

## Roles of plasminogen in the alterations in bone marrow hematopoietic stem cells during bone repair

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### ABSTRACT

We previously revealed that stromal cell-derived factor-1 (SDF-1) is involved in the changes in the number of bone marrow stem cells during the bone repair process in mice. Moreover, we reported that plasminogen (Plg) deficiency delays bone repair and the accumulation of macrophages at the site of bone damage in mice. We investigated the roles of Plg in the changes in bone marrow stem cells during bone repair. We analyzed the numbers of hematopoietic stem cells (HSC) and mesenchymal stem cells (MSCs) within bone marrow from Plg-deficient and wild-type mice after a femoral bone injury using flow cytometric analysis. Plg deficiency significantly blunted a decrease in the number of HSCs after bone injury in mice, although it did not affect an increase in the number of MSCs. Plg deficiency significantly blunted the number of SDF-1- and Osterix- or SDF-1- and alkaline phosphatase-double-positive cells in the endosteum around the lesion as well as matrix metalloproteinase-9 (MMP-9) activity and mRNA levels of SDF-1 and transforming growth factor- $\beta$  (TGF- $\beta$ ) elevated by bone injury. TGF- $\beta$  signaling inhibition significantly blunted a decrease in the number of HSCs after bone injury. The present study showed that Plg is critical for the changes in bone marrow HSCs through MMP-9, TGF- $\beta$ , and SDF-1 at the damaged site during bone repair in mice.

### 1. Introduction

The bone repair process is divided into three phases, including inflammation, repair, and remodeling (Claes et al., 2012). In the acute inflammation phase, leukocytes, including neutrophils and macrophages, are recruited into the damaged site and release cytokines and growth factors. In the repair phase, angiogenesis and endochondral ossification occur in the damaged bone. In the remodeling phase, osteoblasts and osteoclasts participate in bone formation and resorption, respectively. Local ischemia, recruitment of stem cells, and interaction among inflammatory cells are observed in the inflammation phase (Khosla et al., 2008). The cells participating in the bone repair process are derived from bone marrow in the area surrounding the injury site (Gerstenfeld et al., 2003; Nistala et al., 2010). However, the roles of bone marrow stem cells in the bone repair process after bone destruction or fractures have remained unclear. We previously revealed that the numbers of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) in bone marrow were decreased and increased in the damaged site 2 days after femoral bone injury in mice, respectively

(Okada et al., 2016). That study indicated that stromal cell-derived factor-1 (SDF-1) is involved in the changes in the number of bone marrow stem cells during the bone repair process.

Plasminogen (Plg), a zymogen of the fibrinolytic enzyme, is responsible for fibrin thrombolysis by its active-type plasmin in the body (Collen and Lijnen, 1991). Conversion of Plg to plasmin is promoted by two-types of plasminogen activators, urokinase-type and tissue-type plasminogen activators (u-PA and t-PA). Acting in concert with other proteases, active plasmin is supposed to play a role in the degradation of the extracellular matrix in the context of physiological and pathological tissue remodeling and cell migration events, such as wound healing (Singer and Clark, 1999), angiogenesis (Eming et al., 2007), and tumor cell invasion (Bugge et al., 1996). We previously revealed that Plg deficiency delays bone repair and the accumulation of macrophages at the site of bone damage in mice (Kawao et al., 2013). Moreover, we revealed that t-PA and u-PA are related to the bone repair process in mice (Kawao et al., 2014, 2015). These findings suggest that the tissue fibrinolytic system is crucial for the bone repair process. Although our findings suggested that bone marrow HSCs and MSCs

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participate in the early phase of the bone repair process in mice (Okada et al., 2016), the relationships between the tissue fibrinolytic system and bone marrow stem cells during bone repair have remained unknown.

In the present study, we therefore investigated the roles of Plg in changes in the HSCs and MSCs populations in bone marrow during the bone repair process in mice and clarify its mechanism using Plg-deficient mice.

## 2. Materials and methods

### 2.1. Materials

Anti-Osterix and alkaline phosphatase (ALP) antibodies were obtained from Abcam (Cambridge, UK) and Abnova (Taipei, Taiwan), respectively. Violet (V)450/brilliant violet (BV)421-conjugated anti-CD29, Alexa 700-conjugated anti-CD34, allophycocyanin (APC)-Cy7-conjugated anti-CD44, V500/BV510-conjugated anti-CD45, APC-conjugated anti-CD73, phycoerythrin (PE)-conjugated anti-CD105, BV711-conjugated anti-c-Kit, PE-Cy7-conjugated anti-Sca-1 antibodies, and the peridinin chlorophyll protein complex (PerCp)-Cy5.5-conjugated anti-lineage antibodies cocktail (anti-CD3e, anti-CD11b, anti-B220/CD45R, anti-Gr1, and anti-TER-119 antibodies 1) were obtained from BD Biosciences (San Jose, CA, USA). SB431542 was obtained from Sigma-Aldrich (Tocris Cookson Ltd., Bristol, UK). Recombinant human bone morphogenetic protein (BMP)-2 was provided by Pfizer Inc. (Groton, CT, USA).

### 2.2. Animals

Male and female mice with plasminogen gene deficiency (Plg<sup>-/-</sup>) and their wild-type counterparts (Plg<sup>+/+</sup>), provided initially by Prof. D. Collen (University of Leuven, Leuven, Belgium), each weighing 18 to 25 g and 6 to 10 weeks old, with a mixed C57BL/6 J (75%) and 129/SvJ (25%) background, were used. All experiments were performed according to the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kindai University. The protocol was approved by the Experimental Animal Welfare Committee of Kindai University (permit no. KAME-27-025).

### 2.3. Murine bone damage model

Bone injury was induced in mice according to a method previously described (Okada et al., 2016). Briefly, under anesthesia induced by pentobarbital sodium (50 mg/kg, intraperitoneally), the anterior skin over the mid-femur of the right leg was incised longitudinally 5 mm in length. After splitting the muscle, the surface of the femoral bone was exposed. Thereafter, a hole was made using a drill with a diameter of 0.9 mm. The hole was irrigated with saline to prevent thermal necrosis of the margins. The incised skin was then sutured in a sterile manner, and anesthesia was discontinued.

### 2.4. Flow cytometric analysis

Bone marrow stromal cells were obtained from mice as previously described (Okada et al., 2016). HBSS buffer with 2% fetal bovine serum (FBS) was used to flush the bone marrow cells from the harvested femur. Bone marrow cells were then added in an equivalent volume to Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and were harvested by centrifugation for 15 min at 630 × g at 4 °C. Cells were re-suspended in PBS supplemented with 3% FBS.

Bone marrow cells were analyzed using a FACS Aria II cell sorter (BD Biosciences), as previously described (Okada et al., 2016). HSCs and MSCs were identified in bone marrow cell populations using color-conjugated antibodies specific for CD34, c-Kit, Sca-1, and CD29, CD34, CD44, CD45, CD73, CD105, Lin, respectively (Okada et al., 2016). The

numbers of HSCs and MSCs harvested from the bone marrow of the contralateral intact and damaged femurs 2 days after femoral bone injury, as enumerated by flow cytometry, were measured. The results represent experiments performed on 5 mice in each group.

### 2.5. Histological analysis

The mice were anesthetized using pentobarbital sodium (50 mg/kg, intraperitoneally) on day 0, day 1, day 2, day 4, or day 7 after surgery. Femurs were removed, fixed in 4% paraformaldehyde, demineralized in 22.5% formic acid and 340 mM sodium citrate solution for 24 h, and embedded in paraffin. Immunostaining was performed as previously described (Okada et al., 2016). Briefly, the sections were incubated with anti-ALP antibody at a dilution of 1:100, anti-Osterix antibody at a dilution of 1:200, or anti-SDF-1 antibody at a dilution of 1:200 followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Positive signals were visualized using the tyramide signal amplification system (PerkinElmer, Waltham, MA, USA), and sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and photographed using a fluorescence microscope (E800; Canon, Tokyo, Japan) with a CCD camera or a confocal microscope (C2 Si; Nikon, Tokyo, Japan).

### 2.6. Quantitative real-time PCR

Total RNA was isolated from the tissues and cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The incorporation of SYBR Green into double-stranded DNA was assessed by quantitative real-time PCR using an ABI StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) as previously described (Kawao et al., 2013). The PCR primers are listed in Table 1. The mRNA levels of the target genes were normalized with the glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

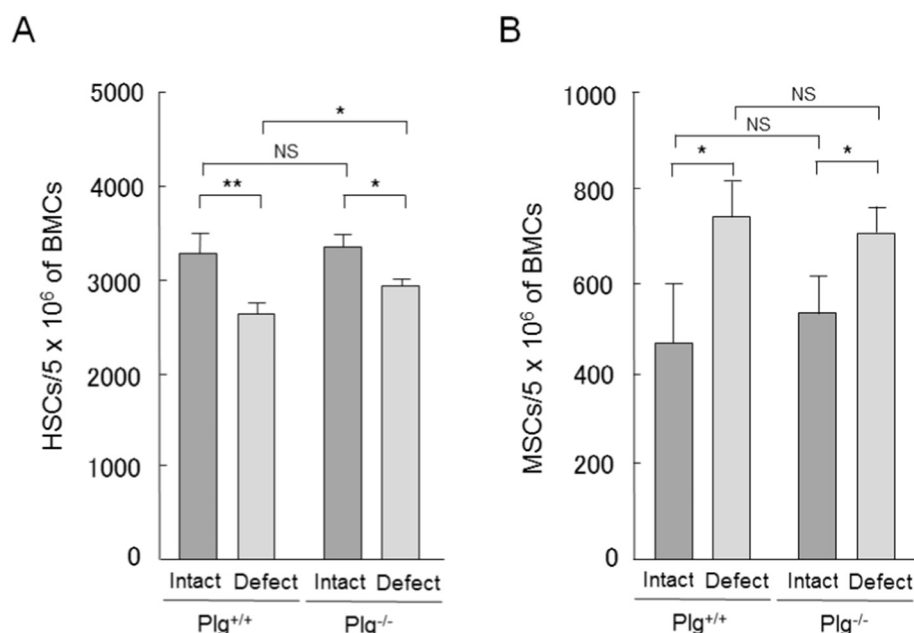
### 2.7. Preparation of primary osteoblasts and bone marrow stromal cells

Calvarial osteoblasts were obtained from Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice according to a previously described method (Kawao et al., 2013). Briefly, calvaria was removed from 3-day-old mice, cleaned to remove soft tissue, and digested four times with 1 mg/ml collagenase and 0.25% trypsin for 20 min at 37 °C with gentle agitation. The cells from the second, third, and fourth digestions were plated and grown in Eagle's minimum essential medium (α-MEM) with 10% FBS. The cells

**Table 1**  
Primers used for real-time PCR experiments.

Gene	Primer sequence
SDF-1	Forward 5'-CTGTGCCCTTCAGATTGTTG-3'
	Reverse 5'-TCAGCCTTCCTCGGGGTTCT-3'
TGF-β1	Forward 5'-CCTCTGTCACTGTCAACA-3'
	Reverse 5'-GATGAATTGGCGTGGAACTCT-3'
PDGF	Forward 5'-CAGTGACCTTGGAGGACCAC-3'
	Reverse 5'-GAATGGTCACCCGAGCTTGA-3'
FGF-2	Forward 5'-CCTTGTATGAAGGAAGATGG-3'
	Reverse 5'-TCCGTGACCCGTAAGTATTGT-3'
HGF	Forward 5'-CACCCCTGGGAGTATTGTG-3'
	Reverse 5'-GGGACATCAGTCTCAITTCACAG-3'
Osterix	Forward 5'-AGCGACCACCTGAGCAAAACAT-3'
	Reverse 5'-GCGCGTATTGGCTTCTTCT-3'
ALP	Forward 5'-ATCTTTGGTCTGGCTCCCATG-3'
	Reverse 5'-TTTCCCGTTCACCGTCCAC-3'
GAPDH	Forward 5'-AGGTCGGTGTGAACGGATTG-3'
	Reverse 5'-GGGTCTGTGATGGAACA-3'

SDF-1, stromal-derived factor-1; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; FGF-2, fibroblast growth factor-2; HGF, hepatocyte growth factor; ALP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



**Fig. 1.** HSCs and MSCs after a femoral bone defect in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. (A) The number of HSCs from the bone marrow of the contralateral intact (Intact) and damaged (Defect) femurs 2 days after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice, as enumerated by flow cytometry. Data represent the mean  $\pm$  standard error of the mean (SEM) of 5 mice. \*\* $p < 0.01$ , \* $p < 0.05$ , NS; not significant. (B) Ratio of HSC number in injured versus intact femurs on day 2 after femoral bone injury in Plg<sup>+/+</sup> mice and Plg<sup>-/-</sup> mice. Data represent the mean  $\pm$  SEM of 5 mice. \* $p \leq 0.05$ . (C) The number of MSCs from bone marrow of the contralateral intact (Intact) and damaged (Defect) femurs 2 days after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice, as enumerated by flow cytometry. Data represent the mean  $\pm$  SEM of 5 mice. \*\* $p < 0.01$ , \* $p < 0.05$ , NS; not significant.

were seeded in a 6-well plate ( $1.0 \times 10^5$  cells/well) and grown until confluent.

Bone marrow stromal cells were obtained from Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice as previously described (Kawao et al., 2013). Briefly, femurs and tibias were removed from mice and cleaned of soft tissue. The bone marrow cells were flushed out into DMEM. After the cells were grown in DMEM with 10% FBS for 24 h, the non-adherent cells were removed using phosphate-buffered saline. For osteogenic differentiation of the bone marrow stromal cells, the medium was changed to  $\alpha$ -MEM with 10% FBS and 100 ng/ml BMP-2 after the cells were passaged once.

## 2.8. SB431542 treatment

SB431542, dissolved in sterile 50% dimethyl sulfoxide in saline at 5 mg/ml, was administered intraperitoneally into Plg<sup>+/+</sup> mice daily 1 day before and 2 days after the surgery at 5 mg/kg (Kawao et al., 2016).

## 2.9. Fibrin and gelatin zymographic analysis

The activity of the plasminogen activator was determined as previously described (Matsuo et al., 1986). Briefly, protein samples were loaded onto a 10% polyacrylamide gel containing 0.5 mg/ml of bovine fibrinogen (Organon Teknika, Bostel, Netherlands) and 0.05 NIH U/ml of thrombin (Sigma Aldrich, St Louis, USA). After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution for 60 min, then incubated in reaction buffer (0.1 M glycine-NaOH, pH 8.4) at 37°C for 18 h. The gels were stained with Coomassie Blue R-250 for 1 h and destained with de-staining solution (30% methanol, 10% acetic acid). The intensity of the lytic band corresponding to u-PA and t-PA was measured with an LAS-1000 system (Fuji Film, Tokyo, Japan), which was calibrated using human standard u-PA (0.1 IU) and t-PA (0.5 IU).

The gelatinase activity was determined using gelatin zymography kits (Sigma Aldrich, St Louis, USA). Briefly, protein samples were loaded onto gelatin gels. After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution for 60 min, then incubated in reaction buffer (0.1 M glycine-NaOH, pH 8.4) at 37°C for 18 h. The gels were stained with Coomassie Blue R-250 for 1 h and destained with de-staining solution (30% methanol, 10% acetic acid). The intensity of the lytic band corresponding to MMP-9 was measured with an LAS-1000 system (Fuji Film, Tokyo, Japan), which was calibrated using human

standard Pro-MMP-9.

## 2.10. Statistical analyses

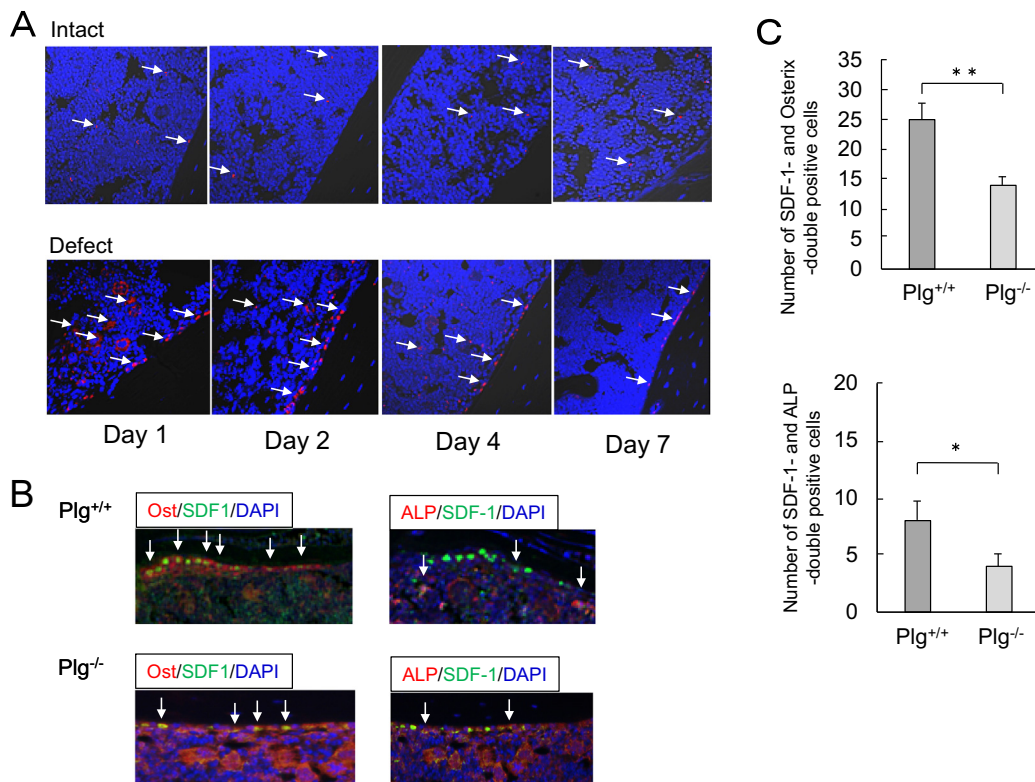
All data were expressed as the means  $\pm$  standard errors of the mean (SEM). Statistical analyses were performed by Mann-Whitney *U* test for comparisons of 2-group. Two-way ANOVA followed by Tukey's test was performed for multiple comparisons. Differences of  $p < 0.05$  were regarded as significant. All statistical analyses were performed using StatView version 5.0 software (Statistical Analysis System (SAS) Institute Inc.; Cary, NC, USA).

## 3. Results

### 3.1. Changes in HSCs and MSCs in bone marrow after femoral bone damage

The changes in HSCs and MSCs in bone marrow of damaged and contralateral intact femurs after femoral bone injury were evaluated by flow cytometric analysis. HSCs were defined as cells that were CD34<sup>-</sup>, c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, and Lin<sup>-</sup> (CD34<sup>-</sup>KSL) cells, and MSCs were defined as CD29<sup>+</sup>, CD34<sup>-</sup>, CD44<sup>+</sup>, CD45<sup>-</sup>, CD73<sup>+</sup>, CD105<sup>+</sup> and Lin<sup>-</sup> cell populations (CD29<sup>+</sup>CD34<sup>-</sup>CD44<sup>+</sup>CD45<sup>-</sup>CD73<sup>+</sup>CD105<sup>+</sup>Lin<sup>-</sup> cells), as described in the Materials and Methods. The numbers of HSCs harvested from bone marrow of the damaged femurs in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice were significantly lower than those of HSCs from contralateral intact femurs on day 2 after femoral bone damage (Supplemental Fig. S1 and Fig. 1A). However, a reduction in the number of HSCs from bone marrow of the damaged femurs in Plg<sup>-/-</sup> mice was significantly lower than that of HSCs from bone marrow in Plg<sup>+/+</sup> mice (Supplemental Fig. S1 and Fig. 1A). The ratio of the number of HSCs in the injured versus the intact femurs in Plg<sup>+/+</sup> mice was significantly lower than that in Plg<sup>-/-</sup> mice (Fig. 1B).

The number of MSCs harvested from bone marrow of the damaged femurs was significantly higher than the number of that from contralateral intact femurs on day 2 after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice (Supplemental Fig. S2 and Fig. 1). The increase in the number of MSCs from bone marrow of the damaged femurs in Plg<sup>-/-</sup> and Plg<sup>+/+</sup> mice was not significantly different (Supplemental Fig. S2 and Fig. 1).



**Fig. 2.** Localization of SDF-1-positive cells at the site of femoral bone injury in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. (A) Microphotographs of SDF-1-positive cells at the contralateral intact (Intact) and damaged (Defect) sites on day 1, 2, 4 and 7 after femoral bone damage in Plg<sup>+/+</sup> mice. The arrows indicate SDF-1-positive cells. (B) Microphotographs of SDF-1- and Osterix- or ALP-positive cells at the damaged site on day 2 after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. The arrows indicate SDF-1- and Osterix- or ALP-double-positive or SDF-1- and ALP-double-positive cells. (C) The number of SDF-1- and Osterix-double-positive or SDF-1- and ALP-double-positive cells at the damaged site on day 2 after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. Data represent the mean  $\pm$  SEM of 5 mice. \*\* $p < 0.01$ , \* $p < 0.05$ , NS; not significant.

### 3.2. Effects of Plg deficiency on SDF-1 expression after femoral bone damage

Our previous study indicated that SDF-1 mediates changes in bone marrow HSCs during the bone repair process in mice (Okada et al., 2016). We therefore examined the effects of Plg deficiency on SDF-1 expression at the injury site after femoral bone injury in mice. SDF-1-positive cells localizing to bone tissues and bone marrow after femoral bone damage were investigated using immunohistochemical staining. SDF-1-expressing cells was increased in bone marrow and endosteum around the damaged site after femoral bone injury in Plg<sup>+/+</sup> mice (Fig. 2A). However, SDF-1-expressing cells of contralateral intact femurs after bone injury were scattered in whole bone marrow. Since the differences of SDF-1 expression in cells between the intact and damaged sites were evident only in the endosteum cells, we examined the effects of Plg deficiency on the expression of SDF-1 in the endosteum osteoblastic cells in mice. Osterix- and ALP-positive cells are considered to be putative preosteoblasts and osteoblasts, respectively. As shown in Fig. 2B and C, SDF-1-expressing cells in the endosteum around the damaged site on day 2 after bone injury seemed to be more present in Osterix-positive cells, compared with ALP-positive cells in Plg<sup>+/+</sup> mice. The number of SDF-1- and Osterix- or SDF-1- and ALP-double positive cells in the endosteum around the damaged site after bone injury was significantly decreased in Plg<sup>-/-</sup> mice, compared to those in Plg<sup>+/+</sup> mice.

Next, we examined SDF-1 mRNA expression in the damaged bone after femoral bone damage using quantitative real-time PCR in mice. The levels of SDF-1 mRNA of the damaged femurs in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice were significantly elevated compared to those of contralateral intact femurs on day 2 after femoral bone damage (Fig. 3A). However, the levels of SDF-1 mRNA in the damaged femurs of Plg<sup>-/-</sup> mice were significantly lower than those of Plg<sup>+/+</sup> mice (Fig. 3A). Since Plg deficiency suppressed the number of SDF-1- and Osterix- or SDF-1- and ALP-double positive cells in the endosteum around the damaged site after bone injury, we examined SDF-1 mRNA expression in mouse primary osteoblasts and bone marrow stromal cell cultures from Plg<sup>+/+</sup>

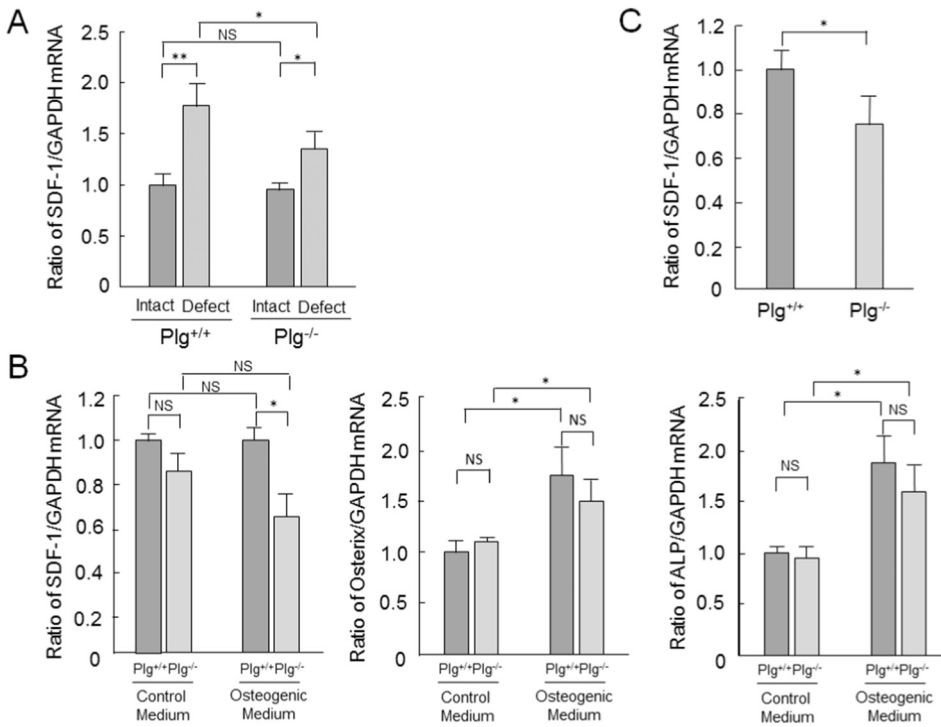
and Plg<sup>-/-</sup> mice with or without osteogenic medium. SDF-1 mRNA levels were not significantly different in bone marrow stromal cells cultured without osteogenic medium from Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice (Fig. 3B). However, SDF-1 mRNA levels were significantly lower in bone marrow stromal cells cultured with osteogenic medium in Plg<sup>-/-</sup> mice, compared to those in Plg<sup>+/+</sup> mice, although the mRNA levels of Osterix and ALP enhanced by osteogenic medium were comparable between Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice (Fig. 3B). SDF-1 mRNA levels of primary osteoblasts in Plg<sup>-/-</sup> mice were slightly lower, compared to those in Plg<sup>+/+</sup> mice (Fig. 3C).

### 3.3. Plasminogen activator activity

The activities of PA of plasma and bone extracts after femoral bone damage were determined by fibrin zymography in mice. Fig. 4A shows fibrin zymography gels of PA activity patterns in plasma. PA activities identified as t-PA and u-PA by their molecular weight were observed in plasma. t-PA and u-PA activities in plasma were increased after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice (Fig. 4B). Fig. 4C shows fibrin zymography gels of PA activity patterns in bone extracts of the damaged sites after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. u-PA activities in bone extracts were increased after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice (Fig. 4D). Plg deficiency did not affect t-PA and u-PA activities in plasma as well as the u-PA activity in bone extracts (Fig. 4B, D).

### 3.4. MMP-9 activity

Collagen, gelatin, and fibronectin are included in the extracellular matrix proteins. The degradation of extracellular matrix by matrix metalloproteinases (MMPs), such as MMP-9, plays an important role in a repair process of tissue injury. MMP-9 is secreted with the inactive form (pro-MMP-9), then activated by protease. The plasmin fibrinolytic system regulates the activity of MMP-9, which is crucial for the degradation of the extracellular matrix and subsequent tissue repair process (Hald et al., 2011). The activities of geratinase in bone extracts



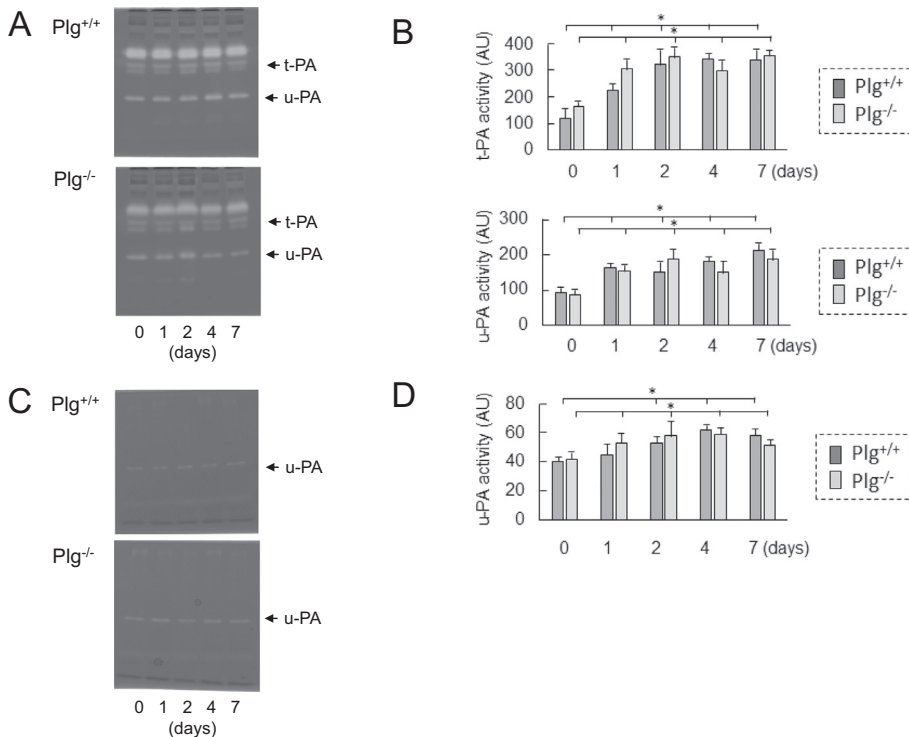
**Fig. 3.** Effects of Plg deficiency on the expression of SDF-1 mRNA in the bones of mice. (A) Relative mRNA levels of SDF-1 at the contralateral intact (Intact) and damaged (Defect) sites on day 2 after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. The results were obtained from experiments performed on 5 mice in each group. Data are expressed relative to GAPDH mRNA values and as the mean ± SEM. \**p* < 0.05, NS; not significant. (B) Mouse bone marrow stromal cells were obtained from Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. Relative mRNA levels of SDF-1, Osterix, and ALP in bone marrow stromal cells cultured with or without osteogenic medium for 5 days. The results were obtained from experiments performed on 5 mice in each group. Data are expressed relative to GAPDH mRNA values and as the mean ± SEM. \**p* < 0.05, NS; not significant. (C) Relative mRNA levels of SDF-1 in primary osteoblasts from Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. The results were obtained from experiments performed on 5 mice in each group. Data are expressed relative to GAPDH mRNA values and as the mean ± SEM. \**p* < 0.05.

after femoral bone damage were determined by geratin zymography in mice. Fig. 5A shows geratin zymography gels of geratinase activity patterns in bone extracts. Geratinase activities identified as pro- and active-MMP-9 by their molecular weight were observed in bone extracts. Active-MMP-9 activity in bone extracts was 12-fold higher, compared to that in bone extracts from the contralateral intact femurs, on day 2 after femoral bone damage in Plg<sup>+/+</sup> mice, and the peak was observed on day 2 after femoral bone damage (Fig. 5B). However, Plg deficiency significantly suppressed active-MMP-9 activity in bone extracts, and the peak of MMP-9 activity was observed on day 4 after

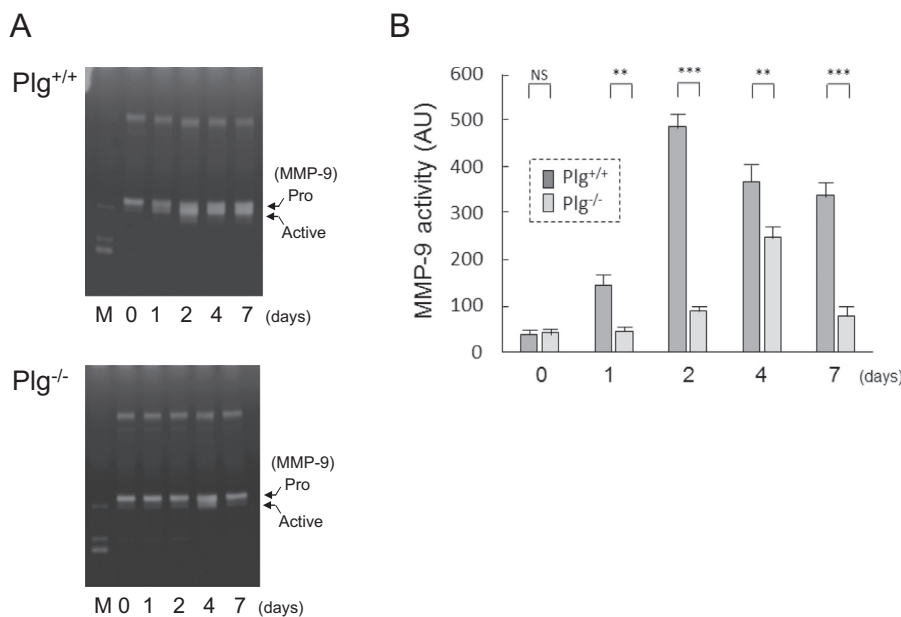
femoral bone damage.

3.5. Effects of plasminogen deficiency on the mRNA levels of growth factors in bone after femoral bone damage

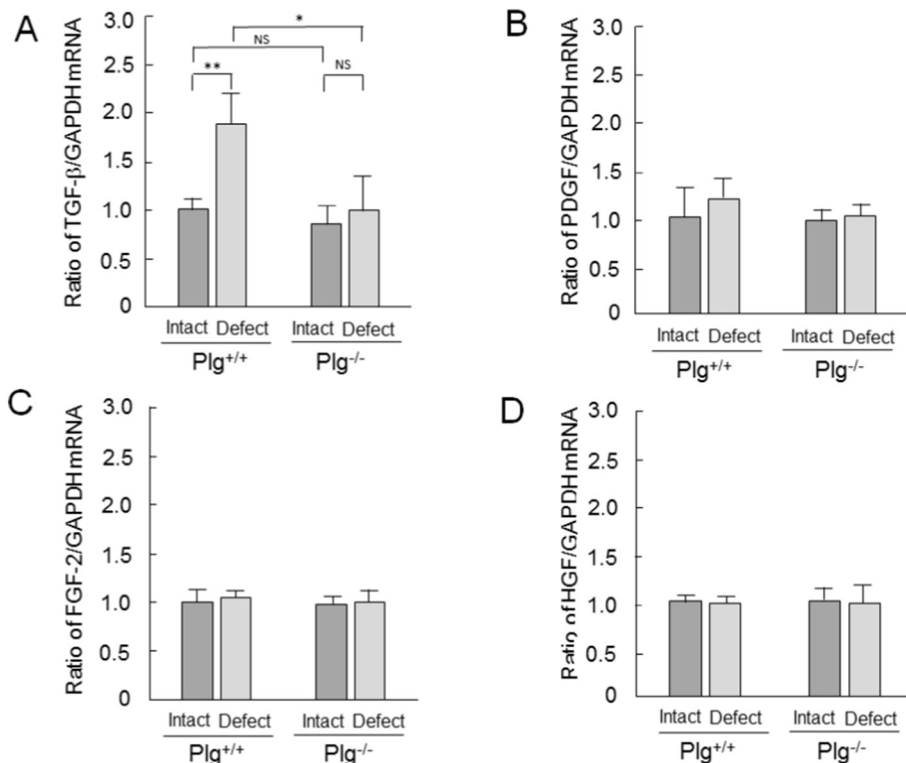
Activation of the tissue fibrinolytic system induces various growth factors, such as TGF-β, PDGF, FGF-2, and HGF (Toriseva and Kahari, 2009). We therefore examined the effects of Plg deficiency on mRNA levels of TGF-β, PDGF, FGF-2, and HGF in the damaged bone after femoral bone injury in mice. Although TGF-β mRNA levels of the



**Fig. 4.** PA activity in plasma or bone extracts of Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice after femoral bone injury. (A) PA activity by fibrin zymography in plasma on day 0, 1, 2, 4, or 7 after femoral bone injury in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. t-PA and u-PA indicate the bands of mouse t-PA and u-PA, respectively. (B) Quantitative analysis of t-PA and u-PA activity in plasma by fibrin zymography is shown, as described in the Materials and methods. The results were obtained from experiments performed on 3 mice in each group. Data are expressed as the mean ± SEM. \**p* < 0.05, compared with the value of day 0. (C) PA activity by fibrin zymography in bone extracts on day 0, 1, 2, 4, or 7 after femoral bone injury in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. (D) Quantitative analysis of t-PA and u-PA activity in bone extracts by fibrin zymography, as described in the Materials and methods. The results were obtained from experiments performed on 3 mice in each group. Data are expressed as the mean ± SEM. \**p* < 0.05, compared with the value of day 0.



**Fig. 5.** MMP-9 activity in bone extracts of Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice after femoral bone injury. (A) MMP-9 activity by geratin zymography in plasma on day 0, 1, 2, 4, or 7 after femoral bone injury in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. The arrows show Pro-MMP-9 and Active-MMP-9. The arrows a, b, and c in lane M (size markers) show Pro-MMP-9, Pro-MMP-2 and Active-MMP-2 of human. (B) Quantitative analysis of MMP-9 activity in bone extracts by geratin zymography, as described in the Materials and methods. The results were obtained from experiments performed on 3 mice in each group. Data are expressed as the mean ± SEM. \**p* < 0.05.



**Fig. 6.** Effects of Plg deficiency on the mRNA expression of growth factors from the damaged and contralateral intact femurs on day 2 after a femoral bone defect in mice. Relative mRNA levels of (A) TGF-β, (B) PDGF, (C) FGF-2, or (D) HGF at the contralateral intact (Intact) and damaged (Defect) sites on day 2 after femoral bone damage in Plg<sup>+/+</sup> and Plg<sup>-/-</sup> mice. The results were obtained from experiments performed on 5 mice in each group. Data are expressed relative to GAPDH mRNA values and as the mean ± SEM. \**p* < 0.05, NS; not significant.

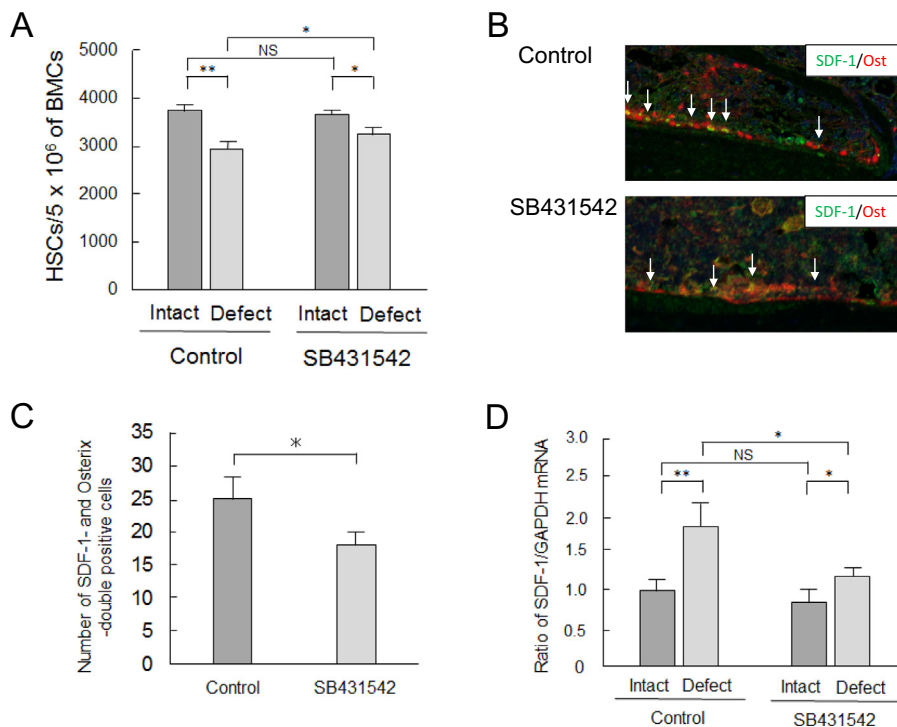
damaged femurs were significantly enhanced, compared with those of contralateral intact femurs on day 2 after femoral bone damage in Plg<sup>+/+</sup> mice, Plg deficiency significantly blunted TGF-β mRNA levels enhanced by bone injury in mice (Fig. 6). On the other hand, Plg deficiency did not affect the mRNA levels of PDGF, FGF-2, and HGF enhanced by bone injury.

Next, we investigated the roles of TGF-β in changes in bone marrow HSC number during bone repair using SB431542, a TGF-β signal inhibitor. Intraperitoneal administration of SB431542 significantly blunted a decrease in the number of HSCs in bone marrow harvested from the damaged femurs on day 2 after bone injury in Plg<sup>+/+</sup> mice (Fig. 7A). Moreover, SB431542 significantly decreased the number of SDF-1- and Osterix-double-positive cells in the endosteum around the

damaged site on day 2 after bone injury in Plg<sup>+/+</sup> mice (Fig. 7B, C). In addition, SB431542 significantly blunted femur SDF-1 mRNA levels enhanced by bone injury (Fig. 7D).

#### 4. Discussion

The present findings that the numbers of HSCs and MSCs in the bone marrow of the damaged site were decreased and increased, respectively, 2 days after femoral bone injury in a bone defect mouse model were compatible with our previous reports (Okada et al., 2016). Since significant changes in the number of bone marrow stem cells were observed only 2 days after bone injury in our study, we evaluated the effects of the changes in bone marrow stem cell populations 2 days after



**Fig. 7.** Effects of a TGF- $\beta$  signal inhibitor on the number of bone marrow HSCs and SDF-1 expression from the damaged and contralateral intact femurs on day 2 after a femoral bone defect in Plg<sup>+/+</sup> mice. (A) The number of HSCs from bone marrow of the contralateral intact (Intact) and damaged (Defect) femurs two days after femoral bone damage with or without SB431542 treatment in Plg<sup>+/+</sup> mice, as enumerated by flow cytometry. The results were obtained from experiments performed on 5 mice in each group. Data represent the mean  $\pm$  SEM. \*\* $p < 0.01$ , \* $p < 0.05$ , NS; not significant. (B) Microphotographs of SDF-1- and Osterix-positive cells at the damaged site on day 2 after femoral bone damage with or without SB431542 treatment in Plg<sup>+/+</sup> mice. The arrows indicate SDF-1- and Osterix-double positive cells. (C) The number of SDF-1- and Osterix-double positive cells at the damaged site on day 2 with or without SB431542 treatment in Plg<sup>+/+</sup> mice. The results were obtained from experiments performed on 5 mice in each group. Data represent the mean  $\pm$  SEM. \*\* $p < 0.01$ , \* $p < 0.05$ , NS; not significant. (D) Relative mRNA levels of SDF-1 at the contralateral intact (Intact) and damaged (Defect) sites on day 2 after femoral bone damage with or without SB431542 treatment in Plg<sup>+/+</sup> mice. The results were obtained from experiments performed on 5 mice in each group. Data are expressed relative to GAPDH mRNA values and as the mean  $\pm$  SEM. \*\* $p < 0.01$ , \* $p < 0.05$ .

bone injury in the present study. In this study, Plg deficiency significantly blunted a decrease in the number of HSCs in bone marrow from the damaged sites 2 days after femoral bone injury in mice, although it did not affect an increase in the number of MSCs in bone marrow from the damaged site. These data indicate that Plg is involved in a decrease in the number of bone marrow HSCs induced by bone injury in mice. Since our previous study suggested that HSCs in bone marrow might be mobilized into the damaged site of the injured bone and participate in bone repair by differentiating into macrophages and osteoclasts, the fibrinolytic system at the damaged site after bone injury might be crucial for the mobilization of HSCs into the cells participating in the bone repair process. Taken together, we can speculate that the impaired change in bone marrow HSC number induced by Plg deficiency might contribute the impaired accumulation of macrophages and osteoclasts, then resulting in the delayed bone repair after bone injury in mice. PAs and plasmin generation in specific microenvironments in bone marrow may be one of the factors orchestrating hematopoiesis (Reiner and Aramaki, 2000). The special microenvironments, known as niches, are responsible for the proliferation and differentiation of hematopoietic stem cells and progenitor cells in bone marrow (Morrison and Scadden, 2014). A previous study suggested that Plg is important for granulocyte colony-stimulating factor (G-CSF)-induced HSCs mobilization and hematopoietic regeneration in mice (Heissig et al., 2007; Gong et al., 2011). Further studies will be necessary to clarify the roles of Plg and the fibrinolytic system in the bone marrow microenvironment such as niches during the bone repair process after bone injury.

SDF-1 induces migration and differentiation of bone marrow stromal and bone marrow cells, which are related to bone repair at the fracture sites (Sugiyama et al., 2006; Dar et al., 2006). Our present study revealed that SDF-1 expression was elevated at the damaged site after femoral bone injury in mice, which were compatible with previous reports (Kidd et al., 2010; Kitaori et al., 2009). Our previous study showed that SDF-1 antibody treatment at the damaged site as well as the antagonist C-X-C chemokine receptor 4 (CXCR4), a SDF-1 receptor, blunted a decrease in the HSCs number in bone marrow of the damaged site after femoral bone injury in mice (Okada et al., 2016). These findings indicated that SDF-1 mediates changes in HSCs during the

bone repair process. Bone damage might induce the expression of SDF-1 at the damaged site, leading to the migration of HSCs from bone marrow and a decrease in number of HSCs. Alternatively, SDF-1 might partly influence the maintenance of HSC niche in bone marrow through circulating or local SDF-1 induced by bone damage, since several studies indicated that SDF-1-CXCR4 signaling is crucial for homing and maintenance of HSCs (Nagasawa et al., 2011). On the other hand, Plg deficiency significantly blunted the SDF-1 mRNA level as well as the number of SDF-1- and Osterix- or SDF-1- and ALP-double-positive cells increased by bone injury in the endosteum around the lesion in mice in our study. In addition, Plg deficiency significantly blunted SDF-1 mRNA levels in mouse primary osteoblasts and bone marrow stromal cells which differentiated into osteoblasts in the osteogenic medium. These data suggest that the tissue fibrinolytic system is crucial for the induction of SDF-1 mainly at preosteoblasts and osteoblasts in the damaged site during the bone repair process after bone injury in mice. The fibrinolytic system might induce local SDF-1 production in osteoblastic cells, which leads to alterations of the HSCs population and their migration into the damaged sites during the bone repair process after bone injury. In our data, Plg deficiency appeared to suppress SDF-1 expression in Osterix-positive cells more potently than in ALP-positive cells as assessed by immunohistochemistry. Moreover, the effects of Plg deficiency appeared to be more potent in mouse bone marrow cells treated with osteogenic medium than in mouse primary calvaria osteoblasts. Taken together, we speculated that preosteoblasts might be more important for SDF-1 expression during the bone repair process after bone injury, compared to mature osteoblasts.

t-PA and u-PA convert the inactive plasminogen to the active plasmin in the fibrinolytic system. Inflammation-related cytokines and growth factors, such as IL-1 $\beta$ , FGF-2, and TGF- $\beta$ , stimulate PA secretion by human bone marrow stromal fibroblasts (Hannocks et al., 1992). In our study, t-PA and uPA activities in plasma as well as u-PA activity in bone extracts from the damaged site were increased during bone repair after femoral bone injury in mice. However, Plg deficiency did not affect both t-PA and uPA activities in plasma as well as u-PA activity in bone extracts from the damaged site during bone repair after femoral bone injury. These findings suggest that Plg deficiency does not affect PA activity at the bone injury site after a femoral bone defect in mice.

Skin wound healing is delayed by Plg deficiency due to impaired degradation of the extracellular matrix (Bezerra et al., 1999). The plasmin fibrinolytic system activates MMPs, especially MMP-9, which is related to the degradation of the extracellular matrix and the subsequent tissue repair and regeneration process (Hald et al., 2011). Moreover, Gong et al. reported that Plg regulates SDF-1-mediated HSC mobilization in response to G-CSF by activation of MMP-9 in mice (Gong et al., 2011). In the present study, Plg deficiency significantly suppressed MMP-9 activity at the bone injury site after a femoral bone defect in mice, although the bone injury enhanced MMP-9 activity at the damaged site and the maximal effects of bone injury on MMP-9 activity were observed 2 days after injury corresponding to the time at which the changes in the HSCs population in the bone marrow were observed. Taken together, the present study suggests that MMP-9 is a downstream factor of fibrinolytic system-induced HSC changes during bone repair after bone injury in mice.

Plasmin induces the production and release of growth factors, such as TGF- $\beta$ , PDGF, FGF-2, and HGF from cells and the extracellular matrix, respectively (Lyons et al., 1988; Falcone et al., 1993; Wilson et al., 1991; Shimizu et al., 2001), which might be involved in the fibrinolytic system-dependent bone repair process after bone injury as well as the changes in HSCs in bone marrow during the bone repair. An increase in pericellular plasmin activity can further increase the activation and/or release of growth factors, and might accelerate the positive cascade of plasmin generation and cytokine activation. In the present study, Plg deficiency significantly suppressed the levels of TGF- $\beta$  mRNA enhanced by bone injury at the damaged site in mice. Moreover, TGF- $\beta$  signal inhibitor treatment significantly blunted the decrease in the HSCs number and SDF-1 expression in preosteoblasts at the damaged site after bone injury in mice. These findings indicate that TGF- $\beta$  is involved in SDF-1 expression at the local damaged site and changes in HSCs population within bone marrow during the bone repair process. Fibrinolysis-dependent TGF- $\beta$  production and release at the damaged site might be responsible for the changes in bone marrow HSCs and subsequent bone repair process after bone injury. Various cells produce TGF- $\beta$ , and active macrophages are important as the major source of TGF- $\beta$  during bone repair (Sugiyama et al., 2006; Arjaans et al., 2012). Our previous study suggests that Plg is crucial for the induction of TGF- $\beta$  in macrophages at the damaged site after femoral bone injury (Kawao et al., 2016). We can therefore speculate that Plg might induce the production of TGF- $\beta$  in macrophages participating in the bone repair process and the release of TGF- $\beta$  from the extracellular matrix through the activation of MMP-9 after bone injury, resulting in the induction of SDF-1 expression in the endosteal preosteoblasts at the damaged site and a decrease and mobilization of bone marrow HSCs.

In conclusion, the present study revealed that Plg is crucial for changes in the HSCs number within bone marrow during bone repair in mice. Our data suggest that the plasmin fibrinolytic system, SDF-1 expression, and subsequent MMP-9 and TGF- $\beta$  induction might contribute to changes in the bone marrow HSCs population induced by bone injury.

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## Conflict of interest

The authors have no conflict of interest.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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## References

- Arjaans, M., Oude Munnink, T.H., Timmer-Bosscha, H., Reiss, M., Walenkamp, A.M., Lub-de Hooge, M.N., de Vries, E.G., Schröder, C.P., 2012. Transforming growth factor (TGF)- $\beta$  expression and activation mechanisms as potential targets for anti-tumor therapy and tumor imaging. *Pharmacol. Ther.* 135 (2), 123–132.
- Bezerra, J.A., Bugge, T.H., Melin-Aldana, H., Sabla, G., Kombrinck, K.W., Witte, D.P., Degen, J.L., 1999. Plasminogen deficiency leads to impaired remodeling after a toxic injury to the liver. *Proc. Natl. Acad. Sci. U. S. A.* 96 (26) (15143–1518).
- Bugge, T.H., Kombrinck, K.W., Flick, M.J., Daugherty, C.C., Danton, M.J.S., Degen, J.L., 1996. Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell* 87 (4), 709–719.
- Claes, L., Recknagel, S., Ignatius, A., 2012. Fracture healing under healthy and inflammatory conditions. *Nat. Rev. Rheumatol.* 8 (3), 133–143.
- Collen, D., Lijnen, H.R., 1991. Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* 78 (12), 3114–3124.
- Dar, A., Kollet, O., Lapidot, T., 2006. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. *Exp. Hematol.* 34 (8), 967–975.
- Eming, S.A., Krieg, T., Davidson, J.M., 2007. Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* 127 (3), 514–525.
- Falcone, D.J., McCaffrey, T.A., Haimovitz-Friedman, A., Vergilio, J.A., Nicholson, A.C., 1993. Macrophage and foam cell release of matrix-bound growth factors. Role of plasminogen activation. *J. Biol. Chem.* 268 (16), 11951–11958.
- Gerstenfeld, L.C., Cullinane, D.M., Barnes, G.L., Graves, D.T., Einhorn, T.A., 2003. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J. Cell. Biochem.* 88 (5), 873–884.
- Gong, Y., Fan, Y., Hoover-Plow, J., 2011. Plasminogen regulates stromal cell-derived factor-1/CXCR4-mediated hematopoietic stem cell mobilization by activation of matrix metalloproteinase-9. *Arterioscler. Thromb. Vasc. Biol.* 31 (9), 2035–2043.
- Hald, A., Ronø, B., Melander, M.C., Ding, M., Holck, S., Lund, L.R., 2011. MMP9 is protective against lethal inflammatory mass lesions in the mouse colon. *Dis. Model. Mech.* 4 (2), 212–227.
- Hannocks, M.J., Oliver, L., Gabrilove, J.L., Wilson, E.L., 1992. Regulation of proteolytic activity in human bone marrow stromal cells by basic fibroblast growth factor, interleukin-1, and transforming growth factor beta. *Blood* 79 (5), 1178–1184.
- Heissig, B., Lund, L.R., Akiyama, H., Ohki, M., Morita, Y., Römer, J., Nakauchi, H., Okumura, K., Ogawa, H., Werb, Z., Danø, K., Hattori, K., 2007. The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell* 1 (6), 658–670.
- Kawao, N., Tamura, Y., Okumoto, K., Yano, M., Okada, K., Matsuo, O., Kaji, H., 2013. Plasminogen plays a crucial role in bone repair. *J. Bone Miner. Res.* 28 (7), 1561–1574.
- Kawao, N., Tamura, Y., Okumoto, K., Yano, M., Okada, K., Matsuo, O., Kaji, H., 2014. Tissue-type plasminogen activator deficiency delays bone repair: roles of osteoblastic proliferation and vascular endothelial growth factor. *Am. J. Physiol. Endocrinol. Metab.* 307 (3), E278–E288.
- Kawao, N., Tamura, Y., Horiuchi, Y., Okumoto, K., Yano, M., Okada, K., Matsuo, O., Kaji, H., 2015. The tissue fibrinolytic system contributes to the induction of macrophage function and CCL3 during bone repair in mice. *PLoS One* 10 (4), e0123982.
- Kawao, N., Yano, M., Tamura, Y., Okumoto, K., Okada, K., Kaji, H., 2016. Role of osteoclasts in heterotopic ossification enhanced by fibrodysplasia ossificans progressiva-related activin-like kinase 2 mutation in mice. *J. Bone Miner. Metab.* 34 (5), 517–525.
- Khosla, S., Westendorf, J.J., Oursler, M.J., 2008. Building bone to reverse osteoporosis and repair fractures. *J. Clin. Invest.* 118 (2), 421–428.
- Kidd, L.J., Stephens, A.S., Kuliwaba, J.S., Fazzalari, N.L., Wu, A.C., Forwood, M.R., 2010. Temporal pattern of gene expression and histology of stress fracture healing. *Bone* 46 (2), 369–378.
- Kitaori, T., Ito, H., Schwarz, E.M., Tsutsumi, R., Yoshitomi, H., Oishi, S., Nakano, M., Fujii, N., Nagasawa, T., Nakamura, T., 2009. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum.* 60 (3), 813–823.
- Lyons, R.M., Keski-Oja, J., Moses, H.L., 1988. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J. Cell Biol.* 106 (5), 1659–1665.
- Matsuo, O., Sakai, T., Bando, H., Okada, K., Nakajima, S., Takagi, O., Izaki, S., 1986. Plasminogen activator in bronchoalveolar fluid. *Haemostasis* 16 (1), 43–50.
- Morrison, S.J., Scadden, D.T., 2014. The bone marrow niche for hematopoietic stem cells. *Nature* 505 (7483), 327–334.
- Nagasawa, T., Omatsu, Y., Sugiyama, T., 2011. Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells. *Trends Immunol.* 32 (7), 315–320.
- Nistala, H., Lee-Arteaga, S., Siciliano, G., Smaldone, S., Ramirez, F., 2010. Extracellular regulation of transforming growth factor beta and bone morphogenetic protein signaling in bone. *Ann. N. Y. Acad. Sci.* 1192, 253–256.
- Okada, K., Kawao, N., Yano, M., Tamura, Y., Kurashimo, S., Okumoto, K., Kojima, K., Kaji, H., 2016. Stromal cell-derived factor-1 mediates changes of bone marrow stem cells during the bone repair process. *Am. J. Physiol. Endocrinol. Metab.* 307 (1),



- E278–288.
- Reiner, A.P., Aramaki, K.M., 2000. Proteolytic modulation of thrombopoietin activity: comparison of thrombin, plasmin, and urokinase. *Thromb. Haemost.* 83 (6), 909–914.
- Shimizu, M., Hara, A., Okuno, M., Matsuno, H., Okada, K., Ueshima, S., Matsuo, O., Niwa, M., Akita, K., Yamada, Y., Yoshimi, N., Uematsu, T., Kojima, S., Friedman, S.L., Moriwaki, H., Mori, H., 2001. Mechanism of retarded liver regeneration in plasminogen activator-deficient mice: impaired activation of hepatocyte growth factor after Fas-mediated massive hepatic apoptosis. *Hepatology* 33 (3), 569–576.
- Singer, A.J., Clark, R.A., 1999. Cutaneous wound healing. *N. Engl. J. Med.* 341 (10), 738–746.
- Sugiyama, T., Kohara, H., Noda, M., Nagasawa, T., 2006. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25 (6), 977–988.
- Toriseva, M., Kahari, V.M., 2009. Proteinases in cutaneous wound healing. *Cell. Mol. Life Sci.* 66 (2), 203–224.
- Wilson, E.L., Rifkin, D.B., Kelly, F., Hannocks, M.J., Gabilove, J.L., 1991. Basic fibroblast growth factor stimulates myelopoiesis in long-term human bone marrow cultures. *Blood* 77 (5), 954–960.