with control, plasmin increased the levels of sVCAM-1 in the conditioned medium by ~1.7-fold, as measured by ELISA. Consistent herewith, plasmin reduced the levels of VCAM-1 on cell extracts by ~15% (measured by ELISA), indicating that plasmin cleaves VCAM-1 *in vitro*.

Figure S1: Effect of tranexamic acid in WT mice after 5-FU. (A, B) Compared with vehicle, administration of tranexamic acid (TA) *via* osmotic minipumps and drinking water [19] from day 0 onwards impaired the WBC recovery (A) and survival (B) of WT mice after 5-FU (250 mg/kg *i.v.*). Data are expressed as percentage *versus* steady-state. These results might be clinically relevant because an anti-fibrinolytic agent is sometimes given alongside cytotoxic drugs. $P < 0.05$ *versus* vehicle ($n = 10$; **anova**). *: $P < 0.05$ *versus* vehicle-treated WT mice per time point (A). $P < 0.05$ *versus* vehicle (Cox regression; B).

Figure S2: Plasmin regulates HPC proliferation after 5-FU. To study the role of plasmin on HPC proliferation after 5-FU, WT and Plg^{-/-} mice were challenged with one injection of 200 mg/kg 5-FU together with 3 times daily administration of 1 mg/kg BrdU *i.p.* [22], and the number of BrdU⁺Lin⁻Sca-1⁺ HPCs in the BM was quantified by flow cytometry 2 days later. Compared with WT mice, the fraction of BrdU⁺Lin⁻Sca-1⁺ cells was significantly reduced in Plg^{-/-} mice (Fig. S2A; *: $P < 0.05$ *versus* WT; $n = 3-4$). Consistent herewith, up to ~85% fewer HPCs were in S-phase of the cell cycle in Plg^{-/-} than WT mice on day 7 after 5-FU (Fig. S2B; *: $P < 0.05$ *versus* WT; $n = 3-4$). However, the number of HPCs in S-phase was similar in WT and Plg^{-/-} mice in steady-state conditions (not shown). As the fraction of TUNEL-positive Lin⁻Sca-1⁺ cells on day 2 after 5-FU was similar in WT and Plg^{-/-} mice (Fig. S2C; $P = \text{NS}$ *versus* WT; $n = 3-4$), the impaired haematopoietic recovery and survival of Plg^{-/-} mice appears to be caused by decreased proliferation and not increased apoptosis of HPCs.

Figure S3: Positive control for the histological presence of microthrombi. As positive control, a section of WT mouse injected with LPS in the footpad to induce inflammatory thrombosis showed a highly organized intravascular thrombus containing fibrin(ogen) and platelets. Magnification bars: 10 μm .

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References

1. Yin T, Li L. The stem cell niches in bone. *J Clin Invest.* 2006; 116: 1195–201.
2. Heissig B, Hattori K, Dias S, *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell.* 2002; 109: 625–37.
3. Levesque JP, Henty J, Takamatsu Y, *et al.* Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by G-CSF or cyclophosphamide. *J Clin Invest.* 2003; 111: 187–96.
4. Tjwa M, Sidenius N, Moura R, *et al.* Membrane-anchored uPAR regulates the quiescence, maintenance, engraftment and mobilization of hematopoietic progenitors. *J Clin Invest.* 2009; 119: 1008–18.
5. Collen D. Ham-Wasserman lecture: role of the plasminogen system in fibrin-homeostasis and tissue remodeling. *Hematology (Am Soc Hematol Educ Program).* 2001: 1–9.
6. Craddock CF, Nakamoto B, Elices M, *et al.* The role of CS1 moiety of fibronectin in VLA mediated haemopoietic progenitor trafficking. *Br J Haematol.* 1997; 97: 15–21.
7. Scott LM, Priestley GV, Papayannopoulou T. Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. *Mol Cell Biol.* 2003; 23: 9349–60.
8. Voermans C, Rood PM, Hordijk PL, *et al.* Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. *Stem Cells.* 2000; 18: 435–43.
9. Qian H, Tryggvason K, Jacobsen SE, *et al.* Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. *Blood.* 2006; 107: 3503–10.
10. Selleri C, Ragno P, Ricci P, *et al.* The metastasis-associated 67-kDa laminin receptor is involved in G-CSF-induced hematopoietic stem cell mobilization. *Blood.* 2006; 108: 2476–84.
11. Hattori K, Heissig B, Wu Y, *et al.* Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med.* 2002; 8: 841–9.
12. Avecilla ST, Hattori K, Heissig B, *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med.* 2004; 10: 64–71.
13. Hatzfeld JA, Hatzfeld A, Maigne J. Fibrinogen and its fragment D stimulate proliferation of human hemopoietic cells *in vitro*. *Proc Natl Acad Sci USA.* 1982; 79: 6280–4.
14. Zhou YQ, Levesque JP, Hatzfeld A, *et al.* Fibrinogen potentiates the effect of interleukin-3 on early human hematopoietic progenitors. *Blood.* 1993; 82: 800–6.
15. Weinstein R, Riordan MA, Wenc K, *et al.* Dual role of fibronectin in hematopoietic differentiation. *Blood.* 1989; 73: 111–6.
16. Larson MK, Watson SP. Regulation of pro-platelet formation and platelet release by integrin alpha IIb beta3. *Blood.* 2006; 108: 1509–14.
17. Bugge TH, Kombrinck KW, Flick MJ, *et al.* Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell.* 1996; 87: 709–19.
18. Heissig B, Lund L, Akiyama H, *et al.* The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell.* 2007; 1: 658–70.
19. Hattori N, Degen JL, Sisson TH, *et al.* Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice. *J Clin Invest.* 2000; 106: 1341–50.
20. Suelves M, Vidal B, Serrano AL, *et al.* uPA deficiency exacerbates muscular dystrophy in MDX mice. *J Cell Biol.* 2007; 178: 1039–51.
21. Inoue A, Seidel MG, Wu W, *et al.* Slug, a highly conserved zinc finger transcriptional repressor, protects hematopoietic progenitor cells from radiation-induced apoptosis *in vivo*. *Cancer Cell.* 2002; 2: 279–88.
22. Passegue E, Wagers AJ, Giuriato S, *et al.* Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med.* 2005; 202: 1599–611.
23. Petit I, Szyper-Kravitz M, Nagler A, *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol.* 2002; 3: 687–94.
24. Chen ZL, Strickland S. Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. *Cell.* 1997; 91: 917–25.
25. Ledoux D, Papy-Garcia D, Escartin Q, *et al.* Human plasmin enzymatic activity is inhibited by chemically modified dextrans. *J Biol Chem.* 2000; 275: 29383–90.
26. Kopp HG, Avecilla ST, Hooper AT, *et al.* Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood.* 2005; 106: 505–13.
27. Ploplis VA, Carmeliet P, Vazirzadeh S, *et al.* Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation.* 1995; 92: 2585–93.
28. Nilsson SK, Debatis ME, Dooner MS, *et al.* Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow *in situ*. *J Histochem Cytochem.* 1998; 46: 371–7.
29. Gu Y, Sorokin L, Durbeej M, *et al.* Characterization of bone marrow laminins and identification of alpha5-containing laminins as adhesive proteins for multipotent hematopoietic FDCP-Mix cells. *Blood.* 1999; 93: 2533–42.
30. Carmeliet P, Moons L, Lijnen R, *et al.* Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet.* 1997; 17: 439–44.
31. Heymans S, Lutun A, Nuyens D, *et al.* Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med.* 1999; 5: 1135–42.
32. Liotta LA, Goldfarb RH, Brundage R, *et al.* Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane. *Cancer Res.* 1981; 41: 4629–36.
33. Carmeliet P, Moons L, Herbert JM, *et al.* Urokinase but not tissue plasminogen activator mediates arterial neointima formation in mice. *Circ Res.* 1997; 81: 829–39.
34. Levesque JP, Takamatsu Y, Nilsson SK, *et al.* Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. *Blood.* 2001; 98: 1289–97.
35. Barthel SR, Johansson MW, Annis DS, *et al.* Cleavage of human 7-domain VCAM-1 (CD106) by thrombin. *Thromb Haemost.* 2006; 95: 873–80.
36. Vidal B, Serrano AL, Tjwa M, *et al.* Fibrinogen drives dystrophic muscle fibrosis *via* a TGFbeta/alternative macrophage activation pathway. *Genes Dev.* 2008; 22: 1747–52.