Genes to Cells

miR126 positively regulates mast cell proliferation and cytokine production through suppressing Spred1

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The protein known as Spred1 (Sprouty-related Ena/VASP homology-1 domain-containing protein) has been identified as a negative regulator of growth factor-induced ERK/mitogen-activated protein kinase activation. Spred1 has also been implicated as the target of micro RNA-126 (miR126), a miRNA located within the *Egfl7* gene, and is involved in the regulation of vessel development through its role in regulating VEGF signaling. In this study, we examined the role of miR126 and Spred1 in the hematopoietic system, as miR126 has been shown to be overexpressed in leukemic cells. miR126 levels were down-regulated during mast cell differentiation from bone marrow cells, whereas Spred1 expression was inversely up-regulated. Overexpression of miR126 suppressed Spred1 expression and enhanced ERK activity in primary bone marrow cells and MC9 mast cells, which were associated with elevated FcεRI-mediated cytokine production. To confirm the effect of Spred1 reduction *in vivo*, we generated hematopoietic cell-specific Spred1-conditional knockout mice. These mice showed increased numbers of mast cells, and Spred1-deficient bone marrow-derived mast cells were highly activated by cross-linking of FcεR stimulation as well as by IL-3 and SCF stimulation. These results suggest that Spred1 negatively regulates mast cell activation, which is modulated by miR126.

Introduction

The Spred/Sprouty family proteins have been identified as negative regulators for growth factor- and cytokine-induced ERK activation. In mammals, four Sprouty and three Spred (Sprouty-related Ena/VASP homology 1 domain-containing proteins) homologues have been identified (Hacohen *et al.* 1998; Minowada *et al.* 1999; Tefft *et al.* 1999; Wakioka *et al.* 2001; Taniguchi *et al.* 2007a). Spreds bind to Ras and Raf, thereby suppressing activation of Raf. Germline loss of human *SPRED1* has been identified in Legius Syndrome, an autosomal dominant disorder that resembles neurofibromatosis type 1 (NF1) (Brems *et al.* 2007; Messiaen *et al.* 2009; Denayer *et al.* 2011). As NF1 is caused by abnormal activation of the Ras-ERK pathway, the existence and nature of this disorder strongly support our notion that Spred is a negative regulator of the RAS-ERK pathway.

Spred1 has also been implicated in hematopoiesis, based on the observation that more colonies formed in cultures of bone marrow (BM) cells from Spred1$^{-/-}$ mice and that megakarocytopoiesis occurred in the spleens of Spred1$^{-/-}$ mice. BM-derived eosinophils and mast cells from Spred1$^{-/-}$ mice were more sensitive to interleukin 5 (IL-5) and IL-3, respectively, than those from wild-type (WT) mice were (Nonami

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et al. 2004). Spred1-deficient mice showed stronger eosinophilia in a murine allergic asthma model (Inoue *et al.* 2005). Nevertheless, the role of Spred1 in hematopoiesis and hematopoietic cell functions has not been completely clarified.

In addition, Spreds and Sproutys have been implicated in lymphangiogenesis and angiogenesis (Taniguchi *et al.* 2007a,b). In the latter process, Spred1 has been identified as a target of microRNA-126 (miR126) (Kuhnert *et al.* 2008; Wang *et al.* 2008; Fish *et al.* 2008). miR126 is 21 nucleotides in length, is located on chromosome 9q34.3, and is contained within intron 5 of its host gene, epidermal growth factor like-7 (*Egfl7*). miR126 has been shown to play functional roles in angiogenesis and to regulate PI3K and ERK signaling by directly targeting PI3KR2 (p85-β) and Spred1, respectively. miR126 has also been implicated in leukemia (Li *et al.* 2008) and asthma. Selective blockade of miR126 has been reported to suppress the asthmatic phenotype, including Th2 responses, inflammation, airway hyperresponsiveness, eosinophil recruitment, and mucus hypersecretion (Mattes *et al.* 2009). However, the role of miR126 with regard to the hematopoietic system has not yet been investigated.

Mast cells can function as effector cells during innate and adaptive immune responses through the direct or indirect action of a wide variety of mast cell–derived products (Galli *et al.* 2008, 2005b). In vertebrates, mast cells are widely distributed throughout the vascularized tissues, particularly near surfaces that are exposed to the environment, including the skin, airways, and gastrointestinal tract, making them well positioned to be among the first immune cells to interact with environmental antigens and allergens. They are long-lived and are similar to monocytes and macrophages; in that, they can reenter the cell cycle and proliferate after appropriate stimulation such as SCF and IL-3. Given their importance in the immune responses and similarity to hematopoietic stem cells, it is important that we understand the molecular basis of mast cell development, proliferation, and functions.

In this study, we investigated the roles of miR126 and Spred1 in mast cell development and activation, as these were inversely regulated during mast cell development. We showed that forced expression of miR126 in hematopoietic cell lines increased proliferation rate and ERK activation in a Spred1-dependent manner. Forced expression of miR126 in bone marrow–derived mast cells (BMMCs) also increased FcεRI-mediated cytokine production. Loss of Spred1 in BMMCs resulted in enhanced proliferation and cytokine production, as did miR126 overexpression. We concluded that Spred1 negatively regulates mast cell proliferation and FcεRI-mediated cytokine production, which is fine-tuned by miR126.

Results

Relationship between miR126 and Spred1 expression in mast cells

Previously, we reported that Spred1 expression is upregulated during mast cell development from BM cells in response to IL-3 (Nonami *et al.* 2004). In this study, we examined the relationship between miR126 and Spred1 expression in mast cell development by measuring the expression levels of miR126 and Spred1 in total RNA of BMMCs (Fig. 1). As reported previously (Garzon *et al.* 2006), BM cells initially expressed high levels of miR126. Immediately after culture with IL-3, however, miR126 was drastically down-regulated, and it remained at low levels thereafter (Fig. 1A). Spred1 mRNA expression levels, in contrast, increased gradually during the full differentiation of BMMCs (Fig. 1B). This raises a possibility that the down-regulation of miR126 triggers the upregulation of Spred1.

To explore this possibility, we constitutively expressed miR126 in a mast cell line, MC9 by retroviral transduction. As shown in Fig. 1C, at steady state levels, MC9 cells that had been stably transformed with miR126 expressed much lower amounts of Spred1, but those that had been exposed to an empty vector did not. Similar results were obtained in different cell preparations (see Fig. 2A,B).

Effect of miR126 on proliferation of BM cells (Fig. 1D) and MC9 cells (Fig. 1E) was examined by GFP-positive ratio measurement with flow cytometry. Infected were GFP positive; GFP-positive ratio was increased when BM cells or MC9 cells were infected with miR126 retrovirus, but the ratio was not altered by empty virus infection (Fig. 1D,E). These results indicate that miR126-expressing mast cells proliferate faster than non-expressing cells.

Forced expression of miR126 resulted in enhanced ERK activity via suppression of Spred1

To examine the effect of miR126 on the activation of ERK more precisely, we stimulated MC9 cell transformants with IL-3. Enhanced ERK phosphorylation was observed in miR126-overexpressing cells

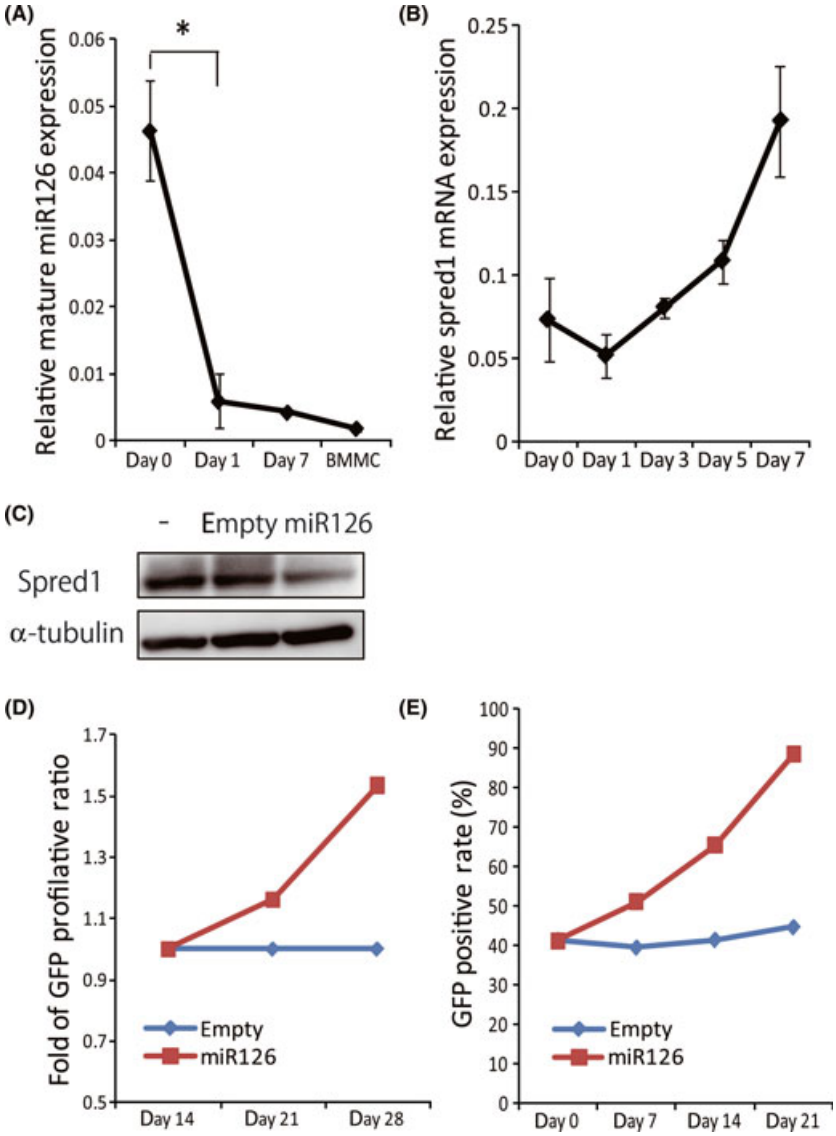

Figure 1 Relationship between miR126 and Spred1 expression in mast cells. (A, B) Time course of the levels of miR126 and Spred1. Bone marrow–derived mast cells (BMMCs) were cultured from BM cells starting on Day 0 for various lengths of time. Total RNA was isolated on each day. miR126 was measured using a TaqMan® MicroRNA Assay (Applied Biosystems), and the mRNA expression of Spred1 was assessed by means of real-time RT-PCR. (A; $n = 6$ for day 0 and day 1, mean ± standard deviation (SD). $n = 1$ for day 7 and $n = 3$ for BMMC, B; $n = 2$ mean of the two independent experiments. $\star P < 0.05$) (C) Total cell lysates from parental MC9 cells (−), empty-infected or miR126 infected MC9 cells were analyzed by Western blotting for Spred1 and tubulin. One representative data from three independent experiments is shown. (D,E) GFP-positive cell fraction (%) was scored by means of flow cytometry. After the transduction, retroviral vector-positive (GFP^+) and negative (GFP^-) BMMCs were cultured together for the indicated periods (D). GFP proliferative ratio = (GFP positive rate)/(GFP positive rate on day14). miR126-positive (GFP^+) and negative (GFP^-) MC9 cells were mixed at a ratio of 1 : 1 and subsequently cultured. Empty vector-infected (GFP^+) MC9 cells were used as a control (E). Data shown are representative of more than three independent experiments on different cells with similar results (D,E).

compared with control cells (Fig. 2A). We then examined ERK activation through FcεRI. MC9 transformants were sensitized with anti-DNP IgE and stimulated with DNP-HSA. Ligation of FcεRI resulted in a rapid, transient activation of ERK in control MC9 cells, whereas ERK activation was prolonged in miR126-expressing cells. These results suggest that miR126 suppresses Spred1 expression and

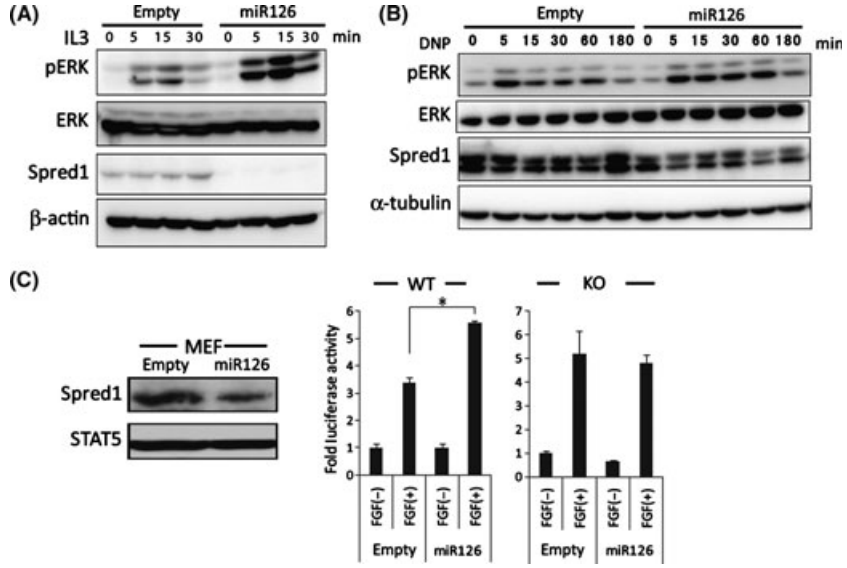

Figure 2 Reduction of Spred1 expression in miR126 forced cell lines. (A, B) miR126-MC9 cells and empty-MC9 cells were stimulated with 5 ng/mL of IL-3 for time indicated periods (A). miR126-MC9 cells and empty-MC9 cells were sensitized with anti-DNP IgE, then stimulated with DNP-HSA for the indicated periods (B). Total cell lysates were analyzed by Western blotting with indicated antibodies. One representative data from three independent experiments is shown (A,B). (C) Mouse embryonic fibroblast (MEFs) were infected with miR126 or empty retrovirus. Total cell lysates were analyzed by means of Western blotting for Spred1 and STAT5 (left). WT and Spred1$^{-/-}$ (KO) MEF cells were transfected with Elk1 promoter and β-galactosidase. After FGF stimulation of the cells, Elk1 luciferase activity was measured and normalized against β-galactosidase activity. The means and SDs of triplicate samples of one representative experiment of three independent experiments are shown (right). $\star P < 0.05$.

enhances ERK activation through the stimulation of various processes in MC9 cells (Fig. 2B).

To confirm whether the enhancement of ERK activation induced by miR126 is really dependent on reduced expression of Spred1, we studied Spred1-deficient mouse embryonic fibroblasts (MEFs). As shown in Fig. 2C (left panel), forced expression of miR126 suppressed Spred1 expression in WT MEFs. ERK activation was monitored via Elk-1 luciferase activity (Kato *et al.* 2003). WT or Spred1-deficient MEF cells were transfected with miR126 expression vector and Elk-1 reporter plasmids, then stimulated with FGF for 6 h. As shown in Fig. 2C (right panels), miR126 increased FGF-mediated ERK activation in WT MEF cells but not in Spred1-deficient MEFs. This strongly supports our idea that the miR126-mediated upregulation of ERK activation is because of the down-regulation of Spred1.

Generation of Spred1 conditional KO mice and assessment of mast cell development

Next, to confirm the effect of Spred1 on mast cell development, we conditionally knocked out the Spred1 gene in hematopoietic stem cells, as Spred1 may function in various tissues. Figure 3A depicts the strategy by which we generated the Spred1$^{flox/flox}$ allele. Mice carrying the targeted Spred1^{neo} allele were generated as described in Fig. 3 and in experimental procedures. In the Spred1^{neo} allele, parts of intron 6 and exon 7 were floxed, and the neomycin resistance (neoR) cassette in intron 6 was flanked by FRT sites (Fig. 3A FRT). Spred1^{neo} heterozygotes were bred to β-actin-FLPe (Rodriguez *et al.* 2000) transgenic mice to obtain offspring carrying the Spred1^{flox} alleles. Proper recombination and deletion in a selected ES clone was confirmed by Southern blotting (Fig. 3B). We subsequently crossed Spred1$^{flox/flox}$ mice with Tie2Cre-transgenic mice to delete the Spred1 gene from all hematopoietic lineages. In the resulting Tie2Cre-Spred1$^{flox/flox}$ (Spred1-cKO) mice, Spred1 mRNA was undetectable in BM cells and lymphocytes from the lymph nodes and spleen, although the expression of Spred1 in the brain was not affected by the Cre transgene (Fig. 3C). The Spred1-cKO mice were fertile and were born at the expected Mendelian frequency (data not shown). Spred1-cKO mice did not show the short-nose

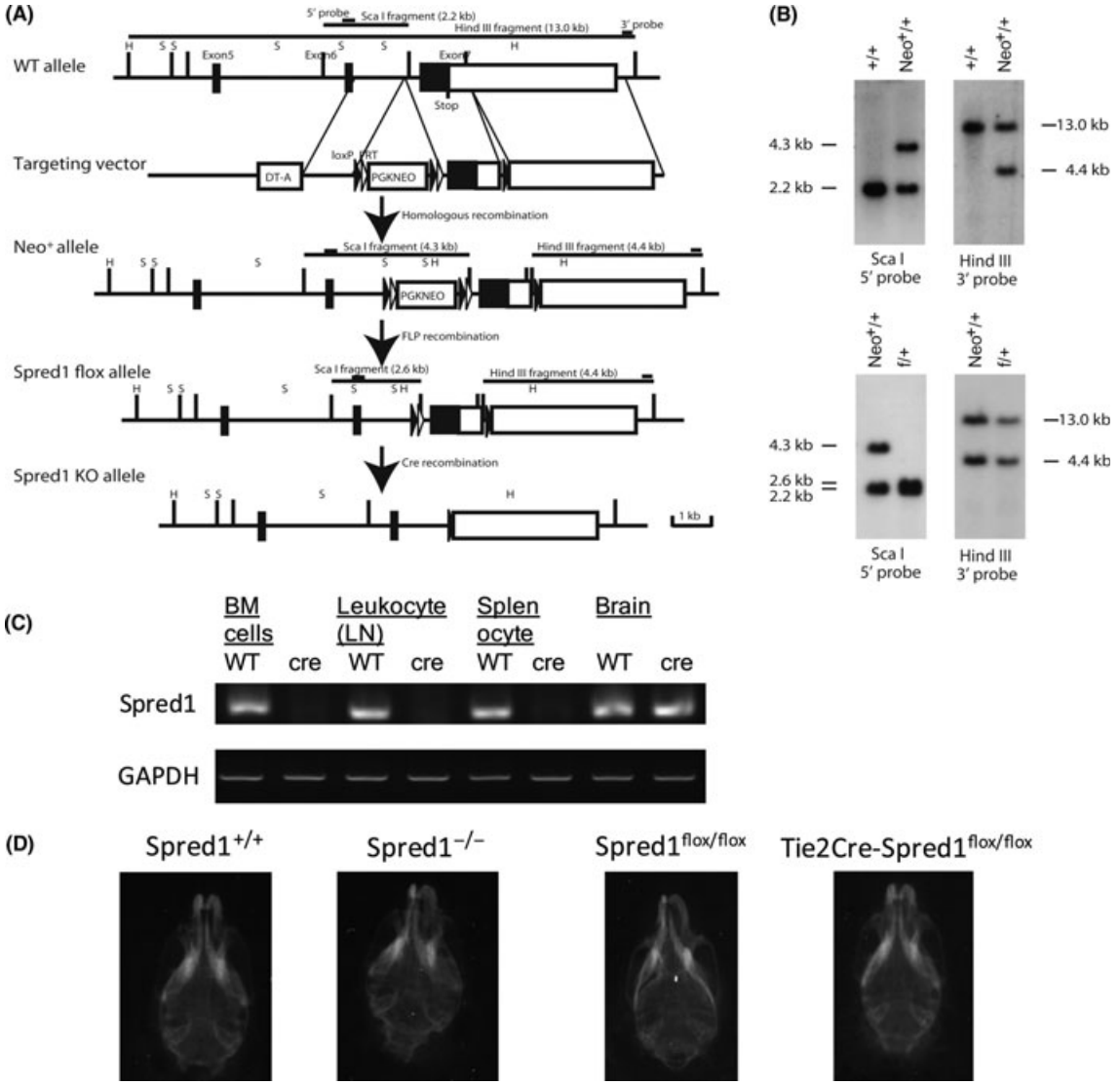

Figure 3 Generation of Tie2Cre spred1$^{flox/flox}$ mice. (A) Schematic representations of wild type and mutant loci of the Spred1 gene together with the targeting vector. The exons (from the 5th to the 7th) for the genes encoding Spred1 are represented by black and white boxes. Neomycin-resistance genes driven by PGK promoters (pgk-neo) are indicated by white boxes. LoxP sites are represented by black arrow heads. FRT sites are represented by white arrow heads. Restriction sites: H, HindIII; S, Sca 1. (B) Representative Southern blot analysis with Sca 1- or HindIII-digested DNA. The positions of probes are shown in A. (C) mRNA contents of BM cells, lymphocytes, splenocytes, and brain cells were determined by RT–PCR. (D) X-ray photography of skulls of indicated mice.

phenotype observed in straight Spred1$^{-/-}$ mice (Fig. 3D). No gross abnormality was observed in major organs or blood vessels (data not shown).

We counted the hematopoietic cells, including mast cells, in Tie2Cre-Spred1$^{flox/flox}$ mice. The numbers of lymphocytes (T cells, B cells, and NK cells) as well as myeloid cells (erythrocytes and granulocytes) in the peripheral blood and spleen were not altered in Spred1-cKO mice (data not shown). The fractions of mast cells in the spleen, bone marrow, and peritoneal cavity in Spred1-cKO mice (information on the peritoneal cavity is shown in Fig. 4A) were similar to those in WT mice. In the subcutaneous tissue of the ear, however, histochemical analysis with toluidine blue staining showed that the number of mast cells in Spred1-cKO mice was increased compared with that

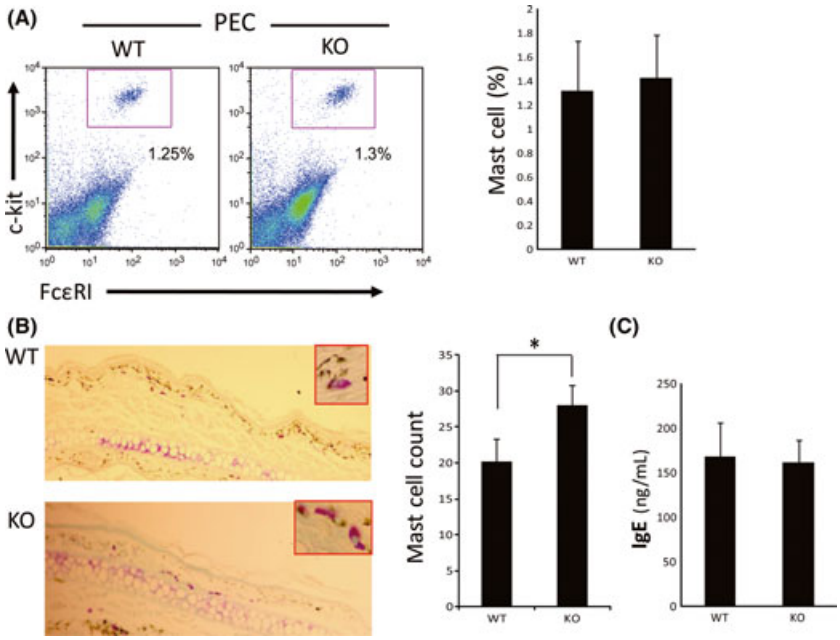

Figure 4 Increased mast cell count in Tie2Cre Spred1$^{flox/flox}$ mice. (A) Flow cytometry of peritoneal exudate cells derived from control or Tie2Cre Spred1$^{flox/flox}$ (KO) mice and stained with mAb to c-Kit and FcεRI (left). Histograms show mean percentage of double-positive cells (right). (n = 6, mean ± SD.) (B) Localization of skin mast cells stained with toluidine blue (left). Histograms show mean mast cell count per field (right). (n = 6. $\star P < 0.05$. Error bars indicate the SDs) (C) Levels of IgE in sera derived from control or KO mice (WT; n = 3, KO; n = 6 mice) were determined by ELISA. Values represent the mean ± SD.

in WT mice (Fig. 4B). Serum IgE levels in Spred1-cKO mice were not different from those in control mice at 6–10 weeks of age (Fig. 4C). Passive cutaneous anaphylaxis (PCA) was examined in sensitized WT and Spred1-cKO mice on i.v. challenge with Ag (DNP-HSA in 0.5% Evans blue dye). IgE-mediated degranulation was only slightly higher in Spred1-cKO mice than in WT mice, as measured through Evans blue dye extravasation (data not shown). Our results show that Spred1 negatively regulates connective tissue mast cell development in the skin, though IgE-mediated degranulation was not altered.

Roles of Spred1 and miR126 in IgE-dependent mast cell activation

To examine the role of Spred1 in IgE-dependent mast cell activation, we compared BMMCs from WT and Spred1-cKO mice. BM cells from Spred1-deficient and WT mice were cultured in conditioned medium and analyzed by flow cytometry for c-kit and IgE receptor expression. Although the number of mast cells after 4 weeks of culture was higher among BM cells from Spred1-KO mice than among those from WT mice (Nonami *et al.* and data not shown), no difference in c-kit and IgE receptor expression was observed between WT and Spred1-deficient BMMCs (Fig. 5A). As expected based on our *in vivo* PCA assay, we did not observe strong differences in IgE-mediated degranulation *in vitro* (data not shown).

FcεRI has been shown to activate various intercellular signaling pathways including NF-κB, PI3k-Akt, and MAP kinases. As shown in Fig. 5B, JNK, p38, and I-kB phosphorylation in response to IgE/DNP stimulation were not significantly different between WT and Spred1-deficient BMMCs, nor was the phosphorylation of Akt (data not shown). A slightly higher level of ERK phosphorylation was observed in Spred1-deficient BMMCs before stimulation (Fig. 5B, 0 min), which was probably due to an increased ERK activation by IL-3 and SCF during culture of BMMCs. However, ERK phosphorylation was apparently increased and prolonged in Spred1-deficient BMMCs compared with WT BMMCs (Fig. 5B). Thus, Spred1 specifically regulates the ERK pathway among FcεRI-mediated signals.

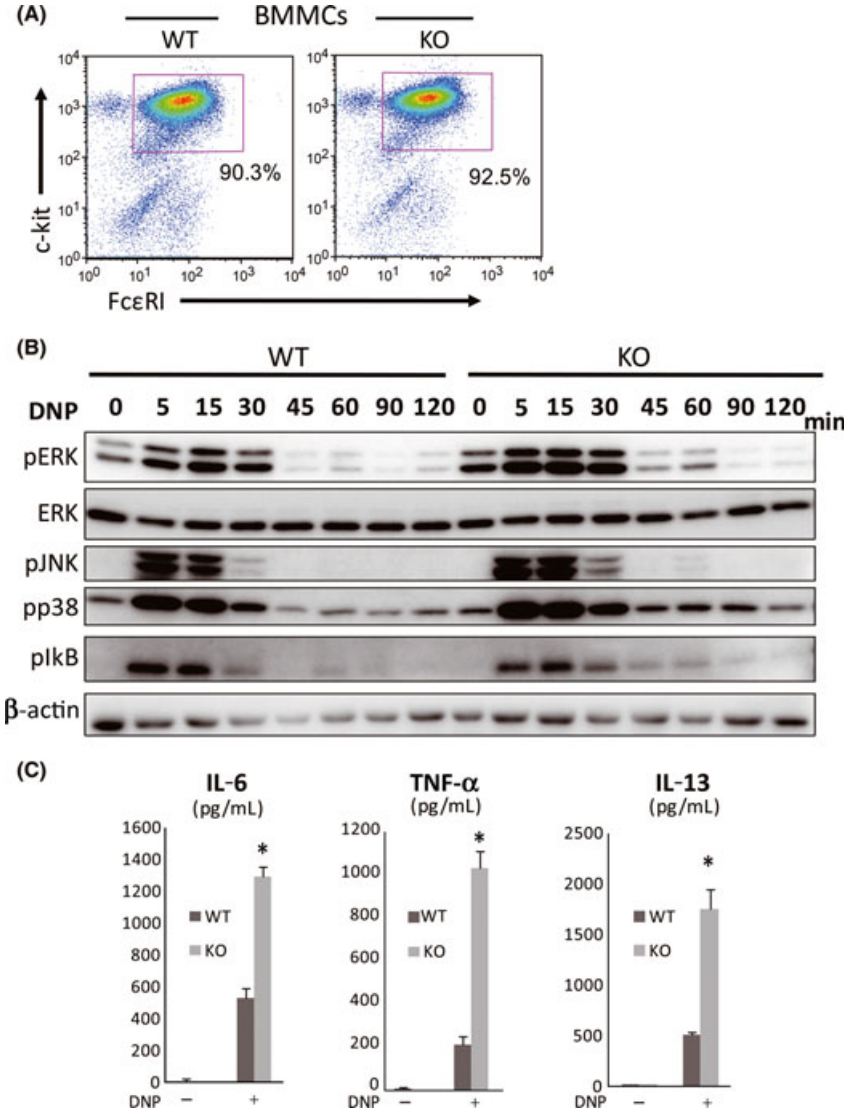

Figure 5 Enhanced anti-IgE-mediated cytokine production in Spred-1$^{-/-}$ bone marrow–derived mast cells (BMMCs). BMMCs derived from control or Tie2Cre Spred1$^{flox/flox}$ mice were stained with mAb to c-Kit and FcεRI. Numbers in top right quadrants indicate percent mast cells. Data shown are representative of three independent experiments on different mice. (B,C) Control and Spred1$^{-/-}$ BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for the indicated periods. (B) Total cell lysates were analyzed by means of Western blotting for various phosphorylated and total proteins. One representative data from three independent experiments is shown. (C) ELISA of cytokines in supernatants of BMMCs stimulated with DNP-HAS for 6 h. Concentrations of cytokines in supernatants were determined by means of ELISA. One representative data from three independent experiments is shown. $*P < 0.05$. Error bars indicate the SDs.

It has been shown that FcεRI-mediated ERK activity is important for cytokine production from mast cells (Galli *et al.* 2005a). To determine whether Spred1 is involved in the regulation of FcεRI-mediated cytokine production, IL-6, IL-13 and TNF-α production patterns were measured by means of ELISA and RT-PCR. Production rates of all these cytokines were significantly enhanced in Spred1-deficient BMMCs compared with WT BMMCs (Fig. 5C; data from RT-PCR not shown). These results indicate that Spred1 negatively regulates IgE-mediated cytokine production.

Finally, we investigated the role of miR126 in the Spred1-mediated regulation of cytokine production. Retroviral miR126-infected (GFP^{+}) BMMCs were FACS-sorted after 4 weeks of culture, sensitized with

anti-DNP IgE and stimulated with DNP-HAS for 6 h. IL-6, IL-13, and TNF-α levels in the culture supernatants were determined by means of ELISA. Reduction of cytokine production was observed in empty-vector infected WT and Spred1-KO BMMCs (Fig. 6. empty) compared with uninfected cells (Fig. 5C), suggesting that virus infection and/or GFP sorting had some effect on cytokine production. However, as shown in Fig. 6, miR126 expression apparently enhanced IgE-mediated cytokine production in WT BMMCs (Fig. 6; WT, miR126). This enhancement was Spred1 dependent, because no or very weak upregulation was observed in Spred1-deficient mast cells. These results strongly support our hypothesis that miR126 regulates FcεRI-mediated cytokine production by directly targeting Spred1 in mast cells.

Discussion

In the present study, we have shown that Spred1 negatively regulates FcεRI-mediated cytokine production in mast cells, whereas miR126 regulates ERK signaling and cytokine production by directly targeting Spred1. *In vitro,* we have shown that Spred1 negatively regulates mast cell proliferation (Nonami *et al.* 2004). Consistently, the numbers of mast cells in the subcutaneous tissue of the ear are increased in Spred1-cKO mice compared with WT mice. Tissue mast cells are derived from hematopoietic stem cells, which ultimately give rise to mast-cell progenitors (MCPs). MCPs circulate in the blood and enter the tissues, where they undergo differentiation and maturation to become mature mast cells. SCF is normally required to ensure mast-cell survival in the tissues (Galli *et al.* 2008). We previously reported that Spred1 negatively regulates IL-3 as well as SCF-induced ERK activation and cell proliferation in BMMCs without affecting STAT or Akt activation (Nonami *et al.* 2004). Thus, the increased concentration of mast cells in the skin of Spred1-cKO mice is probably due to the enhanced sensitivity of Spred1-deficient mast cells to SCF. We did not observe any differences in mast cell numbers in the peritoneal cavity or in other organs, however. The reason for this is not entirely clear at present, though we suspect that skin cells express larger amounts of SCF than cells of other tissues do. A similar increase in cutaneous mast cells has been reported in $NF1^{+/-}$ mice (Ingram *et al.* 2000) as well as in Rab-$GEF1^{-/-}$ mice (Tam *et al.* 2004); significantly, both NF1 and Rab-GEF1 negatively regulate Ras-mediated ERK activation. Mast cells from mice of these two genotypes ($NF1^{+/-}$ and Rab-$GEF1^{-/-}$) showed enhanced ERK activation and mast cell proliferation, as did Spred1-deficient mast cells. These results further support our notion that Spred1 is a specific inhibitor for the Ras-ERK pathway.

It has been reported that the ERK pathway is involved not only in cytokine production but also in the synthesis of prostaglandin D2 (PGD2) from arachidonic acid (Galli *et al.* 2005a). In the present study, we found that Spred1-deficient mast cells were hyperresponsive to IgE, but only in the form of increased cytokine production: we did not observe

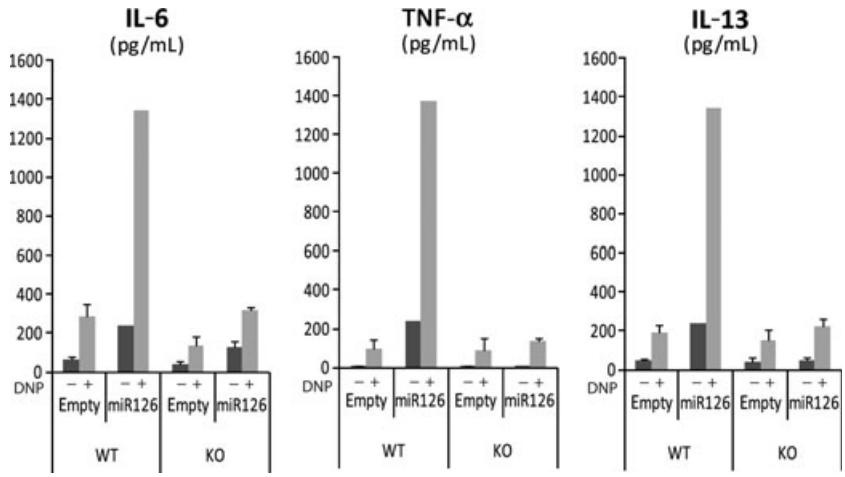

Figure 6 Effect of miR126 expression on cytokine production from WT and $Spred1^{-/-}$ bone marrow–derived mast cells (BMMCs). BMMCs derived from WT or Spred1 cKO mice were transduced with retroviral vectors encoding for IRES-GFP (empty) and miR126-IRES-GFP (miR126). FACS-sorted BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-HSA for 6 h. Concentrations of indicated cytokines in supernatants were determined by means of ELISA. One representative data (mean ± SD) from three independent experiments is shown.

higher levels of degranulation or PGD2 synthesis in Spred1-deficient mast cells in response to IgE. This characteristic places Spred1-deficient mast cells in clear contrast to $NF1^{+/-}$ and Rab-$GEF1^{-/-}$ mast cells, which showed higher levels of degranulation and PGD2 synthesis in response to IgE. One possible explanation for this is that cytokine production is more sensitive to ERK than degranulation and PGD2 synthesis are, and that Spred1 only partially regulates ERK activation. After long-term IgE stimulation, we observed some down-regulation of ERK even in $Spred1^{-/-}$ mast cells. Other negative mechanisms, including NF1 and Rab-GEF1, along with other negative regulators such as SHP-1, SHIP, c-Cbl, and Ras-GAP (Galli *et al.* 2005a) may compensate for the lack of Spred1. Alternatively, as Spred1 does not affect the Akt, JNK and p38 cascades, these kinases could play significant roles in degranulation and PGD2 synthesis.

Although the role of miRNA in development and differentiation is well established (Vasilatou *et al.* 2010), only a few papers have shown its role in mast cells. miRNA array studies have shown that miR221–222 is the only family of miRNAs that is significantly up-regulated on activation of differentiated mast cells, under resting or stimulated conditions (Mayoral *et al.* 2009), but there have been no comparable investigations into whether any functionally important miRNA is down-regulated in mast cells. Here, we focused on miR126 because Spred1 is reported to be among its targets. We suspected that miR126 negatively regulates mast cell differentiation and functions by modulating Spred1 expression levels (Wang *et al.* 2008).

Although Landgraf *et al.* (2007) have reported the qualitative detection of miR126 in the CD34+ pool that contains HSCs, Kotani *et al.* (2010) have investigated the function of miR126 in the hematopoietic system and found that it facilitates B-cell differentiation. We have shown that the relative expression of miR126 decreased rapidly in the process of BMMC development, whereas that of Spred1 increased gradually. Overexpression of miR126 in mast cells enhanced their proliferation in response to IL-3 and cytokine production in response to IgE, in a pattern very similar to that observed in Spred1-deficient mast cells. In addition, we have shown that miR126-mediated enhancement of both ERK activity and cytokine production was Spred1 dependent. These results constituted the first biochemical demonstration of the Spred1-dependent functions of miR126. Thus, miR126 probably regulates ERK signaling by directly targeting Spred1, not only in angiogenesis but also in the immune system.

We concluded that Spred1 negatively regulates mast cell proliferation and IgE-mediated cytokine production, and that Spred1 itself is fine-tuned by miR126. We have previously reported that $Spred1^{-/-}$ mice showed exaggerated allergen-induced airway hyperresponsiveness, eosinophilia, and mucus production in a murine allergic asthma model (Inoue *et al.* 2005). Selective blockade of miR126 suppressed the asthmatic phenotype, resulting in diminished Th2 responses, inflammation. (Mattes *et al.* 2009). In future studies, it will be of interest to measure miR126 levels in human asthma and allergic diseases. Spred1 has also been reported to be a target of miR212, which amplifies CREB signaling through Raf-1 and thus adjusts the effects of cocaine intake (Hollander *et al.* 2010). As miR212 is relatively highly expressed in BMMCs (Monticelli *et al.* 2005), the role of miR212 in mast cells is worthy of future investigation.

Experimental procedures

Generation of conditional $Spred1^{-/-}$ mice

To generate the Spred1 targeting vector, a 1.4-kb fragment containing the sixth intron, a 1.6-kb fragment containing part of the open reading frame that codes the c-Kit-binding domain (KBD) and the cysteine-rich domain related to Sprouty (SPR) on the seventh exon, and a 4.0-kb fragment containing a non-coding region on the seventh exon were inserted into the *Cla* I-*Eco*R V sites, the *Nhe* I site, and the *Not* I-*Sac* II sites of the LFNeo/DT-ApBluescript vector, respectively. The targeting vector was linearized and transfected into 129/Sv embryonic stem (ES) cells by electroporation. The ES cells that were positive for homologous recombination were screened by PCR and confirmed by Southern blot analysis. ES cell lines with proper homologous recombination were injected into C57/B6 blastocysts. Two chimeric mice successfully transmitted the germline. Heterozygous ($Spred1^{neo}$) F1 mice were crossed with hemizygous FLPe deleter mice (Rodriguez *et al.* 2000) to remove the FRT-flanked neo cassette *in vivo*. The resulting $Spred1^{+/flox}$ FLPe mice were then backcrossed to wild-type C57BL/6 mice to generate $Spred1^{+/flox}$ mice, which lacked the FLPe transgene. Finally, $Spred1^{+/flox}$ mice were intercrossed to produce $Spred1^{flox/flox}$ mice, which were maintained on a mixed 129/Sv × C57BL/6 genetic background through brother–sister mating.

Tie2Cre mice with a C57BL/6 background expressing Cre recombinase under the control of the mouse proximal Tie2 gene were crossed with $Spred1^{flox/flox}$ mice. Offspring carrying both Tie2Cre and floxed Spred1 genes were used for intercrossing and further analysis. Littermates without the Tie2Cre allele ($Spred1^{flox/flox}$) were used as control mice. All experiments using mice were approved by and carried out according

to the guidelines of the Animal Ethics Committee of Keio University.

Cell lines

HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells from the murine mast-cell-derived line MC9 were cultured in RPMI medium containing 5% fetal bovine serum, 50 μM 2-mercaptoethanol, and 5 ng/mL IL-3, as described previously (Nonami *et al.* 2004, 2005).

Mast cell culture and activation

BMMCs were obtained from 4- to 8-week cultures of bone marrow cells in RPMI 1640 supplemented with 5 ng/mL murine IL-3, 10 ng/mL murine SCF, 10% fetal calf serum, non-essential amino acids, 100 IU/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B, and 10 μM 2-mercaptoethanol. BMMCs were passively sensitized with 1 ng/mL anti-DNP IgE for 12 h. Cells were stimulated with 1 μg/mL DNP-HSA.

Retrovirus construction and transduction

miR126 expression vector of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was kindly provided by Dr C. Kuo and Dr M.Mancuso (Stanford University)(Kuhnert *et al.* 2008). BamHI-HindIII fragments containing miR126 (280 bp) were subcloned into a pMX-IRES-EGFP vectors. pMX-IRES-EGFP vectors and retrovirus packaging vectors were co-transfected into HEK293 cells according to the polyethyleneimine method to obtain the viruses. Cells were added to fresh retrovirus supernatant and spun at 1200 *g* for 1.5 h at 35 °C, as described previously (Ichiyama *et al.* 2009). Cell proliferation was determined with GFP positivity, as described (Nonami *et al.* 2005).

Luciferase assay

FGF- or EGF-induced ERK activation was measured by means of a luciferase assay using an Elk1-responsive reporter, as described previously (Kato *et al.* 2003). In all reporter assays, MEF cells were plated on six-well dishes, and plasmids were transfected according to the polyethyleneimine method. The luciferase assay was carried out using a luciferase substrate kit (Promega, Madison, WI, USA), and luciferase activity was read with a Packard luminometer and normalized to β-galactosidase as an internal control.

ELISA

Supernatants were collected after the indicated periods of cell culture and were analyzed for IL-6, TNF-α, IL-4, IL-10, and IL-13 with an ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Real-time RT-PCR

cDNA was synthesized and analyzed by real-time quantitative PCR, as described previously. Gene expression was examined with a Bio-Rad iCycler Optical System with the iQ SYBR green real-time PCR kit (Bio-Rad Laboratories, Hercules, CA, USA). The results were normalized to HPRT. The primers have been described previously (Ivanov *et al.* 2006). The relative expression of the indicated gene to that of HPRT was calculated as $(2^{-[\text{experimental CT}-\text{HPRT CT}]}) \times 1000$, where CT is the cycle threshold of signal detection.

MicroRNA analysis

Total RNA was isolated using a mirVana miRNA isolation kit (Ambion, Austin, TX, USA). Expression levels of mature miR126 were measured using a TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The Student's paired two-tailed *t*-test was used. Values of $P < 0.05$ were considered significant.

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Disclosure

We have no conflict of interest.

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