論文の内容の要旨

EGFL7 expands hematopoietic stem cells through modulating Notch signaling

（EGFL7 は、Notch シグナル伝達を通じて造血幹細胞増殖を制御する）

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Adult hematopoietic stem cells (HSCs) reside in a specialized microenvironment called the bone marrow (BM) niche where they are subjected to regulatory signals coming from the surrounding cells orchestrating their quiescence during homeostasis or their activation resulting in proliferation, self-renewal and differentiation (Mendelson and Frenette, 2014). The secreted, extracellular matrix associated angiogenic factor epidermal growth factor-like protein 7 (Egfl7) is expressed at high levels in the vasculature associated with tissue proliferation, like during cancer growth. Egfl7, expressed at low levels under hemostasis, but upregulated in BM cells during hematopoietic stress (after irradiation, chemotherapy). Although angiogenic factors can support HSC maintenance (Hattori et al., 2001; Heissig et al., 2002; Takakura et al., 2000), the effect of Egfl7 on HSC expansion and hematopoietic lineage differentiation is not yet well characterized.

Expression studies on cellular subsets within the BM revealed that Egfl7 expression was highest in murine HSC ($CD34^-KSL$) as determined by qPCR and by immunofluorescence microscopy on single sorted cells, while Egfl7 was downregulated during hematopoietic differentiation starting at the progenitor stage. Egfl7 knockdown (KD) in KSL cells led to a reduction in the number of KSL and progenitors after 4 days, and an increase in Annexin V^+ cells indicated that endogenous Egfl7 is critical for HSC survival. The CRU assay detects transplantable mouse HSC with the capacity to regenerate all of the blood cell lineages for extended time periods in vivo, and is a functional assays for the quantification of mouse HSC and progenitor cells. When Egfl7KD KSL were transplanted into wild-type recipients, the competitive repopulating ability was the same as shown for wild-type KSL,

indicating that the observed survival disadvantage of Egfl7KD KSL cells could be compensated in vivo by non-KSL cells (e.g. BM niche cells).

We next studied how Egfl7 could improve KSL cell survival. Cytokines like Kit ligand (KitL) and its receptor c-Kit convey survival signals in HSC. Spred1 is a negative regulator of c-Kit (Tadokoro et al.) and is a target of miR126 (Ji et al., 2016). Mechanistically, we show that Egfl7 enhanced c-Kit signaling in KSL and in the human erythroleukemic cell line HEL.

Integrin Beta 3 (*Itgb3*) KD in leukemic cells downregulated c-Kit (Miller et al., 2013). Megakaryocytes, macrophages and quiescent HSCs express integrin β_3 (Umemoto et al., 2006) (Umemoto et al., 2008). I confirmed that Similar to endothelial cells (Nikolic et al., 2013), I could show that mouse $CD34^-KSL$ cells express β3 integrin, and that Egfl7 binds to β3 on KSL. Egfl7 binding to β_3 integrin on Lin^- cells and (HEL) led to the upregulation of c-Kit expression, while β_3 knockdown on HEL cells impaired Egfl7-mediated c-Kit expression. The Egfl7/β3 binding on HEL cells enhanced the phosphorylation of AKT, STAT3, JAK-2, ERK1/2 and Tyr747 (β3), as shown using β_3 knockdown cells. These data indicated that Egfl7 upregulates c-Kit expression via β3 integrin.

Kit ligand (KitL) binds to the c-Kit receptor and enhances e.g. HSC survival. To establish the role of c-Kit signaling for Egfl7-mediated effects on HSC, I determined KSL cell survival after Egfl7KD in $c\text{-}Kit^{-/-}$ cells. Egfl7KD in wild-type, but not $c\text{-}Kit^{-/-}$ KSL cells induced KSL cell death in vitro. These data establish the significance of the c-Kit/KitL pathway for the improved cell survival of Egfl7 expressing cells. In addition, c-Kit was important for Egfl7-mediated thrombopioesis and myelopoiesis as shown in $c\text{-}Kit^{-/-}$ mice. Enforced Egfl7 expression increased the frequency of marrow competitive repopulation units (CRUs) in murine $CD34^-KSL$ HSCs after transplantation. Furthermore, Egfl7 enhanced KSL cell cycle progression. In contrast to the importance of the c-Kit pathway for thrombopoiesis and myelopoiesis, c-Kit was indispensable for Egfl7-mediated HSC expansion.

To test whether recombinant Egfl7 treatment augmented the number of functional long-term HSCs (LT-HSCs) in BM cells, we performed CRU assay. BM cells derived from Eglf7-treated exhibited mice higher reconstitution ability in the primary recipients when compared to control-treated mice. Reconstitution of BM cells in the secondary recipients was higher in mice transplanted with BM cells derived from Eglf7-treated compared to *non-Egfl7* treated cells, at 4 months post transplantation. These data suggest that Egfl7 expands adult BM repopulating HSCs *in vivo*. Furthermore, increases of Egfl7 achieved in suspension cultures by adding recombinant Egfl7 or achieved by overexpressing Egfl7 on endothelial cells similarly expanded murine KSL *in vitro*. I demonstrate that Egfl7-mediated HSC expansion was even higher in $\beta_3^{-/-}$ when compared to wild-type mice. Mechanistically, Egfl7 in the absence of b3 upregulates Notch and downregulates c-Kit signaling. These studies demonstrate for the first time that Egfl7 expands HSC through the alteration of three key stem cell active signaling pathways. BM regeneration after myelosuppression requires the reentry of HSC into the cell cycle, their expansion and terminal differentiation into all three hematopoietic lineages. I show that Egfl7 is upregulated under conditions of hematopoietic stress like after chemotherapy, and that enforced Egfl7 expression accelerated hematopoietic regeneration after myelosuppression.

Egfl7 is a promiscuous molecule, as it can bind to other receptors like platelet-derived growth factor and Notch1-4 receptors. Egfl7 antagonizes Notch receptor/ligand interaction by either binding to the receptor or its corresponding ligand (Durrans and Stuhlmann, 2010; Schmidt et al., 2009). The Notch1 receptor is found in immature $CD34^+$ cells (Milner and Bigas, Blood 1999). Next I examined whether Egfl7 altered Notch signaling in HSC. Similar to gamma secretase inhibitor treatment, Egfl7 overexpression in vivo inhibited Notch target gene expression (*Hes1* and *Hey1*) in KSL cells. Hes1 was reported to control FMS-Like Tyrosine Kinase gene (FLT3) expression on leukemic cells. I show that Egfl7 enhances Flt3 expression in KSL. These data implicate that Egfl7 enhances tyrosine kinase activity in HSC.

Thymic regeneration is a crucial function that allows for the generation of mature T cells after irradiation. Here, I report that Egfl7 is expressed on steady state thymic endothelial cells and further upregulated under stress like post-irradiation. Egfl7 overexpression increased intrathymic early thymic precursors (ETPs) and expanded thymic $Lin^{-}Sca1^{+}$ stromal, endothelial, and epithelial niche cells. Mechanistically, Egfl7 through Notch blockade enhances Flt3 expression in ETPs, and $Lin^{-}Sca1^{+}$ stromal, thymic epithelial and thymic endothelial cells. Thymic endothelial cells are a source of Flt3 ligand. Egfl7 activates the Flt3/Flt3 ligand pathway, and selective Flt3 blockade prevented the Egfl7-driven ETP and thymic niche cell expansion.

We identify Egfl7 as a critical factor that under hematopoietic stress conditions controls HSC fate within the BM and early progenitors in the thymic niche by highjacking and controlling critical stem cell active cytokine pathways that allow for hematopoietic and thymus regeneration. We propose that Egfl7 links endothelial biology to the production of T cell-based adaptive immunity and stem cell maintenance. We summarize our findings in the model below: