博士論文（要約）

Doctoral Thesis (Abridged)

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）

サラマ　ユセフ

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ABBREVIATIONS

Ab	antibody
Itgb3	integrin β3
BM	Bone marrow
BMMNC	bone marrow mononuclear cells
CB	cord blood
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
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 alters Notch signaling, thereby fine-tuning early progenitor fate dependent on the niche requirements. I demonstrate that ECs, and early stem and progenitor cells in the thymus (early thymic progenitors (ETPs) express Egfl7 especially during tissue regeneration.

Egfl7 expands ETPs and enhances their self-renewal capacity by activating Notch signaling and Flt3 signaling. Egfl7 enhances ETP expanson by inducing Flt3 receptor expression and signaling.

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

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.

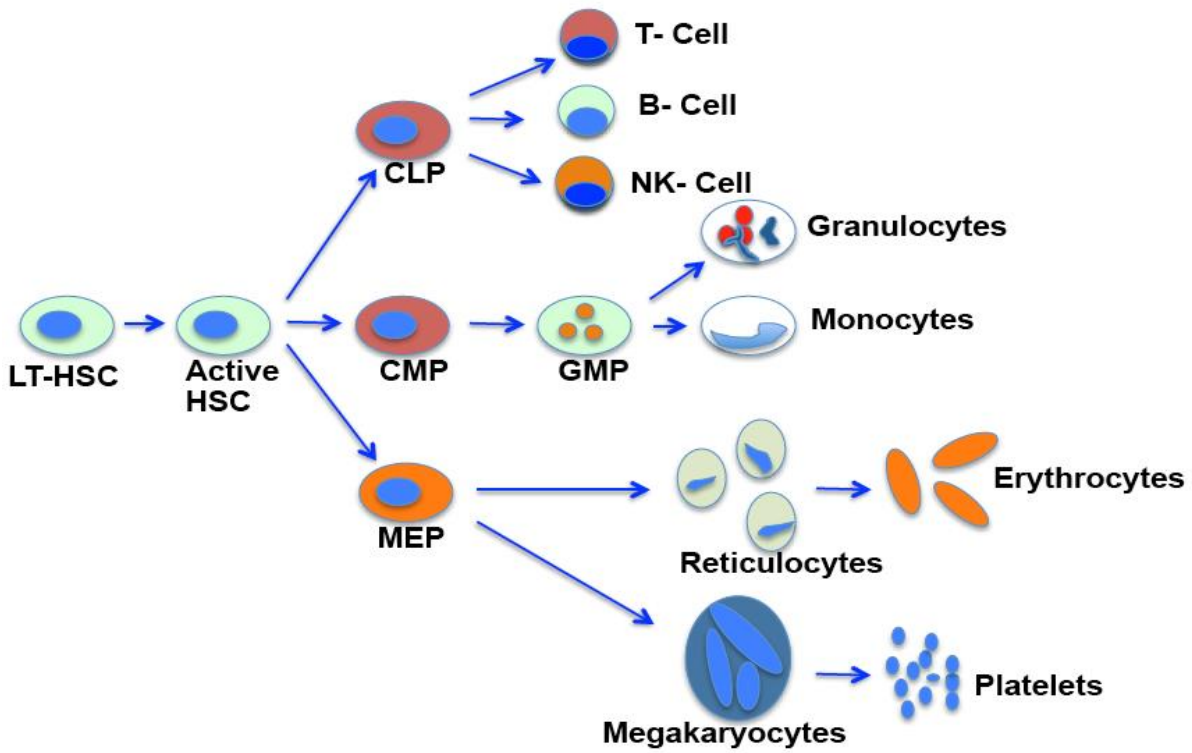

Figure 0. **Impaired proliferation of murine *Egfl7* knockdown KSL/progenitors**
During hematopoiesis, long-term HSC (LT-HSC) can respond to extracellular singals and differentiate into cells of the erythroid, lymphoid, and myeloid lineages.

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 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., 2010). 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).

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$^{w\text{-}v}$)	*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$^{W\text{-}v}$* 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

Thymopoiesis allow for the generation mature lymphocytes both under steady state and following stress situations like ischemia, myelosuppression, or during cancer growth. ECs located e.g. in the stem cell niche release angiogenic factors that can regulate steady state and stress-induced thymogenesis. The angiogenic factor epidermal growth factor–like domain 7 (Egfl7) is a promiscuous molecule, because it can bind to stem cell-associated receptors like the platelet-derived growth factor-beta receptor, Itgb3 and Notch1-4 receptors.

Here, I hypothesized that Egfl7 dictates early progenitor fate by fine-tuning Notch signaling through its ability to bind to some receptor, thereby altering critical stem cell 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.

In vivo manipulation of mice

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 Flt3 inhibitor (Tandutinib) (30mg/kg) or PBS orally daily at d 0, 1, and 2.

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.

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.

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 cultures

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.

Some cultures were supplemented with inhibitory reagents: Inhibitors for 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.

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.

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

VIII. P*erivascular 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.

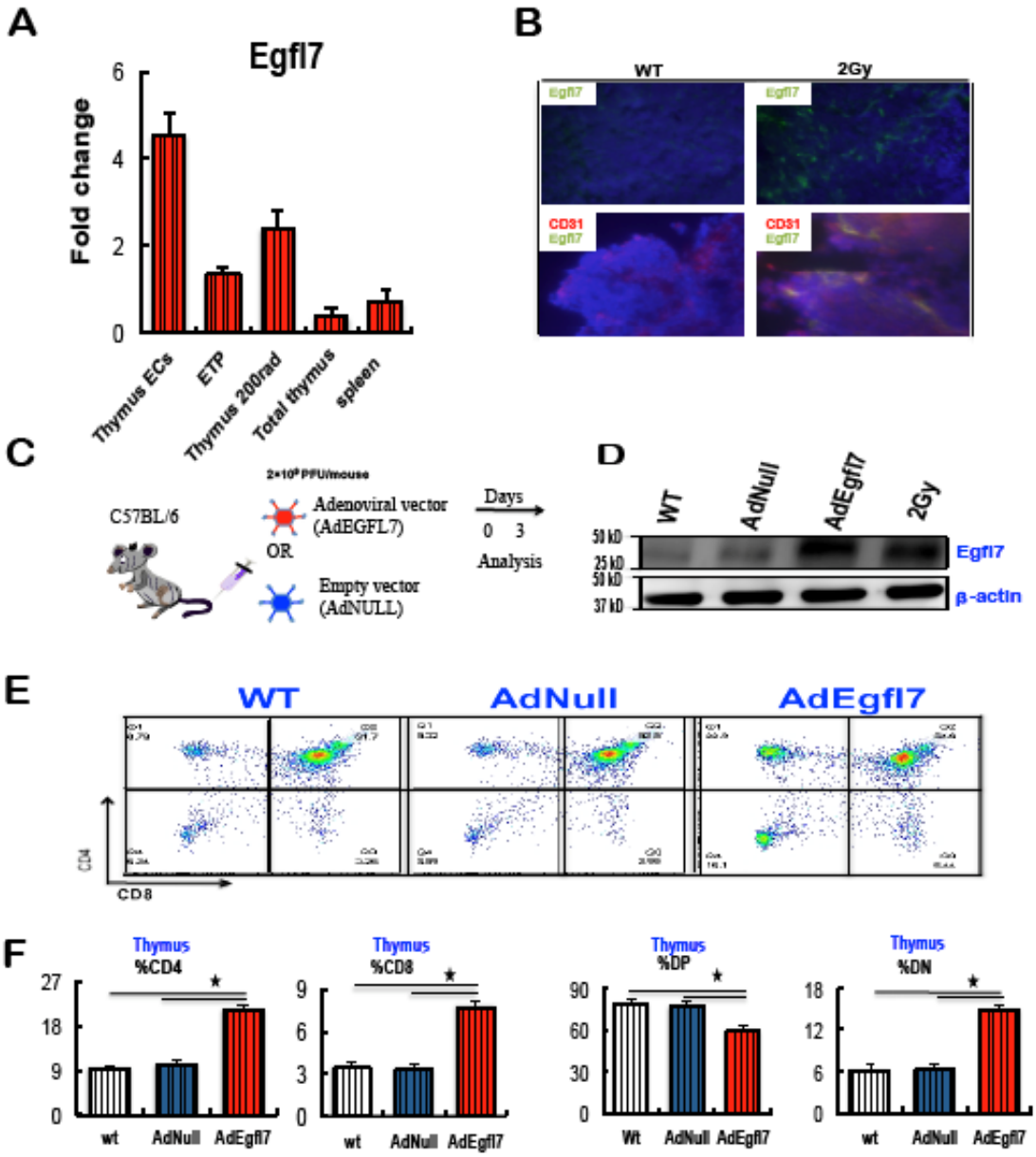

Figure 9. **Egfl7 is upregulated after irradiation and expands immature thymic progenitors**

(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.

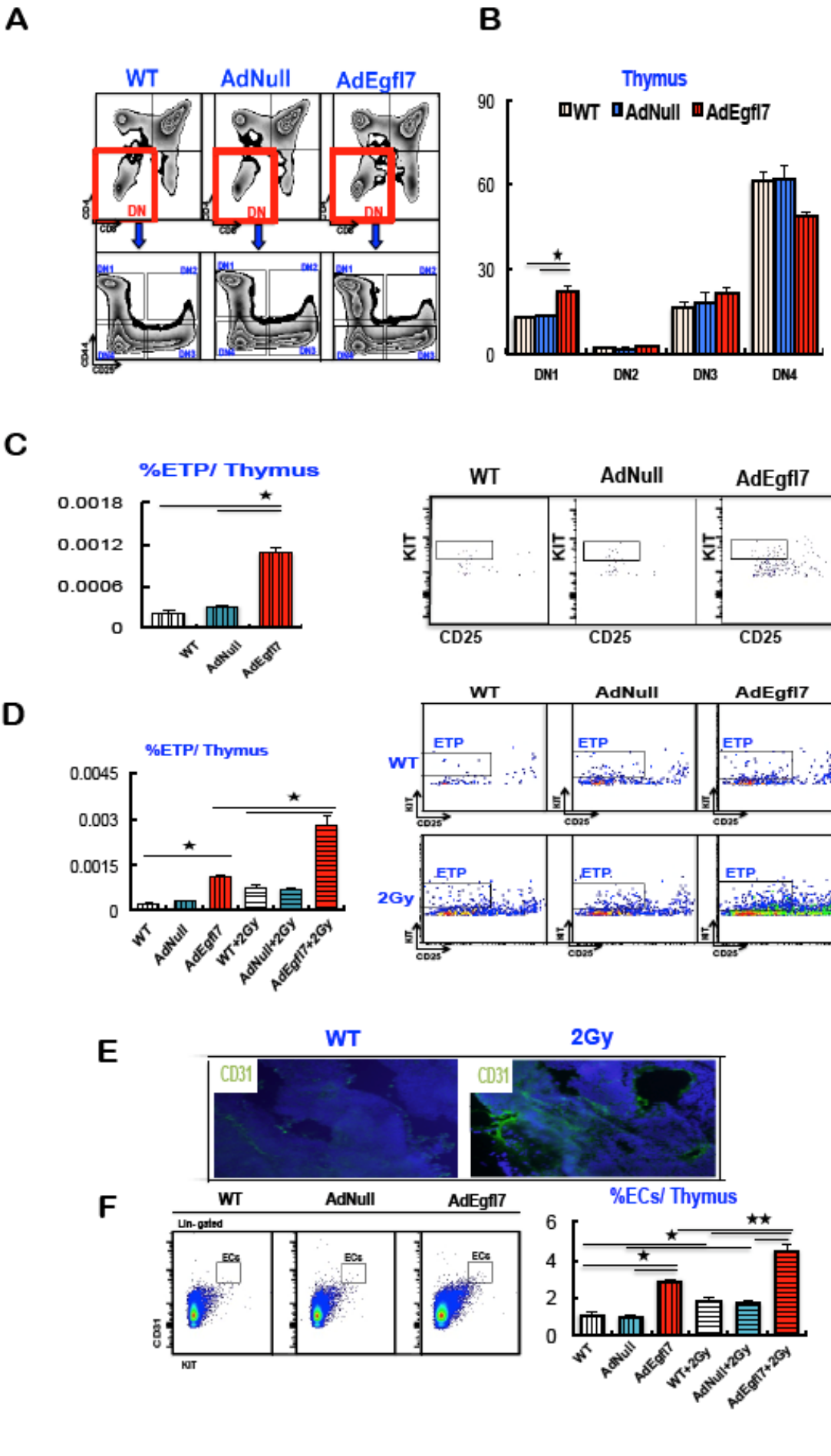
A
WT
AdNull
AdEgfl7
DN
B
Thymus
WT
AdNull
AdEgfl7
90
60
30
0
DN1
DN2
DN3
DN4
C
%ETP/ Thymus
0.0018
0.0012
0.0006
0
WT
AdNull
AdEgfl7
KIT
CD25
D
%ETP/ Thymus
0.0045
0.003
0.0015
0
WT
AdNull
AdEgfl7
WT+2Gy
AdNull+2Gy
AdEgfl7+2Gy
ETP
2Gy
E
WT
2Gy
CD31
F
Lin- gated
ECs
CD31
KIT
%ECs/ Thymus
6
4
2
0

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).

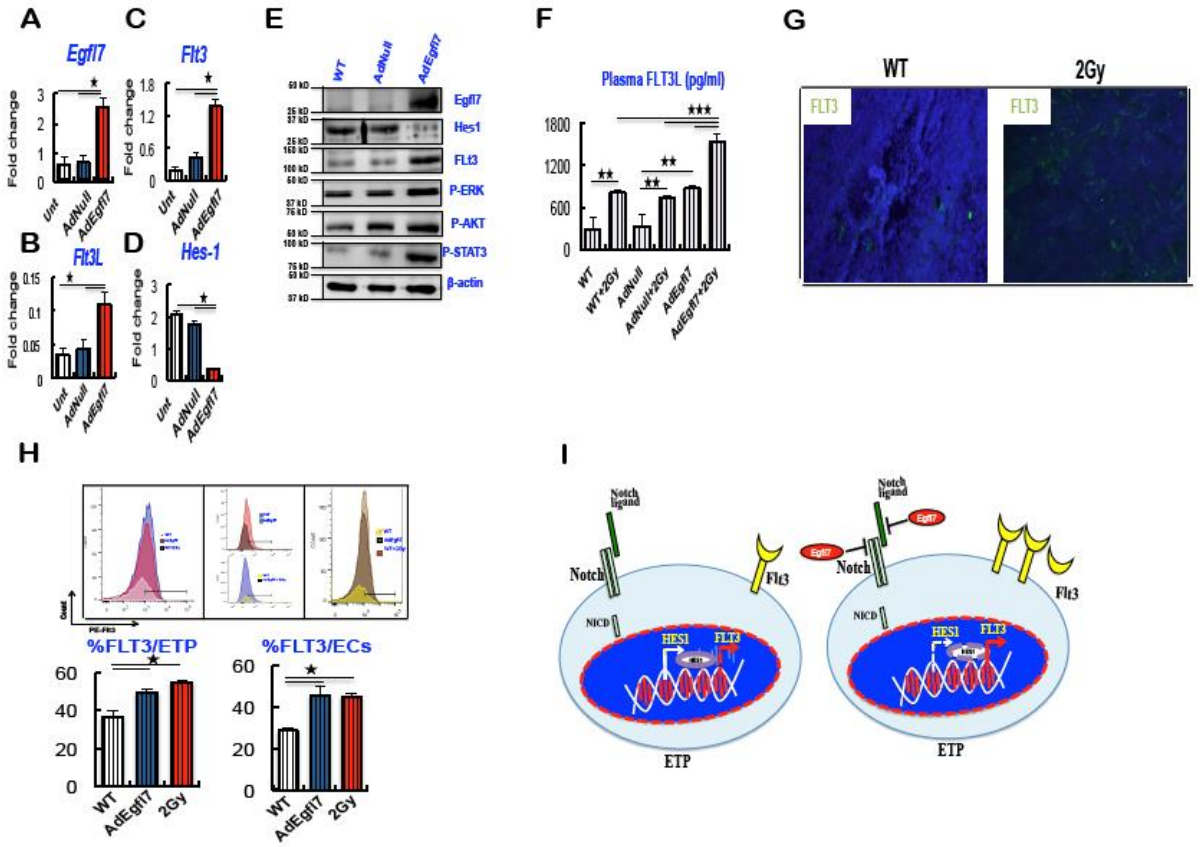

Figure 11. **Enhanced Flt3-Flt3 ligand signaling after Egfl7 overexpression**
(A-D) *Egfl7 (A), Flt3 ligand* (C) *Flt3*, and (F) *Hes-1* mRNA induction was measured by qPCR in thymocytes isolated from AdNull and AdEgfl7 or untreated animals at d 3. (E) Adenovirus-treated HUVEC cells were collected after 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.

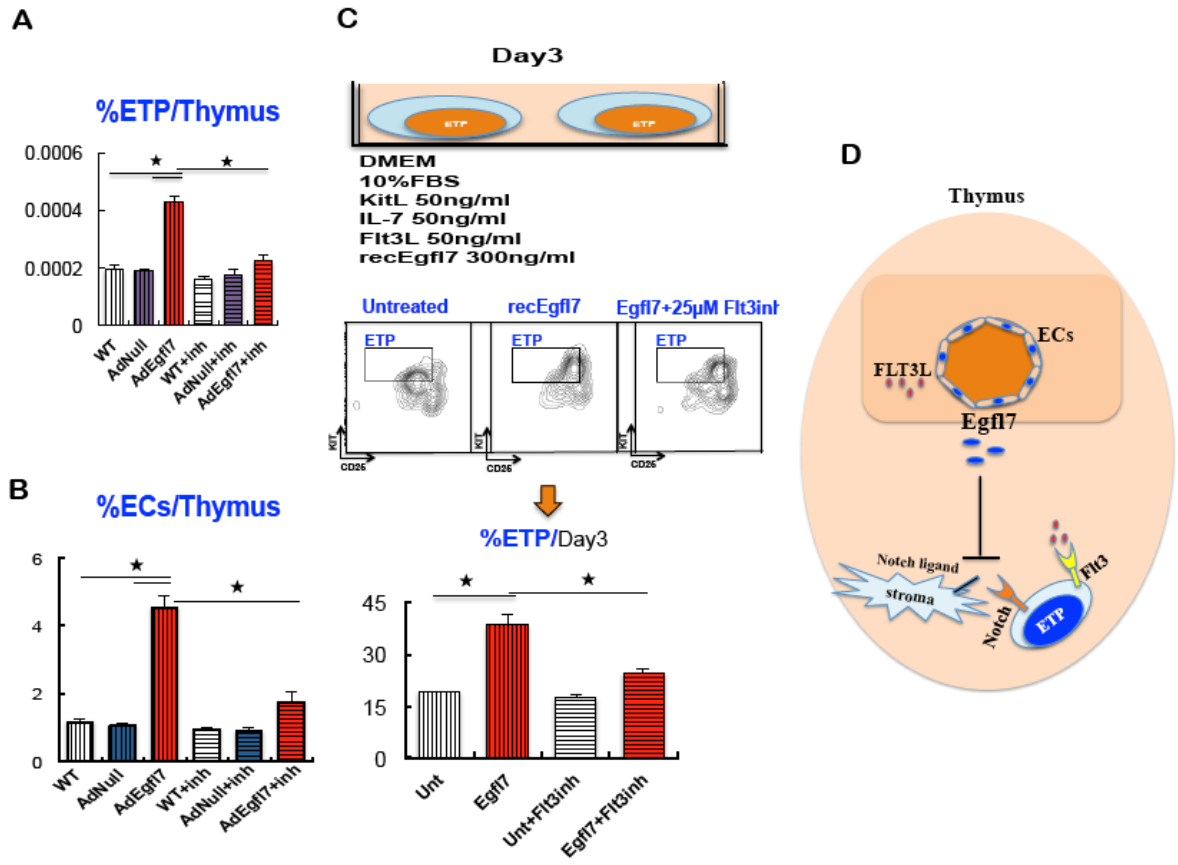

Figure 12: **Pharmacological inhibition of Flt3 prevented Egfl7-mediated ETP and EC expansion**

(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., 2010; Heissig et al., 2002; Kiel and Morrison, 2006).

Egfl7 blocked Notch signaling with down-regulation of Hes1 expression that induced Flt3 signaling on ETPs. Flt3 receptor expression was induced on 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 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.

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

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). I indeed showed that Hes-1 in thymocytes fter 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).

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).

My findings might also have important implications for blood-borne and solid cancer treatment. 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 the thymic niche by fine-tuning Notch signaling and high-jacking various critical stem cell active cytokine receptors/tyrosine kinases that can promote thymus regeneration.

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'

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I would like to thank Dr. Koichi Hattori for his support and guidance. He has a special sense for science and taught me how to build up knowledge. Beside that, he always took care of me when I had health or physical issue in the hospital.

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My deep thanks for the Japan government representing by MEXT for giving me the chance to do my research here in Japan by providing me with a scholarship that covered every things related to my study. Without their kind support and trust my research was difficult to do. I wish this country and the people of Japan to always have all of the best and prosperity.

Finally and not finally, I dedicate this thesis to my beloved family, for their constant support and unconditional love. They were patience during my absence from the home for 6 years. They missed me all of the time, but never make me felt I am absent.

References

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Mönch, K., Åstrand-Grundström, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E.W. (2001). Upregulation of Flt3 Expression within the Bone Marrow Lin−Sca1+c-kit+ Stem Cell Compartment Is Accompanied by Loss of Self-Renewal Capacity. Immunity *15*, 659-669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A*., et al.* (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295-306.

Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A., and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. Nat Immunol *4*, 168-174.

Avecilla, S.T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D.K., Dias, S., Zhang, F., Hartman, T.E*., et al.* (2004). Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nature medicine *10*, 64-71 Epub 2003 Dec 2021.

Berent-Maoz, B., Montecino-Rodriguez, E., Fice, M., Casero, D., Seet, C.S., Crooks, G.M., Lowry, W., and Dorshkind, K. (2015). The Expansion of Thymopoiesis in Neonatal Mice Is Dependent on Expression of High Mobility Group A 2 Protein (Hmga2). PloS one *10*, e0125414.

Butler, J.M., Kobayashi, H., and Rafii, S. (2010). Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nature reviews Cancer *10*, 138-146.

Chu, S.H., Heiser, D., Li, L., Kaplan, I., Collector, M., Huso, D., Sharkis, S.J., Civin, C., and Small, D. (2012). FLT3-ITD Knock-in Impairs Hematopoietic Stem Cell Quiescence/Homeostasis, Leading to Myeloproliferative Neoplasm. Cell stem cell *11*, 346-358.

Ciofani, M., Knowles, G.C., Wiest, D.L., von Boehmer, H., and Zúñiga-Pflücker, J.C. (2006). Stage-Specific and Differential Notch Dependency at the αβ and γδ T Lineage Bifurcation. Immunity *25*, 105-116.

Copley, M.R., Babovic, S., Benz, C., Knapp, D.J.H.F., Beer, P.A., Kent, D.G., Wohrer, S., Treloar, D.Q., Day, C., Rowe, K*., et al.* (2013). The Lin28b–let-7–Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. Nature cell biology *15*, 916-925.

de Koning, C., Nierkens, S., and Boelens, J.J. (2016). Strategies before, during, and after hematopoietic cell transplantation to improve T-cell immune reconstitution. Blood *128*, 2607.

de Leeuw, D.C., Denkers, F., Olthof, M.C., Rutten, A.P., Pouwels, W., Schuurhuis, G.J., Ossenkoppele, G.J., and Smit, L. (2014). Attenuation of microRNA-126 expression that drives CD34+38- stem/progenitor cells in acute myeloid leukemia leads to tumor eradication. Cancer research *74*, 2094-2105.

Delgado, A.C., Ferron, S.R., Vicente, D., Porlan, E., Perez-Villalba, A., Trujillo, C.M., D'Ocon, P., and Farinas, I. (2014). Endothelial NT-3 delivered by vasculature and CSF promotes quiescence of subependymal neural stem cells through nitric oxide induction. Neuron *83*, 572-585.

Dhahri, D., Sato-Kusubata, K., Ohki-Koizumi, M., Nishida, C., Tashiro, Y., Munakata, S., Shimazu, H., Salama, Y., Eiamboonsert, S., Nakauchi, H*., et al.* (2016). Fibrinolytic crosstalk with endothelial cells expands murine mesenchymal stromal cells. Blood *128*, 1063-1075.

Doliana, R., Bot, S., Bonaldo, P., and Colombatti, A. (2000). EMI, a novel cysteine-rich domain of EMILINs and other extracellular proteins, interacts with the gC1q domains and participates in multimerization. FEBS letters *484*, 164-168.

Dorrance, A.M., Neviani, P., Ferenchak, G.J., Huang, X., Nicolet, D., Maharry, K.S., Ozer, H.G., Hoellarbauer, P., Khalife, J., Hill, E.B*., et al.* (2015). Targeting leukemia stem cells in vivo with antagomiR-126 nanoparticles in acute myeloid leukemia. Leukemia *29*, 2143-2153.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N*., et al.* (2005). Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol *6*, 314-322.

Durrans, A., and Stuhlmann, H. (2010). A role for Egfl7 during endothelial organization in the embryoid body model system. J Angiogenes Res *2*, 4.
Fish, J.E., Santoro, M.M., Morton, S.U., Yu, S., Yeh, R.F., Wythe, J.D., Ivey, K.N., Bruneau, B.G., Stainier, D.Y., and Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. Developmental cell *15*, 272-284.
Fitch, M.J., Campagnolo, L., Kuhnert, F., and Stuhlmann, H. (2004). Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. Dev Dyn *230*, 316-324.
Fleishman, R.A. (1996). Engraftment of W/c-kit mutant mice is determined by stem cell competition, not by increased marrow 'space'. Exp Hematol *24*, 209-213.
Fleming, R.J. (1998). Structural conservation of Notch receptors and ligands. Seminars in cell & developmental biology *9*, 599-607.
Garbe, A.I., Krueger, A., Gounari, F., Zuniga-Pflucker, J.C., and von Boehmer, H. (2006). Differential synergy of Notch and T cell receptor signaling determines alphabeta versus gammadelta lineage fate. The Journal of experimental medicine *203*, 1579-1590.
Hassanein, M., Almahayni, M.H., Ahmed, S.O., Gaballa, S., and El Fakih, R. (2016). FLT3 Inhibitors for Treating Acute Myeloid Leukemia. Clinical Lymphoma Myeloma and Leukemia *16*, 543-549.
Hattori, K., Heissig, B., Wu, Y., Dias, S., Tejada, R., Ferris, B., Hicklin, D.J., Zhu, Z., Bohlen, P., Witte, L.*, et al.* (2002). Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. Nature medicine *8*, 841-849.
Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.*, et al.* (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell *109*, 625-637.
Heissig, B., Rafii, S., Akiyama, H., Ohki, Y., Sato, Y., Rafael, T., Zhu, Z., Hicklin, D.J., Okumura, K., Ogawa, H.*, et al.* (2005). Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. The Journal of experimental medicine *202*, 739-750 Epub 2005 Sep 2012.
Henry, C.J., Marusyk, A., Zaberezhnyy, V., Adane, B., and DeGregori, J. (2010). Declining lymphoid progenitor fitness promotes aging-associated leukemogenesis. Proceedings of the National Academy of Sciences of the United States of America *107*, 21713-21718.
Hodivala-Dilke, K.M., McHugh, K.P., Tsakiris, D.A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F.P., Coller, B.S., Teitelbaum, S., and Hynes, R.O. (1999). Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. The Journal of clinical investigation *103*, 229-238.
Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K.*, et al.* (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. Cell stem cell *4*, 263-274.
Huang, E., Nocka, K., Beier, D.R., Chu, T.Y., Buck, J., Lahm, H.W., Wellner, D., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell *63*, 225-233.
Johnson, L., Huseni, M., Smyczek, T., Lima, A., Yeung, S., Cheng, J.H., Molina, R., Kan, D., De, M., xE*, et al.* (2013). Anti-EGFL7 antibodies enhance stress-induced endothelial cell death and anti-VEGF efficacy. The Journal of clinical investigation *123*, 3997-4009.
Kanegae, Y., Makimura, M., and Saito, I. (1994). A simple and efficient method for purification of infectious recombinant adenovirus. Jpn J Med Sci Biol *47*, 157-166.
Kato, T., Sakata-Yanagimoto, M., Nishikii, H., Ueno, M., Miyake, Y., Yokoyama, Y., Asabe, Y., Kamada, Y., Muto, H., Obara, N.*, et al.* (2015). Hes1 suppresses acute myeloid leukemia development through FLT3 repression. Leukemia *29*, 576-585.
Kenins, L., Gill Jw Fau - Hollander, G.A., Hollander Ga Fau - Wodnar-Filipowicz, A., and Wodnar-Filipowicz, A. (2010). Flt3 ligand-receptor interaction is important for maintenance of early thymic progenitor numbers in steady-state thymopoiesis. Eur J Immunol *40 (1)*, 81-90.

Kiel, M.J., and Morrison, S.J. (2006). Maintaining Hematopoietic Stem Cells in the Vascular Niche. Immunity *25*, 862-864.
Kobayashi, T., and Kageyama, R. (2010). Hes1 regulates embryonic stem cell differentiation by suppressing Notch signaling. Genes to Cells *15*, 689-698.
Kuhnert, F., Mancuso, M.R., Hampton, J., Stankunas, K., Asano, T., Chen, C.Z., and Kuo, C.J. (2008). Attribution of vascular phenotypes of the murine Egfl7 locus to the microRNA miR-126. Development *135*, 3989-3993.
Kumar, C.C. (2003). Integrin alpha v beta 3 as a therapeutic target for blocking tumor-induced angiogenesis. Current drug targets *4*, 123-131.
Le Bras, A., Samson, C., Trentini, M., Caetano, B., Lelievre, E., Mattot, V., Beermann, F., and Soncin, F. (2010). VE-statin/egfl7 expression in endothelial cells is regulated by a distal enhancer and a proximal promoter under the direct control of Erg and GATA-2. PloS one *5*.
Lechman, E.R., Gentner, B., van†Galen, P., Giustacchini, A., Saini, M., Boccalatte, F.E., Hiramatsu, H., Restuccia, U., Bachi, A., Voisin, V.*, et al.* (2012). Attenuation of miR-126 Activity Expands HSC In†Vivo without Exhaustion. Cell stem cell *11*, 799-811.
Li, X., and von Boehmer, H. (2011). Notch Signaling in T-Cell Development and T-ALL. ISRN Hematology *2011*, 9.
Li, Z., Chen, P., Su, R., Li, Y., Hu, C., Wang, Y., Arnovitz, S., He, M., Gurbuxani, S., Zuo, Z.*, et al.* (2015). Overexpression and knockout of miR-126 both promote leukemogenesis. Blood *126*, 2005-2015.
Li, Z., Lu, J., Sun, M., Mi, S., Zhang, H., Luo, R.T., Chen, P., Wang, Y., Yan, M., Qian, Z.*, et al.* (2008). Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. Proceedings of the National Academy of Sciences of the United States of America *105*, 15535-15540.
Linton, P.J., and Dorshkind, K. (2004). Age-related changes in lymphocyte development and function. Nat Immunol *5*, 133-139.
Liu, Z., Wang, F., and Chen, X. (2008). Integrin α(v)β(3)-Targeted Cancer Therapy. Drug development research *69*, 329-339.
Mackarehtschian, K., Hardin, J.D., Moore, K.A., Boast, S., Goff, S.P., and Lemischka, I.R. (1995). Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoietic progenitors. Immunity *3*, 147-161.
Mayer, T.C., and Green, M.C. (1968). An experimental analysis of the pigment defect caused by mutations at the W and S1 loci in mice. Developmental biology *18*, 62-75.
Moore, T.A., and Zlotnik, A. (1997). Differential effects of Flk-2/Flt-3 ligand and stem cell factor on murine thymic progenitor cells. J Immunol *158*, 4187-4192.
Murphy, M., Reid, K., Williams, D.E., Lyman, S.D., and Bartlett, P.F. (1992). Steel factor is required for maintenance, but not differentiation, of melanocyte precursors in the neural crest. Developmental biology *153*, 396-401.
Nichol, D., Shawber, C., Fitch, M.J., Bambino, K., Sharma, A., Kitajewski, J., and Stuhlmann, H. (2010). Impaired angiogenesis and altered Notch signaling in mice overexpressing endothelial Egfl7. Blood *116*, 6133-6143.
Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., and Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. Genes & development *3*, 816-826.
Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science (New York, NY) *273*, 242-245.
Palomero, T., Lim, W.K., Odom, D.T., Sulis, M.L., Real, P.J., Margolin, A., Barnes, K.C., O'Neil, J., Neuberg, D., Weng, A.P.*, et al.* (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proceedings of the National Academy of Sciences of the United States of America *103*, 18261-18266.

Parker, L.H., Schmidt, M., Jin, S.W., Gray, A.M., Beis, D., Pham, T., Frantz, G., Palmieri, S., Hillan, K., Stainier, D.Y.*, et al.* (2004). The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. Nature *428*, 754-758.
Picuric, S., Friedrich, M., and Oess, S. (2009). Expression and purification of recombinant human EGFL7 protein. Protein Expr Purif *68*, 1-6.
Poulos, M.G., Crowley, M.J., Gutkin, M.C., Ramalingam, P., Schachterle, W., Thomas, J.L., Elemento, O., and Butler, J.M. (2015). Vascular Platform to Define Hematopoietic Stem Cell Factors and Enhance Regenerative Hematopoiesis. Stem Cell Reports *5*, 881-894.
Puissant, A., Fenouille, N., Alexe, G., Pikman, Y., Bassil, C.F., Mehta, S., Du, J., Kazi, J.U., Luciano, F., Rönnstrand, L.*, et al.* (2014). SYK Is a Critical Regulator of FLT3 In Acute Myeloid Leukemia. Cancer cell *25*, 226-242.
Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity *10*, 547-558.
Russell, E.S. (1949). Analysis of pleiotropism at the W-locus in the mouse; relationship between the effects of W and Wv substitution on hair pigmentation and on erythrocytes. Genetics *34*, 708-723.
Russell, E.S., and Bernstein, S.E. (1968). Proof of whole-cell implant in therapy of W-series anemia. Archives of biochemistry and biophysics *125*, 594-597.
Salama, Y., Hattori, K., and Heissig, B. (2017). The angiogenic factor Egfl7 alters thymogenesis by activating Flt3 signaling. Biochemical and biophysical research communications.
Sambandam, A., Maillard, I., Zediak, V.P., Xu, L., Gerstein, R.M., Aster, J.C., Pear, W.S., and Bhandoola, A. (2005). Notch signaling controls the generation and differentiation of early T lineage progenitors. Nat Immunol *6*, 663-670.
Schmidt, M.H.H., Bicker, F., Nikolic, I., Meister, J., Babuke, T., Picuric, S., Muller-Esterl, W., Plate, K.H., and Dikic, I. (2009). Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. Nature cell biology *11*, 873-880.
Seguin, L., Kato, S., Franovic, A., Camargo, M.F., Lesperance, J., Elliott, K.C., Yebra, M., Mielgo, A., Lowy, A.M., Husain, H.*, et al.* (2014). A β3 integrin-KRAS-RalB complex drives tumor stemness and resistance to EGFR inhibition. Nature cell biology *16*, 457-468.
Seita, J., and Weissman, I.L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. Wiley interdisciplinary reviews Systems biology and medicine *2*, 640-653.
Shen, Q., Goderie, S.K., Jin, L., Karanth, N., Sun, Y., Abramova, N., Vincent, P., Pumiglia, K., and Temple, S. (2004). Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science (New York, NY) *304*, 1338-1340. Epub 2004 Apr 1301.
Sitnicka, E., Buza-Vidas, N., Ahlenius, H., Cilio, C.M., Gekas, C., Nygren, J.M., Månsson, R., Cheng, M., Jensen, C.T., Svensson, M.*, et al.* (2007). Critical role of FLT3 ligand in IL-7 receptor–independent T lymphopoiesis and regulation of lymphoid-primed multipotent progenitors. Blood *110*, 2955.
Solanilla, A., Grosset, C., Lemercier, C., Dupouy, M., Mahon, F.X., Schweitzer, K., Reiffers, J., Weksler, B., and Ripoche, J. (2000). Expression of Flt3-ligand by the endothelial cell. Leukemia *14*, 153-162.
Soncin, F., Mattot, V., Lionneton, F., Spruyt, N., Lepretre, F., Begue, A., and Stehelin, D. (2003). VE-statin, an endothelial repressor of smooth muscle cell migration. The EMBO journal *22*, 5700-5711.
Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D.T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood *99*, 2369-2378.
Umemoto, T., Yamato, M., Shiratsuchi, Y., Terasawa, M., Yang, J., Nishida, K., Kobayashi, Y., and Okano, T. (2006). Expression of Integrin β3 Is Correlated to the Properties of Quiescent Hemopoietic Stem Cells Possessing the Side Population Phenotype. The Journal of Immunology *177*, 7733-7739.
Wang, S., Aurora, A.B., Johnson, B.A., Qi, X., McAnally, J., Hill, J.A., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. Developmental cell *15*, 261-271.

Weinberg, K., Blazar, B.R., Wagner, J.E., Agura, E., Hill, B.J., Smogorzewska, M., Koup, R.A., Betts, M.R., Collins, R.H., and Douek, D.C. (2001). Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. Blood *97*, 1458-1466.
Yang, R.H., Qi, S.H., Ruan, S.B., Lin, Z.P., Lin, Y., Zhang, F.G., Chen, X.D., and Xie, J.L. (2016). EGFL7-overexpressing epidermal stem cells promotes fibroblast proliferation and migration via mediating cell adhesion and strengthening cytoskeleton. Molecular and cellular biochemistry *423*, 1-8.
Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q.*, et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature *425*, 836-841.
Zhang, Y., Yang, P., Sun, T., Li, D., Xu, X., Rui, Y., Li, C., Chong, M., Ibrahim, T., Mercatali, L.*, et al.* (2013). miR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis. Nature cell biology *15*, 284-294.
Zsebo, K.M., Williams, D.A., Geissler, E.N., Broudy, V.C., Martin, F.H., Atkins, H.L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock, D.C.*, et al.* (1990). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell *63*, 213-224.