Doctoral Thesis

博士論文

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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CONTENTS

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ABBREVIATIONS

Ab	antibody
Itgb3	integrin β3
BM	Bone marrow
BMMNC	bone marrow mononuclear cells
CB	cord blood
CGD	Cilengitide
DMSO	Dimethyl Sulphoxide
EC	endothelial cell
ECM	Extracellular matrix
EGF	epidermal growth factor
EGFL7	epidermal growth factor–like domain 7
ETP	early thymic progenitor
FBS	fetal bovine serum
Flt3	FMS-like tyrosine kinase 3
FN	fibronectin
HSPCs	hematopoietic stem and progenitor cells
HSC	hematopoietic stem cells
HUVEC	Human Umbilical Vein Endothelial Cells
IL	Interleukin
KD	knockdown
KitL	Kit ligand
KSL	c-Kit^{+} Sca-1^{+} Lin^{-}
miRNA	micro RNA
DLL1	Delta-like-1
P/S	penicillin/streptomycin
qPCR	quantitative polymerase chain reaction
Rec.	recombinant
RT	room temperature
TKF	Thrombopoietin, KitL), Flt-3 ligand
VEGF	vascular endothelial growth factor

Abstract

Endothelial cells (ECs) and their growth factors are required for tissue regeneration, cancer progression, and stem cell maintenance. How these angiogenic factors modulate hematopoietic stem cell (HSC) fate is widely unknown. Epidermal growth factor–like domain 7 (*Egfl*7) is released by endothelium and deposited into the matrix. Here, I hypothesized that Egfl7 through its cellular binding to β3 integrin (Itgb3) context dependent titrates Notch signaling, thereby fine-tuning HSC and early progenitor fate dependent on the niche requirements. I demonstrate that ECs, and early stem and progenitor cells in the BM (HSC) and thymus (early thymic progenitors (ETPs), express Egfl7 especially during tissue regeneration.

Egfl7 expands HSCs and enhances their self-renewal capacity by activating Notch signaling under Itgb3 deficient conditions. In contrast, when tissue regeneration is required Egfl7 enhances proliferation and differentiation of Itgb3-expressing HSC and progenitors by inhibition of Notch signaling. Egfl7 drives myeloid differentiation and enhances HSC survival of Itgb3 positive HSCs and progenitors by inducing c-Kit and Flt3 receptor expression and signaling.

My results highlight the impact of the ECM-bound molecule Egfl7 on the regulation of stem cell fate by fine-tuning Notch and stem cell-active cytokine signaling pathways dependent on the cellular Itgb3 status.

Introduction

I. The angiogenic factor Egfl7

Epidermal growth factor–like domain 7 (*Egfl*7, also known as vascular endothelial statin - *VE-statin*) is expressed in the endothelium during embryogenesis and during cancer growth. The mouse and human *Egfl7* genes are located on chromosome 2 and 9, respectively (Fitch et al., 2004). The mouse gene spans 11.5 kb and consists of 11 exons (Soncin et al., 2003) with a micro RNA (miRNA), miR126, located between exons 7 and 8 (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008). The Egfl7 protein (29 kDa) is composed of several putative domains: a cleavable signal peptide at the N-terminal end, an EMI domain, found on extracellular matrix (ECM) proteins (Doliana et al., 2000), two epidermal growth factor (EGF)-like domains a leucine and valine rich C-terminal region. The Eglf7 protein is secreted from endothelial cells (ECs) and is deposited in the ECM perivascular. Overexpression of *Egfl7* in mice results in abnormal patterning and remodeling of blood vessels (Nichol et al., 2010). *Tie2-Egfl7* transgenic mice overexpressing endothelial Egfl7 mRNA, without altering miR126 levels, exhibit partial embryonic lethality, which is accompanied by hemorrhaging and abnormal vascular patterning. Antibodies targeting Egfl7 prevented tumor neoangiogenesis by targeting Itgb3 expressing tumor ECs (Johnson et al., 2013; Parker et al., 2004).

The first EGF-like domain of Egfl7 has a region similar to the Delta/Serrate/Lag-2 domain found in ligands of the Notch receptors family (Fleming, 1998), the second EGF-like domain is predicted to bind Ca^{2+}. Egfl7 was shown to antagonize Notch receptor/ligand interaction by binding to the receptor or its corresponding ligand on neuronal stem cells (Durrans and Stuhlmann, 2010; Schmidt et al., 2009).

Egfl7 or miR126 change the cellular behavior of cancer niche cells like EC, mesenchymal stem cells, and fibroblasts (Yang et al., 2016; Zhang et al., 2013). miR-126/miR-126* inhibit stromal cell-derived factor-1 alpha (Sdf-1α) expression suppressing mesenchymal stem cell recruitment by lung cancer cells in a mouse xenograft model (Zhang et al., 2013). mir-126* is the complementary strand to mir-126 which forms once the double stranded pri-miRNA is cleaved and the two strands denature, separating. mir-126* is less abundantly found in organisms than mir-126 and fewer roles in regulating gene expression have been identified.

miR-126/miR-126* expression is downregulated in cancer cells by promoter methylation of their host gene *Egfl7*.

GATA transcription factors can regulate gene expression in hematopoietic cells.
The *Egfl7* promoter also contains a GATA-2 consensus binding site (Le Bras et al., 2010), suggesting that Egfl7 could play a role in hematopoiesis. miR-126 knockdown (KD) in normal hematopoietic stem/progenitor cells (HSPCs) was reported to expand long-term HSCs by controlling multiple targets within the PI3K/AKT/GSK3β pathway in HSC/early progenitors (Lechman et al., 2012). During malignant hematopoiesis, like in acute myeloid leukemia (AML), miR-126 likely functions as an oncogene (de Leeuw et al., 2014; Li et al., 2008) (Dorrance et al., 2015). However, miR-126 function seems to 2-faceted in leukemia where it either can promote or suppress progression of leukemic growth (Li et al., 2015).

During my PhD studies, I examined the role of Egfl7 for normal hematopoietic stem and progenitor cell (HSPC) development.

II. Hematopoiesis

Within the hematopoietic system, only HSCs possess the ability of both multipotency and self-renewal (Seita and Weissman, 2010). Multipotency is the ability to differentiate into all functional blood cells. Self-renewal is the ability to give rise to HSC itself without differentiation. Since mature blood cells are predominantly short-lived, HSCs continuously provide more differentiated progenitors while properly maintaining the HSC pool size throughout life by precisely balancing self-renewal and differentiation (Figure 0). But signals that regulate this balance of self-renewal and differentiation are not well defined.

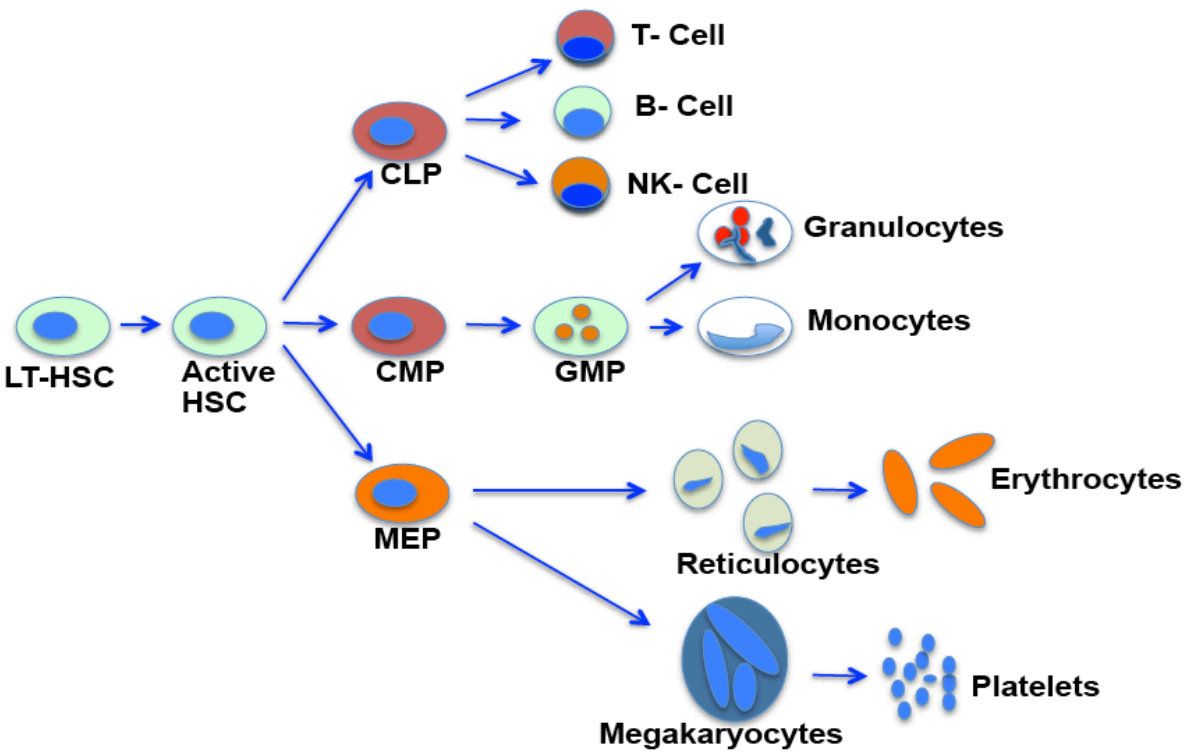


Figure 0. **Impaired proliferation of murine *Egfl7* knockdown KSL/progenitors**
During hematopoiesis, long-term HSC (LT-HSC) can respond to extracellular singals and differentiate into cells of the erythroid, lymphoid, and myeloid lineages.

Although several markers have been used to identify human HSCs, CD34 is most commonly used for clinical use and the enrichment of human HSCs. HSCs from adult mouse bone marrow (BM) are c-Kit^{+} Sca-1^{+} Lin^{-} (KSL) cells and show low or no expression for CD34.

These CD34-KSL cells show myelolymphoid long-term reconstitution in lethally irradiated recipients after a single cell transplant (Osawa et al., 1996).

The low number of HSC in the adult BM hinders their use in the clinic. HSCs are rare with estimated frequencies of 1 in 10,000 BM cells and 1 in every 100,000 blood cells, suggesting a limiting number of true niches. A small pool of HSCs exists in the BM during adulthood in a quiescent state. This process is tightly controlled by a specific microenvironment, the so called BM niche that consists of the ECM and a plethora of stromal cells and non-hematopoietic cells like ECs, osteoblasts and fibroblasts (Morrison and Scadden, 2014). A crosstalk of tissue-specific stem cells and their progenitors and ECs has been reported in stem cell niches harboring neuronal (Delgado et al., 2014; Shen et al., 2004), mesenchymal (Dhahri et al., 2016), or HSCs (Poulos et al., 2015).

The niche cell composition dictates HSC numbers as shown e.g. in genetically engineered mice with increases in osteoblast numbers that led to elevated HSC numbers without changes in committed progenitor populations (Zhang et al., 2003). ECs are another niche cell type important for HSC maintenance. Others and I have demonstrated that HSC can be found in close vicinity to ECs, especially after myelosuppression (Heissig et al., 2002), and that EC-derived growth factors like vascular endothelial growth factor (VEGF-A), placental growth factor or Kit ligand (KitL, also known as stem cell factor) can enhance hematopoiesis (Avecilla et al., 2004; Hattori et al., 2002).

HSCs proliferate and differentiate following hematological stress like bleeding, irradiation or chemotherapy in order to regenerate the blood cell pool. In the BM HSCs are anchored through cell-matrix adhesion and maintained by growth factors. A few growth factor receptors/ligands have been shown to play essential roles in HSC function, namely c-Kit ligand (KitL)/c-Kit, TPO/c-Mpl, and FMS-like tyrosine kinase 3 (Flt3). Investigations of two

mast cell-deficient spontaneous mutant mouse strains, the W strain (Russell, 1949), and the Sl strain (Murphy et al., 1992), lead to the concept that the W locus was critical for HSCs while the Sl locus was important for signals from HSC niche cells (Mayer and Green, 1968; Russell and Bernstein, 1968). In 1990, the critical gene in the W locus, the cytokine receptor-tyrosine kinase c-Kit was identified (Nocka et al., 1989). At approximately the same time, the important gene in the Sl locus was shown to encode KitL or Stem Cell Factor (Zsebo et al., 1990).

Another important stem cell active cytokine receptor is FMS-like tyrosine kinase 3 (Flt3), which is expressed on approximately 60% of KSL cells. KSL $Flt3^+$ cells rapidly and efficiently reconstituted B and T lymphopoiesis, whereby myeloid reconstitution was short term. Activation of Flt3 failed to support survival of HSC.

Notch signaling is an important cell-cell communication system. The receptor is normally triggered via direct cell-to-cell contact, in which the transmembrane proteins of the cells in direct contact form the ligands that bind the Notch receptor. Notch signaling appears to inhibit differentiation programs that accompany Wnt-induced proliferation (Duncan et al., 2005). However, genetic ablation studies suggest that at least some aspects of these pathways may be dispensable for *in vivo* HSC function (Duncan et al., 2005).

Although HSC fate is dictated by external signals from the microenvironment through cell-cell and cell-matrix interactions, factors to attune these cell-cell or cell-matrix interactions are not well defined. Active self-renewal and differentiation of HSCs is probably more likely to happen in the vascular niche, and was shown to be partly mediated by Notch signaling (Butler et al., 2010a). The EC-derived Egfl7 is a promiscuous molecule. It can mediate integrin (cell-matrix) and Notch (cell-cell)-mediated signals.

III. Thymogenesis

Impaired thymic function occurs in elderly people, after myelosuppression like chemotherapy or irradiation for cancer patients, and after myelosuppression used in preparation for HSC transplantation that leads to a malfunction of the immune system with an increased risk of infection, and ultimately death. Determining factors that control thymogenesis in the regenerating thymus will lead to a better understanding of the mechanisms underlying the limited organ regenerative capacity during aging, and after allogeneic hematopoietic stem cell transplantation. Strategies to improve thymic functions are therefore desirable. T cell reconstitution of the recipient immune system after allogeneic hematopoietic cell transplantation is largely dependent on replication of donor T cells infused with the BM (Weinberg et al., 2001), and can take several months to even years (de Koning et al., 2016).

The thymic microenvironment is the cradle of T cell development. It represents a spectrum of developing T lymphoid cells, hematopoietic (mainly B cells, macrophages, and dendritic cells) and stromal cells like thymic epithelial cells, and thymic ECs. Myelosuppression after total body irradiation augments the release of other endothelial-derived factors like VEGF-A, and the Notch ligand Jagged-1 (Heissig et al., 2005; Hooper et al., 2009). Thymic ECs are resistant to damage as induced by chemotherapy, and sublethal total body irradiation. Yet, the role of thymic ECs and/or their released factors for steady state and stress-induced thymogenesis is unclear. The $Flt3^+$ fraction of Lin^- $CD25^-$ Kit^+ early thymic progenitor (ETP) (Lin^- $CD25^-$ Kit^+ $Flt3^+$) harbors canonical intrathymic T cell progenitors (Adolfsson et al., 2005; Allman et al., 2003). Flt3 or Flt3 ligand (Flt3L) deficient mice display defects in very early T cell development (Adolfsson et al., 2001; Sitnicka et al., 2007). In addition, Flt3 has been shown to activate Stat3 and Stat5a signaling mediators.

Notch signaling is essential for T lineage cell differentiation including T versus B and αβ versus γδ lineage specification (for review see (Li and von Boehmer, 2011)). The c-Myc proto-oncogene has been identified as a critical direct downstream target of Notch in leukemogenesis (Palomero et al., 2006). A stage-specific requirement for Notch signaling at the αβ and γδ T lineage bifurcation has been reported (Ciofani et al., 2006; Garbe et al., 2006).

Notch receptor-ligand interactions are necessary for early T cell development. The earliest intrathymic T-cell precursors, which are characterized by high expression of c-Kit receptor and low expression of the interleukin 7 receptor alpha chain (IL7R), are found in the double-negative DN1 thymocyte subset ($CD4^{-}CD8^{-}CD25^{-}CD44^{+}$) (Sambandam et al., 2005). Notch signaling is required not only for generation of the ETP population but also for transitions of ETP-to-DN2 and ETP-to-DN3 suggesting that Notch1 activation is needed continuously to promote survival or proliferation throughout the early stages of intrathymic T-cell development (Sambandam et al., 2005).

IV. αvβ3 integrin

Integrins connect the cytoskeleton with the ECM. They consist of two subunits, a and b, which are noncovalently associated with each other. Upon ligand binding, the cytoplasmic tail contacts cytoskeletal filaments and proteins to initiate a signaling cascade, including a series of intracellular signaling events that start from the recruitment and activation of Src kinases via phosphorylation of focal adhesion kinase (FAK).

avb3 integrin (vitronectin receptor) binds to ECM molecules like fibronectin, fibrinogen, vitronectin, and associate with fibroblast growth factor-2, matrix metalloproteinase-2, activate PDGF, insulin and VEGF receptors, thereby facilitating cell proliferation invasion and preventing apoptosis (Kumar, 2003). Integrin $\alpha_v\beta_3$ is highly expressed on activated ECs and new-born vessels, but is absent in resting ECs and most normal organ systems, making it

a suitable target for anti-angiogenic cancer therapy. αvβ3 seems to be the most important integrin during tumor angiogenesis. The inhibition of integrin αvβ3 signaling with antibodies (e.g. Avastin^{R}, Centocor^{R}), peptides, peptidomimetics, and other antagonists has shown great potential in the treatment of cancer. There are antagonists that specifically bind to integrin $\alpha_v\beta_3$, such as disintegrins, peptides (mostly RGD-based) and nonpeptidic molecules. Among the integrin inhibitors, the RGD mimetics are the best known. One of them is a head-to-tail cyclized RGD-containing pentapeptide, c(RGDf[NMe]V) (also known as Cilengitide), can bind to integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ with high affinity (Liu et al., 2008). Integrin $\alpha_v\beta_3$ expression in multiple types of solid tumor stem cells is now shown to control a pro-survival pathway that contributes to therapy resistance (Seguin et al., 2014). Expression of Integrin beta3 is correlated to the properties of quiescent HSCs (Umemoto et al., 2006). Genetic knockout models have been created and helped to understand the role of angiocrine factors and growth factors in stem cell biology as well as during development and disease.

Genotype	Phenotype
miR126 $^{-/-}$	EC-restricted microRNA (miR-126) miR-126 enhances the proangiogenic actions of VEGF and FGF and promotes blood vessel formation by repressing Spred-1 expression (Wang et al., 2008)
Egfl7transgenic	Egfl7 and EC-specific NOTCH physically interact *in vivo* and strongly suggest that Egfl7 antagonizes Notch in both the postnatal retina and in primary ECs (Nichol et al., 2010)
c-kit $^{-/-}$ (Kit-Kit^{w-v})	*Kit* mice possess pleiotropic defects in pigment-forming cells, germ cells, RBC's and mast cells. In addition, they exhibit impaired resistance to parasitic infection and an intrinsic progenitor cell defect. *Kit^{W-v}* homozygotes resemble *Kit^{W}* homozygotes in color, anemia, and germ cells, but many of them survive to maturity (Huang et al., 1990) (Fleishman, 1996)
FLT3$^{-/-}$	Mutants developed into healthy adults with normal mature hematopoietic populations. However, they possessed specific deficiencies in primitive B lymphoid progenitors. Bone marrow transplantation experiments revealed a further deficiency in T cell and myeloid reconstitution by mutant stem cells (Mackarehtschian et al., 1995)
Beta3 $^{-/-}$	Mice are viable at birth with bleeding diathesis due to a platelet defect, the murine homologue of Glanzmann's thrombasthenia. Osteoclast functional defect in the bones of mutant mice were reported (Hodivala-Dilke et al., 1999)
Notch1-/-	Competitive repopulation of lethally irradiated wild-type hosts with wild-type- and Notch1-deficient BM revealed a cell autonomous blockage in T cell development at an early stage, before expression of T cell lineage markers (Radtke et al., 1999). Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome(Stier et al., 2002)

Table 1: Summary of genes important for this study and the murine knockout phenotype, with special focus on the hematopoietic phenotype.

Hypothesis

Hematopoiesis and thymopoiesis allow for the generation mature blood cells both under steady state and following stress situations like ischemia, myelosuppression, or during cancer growth. αvβ3 integrin expression in multiple types of solid tumor *stem cells* is now shown to control a pro-survival pathway. ECs located e.g. in the stem cell niche release angiogenic factors that can regulate steady state and stress-induced hematopoiesis and thymogenesis. The angiogenic factor epidermal growth factor–like domain 7 (Egfl7) is a promiscuous molecule, because it can bind to to stem cell-associated receptors like the platelet-derived growth factor-beta receptor, Itgb3 and Notch1-4 receptors.

Here, I hypothesized that Egfl7 dictates HSC and early progenitor fate by fine-tuning Notch signaling through its ability to bind to b3 integrin, thereby altering critical stem cell signaling pathways, like the c-Kit, Flt3 signaling pathways.

Material and Methods

Experimental Animals and human cord blood samples

C57BL/6 mice (6-8 weeks old) were purchased from SLC, Inc. (Shizuoka, Japan). Animal experiments were conducted in accordance with the guidelines and approval of the Institutional Animal Care and Usage Committee at the Institute of Medical Science, The University of Tokyo. WBB6F1/Kit-Kitw-v/Slc (c-Kit$^{-/-}$) and wildtype control mice were purchased from Nihon Clear. Itgb3-/- mice on a c57/Bl6 background were kindly provided by Terumasa Umemoto (Kumamoto University, Japan). All human cell-associated experiments were approved under a protocol from the ethics committee of The Institute of Medical Science, The University of Tokyo.

In vivo manipulation of mice

C57/BL6 were injected with a single intraperitoneal dose of 5-fluorouracil (5-FU) at 150 mg/kg of body weight.

Groups of whole-body irradiated mice (6-8 wks old; 2 Gy using ^{137}C) were given AdEgfl7 or AdNull in the tail vein. Thymic recovery was determined 3 days (d) after irradiation of the mice. At d 0, mice were irradiated with a single dose of 2 Gy.

Overexpression of Egfl7 using adenoviral injections

Adenoviral vectors expressing Egfl7 or containing no transgene were kindly provided by Matthias Friedrich and Dirk Dikic (Institute of Biochemistry II, Johann Wolfgang Goethe University School of Medicine) (Picuric et al., 2009; Schmidt et al., 2009). In brief, this replication-deficient adenovirus is based on adenovirus type 5, which lacks the EIA, E1B, and E3 regions of the virus and contains the SRa promoter, human *Egfl7* cDNA, and SV40

poly(A) signal sequences inserted into the El-deleted region. Null Adenovirus (AdNull, empty vector), contains the SRa promoter and SV40 poly(A) signal. Purified virus stocks were prepared through CsCl step gradient centrifugation as described (Kanegae et al., 1994). Mice were intravenously injected with 2 x 10^9 plaque-forming units of AdEgfl7 and AdNull in the tail vein. Mice were sacrificed on d3 for thymus collection or at indicated time points for BM collection. Peripheral blood samples obtained were collected using heparin-coated capillaries.

Blocking experiments in vivo

AdEgfl7 or AdNull was co-injected with the β3 inhibitor Cilengitide (EMD 121974) (Selleckchem) by i.p. at a concentration of 1mg/kg for three times per week. In some experiments, mice received Flt3 inhibitor (Tandutinib) (30mg/kg) or PBS orally daily at d 0, 1, and 2.

Competitive Transplantation Experiments and long-term engraftment

Egfl7 KD KSL transplantation. c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) cells were sorted and tranduced using shRNAs targeting *Egfl7* (see below for details) and cultured in 96-well plates (4×10^2 cells per well) with serum-free medium (S-CLONE SF-O3; Iwai North America Inc., cat. No. SJU: 1303) supplemented with thrombopoietin (TPO, 50 ng/mL), Kit ligand (KitL, 100 ng/mL), and Flt-3 ligand (Flt-3L, 50 ng/mL) (PeproTech, Rocky Hill, NJ). Cells were cultured further for 4 days. Next, the lentivirus-transfected KSL cells (2×10^2) were mixed with normal BM cells (5×10^5) and transplanted into lethally irradiated mice (9.5 Gy) retro-orbital. Peripheral blood cells of the recipient mice were analyzed 2 months after

transplantation. The percentage of donor-derived lineage contributions in PBMCs was assessed using antibodies against CD45.2, Gr-1/CD11b, B220, CD19, CD4, and CD8. Total chimerism of more than 1% for all antibodies tested using PBMCs was considered as long-term reconstitution. The frequencies were determined using FlowJo software (TreeStar, Ashland, OR, USA).

Cell culture and transfection

Human erythroleukemic (HEL) cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), and P/S. HEL cells (1×10^5) were seeded in 24-well plates for 24 h. HEL cells were transiently transfected by using Lipofectamine RNAiMAX (Invitrogen). siRNA targeting β3 integrin was designed using BLOCK-iT™ RNAi Designer (Invitrogen). *Itgb3*-Sense: CCAAGACUCAUAUAGCAUU, *Itgb3*-Antisense: AAUGCUAUAUGAGUCUUGG, control-Sense: CCACUCAUAUACGAGAAUU, control-anti-sense: AAUUCUCGUAUAUGAGUGG.

Lentiviral production

shRNA cloning. shRNAs was designed using BLOCK-iT™ RNAi Designer. shRNAs targeting *Egfl7* (Fasmac) *Egfl7*shRNA-

5'-CACCGCTTGTGGAGCAGCAATATGCCGAAGCATATTGCTGCTCCACAAGC -3'

and Bottom strand-m*Egfl7*shRNA-

5'-AAAAGCTTGTGGAGCAGCAATATGCTTCGGCATATTGCTGCTCCACAAGC -3'

Top Strand-scramble-

5'- CACCGGAGACGGAGGATAGTCTTCGAAAAGACTATCCTCCGTCTCC-3'

Bottom Strand-scramble-5'-

AAAAGGAGACGGAGGATAGTCTTTTCGAAGACTATCCTCCGTCTCC-3' were cloned into CS-Ubc-GFP vector. Gene knockdown efficiency in LSK cells was quantified by qRT-PCR.

Vesicular stomatitis virus glycoprotein–pseudotyped lentivirus was prepared as previously described via a three-plasmid system (Target vector, pMDL, and vesicular stomatitis virus glycoprotein envelope plasmid) by co-transfection of 293T cells using polyethylenimine (PEI, Takara, Japan). Viral supernatant was collected 48 hrs later, cleared, and stored at −80°C. Viral titration was performed using 293T cells.

Adhesion assay.

Preparation of decellularized, Egfl7-coated plates: To generate protein deposition on precoated dishes, HEK293 cells were infected with adenovirus expressing the full-form (AdEgfl7), the RGD deletion form (AdRGD del) of Egfl7, or a control vector (AdNull). Cells were trypsinized leaving behind deposited Egfl7 protein variants on the dishes after 16 hrs.

Adhesion: Lin- cells were cultured for 4 hrs on the described pre-coated decellularized 96 well microplates (IWAKI, Japan). Then, cells were fixed with 4% formaldehyde in PBS for 10 min, followed by a washing step using 0.1% BSA/Dulbecco's Modified Eagle's Medium (DMEM) buffer. Then, cells were stained with 0.5% crystal violet for 10 min, washed with tap water. 2% SDS was added to dried plates. The plate were incubated at RT for 30 min, and the absorbance was detected at 550nm using a mircoplate reader (Molecular Devices, USA).

Flow cytometry and cell sorting

Tissue preparation: Thymi were minced, and thymus single cell suspensions were blocked with 2% FBS, washed and stained. Murine BM cells were obtained after flushing femurs and tibiae.

Murine KSL analysis by FACS: PBMNCs and BMMNCs were incubated with the following antibodies: CD45-Pacific Blue (clone 30-F11; BioLegend, San Diego California), CD11b-FITC (clone M1/70; BD Biosciences, San Jose, California), F4/80-PE (clone BM8; BioLegend), Gr1-APC (clone RB6-8C5; BD Biosciences), c-kit (CD117)-APC (clone 2B8; BioLegend), biotinylated lineage cocktail antibodies (Miltenyi Biotec), Sca-1-PE.Cy7 (clone D7; eBioscience), CD16/32 (FCγRII)-PE (clone 93; eBioscience), CD34-FITC (clone RAM34; BD Biosciences), CD61-FITC(BD Biosciences). PI fluorescence was measured, and PI positive cells (dead cells) were excluded. Cells were then analyzed with the use of a BD FACS AriaII (Becton Dickinson, Franklin Lakes, New Jersey). Detection and gating protocol of $CD34^-KSL$ cells was previously reported (Morita et al., 2006).

MACS sorting. BMMNCs were stained using a lineage cell depletion kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After running through 2 MACS columns (Miltenyi Biotec) more than 90% of the separated cells were lineage negative (Lin^-) by FACS.

For thymic EC isolation thymocytes were stained with CD45 and CD31 magnetic beads and isolated by MACS (Miltenyi Biotec).

FACS sorting. MACS-isolated Lin- cells were stained with above-mentioned specific antibodies. FACS sorting was performed to isolate the following cell populations: $CD34^-$ c-$kit^+Sca\text{-}1^+Lin^-$ ($CD34^-KSL$) cells, $CD34^+KSL^-$ cells.

Flow cytometry analysis and sorting were performed on a FACS Verse and Area (BD Biosciences) flow cytometers. Data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

Cell cycle analysis. $5x10^5$ BMMNCs were suspended in 1ml of warm medium at 10^6 cells/ml and mixed with Hoechst 33342 (Dojindo, Japan) at 10 μg/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 20 min, centrifuged and resuspended in FACS buffer for subsequent flow cytometry analysis.

Cell cultures

Egfl7 overexpression on HUVEC. Human umbilical vein ECs (HUVECs) were cultured on 0.1% gelatin (Wako Pure Chemicals, Japan)-coated culture plates (Falcon) in endothelial growth medium EGM-2 (Lonza, cc4176) supplemented with 100 U/ml penicillin and 100 ug/ml streptomycin (P/S; WAKO, Osaka, Japan) at 37°C, 5% CO2. HUVEC were transfected with AdEgfl7 or AdNull containing no transgene at multiplicity of infection (MOI) of 30.

Human and mouse stem cell and progenitor proliferation assay

ETP Proliferation assay. 1×10^3 FACS sorted ETP cells per well were cultured in Dulbecco's modified Eagle's medium containing 20% FBS supplemented recombinant mouse Kit ligand (PeproTech, 50 ng/ml), mouse IL-7 (PeproTech, 50 ng/ml), mouse Flt3 ligand (PeproTech, 50 ng/ml) with or without recombinant Egfl7 (Abnova, 300 ng/ml) at the start of the culture. The Flt3 inhibitor (Tandutinib, ChemScene) was added at a concentration of 25uM to

indicated cultures. On d 3 of culture, cells were counted, and the percentage of ETP was determined using FACS.

Murine KSL Proliferation assay: I supplemented liquid suspension cultures of bone marrow murine $CD34^{-}$KSL cells (200 per 96-well plate) with Iscove's modified Dulbecco's medium plus 10% FBS, 1% P/S, 50 ng/ml thrombopoietin, 100 ng/ ml Kit ligand and 50 ng/ml Flt-3 ligand (from now on abbreviated as TKF) with or without recombinant murine Egfl7 at indicated concentration for the indicated time. If the Egfl7 concentration was not specifically mentioned, rec. Egfl7 was added at 300 ng/ml (Abnova).

Some cultures were supplemented with inhibitory reagents: Inhibitors for Itgb3 (Cilengitide; 5μM), and Flt3 (Tandutinib, ChemScene; 25uM) were added at day 0. Cells after 5 days in culture were collected and either analyzed by FACS or samples were prepared for qPCR and Western Blot.

Human cord blood Proliferation assay: Human cord blood (CB) units were obtained from the Red Cross Japan. MACS isolated human $CD34^{+}$ cells were cultured (5×10^{3} CB $CD34^{+}$ cells) in 200 ul medium supplemented with TKF cytokines. Rec. Egfl7 was added at a concentration of 300 ng/ml. Phenotypic analysis of cultured cells was performed after 5 days in culture using FACS. Cells were subjected to colony assays, and 14-d CFC progenitor cell counts were performed.

Colony Formation Assays. Colony formation was examined with the use of Methocult medium (Stemcell Technologies, Vancouver, British Columbia, Canada).

Op9-DLL1 and HSC co-cultures: Op9 mouse fibroblasts and Op9-Delta-like-1 (DLL1) cells (provided by RIKEN, Japan) were maintained using Minimum Essential Medium-Alpha (α-MEM) culture medium supplemented with 10% FBS, and 1% P/S.

To investigate the effects of the Notch activation on stem cells in vitro, OP9 and OP9-DLL1 cells were plated at 95% confluency, and cells were used as a feeder layer. KSL cells were cocultured for 1 week (initial cell input 5 x 10^2 cells) with OP9 stromal cells in a 12-well plate containing α-MEM (Sigma) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). The cocultures were performed in the presence or absence of Flt3 inhibitor (Tandutinib, ChemScene; 25uM). The KSL cells were collected after 1 week and assayed for FACS.

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.

Western blot analysis

Cultured HUVEC transfected with 30 MOI AdEgfl7 or AdNull were lysed with lysis buffer (Cell Signaling Technology). In some experiments BMMNCs were lysed. Protein crude was recovered by acetone. Cell lysates (2~50μg proteins) were applied on 10% acrylamide gel, transferred to PVDF membrane (Millipore, Immobilon), blocked, then stained overnight at 4°C for Egfl7 (Santa Cruz Biotech, sc-34416), c-Kit (Santa Cruz Biotech, sc-5535), FAK (Merk Millipore, #07-832), Hes1 (Santa Cruz Biotech, sc-25392), ß-actin (Cell Signaling, #4967), p-ERK (Cell Signaling, #4370), p-AKT (Cell Signaling, #9271), Tyr747 (Santa Cruz Biotech, sc-20234), p-JAK-2 (Merk Millipore, #07-706), p-STAT3 (Cell Signaling, #9131),

FLT3 (Proteintech, 21049-1-AP), and NICD (cleaved Notch1 (Val1744) (D3B8) rabbit mAb #4147, Cell signaling). Membranes were stained with secondary antibody conjugated with horseradish 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

Flt3 ligand plasma levels were measured using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's protocol.

Immunostaining

Thymi were embedded in OCT compound (Sakura), and frozen. Tissue sections (5μm) were cut with an OM cryostat (HM500; Microm) and collected onto Superfrost/Plus slides (Fisher Scientific). For immunohistochemical staining of mouse thymus, 4% formaldehyde fixed cryo-sections were blocked with 5% BSA in PBS solution, and stained over night at 4°C with goat anti-Egfl7 (Santa Cruz Biotech, sc-34416), anti-Flt3 (proteintech, 21049-1-AP), and anti-CD31 (Santa Cruz Biotech, sc-28188). After two washing steps, tissue sections were incubated for 1 hrs at RT with Alexa Fluor 488 rabbit anti-goat IgG, Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 goat anti-rabbit IgG and Alexa Fluor 488 donkey anti-rabbit IgG, respectively. Sections were counterstained with DAPI.

In droplet immunostaining of KSL cells. 10% poly-L-lysine coated glass slides with 4 mm diameter holes (Matsunami TF2404) were washed with distilled water and dried. 20 μl of S-CLONE SF-O3 medium (Iwai, Tokyo, Japan) was applied each hole and CD34-KSL cells were sorted onto the slide by FACS sorting. After 60 min, cells were fixed with 4%

paraformaldehyde for 10 minat RT. Slides were then washed twice with PBS, and Max Block Blocking (Active Motif) applied for 30 minutes at RT. Cells were incubated with diluted antibodies against Egfl7 (Santa Cruz Biotech, sc-34416), c-Kit (Santa Cruz Biotech,sc-5535), and Biotin mouse Ly-6A/E (Sca-1) (Biolegend) overnight at 4 °C. The next day, the cells were washed three times with PBS and incubated for 60 minutes at RT. with diluted Alexa Fluor 488 rabbit anti-goat IgG, Alexa Fluor 546 donkey anti-rabbit IgG, PE-Streptavidin, respectively. Slides were then mounted with Fluorescent Mounting Medium (Dako), and imaged by confocal microscopy (Olympus).

Statistical analysis

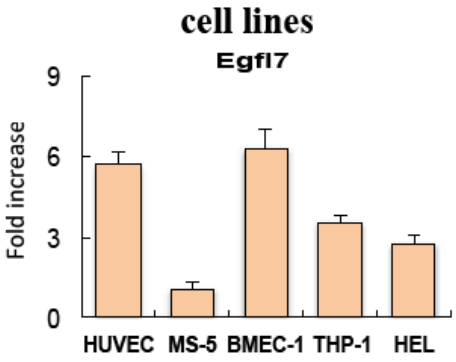
Results are presented as means ±SEM. Statistical comparisons were based on Student's *t* test or ANOVA with Tukey HSD posthoc tests using R program. P value level of <0.05 was considered significant.

Results

I. HSCs express Egfl7

Epidermal growth factor–like domain 7 (Egfl7) affects endothelial cells (ECs), neuronal and embryonic stem cell behavior (Durrans and Stuhlmann, 2010; Schmidt et al., 2009). To define the hematopoietic and stromal cell types within the BM niche, primary cells and cell line were tested by qPCR. High *Egfl7* expression was detected in the human BM EC line (BMEC-1), in Human Umbilical Vien Endothelial Cells (HUVEC, positive control), the human macrophage cell line THP-1, and the human erythroleukemica cell line HEL, while low *Egfl7* expression was found on the murine stromal cell line MS-5 (Figure 1A). qPCR analysis of murine primary BM cells revealed that *Egfl7* expression was highest in the most primitive HSC fraction, the CD34- c-Kit^{+} Sca1^{+}Lin^{neg} (KSL) cells, and was gradually downregulated on FACS-purified hematopoietic progenitors like common lymphoid progenitors (CLP), megakaryocyte erythroid progenitor (MEP), common myeloid progenitors (CMP) and granulocyte macrophage progenitor (GMP), which were identified by the antigen marker profile as mentioned in the figure legend of Figure 1, and total BM (Figure 1B). Egfl7 protein expression on CD34-KSL cells was confirmed on FACS-sorted KSL cells by immunofluorescence (Figure 1C). To understand whether Egfl7 can be up-regulated within the BM after myelosuppressive stress, C57/Bl6 mice were treated with 5-FU. During hematopoietic recovery, peaking at day 5 after 5-FU injection, *Egfl7* mRNA expression was high in BM cells (Figure 1D) These data suggested that Egfl7 is expressed on the earliest progenitors and HSCs, is upregulate after myelosuppression, and therefore might have a potential function in HSC.

A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6
3
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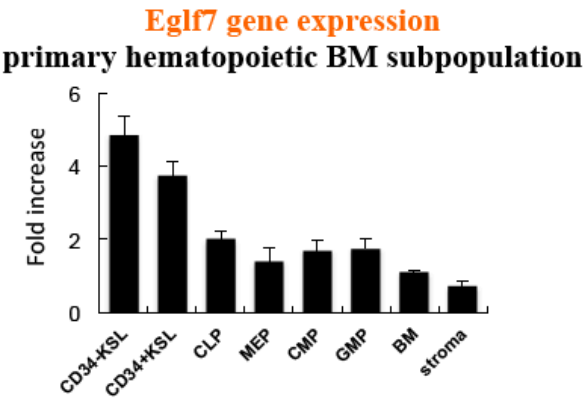
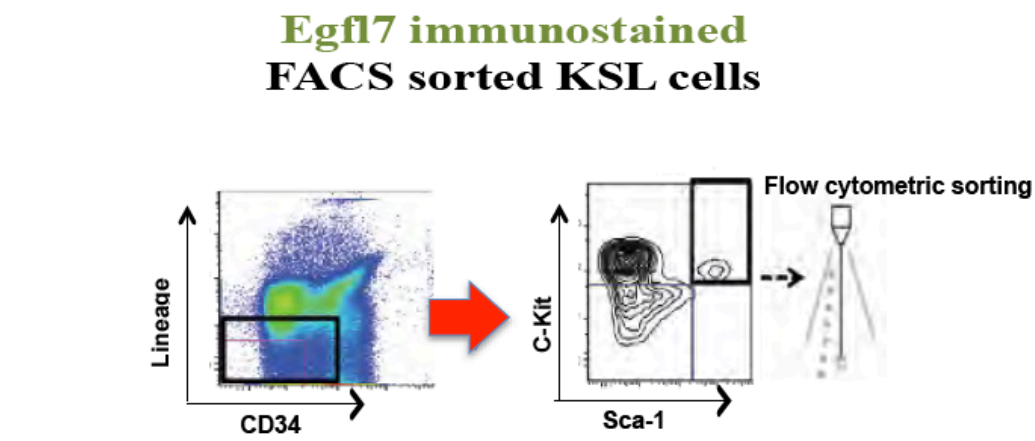
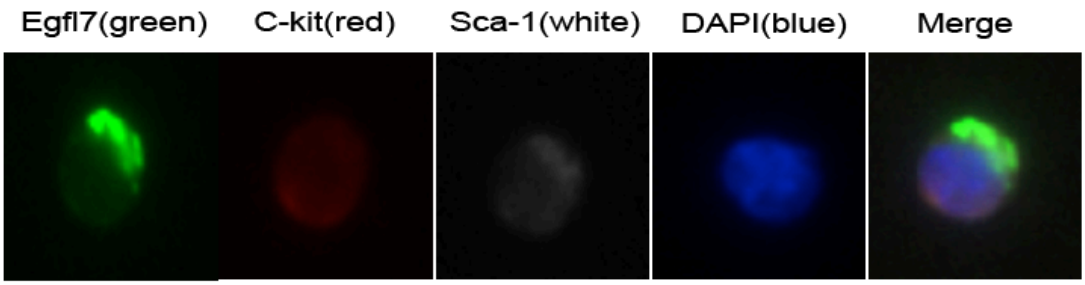
HUVEC
MS-5
BMEC-1
THP-1
HEL

B


Eglf7 gene expression
primary hematopoietic BM subpopulation
Fold increase
6
4
2
0
CD34-KSL
CD34+KSL
CLP
MEP
CMP
GMP
BM
stroma

C


Egfl7 immunostained
FACS sorted KSL cells
Lineage
CD34
C-Kit
Sca-1
Flow cytometric sorting


Egfl7(green)
C-kit(red)
Sca-1(white)
DAPI(blue)
Merge

D

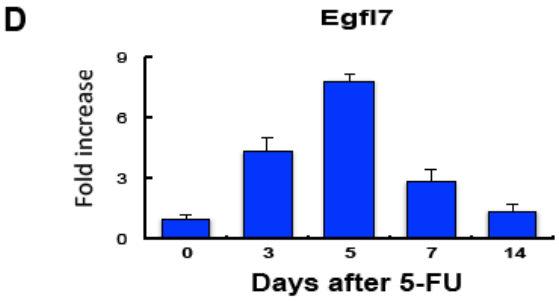

Egfl7
Fold increase
9
6
3
0
0
3
5
7
14
Days after 5-FU

Figure 1. **Impaired proliferation of murine *Egfl7* knockdown KSL/progenitors**
(A) *Egfl7* mRNA expression on human HUVEC, BMEC-1, the murine stromal cell line MS-5, the monocytic cell line THP-1 and erythroleukemia cell line HEL cells, and as determined by qPCR. Normalization of gene expression using β-actin (n=2). (B) Murine BM-derived KSL cells were FACS-isolated into distinct cell population based on differential antigen expression profile: GMP (Lin^- c–Kit^+ Sca–1^- $CD16/32^+$ $CD34^+$), CMP (Lin^- c–Kit^+ Sca–1^- $CD16/32^+$ $CD34^+$) and MEP (Lin^- c–Kit^+ Sca–1^- $CD16/32^+$ $CD34^-$). *Egfl7* expression on indicated FACS-isolated subpopulations, total BM and stromal cells was determined by qPCR. Data presented are from two independent experiments (n=2). (C) Representative fluorescence images of FACS-sorted $CD34^-$KSL cells stained with antibody against EGFL7 (green), c-kit (red) and with antibodies against Sca-1 (white), DAPI (blue) and lineage markers. (D) *Egfl7* gene expression on BM cell samples isolated from 5-FU treated mice (n-2/time point).

II. Egfl7 expands transplantable, clonogenic HSCs

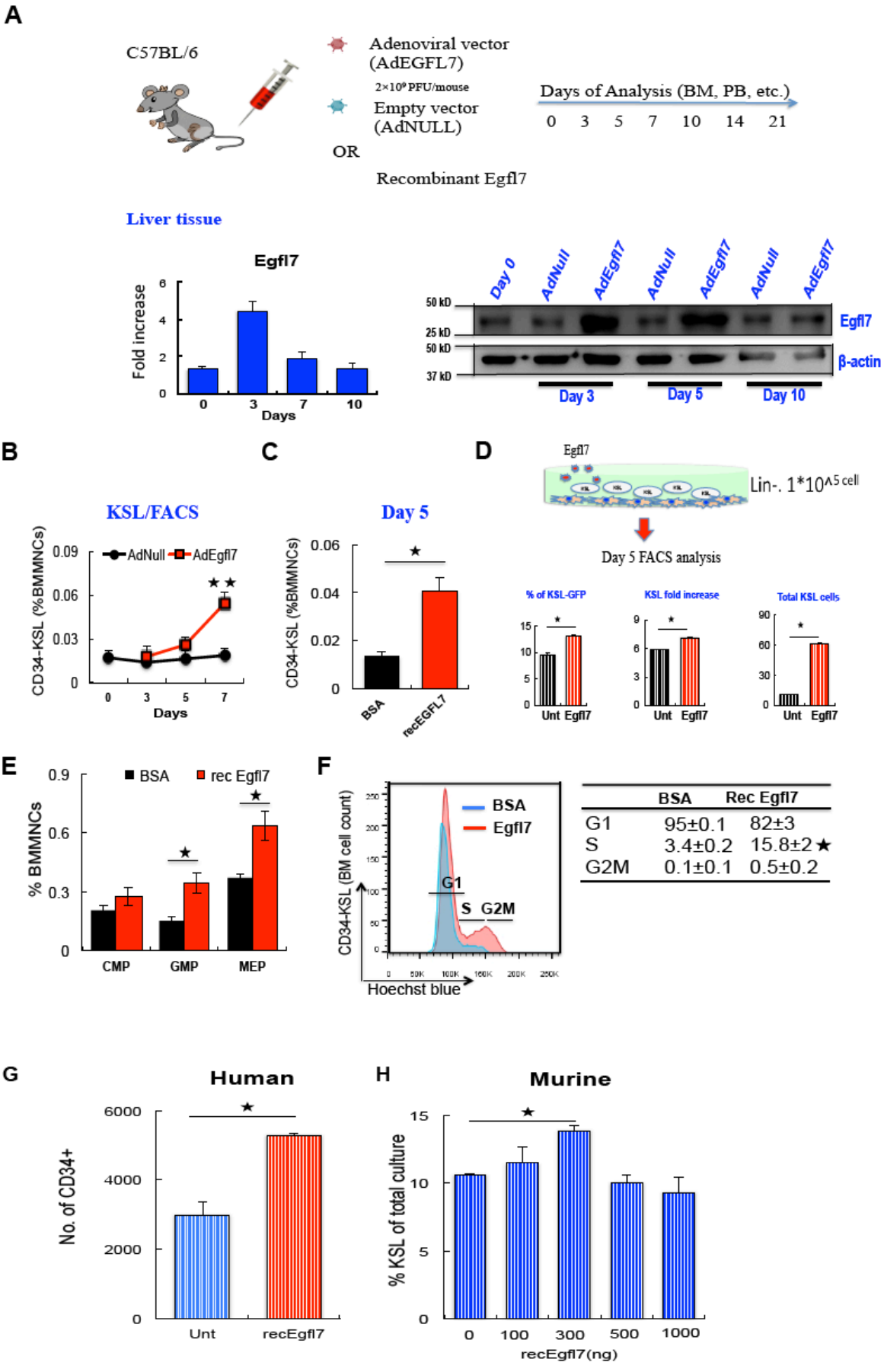
To determine whether Egfl7 overexpression effects hematopoiesis *in vivo*, mice were injected with an adenovirus expressing human Egfl7 or no transgene (AdEgfl7 or AdNull, respectively), or recombinant (rec.) human Egfl7 protein. Egfl7 overexpression was confirmed on liver cell lysates derived from AdEgfl7-treated mice showing high *Egfl7* expression at day 3 and 5, while returning to baseline levels at day 10 after virus injection (Figure 2A). The frequency of CD34-KSL cells increased in BM mononuclear cells (BMMNCs) of Egfl7 overexpressing animals (Figure 2B and Figure 2C), suggesting that Egfl7 can expand HSCs.

Egfl7 is an EC-derived factor, that can block Notch signaling on neuronal stem cells (Schmidt et al., 2009). Given that ECs express Notch ligands that promote proliferation and prevent exhaustion of long term-HSCs (Butler et al., 2010a), I quantified KSL expansion after Egfl7 treatment in EC-cocultures (Figure 2D). Confirming reports by others, KSL expansion was high in EC cocultures, but the KSL expansion could be further amplified in Egfl7 supplemented EC cocultures (Figure 2D).

To further characterize Egf7 expanded KSL cells, various progenitor and stem cell assays were carried out. Administration of rec. Egfl7 augmented the frequency of BM-derived GMP and MEP progenitor sub-fractions (Figure 2E).

HSCs are normally maintained in an undifferentiated quiescent (G0 phase of cell cycle) state, with this quiescence protecting the cells against loss of self-renewal capacity. Cell-cycle analysis revealed that more KSL cells derived from Egfl7-treated mice were in the S-phase (15% of KSL cells derived from rec. Egfl7-treated mice) of the cell cycle, while only 3% of control KSL cells were positive in S-phase of the cell cycle (Hoechst blue positive) indicating that Egfl7 enhances cell cycle entry (Figure 2F).

In order to determine if Egfl7 is a growth factor for human $CD34^+$ cells, cord blood derived $CD34^+$ cells were placed alone or with various concentrations of murine rec. Egfl7 in cultures with the cytokine cocktail Thrombopoietin, Kit ligand and Flt3 (TKF). Rec. Egfl7 at a concentration of 300 ng/ml increased the number of $CD34^-$KSL cells in 5-day cultures (Figure 2G), and expanded murine $CD34^-$KSL cells (Figure 2H). These data suggest that Egfl7 expands murine and human adult BM repopulating HSCs.


A
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2×10⁹ PFU/mouse
Empty vector (AdNULL)
OR
Recombinant Egfl7
Days of Analysis (BM, PB, etc.)
0 3 5 7 10 14 21
Liver tissue
Egfl7
Fold increase
Days
Day 0
AdNull
AdEgfl7
50 kD
25 kD
37 kD
β-actin
Day 3
Day 5
Day 10
B
KSL/FACS
CD34-KSL (%BMMNCs)
AdNull
AdEgfl7
Days
C
Day 5
BSA
recEGFL7
D
Egfl7
Lin-. 1*10^5 cell
Day 5 FACS analysis
% of KSL-GFP
KSL fold increase
Total KSL cells
Unt
Egfl7
E
% BMMNCs
BSA
rec Egfl7
CMP
GMP
MEP
F
CD34-KSL (BM cell count)
BSA
Egfl7
G1
S G2M
Hoechst blue
BSA Rec Egfl7
G1 95±0.1 82±3
S 3.4±0.2 15.8±2★
G2M 0.1±0.1 0.5±0.2
G
Human
No. of CD34+
Unt
recEgfl7
H
Murine
% KSL of total culture
recEgfl7(ng)

Figure 2. **Egfl7 expands phenotypic HSC**
(A) Experimental schedule of Egfl7 administration either using adenovirus expressing Egfl7 (AdEgfl7), an empty control vector (AdNull) or rec. Egfl7. Lower left panel: *Egfl7* expression in liver tissues after virus administration by qPCR with normalization using b-actin. Lower right panel: Western blot analysis of human Egfl7 and b-actin at indicated time points. (B,C) HSC frequencies were evaluated using FACS by measuring $CD34^-$KSL cells. KSL frequency was tested on day 7 after AdEgfl7/AdNull injection (A; n=5), and on day 5 after administration of rec. Eglf7 (C; n=3). (D) FACS-sorted CD34-KSL cells were cultured on HUVEC with/without rec. Egfl7 (n=5). KSL cell frequency (lower left panel), KSL fold increase compared to non-EC containing cultures (middle panel), and the total no. of KSL per well (lower right panel) were determined in 4 day-cocultures by FACS (cultured in triplicates). (E) Frequency of CMP, GMP and MEP in BMMNCs of mice treated with/without rec. Egfl7 on day 7 (n=5) as determined by FACS. (F) CD34-KSL cells retrieved from BM of rec. Egfl7 treated mice or controls on day 5 were costained with Hoechst blue. Left panel: Representative histograms of Hoechst blue stained KSL cells. Right panel: Quantification of cell cycle status of KSL cells. Percentage of cells that efflux the Hoechst 33342 dye that shows the distribution of KSLs in the G0/G1, S and G2M phase (n=3 independent mice for each group). (G) Human $CD34^+$ cells were placed in culture with TKF alone or TKF plus rec. Egfl7 for 5 days (200 cells input/well, n=5). Abs. no. of $CD34^+$ cells as determined by FACS per well. (H) CD34-KSL cells were cultured with different concentration of rec. murine Egfl7 for 5 days. The percentage of KSL cells was determined in retrieved cells after 5 days in culture. p*<0.05; T-test.

III. ***Endogenous Egfl7 is a survival factor for HSCs***

To functionally explore the role of endogenous Egfl7 in regulating HSCs fate, shRNA lentivirus strategies were applied to KD *Egfl7* in KSL cells. KSL cells were transduced with shRNA targeting *Egfl7* or scramble siRNA (*Scr*). Following a four day culture period in TKF-supplemented cultures CD34-KSL cells were resorted (Figure 3A). An aliquot of transduced cells was maintained and analyzed for transduction efficiency. Mean transduction efficiency was 60 %. *Egfl7* KD in GFP^+ resorted KSL cells after 4 days culture was confirmed by qPCR (Figure 3B). After the 4 days culture, the number of recovered *Egfl7* KD KSL cells was lower when compared to *Scr* KSL cells (Figure 3C). Less colonies generated after 10 days in methylcellulose-based cultures were found when *Egfl7* KD KSL cells had been plated (Figure 3D). These data suggested that endogenous Egfl7 is critical for HSC survival *in vitro*.

In vivo repopulation studies, where HSCs reside in a physiological niche, remain the gold standard to prove that HSC self-renewal and differentiation are affected. The competitive repopulation units (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 an assay to identify bona fide, transplantable HSCs.

Resorted *Egfl7* KD or *Scr* KSL cells were also transplanted into ablated Ly5.1 mice with $1x10^5$ competitor BMMNCs. Recipient peripheral blood (PB) was analyzed for test cell versus competitor cell contribution 8 wk after transplantation. *Egfl7* KD KSL cells were transplanted into wild-type recipient mice. *Egfl7* KD and scr KSL cells showed a similar CRU ability with three-lineage cell differentiation potential and normal KSL recovery (Figure 3E), indicating that the observed survival disadvantage of *Egfl7* KD KSL cells *in vitro* was compensated *in vivo*.

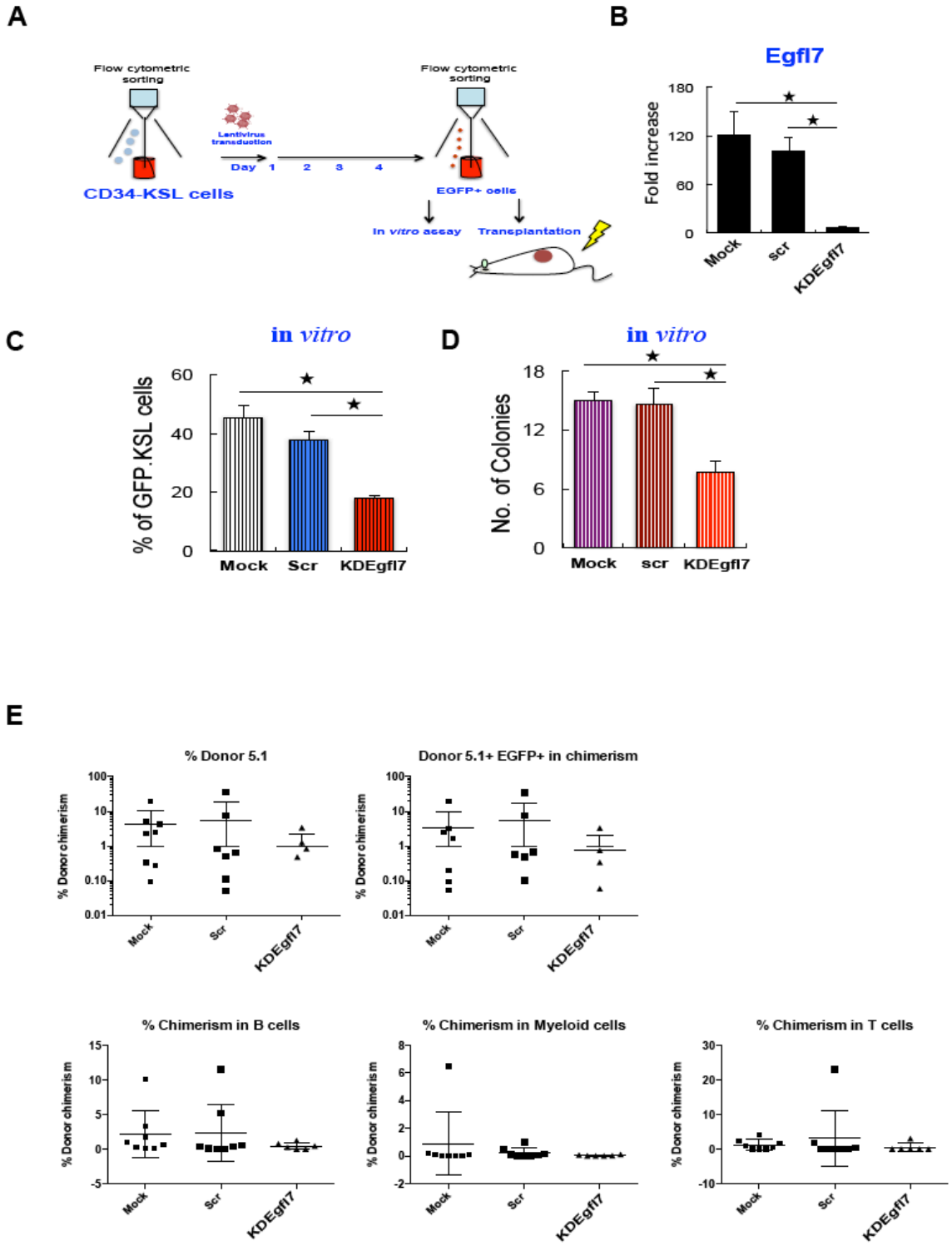


Figure 3. **Impaired proliferation of murine *Egfl7* KD KSL cells**
(A-E) Experimental outline of knockdown of *Egfl7* in murine GFP+ CD34-KSL cells transduced with lentiviral shvector. GFP+ cells were not infected with virus (mock), or infected with lentivirus to knockdown (*Egfl7* KD) KSL cells or scr KD derived from GFP mice were collected and cultured for four days in a suspension culture. Cultured cells were isolated, and resorted for CD34-KSL. Resorted cells were subjected to the various assays. Data presented are from two independent experiments (n=2). (B) *Egfl7* expression as determined by qPCR (n=2/group) of FACS-sorted GFP+ cells of mock, scr or *Egfl7* KD KSL cells. Transcript levels were normalized to β-actin. (C) Percentage of CD34-KSL in recovered cells. (D) In vitro colony formation of KSL cells from mock, *Scr* and *Egfl7* KD

KSL cells (n=2, with each assay done in triplicate). (E) *Egfl7* KD KSL derived from CD45.1$^+$ test cells that were then transplanted into CD45.2+ mice with CD45.1/2$^+$ competitor cells. Recipient peripheral (PB) was analyzed after 8 wk for the presence of donor CD45.1 cells. Trilineage engraftment: Scatter plots showing the percentages of total CD45.1$^+$ donor cells and donor-derived B220$^+$ (B lymphoid), Mac-1$^+$/Gr-1$^+$(myeloid) and CD4$^+$, CD8$^+$ (T cell) populations in the PB of mice transplanted with 200 BM CD34$^-$KSL cells or their progeny (*n*=8–10 mice per group). Horizontal lines represent the mean engraftment levels for each group.

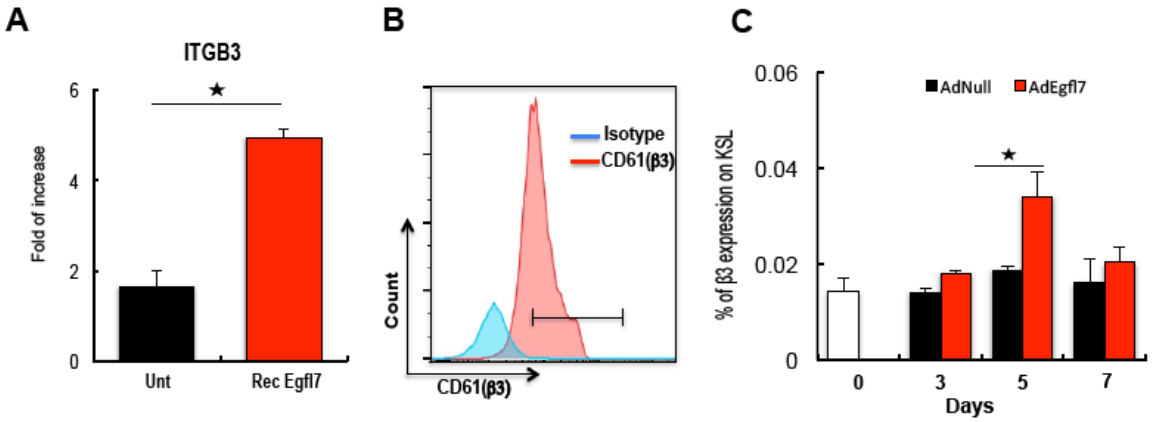
IV. ***Egfl7-mediated HSC expansion requires Itgb3 signaling***

Egfl7 enhances Itgb3 signaling on ECs (Nikolic et al., 2013). Treatment of HEL cells with rec. human Egfl7 increased *Itgb3* mRNA expression after 24 hrs (Figure 4A), indicating that Egfl7 upregulates one of its own receptors. Murine BM-derived CD34-KSL cells express Itgb3 as determined by FACS (Figure 4B). The percentage of Itgb3 expressing KSL cells increased in BMMCs by day 5 after AdEgfl7 injection in mice (Figure 4C). To interrogate a role of Itgb3 for Egfl7-mediated KSL expansion *in vitro*, rec. murine Egfl7 was added to short-term TKF-containing KSL cultures. β_3 inhibition using the CGD Itgb3 inhibitor, or rec. Egfl7 treatment alone increased the frequency of KSL cells after 5 days in culture (Figure 4D). Highest KSL expansion was achieved in cultures supplemented with rec. Egfl7 and the Itgb3 (Figure 4D), indicating that Egfl7 independent of the presence of Itgb3 led to highest HSC expansion *in vitro*.

Egfl7 can bind to platelet derived growth factor-receptor β, Itgb3, or Notch receptors. HSCs, like murine KSL cells express at the two Egfl7 receptors Itgb3 and Notch1, and 2. Often integrins recognize the Arg-Gly-Asp motif (RGD) in their ligands. To assess whether Egfl7-mediated HSC expansion was due to Egfl7 binding to Itgb3, or Notch receptor, an adenovector overexpressing an Egfl7 mutant protein lacking the RGD domain was constructed (referred to as AdRGDdel). The sequence is presented in Figure 4E. The AdRGDdel infection of cells results in the generation of a protein that had lost its Itgb3 binding ability, but still could interact with Notch receptors.

The full-form of Egfl7 promotes ECs adhesion through binding of Itgb3 (Nikolic et al., 2013). Egfl7 full-form and fibronectin (FN, a known ligand for Itgb3), but not the Egfl7 deletion mutant protein (RGDdel) coating of culture plates enhanced adhesion of Lin- cells (Figure 4F), indicating that the newly generated RGDdel protein was unable to bind KSL cells, but other Itgb3 ligands could (Figure 4F).

Given that loss/blockade of Itgb3 favored Egfl7-mediated KSL expansion *in vitro*, I next tested whether the same was true *in vivo*. Loss of Itgb3 signaling either by injection of AdRGDdel or due to genetic Itgb3 deficiency in mice increased Egfl7-mediated KSL expansion within the BM (Figure 4G). My data set forward the idea that Egfl7 enhances KSL proliferation in the absence of cellular Itgb3 possibly by inducing a different signaling pathway.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Isotype
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0.02
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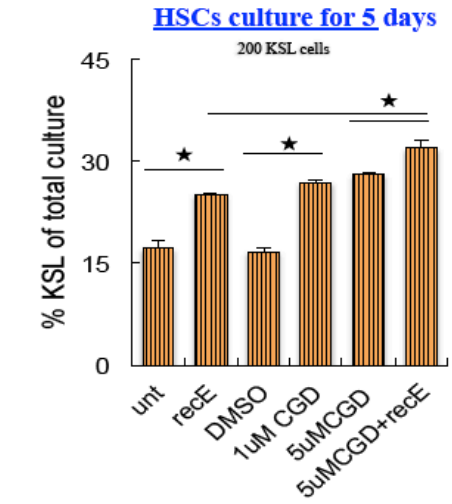
3
5
7
Days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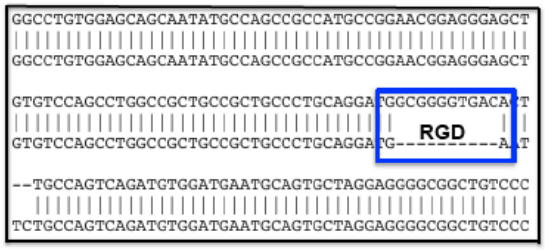
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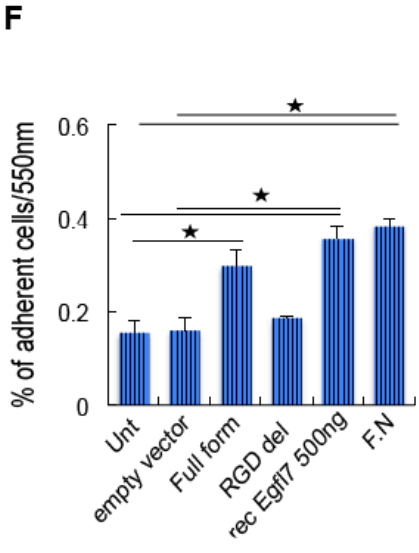
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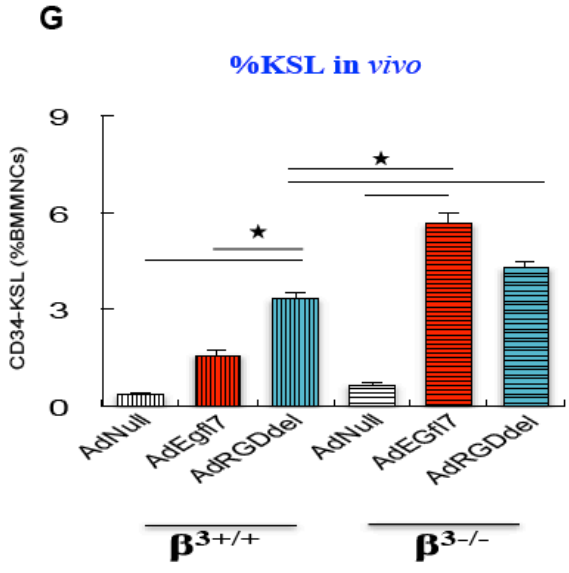
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6
3
0
AdNull
AdEgfl7
AdRGDdel
AdNull
AdEGfl7
AdRGDdel
β3+/+
β3-/-

Figure 4. **Itgb3 inhibition amplifies Egfl7-mediated HSC expansion**
(A) *Itgb3* expression as determined by qPCR (n=2/group) of FACS-sorted EGFP+ cells of Mock, Scr or *Egfl7* KD KSL cells. Transcript levels were normalized to b-actin. (B) Representative histogram shows Itgb3 expression on KSL cells after FACS analysis (blue: isotype; red: Itgb3). (C) Percentage of Itgb3-expressing KSL cells retrieved at indicated days from AdEgfl7 and AdNull injected mice (n= 3/group). (D) The frequency of KSL cells after 5 day culture of highly purified $CD34^{-}$KSL cells (>97%) with cytokines (TKF) in the presence of rec. Egfl7 with or without Itgb3 inhibitor (CGD). (E) DNA sequence of RGDdel is shown. (F) Percentage of adhesion at 4 hr on precoated plates with deposited ECM from cells infected with AdNull (AdNull), AdEgfl7 full-form (AdEgfl7), or AdRGDdel. Precoating was also performed using rec. murine Egfl7 or rec. fibronectin (FN). Cumulative data of 2 independent experiments are shown (n=5/condition/experiment). (G) Frequency of KSL cells isolated after 7 days from AdEgfl7 or Null injected wt or $Itgb3^{-/-}$ mice (n=6). Data shown represent means ± s.e.m. $P < 0.05$. All comparisons were one-tailed *t* tests.

*V. **Egfl7 enhances c-Kit signaling in Itgb3 expressing cells***

The zinc-finger transcription factor GATA-2 is required for the expansion of multipotential hematopoietic progenitors and the formation of mast cells, but dispensable for the terminal differentiation of erythroid cells and macrophages (Tsai and Orkin, 1997). Furthermore, GATA-2 had been reported to regulate the c-Kit promoter activity in mast cells together with Sp1 (Maeda et al., 2010). Given that the proangiogenic miR-126, which is hosted by Egfl7, was regulated by GATA2 transcriptionally and targeted antiangiogenic SPRED1 and FOXO3a as shown by global miRNAnome-screening in ECE (Hartmann et al., 2016), I hypothesized that Egfl7 binding to Itgb3 regulates GATA-2 and c-Kit expression. The highly expressing Itgb3 erythroleukemic cell line HEL was chosen for analysis to further delineate the mechanism how Itgb3 (also known as CD61) alters cell proliferation of Egfl7. *Itgb3* KD was achieved in HEL cells using siRNA. *Itgb3* KD was confirmed in transfected cells by qPCR and Western blot (Figure 5A and B). Indeed, *Itgb3* KD HEL cells showed a decreased GATA-2 (Figure 5A) and c-Kit expression (Figure 5B) when compared to *Scr* HEL cells by qPCR and/or Western blotting.

To investigate the induction of signaling pathways after ligand binding to Itgb3, HEL cells were cultured on FN (pos. control for Itgb3 activation), BSA (neg. control), or rec. Egfl7

(Figure 5B and 5C). Cells cultured in the presence of Egfl7 and FN, but not BSA (control) showed enhanced Itgb3 signaling as determined by Tyr 747 phosphorylation of human Itgb3 by Western blotting (Figure 5B). *Itgb3* KD HEL cells when compared to *Scr* HEL cells showed lower expression of *Egfl7* on the mRNA and protein level (Figure 5A, and 5B), suggesting that Egfl7 amplifies itself in an Itgb3 dependent manner (autocrine amplification loop).

Phosphorylation of cytoplasmic tyrosine kinase focal adhesion kinase (FAK) plays critical roles in integrin-mediated signal transductions. *Scr*, but not *Itgb3* KD HEL cells cultured on plates coated with Egfl7, and FN-, but not BSA enhanced phosphorylation of signal transducer and activator of transcription 3 (STAT3), and extracellular signal-regulated kinase 1/2 (ERK1/2), as already shown on ECs (Chim et al., 2015). Phosphorylation was slightly changed for AKT, and JAK-2 following *Itgb3* KD when compared with control *Scr* cells after 4 hrs, indicating that Egfl7-driven ERK1/2, STAT-3 and AKT activation requires Itgb3 signaling (Figure 5B). Scr, but not *Itgb3* KD HEL cells cultured on Egfl7- and FN-coated plates upregulated Egfl7, FAK, and c-Kit expression after 24 hrs (Figure 5C). These data indicate that Itgb3 was required for Egfl7-mediated c-Kit, and endogenous Egfl7 induction (amplification loop), and led to the activation of typical cytokine signaling pathways, like STAT-3.

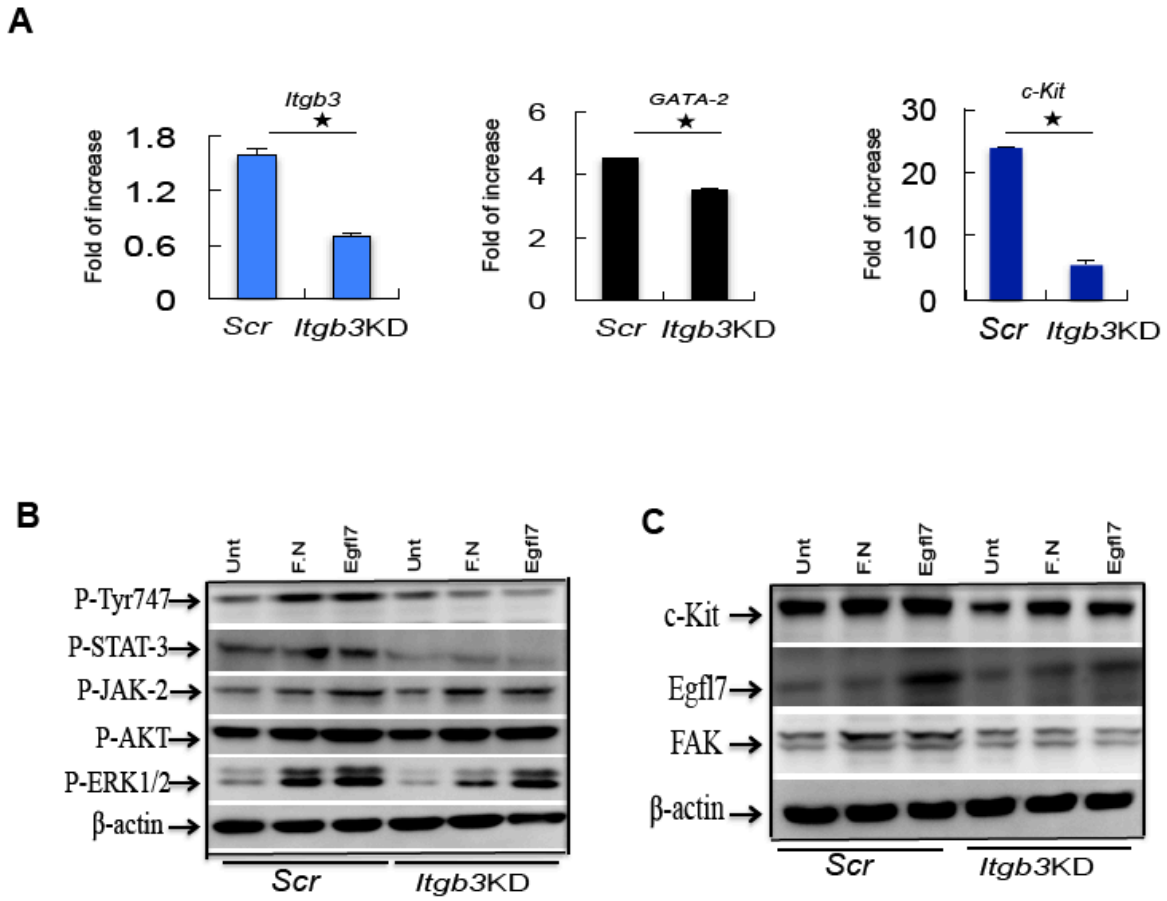


Figure 5. **Egfl7 through β_3 enhances c-Kit signaling and upregulates Egfl7 in HEL cells**

(A) *Itgb3* KD was achieved in human HEL cells by transient transfection with siRNA targeting Itgb3. Control cells were transfected with a scramble siRNA (scr). Gene expression of *Itgb3*, *GATA-2* and *c-Kit* was determined on *Scr* HEL and *Itgb3* KD HEL cells by qPCR (n=2), (B) Phosphorylation levels of STAT-3, AKT, ERK1/2 as determined by Western blotting of *Scr* transfected and *Itgb3* KD HEL cells cultured for 4 hrs on plates coated with rec. Egfl7, Fibronectin (FN) or BSA as determined by Western blotting. (C) Protein expression of c-Kit, Egfl7 and FAK in *Scr* HEL or *Itgb3* KD HEL cells cultured for 24 hrs in plates coated with rec. Egfl7, FN or BSA. *β-actin* served as a loading control (2 independent experiments). Data shown represent means ± s.e.m., $P < 0.05$. All comparisons were one-tailed *t* tests.

*VI. **Egfl7 upregulates c-Kit expression and enhances HSC survival***

In the hematopoietic system, the c-Kit tyrosine kinase receptor is expressed on HSCs and progenitors, where it regulates HSC cycling, maintenance, differentiation and expansion (Heissig et al., 2002; Ku et al., 2012; Thoren et al., 2008). c-Kit is expressed on various BM cells, like KSL cells, mast cells, myeloid cells, and megakaryocytes. A recent report

demonstrated that Spred1 is targeted by miR126, the miRNA found within the *Egfl7* gene (Ji et al., 2016). Of interest, Spred1 is a negative regulator of c-Kit (Tadokoro et al., 2014).

To further investigate the role of c-Kit for Egfl7 function, c-Kit expression was determined in hematopoietic cells after in vitro and in vivo exposure to *Egfl7*. c-Kit expression was upregulated on Egfl7-treated HEL cells (Figure 6A). Similarly, BM cells from AdEgfl7-treated, but not from AdNull-treated mice showed higher c-Kit expression (Figure 6B). Higher c-Kit expression was found on KSL cells derived from AdEgfl7-treated, but not AdNull-treated mice as determined by FACS (Figure 6C).

Because c-Kit/KitL pathway is critical for HSC expansion, and my data indicated that Egfl7 alters c-Kit signaling, I next studied the role of c-Kit for Egfl7-mediated KSL expansion. Kit-Kit$^{W\text{-}v}$ mice have a mutation in the c-Kit receptor leading to a functionally inactive c-Kit receptor, which allows mice to survive. While, no gross changes were found in H&E stained BM sections of AdEgfl7 injected c-Kit$^{+/+}$ mice of treated mice, AdEgfl7-injected c-Kit$^{-/-}$ mice showed impaired BM cellularity (Figure 6D).

More CD34-KSL cells in AdEgfl7 treated c-Kit$^{-/-}$ (Kit-Kit$^{W\text{-}v}$) when compared to wild-type mice. This effect was further amplified in Itgb3$^{-/-}$ mice, or when the Egfl7 deletion vector (AdRGDdel) had been used (Figure 6F). These data suggest that efficiently Egfl7 expands when c-Kit and Itgb3 signaling were blocked.

I next utilized short-term cultures to confirm the importance of c-Kit signaling for Egfl7-mediated HSC expansion. Similarly, rec. Egfl7 addition to short-term cultures of c-Kit deficient KSL expanded KSL cells more than two-fold in 7 day cultures (Figure 6E), while the Egfl7-mediated KSL expansion was less pronounced in wild-type KSL cells (see Figure 2H). Collectively, these data suggest Egfl7 activates c-Kit signaling in the presence of Itgb3 on HSC, whereby HSC differentiate rather than self-renewal, while Egfl7 biases KSL cells to self-renew rather than differentiation in the absence of c-Kit.

c-Kit signaling is important for HSC survival. To evaluate whether c-Kit loss accounts for the impaired HSC survival observed in *Egfl7* KD KSL cells (see Figure 3C and 3D), *Egfl7* KD was achieved in c-Kit$^{-/-}$ KSL cells. *Egfl7* KD in c-Kit$^{-/-}$ KSL cells rescued the impaired survival that had been documented in c-Kit$^{+/+}$ KSL cells (Figure 6G), indicating that Egfl7 in an Itgb3 dependent manner regulates c-Kit expression necessary for HSC survival and self-renewal.

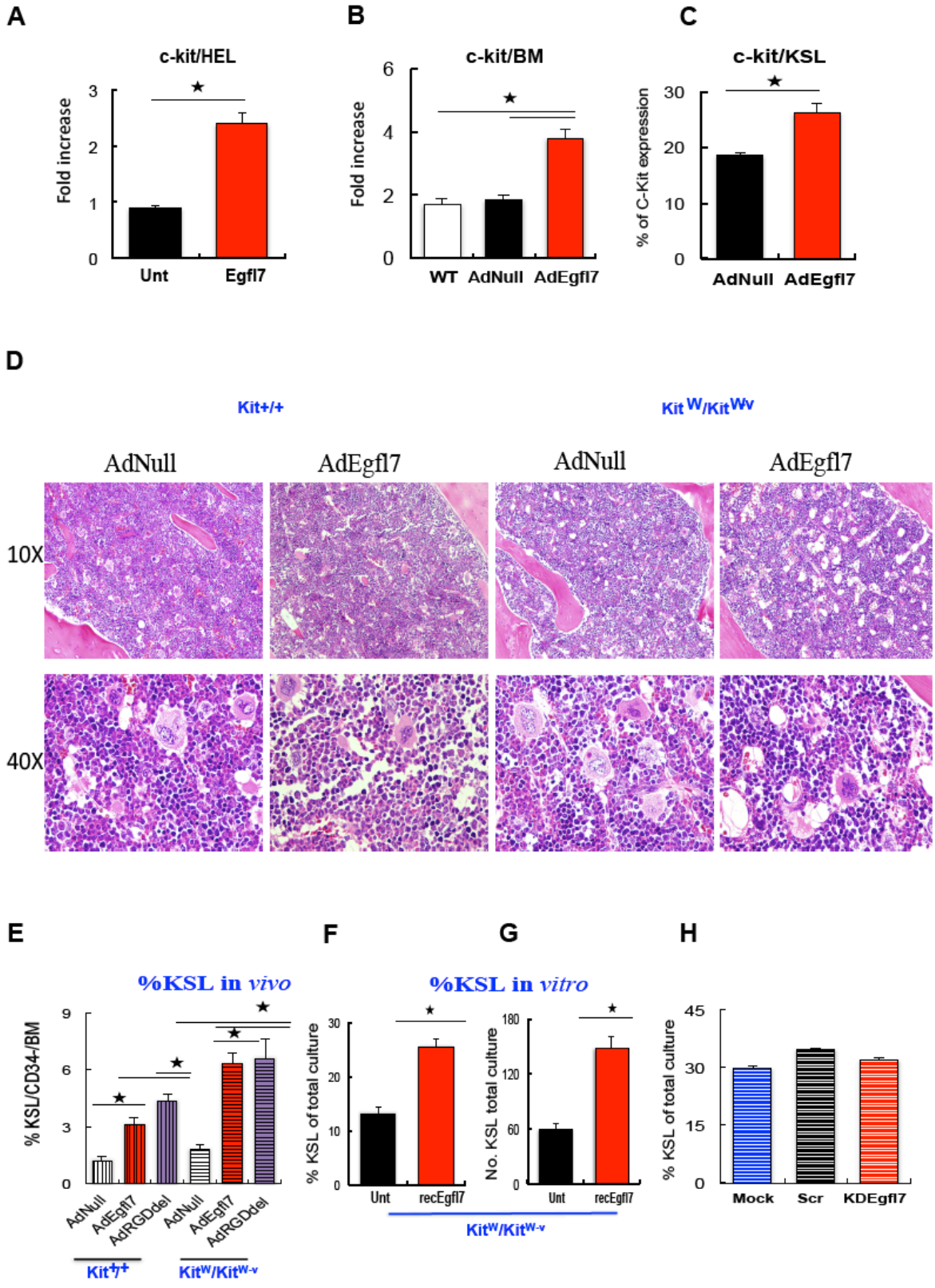


Figure 6. **c-Kit deficiency enhances Egfl7-driven HSC expansion**
(A-B) *c-Kit* expression by qPCR on HEL cells (A), and on total BMMNCs (B) isolated from AdEgfl7 or AdNull treated mice, or cultured with rec. Egfl7. *c-Kit* gene expression as fold increase compared to non-virus injected controls on BM cells by qPCR; Gene normalization using *β-actin* (B; n=2). (C) Percentage of c-Kit expressing KSL cells in BM of AdEgfl7 or AdNull-treated mice was determined by FACS (n=3/group). (D-E) c-Kit$^{+/+}$ and c-Ki$^{t-/-}$ mice were injected with AdEglf7 (full form),

AdRGDdel, and AdNull. (D) Representative H&E stained BM sections are shown. (E) Collected BMMNCs at day 7 were stained for c-Kit, and analyzed by FACS (n=5/group). (E) The frequency of CD34-KSL cells was determined by FACS in BM cells (n=5/group). (F) c-Kit$^{-/-}$ FACS-sorted KSL cells were plated in cytokine supplemented cultures in the presence/absence of rec. Egfl7. (G) KSL cell frequency (left column) and absolute number (right column) per total culture after FACS of BMMNCs (n=5/condition) were determined. Experiment was repeated twice, and combined data from both experiments are presented (H) *Egfl7* KD was achieved by retroviral infection in c-Kit$^{-/-}$ KSL cells were retrovirally transfected with *Scr* or *Egfl7* KD vectors. After cells were subjected to cytokine supplemented cultures for 4 days, the frequency of KSL cells was determined by FACS (n=3/condition). Experiments were repeated twice. Data shown represent means ± s.e.m., $P < 0.05$. All comparisons were one-tailed *t* tests.

VII. ***Egfl7 in the absence of Itgb3 activates Notch and Flt signaling in HSCs***

My data indicated that Egfl7 mediates HSC self-renewal and expansion in the absence of Itgb3. Egfl7 antagonizes Notch receptor/ligand interaction by either binding to the receptor or its corresponding ligand in certain cells (Durrans and Stuhlmann, 2010; Schmidt et al., 2009). Egfl7 can antagonize Notch signaling in neuronal stem cells. Notch1 receptor is expressed on immature CD34^{+} hematopoietic lymphoid, myeloid and erythroid precursors (reviewed by Milner and Bigas, Blood 1999). To test whether Egfl7 modulated Notch signaling on HSCs, I examined the Notch signaling downstream genes *Hairy enhancer of split-1* (*Hes-1)* (Figure 7A) and *Hey1* (not shown here). Hes-1 mRNA was down-regulated in BM cells of AdEgfl7-treated BM cells, and in BM cells from Notch inhibitor (gamma secretase inhibitor, GSI) treated mice (Figure 7A), indicating that Egfl7 blocked Notch signaling within BM cells.

To investigate the effects of Egfl7 for ligand-induced Notch signaling on HSC, KSL cells were cocultured with OP9 control or OP9 stromal cells overexpressing the Notch ligand DLL1 (OP9-DLL1) in the presence or absence of rec. Egfl7 daily for 7 days (Figure 7B). Although the Notch inhibitor DAPT blocked Egfl7-mediated KSL cell increase in the presence of Notch ligand DLL1, highest KSL expansion after Egfl7 addition was observed in OP9 cultures lacking the Notch ligand (Figure 7B). These data suggested that Egfl7-mediated effects on KSL cell expansion did not require the presence of Notch ligands.

A recent study showed that Notch inhibition downregulates *Hes-1* expression in leukemic cells (Kato et al., 2015). Hes-1 binds to the promoter region of the *FMS-like tyrosine kinase 3* (*Flt3*) gene and leads to the downregulation of Flt3 promoter activity (Kato et al., 2015). Increased Flt3 expression was found in total BMMNCs of AdEgfl7 treated mice, and on KSL cells extracted 5 days after virus injection as determined by qPCR and by FACS analysis (Figure 7C). Next, I unequivocally demonstrate that Flt3 signaling was required for HSCs expansion *in vitro*, because Flt3 inhibitor treatment prevented Egfl7-mediated KSL expansion (Figure 7D).

To further characterize the signaling pathways involved after Egfl7, total BMMNC lysates of non-vector treated (wt), AdNull and AdEgfl7 mice were tested. Flt3 receptor up-regulation was confirmed in BMMNCs of AdEgfl7 treated mice at day 5.

BMMNCs of AdEgfl7 treated mice showed robust up-regulation of Egfl7 (Figure 7E), again supporting my initial findings on HEL cells that Egfl7 can amplify its own gene in hematopoietic cells.

Similar to my data using the HEL expressing cells, phosphorylation of AKT, ERK and STAT3 was detected in BMMNCs of AdEgfl7 treated mice. Interestingly, STAT3 activation has been to be required for self-renewal of embryonic stem cells (Matsuda et al., 1999).

Similar to HEL cells, enhanced Itgb3 signaling as determined by Tyr 747 phosphorylation of human Itgb3 was found on Egfl7 overexpressing BMMNCs (Figure 7E).

Following the binding of the specific ligand, the intracellular part of the Notch receptor, the intracellular domain Notch-IC (NICD) translocates into the nucleus, forms a complex with the DNA-binding protein RBPj and induces the expression of downstream effectors such as the transcriptional repressor genes *Hes1* and *Hes5* (Fortini, 2009). Blockade of Notch

signaling was found in AdEgfl7 derived wildtype mice, as evidenced by downregulation of Hes-1, and NICD in BMMNCs or KSL cells (Figure 7E-G).

The presence of Itgb3 on HSC was important for Egfl7-mediated changes in c-Kit/Kit ligand signaling. I therefore, investigated whether c-Kit or Itgb3 deficiency altered Egfl7-induced Notch signaling. Eglf7 blocked Notch signaling in wt type cells or c-Kit$^{-/-}$ BMMNCs as demonstrated by impaired *Hes-1* mRNA expression and a reduction in NICD using Western blot analysis (Figure 7E-G). In contrast, Egfl7 in Itgb3$^{-/-}$ cells activated Notch signaling (Figure 7G).

These data indicate that Egfl7 dependent on the Itgb3 expression status fine-tunes Notch signaling on HSC.

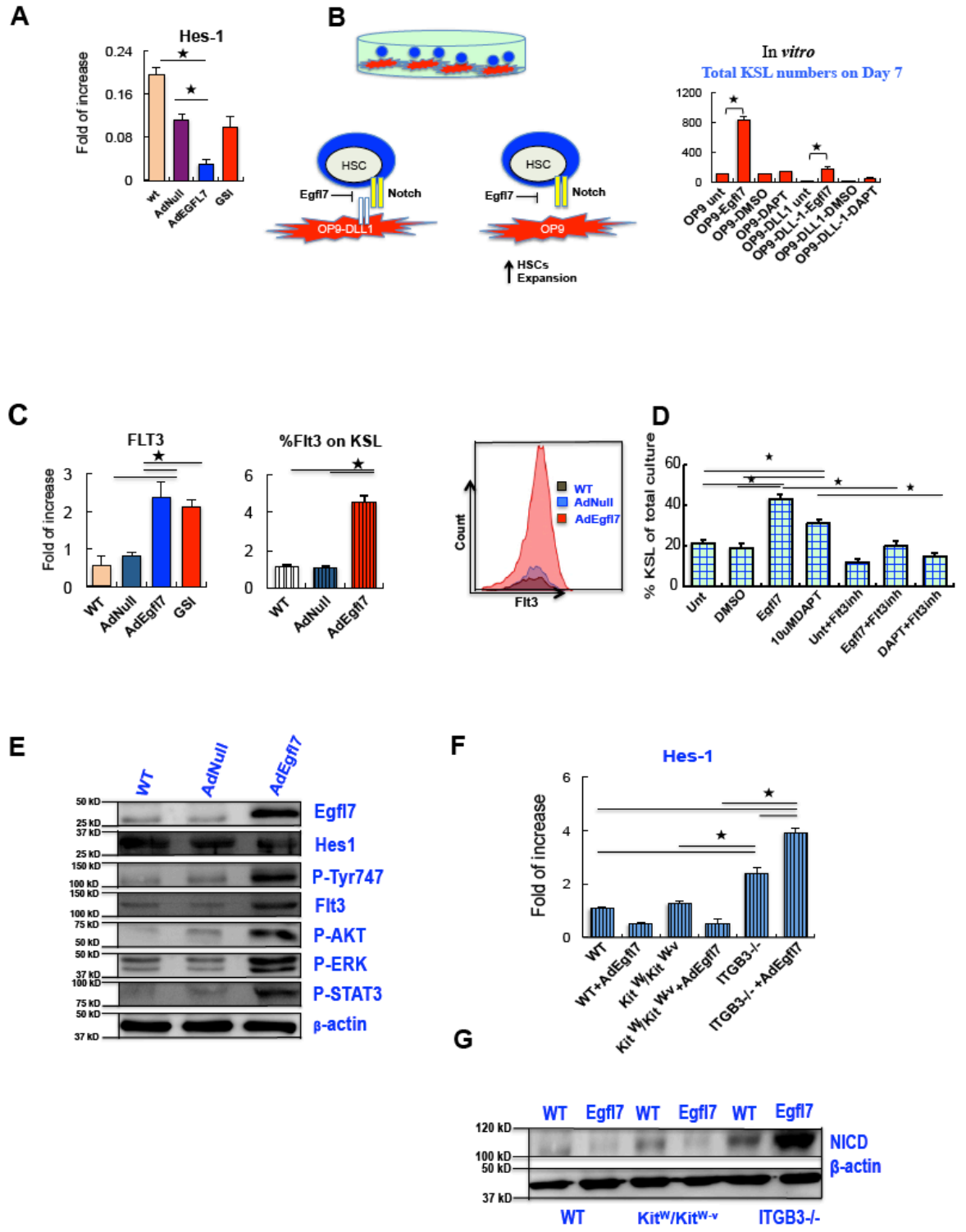


Figure 7. **Egfl7 in the absence of Itgb3 activates Notch leading to HSC expansion**

(A) *Hes-1* expression by qPCR on BMMNCs of AdEgfl7, AdNull, or γ-secretase inhibitor-I (GSI) treated mice retrieved at day 5 after vector injection (n=2). Data represent rel. mRNA expression normalized to *β-actin*. (B) Absolute numbers of KSL cells of coculture with OP9 or OP9-DLL1 cells treated with/without rec. murine Egfl7 after 7 days (n=3/condition). (C) Mice were injected with AdEgfl7, AdNull or GSI (n=3/group). *Flt3* expression was determined on isolated KSL cells. Data

represent rel. mRNA expression normalized to *β-actin*. (n=x; left panel). Frequency of KSL cells coexpressing Flt3 tested by FACS (n=5; middle panel). Representative histogram shows Flt3 expression on KSL cells of indicated groups (right panel). (D) FACS-sorted KSL cells were cultured in medium supplemented with TKF in the presence of rec. Egfl7, the Notch inhibitor DAPT with or without the Flt3 inhibitor. Expanded cells were analyzed by FACS at day 5 (n=5). (E-G) Mice were injected with AdEgfl7 or AdNull. (E) BMMNCs of wildtype mice treated with or without vectors were isolated by day 3. Western blotting was performed on indicated proteins. Two independent experiments. (F,G) c-Kit, Itgb3 deficient and control mice were injected with or without virus. (F) *Hes-1* expression was tested in KSL cells by qPCR (n=3/group). (G) Western blot analysis of NICD in BMMNCs of indicated groups at day 5. Loading control: β-actin. Data shown represent means ± s.e.m., $P < 0.05$. All comparisons were one-tailed *t* tests.

In summary, I demonstrate that *Egfl7* is expressed at rather low levels under steady state conditions in the BM niche. Endogenous *Egfl7* expression in HSC maintains high c-Kit expression, while Flt3 expression is rather low. Following stress like myelosuppression, Egfl7 increases in the niche, which not only can amplify its own expression, but also upregulates Itgb3 on HSC. Dependent on the Itgb3 expression status of the cell, Egfl7 changes the expression of stem cell-active receptor like *c-Kit* and *Flt3*. Egfl7 in Itgb3 positive HSC up-regulates c-Kit and Flt3 expression, while blocking Notch signaling, which ultimately drives HSC expansion, and differentiation. In contrast, in low expressing Itgb3 positive HSCs, a characteristic of quiescent HSC Egfl7 activates the Notch pathway causing HSC expansion and self-renewal (Figure 8).

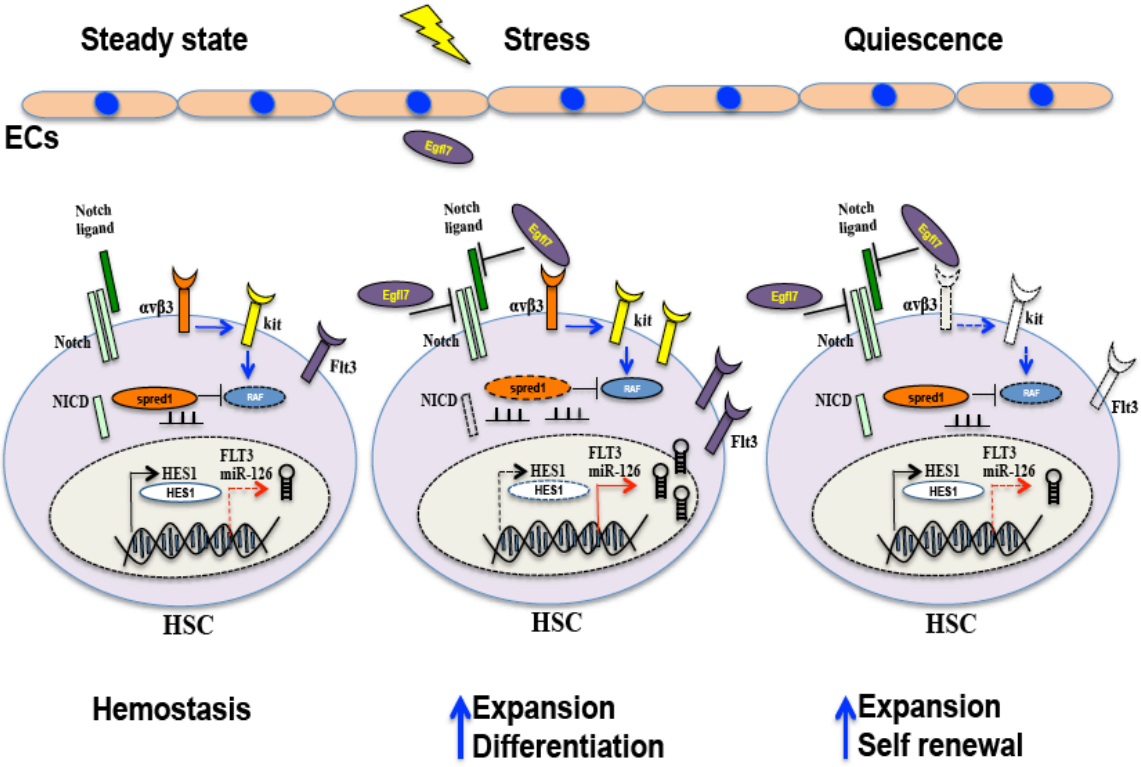


Figure 8. **Egfl7 regulates stem cell fate dependents on β3 integrin status of HSC**

My data led to the following model of stem cell fate determination by Egfl7 dependent the following model of HSC expansion. Egfl7 dependent on the presence or absence of β3 integrin fine-tunes Notch signaling, by rather activating or downregulating the signal.

Up-regulation of Flt3 expression on KSL cells is accompanied by loss of self-renewal capacity but sustained lymphoid-restricted reconstitution potential (Adolfsson et al., 2001). Having established a role for Egfl7 in regulating HSC fate in the BM niche, I further explored the possibility that Egfl7 alters the fate of stem cells in other niches. Notch, c-kit and Flt3 signaling are indispensable for T cell lineage commitment, and are critical for thymogenesis, the generation of T cells in the thymus, I next studied the role of Egfl7 in the thymic niche and for thymogenesis.

VIII. ***Perivascular Egfl7 deposition in the irradiated thymus***

Egfl7 transcripts were found by qPCR in lymphoid organs like the thymus, and spleen (Figure 9A). High *Egfl7* expression was detected on MACS-sorted $CD45^{-}CD31^{+}$ ECs, and FACS-sorted $CD44^{+}$c-$Kit^{+}CD25^{-}$ ETP (Figure 9A). Impaired thymic function after myelosuppressive irradiation or during aging can deplete the thymus of lymphoid cells and damage thymic stroma cells. *Egfl7* mRNA was upregulated in thymi 3 d after sublethal irradiation with 2 Gy (Figure 9A). Immunoreactive Egfl7 was found perivascular in thymi of irradiated, but not in non-irradiated mice (Figure 9B). These data indicate that Egfl7 is expressed in a thymocyte subpopulation and thymic EC, and its expression is upregulated after sublethal irradiation.

IX. ***Egfl7 augments early thymic progenitors and ECs***

To investigate Egfl7's role for T cell development *in vivo*, C57/BL6 mice were injected intravenously AdEgfl7 or AdNull (Figure 9C). Egfl7 overexpression was confirmed in liver cell lysates from adenovirus-injected mice by Western blotting (Figure 9D). Next, I determined the thymic T cell differentiation that can be divided into discrete stages characterized by the expression pattern of CD4 and CD8. CD4 and CD8 double negative (DN) cells are the early T cell progenitors, that can differentiate into CD4 and CD double positive (DP) and then give rise to CD4 or CD8 single-positive (SP) cells. T cell differentiation in spleen and peripheral blood was unchanged (data not shown). While the frequency of $CD4^{+}$ and $CD8^{+}$ SP and DN increased, the percentage of DP decreased (Figure 9F) in thymocytes retrieved from AdEgfl7-injected mice at d 3. This translated into a two-fold decrease in thymic cellularity, a 50% reduction in thymus size and thymocyte cell number in AdEgfl7 injected (data not shown). These results suggest that Egfl7 is a modulator of early T cell development.

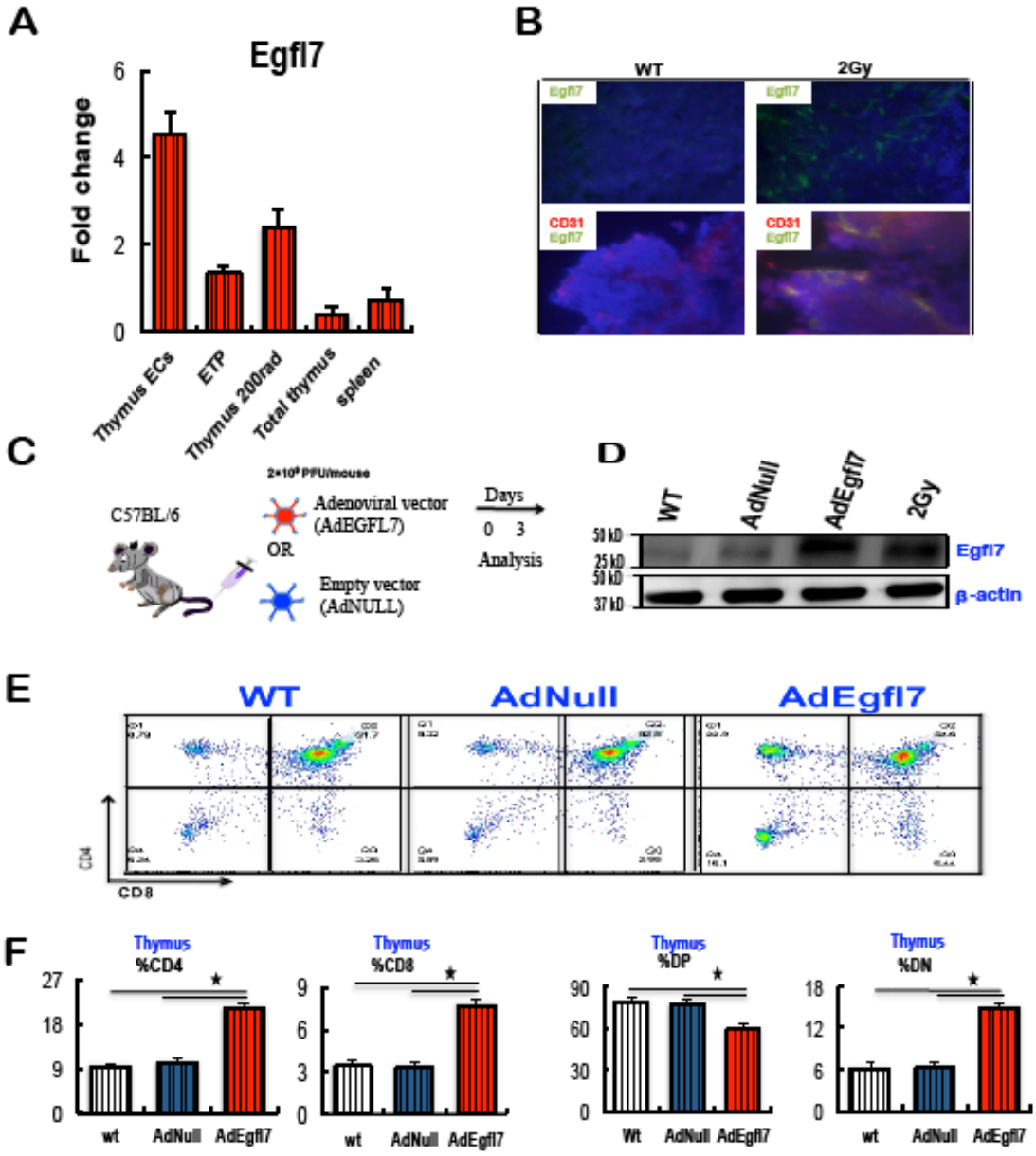


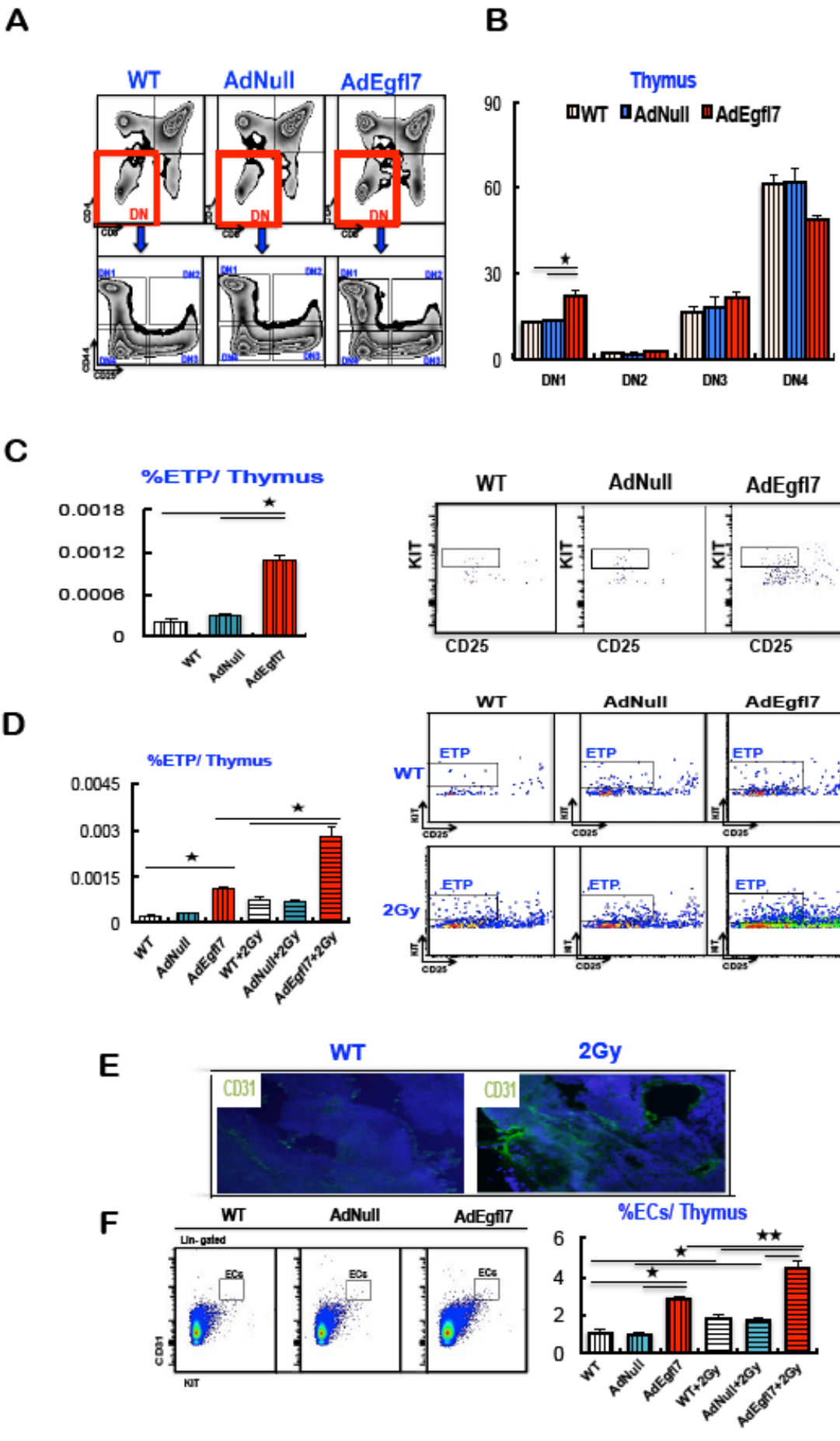
Figure 9. **Egfl7 is upregulated after irradiation and expands immature thymic progenitors**

(A) *Egfl7* expression as determined by qPCR (n=3/group) of total thymocytes from non-irradiated and mice irradiated with 2 Gy, splenocytes, FACS-sorted ETP based on CD45, CD44, c-Kit and CD25 expression, and MACS-sorted $CD45^{-}CD31^{+}$ endothelial cells (ECs) (n=2). Transcript levels were normalized to *β-actin*. Graphs represent averages from three to seven independently prepared templates. The data represent three independent experiments with similar results. (B) Thymus sections isolated from mice before (WT) and 3 d after irradiation were stained with antibodies against Egfl7 (shown in green) and endothelial cell-associated CD31 (shown in red). (C-F) Schematic representation of the experimental design: C57/BL6 mice were injected intravenously (i.v.) with AdEgfl7 or AdNull (no transgene) on d 0. (D) Egfl7 expression was determined in liver cell lysates by Western blotting 3 d after injection. (E) Representative FACS blots of CD4 and CD8 stained thymocytes isolated from vector-treated and non-treated mice. (F) Frequency of $CD4^{+}$ and $CD8^{+}$ single positive (SP), double positive (DP), and CD8 and CD4 double negative (DN) thymocytes was assessed by FACS (n=6). Values are the mean ± SEM of duplicate data points. *$p<0.05$, ** $p<0.01$ for all experiments.

X. ***Egfl7 augments ETPs under steady state condition and after irradiation***

The increase in the DN fraction (Figure 10A and 10B) was due to an accumulation of DN1 thymocytes (Figure 10B) that also harbors the most primate thymic progenitor ETP population ($Lin^{low}CD44^{+}CD25^{-}c\text{-}kit^{+}$), a fraction that was increased in thymocytes of AdEgfl7-treated mice (Figure 10C).

To understand if Egfl7 upregulation after irradiation might have functional consequences for thymic regeneration, groups of irradiated mice were injected with AdEgfl7 or AdNull. Thymi retrieved from AdEgfl7-treated mice showed a 2.5-fold increase in ETPs around 2.5-fold after irradiation compared to AdNull controls (Figure 10D). I show increased CD31 expression on EC after irradiation (Figure 10E). Similarly, an increase in $Lin^{-}c\text{-}Kit^{+}CD31^{+}$ ECs was observed after Egfl7 overexpression in thymi isolated 3 d after irradiation (Figure 10F). These data indicate that forced overexpression of Egfl7 expanded ETP and thymic ECs even after irradiation.


A
WT
AdNull
AdEgfl7
DN
B
Thymus
WT
AdNull
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90
60
30
0
DN1
DN2
DN3
DN4
C
%ETP/ Thymus
0.0018
0.0012
0.0006
0
WT
AdNull
AdEgfl7
KIT
CD25
D
%ETP/ Thymus
0.0045
0.003
0.0015
0
WT+2Gy
AdNull+2Gy
AdEgfl7+2Gy
ETP
2Gy
E
WT
2Gy
CD31
F
Lin- gated
ECs
%ECs/ Thymus
6
4
2
0

Figure 10. **Egfl7 expands early thymic progenitors and thymic ECs after irradiation**
(A-C) Thymocytes were isolated from nonirradiated (A-C) or irradiated (D-F) mice injected with AdEgfl7 or AdNull. (A-B) DN thymocytes were characterized using CD44 and CD25 as demonstrated in the representative gating schemes (A): DN1, $CD25^{-}CD44^{+}$; DN2, $CD25^{+}CD44^{+}$; DN3, $CD25^{+}CD44^{-}$; DN4, $CD25^{-}CD44^{-}$), and their percentage in each subpopulation is shown (B) (n=9). (C) Frequency of $CD44^{+}$c-$Kit^{+}CD25^{-}$ ETPs (left panel; n=9). Right panel show representative FACS plots of CD25 and c-Kit expression of pregated $CD44^{+}$ thymocytes. (D-F) 2 Gy irradiated mice were injected with AdEgfl7 or AdNull i.v. on d 0 (n=6/group). Thymi were analyzed 3 d after irradiation. (D) Intrathymic frequency of ETP is given (left panel; n=9). Right panel shows representative FACS plots of CD25 and c-Kit expression of pregated $CD44^{+}$ thymocytes. (E) Representative images of immunoreactive CD31 on thymic tissue sections in non-irradiated (WT) and irradiated mice (2Gy). (F) Representative FACS blot showing c-Kit and CD31 expression of pregated lin- thymocytes isolated from control and vector-injected mice at day 3 (left panel). Frequency of thymic Lin^{-}c-$Kit^{+}CD31^{+}$ ECs isolated from non-irradiated and irradiated mice (n=5/group). Values are mean ± SEM. All data are representative of at least two independent experiments. *$p<0.05$, ** $p<0.01$ for all experiments.

XI. ***Egfl7 upregulates Flt3 receptor on ECs***

Because Flt3 receptor signaling is important for ETP maintenance during steady-state thymopoiesis (Kenins et al., 2010), I investigated whether Egfl7 mediates cellular changes in the thymus by altering the Flt3/Flt3-ligand (Flt3L) pathway. Thymic tissues retrieved from mice after AdEgfl7 injection showed high *Egfl7*, *Flt3* and *Flt3L* (Figure 11A-C), but low *Hes1* expression by qPCR (Figure 11D). Egfl7 and Flt3 up- and Hes1 down-regulation was confirmed by Western blotting in AdEgfl7 infected human umbilical vein EC (HUVEC) (Figure 11E). Egfl7 overexpression led to the phosphorylation of Flt3 downstream signaling molecules of receptor tyrosine kinases like including AKT, STAT-3 and ERK1/2 in HUVEC (Figure 11E). Because Flt3L is produced by ECs (Solanilla et al., 2000), I reasoned that Flt3L plasma levels also might be elevated, which indeed was the case in AdEgfl7-treated mice (Figure 11F). Immunoreactive Flt3 was higher in irradiated thymic tissues (Figure 11H). Flt3 expression increased in thymic ETP, and ECs derived from AdEgfl7 treated or irradiated mice as determined by FACS (Figure 11H). These data demonstrate that Egfl7 upregulates Flt3 expression on ETP, and thymic ECs, and enhances the release of Flt3L from ECs (Figure 11I).

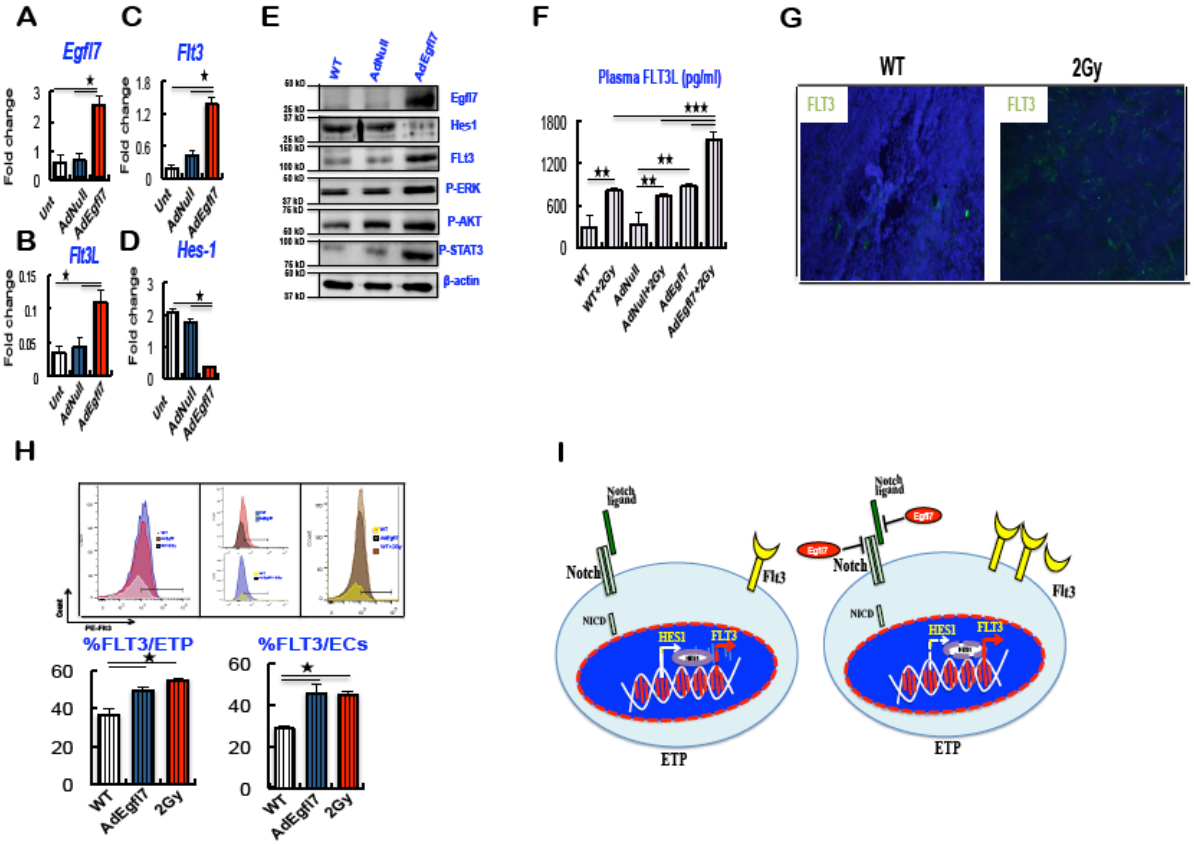

Figure 11. **Enhanced Flt3-Flt3 ligand signaling after Egfl7 overexpression**
(A-D) *Egfl7 (A)*, *Flt3 ligand* (C) *Flt3,* and (F) *Hes-1* mRNA induction was measured by qPCR in thymocytes isolated from AdNull and AdEgfl7 or untreated animals at d 3. (E) Adenovirus-treated HUVEC cells were collected after 24 h. Proteins were immunoblotted with the indicated antibodies. Representative blots are shown (n=2). (F) Flt3 ligand was assayed in plasma of mice with or without total body irradiation that were cotreated with AdEgfl7 by ELISA (n=5/group). (G) Representative images of immunoreactive Flt3 of thymi from non-irradiated (WT) and irradiated mice. (H) Flt3 expression in indicated thymic cell populations isolated from AdEgfl7 treated mice with or without irradiation in vivo as determined by FACS. Upper panel: FACS histograms showing Flt3 expression on $CD44^{+}$c-$Kit^{+}CD25^{-}$ ETPs, and Lin^{-}c-Kit^{+} $CD31^{+}$ EC. One representative of 3 experiments is shown. Lower panel: % expression of Flt3 on pregated ETP, and EC populations (n=6). (I) The depiction is a working model that implicates how Egfl7 might alter Flt3 expression. Egfl7-mediated Notch signaling blockade lead to *Hes1* down-regulation leading to the induction of Flt3 expression. Values are mean ± SEM. *p<0.05, ** p<0.01 for all experiments.

XII. ***Egfl7 expands ETP and ECs by activating Flt3 signaling***

Finally, to establish whether Flt3 signaling is critical for the observed ETP and thymic EC expansion, a Flt3 inhibitor was exploited. Flt3 inhibition prevented Egfl7-mediated ETP and thymic EC expansion *in vivo* (Figure 12A, 12B). Finally, I asked whether rec. Egfl7 was able to expand ETP *in vitro*. The addition of Egfl7 to cytokine-supplemented cultures readily

expanded ETP in stroma-free cultures after 3 d, a process that could be blocked in the presence of an Flt3 inhibitor (Figure 12E). Egfl7 enhances Flt3 signaling (Figure 12F). I propose that Egfl7 enhances the lympho-stromal cross talk that might have important implication for thymus organogenesis and regeneration that is necessary to maintain the thymic progenitor pool. In summary, the angiogenic factors like Egfl7 provide key molecular drivers enforcing thymus progenitor generation and thereby directly link endothelial biology to the production of T cell-based adaptive immunity.

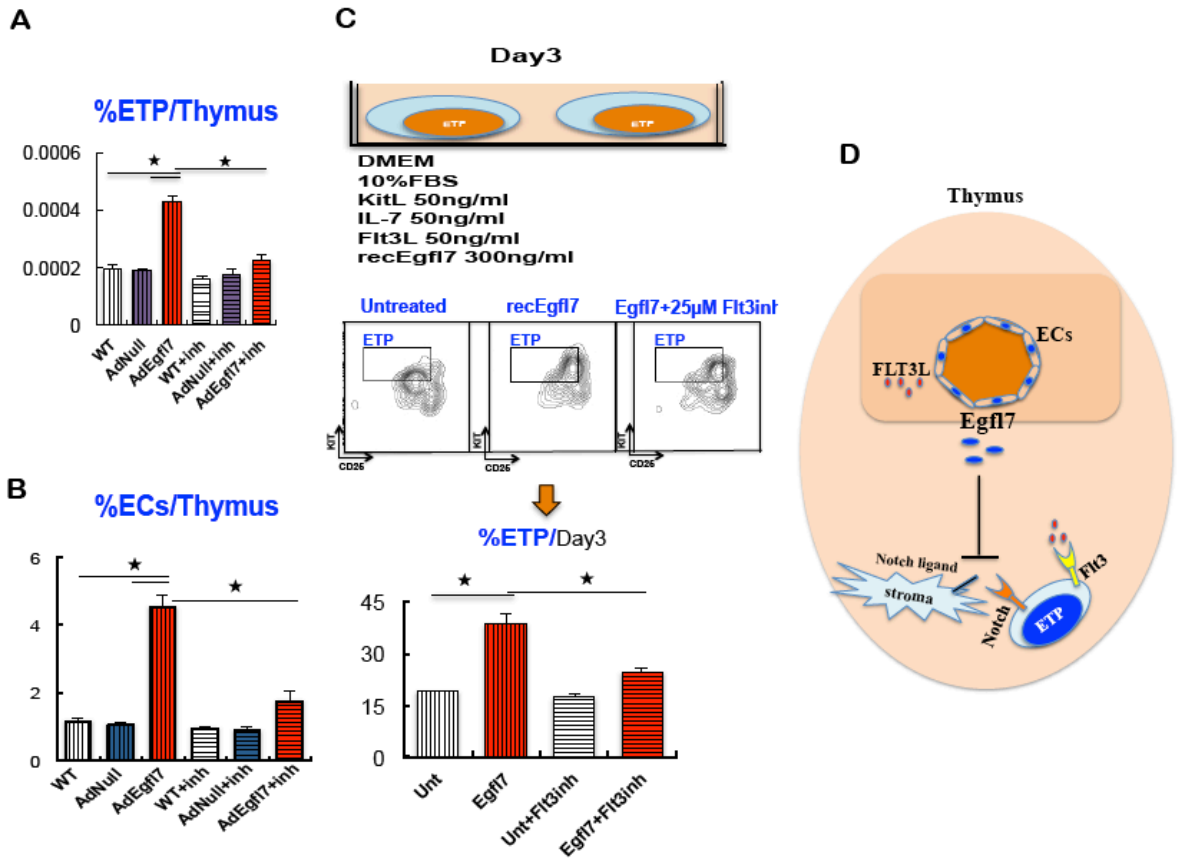

Figure 12: **Pharmacological inhibition of Flt3 prevented Egfl7-mediated ETP and EC expansion**

(A-B) C57Bl/6 mice were injected with adenovirus expressing *Egfl7* or no transgene and cotreated daily with Flt3 inhibitor. The percentage of $CD44^{+}c\text{-}Kit^{+}CD25^{-}$ ETP (A), and $Lin^{-}c\text{-}Kit^{+}CD31^{+}$ EC (B) was determined (n=5/group). (C) FACS-isolated $CD44^{+}c\text{-}Kit^{+}CD25^{-}$ ETP were cultured for 3 d in the mentioned cytokines in the presence or absence of rec. Egfl7 (n=5/group). (D) Model of Egfl7 effects in the thymic niche. Egfl7 induced in cells e.g. after irradiation blocks intracellular Notch signaling in target cells leading to expansion of ETP and EC through upregulation of Flt3 receptor on target cells and the release of Flt3 ligand. Values are mean ± SEM. *p<0.05, ** p<0.01 for all experiments.

Discussion

EC-derived (angiocrine) factors modulate stem cell fate in the vascular stem cell niche (Avecilla et al., 2004; Butler et al., 2010a; Heissig et al., 2002; Kiel and Morrison, 2006). My data establish that angiocrine factor Egfl7 differentially expressed in HSPC is a key regulator of stem cell and early progenitor fate. By using knockout and knockdown strategies, I unravel a mechanism whereby Egfl7 fine-tunes Notch signaling in a β3 integrin dependent manner (Figure 8). Egfl7 in the presence of β3 integrin increases HSC proliferation and differentiation by blocking Notch signaling. In contrast, Egfl7 in the absence of β3 integrin (Itgb3) enhances HSC self-renewal by activating Notch signaling. I identify critical stem-cell active cytokine pathways including c-Kit/KitL and Flt3 receptor/Flt3 ligand as direct targets following Egfl7 stimulation in Itgb3-expressing cells that enhances progenitor proliferation and cell differentiation.

The Notch pathway is classically activated following cell-cell interactions. ECs provide Notch ligands and expande HSC without exhaustion in a Notch dependent manner (Butler et al., 2010b). Notch1 activation keeps HSCs in a more immature state (Duncan et al., 2005; Varnum-Finney et al., 2000), but confusion persists over the role of Notch signaling in HSCs, as gain of function studies showed that Notch signaling (through conditional deletion of Notch1 or the ligand Jag1) was dispensable for HSC maintenance. Itgb3 is highly expressed on ECs and a marker/driver of stemness on quiescent HSCs (Umemoto et al., 2006; Umemoto et al., 2008), on solid cancer stem cells, and on leukemic stem cells (LSCs) (Seguin et al., 2014), where it mediates cell-matrix interactions.

Egfl7 is an extracellular HSC modulator due to its promiscuous ability to bind to both β3 integrin and Notch receptors, that can modulate cell-cell and cell-matrix signaling pathways in the niche. The function of HSCs during adulthood is to maintain the blood cell pool in balance (Dhahri et al., 2016; Heissig et al., 2009). Egfl7 is induced following

myelosuppression like chemotherapy or irradiation under conditions when the niche is in demand for replenishment of mature cells.

Integrin-cytokine signals have been reported: Thrombopoietin-mediated HSC maintenance depended on inside-out signaling through activation of the integrin-αvβ3 (Umemoto et al., 2012). Numerous studies demonstrated that Flt3 ligand and KitL are increased in the recovery phase after myelosuppression (Butler et al., 2010b; Heissig et al., 2002; Heissig et al., 2007; Kenins et al., 2008; Kenins et al., 2010). Egfl7 enhances HSC proliferation and differentiation in Itgb3-expressing cells through down regulation of Notch signaling. Egfl7 in the presence of Itgb3 pushes quiescent HSCs or early progenitors like ETPs towards proliferation and differentiation by increasing c-Kit and/or Flt3 receptor expression. Genetic or pharmacological blockade of Kit and Flt3 signaling prevented HSC proliferation and myeloid differentiation in Itgb3-expressing cells. Egfl7 blocked Notch signaling with down-regulation of Hes1 expression that induced Flt3 signaling on HSCs and ETPs. Flt3 receptor expression was induced on HSCs, ETPs after Egfl7 treatment (Salama et al., 2017), and ECs. Flt3/FL signaling is critical for T cell progenitor expansion *in vitro* (Moore and Zlotnik, 1997) and *in vivo* (Mackarehtschian et al., 1995). In accordance with my data it was reported that Hes1 binds to the promoter region of the Flt3 gene and enhances the promoter activity of Flt3 in leukemic cells (Kato et al., 2015). Pharmacological Flt3 blockade abolished Egfl7-mediated HSC and ETP proliferation. In support for a role of Egfl7 to block the Notch pathway, I observed in the thymus expansion of early thymocytes at the DN1 stage, and a differentiation block. This phenotype is similar to the one reported for mice with loss of Notch signaling.

c-Kit receptor regulates cell differentiation and proliferation, conveys signals to resist cell apoptosis, and plays a key role in tumor occurrence through activating the downstream signaling molecules after interaction with KitL (stem cell factor). I demonstrate that *Egfl7*

expression is highest in the human and murine HSC and early progenitors. *Egfl7* KD in HSCs led to impaired HSC survival by downregulation of c-Kit expression on HSC. miR126 is located in intron 7 of *Egfl7*. It was demonstrated that miR126 KD in HSCs expanded long-term HSC without exhaustion and reduced lymphopoiesis in vivo (Lechman et al., 2012). My data on the KD of *Egfl7* seem to be in contrast to the KD of miR126 in HSCs, because *Egfl7* KD in HSC impaired HSC survival. But overexpression of Egfl7 led to HSC expansion in Itgb3+ cells. This discrepancy might be due to the fact that the most important target gene of miR126 is the host gene *Egfl7* itself (Sun et al., 2010). Transcription of both occur, however mature mir-126 binds to a complementary sequence within Egfl7 preventing translation of the mRNA resulting in a decrease of Egfl7 protein levels. miR126 KD might have caused endogenous upregulation of Egfl7 that similar my study showing Egfl7 overexpression led to HSC survival and HSC expansion. My observation of increased AKT activation of AKT after Egfl7 treatment of hematopoietic cells indeed is similar to the phenotype observed in miR126 KD HSC.

Supposed discrepancies between the cellular Egfl7 and miR126 knockout phenotype is not new in the history of Egfl7 and miR126 (Schmidt et al., 2007). Like the hematopoietic phenotype, the vascular phenotypes of Egfl7 and miR126 deficient mice are grossly different. One reason might be that miR126 not only targets Egfl7, but also other critical genes that are required for normal HSC function, incl. c-Myc (Min et al., 2013), the chemokine SDF1, VEGF and others yet to be discovered (Kuhnert et al., 2008; Nichol and Stuhlmann, 2012; Parker et al., 2004)

The number of naive T cells decreases considerably with age, which is partly linked to the involution of the thymus (Linton and Dorshkind, 2004).

The ECM molecule Periostin, which like Egfl7 binds to Itgb3 (Gillan et al., 2002), is expressed in endothelial progenitors (Khurana et al., 2016). The Periostin-integrin av

interaction was shown to inhibit the FAK/PI3K/AKT pathway in HSCs, resulting in improved maintenance of quiescent HSCs (Khurana et al., 2016). Although Egfl7 and periostin target the same downstream integrin-mediated signals they show opposing effects on downstream signal transduction proteins like FAK and AKT. Further studies will be necessary to understand how both perostin and Egfl7 interact or modify each others binding to αvβ3 integrin.

Interestingly, Egfl7 is highly expressed in fetal and prenatal ETPs and downregulated in ETPs from older mice (Berent-Maoz et al., 2015). In ETP, Egfl7 expression was associated with a "young progenitor cell phenotype" that correlated with the expression of the high mobility group A2 protein, a factor shown to regulate HSC self-renewal potential (Copley et al., 2013). My data imply that blocking Itgb3 prevents stem cell aging by activating Notch signaling. Compelling evidence shows that muscle stem cells can be rejuvenated by enhancing the activation of the Notch-Delta pathway and by restoring satellite cell proliferation (Conboy et al., 2005). I confirmed reports that the number of Itgb3$^{-/-}$ HSCs under steady state conditions is similar to wild type HSCs (Miller et al., 2013). Egfl7 treatment of either Itgb3$^{-/-}$ mice or HSC cytokine cultures supplemented with an Itgb3 inhibitor dramatically expanded HSCs. Under these conditions, the Notch pathway was activated in HSCs. I indeed showed that Hes-1 in Itgb3 BMNCS after Egfl7 overexpression led to a dramatic increase in Hes1, leading to increased detection of NICD, a processed active form of Notch1. While in my tested total BMMNCs Hes1 lead to increases in NICD, Hes1 was shown to act as an inhibitor rather than an effector of Notch signaling in embryonic stem cells that delays embryonic stem cell differentiation and promotes preference for the mesoderm rather then neuronal fate (Kobayashi and Kageyama, 2010).

Egfl7-mediated Notch activation in Itgb3$^{-/-}$ HSCs is keeping HSC immaturity and enhances HSC maintenance. Only few data show pharmacological approaches to rejuvenate mammalian stem cell function by targeting HSCs or niche cells.

Loss of periostin-av interactions caused poor HSC function similar to that of "old" HSCs, while loss of Egfl7 or Itgb3 increased HSC self-renewal, a phenotype associated with young HSCs. Here, I propose that blocking Itgb3 during aging, where Egfl7 decreases will rejuvenate HSC or other tissue stem cells by activating Notch signaling. Itgb3 inhibitor treatment will stop or decelerate the aging process by Notch activation in stem cells. But further studies will be required to demonstrate this.

There is now evidence body that aged cells show higher Itgb3 expression and that regulates senescence by activating TGF-β in a paracrine and autocrine fashion (Rapisarda et al., 2017). It was reported that the αvβ3 antagonist cliengitide blocks the senescence-associated secretory phenotype without affecting proliferation. Overexpression of Egfl7 in Itgb3 wildtype mice indeed indicated that BM HSC and thymic ETP showed an aged hematopoietic phenotype: HSCs increase with age, they show impaired homing functions, but can be mobilized easier than young HSCs, and most importantly they are skewed towards myeloid cell differentiation, with fewer lymphoid progenitors compared with young HSC.

I set forward the idea that Egfl7 downregulation during aging leads to Notch activation, causing the decline of lymphoid progenitor fitness that can lead to aging-associated leukemogenesis (Henry et al., 2010). It will be important to see how aging alters the Itgb3 expression on HSCs or other stem cells.

My findings might also have important implications for blood-borne and solid cancer treatment. Deregulation of c-Kit has been reported in cancers like gastrointestinal tumors, acute myeloid leukemia, melanoma encouraging clinical studies to use c-Kit inhibitors (Abbaspour Babaei et al., 2016). Itgb3 expression is often found on cancer stem cells, and

was claimed to be responsible for chemotherapy-resistance. Both cytokine receptors, and Notch signaling are also critical pathways involved in the pathogenesis of acute leukemia and solid cancer like colon cancer. Internal tandem duplication mutations within FLT3 render the receptor constitutively active, driving proliferation and survival in leukemic blasts occurring in around 25% of AML (Chu et al., 2012) it will be important to understand the regulation of Egfl7 in AML cells. FLT3 inhibitors together with conventional chemotherapy have shown good treatment outcome in patients with FLT3-positive AML (Hassanein et al., 2016).

In AML cells Syk was shown to be a critical regulator of FLT3 (Puissant et al., 2014). Clustering of Itgb3 promotes transphosphorylation in the kinase domain of Src, which is followed by the recruitment and phosphorylation of Syk, which can occur in concert with signaling downstream from growth-factor receptors. Further studies will be important to understand the involvement of Egfl7 for these signaling pathways.

Conclusion

Collectively, I identify the ECM-bound protein Egfl7 as a critical factor that controls stem cell or progenitor fate within the BM and the thymic niche by fine-tuning Notch signaling and high-jacking various critical stem cell active cytokine receptors/tyrosine kinases that can promote BM or thymus regeneration (Figure 8). Egfl7 is a “chameleon-like protein” that dependent on the cellular integrin make-up.

My data demonstrate that biological effects of Egfl7 are dependent on Itgb3 signaling in the target cell, which will have important therapeutic implications both for tissue-specific stem cell expansion, but also to control cancers/cancer stem cell fate, and for age-related tissue regeneration. I hereby establish a directly link between endothelial biology and normal and malignant stem cell fate.

Annex

Primer sequences

Table 1. Primer list for quantitative polymerase chain reaction assay.		
gene name	Forward primer sequence	Reverse primer sequence
hβ-actin	5'-GACGACATGGAGAAAATCTG-3'	5'-AGGTCTCAAACATGATCTGG-3'
hEgfl7	5'-TGTAGCCAGGATGAGCAGTG-3'	5'-GCGGAGGAGAATCAGTCATC-3'
mB-actin	5'-CCAACCGTGAAAAGATGACC-3'	5'-ACCAGAGGCATACAGGGACA-3'
mEgfl7	5'-GCGCTGCCTGTCTAAGGA-3'	5'-CCTCTCTCGCCATGCTGT-3'
mHes1	5'-GTGGGTCCTAACGCAGTGTC-3'	5'-ACAAAGGCGCAATCCAATATG-3'
mFLT3	5'-GCCTCATTTCCTTGTGAACAG-3'	5'-GCTTGTTCTTATGATCGCAAAAT-3'
mC-kit	5'-GATCTGCTCTGCGTCCTGTT-3'	5'-CTTGCAGATGGCTGAGACG-3'
hGATA-2	5'-TAGGGGAGGGAACGGTCT-3'	5'-AGGAGCTGGGGGTAGAGTG-3'
hItgb3	5'-CGCTAAATTTGAGGAAGAACG-3'	5'-GAAGGTAGACGTGGCCTCTTT-3'
hC-kit	5'-TCAGCAAATGTCACAACAACC-3'	5'-TCTCCATCGTTTACAAATACTGTAGTG-3'

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