Doctoral Thesis（Abridged）

The fibrinolytic pathway expands mesenchymal stem cells through a crosstalk with endothelial cells

（血液線維素溶解系による血管内皮細胞との相互作用を通じた間葉系幹細胞動態の制御機構）

ダヒリ ドゥアー

Dhahri Douaa

Doctoral Thesis

The fibrinolytic pathway expands mesenchymal stem cells through a crosstalk with endothelial cells

(血液線維素溶解系による血管内皮細胞との相互作用を通じた間葉系幹細胞動態の制御機構)

ダヒリ　ドゥアー　　Dhahri Douaa

Supervisor: Dr. Beate Heissig

Contents

List of Figures

Abstract

Following injury, the tissue plasminogen activator (tPA) is upregulated to maintain tissue hemostasis through its main product, plasmin. However, many reports have shown that tPA can act as a cytokine by itself and exerts many effects controlling cell fate. Therefore, we investigated its role in modulating one of the main cellular components of the bone marrow niche, the mesenchymal stem cells. In this study, we demonstrate that tPA expands murine bone marrow-derived $CD45^-$ $TER119^-$ $Sca\text{-}1^+$ PDGF-$R\alpha^+$ mesenchymal stem cells (MSCs) *in vivo* through a crosstalk with endothelial cells. Mechanistically, by driving conversion of plasminogen into plasmin, tPA activates matrix metalloproteinases (MMPs). In turn, ….These data show a novel mechanism to expand the pro-homeostasis MSCs *in vivo* which can arguably lead to faster recovery following injury.

Introduction

I. The fibrinolytic/protease pathway: tissue plasminogen activator and MMPs

Tissue injury leads to the activation of the coagulation system resulting in thrombus formation by thrombin. The fibrinolytic system with its main player, plasmin, degrades fibrin within the thrombus. Plasmin is a serine protease generated by the conversion of plasminogen (Plg) by the tissue and urokinase plasminogen activators (tPA and uPA, respectively), and kallikrein.

tPA consists of 527 amino acids containing 5 domains: a fibronectin-like finger, an epidermal growth factor-like cassette, two kringle structures and a serine protease domain. Plasmin converts tPA from a single-chain tPA to a more active two-chain polypeptide by the cleavage of the Arg275-Ile276 peptide bond (Pennica et al, 1983). Endothelial cells lining up veins and arteries are the main source of tPA. Several stimuli regulate the gene expression of tPA and its release such as thrombin, adrenaline, histamine and arterial shear stress level. tPA activity is inhibited by its endogenous inhibitors: plasminogen activator inhibitor -1 (PAI-1) and PAI-2. PAI-1, which is the most potent inhibitor of tPA, is synthesized by platelets, endothelial and hepatic cells and its expression is regulated by thrombin and growth factors like the transforming growth factor beta (TGF-ß), proinflammatory cytokines such as tumor necrosis factor-α and fibroblast growth factor-2 (FGF-2) (Hajjar, 2003). PAI-1 exists in the blood (2µM) in both active and latent form accounting for 90% of circulating PAI-1. It also exists bound to the extracellular matrix (ECM) in an active form that can be released upon binding to plasminogen activators (PAs). Hence, this reservoir of matrix-bound PAI-1 is essential to rapidly neutralize tPA which is known to have a very short half-life of 5 minutes (as for human endogenously occurring tPA) and is accumulated in the liver and rapidly cleared from the blood system. Many mutant proteins have been engineered to increase the half-life of tPA leading to an up to 50 minutes half-life (Gerard and Meidell., 1989).

The Plg/plasmin cascade and matrix metalloproteinases (MMPs) can induce extracellular proteolysis that is critical for development, tissue repair, and the control of stem cells. They are essential players in physiological events like inflammation, angiogenesis. Plasmin can catalyze the activation/inactivation of cytokines, chemokines, cell surface receptors, growth factors and other proteases like (MMPs) which, together with plasmin can accelerate local ECM degradation thus leading to its remodeling and the release of ECM-bound signaling molecules (Cauwe et al., 2007; Heissig et al., 2012). The Plg/plasmin cascade is depicted in the diagram below (Diagram 1). MMPs, synthesized as zymogens, are a large family of zinc-dependent endoproteinases. The enzymatically inactive state of MMPs is due to the interaction of the cysteine residue of the pro-domain with the zinc ion localized in the catalytic site. Therefore a cysteine switch is required for their activation. MMPs are activated by the removal of the pro-domain by furin, other MMPs or serine proteinases like plasmin. In total, there are 23 MMPs in humans that, according to their structure, can be organized into 4 different groups including gelatinases. MMP-2 and MMP-9 are two types of gelatinases constitutively expressed by many cells including the fibroblasts and endothelial cells. Thanks to their fibronectin catalytic domain, these gelatinases are able to degrade a broad spectrum of ECM components such as collagen type I, IV, V, X, fibronectin, elastin, aggrecan and laminin as well as activating several growth factors including TNF-α, TGF-ß and IL-1ß (Werb, 1997; Kessenbrock et al., 2010).

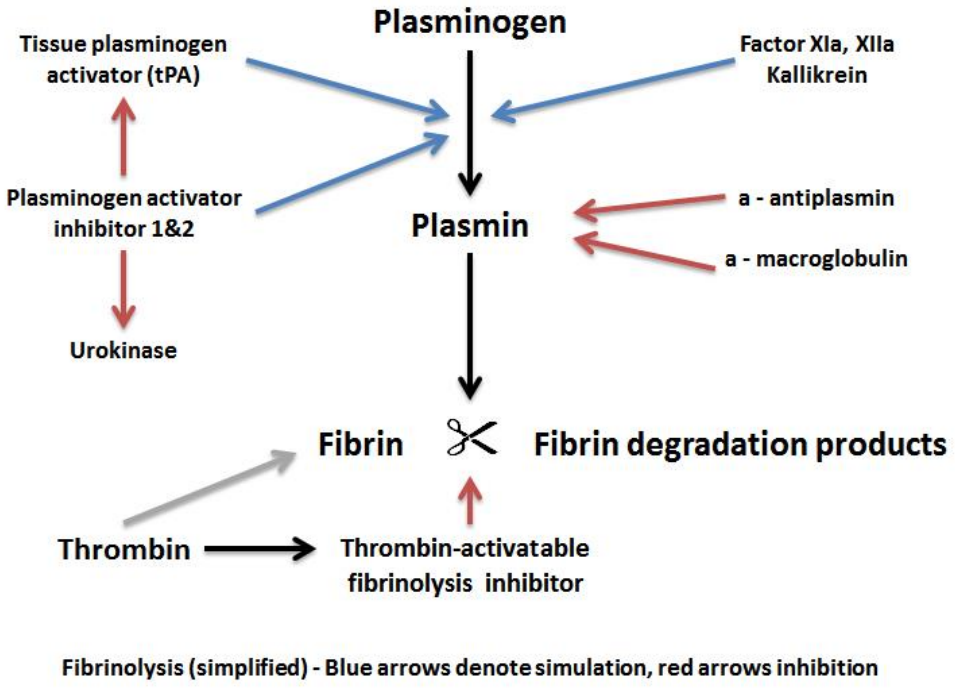

Diagram1: **The fibrinolytic system**; Adopted from CoaguCheck® (Roche)

Several genetic knockout models have been created and helped to understand the role of fibrinolytic factors and MMPs in many physiological events as well as during development and disease. Below is a summary of few established models:

Genotype	Phenotype
Plg $^{-/-}$	Spontaneous thrombosis, runting, premature death; Fibrin in liver, lungs, stomach; Gastric ulcers; Impaired wound healing; Ligneous conjunctivitis; Impaired monocyte recruitment; Impaired neointima formation after electrical injury; Impaired dissemination of Borrelia burgdorferi; Reduced excitotoxic neuronal cell death in brain
tPA $^{-/-}$	Reduced lysis of fibrin clot; Increased endotoxin-induced thrombosis
uPA $^{-/-}$	Occasional fibrin in liver/intestine; Rectal prolapse, ulcers of eyelids, face, ears; Reduced macrophage degradation of fibrin; Increased endotoxin-induced thrombosis

uPA$^{-/-}$/ tPA$^{-/-}$	Reduced growth, fertility and lifespan; cachexia; Fibrin deposits in liver, gonads, lungs; Ulcers in intestine, skin, ears; rectal prolapse; Impaired clot lysis
PAI $^{-/-}$	Mildly increased lysis of fibrin clot; Resistance to endotoxin-induced thrombosis
MMP2 $^{-/-}$	Reduced body size; reduced neovascularization; decreased primary ductal invasion in the mammary gland; reduced lung saccular development
MMP9 $^{-/-}$	Bone-development defects; defective neuronal remyelination after nerve injury; delayed healing of bone fractures; impaired vascular remodelling; impaired angiogenesis

Table 1: **Selected fibrinolytic factors and MMP mutant phenotypes**; Adopted from Cesarman-Maus and Hajjar., 2005; Page-McCaw et al., 2007

II. The role of fibrinolytic factors in the Bone Marrow (BM)

Hematopoiesis is the process by which all blood cells are generated from a small pool of multipotent hematopoietic stem cells (HSC). These cells exist in the BM during adulthood in a quiescent state and are induced to proliferate and differentiate following hematological stress like bleeding, irradiation or chemotherapy in order to regenerate injured tissues. This process is tightly controlled by a specific microenvironment in the BM called the BM niche that consists of the ECM and a plethora of stromal cells and non-hematopoietic cells like endothelial cells, osteoblasts and fibroblasts (Morrison and Scadden, 2014).

MMPs like MMP-9 were shown to be essential for BM regeneration following myelosuppression by the 5-fluorouracil (5-FU) drug as mice deficient in MMP-9 showed a lower survival rate due to a delayed hematopoietic recovery (Heissig et al., 2002). Moreover, an accumulation of plasminogen/plasmin and an upregulation in tPA was observed in the BM

stromal compartment following myelosuppression. Plasminogen was also shown to be important for hematopoietic recovery because Plg deficient mice could not survive the myelosuppression due to a failure in cell cycle activation of dormant HSCs (Heissig et al., 2007). Mechanistically, it was shown that Plg effect was mediated through the activation of MMP-9 and the release of the hematopoietic maintenance factor kit ligand (KitL; also known as stem cell factor/SCF). Several studies have shown that the lack of the membrane form of KitL impairs hematopoiesis (Brannan et al., 1991). Initially it was suggested that KitL is secreted by many cell types of the BM like stromal cells, but a recent study demonstrated that endothelial cells and perivascular mesenchymal stem cells are the main source of KitL (Ding et al., 2012).

III. Mesenchymal Stem Cells:

Mesenchymal stem cells (MSCs) are a subset of BM stromal cells that have a prominent role in local tissue remodeling and regeneration. MSCs were first identified by Friedenstein in the early 70's (Friedenstein et al., 1974). They were isolated from BM and characterized as fibroblast-like shaped cells, able to form colonies *in vitro* (colony forming unit-fibroblast, CFU-F) and showed multipotency, i.e. the ability to differentiate into different mesenchymal tissues including osteoblasts, adipocytes and chondrocytes, as well as hematopoietic stromal cells (Horwitz et al., 2005; Bianco et al., 2008). They are a rare population of cells forming only 0.001%- 0.01% of the human adult BM cells (Pittenger et al., 1999) and are mainly perivascular (Crisan et al., 2008).

Other than the BM, MSCs virtually exist in every type of connective tissue such as fat (Zuk et al., 2001), muscle (Poulsom et al., 2002), skin (Toma et al., 2001) and placenta (Fukuchi et al., 2004).

MSCs are further characterized by the lack of expression of endothelial and hematopoietic markers (e.g. CD45, Ter119, CD31) and a positive expression of mesenchymal markers like CD146 for human BM MSCs (Sacchetti et al., 2007) or Nestin (Mendez-Ferrer et al., 2010), Leptin receptor (Zhou et al., 2014), Platelet-Derived Growth Factor Receptor α (PDGFRα) and Stem cell antigen-1 (Sca-1) for mouse (Morikawa et al., 2009). Although the origin and relationship between these recently reported subpopulations of MSCs remain unclear, they all showed the common potential of supporting hematopoiesis *in vivo* by generating a stromal cell niche important for HSC maintenance.

Many techniques have been developed in order to prove the true functional properties of MSCs, like their ability for self-renewal and differentiation. When transplanted *in vivo,* these cells can generate a bone organoid composed of bone, fat, cartilage and hematopoietic stroma that allows HSCs of the recipient mouse to home to and to engraft, thus forming a site of extramedullar hematopoiesis (Bianco, 2014).

MSCs offer many advantages in the field of autologous cell therapies thanks to their immunomodulatory potential, low immunogenicity and homing capacity to sites of injury (Wei et al., 2013). Even though they are easy to isolate, their low numbers in the adult BM hinders their use in the clinic. Moreover, MSCs fate is dictated by external signals from the microenvironment through cell-cell and cell-matrix interactions which are hardly replicated *in vitro,* making the expansion of true MSCs *ex vivo* a difficult process (Liechty et al., 2000). Therefore, finding a mechanism by which MSCs can be expanded *in vivo* is of utmost importance.

Hypothesis

The fibrinolytic system is activated under stress situations like ischemia, myelosuppression, or during cancer growth (Heissig et al., 2007; Ohki et al., 2010; Tashiro et al., 2012). Previously, we reported that tPA is upregulated following myelosuppression resulting in hematopoietic regeneration through KitL release which results in hematopoietic stem cells (HSCs) activation and cell cycle entry (Heissig et al., 2007). Many other studies have addressed the importance of several cell types in creating a specialized hematopoietic microenvironment, or niche, which will ensure the maintenance and proliferation of HSCs. A recent study has shown that the ablation of perivascular Nestin-expressing mesenchymal stem cells (MSCs) reduces the HSCs number in the BM and impairs their homing upon transplantation (Mendez-Ferrer et al., 2010).

Although, plasmin or MMPs have already been shown to direct BM regeneration and that MSCs are also important for BM maintenance, no studies have yet characterized how plasmin or MMPs can influence other BM niche cells like the stromal MSCs. Therefore, it is important to understand how the fibrinolytic/ protease systems regulate other niche cells.

Here, we hypothesize that plasmin in part by modulating MMPs can influence MSCs fate i.e. proliferation and differentiation.

Material and Methods

Experimental Animals

C57BL/6 (WT B6) mice were purchased from SLC. WT tissue-type plasminogen activator *(tPA)*$^{+/+}$ and knockout tPA (*tPA*$^{-/-}$), plasminogen *(Plg)* $^{+/+}$, *Plg*$^{-/-}$, plasminogen activator inhibitor-1 *(PAI-1)*$^{+/+}$, *PAI-1* $^{-/-}$, matrix metalloproteinase-9 *(MMP9*$^{+/+}$*)* and *MMP9*$^{-/-}$ mice were used after more than 10 back crosses onto a C57BL/6 background. Animal procedures were approved by the Experimental Animal Care and Use Committee in the Animal Review Board of Institute of Medical Science, University of Tokyo.

In vivo treatment regiments

Blocking experiments *in vivo*:

Preparation of bone marrow mesenchymal stem cells

MSCs were prepared as previously described (Morikawa et al., 2009): The bone fragments were collected from femurs and tibias and incubated for 1h at 37°C in 20ml of DMEM (Invitrogen) containing 40mg collagenase (Wako Chemicals, Inc.), 10mM Hepes (pH7.2) and 1% Penicillin/Streptomycin with gentle shaking. The cell suspension was filtered with a cell strainer (BD Falcon 2350, 70μm mesh) to remove debris and bone fragments and centrifuged at 1200 rpm for 7 min at 4°C. The pellet was resuspended into 1ml of cold sterile water for 5 to 10 s to lyse the red blood cells, followed by addition of 1ml of cold 2x PBS containing 4% FBS. The suspension was filtered through a cell strainer and centrifuged again. The pellet was resuspended in 1ml of ice-cold HBSS/2%FBS and stained for 30 min with the following mAbs:

APC-conjugated PDGFRα (APA5), FITC-conjugated Sca-1 (Ly6A/E), Pacific blue-conjugated CD45 (30-F11) and PE-TER119 (TER-119). All mAbs were purchased from eBioscience.

Another set of antibodies was used to identify the LeptinR+ MSC subpopulation: FITC-conjugated CD31+ (PECAM-1) and biotinylated LeptinR (R&D systems, BAF497) which was visualized with APC-conjugated streptavidin (Invitrogen).

Flow cytometry analysis and sorting were performed on a FACS Verse and Area (BD Biosciences) flow cytometers. PI fluorescence was measured, and PI positive cells (dead cells) were excluded.

Femoral muscle and subcutaneous fat were similarly collagenase-digested and single cells were stained similarly.

Cell cycle analysis

$5x10^6$ MSCs were suspended in warm medium at 106 cells/ml and mixed with Hoechst 33342 (Dojindo, Japan) at 10µg/ml. Verapamil was added at 40µg/ml to block dye flux. Cells were incubated for 40min at 37°C with occasional shaking. Pyronin Y (Sigma-Aldrich) was added at 0.5µg/ml. Cells were incubated for 20min, centrifuged and resuspended in FACS buffer for subsequent flow cytometry analysis.

Colony forming Unit-Fibroblast assay

To determine the clonogenic potential of MSCs, single cells from MSCs suspension were plated in a 96-well plate by FACS sorter (Aria, BD Biosciences) and maintained in MSCs

growth medium (10% fetal bovine serum in MEMα GlutaMAX, Gibco) for 2 weeks. Adherent visible colonies (more than 50 cells) were counted under a microscope.

Differentiation cultures

To induce adipogenic differentiation, confluent cells were cultured for 2 days, replaced with Adipogenic Induction Medium for 2 days followed by Adipogenic Maintenance Medium (Lonza) for 4 days, supplemented with 1.7μM (1μg/ml) insulin, 0.25μM dexamethazone (DEX) and 1mM isobuthylmethylxisantin (IBMX). Medium was changed every other day. After 8 days, the cells were fixed with 4% paraformaldehyde for 15min, and stained with Oil Red O (MutoPure Chemicals).

For chondrogenic differentiation, cultured cells were harvested by trypsinization. The 1~2.5 $x10^5$ cells were washed with MEMα + GlutaMAX (Invitrogen). The tube was spun at 150x *g* for 5min at room temperature and the supernatant was aspirated. The cells were resuspended in 1ml Differentiation Basal Medium Chondrogenic (Lonza), spun at 150x g for 5 min, and the medium was aspirated. The cells were resuspended in 1ml of Differentiation Basal Medium Chondrogenic, supplemented with Chondrogenic SingleQuots kit, TGF-β3 (10ng/ml; Lonza) and BMP-6 (500ng/ml; R&D Systems), and spun at 150x *g* for 4min, 300 x g for 4min, and 450x g for 4min at room temperature. The pellet was maintained with Differentiation Medium changed every 2 days for 2-3 weeks. After 3 weeks, cell clumps were harvested, washed in 4% paraformaldehyde, and their tissue sections were stained with Toluidine Blue (Sigma-Aldrich). To induce osteogenic differentiation, subconfluent cells were cultured with differentiation Basal Medium Osteogenic, supplemented with Osteogenic SingleQuots (Lonza) for 8 days. The cells were then fixed with 4% paraformaldehyde for 15min and stained with Alizarin Red S (Sigma-Aldrich).

Cell cultures

MSC single cell cultures: Isolated $CD45^-$ $Ter119^-$ $Sca\text{-}1^+PDGFR\alpha^+$ cells were cultured in 10% FBS/ MEMα/ Glutamax medium. Cells were passaged by trypsinization every 3 days. Cells from 2-10 passages were used for analysis.

Endothelial single cell cultures: Both murine and human endothelial cells (ECs) were used in this study: Magnetic-Activated Cell Sorting (MACS)-isolated murine $CD45^-CD31^+$ ECs and human umbilical vein endothelial cells (HUVECs; purchased from Gibco) were cultured at 37°C and 5% CO_2 on 0.1% gelatin (Wako Pure Chemicals, Tokyo Japan)-coated plastic culture plates (Falcon) in EGM-2 medium (Lonza, cc4176).

Cytokine treatment of cultured cells:

Transwell cultures

MSC proliferation assay

$CD45^-$ $Ter119^-$ $Sca\text{-}1^+PDGFR\alpha^+$ MSCs (5000 cells/well) were cultured for 3 days in 96-well plate with MSC growth medium in triplicate. Culture medium was changed once and replaced with Cell Counting Kit-8 containing fresh medium (x20 dilution, 100μl) (Dojin-Wako Pure Chemical, Tokyo, Japan). Cells were cultured for an additional 1-2h. The absorbance at 450 nm was measured by an ELISA plate reader (Molecular Devices, microplate reader with SoftMax Pro, Tokyo, Japan).

BrdU assay: FACS-sorted PαS-MSCs derived from control and tPA-treated mice were analyzed using BrdU Cellomics® proliferation kit (Thermo Scientific). Cells were fixed, permeabilized and stained according to manufacturer's instructions. Ten images/group were analyzed using a fluorescent microscope.

Quantitative RT-PCR Analysis

Total RNA was prepared from cell pellets using TRIzol Reagent (Ambion by Life Technologies, #15596018) according to the manufacturer's instructions. First-strand cDNA was synthesized from 0.2-2µg of total RNA using a High Capacity Reverse Transcriptase kit (Applied Biosystems). The cycle number for PCR was 40 for all samples using a qPCR machine Step One Plus (Applied Biosystems) with SYBR Premix Ex Taq II (x2) Tli RNaseH Plus (Takara, #RR820). Primer sequences are provided in annex.

RTK array

In order to determine the activated tyrosine kinase receptors in MSCs following tPA treatment, MSCs were sorted from PBS or tPA treated mice and cultured for 6h in 6 well plates at a cell density of 5 x 10^4 cells/well. Medium was changed and cells were cultured for another 24h. Then cells were lysed and analyzed using RTK array kit according to the manufacturer's protocol (R&D systems, #ARY014). The band intensity was measured using an Epson scanner and analyzed using ImageJ.

Western blot analysis

Cultured cells were lysed with ice-cold 1% TritonX-100 buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% TritonX-100 w/v, 2mM EDTA, 1mM Na3VO4, 10mM NaF) and 1mM PMSF (added freshly) for 10min on ice. Remaining supernatants were centrifuged at 15,000rpm for 10min at 4°C, and supernatants were collected. The cell lysates (2~50µg proteins) were applied on 8% or 10% acrylamide gel, transferred to a PVDF membrane (Millipore, Immobilon), blocked with 5% skim-milk in PBST (PBS containing 0.05% Tween20), and stained overnight at 4°C with antibodies. Membranes were stained with secondary antibody conjugated with horse radish peroxidase (Nichirei, rabbit-HRP or goat-HRP), and developed with the ECL Plus

detection system (Amersham Life Science, RPN2132) using image analyzer Image-Quant LAS4000 (GE-healthcare).

ELISA

Mouse serum and plasma taken at indicated time points were used for ELISA analysis according to the manufacturer's protocol.

Immunohistochemistry

Mouse femur samples were snap frozen with Tissue-Tek OCT Compound (Sakura) in liquid nitrogen. Transverse tissue sections of the whole femur were prepared. Tissue sections were blocked with BSA/serum buffer (5% BSA and 5% normal goat serum in PBS(-), pH 7.2), and stained with the primary antibodies (1µg/ml solved in BSA/serum buffer) overnight at 4°C. Respective mouse, rat, and rabbit IgG (Santa Cruz Biotsch) isotype controls followed by Alexa488 or Alexa594-conjugated secondary antibodies (IgG H+L) (Invitrogen, A11072, A11017). Nuclei were counterstained with DAPI (Molecular Probes). Immunoreactions were visualized with the use of an Olympus fluorescence microscope IX71 and BX51.

Gelatin-based Zymography

To determine MMP activity in plasma or culture samples, 1 mL cell culture of culture supernatants or 10µl of plasma (+990µl PBS) was mixed with 20 µL gelatin-agarose beads (Sigma-Aldrich, G5384) and incubated overnight at 4°C. Samples were run through SDS-PAGE acrylamide gels containing 1% gelatin. Gels were subsequently incubated in renaturing buffer (1M Tris-HCl, 10% NaCl, 2.5% Triton X-100) for 2 hours at room temperature, rinsed in distilled water and placed in low-salt collagenase buffer (5% Tris-HCl, 1% CaCl2) at 37°C

for 24 hours. Gels were stained with 0.2% Coomassie blue solution for 30min, and then destained in bleaching solution (DW, 5% methanol and 7% acetic acid) until bands are clear. The density of each lytic band was quantified using image analysis software (ImageJ).

Plasmin concentration assay

Plasma samples were incubated with thrombin (1U/ml) and fibrinogen (5mg/ml) at 37°C for 30min then put on ice for 2 minutes. Samples were centrifuged at 15,000rpm for 5min. Supernatants were recovered and protein content (which is the result of product degradation by plasmin) was measured at 280nm.

Knockdown experiments in B16F10 melanoma

Statistical analysis

Results are presented as means ±SEM. Statistical comparisons were based on Student's *t* test or ANOVA with Tukey HSD posthoc tests using R program. P value level of <0.05 was considered significant.

Results

Discussion

Conclusion

References

Ball, SG.,Shuttleworth, CA., Kietly, CM., 2010. Platelet-derived growth factor receptors regulate mesenchymal stem cell fate: implications for neovascularization. *Expert Opin Biol Ther* 10 :57-71.

Bianco, P., P.G. Robey, and P.J. Simmons. 2008. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2:313-319.

Broudy, V.C. 1997. Stem cell factor and hematopoiesis. *Blood* 90:1345-1364.

Cauwe, B., P.E. Van den Steen, and G. Opdenakker. 2007. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit Rev Biochem Mol Biol* 42:113-185.

Corselli, M., C.J. Chin, C. Parekh, A. Sahaghian, W. Wang, S. Ge, D. Evseenko, X. Wang, E. Montelatici, L. Lazzari, G.M. Crooks, and B. Péault. 2013. Perivascular support of human hematopoietic stem/progenitor cells. *Blood* 121:2891-2901.

Coutu, D.L., M. François, and J. Galipeau. 2011. Inhibition of cellular senescence by developmentally regulated FGF receptors in mesenchymal stem cells. *Blood* 117:6801-6812.

Crisan, M., Yap, S., Casteilla, L., Chen CW., Corselli M., Park TS., Andriolo G., Sun B., Zheng B., Zhang L., Norotte C., Teng PN., Traas J., Schugar R., Deasy BM., Badylak S., Buhring HJ., Giacobino JP., Lazzari L., Huard J., Péault B. 2008. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3, 301–313.

Ding, L., T.L. Saunders, G. Enikolopov, and S.J. Morrison. 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481:457-462.

Friedenstein, A.J. 1980. Stromal mechanisms of bone marrow: cloning in vitro and retransplantation in vivo. *Haematol Blood Transfus* 25:19-29.

Fukuchi, Y., Nakajima, H., Sugiyama, D., Hirose I., Kitamura T., Tsuji K. 2004. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 22, 649–658.

Gerard, R.D. Meidell, R.S. 1989. Regulation of tissue plasminogen activator expression. *Annu. Rev. Physiol.* 51: 245–262

Hajjar, K.A.2003. The molecular basis of fibrinolysis. In: *Hematology of Infancy and Childhood* pp. 1497–1514

Heissig, B., K. Hattori, S. Dias, M. Friedrich, B. Ferris, N.R. Hackett, R.G. Crystal, P. Besmer, D. Lyden, M.A. Moore, Z. Werb, and S. Rafii. 2002. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109:625-637.

Heissig, B., L.R. Lund, H. Akiyama, M. Ohki, Y. Morita, J. Romer, H. Nakauchi, K. Okumura, H. Ogawa, Z. Werb, K. Dano, and K. Hattori. 2007. The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell Stem Cell* 1:658-670.

Heissig, B., M. Ohki, M. Ishihara, Y. Tashiro, C. Nishida, I. Gritli, J. Rosenkvist, and K. Hattori. 2009. Contribution of the fibrinolytic pathway to hematopoietic regeneration. *Journal of cellular physiology* 221:521-525.

Heissig, B., M. Ohki-Koizumi, Y. Tashiro, I. Gritli, K. Sato-Kusubata, and K. Hattori. 2012. New functions of the fibrinolytic system in bone marrow cell-derived angiogenesis. *Int J Hematol* 95:131-137.

Horwitz, E.M., K. Le Blanc, M. Dominici, I. Mueller, I. Slaper-Cortenbach, F.C. Marini, R.J. Deans, D.S. Krause, and A. Keating. 2005. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 7:393-395.

Hu K., Yang J., Tanaka S., Gonias SL., Mars WM., and Liu Y.2006. Tissue-type plasminogen activator acts as a cytokine that triggers intracellular signal transduction and induces matrix metalloproteinase-9 gene expression. *Journal of Biological Chemistry*.;281(4):2120-7.

Ibrahim, A.A., T. Yahata, M. Onizuka, T. Dan, C. Van Ypersele De Strihou, T. Miyata, and K. Ando. 2014. Inhibition of plasminogen activator inhibitor type-1 activity enhances rapid and sustainable hematopoietic regeneration. *Stem Cells* 32:946-958.

Itkin, T., A. Ludin, B. Gradus, S. Gur-Cohen, A. Kalinkovich, A. Schajnovitz, Y. Ovadya, O. Kollet, J. Canaani, E. Shezen, D.J. Coffin, G.N. Enikolopov, T. Berg, W. Piacibello, E. Hornstein, and T. Lapidot. 2012. FGF-2 expands murine hematopoietic stem and progenitor cells via proliferation of stromal cells, c-Kit activation, and CXCL12 down-regulation. *Blood* 120:1843-1855.

Kessenbrock, Kai . Plaks, Vicki .and Werb Zena. 2010. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell.* 141(1): 52–67.

Lozito, T.P., and R.S. Tuan. 2011. Mesenchymal stem cells inhibit both endogenous and exogenous MMPs via secreted TIMPs. *Journal of cellular physiology* 226:385-396.

Lund, L.R., J. Romer, T.H. Bugge, B.S. Nielsen, T.L. Frandsen, J.L. Degen, R.W. Stephens, and K. Dano. 1999. Functional overlap between two classes of matrix-degrading proteases in wound healing. *Embo J.* 18:4645-4656.

Mendez-Ferrer, S., T.V. Michurina, F. Ferraro, A.R. Mazloom, B.D. Macarthur, S.A. Lira, D.T. Scadden, A. Ma'ayan, G.N. Enikolopov, and P.S. Frenette. 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466:829-834.

Morikawa, S., Y. Mabuchi, Y. Kubota, Y. Nagai, K. Niibe, E. Hiratsu, S. Suzuki, C. Miyauchi-Hara, N. Nagoshi, T. Sunabori, S. Shimmura, A. Miyawaki, T. Nakagawa, T. Suda, H. Okano, and Y. Matsuzaki. 2009. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J Exp Med* 206:2483-2496.

Morrison, Sean, J and Scadden, David, T. 2014. The bone marrow niche for haematopoietic stem cells. *Nature* 505: 327–334.

Murakami, M., Nguyen, L. T., Hatanaka, K., Schachterle, W., Chen, P. Y., Zhuang, Z. W., Black, B. L. and Simons, M. 2011. FGF-dependent regulation of VEGF receptor 2 expression in mice. *Journal of Clinical Investigation* 121: 2668–2678.

Ng, F., S. Boucher, S. Koh, K.S. Sastry, L. Chase, U. Lakshmipathy, C. Choong, Z. Yang, M.C. Vemuri, M.S. Rao, and V. Tanavde. 2008. PDGF, TGF-beta, and FGF signaling is important for differentiation and growth of mesenchymal stem cells (MSCs): transcriptional profiling can identify markers and signaling pathways important in differentiation of MSCs into adipogenic, chondrogenic, and osteogenic lineages. *Blood* 112:295-307.

Nissen, L.J., R. Cao, E.M. Hedlund, Z. Wang, X. Zhao, D. Wetterskog, K. Funa, E. Brakenhielm, and Y. Cao. 2007. Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis. *The Journal of clinical investigation* 117:2766-2777.

Nombela-Arrieta C., Pivarnik G., Winkel B., Canty K.J., Harley B., Mahoney J.E., Park S.Y., Lu J., Protopopov A., Silberstein L.E. 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat. Cell Biol*.;15:533–543

Oh, D.Y., P. Cui, H. Hosseini, J. Mosse, B.H. Toh, and J. Chan. 2012. Potently immunosuppressive 5-fluorouracil-resistant mesenchymal stromal cells completely remit an experimental autoimmune disease. *J Immunol* 188:2207-2217.

Ohki, M., Y. Ohki, M. Ishihara, C. Nishida, Y. Tashiro, H. Akiyama, H. Komiyama, L.R. Lund, A. Nitta, K. Yamada, Z. Zhu, H. Ogawa, H. Yagita, K. Okumura, H. Nakauchi, Z. Werb, B. Heissig, and K. Hattori. 2010. Tissue type plasminogen activator regulates myeloid-cell dependent neoangiogenesis during tissue regeneration. *Blood* 115:4302-4312.

Ornitz, D.M., and P.J. Marie. 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes & development* 16:1446-1465.

Pennica, D. W.E. Holmes, W.J. Kohr, R.N. Harkins, G.A. Vehar, C.A. Ward, W.F. Bennett, E. Yelverton, P.H. Seeburg, H.L. Heyneker, D.V. Goeddel, D. Collen. 1983. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. *Nature* 301: 214–221

Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143-147.

Rey S. and Semenza GL. 2010. Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodeling. Cardiovascular research

Sacchetti, B., A. Funari, S. Michienzi, S. Di Cesare, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P.G. Robey, M. Riminucci, and P. Bianco. 2007. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131:324-336.

Sugrue T, Lowndes NF, Ceredig R. 2013. Mesenchymal stromal cells: radio-resistant members of the bone marrow. *Immunol Cell Biol*; 91(1):5-11.

Tashiro, Y., C. Nishida, K. Sato-Kusubata, M. Ohki-Koizumi, M. Ishihara, A. Sato, I. Gritli, H. Komiyama, Y. Sato, T. Dan, T. Miyata, K. Okumura, Y. Tomiki, K. Sakamoto, H. Nakauchi, B. Heissig, and K. Hattori. 2012. Inhibition of PAI-1 induces neutrophil-driven neoangiogenesis and promotes tissue regeneration via production of angiocrine factors in mice. *Blood* 119:6382-6393.

Tokunaga, A., T. Oya, Y. Ishii, H. Motomura, C. Nakamura, S. Ishizawa, T. Fujimori, Y. Nabeshima, A. Umezawa, M. Kanamori, T. Kimura, and M. Sasahara. 2008. PDGF receptor beta is a potent regulator of mesenchymal stromal cell function. *J Bone Miner Res* 23:1519-1528.

Toma, J.G., Akhavan, M., Fernandes, K.J.L., et al. 2001. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nature Cell Biology* 3: 778–784.

Tsuji, W., J.P. Rubin, and K.G. Marra. 2014. Adipose-derived stem cells: Implications in tissue regeneration. *World journal of stem cells* 6:312-321.

Wang, X., S.R. Lee, K. Arai, K. Tsuji, G.W. Rebeck, and E.H. Lo. 2003. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. *Nature medicine* 9:1313-1317 Epub 2003 Sep 1317.

Werb Z. 1997. ECM and cell surface proteolysis: regulating cellular ecology.*Cell*. 91(4):439-42.

Zhou, Bo O., R. Yue, Malea M. Murphy, J.G. Peyer, and Sean J. Morrison. 2014. Leptin-Receptor-Expressing Mesenchymal Stromal Cells Represent the Main Source of Bone Formed by Adult Bone Marrow. *Cell Stem Cell*

Acknowledgments

I would like to thank my supervisor Dr. Beate Heissig for her guidance and inspiration throughout the project. This project would not have been possible without her valuable input.

I would like to extend my appreciation for Dr. Koichi Hattori for his constant presence and guidance. His willingness to discuss about technical issues and future directions has pushed this project further.

My appreciation also goes to Prof. Hiromitsu Nakauchi for allowing me to be part of the centre for stem cell biology and regenerative medicine. I thank him for his constant support

A special thanks to the members of the FACS Core Laboratory for their support and technical assistance. The guidance of Ishii-san have boosted my confidence in the presented data.

Many thanks to the Stem Cell Dynamics members. Their comments and countless suggestions have contributed hugely to the quality of this project. Working with them has been an absolute pleasure.

A special thanks to my little Buddha: Salita. Her kind character has helped me countless times to restore my faith in a better tomorrow. Her patience with my mood swings and her willingness to listen to me have helped me keep my spirit up. I cannot thank her enough.

To the nice people I met in Japan and the ones that became like a second family to me: I cannot thank them enough for their friendship, the nice memories we created, for giving me a "home" feeling in Japan.

To MEXT for granting me the scholarship to conduct my studies in Japan. I cannot thank them enough for the trust they put in me to have the opportunity to study in one of the top universities in this beautiful country.

Finally, to my parents and loved ones: For their constant support, unconditional love and care. I thank them for always believing in me and listening to me. Your understanding has been a blessing. To my sister in Japan who is always offering a helping hand, her presence had given me such peace and strength. To my man, for his presence and support. I cannot thank him enough for always bringing the best in me, for seeing my light when I am blinded.

In loving memory of my Grandfather, my mentor, He will never be forgotten.