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The angiogenic factor Egfl7 alters thymogenesis by activating Flt3 signaling

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ABSTRACT

Thymic regeneration is a crucial function that allows for the generation of mature T cells after myelosuppression like irradiation. However molecular drivers involved in this process remain undefined. Here, we report that the angiogenic factor, epidermal growth factor-like domain 7 (Egfl7), is expressed on steady state thymic endothelial cells (ECs) and further upregulated under stress like post-irradiation. Egfl7 overexpression increased intrathymic early thymic precursors (ETPs) and expanded thymic ECs. Mechanistically, we show that Egfl7 overexpression caused Flt3 upregulation in ETPs and thymic ECs, and increased Flt3 ligand plasma elevation *in vivo*. Selective Flt3 blockade prevented Egfl7-driven ETP expansion, and Egfl7-mediated thymic EC expansion *in vivo*. We propose that the angiogenic factor Egfl7 activates the Flt3/Flt3 ligand pathway and is a key molecular driver enforcing thymus progenitor generation and thereby directly linking endothelial cell biology to the production of T cell-based adaptive immunity.

1. Introduction

T cell reconstitution of the recipient immune system after allogeneic hematopoietic cell transplantation is largely dependent on homeostatic replication of donor T cells infused with the bone marrow (BM) [1], and can take several months to even years [2].

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 endothelial cells (EC). Myelosuppression after total body irradiation augments the release of other endothelial-derived factors like vascular endothelial growth factor-A, and the Notch ligand Jagged-1 [3,4]. Thymic ECs have been reported to be 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.

Epidermal growth factor–like domain 7 (*Egfl7*, also known as vascular endothelial statin - *VE-statin*) is expressed in the endothelium during embryogenesis [5] and during cancer growth. Egfl7 antagonizes Notch receptor/ligand interaction by binding to the receptor or its corresponding ligand [6,7]. Crosstalk of tissue-specific stem cells and ECs has been reported for neuronal cells [8,9], mesenchymal stem cells within the BM [10], or hematopoietic stem and progenitor cells [11].

The $Flt3^+$ fraction (Lin^- $CD25^-$ Kit^+ $Flt3^+$) of Lin^- $CD25^-$ Kit^+ ETP harbors canonical intrathymic T cell progenitors [12,13]. Flt3 or Flt3 ligand (Flt3L) deficient mice display defects in very early T cell development [14,15].

Here, we reveal that Egfl7 is expressed by thymic ECs, and by ETPs under steady state conditions, and that Egfl7 was rapidly upregulated after irradiation. The importance of Flt3 activation for Egfl7-mediated expansion of ETP and thymic niche cells was shown using Flt3 antagonists.

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2. Materials and methods

2.1. Mice

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. 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.

2.2. 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) [7,16]. 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 [17]. Mice were intravenously injected with 2×10^9 plaque-forming units of AdEgfl7 and AdNull in the tail vein. In some experiments, mice received Flt3 inhibitor (Tandutinib) (30 mg/kg) or PBS orally daily at d 0, 1, and 2. Mice were sacrificed on d3 for thymus collection. Thymus and peripheral blood samples obtained using heparin-coated capillaries were collected.

2.3. Quantitative PCR

Total RNA was prepared using TRIzol Reagent (Ambion by Life Technologies, #15596018). First-strand cDNA was synthesized from total RNA using a High Capacity Reverse Transcriptase kit (Applied Biosystems) and run using the Step One Plus qPCR machine (Applied Biosystems) with SYBR Premix Ex Taq II (×2) Tli RNaseH Plus (Takara, #RR820). Quantitative PCR was performed with TaqMAn probes as follows:

hβ-actin 5′-GACGACATGGAGAAAATCTG-3′, 5′-AGGTCTCAAACATGATCTGG-3'.
hEgfl7 5′-TGTAGCCAGGATGAGCAGTG-3′, 5′-GCGGAGGAGAATCAGTCATC-3'.
hHes1 5′-GAAGCACCTCCGGAACCT-3′, 5′-GTCACCTCGTTCATGCACTC-3'.
hFLT3 5′-TGGAATTTCTGGAATTTAAGTCG-3′,5′-TTTCCCGTGGGTGACAAG-3.
hFLT3L 5′-GGCCGAAATGACAGTGCT-3′, 5′-AGCAGCAGGAGGAGATAGGTT-3'.
mβ-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'.
mFlt3L 5′-CCTAGGATGCGAGCCTTGT-3′, 5′-TGTTTTGGTTCCCAACTCG-3'.

2.4. Western blotting analysis

Cultured HUVEC transfected with 30 MOI AdEgfl7 or AdNull were lysed with lysis buffer (Cell Signaling Technology). Protein crude was recovered by acetone. Cell lysates (2–50 μg 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), Hes1 (Santa Cruz Biotech, sc-25392), β-actin (Cell Signaling, #4967), p-ERK (Cell Signaling, #4370), p-AKT (Cell Signaling, #9271) and p-STAT3 (Cell Signaling, #9131), FLT3 (Proteintech, 21049-1-AP). 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).

2.5. Flow cytometry and cell sorting

Thymi were minced, and thymus single cell suspensions were blocked with 2% fetal bovine serum (FBS), washed and stained. Single cell suspensions (1×10^6 cells in 100ul FACS buffer) were stained at 4 °C for 15 min with directly fluorochrome-labeled antibodies. Antibody details: FITC-conjugated anti-mouse CD44. The following antibodies were PE-conjugated: anti mouse CD8, CD4, CD25, FLT3, Lin-; PE-CY7-conjugated anti mouse CD45, and PE-CY7-conjugated anti mouse SCA1; APC-CY7-conjugated anti-mouse c-KIT, APC-conjugated anti-mouse Lin-, APC-conjugated anti-mouse c-KIT, and APC-conjugated anti-mouse CD4; Pacific Blue-conjugated anti-mouse CD4, and PerCP-CY5.5-conjugated anti-mouse c-KIT. Dead cells were excluded using PI staining. Flow cytometric analysis was performed using a FACSAria machine. Data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). Thymic EC were isolated using MACS sorting of $CD45^-CD31^+$ cells (Miltenyi Biotec).

2.6. Immunostaining

Thymi were embedded in OCT compound (Sakura), and frozen. Tissue sections (5 μm) 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 crytosections 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 h 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.

2.7. ELISA

Flt3 ligand plasma levels were measured using commercially available ELISA kits (R&D Systems Inc., Minneapolis, MN).

2.8. Egfl7 overexpression in 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 μg/ml streptomycin (P/S; WAKO, Osaka, Japan) at 37 °C, 5% CO2. HUVEC were transfected with AdEgfl7 or AdNull containing no transgene at an MOI of 30.

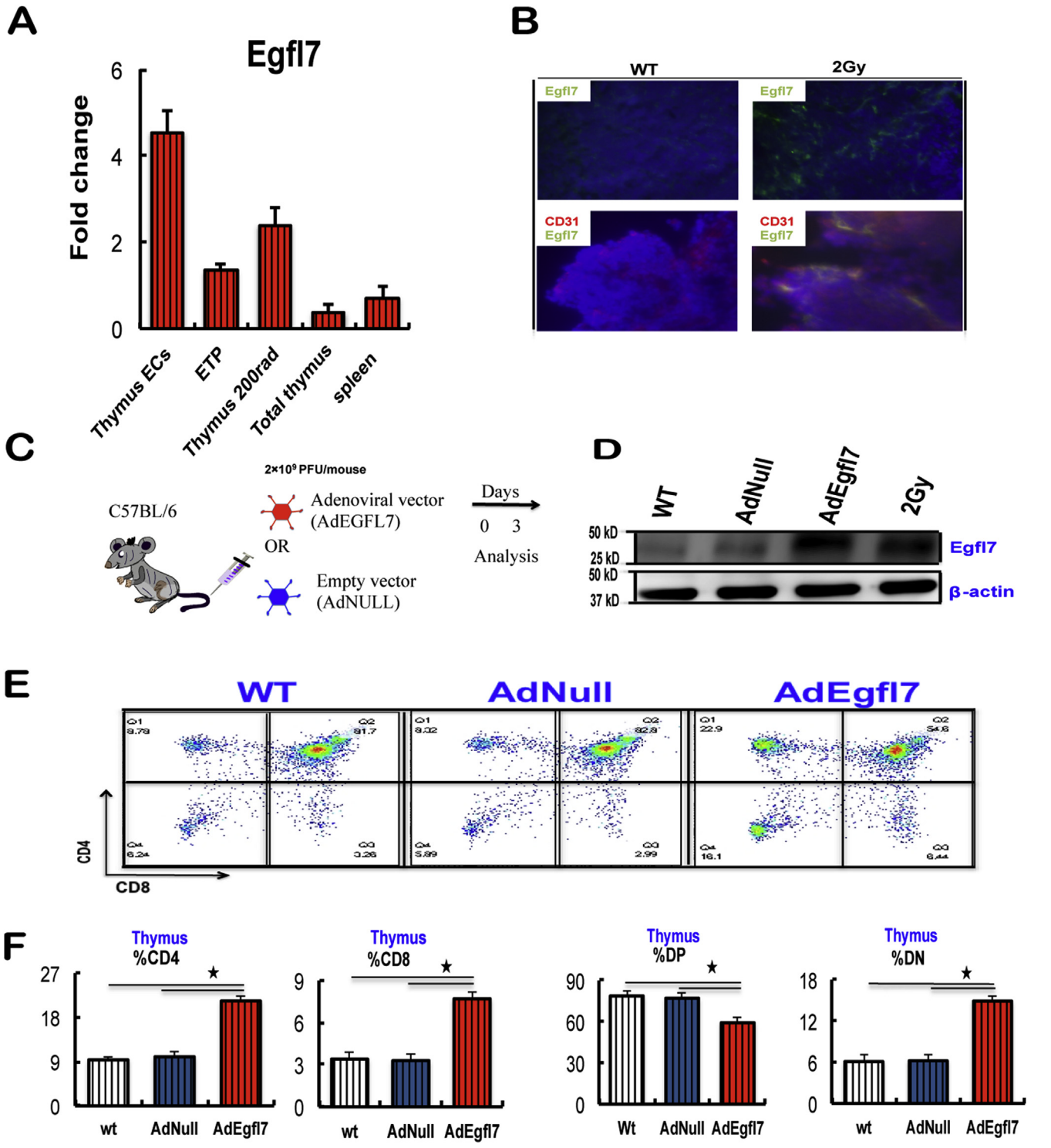
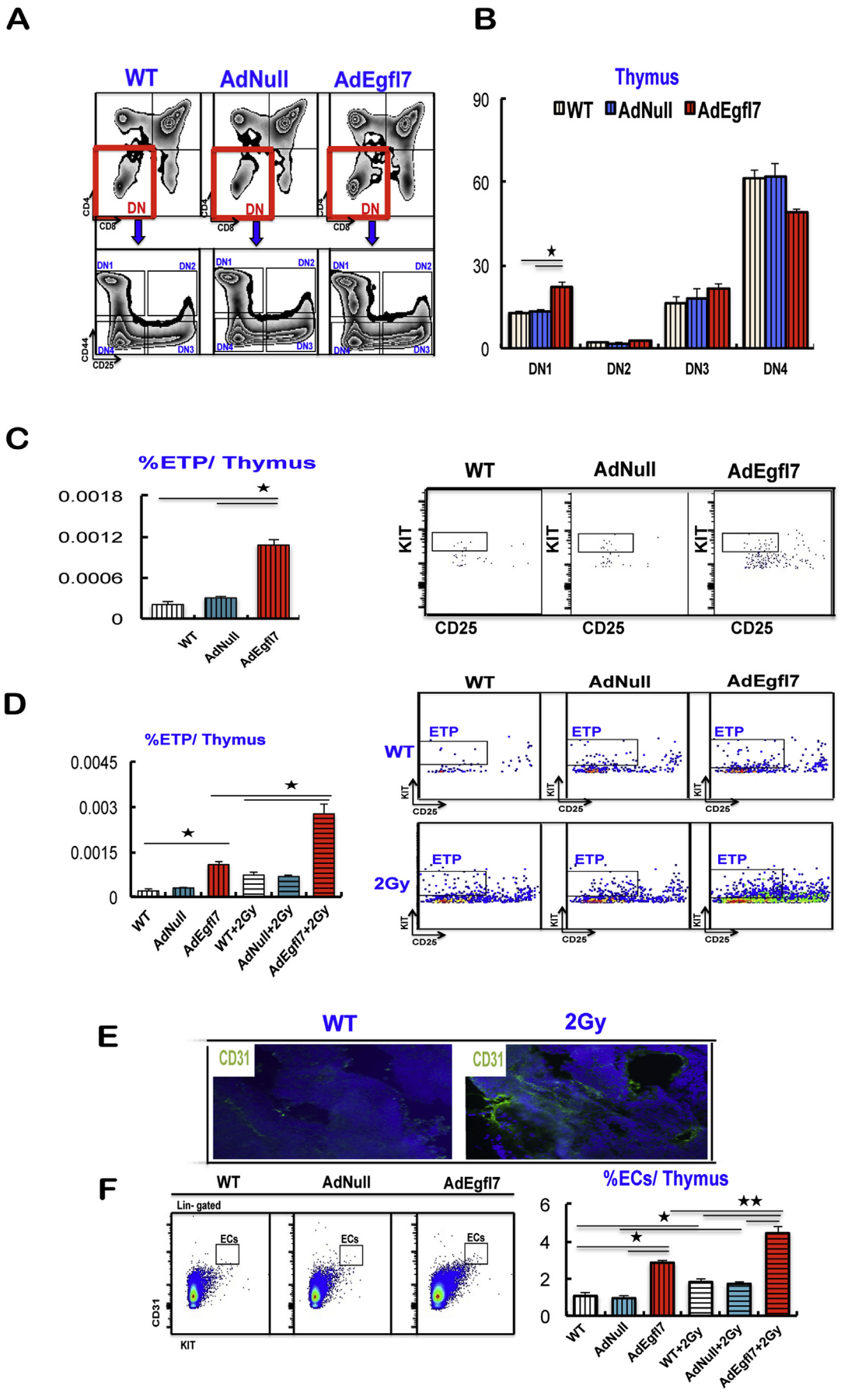


Fig. 1. Egfl7 is upregulated after irradiation and expands immature thymic progenitors. (A) *Egfl7* expression as determined by qPCR (n = 3/group) of total thymocytes from non-irradiated and mice irradiated with 2 Gy, splenocytes, FACS-sorted ETP based on CD45, CD44, c-Kit and CD25 expression, and MACS-sorted $CD45^{-}CD31^{+}$ 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.

2.9. 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 with recombinant mouse Kit ligand (PeproTech, 50 ng/ml), mouse IL-7 (PeproTech, 50 ng/ml), mouse Flt3 ligand (PeproTech,


A
WT
AdNull
AdEgfl7
DN
CD4
CD8
DN1
DN2
DN3
DN4
CD44
CD25
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

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.

2.10. Statistics

Data are reported as the mean ± standard error of the mean (SEM). Student *t*-tests were performed. $P < 0.05$ were considered significant.

3. Results

3.1. Perivascular Egfl7 deposition in the irradiated thymus

Epidermal growth factor-like 7 (Egfl7) transcripts were found by quantitative PCR (qPCR) in lymphoid organs like the thymus, and spleen (Fig. 1A). High Egfl7 expression was detected in MACS-sorted $CD45^{-}CD31^{+}$ endothelial cells (EC), and FACS-sorted $CD44^{+}c\text{-}Kit^{+}CD25^{-}$ early thymocyte progenitors (ETP) (Fig. 1A). Impairs 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 (Fig. 1A). Immunoreactive Egfl7 was found perivascular in thymi of irradiated, but not in non-irradiated mice (Fig. 1B). These data indicate that Egfl7 is expressed in a thymocyte subpopulation and thymic EC, and its expression is upregulated after sublethal irradiation.

3.2. Egfl7 augments early thymic progenitors and ECs

To investigate Egfl7's role for T cell development *in vivo*, C57/BL6 mice were injected intravenously with adenovirus expressing Egfl7 (AdEgfl7) or adenovirus with no transgene (AdNull) (Fig. 1C). Egfl7 overexpression was confirmed in liver cell lysates from adenovirus-injected mice by Western blotting (Fig. 1D). Next, we 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 (Fig. 1F) 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), indicating that Egfl7 is a modulator of early T cell development.

3.3. Egfl7 augments ETPs under steady state condition and after irradiation

The increase in the DN fraction (Fig. 2A and B) was due to an accumulation of DN1 thymocytes (Fig. 2B) that also harbors the most primate thymic progenitor ETP population ($Lin^{low}CD44^{+}CD25^{-}c\text{-}Kit^{+}$), a fraction that also was increased in thymocytes of AdEgfl7-treated mice (Fig. 2C).

To understand if Egfl7 upregulation after irradiation might have a role in 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 (Fig. 2D). We show increased CD31 expression in EC after irradiation (Fig. 2E). Similarly, an increase in $Lin^{-}c\text{-}Kit^{+}CD31^{+}$ ECs was observed after Egfl7 overexpression in thymi isolated 3 d after irradiation (Fig. 2F). These data indicate that forced overexpression of Egfl7 expanded ETP and thymic ECs even after irradiation.

3.4. Egfl7 upregulates Flt3 receptor on ECs

Notch signaling activates target genes like Hairy enhancer of split 1 (*Hes1*). Hes1 can bind to the promoter region of the *FMS-like tyrosine kinase 3* (*Flt3*) gene that downregulates Flt3 promoter activity [18]. Because Flt3 receptor signaling is important for ETP maintenance during steady-state thymopoiesis [19], we 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* (Fig. 3A–C), but low *Hes1* expression by qPCR (Fig. 3D). Egfl7 and Flt3 up- and Hes1 down-regulation was confirmed by Western blotting in AdEgfl7 infected human umbilical vein EC (HUVEC) (Fig. 3E). Egfl7 overexpression led to the phosphorylation of Flt3 downstream signaling molecules of receptor tyrosine kinases like including AKT, STAT-3 (signal transducers and activators of transcription) and extracellular signal-regulated kinase (ERK1/2) in HUVEC (Fig. 3E). Because Flt3L is produced by ECs [20], we reasoned that Flt2L plasma levels also might be elevated, which indeed was the case in AdEgfl7-treated mice (Fig. 3F). Immunoreactive Flt3 was higher in irradiated thymic tissues (Fig. 3H). Flt3 expression increased in thymic ETP, and ECs derived from AdEgfl7 treated or irradiated mice as determined by FACS (Fig. 3H). These data demonstrate that Egfl7 upregulates Flt3 expression on ETP, and thymic ECs, and enhances the release of Flt3L from ECs (Fig. 3I).

3.5. 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* (Fig. 4A and B). Finally, we asked whether recombinant 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 (Fig. 4E). These data are consistent with a mechanism whereby Egfl7 enhances Flt3 signaling (Fig. 4F).

Fig. 2. Egfl7 expands early thymic progenitors and thymic endothelial cells 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 $CD44^{+}c\text{-}Kit^{+}CD25^{-}$ 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 $Lin^{-}c\text{-}Kit^{+}CD31^{+}$ 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.

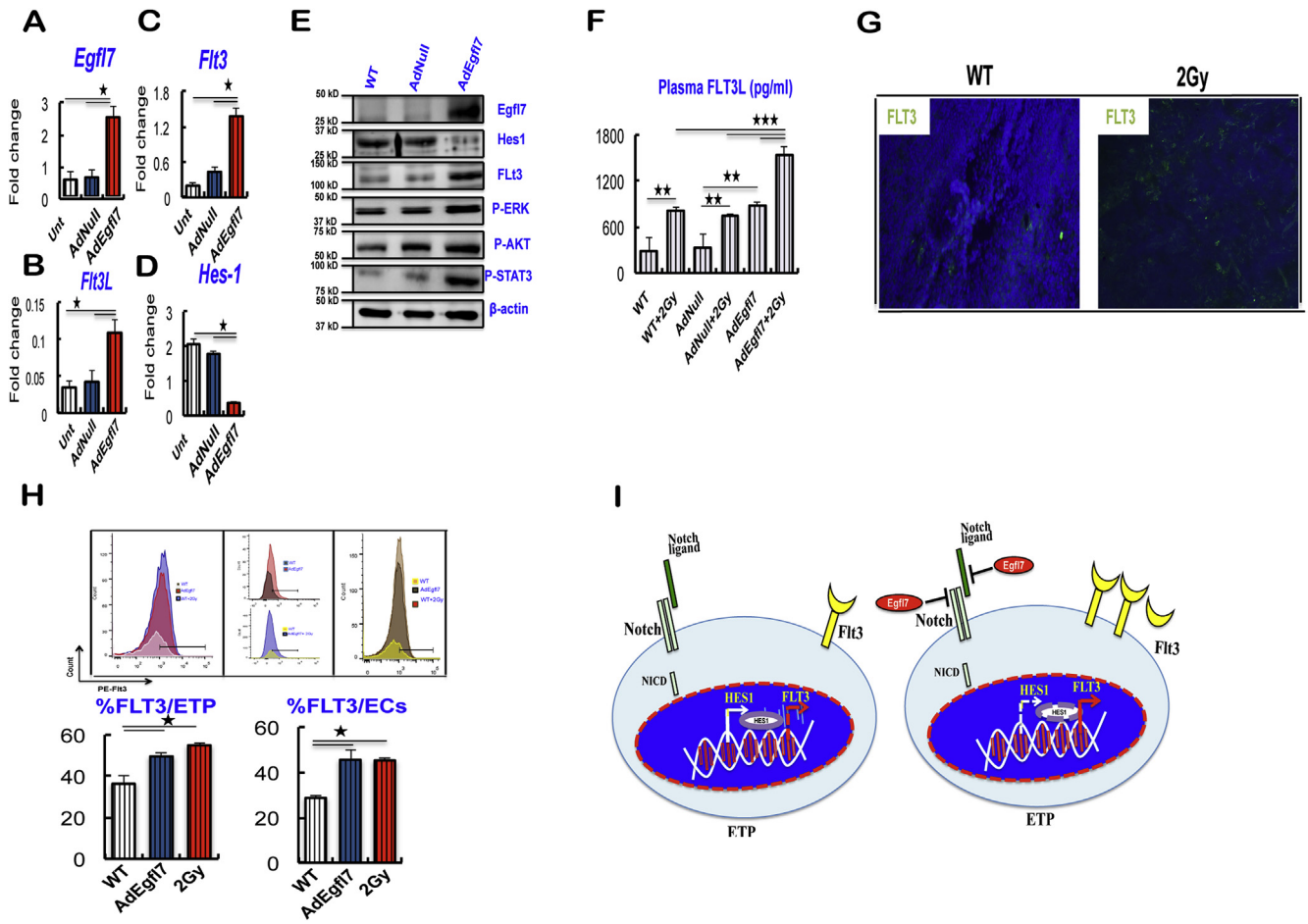


Fig. 3. Enhanced Flt3-Flt3 ligand signaling after Egfl7 overexpression. (A–D) *Egfl7 (A)*, *Flt3 ligand* (C) *Flt3*, and (F) *Hes-1* mRNA induction was measured by qPCR in thymocytes isolated from AdNull and AdEgfl7 or untreated animals at d 3. (E) Adenovirus-treated HUVEC cells were collected after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

4. Discussion

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. Here, we show that the angiogenic factor Egfl7 is upregulated in the irradiated thymus, and can expand ETPs and thymic ECs by activating the Flt3/Flt3L pathway.

Egfl7 upregulated Flt3 expression on ETPs, and thymic EC. Importantly, pharmacological Flt3 blockade abolished Egfl7-mediated ETP expansion both *in vitro* and *in vivo*. Given that Egfl7 expanded ECs, and ECs are known to release large amounts of soluble Flt3L [20], it was not surprising to detect increased FL in plasma of Egfl7 overexpressing mice. We showed that Egfl7 suppressed the expression of the Notch downstream target Hes1 in mouse thymocytes and EC. Egfl7 by blocking Notch signaling leads to Hes1 downregulation. Hes1 binding to the promoter region of the Flt3 gene enhances the promoter activity of Flt3 in leukemic cells [18]. We propose that a similar mechanism leads to Flt3 upregulation on ETP and ECs.

A recent report indicated that intrathymic FL expression on the surface of perivascular fibroblasts enhances thymic recovery after irradiation [21]. Higher Flt3L availability correlated with an enhanced recovery of thymic cellularity [22]. We showed that Egfl7 lead to increased Flt3L plasma levels after irradiation, in a condition of impaired thymic function, a fact important if one would want to treat patients with Egfl7 to improve T cell function.

Our data are in accordance with studies demonstrating the importance of Flt3/FL signaling for T cell progenitor expansion *in vitro* [23] and *in vivo* [24]. Flt3 often interacts with other growth factors that enhance downstream signaling. It is conceivable that Egfl7 can highjack other growth factor pathways that ultimately can lead to thymic regeneration. Egfl7 by enhancing Flt3 signaling also changed the thymic niche, and therefore could have changed the production of Notch ligands, adding more complexity to the observation that Egfl7 overexpression expands ETPs and EC within the thymus.

Thymic portal ECs via lymphotoxin beta receptor signaling control T-cell regeneration [25]. Our results reveal a hitherto uncharacterized function of Egfl7 in expanding thymic early DN1 and the ETP progenitors. Similar to our data, it was reported that Egfl7 is expressed in ETP [26].

The decrease in total thymocytes after Egfl7 treatment was

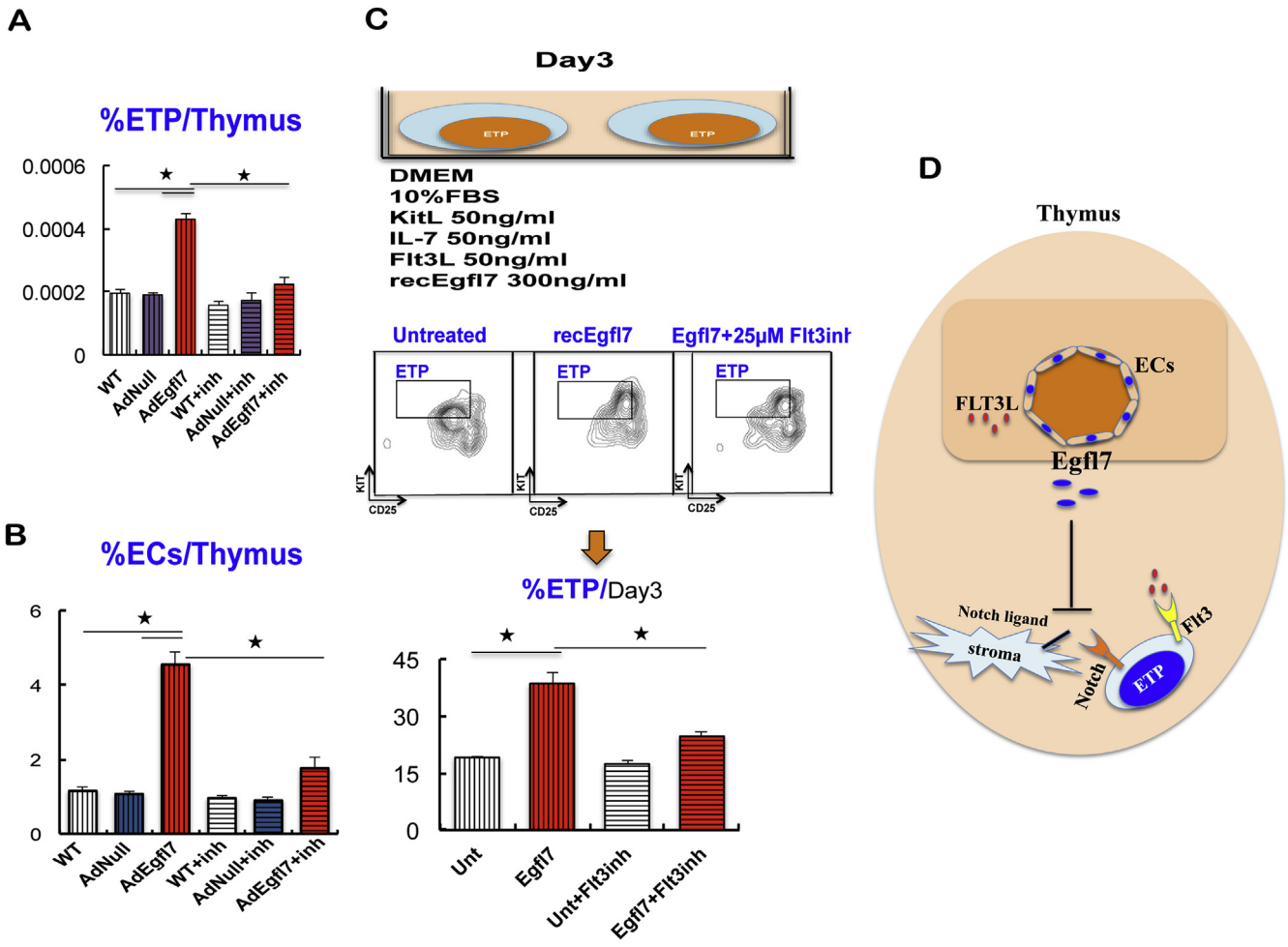


Fig. 4. Pharmacological inhibition of Flt3 prevented Egfl7-mediated ETP and EC expansion. (A–B) C57Bl/6 mice were injected with adenovirus expressing Egfl7 or no transgene and cotreated daily with Flt3 inhibitor. The percentage of $CD44^{+}c\text{-}Kit^{+}CD25^{-}$ ETP (A), and $Lin^{-}c\text{-}Kit^{+}CD31^{+}$ 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 recombinant 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.

mainly due to a reduction in DP cells. The impact of Egfl7 on ETPs and DN1 thymocytes is likely to be direct as it could be reproduced in in vitro cultures. The observed phenotype of expansion of early thymocytes at a DN stage and a maturation block resembled the phenotype observed in mice with loss of Notch signaling, but further studies will be required to understand whether Egfl7-mediated Flt3 alterations are dependent or can also occur independent of Notch signaling.

Our data demonstrate that Egfl7 enhances Flt3-FL signaling in the thymus by inducing Flt3 expression in ETPs and by concomitant upregulation of its ligand FL in ECs that ultimately most likely in combination with other factors will induce thymopoiesis. We 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.

Conflict of interest

The authors declare no conflict of interest.

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