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Regulatory role of mouse epidermal growth factor-like protein 8 in thymic epithelial cells

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ABSTRACT

Unlike epidermal growth factor-like protein 7 (EGFL7), which is a secreted protein implicated in the regulation of blood vessel formation and cell migration, little is known about the physiological function of EGFL8. Thymic epithelial cells (TECs) play a pivotal role in T-cell development by regulating cellular interactions and expression of growth factors, cytokines, and chemokines. In order to investigate the functional role of EGFL8 in TECs, we transfected TECs with an EGFL8-expressing vector to overexpress EGFL8 protein and with an EGFL8 siRNA to knockdown EGFL8 expression. EGFL8-silenced TECs showed significant increase in the number of adherent thymocytes by enhancing the expression of intercellular adhesion molecule-1 (ICAM-1), while the overexpression of EGFL8 inhibited the adherence of TECs to thymocytes by suppressing ICAM-1 expression. Furthermore, in vitro co-culture study revealed that knockdown of EGFL8 facilitated the maturation of thymocytes to $CD4^+$ and $CD8^+$ single-positive populations. These regulatory effects of EGFL8 in T-cell development were further confirmed by the results that knockdown of EGFL8 enhanced the expression of genes involved in thymopoiesis, such as interleukin-7 (IL-7), granulocyte/macrophage-colony stimulating factor (GM-CSF), and thymus-expressed chemokine (TECK). Our data show that EGFL8 exerts inhibitory effects on TECs and thymocytes, suggesting that EGFL8 acts as a negative regulatory molecule in the development of T cells in the mouse thymus.

1. Introduction

The thymus is the central lymphoid organ that provides a unique microenvironment for T-cell development and maturation [1]. Thymic epithelial cells (TECs) constitute a key component of the thymic stroma and facilitate T-cell differentiation and maturation from lymphoid progenitor cells. The thymic stromal cells play an essential role in this process by direct cellular interaction as well as by producing various molecules including growth factors, cytokines, and chemokines. The proper differentiation and organization of different TECs is pivotal for both thymocyte development and T-cell repertoire selection [2].

Epidermal growth factor (EGF)-like domain is an evolutionary conserved protein domain, which is found in a large number of vertebrate proteins that are involved in several cellular activities, such as blood coagulation, fibrinolysis, cell adhesion, and neural and vertebrate development [3]. For example, EGF-like repeats located in extracellular domains of Notch receptors play a central role in controlling the Notch signaling pathway [4], and these repeats are implicated in the development of many serious diseases such as symptomatic ischemic cerebrovascular disease that is caused by mutations and polymorphisms in the EGF-like domains of Notch3 protein [5].

Epidermal growth factor-like protein 7 (EGFL7), a secretory protein expressed by endothelial cells as well as by a subset of neurons, plays a role in controlling blood vessel formation during angiogenesis [6] and is involved in regulating proliferation and self-renewal of neural stem cells [7]; however, the functional role of epidermal growth factor-like protein 8 (EGFL8) is currently unknown. Only EGFL8 has been known as a potential paralog of EGFL7, and EGFL8 has a similar expression pattern as that of EGFL7 with a high expression level in various tissues including the thymus [8].

In this study, for the first time, we investigated the regulatory effects of EGFL8 on TECs by using gain-of-function and loss-of-function techniques.

2. Materials and methods

2.1. Cell lines and cell culture

The generation, maintenance, and functional characterization of the mouse thymic subcapsular cortex or thymic nurse epithelial

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cells (427.1; SNECs) have been described by Faas et al. [9]. This cell line constitutively expresses the SV40 T antigen transgene and the class I antigens of the major histocompatibility complex (MHC), and they can be induced to express MHC class II antigens upon stimulation with recombinant interferon (IFN)-γ and produce granulocyte–macrophage colony-stimulating factor (GM-CSF) [9]. These cell lines were kindly provided by Dr. Barbara B. Knowles (The Jackson Laboratory, Bar Harbor, ME, USA). All cell lines in this study were cultured and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco, Invitrogen), 2 mM glutamine (Sigma, St. Louis, MO, USA), 100 U/ml penicillin (Gibco, Invitrogen), and 100 μg/ml streptomycin (Gibco, Invitrogen) at 37 °C in 5% CO_2-enriched atmosphere.

2.2. Plasmid constructs and transfection

For EGFL8 plasmid construction, full-length mouse EGFL8 cDNA was amplified with total thymic RNA and cloned into a pcDNA3.1 mammalian expression vector. SNECs were transfected with pcDNA3.1-EGFL8 or pcDNA3.1-EGFL8-c-myc expression constructs using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. After 48 h incubation, if necessary, cells were selected by G418 sulfate (Invitrogen) at 750 μg/ml for 14 days.

2.3. siRNA transfection

For knockdown of EGFL8 gene, a 21-bp siRNA duplex and negative control siRNA with an overhang of dTdT were synthesized by Bioneer (Daejeon, Korea). The siRNA sequences for EGFL8 are as follows: 5′-GGAUCUUUCAAAGAGAGUU-3′. SNECs were seeded into 6-well microplates and transfected with 100 pmol of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

2.4. Semiquantitative reverse transcription-polymerase chain reaction analysis

The total RNA was isolated from culture cells using Trizol reagent (Ambion, Invitrogen) following the manufacturer's recommendation. For reverse transcription–polymerase chain reaction (RT–PCR), single-strand cDNA was synthesized using 2 μg of the total RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) and an oligo(dT) primer. All PCR reactions were performed in 22 to 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with the following primers: EGFL8 forward, 5′-TTT CAA AGA GAG TTT GGG AGT G-3′; EGFL8 reverse, 5′-CAC CAC GTG TGT CTG TGG TA-3′; ICAM-1 forward, 5′-GAG AGT GGA CCC AAC TGG AA-3′; ICAM-1 reverse, 5′-CTT TGG GAT GGT AGC TGG AA-3′; IL-7 forward, 5′-GCC CTG TCA CAT CAT CTG AGT GCC-3′; IL-7 reverse, 5′-CAG GAG GCA TCC AGG AAC TTC TG-3′; GM-CSF forward, 5′-GTC ACC CGG CCT TGG AAG CAT-3′; GM-CSF reverse, 5′-ACA GTC CGT TTC CGG AGT TGG-3′; TECK forward, 5′-AAA CTG TGG CTT TTT GCC TG-3′; TECK reverse, 5′-CCT CTG GAT TCC CAC ACA CT-3′; GAPDH forward, 5′-TGG AGA AAC CTG CCA AGT ATG-3′; GAPDH reverse, 5′-TTG TCA TAC CAG GAA ATG AGC-3′. After PCR reactions, samples were analyzed by agarose gel electrophoresis.

2.5. Western blot analysis

The total proteins were extracted from culture cells by using a protein extraction solution (Intron, Seoul, Korea) supplemented with Protease Inhibitor Mixture (Roche Applied Science, Indianapolis, IN, USA). Protein concentrations were measured using Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein samples were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and then electroblotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA). After blocking with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T), the membrane was incubated overnight at 4 °C with indicated primary antibody and subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was detected by enhanced chemiluminescent substrate kit (Pierce, Rockford, IL, USA), and images were captured and quantified with a LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

2.6. Quantitation of thymocyte adhesion to TECs

An assay of adherence of thymocytes to TECs was conducted as described previously [10,11]. Briefly, freshly isolated thymocytes from C57BL/6 mice were seeded onto a layer of SNECs in 6-well microplates at 1×10^7 cells/well in DMEM containing 10% FBS, and the plates were then incubated for 4 h. After removing the non-adherent thymocytes by gentle washing with phosphate-buffered saline (PBS), the adherent thymocytes to SNECs were collected by treatment with 0.5 mM EDTA (Sigma) in PBS for 2–3 min and were counted using a hemocytometer after trypan blue staining. For in vitro T-cell development study, thymocytes overlaid onto SNECs were further incubated for 1–2 days, harvested, and analyzed by flow cytometry.

2.7. Flow cytometric analysis

Cells were pre-incubated with anti-FcγRII/III (2.4G2, hybridoma supernatant) to avoid unspecific binding of antibodies and stained with indicated fluorescence-conjugated antibodies on ice for 30 min in Hank's balanced salt solution (HBSS; Gibco, Invitrogen) containing 2% FBS. Background fluorescence was determined on cells stained with isotype-matched nonreactive antibodies. Flow cytometric analysis was performed with a FACSCanto II (BD Biosciences, San Jose, CA, USA), and the acquired data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

2.8. Statistical analysis

Data are expressed as mean ± SD for each condition. For comparisons of multiple groups, a one-way analysis of variance (ANOVA) followed by a Scheffe's post hoc test was performed. Statistical significance was achieved when $p < 0.05$.

3. Results

3.1. Overexpression of EGFL8 inhibits thymocyte adhesion to TECs

Since structure analysis of EGFL8 protein predicted that it is a secretory protein [8], we first cloned EGFL8 cDNA from mouse thymus, generated expression vector pcDNA3.1-EGFL8-c-myc, and transiently transfected HEK293 cells with the vector. One day after transfection, we analyzed both supernatant and cell lysates of EGFL8-transfected HEK293 cells by Western blot with an anti-c-myc antibody. As shown in (Fig. 1A), transient transfection of HEK293 cells with pcDNA3.1-EGFL8-c-myc expression vector showed that EGFL8 protein was present in cell culture supernatant and cell lysates, suggesting that EGFL8 is a secretory protein.

To validate the potential role of EGFL8 in mouse TECs, we transfected SNECs with pcDNA3.1-EGFL8 or empty pcDNA3.1 vector and established stable cell lines after G418 selection. The effectiveness of EGFL8 expression in SNECs was confirmed by RT–PCR analysis (Fig. 1B). Then, the effects of EGFL8 on the adherence of thymo-

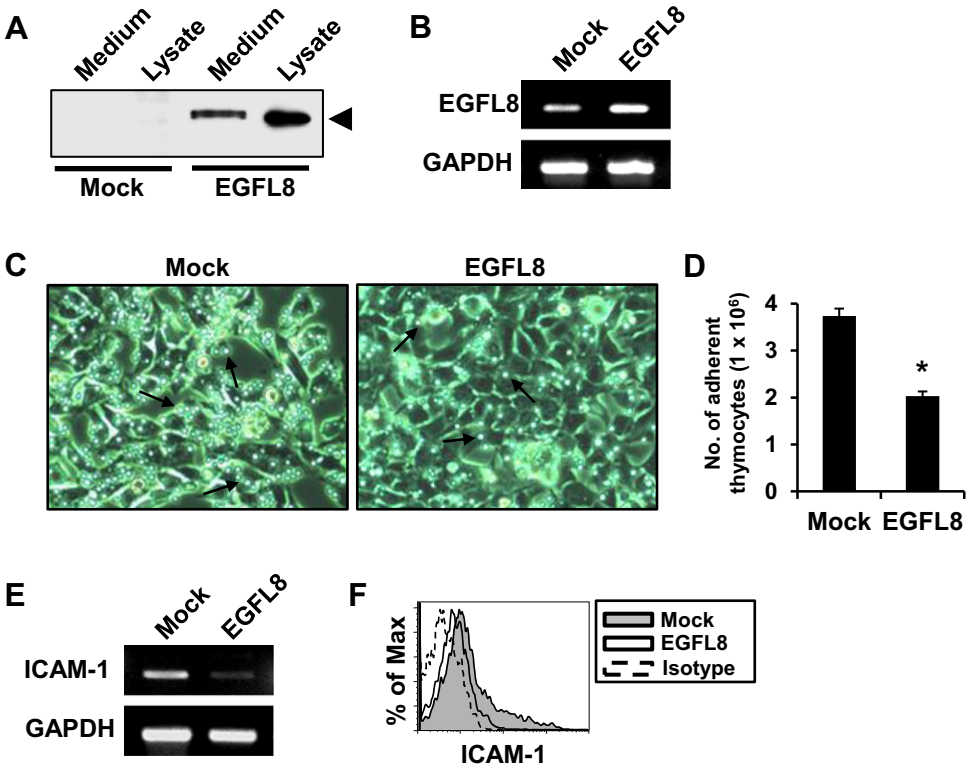

Fig. 1. Overexpression of EGFL8 in TECs down-regulates intercellular adhesion molecule-1 (ICAM-1) expression and inhibits the adhesion of thymocytes to TECs. (A) HEK293 cells transfected with an empty vector or a pcDNA3.1-EGFL8-c-myc expression vector. Culture supernatants and cell lysates were analyzed by Western blotting with an anti-c-myc antibody. (B) Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis of EGFL8 in mock-transfected or EGFL8-overexpressing SNECs. (C-D) Quantitative adherence assay of thymocytes to mock-transfected or EGFL8-overexpressing SNECs. (C) Adherent thymocytes to SNECs were observed under a light microscope at 400× magnification. Arrows indicate the adherent thymocytes. (D) The number of thymocytes adhered to SNECs was determined by cell counting. Data are expressed as the mean ± standard deviation (SD). *$P < 0.05$. (E) RT–PCR analysis of ICAM-1 in mock-transfected and EGFL8-overexpressing SNECs. (F) Flow cytometric analysis of ICAM-1 on the cell surface of mock-transfected and EGFL8-overexpressing SNECs.

cytes to SNECs were determined by cell adhesion assay. In contrast to the mock-transfected SNECs, EGFL8-overexpressing SNECs showed a significant decrease in the number of thymocytes adhered to TECs (Fig. 1C and D). To further determine whether EGFL8 could modulate the expression of ICAM-1, which is one of the key molecules involved in thymocyte-TEC adhesion [12], we determined the level of ICAM-1 mRNA in EGFL8-transfected SNECs by RT–PCR. As shown in Fig. 1E, EGFL8 almost abolished the expression of ICAM-1 in SNECs. Consistent with this finding, down-regulation of ICAM-1 on the surface of SNECs was confirmed by flow cytometric analysis (Fig. 1F). These results suggest that EGFL8 reduces the adhesion of thymocytes to TECs by down-regulating ICAM-1 expression.

3.2. EGFL8 knockdown promotes thymocyte adhesion to TECs

To further define the role of EGFL8 in the adherence of thymocytes to TECs, we used EGFL8 specific siRNA (EGFL8 siRNA) to knockdown EGFL8 expression in SNECs and then examined its effects on the adhesion between thymocytes and TECs. The efficacy of siRNA to suppress EGFL8 expression was determined by RT–PCR. In contrast, the control siRNA treatment had no effect on EGFL8 expression (Fig. 2A), indicating that EGFL8 siRNA effectively suppressed EGFL8 expression in SNECs. We confirmed the role of EGFL8 in the adherence of thymocytes to SNECs by specifically down-regulating its expression through RNA interference in vitro. After transfection with EGFL8 siRNA, we examined the effects of EGFL8 on thymocyte adhesiveness of SNECs by cell adhesion assay. As shown in Fig. 2B and C, knockdown of EGFL8 expression with siRNA significantly increased the number of adherent thymocytes to TECs. To evaluate if siRNA affects the expression of ICAM-1 in SNECs, the level of ICAM-1 mRNA and protein was determined by RT–PCR and flow cytometry, respectively. As shown in Fig. 2D and E, the expression of ICAM-1 was markedly up-regulated in EGFL8 siRNA-transfected SNECs.

3.3. EGFL8 in TECs inhibits T-cell development in vitro

Our findings that EGFL8 reduces the adhesion of thymocytes to mouse TECs by down-regulating ICAM-1 expression prompted us to investigate whether EGFL8 modulates T-cell development in vitro. Therefore, fleshly isolated thymocytes were co-cultured with EGFL8-overexpressing or siRNA-transfected SNECs by overlaying thymocytes onto SNEC monolayers. After co-cultivation, thymocytes were stained with monoclonal antibodies against mouse CD4 and CD8, and subsequently flow cytometry analysis was performed. In contrast to the mock-transfected SNECs, thymocytes co-cultured with EGFL8-overexpressing SNECs exhibited reduced proportions of $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes (Fig. 3A and B). In addition, after co-culture of thymocytes with siRNA-transfected SNECs, the proportion of $CD4^+$ and $CD8^+$ SP populations was increased as compared to the scrambled siRNA-transfected SNECs (Fig. 3A and B).

We next hypothesized whether EGFL8 modulated the expression of thymopoietic factors. To address this issue, we compared the expression levels of IL-7, GM-CSF, and TECK in EGFL8-overexpressing and EGFL8-silenced SNECs by semiquantitative RT–PCR. As shown in Fig. 3C, the expression of IL-7, GM-CSF, and TECK in SNECs was markedly up-regulated by knockdown of EGFL8, while the overexpression of EGFL8 suppressed the expres-

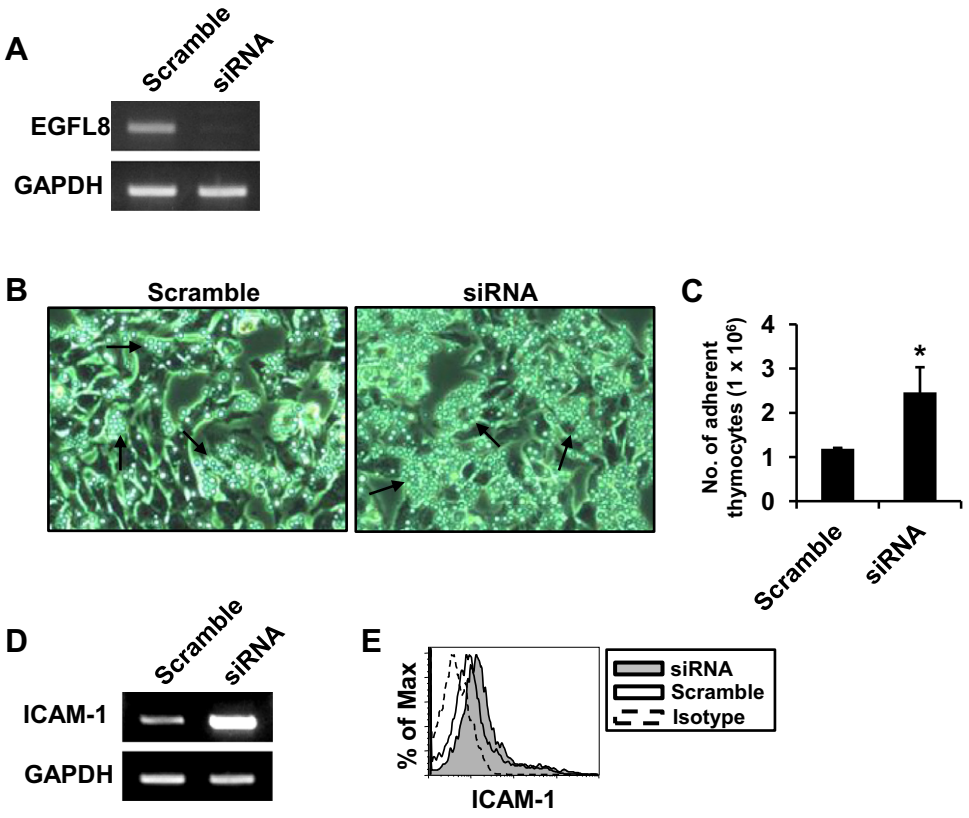

Fig. 2. Knockdown of EGFL8 in TECs enhances the adhesion of thymocytes to TECs by up-regulating ICAM-1 expression. (A) RT–PCR analysis of EGFL8 in scrambled siRNA-transfected or EGFL8 siRNA-transfected SNECs. (B and C) Quantitative adherence assay of thymocytes to scrambled siRNA-transfected or EGFL8 siRNA-transfected SNECs. (B) Adherent thymocytes to SNECs were observed under a light microscope at 400× magnification. Arrows indicate the adherent thymocytes. (C) The number of adherent thymocytes to SNECs was determined by cell counting. Data are expressed as the mean ± SD. $^{*}P < 0.05$. (D) RT–PCR analysis of ICAM-1 in scrambled siRNA-transfected or EGFL8 siRNA-transfected SNECs. (E) Flow cytometric analysis of ICAM-1 on the cell surface of scrambled siRNA-transfected or EGFL8 siRNA-transfected SNECs.

sion of these thymopoietic factors. Taken together, these results suggest that EGFL8 is a negative regulator of T-cell development in vitro.

4. Discussion

EGFL8 was first identified as a paralog of EGFL7 by a BLAST search of the mouse genome using the EGFL7 amino acid sequence [8]. While the overall homology of amino acid sequences between EGFL8 and EGFL7 is relatively low (35%), these two proteins share a highly similar domain structure including an EGF-like domain, a Ca^{2+} binding EGF-like domain, and an EMI domain [8]. EGFL7 has been extensively studied and is involved in blood vessel development by promoting endothelial cell migration, proliferation, sprouting, adhesion, and possibly invasion [6], and in the differentiation of adult neuronal stem cells into neurons and oligodendrocytes by negative regulation of Notch signaling pathway [7]; however, the physiological function of EGFL8 remains unknown. Only one research group has recently reported that EGFL8 expression is significantly down-regulated in the tissues of colorectal cancer and gastric cancer, proposing that EGFL8 may be used as a novel biomarker for these cancers [13,14].

In this study, we showed for the first time that EGFL8 inhibits the adhesion of thymocytes by regulating the expression of ICAM-1 in mouse TECs. The inhibitory effect of EGFL8 was not limited to cell adhesion. Indeed, it reduced the proportions of mature CD4 and CD8 SP thymocytes in the in vitro experimental model of mouse T-cell development. Moreover, RNA interference experiments showed that the knockdown of EGFL8 in TECs enhanced the number of adherent thymocytes to TECs and the proportion of mature CD4 and CD8 SP thymocytes after co-culture, further supporting the regulatory function of EGFL8 in TECs.

Thymopoiesis is an essential process for the development and maintenance of a robust and healthy immune system, and multiple factors including cytokines, hormones, and chemokines regulate this process [15]. IL-7, an essential thymopoietic cytokine predominantly expressed by a subset of TECs that express major histocompatibility complex (MHC) class II, is critical for T-cell development, homeostasis, and effector functions [16,17]. GM-CSF also facilitates T-cell development via enhancing the expression of IL-6 involved in thymocyte activation [18]. TECK (CCL25) is a CC chemokine that is present in the cortical epithelial cells and dendritic cells in the thymus and shows chemotactic activity in activated macrophages, dendritic cells, and thymocytes, enhancing T-cell development [19]. Our results suggest that EGFL8 exerts an inhibitory function in the in vitro model of mouse T-cell development via the inhibition of ICAM-1 expression as well as down-regulation of the expression of genes involved in thymopoiesis. In addition, the findings that knockdown of EGFL8 up-regulated the expression levels of IL-7, GM-CSF, and TECK in TECs, further supported that EGFL8 plays a negative regulatory role in TECs. Taken together, these data suggest that EGFL8 may play a regulatory role in T-cell development by modulating the expression of ICAM-1 as well as that of various thymopoietic factors.

The mechanisms underlying the down-regulation of ICAM-1 expression by EGFL8 need to be elucidated. Recent studies have described that EGFL7 inhibits NF-κB activation, which may be the mechanism by which EGFL7 down-regulates ICAM-1 expression

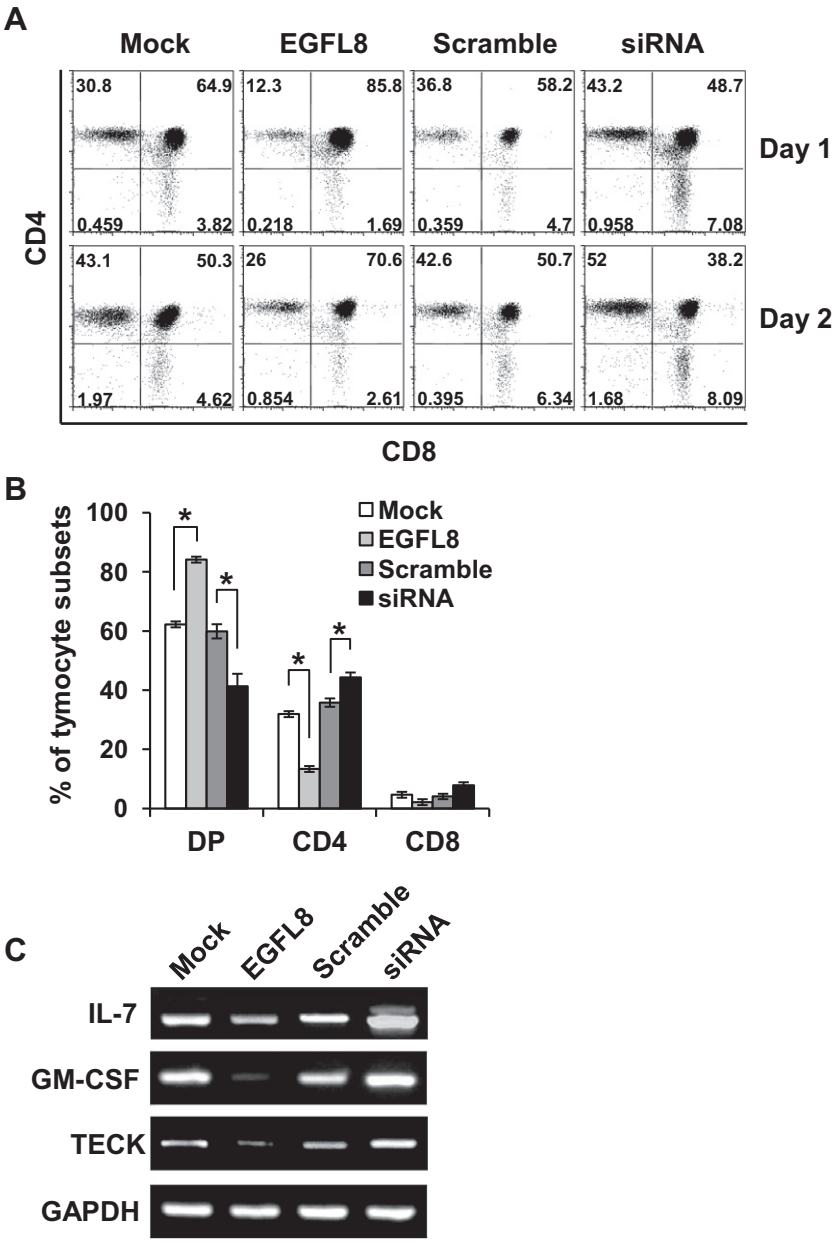

Fig. 3. EGFL8 regulates T-cell development in vitro. (A) Flow cytometric analysis of thymocytes subsets after co-culture with EGFL8-overexpressing or EGFL8 siRNA-transfected SNECs. Thymocytes were stained with monoclonal anti-CD4 and anti-CD8 antibodies. (B) Bar graphs show the mean ± SD for the proportion of $CD4^{-}CD8^{-}$ double-negative (DN), $CD4^{+}CD8^{+}$ double-positive (DP), $CD4^{+}$ single-positive (SP), and $CD8^{+}$ SP thymocytes. $^{*}P < 0.05$. (C) RT–PCR analysis of thymopoietic genes in EGFL8-overexpressing or EGFL8 siRNA-transfected SNECs.

in response to hypoxia/reoxygenation-induced and calcineurin inhibition-induced endothelial injury [20,21]. In this respect, EGFL8 might play a regulatory role in the NF-κB signaling pathway. However, further studies are necessary to define the precise mechanisms of EGFL8 in controlling ICAM-1 expression in TECs. In conclusion, our results suggest that EGFL8 has a novel function in TECs, i.e., it inhibits cellular interactions between thymocytes and TECs by down-regulating ICAM-1 expression, thereby inhibiting thymocyte adhesion to TECs; suppressing thymopoietic gene expression such as IL-7, GM-CSF, and TECK; and interfering with mouse thymocyte development into mature $CD4^{+}$ or $CD8^{+}$ SP thymocytes. Future studies focusing on the signaling events downstream of EGFL8 would provide new insight into the molecular pathways that regulate the thymopoiesis process and consequently shed a light on the precise role of EGFL8 in the development of T cells in the thymus.

Acknowledgments

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