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The Transcription Factor Ehf Is Involved in TGF- β -Induced Suppression of Fc ϵ RI and c-Kit Expression and Fc ϵ RI-Mediated Activation in Mast Cells

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Fc ϵ RI, which is composed of α , β , and γ subunits, plays an important role in IgE-mediated allergic responses. TGF- β 1 has been reported to suppress Fc ϵ RI and stem cell factor receptor c-Kit expression on mast cell surfaces and to suppress mast cell activation induced by cross-linking of Fc ϵ RI. However, the molecular mechanism by which these expressions and activation are suppressed by TGF- β 1 remains unclear. In this study, we found that the expression of Ets homologous factor (Ehf), a member of the Ets family transcriptional factors, is upregulated by TGF- β /Smad signaling in mouse bone marrow–derived mast cells (BMMCs). Forced expression of Ehf in BMMCs repressed the transcription of genes encoding Fc ϵ RI α , Fc ϵ RI β , and c-Kit, resulting in a reduction in cell surface Fc ϵ RI and c-Kit expression. Additionally, forced expression of Ehf suppressed Fc ϵ RI-mediated degranulation and cytokine production. Ehf inhibited the promoter activity of genes encoding Fc ϵ RI α , Fc ϵ RI β , and c-Kit by binding to these gene promoters. Furthermore, the mRNA levels of *Gata1*, *Gata2*, and *Stat5b* were lower in BMMCs stably expressing Ehf compared with control cells. Because GATA-1 and GATA-2 are positive regulators of Fc ϵ RI and c-Kit expression, decreased expression of GATAs may be also involved in the reduction of Fc ϵ RI and c-Kit expression. Decreased expression of Stat5 may contribute to the suppression of cytokine production by BMMCs. In part, mast cell response to TGF- β 1 was mimicked by forced expression of Ehf, suggesting that TGF- β 1 suppresses Fc ϵ RI and c-Kit expression and suppresses Fc ϵ RI-mediated activation through upregulation of Ehf. *The Journal of Immunology*, 2015, 195: 3427–3435.

Mast cells are widely recognized as the major effector cells in IgE-dependent immediate hypersensitivity. They are characterized by expression of Fc ϵ RI and the stem cell factor (SCF) receptor c-Kit on the cell surface. Fc ϵ RI, which is composed of one α , one β , and two γ subunits, plays a critical role in IgE-mediated hypersensitivity reactions. The expression of Fc ϵ RI is regulated by multiple transcription factors in mast cells. Previous studies have shown that the transcription of the Fc ϵ RI α subunit–encoded gene *Fcer1a* is positively regulated by the transcription factors PU.1 and GATA-1 in both human and mouse mast cells, and that the transcription of the Fc ϵ RI β subunit–encoded gene *Ms4a2* in humans and mice is positively regulated by GATA-2 and GATA-1, respectively (1–7). Alternately,

c-Kit is essential for the development of mast cells (8–10). GATA-2 is also involved in the transcription of the c-Kit–encoding gene *Kit* in mast cells (11). Several studies have demonstrated that TGF- β 1 suppresses mast cell Fc ϵ RI and c-Kit expression, and also mast cell activation induced by cross-linking of Fc ϵ RI (12–14). TGF- β 1 acts as a negative regulator of mast cell function, but it induces the expression of mouse mast cell protease (mMCP)-1 and -2 and integrin α_E (CD103), markers of mucosal mast cells (15–19). TGF- β signaling is mediated by Smads, which accumulate in the nucleus to transcriptionally regulate TGF- β target genes. The promoter and enhancer element of *Itgae*, encoding CD103, are known to have Smad-binding elements (20). Thus, CD103 expression is directly regulated by TGF- β /Smad signaling. However, the molecular mechanism by which TGF- β suppresses Fc ϵ RI and c-Kit expression, in addition to Fc ϵ RI-mediated activation in mast cells, is poorly understood.

In this study, we found that the expression of Ets homologous factor (Ehf) is significantly upregulated by TGF- β 1 in mouse bone marrow–derived mast cells (BMMCs). Ehf, also known as Ese-3, is a member of the Ets family of transcription factors that play a crucial role in development (21, 22). Ehf is expressed in various epithelial cells and tumor cells, and it is involved in the control of epithelial differentiation or tumor development (23–28). Functionally, Ehf is a transcriptional repressor of several genes regulated by MAPK signaling with a dependency on an Ets binding site (29). Recently, Ehf has been reported to be involved in the development of monocyte-derived dendritic cells (30–32).

In this study, we show that the transcription factor Ehf contributes to TGF- β –induced suppression of mast cell Fc ϵ RI and c-Kit expression through inhibition of the transcriptional activity of the promoters of genes encoding Fc ϵ RI α , Fc ϵ RI β , and c-Kit, as well

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Abbreviations used in this article: BMMC, bone marrow–derived mast cell; ChIP, chromatin immunoprecipitation; Ehf, Ets homologous factor; mMCP, mouse mast cell protease; qPCR, quantitative real-time PCR; SCF, stem cell factor; siRNA, small interfering RNA.

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as downregulation of GATA-1 and GATA-2 expression. Additionally, our results also indicate that Ehf is probably involved in TGF- β -induced suppression of Fc ϵ RI-mediated mast cell activation through downregulation of Fc ϵ RI and Stat5 expression.

Materials and Methods

BMMCs

BMMCs were generated from femoral bone marrow cells of BALB/c mice as described previously (33). Cells were cultured for 2–4 wk in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Life Technologies, Carlsbad, CA), 100 U/ml penicillin, 100 μ g/ml streptomycin, 100 μ M 2-ME, 10 mM sodium pyruvate, and 10 μ M MEM nonessential amino acid solution (Life Technologies) in the presence of 10 ng/ml recombinant murine IL-3 (Wako Pure Chemical Industries, Osaka, Japan) and 10 ng/ml recombinant murine SCF (Wako Pure Chemical Industries) at 37°C in a humidified atmosphere in the presence of 5% CO₂. Mast cells were identified by flow cytometric analysis of cell surface expression of c-Kit and the Fc ϵ RI α -chain.

TGF- β treatment

Two-week cultured bone marrow cells were cultured in BMMC medium in the presence of 10 ng/ml IL-3, 10 ng/ml SCF, and 5 ng/ml recombinant murine IL-9 (PeproTech, Rocky Hill, NJ) with or without 1 or 5 ng/ml recombinant human TGF- β 1 (Wako Pure Chemical Industries).

Retroviral constructs and transfection of BMMCs

The full-length mouse Ehf (NM_007914.3) cDNA was subcloned into the retrovirus vector pMXs-puro, which was a gift from Dr. Toshio Kitamura (University of Tokyo). A retrovirus-packing cell line, PLAT-E, was transfected with a retroviral vector encoding mouse Ehf or mock vector using FuGENE 6 (Promega, Madison, WI) to generate recombinant retroviruses. The nucleotide sequences of the primer set for mouse Ehf cDNA are listed in Supplemental Table I. Forty-eight hours after transfection, the virus-containing medium was collected and passed through a 0.45- μ m filter membrane. The supernatants were incubated for 6 h at 37°C in RetroNectin (Takara Bio, Shiga, Japan)-coated plates to attach the virus particles onto the RetroNectin. After removing the supernatants, BMMCs were plated onto the virus-attached plates. The transduced cells were selected by additional culture in the presence of 1.6 μ g/ml puromycin for 14 d.

Western blot analysis

Cells were collected and lysed by the direct addition of sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.1 mg/ml bromophenol blue dye, and 10% 2-ME). The cell lysates were electrophoretically resolved on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). Abs against FLAG (M2) and Ehf, which were purchased from Sigma-Aldrich, were used as primary Abs. Alexa Fluor 680- or IRDye 800-conjugated anti-mouse or anti-rabbit IgG Abs (Life Technologies) were used as secondary Abs. Infrared fluorescence on the membrane was detected by the infrared imaging system Odyssey (LI-COR Biosciences, Lincoln, NE).

Flow cytometric analysis

BMMCs were stained with FITC-conjugated anti-mouse Fc ϵ RI α mAb (MAR-1; eBioscience, San Diego, CA), PerCP (PerCP complex)/Cy5.5 or PE-conjugated anti-mouse c-Kit mAb (2B8; BioLegend, San Diego, CA), and PE-conjugated anti-mouse CD103 mAb (2E7; BioLegend) after blocking Fc receptors with anti-mouse CD16/CD32 mAb (2.4G2). The expression of cell surface markers was analyzed on a FACSCalibur (BD Biosciences, San Jose, CA).

Quantitative real-time PCR

Total cellular RNA was purified from cells using an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 500 ng total RNA using a ReverTra Ace quantitative real-time PCR (qPCR) RT kit (Toyobo, Osaka, Japan). Real-time quantitative PCR (real-time PCR) was performed with the Eco real-time PCR system (Illumina, San Diego, CA) using TaqMan Universal PCR master mix and Assays-on-Demand gene expression products for *Ehf* (Mm00468193_m1), *Fcer1a* (Mm00438867_m1), *Ms4a2* (Mm00442778_m1), *Fcer1g* (Mm02343757_m1), *Kit* (Mm00445212_m1), *Itgae* (Mm00434443_m1), *Gata1* (Mm01352636_m1), *Gata2*

(Mm00492301_m1), and *Spil* (Mm00488142_m1), which were purchased from Life Technologies. The mRNA expression levels were quantified with the comparative method using Eco software and normalized against the housekeeping gene *Gapdh*.

RNA interference

Transient silencing of the *Smad4* gene was achieved using small interfering RNAs (siRNA) targeted against *Smad4* (no. 1, Stealth RNA interference siRNA MSS206435; no. 2, MSS206437), which were purchased from Life Technologies. A nontargeting siRNA was used as a negative control. Cells (2×10^6) were transfected with 500 nM siRNA using a mouse macrophage Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions with a Nucleofector II device (Lonza).

Luciferase reporter assay

Fcer1a and *Fcer1g* promoter regions were amplified from BALB/c mouse genomic DNA by using PCR and inserted into the multicloning site of pGL4.10 (Promega) to generate luciferase reporter plasmids. The nucleotide sequences of primer sets are listed in Supplemental Table I. The luciferase reporter plasmids pGL3-Basic (Promega) encoding *Ms4a2* promoter region –763 to +103 relative to the transcription start site (+1) (3, 5) and *Kit* promoter region –622 to +22 (11) were generated as in our previous studies. The expression plasmid pCR3.1 (Life Technologies) encoding FLAG-tagged mouse *Ehf* cDNA and its empty plasmid were used in this study. BMMCs (4×10^5 cells) were transfected with 1.6 μ g reporter plasmid, 16 ng pRL-null vector (Promega), and 10 ng expression plasmid with a Neon 10 μ l kit using a Neon transfection system (Life Technologies) set at program 5. Twenty hours after transfection, luciferase activity was determined using MicroLumat Plus (Berthold Technologies, Bad Wildbad, Germany) and a Dual-Luciferase assay kit (Promega) as described previously (5). Firefly luciferase activity derived from the reporter plasmid was normalized to *Renilla* luciferase activity derived from pRL-null.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described in our previous reports (5, 34). Anti-FLAG tag mAb (M2) purchased from Sigma-Aldrich was used for immunoprecipitation, and mouse IgG (Life Technologies) was used as a control Ab. The amount of chromosomal DNA, including promoters of *Fcer1a*, *Ms4a2*, and *Kit* genes, were determined by the TaqMan system using the primers and TaqMan probes listed in Supplemental Table II.

Measurement of cytokine concentrations

Cells were sensitized with mouse IgE as described previously (35). To assess cytokine production, IgE-sensitized cells were incubated at 1×10^6 cells/ml in the presence or absence of 1 μ g/ml anti-mouse IgE mAb (R35-72; BD Biosciences) for 6 h. Cytokine concentrations in culture supernatants were quantified using a corresponding ELISA kit (R&D Systems, Minneapolis, MN).

β -Hexosaminidase release assay

The β -hexosaminidase release assay was performed as described previously (36). Briefly, BMMCs (4×10^6 cells/ml) were incubated for 1 h at 4°C with 1 μ g/ml mouse IgE mAb (BD Biosciences) and then were stimulated for 40 min at 37°C with 1 μ g/ml anti-mouse IgE (BD Biosciences). After centrifugation, the supernatants were collected and total cell lysates were obtained using 1% Triton X-100. The percentage of β -hexosaminidase release was calculated as described previously (36).

Statistical analyses

The unpaired Student *t* test was used as appropriate for parametric differences. One-way ANOVA or two-way ANOVA with the Dunnett method was used for multiple testing of data. A *p* value <0.05 was considered significant.

Results

TGF- β 1 suppresses Fc ϵ RI and c-Kit expression in mast cells

TGF- β 1 suppresses the expression of Fc ϵ RI and c-Kit and promotes the expression of CD103 in human and mouse mast cells (12–14, 17, 19, 37). We previously demonstrated that the transcription of genes encoding Fc ϵ RI α and Fc ϵ RI β is positively

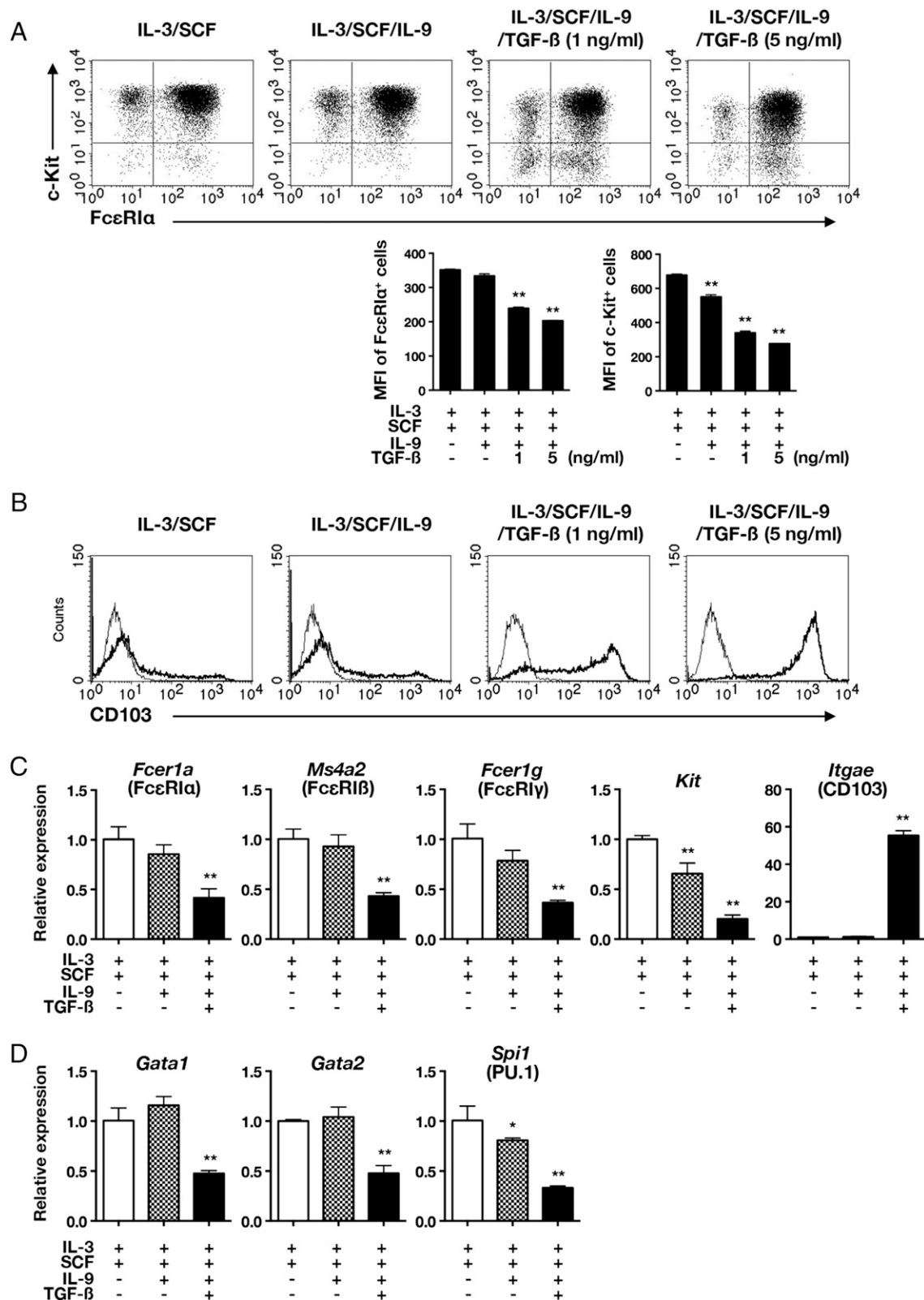


FIGURE 1. TGF-β downregulates FcεRI and c-Kit expression and upregulates CD103 expression on mast cell surfaces. Two-week cultured bone marrow cells were cultured for a further 14 d in media containing IL-3/SCF, IL-3/SCF/IL-9, IL-3/SCF/IL-9/TGF-β (1 ng/ml), or IL-3/SCF/IL-9/TGF-β (5 ng/ml). **(A)** Cell surface expressions of FcεRIα and c-Kit on BMMCs were assessed by FACS analysis. The mean fluorescence intensities (MFI) of FcεRIα or c-Kit are shown ± SD. **(B)** Cell surface expression of integrin α_E (CD103) on BMMCs assessed by FACS analysis is shown as a histogram. **(C and D)** Expressions of mRNAs for the indicated genes encoding cell surface markers (C) and transcription factors (D) in BMMCs were assessed by qPCR. Values represent mean ± SD and are expressed relative to the value of IL-3/SCF. **p* < 0.05, ***p* < 0.005, compared with the result for cells cultured in the presence of IL-3 and SCF, one-way ANOVA.

regulated by the transcription factors PU.1, GATA-1, and GATA-2 in mast cells (3, 5, 7, 38–41). Additionally, GATA-2 also plays an important role in the transcription of the *Kit* gene (11). Therefore, we first investigated the expression of those transcription factors in BMMCs cultured in media supplemented with IL-3 plus SCF, IL-3 plus SCF plus IL-9, or IL-3 plus SCF plus IL-9 plus TGF- β 1 for 14 d. These systems have been widely used to study mast cell development (17, 18). Cell surface expression of Fc ϵ RI and c-Kit was downregulated in BMMCs cultured in the presence of TGF- β 1 (Fig. 1A), whereas expression of CD103 on the cell surface was upregulated by TGF- β 1 (Fig. 1B). qPCR assays revealed that the mRNA levels of Fc ϵ RI subunits *Fcer1a* (encoding Fc ϵ RI α), *Ms4a2* (encoding Fc ϵ RI β), and *Fcer1g* (encoding Fc ϵ RI γ), as well as *Kit* (encoding c-Kit), were significantly downregulated, and the mRNA level of *Itgae* (encoding CD103) was markedly upregulated by TGF- β 1 (Fig. 1C). Mokrani et al. (20) reported that CD103 expression is induced by signaling through Smad and NFAT pathways in human CD8 T cells. However, the mechanism by which TGF- β 1 suppresses the expression of Fc ϵ RI and c-Kit on the mast cell surface is unknown. The present results of gene expression showed that the transcription levels of *Gata1* (encoding GATA-1), *Gata2* (encoding GATA-2), and *Spi1* (encoding PU.1) were significantly lower in BMMCs cultured in the presence of TGF- β 1 compared with BMMCs cultured in the absence of TGF- β 1 (Fig. 1D). These results suggest that a decrease in expression levels of GATA-1, GATA-2, and PU.1 contributes to the TGF- β 1-induced downregulation of Fc ϵ RI and c-Kit expression in mast cells.

Ehf expression is upregulated by TGF- β 1/Smad signaling in mast cells

Ehf is a member of the Ets family of transcription factors. In this study, we found that the level of *Ehf* mRNA was markedly higher in BMMCs cultured for 14 d in the presence of TGF- β 1 compared with cells cultured in the absence of TGF- β 1 (Fig. 2A). A significant increase in the level of *Ehf* mRNA was observed at 6 h after TGF- β 1 stimulation (Fig. 2B). TGF- β 1 signaling is transmitted to the nucleus to activate or repress gene expression by activated intracellular messengers Smad2 and Smad3, which form heteromeric complexes with Smad4. Therefore, to demonstrate that Ehf expression is induced in BMMCs by TGF- β 1 signaling, we blocked the TGF- β 1/Smad signaling pathway using Smad4-specific siRNAs. In this experiment, two siRNAs for *Smad4* were used to exclude the possibility of any off-target effects of siRNAs. The expression of *Smad4* mRNA in BMMCs transfected with siRNA nos. 1 and 2 was reduced by ~32 and 76%, respectively (Fig. 2C). Knockdown of Smad4 significantly reduced the levels of *Ehf* and *Itgae* mRNAs in TGF- β 1-treated cells (Fig. 2C). The mRNA levels of *Ehf* and *Itgae* in BMMCs treated with TGF- β 1 are most likely to be proportional to the level of Smad4 expression. Smads have been shown to directly activate transcription of the *Itgae* gene (20). Alternatively, it is unclear whether Smads directly bind to the *Ehf* promoter. Our data indicate that TGF- β 1/Smad signaling directly or indirectly upregulates the expression of Ehf in mast cells.

Forced expression of *Ehf* decreases the expression of Fc ϵ RI and c-Kit on the mast cell surface

Previous studies have demonstrated that some Ets family transcription factors, such as PU.1 and Elf-1, regulate expression of Fc ϵ RI in mast cells (6, 7, 42). Although Ehf is also a member of the Ets family of transcription factors, the role of Ehf in mast cells has been unknown. In this study, we hypothesized that Ehf contributes to the suppression of Fc ϵ RI and c-Kit expression induced

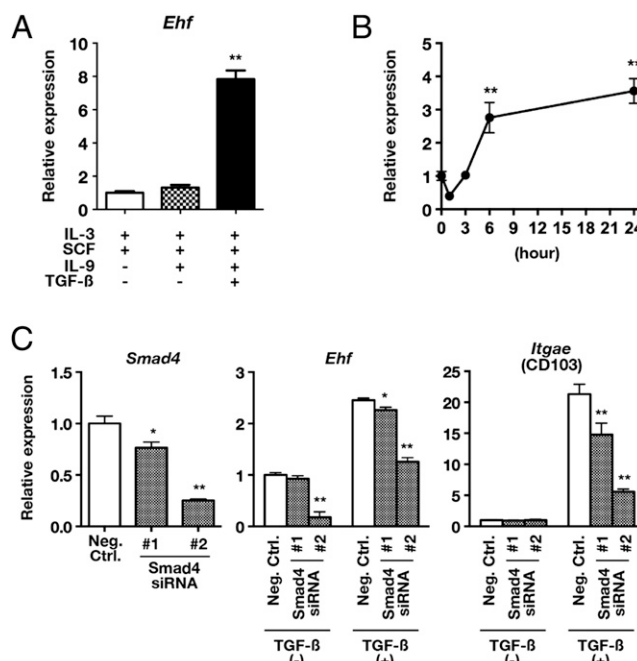


FIGURE 2. TGF- β 1/Smad signaling upregulates Ehf expression in BMMCs. **(A)** Two-week cultured bone marrow cells were cultured for a further 14 d in media containing IL-3/SCF, IL-3/SCF/IL-9, or IL-3/SCF/IL-9/TGF- β 1 (1 ng/ml). Expression of mRNA for the *Ehf* gene in BMMCs was assessed by qPCR. Values represent mean $\pm$ SD and are expressed relative to the value of IL-3/SCF. $^{**}p < 0.005$, compared with the result for cells cultured in the presence of IL-3 and SCF, one-way ANOVA. **(B)** The mRNA expression kinetic of Ehf in 2-wk cultured bone marrow cells treated with TGF- β 1. Values represent mean $\pm$ SD and are expressed relative to the value of TGF- β 1-untreated cells. $^{**}p < 0.005$ compared with the result for TGF- β 1-untreated cells, one-way ANOVA. **(C)** Effects of Smad4 siRNA on *Ehf* and *Itgae* mRNA expression. Two-week cultured bone marrow cells were transfected with siRNA. After 24 h of transfection, cells were incubated for a further 48 h in media containing IL-3/SCF/IL-9 or IL-3/SCF/IL-9/TGF- β 1 (1 ng/ml). Expression of mRNA for *Smad4*, *Ehf*, and *Itgae* in BMMCs was assessed by qPCR. Values represent mean $\pm$ SD and are expressed relative to the value of each negative control (Neg. Ctrl.). $^{*}p < 0.05$, $^{**}p < 0.005$, compared with the result for each negative control, one-way or two-way ANOVA.

by TGF- β 1 in mast cells. To test our hypothesis, we generated BMMCs stably expressing Ehf. A retroviral vector encoding FLAG-tagged mouse Ehf or the empty vector (mock) was transfected into bone marrow cells cultured for 2 wk in medium supplemented with IL-3 and SCF, and then transfectants were selected by culturing for 2 wk in the presence of puromycin. The expression of FLAG-tagged Ehf protein was observed in Ehf-transfected BMMCs in the absence of TGF- β 1 (Fig. 3A, 3B). The cell surface expressions of Fc ϵ RI and c-Kit were markedly suppressed in BMMCs stably expressing Ehf compared with mock-transfected BMMCs (Fig. 3C). Forced expression of Ehf significantly decreased the levels of mRNA for *Fcer1a* and *Ms4a2*, and for *Kit* in BMMCs, but it did not affect the mRNA level of *Fcer1g* (Fig. 3E). Although CD103 expression is upregulated by TGF- β 1 treatment, the expression levels of protein and mRNA were decreased in BMMCs stably expressing Ehf compared with mock-transfected BMMCs (Fig. 3D, 3E). These results suggest that Ehf contributes to TGF- β 1-induced downregulation of Fc ϵ RI α , Fc ϵ RI β , and c-Kit, but not upregulation of CD103, in BMMCs.

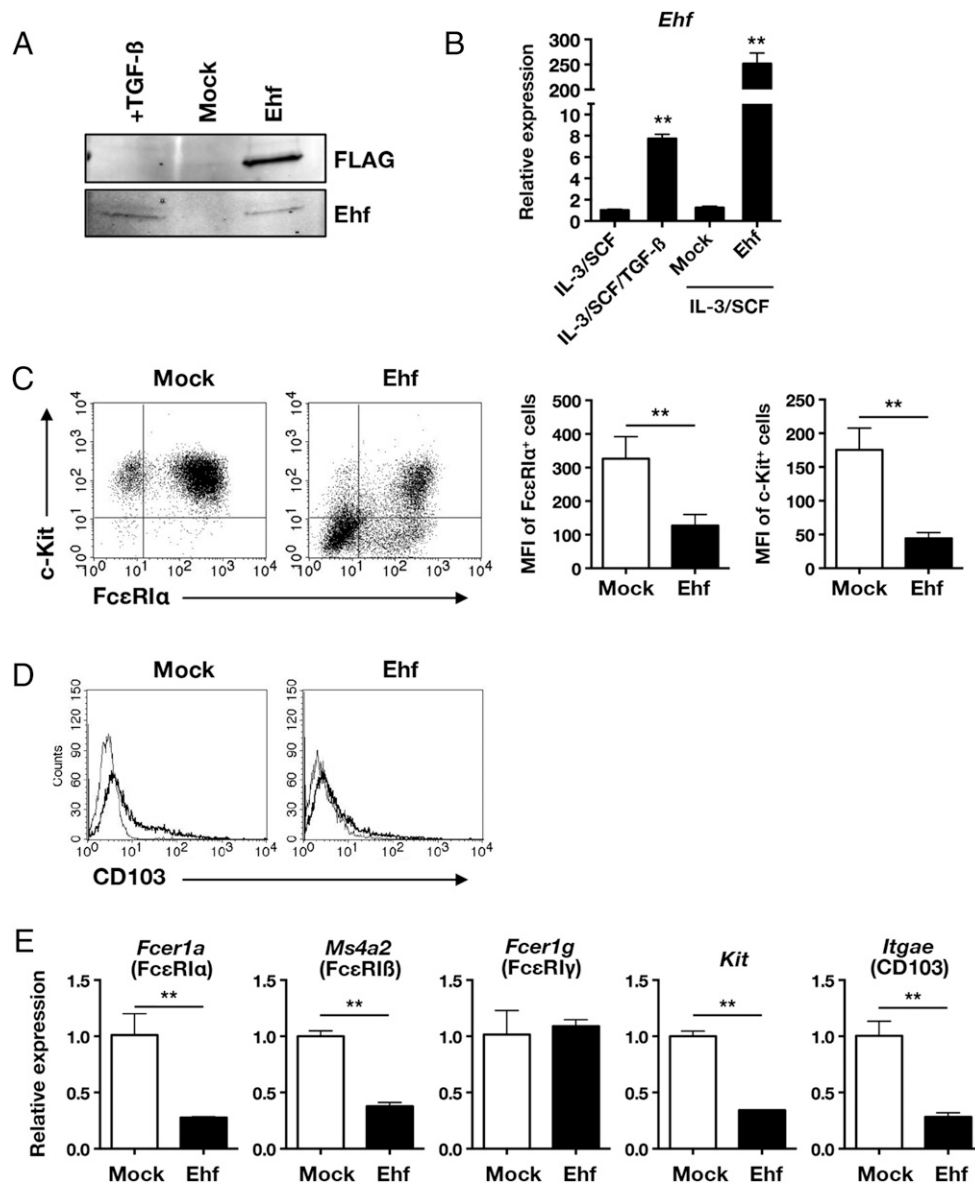


FIGURE 3. Cell surface expressions of FcεRI and c-Kit are suppressed by forced expression of Ehf in BMMCs. Two-week cultured bone marrow cells were cultured for a further 14 d in media containing IL-3/SCF, IL-3/SCF/TGF-β1 (1 ng/ml), or were transfected with a retroviral vector encoding the FLAG-tagged mouse Ehf cDNA or mock vector. Retrovirus-infected cells were cultured for a further 14 d in the presence of IL-3, SCF, and puromycin. **(A)** SDS-lysed total cell lysates were subjected to Western blot analysis as indicated. **(B)** Expression of *Ehf* mRNA in BMMCs was assessed by qPCR. Values represent mean $\pm$ SD and are expressed relative to the value of cells cultured in the presence of IL-3 and SCF. ** $p < 0.005$, compared with the result for cells cultured in the presence of IL-3 and SCF, one-way ANOVA. **(C)** Cell surface expressions of FcεRIα and c-Kit on the transfected cells were assessed by FACS analysis. The mean fluorescence intensities (MFI) of FcεRIα or c-Kit are shown $\pm$ SD. ** $p < 0.005$ as determined by unpaired Student *t* test. **(D)** Cell surface expression of integrin α_E (CD103) on the transfected cells assessed by FACS analysis is shown as a histogram. **(E)** Expression of mRNA for the indicated genes encoding cell surface markers in BMMCs was assessed by qPCR. Values represent mean $\pm$ SD and are expressed relative to the value of mock-transfected cells. ** $p < 0.005$ as determined by unpaired Student *t* test.

Transcriptional activities of *Fcer1a*, *Ms4a2*, and *Kit* gene promoters are downregulated by binding of Ehf

Our previous studies have shown that the transcriptional activity of the *Fcer1a* gene promoter is upregulated by binding of PU.1, an Ets family transcription factor, to an Ets binding site on the promoter region (2, 6, 7). *Ms4a2* and *Kit* genes also have some canonical Ets binding sites on their promoter regions (data not shown). Thus, to examine the mechanism by which the transcription of *Fcer1a*, *Ms4a2*, and *Kit* genes is selectively repressed by forced expression of Ehf, an Ets family transcription factor, we analyzed the influence of Ehf on their promoter activities in

BMMCs. First, the promoter activity of the genes encoding FcεRI subunits and c-Kit were measured by luciferase reporter assays in BMMCs. The luciferase activity of reporter plasmids containing the promoter segments of *Fcer1a*, *Ms4a2*, *Fcer1g*, or *Kit* genes was significantly higher than that of a promoterless reporter plasmid (Fig. 4A). The luciferase activities driven by *Fcer1a*, *Ms4a2*, and *Kit* promoters were strongly repressed by transient expression of Ehf, whereas that of *Fcer1g* was only modestly decreased by Ehf expression (Fig. 4A). These results indicate that Ehf represses the transcription of *Fcer1a*, *Ms4a2*, and *Kit* genes, but not the *Fcer1g* gene, in BMMCs through downregulation of these promoter activities.

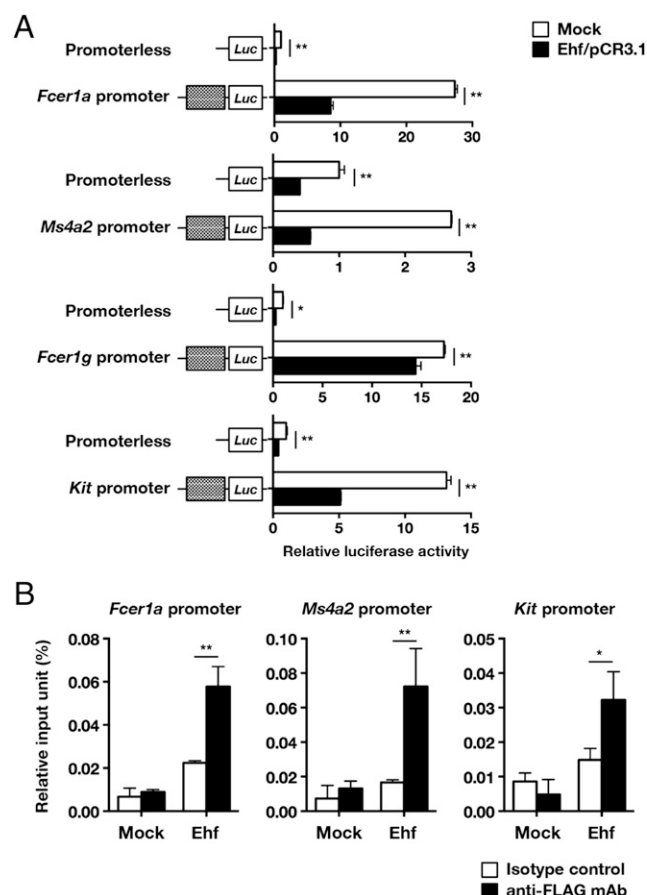


FIGURE 4. Promoter activities of *Fcer1a*, *Ms4a2*, and *Kit* are down-regulated by Ehf in BMMCs. **(A)** BMMCs were transfected with each reporter plasmid and expression plasmid by electroporation. The luciferase activity of each transfection was normalized to the activity of *Renilla* luciferase derived from pRL-null. Data are expressed as relative luciferase activity to the activity driven by a promoterless reporter plasmid and mock-expression vector, which is assigned a value of 1.0, and represent mean $\pm$ SD. **(B)** Binding of Ehf to the promoters of *Fcer1a*, *Ms4a2*, and *Kit* genes was confirmed by ChIP-qPCR. ChIP analyses were performed in BMMCs transfected with a retroviral vector encoding the FLAG-tagged mouse Ehf cDNA or mock vector using anti-FLAG Ab. Immunoprecipitated DNA qPCR data are expressed relative to input DNA within the same reaction and represent mean $\pm$ SD. * p < 0.05, ** p < 0.005 as determined by one-way ANOVA.

We next used ChIP assays to confirm association of Ehf with the promoter regions of *Fcer1a*, *Ms4a2*, and *Kit* genes. A significantly higher amount of chromatin containing the promoter segment of these genes was immunoprecipitated by anti-FLAG mAb compared with isotype control IgG in BMMCs expressing FLAG-tagged Ehf (Fig. 4B), demonstrating that Ehf represses the transcriptional activities of *Fcer1a*, *Ms4a2*, and *Kit* genes by directly binding to these promoter regions. It therefore follows that Ehf acts as a transcriptional repressor of the *Fcer1a*, *Ms4a2*, and *Kit* genes.

Forced expression of Ehf results in a decrease in the transcription level of *Gata1* and *Gata2* genes in BMMCs

Interestingly, forced expression of Ehf significantly suppressed the transcription levels of *Gata1* and *Gata2* in BMMCs but did not affect the transcription of *Spi1* (Fig. 5). Previously, we demonstrated that knockdown of GATA-1 and/or GATA-2 by siRNA suppresses the transcription of *Fcer1a* and *Ms4a2*, but not *Fcer1g*, in mast cells (7). Additionally, knockdown of GATA-2 also suppresses the transcription of *Kit* in mast cells (11). The data

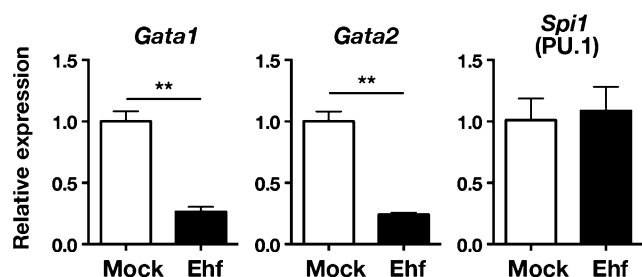


FIGURE 5. Transcription of *Gata1* and *Gata2* genes is repressed in BMMCs expressing Ehf. Expression of mRNA for the indicated genes encoding transcription factors in BMMCs transfected with a retroviral vector encoding the FLAG-tagged mouse Ehf cDNA or mock vector was assessed by qPCR. Values represent mean $\pm$ SD and are expressed relative to the value of mock-transfected cells. ** p < 0.005 as determined by unpaired Student t test.

shown in Fig. 5 are consistent with the previous results. Therefore, the downregulation of GATA-1 and GATA-2 expression may also contribute to the suppression of Fc ϵ RI and c-Kit expression on BMMCs expressing Ehf. However, the detailed molecular mechanism of the downregulation of transcription of *Gata1* and *Gata2* by Ehf remains unknown, because, in this study, we were not able to identify the functional promoter regions of *Gata1* and *Gata2* genes in BMMCs.

Ehf inhibits mast cell activation by Fc ϵ RI cross-linking

Mast cell activation by cross-linking of Fc ϵ RI results in degranulation and de novo synthesized cytokines. TGF- β 1 has been shown to inhibit Fc ϵ RI-mediated degranulation and synthesis of cytokines in mast cells (12, 14). To investigate the role of Ehf in Fc ϵ RI-mediated mast cell activation, we measured the Fc ϵ RI-mediated release of β -hexosaminidase as an index of degranulation and production of cytokines IL-6 and IL-13 by BMMCs stably expressing Ehf. The β -hexosaminidase release by BMMCs treated with TGF- β 1 and BMMCs stably expressing Ehf was reduced by ~60 and 58% compared with that by control cells cultured with IL-3 and SCF, respectively (Fig. 6A). The low levels of degranulation in these BMMCs may be explained by reduced expression of Fc ϵ RI (Figs. 1A, 3C). Additionally, Fc ϵ RI-mediated productions of IL-6 and IL-13 were reduced by >70% in both BMMCs treated with TGF- β 1 and those stably expressing Ehf compared with control cells cultured with IL-3 and SCF (Fig. 6B). However, the marked reduction in Fc ϵ RI-mediated cytokine production cannot be explained only by reduced expression of Fc ϵ RI. Fernando et al. (14) showed that TGF- β 1 reduces the expression of Stat5, which is a critical regulator of cytokine induction, in mast cells. The expression level of *Stat5b* mRNA in BMMCs treated with TGF- β 1 and in BMMCs stably expressing Ehf was reduced by ~45 and 62% compared with that in control cells cultured with IL-3 and SCF, respectively (Fig. 6C). Thus, reduced expression of Fc ϵ RI and Stat5 may contribute to the inhibition of Fc ϵ RI-mediated cytokine production by treatment with TGF- β 1 or forced expression of Ehf. These results suggest that upregulation of Ehf is involved in TGF- β 1-induced inhibition of Fc ϵ RI-mediated mast cell activation.

Discussion

Short-term exposure to TGF- β inhibits mast cell Fc ϵ RI expression through a reversible pathway that diminishes protein but not mRNA expression of α , β , and γ subunits (12). In contrast, long-term exposure to TGF- β during development of mast cells inhibits not only Fc ϵ RI expression but also c-Kit expression (13). Additionally, TGF- β is considered to be a key cytokine in muco-

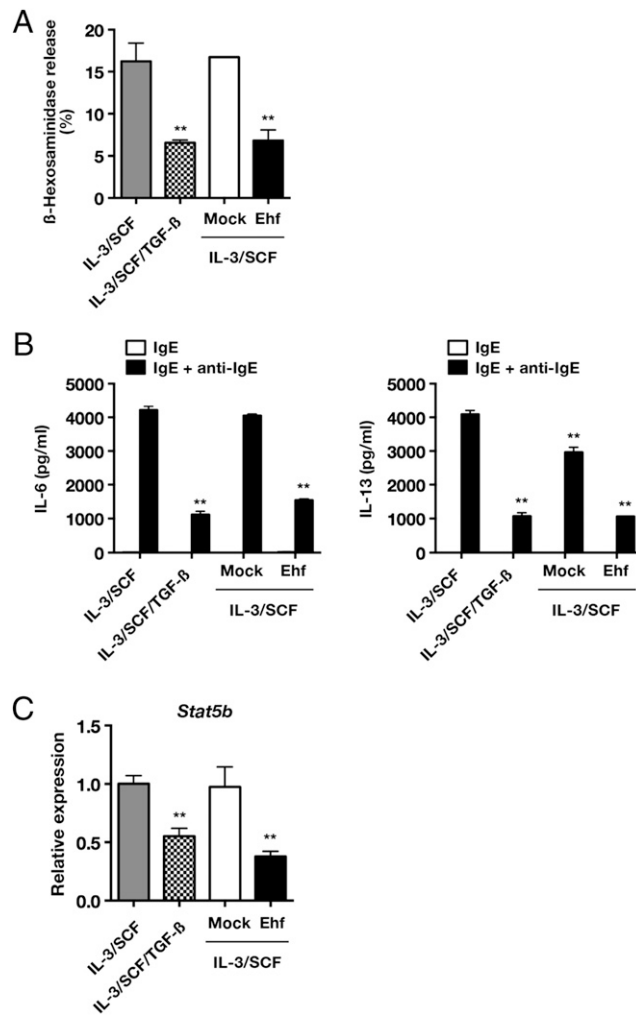


FIGURE 6. FcεRI-mediated mast cell activation is inhibited by forced expression of Ehf. Two-week cultured bone marrow cells were cultured for a further 14 d in media containing IL-3/SCF, IL-3/SCF/TGF-β1 (1 ng/ml), or were transfected with a retroviral vector encoding the FLAG-tagged mouse *Ehf* cDNA or mock vector. Retrovirus-infected cells were cultured for a further 14 d in the presence of IL-3, SCF, and puromycin. Cells were sensitized with IgE and then were stimulated with anti-IgE. Release of β-hexosaminidase (**A**), IL-6, and IL-13 (**B**) by BMMCs cultured in the presence or absence of TGF-β1 and transfected with FLAG-tagged mouse Ehf or the mock vector. Values represent mean ± SD. (**C**) Expression of *Stat5b* mRNA in BMMCs was assessed by qPCR. Values represent mean ± SD and are expressed relative to the value of cells cultured in the presence of IL-3 and SCF. ***p* < 0.005, compared with the result for cells cultured in the presence of IL-3 and SCF, one-way ANOVA.

sal mast cell differentiation because it induces expression of mMCP-1, mMCP-2, and CD103, marker molecules of mature mucosal mast cells, in mice (15–19). Indeed, the levels of cell surface FcεRI and c-Kit expression are lower in mucosal mast cells than in connective tissue-type mast cells (43). We showed that the mRNA levels of c-Kit and each subunit composed of FcεRI were lower in BMMCs cultured in the presence of TGF-β1 for 14 d than in BMMCs cultured in the absence of TGF-β1 during the same period (Fig. 1A). The decreased expression of FcεRI and c-Kit may be explained, in part, by the decrease in the transcription factors GATA-1, GATA-2, and PU.1 (Fig. 1D), because these transcription factors play a crucial role in mast cell FcεRI and c-Kit expression. In particular, mouse FcεRI α and β subunits are positively regulated by GATA-1/PU.1 and GATA-1, respectively, and mouse c-Kit is positively regulated by GATA-2 (3, 5, 6).

However, the molecular mechanism by which TGF-β1 inhibits the expression of these transcription factors in mast cells is not clear. Moreover, an unknown mechanism to directly repress the expression of genes encoding FcεRI subunits and c-Kit may be involved.

In this study, we found that TGF-β1 induces upregulation of Ehf in BMMCs (Fig. 2A, 2B). The upregulation of Ehf was significantly suppressed by knockdown of Smad4 using specific siRNA (Fig. 2C), and this almost certainly depends on TGF-β/Smad signaling in mast cells. However, it remains unclear whether the transcription of the *Ehf* gene is directly regulated by Smads, because there is insufficient information regarding the promoter element of the *Ehf* gene. Ehf is an Ets family transcription factor, a family that includes PU.1. Although PU.1 plays an important role in the activation of the *Fcer1a* gene promoter in mast cells through binding to Ets binding sites, the function of Ehf in mast cells has been unknown. In this study, we showed that forced expression of Ehf suppressed the cell surface expression of FcεRI and c-Kit on BMMCs (Fig. 3C). The levels of mRNA encoding c-Kit and FcεRI subunits α and β, but not γ, were lower in BMMCs stably expressing Ehf than in the control cells (Fig. 3E). Ehf functions as a transcriptional repressor of several genes through Ets binding sites (29). Ets binding motifs are present not only in the *Fcer1a* gene promoter but also in *Ms4a2* and *Kit* gene promoters. Luciferase reporter assays and ChIP assays showed that the promoter activities of *Fcer1a*, *Ms4a2*, and *Kit* genes were directly downregulated by binding of Ehf to these gene promoter regions (Fig. 4). These results demonstrate that Ehf functions as a transcriptional repressor of *Fcer1a*, *Ms4a2*, and *Kit* genes in mast cells. Moreover, forced expression of Ehf in BMMCs resulted in a decrease in mRNA expression of *Gata1* and *Gata2*, which act as transcriptional activators of *Fcer1a*, *Ms4a2*, and/or *Kit* genes (Fig. 5). Therefore, Ehf not only directly represses the transcription of *Fcer1a*, *Ms4a2*, and *Kit* genes as a transcriptional repressor, but it may also indirectly repress them by downregulating the expression of GATA-1 and GATA-2. Alternatively, the transcriptions of *Fcer1g* and *Spil* genes were not repressed in BMMCs stably expressing Ehf (Figs. 3E, 4), although they were repressed in BMMCs treated with TGF-β1 (Fig. 1C, 1D). Thus, the repressions of FcεRIγ and PU.1 expression were probably induced by a mechanism that is not mediated by Ehf in BMMCs treated with TGF-β1.

The reduction of FcεRI expression leads to the inhibition of FcεRI-mediated mast cell activation. Our data showed that treatment with TGF-β or forced expression of Ehf downregulates FcεRI-mediated degranulation and markedly downregulates FcεRI-mediated cytokine production (Fig. 6A, 6B). Recently, the Fyn-Stat5B pathway has been reported to be a novel mechanism for FcεRI-mediated cytokine production (44). The marked suppression of FcεRI-mediated cytokine production may be due not only to reduced FcεRI expression, but also to reduced transcription factor Stat5B expression (Fig. 6C). These findings indicate that the upregulation of Ehf is probably involved in the downregulation of GATA-1, GATA-2, and Stat5B in BMMCs, although it remains unclear whether Ehf acts as a direct transcriptional repressor of these genes. Further investigation is needed to understand the function of Ehf in mast cells. Additionally, the generation and phenotypic analysis of Ehf knockout mice will be of use in evaluating the biological significance of Ehf in mast cells.

In this study, some mast cell responses to TGF-β1 were mimicked by forced expression of Ehf. Therefore, we suggest that Ehf is involved in the suppression of FcεRI and c-Kit expression and FcεRI-mediated mast cell activation caused by TGF-β1/Smad signaling. These findings provide mechanistic insights into

TGF- β 1-mediated mast cell responses. This novel insight may contribute to understanding the mechanisms of mucosal mast cell development and regulation of mast cell functions.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Table S1. Nucleotide sequences of primers used for gene cloning.

Gene		Sequence* (5'→3')	RE
<i>Ehf</i>	Forward	gggaattcATGATTCTGGAAGGAAGTGGTGTAAT	EcoRI
	Reverse	ggctcgagTCAGTTCTCATTTTCTCTCCATCCACG	XhoI
<i>Fcer1a</i> promoter (-652 to +2)	Forward	aaggtaccTGGCAAGGAACATGAAGTGAAACTCTGG	KpnI
	Reverse	aactcgagTAGGCTTGAAACCACAAAGGAGAAGG	XhoI
<i>Fcer1g</i> promoter (-500 to +86)	Forward	ctcgagGTAATGTGTCTTTCTTGGTGTCTTGCTGCC	XhoI
	Reverse	aagcttTGCGGCGCTGGGAGCTGGTGATCG	HindIII

*The inserted nucleotides are shown with small letters and the restriction enzyme (RE) recognition sites are underlined.

Supplemental Table S2. Nucleotide sequences of synthesized oligonucleotides for ChIP assay.

Target		Sequence (5' →3')
<i>Fcer1a</i> promoter	Forwad	GCCCAGGCATAGCTGATGAG
	Reverse	CTTCGAAAATAGGCTTGAAACCA
	TaqMan Probe*	FAM-TTAACCAGATACAACACAGAAG-MGB
<i>Ms4a2</i> promoter	Forwad	ACAGCAAGAGAAAGGAGTCACTGAT
	Reverse	CATGCGGAACCTACTTGTCAGA
	TaqMan Probe	FAM-CAATCAGCCTGGAGACT-MGB
<i>Kit</i> promoter	Forwad	GGGCGCTGTGCCCTCT
	Reverse	CTGAAGGACCACCGATGGA
	TaqMan Probe	FAM-CCCTGGATCCTCT-MGB

*FAM, FAM reporter dye; MGB, Minor Groove Binder.