論文の内容の要旨

論文題目　Recruitment of Hematopoietic Progenitor and Stem Cells is Regulated by the Angiogenic Factor Epidermal Growth Factor-like Domain 7
（血管新生因子 Epidermal Growth Factor-like Domain 7 による造血幹細胞及び前駆細胞動態制御機構）

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Introduction: The ability of hematopoietic stem cells (HSCs) to provide for the sustained production of all blood lineages is accomplished by a balance between extensive HSC expansion characterized by purely symmetrical self-renewal divisions that occur during embryogenesis and at times of hematopoietic stress in the adult, and the homeostatic maintenance of HSC numbers that likely reflect asymmetrical self-renewal divisions. Epidermal growth factor-like domain 7 (Egfl7) is a factor expressed in the endothelium during embryogenesis. Egfl7 is a secreted protein that has been implicated in cell migration and blood vessel formation. Egfl7 has been shown to affect stem cell behavior, e.g. it stimulates embryonic stem cell proliferation. Furthermore, overexpression of Egfl7 or addition of recombinant Egfl7 decreased neural stem cell (NSC) proliferation and self-renewal and promotes differentiation of adult NSCs into neurons. The contribution of Egfl7 to hematopoiesis has not yet been described. In this study, I provided novel mechanistic data demonstrating that overexpression of Egfl7 accelerated hematopoietic cell recovery, especially of myeloid cells and platelets after Fluorouracil (5-FU). In addition, forced expression of Egfl7 in non-myelosuppressed mice resulted in leukocytosis, thrombocytosis and the mobilization of hematopoietic progenitor cells into circulation.

Results: I examined the expression of *Egfl7* in normal murine hematopoietic cells within the bone marrow (BM). *Egfl7* was expressed in primary murine BM mononuclear cells (MNCs), especially within the lineage-negative (Lin^-) cell fraction, and high expression

was detectable within the most immature (c-Kit$^+$/Sca-1$^+$/Lin$^-$/CD34$^+$) KSL$^-$/CD34$^+$ and KSL$^-$/CD34$^-$ fractions. The *Egfl7* expression profile in human and murine malignant hematopoietic cell lines varied from non-detectable in the promyelocytic leukemic cell lines to high expression levels in the megakaryocytic and myeloblastic cell lines. Mice were injected I.V. with adenoviral vector expressing Egfl7 (AdEgfl7) or an empty vector (AdNull). The AdEgfl7-treated mice had peak leukocyte levels, corresponding with an increase in granulocytes. Using flow cytometric analysis, I confirmed a rapid increase in the percentage of CD11b$^+$/Gr-1$^+$ neutrophils, but not CD11b$^+$/F4/80$^+$ monocytes in the PB and the BM of AdEgfl7-treated mice. To examine whether Egfl7 acts merely on committed granulocyte progenitors or acts on earlier HSC populations, I investigated the frequencies of more primitive progenitor populations within BM cells, including HSC-enriched KSL$^-$/CD34$^-$ cells using a flow cytometer. Forced expression of Egfl7 resulted in an increased frequency of HSC-enriched KSL$^-$/CD34$^-$cells within BM cells. I stained BM cells to identify the three myeloid progenitors by flow cytometry: Common-Myeloid Progenitor (CMP), Granulocyte/Monocyte Progenitor (GMP), Megakaryocyte/Erythroid Progenitor (MEP) cells. Associated with the HSC increase, there was an increase in the frequency of GMP and a decrease in the frequency of CMP within BMMNCs. To determine if the increase in peripheral blood leukocytes was due to an expansion of hematopoietic progenitor populations within the BM, standard colony assays in methylcellulose were performed. BM-derived colony forming unit (CFU) cells increased in AdEgfl7-treated mice. Most notably, predominantly immature CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) increased compared with AdNull-treated mice. Since Egfl7 increased the number of CFU-GEMM, which is a progenitor cell responsible for the generation of megakaryocytes, I next investigated the effect of Egfl7 on megakaryocytic (MK) development. I observed an increase in the number of MKs in H&E-stained and vWF-stained BM sections of AdEgfl7-treated mice. AdEgfl7 resulted in an increase in the number of platelets in circulation associated with an increased number of immature CFU-

MK progenitors within the BM. On the protein level, cultured BM cells derived from AdEgfl7-treated mice showed an increased release of total MMP-9 and KitL in culture supernatants and plasma. In search for factors released from endothelial cells, which might explain the hematopoietic phenotype observed after forced overexpression of Egfl7, I examined the expression of VEGF-A, and SDF-1α. BMMNCs derived from AdEgfl7-treated mice showed high gene expression for VEGF-A and SDF-1 α. Increased release of SDF-1α was also detected in supernatants of AdEgfl7-derived BMMNCs. Plasma SDF-1α levels rose rapidly in AdEgfl7-treated animals, which had been reported by to cause hematopoietic stem and progenitor cells (HSPCs) mobilization. Of interest, forced overexpression of Egfl7 mobilized hematopoietic progenitors, especially multi-lineage CFU-GEMMs, into circulation. Since Egfl7 overexpression increased KitL levels *in vivo*, and activation of the c-Kit/KitL pathway is required for hematopoietic regeneration after myelosuppression, I determined the role of Egfl7 in hematopoietic regeneration after myelosuppression. Myelosuppression induced by 5-FU results in apoptosis of cycling HSPCs, but does not affect HSCs in the G_0 stage of the cell cycle. During hematopoietic regeneration, quiescent (G_0/G_1) stem cells enter the cell cycle and differentiate. 5-FU-treated mice coinjected with AdEgfl7 showed enhanced recovery of white blood cells, platelets, and circulating $CD11b^+/Gr\text{-}1^+$ neutrophils, but not $CD11b^+/F4/80^+$ monocytes. Similarly, BM cell recovery as determined by BM cell counts and FACS analysis of $CD45^+$ hematopoietic cells was faster in AdEgfl7-treated 5-FU-mice. Egfl7 overexpressing mice showed a faster recovery of $CD11b^+/Gr\text{-}1^+$ cells and $CD11b^+/F4/80^+$ monocytes in BM cells. The percentage of polyploid megakaryocytes of >64N ploidy increased in AdEgfl7-treated mice as compared to controls, indicating stimulation of the megakaryocytic pathway. Cell cycle analysis revealed that the BM of AdEgfl7 treated myelosuppressed mice contained more cells that had been shifted into S and G2M phase of the cell cycle 7 days after myelosuppression. AdEgfl7 administration during hematopoietic recovery, as already shown for the steady state situation, resulted in a rise in plasma KitL,

exceeding the KitL elevation naturally found during hematopoietic recovery. Overexpression of Egfl7 during hematopoietic recovery increased the absolute number of primitive $KSL^+/CD34^-$ cells per femur and elevated the frequencies and numbers of GMP populations and MEP populations in BM cells. Associated with the GMP, MEP, and $KSL^+/CD34^-$ cell increase was a parallel increase in HSPCs as determined using a methylcellulose-based CFU assay.

Conclusion: Endothelial cell-derived stem cell regulators have been continuously emerging through the last decade, including Ang1/Tie2, *VEGF-A*, and N-Cadherin. In this study I show that the novel angiogenic factor Egfl7, is an important player in regulating the fate of HSCs by promoting HSPC expansion and mobilization. I used an adenoviral vector system to transiently overexpress the gene *in vivo* to understand the functional short-term effects of Egfl7 overexpression. Egfl7 overexpression by adenoviral delivery increased the number of leukocytes, especially of granulocytes, and circulating platelets. This pattern of enforced myeloid/megakaryocytic cell differentiation was also found on the progenitor cell levels. I showed that forced expression of Egfl7 increased plasma levels of soluble KitL and promoted HSPCs proliferation and differentiation. This is the first report to show that Egfl7 overexpression upregulates MMP-9 in the BM niche. I show that forced expression of Egfl7 elevates SDF-1α plasma levels. Taken all together, my data suggest that Egfl7 is a stem cell-associated factor and has a dual effect on HSCs where it induces both mobilization and differentiation most likely through here newly identified downstream targets of Egfl7 including SDF-1α and KitL. Moreover, my data indicate that Egfl7 is involved in the hematopoietic BM cell recovery after myelosuppression.

My findings might be important in the cancer field, where MMP-9 and SDF-1α are known critical players involved in the growth and metastasis of certain tumors. These promising results raise the possibility that Egfl7 can be clinically used to improve BM recovery after myelosuppression and can potentially be used for stem cell harvesting.