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The Transcription Factor Zeb2 Regulates Signaling in Mast Cells

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Mast cell activation results in the release of stored and newly synthesized inflammatory mediators. We found that Zeb2 (also named Sip1, ZBHx1b), a zinc finger transcription factor, regulates both early and late mast cell responses. Transfection with small interfering RNA (siRNA) reduced Zeb2 expression and resulted in decreased FceRI-mediated degranulation, with a parallel reduction in receptor-induced activation of NFAT and NF-κB transcription factors, but an enhanced response to the LPS-mediated activation of NF-κB. There was variable and less of a decrease in the Ag-mediated release of the cytokines TNF-α, IL-13, and CCL-4. This suggests that low Zeb2 expression differentially regulates signaling pathways in mast cells. Multiple phosphorylation events were impaired that affected molecules both at early and late events in the signaling pathway. The Zeb2 siRNA-treated mast cells had altered cell cycle progression, as well as decreased expression of several molecules including cell surface FcεRI and its β subunit, Gab2, phospholipase-Cγ1, and phospholipase-Cγ2, all of which are required for receptor-induced signal transduction. The results indicate that the transcription factor Zeb2 controls the expression of molecules thereby regulating signaling in mast cells. The Journal of Immunology, 2012, 188: 000–000.

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Abbreviations used in this article: BMMC, bone marrow-derived mast cell; miRNA, microRNA; MRE, microRNA response element; PLCγ, phospholipase Cγ; SCF, stem cell factor; siRNA, small interfering RNA.

We report in this study that the zinc finger transcription factor Zeb2 (also called Sip1 for Smad-interacting protein) regulates FceRI-mediated responses in mast cells. Zeb2 was initially identified as a protein that interacted with receptor-activated Smads in the pathway associated with signaling of activin and other members of the TGF-β superfamily (16). Zeb2 is now recognized as a zinc finger DNA-binding protein that functions as a transcriptional regulator (17, 18). Structurally, Zeb2 has two separate clusters of zinc fingers, one at the N terminus, the other at the C terminus, and a central region with a homeodomain, thought to be involved in protein–protein interactions, a SMAD binding domain, and a domain that binds the coactivator CtBP. Binding with its zinc finger to regulatory elements of genes causes Zeb2 to act mainly as a transcriptional repressor, although there have been reports that it can also be an activator of gene transcription. Zeb2 functions to regulate epithelial to mesenchymal transition processes in cells; its expression in epithelial cells causes these polarized cells to change morphology by losing their cell–cell contacts and to acquire the motile, migratory properties of mesenchymal cells. Zeb2 therefore is essential in embryogenesis: knockout results in embryonic lethality by day 9.5 due to multiple defects (18). Haploinsufficiency of Zeb2 in humans results in the Mowat–Wilson syndrome with typical facial features and variable symptoms depending on the mutation but generally with mental retardation and neurologic defects (19). Zeb2 is overexpressed in a number of cancers in which there is a epithelial to mesenchymal shift resulting in a more aggressive phenotype (20).

Zeb2 was identified during a small interfering RNA (siRNA) library screen for molecules that regulate Ag-induced NFAT or NF-κB activation in mast cells. In this study, we used an RNA interference approach to investigate the function of Zeb2 in mast cells. Cells were transfected with siRNA, and changes in the cellular responses and signaling events were monitored for 3 d posttransfection. A decrease in Zeb2 expression resulted in reduced FcεRI-mediated activation of the transcription factors NFAT and NF-κB but in a substantial increase in the LPS-mediated NF-κB activation. Zeb2 knockout also decreased de-
granulation at all 3 d of testing; however, it resulted in increased release of TNF-α, IL-13, and CCL-4 at day 1 but decreased responses by day 2 or 3. Decreased expression of Zeb2 resulted in changes in IgE–FceRI–mediated signaling events with reduced phosphorylation of several proteins. There were also changes in ionomycin-mediated degranulation and cytokine release indicating that Zeb2 knockdown altered signaling events both upstream and downstream of the calcium response. There were also effects on the expression of proteins implicated in signal transduction. These results indicate that Zeb2 by regulating the expression of several proteins controls mast cell responses.

Materials and Methods

Abs and reagents

Mouse IL-3, stem cell factor (SCF), and propidium iodide were purchased from Invitrogen (Carlsbad, CA). Ionomycin and PMA were from Calbiochem (La Jolla, CA). The anti-mouse IgE PE was from eBioscience (San Diego, CA). RNase was from Sigma Aldrich (St. Louis, MO). The Abs for SHIP-1 (PICI), Syk (N-19), Gab2 (M-19), PLCγ2 (Q-20), Zeb2 (SIP1, H-260), and E-cadherin (H-108) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-p38, anti–phospho-Erk2 (Thr180/Tyr182), anti–phospho-p44/42 MAPK, anti–phospho–Syk (S525/Tyr525), anti–phospho-PLCγ2 (Y1217), and anti–phospho-LAT (Y191) were from Cell Signaling Technology (Beverly, MA); anti–SNAI1 (N-term R8) was from Abgent (San Diego, CA). The Abs for PLCγ1 and the activation of NFAT and NF-κB were from CellQuest software (BD Biosciences, San Jose, CA); the Abs and reagents specified in the methods or figure legends.

Cell culture

The growth factor-dependent MC9 mouse mast cell lines stably expressing NFAT binding sites fused to enhanced GFP or NF-κB binding sites fused to GFP were described previously (23). Bone marrow cells were collected from 2- to 4-mo-old C57BL/6 females and cultured for up to 8 wk in DMEM supplemented with 20% heat-inactivated FBS, 2 mM l-glutamine, 5 × 10⁻⁵ M 2-mercaptoethanol, 10% NCTC 109 medium, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotics, 30 mg/ml IL-3, and 25 ng/ml SCF. Bone marrow–derived mast cells (BMMC) were used for experiments starting with the fourth week of culture.

Transient transfection with siRNA

An initial screen was with a library containing pools of four siRNA duplexes per gene that target 198 mouse enzymes with known or predicted phosphatase activity purchased from Dharmacon (Lafayette, CO). The characteristics of this library are described in our previous publication where it was used to study degranulation in mast cells (22). The siRNA targeting Zeb2 (NM_015753) containing either a pool of four siRNA duplexes or the single duplexes was from Dharmacon. For negative controls, a pool containing four scrambled siRNAs with minimal effect on known mouse genes (ON-TARGET plus siCONTROL, nontargeting pool, cat. no. D-001810-10-20) was also from Dharmacon. Mouse Syk (NM_011518) OGENOME SMARTpool (Dharmacon) was used in some transfections as a positive control. Transfection was by use of Amaxa Nucleofector 96-well Shuttle System as previously described (23). After transfection, cells were transferred into 96-well plates and assayed for 3 d. Cell pellets in each experiment were analyzed by immunoblotting to determine changes in the level of expression of proteins.

Flow cytometric measurement of NF-κB and NFAT activation

Fluorescence of the GFP reporter was used as a marker of FceRI–induced NF-κB or NFAT activation. Cells were sensitized for 24 h with 0.3 μg/ml Ag-specific IgE (anti–TNP mAb) and then stimulated with Ag for 5 h (for NF-κB activity) or 18 h (for NFAT activity) at which times there was maximal GFP expression. The mean fluorescence intensity of treated and control cells was determined by a FACScan three-color analysis cytometer and processed by CellQuest software (BD Biosciences, San Jose, CA). The activation in the treated cells is presented as a percentage or fraction of that in cells transfected with control siRNA.

Degranulation measurements

The β-hexosaminidase release was determined in transfected BMMC that were sensitized for 24 h with 0.3 μg/ml Ag-specific IgE (anti–TNP mAb TIB-142) as described previously (23). In some experiments, cells were stimulated with 1 μM ionomycin. Degranulation of cells transfected with the experimental siRNA is presented as percent or fraction of that in cells transfected with siCONTROL. Ag–induced release in control transfected cells was 27 ± 8% on day 1, 27 ± 7% on day 2, and 23 ± 11% on day 3 (n = 7).

Cytokine release measurements

BMMC transfected with siRNA were sensitized overnight with Ag-specific IgE and then challenged for 3 h in complete culture medium with Ag or a combination of 20 nM PMA and 1 μM ionomycin. Quantitative measurements of TNF-α, IL-13, and CCL-4 (MIP-1β) in the supernatants were performed with cytokine-specific Quantikine ELISA Kits (R&D Systems) according to the manufacturer’s instructions. Ag–induced release in cells transfected with experimental siRNA is expressed as a fraction of that in control-treated cells.

Other cellular analyses

For FceRI expression, transfected BMMC (7 × 10⁵) were cultured overnight with 0.6 μg/ml Ag-specific IgE. After washing, the cells were incubated for 1 h at 4°C with 2 μg FITC-conjugated anti-mouse IgE. The cells were then washed, and the fluorescence was read by FACSscan.

For cell cycle analysis, BMMC (7 × 10⁵) were washed with PBS and fixed overnight with ice-cold 70% ethanol. After centrifugation, the pellet was resuspended in 100 U RNase and incubated at 37°C for 30 min. Propidium iodide solution (500 μl of 50 μg/ml in DPBS) was then added, and samples were further incubated for 30 min at 4°C. Percent of events gated in the cell cycle phases (G0-G1, S, G2-M) was determined by a FACSscan.

Immunoblotting and densitometry

Cell pellets from transfections with control or experimental siRNA were used for immunoblotting to determine changes in the level of expression of proteins. Phosphorylation of Akt was determined in cells starved of SCF as described previously (23). Proteins were resolved under reducing conditions on precast minigels, electrophoretically transferred onto polyvinylidene fluoride Immobilon membranes, probed with specific Abs, and detected using ECL. The blots were scanned and processed on a Kodak Image Station 4000MM Digital Imaging System (Carestream Molecular Imaging, Rochester, NY). The densitometry of the bands was used to calculate the changes as a fraction of that in control cells transfected with nontargeting siRNA using the values of the anti-actin or specific protein to correct for gel loading.

Data analysis

Statistical analysis was by nonparametric t tests using GraphPad Prism 5, and the p value is represented in the figures as *p < 0.1, **p < 0.01, and ***p < 0.005. The number of repeats for each set of experiments is specified in the methods or figure legends.

Results

Zeb2 regulated FceRI-induced activation of NFAT and NF-κB in mast cells

To screen for new molecules involved in FceRI–induced gene expression, an siRNA screen was developed using NFAT and NF-κB reporter-expressing mast cells. These reporter cells were derived from MC9 growth factor-dependent mouse mast cells in which expression of GFP was regulated either by NFAT or NF-κB activation. After FceRI stimulation, there was expression of GFP that was determined by FACS analysis. Previously, a screen using an siRNA library targeting known or predicted mouse phosphatase genes in a mast cell degranulation assay identified several potential new targets (22). When the same siRNA library was used with the NFAT and NF-κB reporter cells, one of the strongest positives was for the transcription factor Zeb2, which, although included in this library, is not a phosphatase. In this assay, the reporter cells were transfected with siRNA duplex pools composed of four distinct siRNA species targeting Zeb2 and then sensitized with IgE and challenged with Ag. The Ag-induced activation of NFAT and NF-κB was determined 1, 2, or 3 d after siRNA treatment. Compared to cells transfected with control
cells. The decrease in Zeb2 expression was accompanied with specific and efficiently reduced by siRNA treatment in mouse mast cells. Although there was already a dramatic decrease in Zeb2 expression by day 1, the phenotypic changes were more variable. Notably, whereas there was an intermediate level of Zeb2 expression, changes in phenotype were stronger. Notably, Zeb2 knockdown caused an increase in NF-κB activation through the LPS–TLR4 pathway, having the opposite effect to that with Ag. Thus, at day 3 posttransfection, LPS-stimulated NF-κB activity increased by 213% in cells treated with Zeb2-3, by 80% in cells treated with Zeb2-4, and by 166% in cells treated with the pooled siRNA (Fig. 1A). These results indicate that siRNAs to Zeb2 have profound effects on signaling in mast cells.

After FACS analysis, the remaining cells were used for immunoblotting to confirm that the observed functional changes were due to siRNA-induced decrease in protein expression (Fig. 1B–D). The pooled siRNA decreased Zeb2 expression by >90% on all 3 d of testing. All the single siRNAs decreased Zeb2 expression to some extent; the most active was Zeb2-3, which was also the most active by phenotypic effects, whereas Zeb2-2 had minimal effects on protein expression and phenotype. The other two singles, Zeb2-1 and Zeb2-4, had an intermediate effect on protein expression. In general, there was correlation between the level of protein expression and the phenotype; although when there was an intermediate level of Zeb2 expression, changes in phenotype were more variable. Notably, whereas there was already a dramatic decrease in Zeb2 expression by day 1, the phenotypic changes were greater at day 2 and 3 suggesting that the change in Zeb2 levels required time to have effects on cell signaling.

These results indicate that the expression of Zeb2 can be specifically and efficiently reduced by siRNA treatment in mouse mast cells. The decrease in Zeb2 expression was accompanied with changes in the FcγRI-induced NFAT and NF-κB activation demonstrating that these were related events. This strongly suggested that Zeb2 was involved in the regulation of the signaling pathway leading to synthesis and release of cytokines by stimulated mast cells.

Zeb2 regulated FcγRI- and ionophore-mediated cellular responses in primary mast cells

Because cell lines such as MC9, although growth factor dependent, could still have mutations, Zeb2 function was validated in primary mast cells. Mouse BMMC were treated with the pool of Zeb2 siRNA, and IgE–FcγRI-mediated degranulation and cytokine release were determined for 3 d posttransfection. The release of β-hexosaminidase was used to measure degranulation, and TNF-α, IL-13, and CCL-4 release was used to evaluate changes in cytokine responses (Fig. 2A). Knockdown of Zeb2 decreased β-hexosaminidase release at all 3 d posttransfection with the maximum reduction of ~65 ± 7.5% (n = 7) on day 3. However, the effect of decreased Zeb2 expression on release of the three cytokines was more variable; release was enhanced at the early time points, but by day 3 it was inhibited. On all 3 d after transfection there was 90% decrease in Zeb2 protein expression (Fig. 2B). In Ag dose-response curves, the results were similar with more dramatic effects on degranulation than on cytokine release (Supplemental Fig. 1). Therefore, decrease in Zeb2 expression results in reduction in degranulation and release of cytokines.

Mast cells can also be activated by calcium ionophores and PMA, which bypasses the early steps in the FcγRI-Ag pathway. Treatment with ionomycin induces an increase in intracellular calcium, which results in degranulation. This ionomycin-induced degranulation was inhibited similar to that with Ag by the decrease in Zeb2 expression (Fig. 2C). BMMC stimulated with ionomycin together with PMA result in the release of cytokines; again, the changes in TNF-α and IL-13 release with Ag were similar to that with ionomycin–PMA in the treated cells. These results indicate that decreased Zeb2 levels have effects at late stages of the BMMC response.

To validate these results further, the FcγRI-mediated degranulation and cytokine responses were measured in BMMC treated with four single siRNAs from the original pool (Fig. 3). Syk-specific siRNA was used as a control that efficiently inhibits

FIGURE 1. Zeb2 regulates FcγRI-induced activation of NFAT and NF-κB transcription factors in mast cells. (A) MC9-NFAT-GFP or MC9-NF-κB-GFP cells were treated with individual and pooled siRNA for Zeb2 (indicated by 1 to 4 and P) or with control nontargeting siRNA. FcγRI- or LPS-induction of the transcription factors was determined by FACS analysis of GFP expression on days 1, 2, and 3 posttransfection. The results are expressed as a percentage of that in controls and are the average from three experiments. Cell lysates were analyzed by immunoblotting for expression of Zeb2 at day 1 (B), day 2 (C), and day 3 (D) in MC9-NFAT-GFP treated with control siRNA (lane C) or individual and pooled siRNAs for Zeb2 (lanes 1 to 4 and P). Anti-actin was used as loading control. Fraction of protein still expressed in cells is shown under each lane. Similar immunoblots were observed in six experiments (three each for NFAT and NF-κB).
IgE-mediated responses (22). With the Zeb2-treated cells, the largest decrease in β-hexosaminidase release was at day 2 and 3 with all four siRNAs, with Zeb2-3 being the most effective (Fig. 3A). There was less inhibition of degranulation when the cells were tested 24 or 48 h after transfection. In the cytokine response, there was a decrease in FceRI-induced release of TNF-α, IL-13, and CCL-4 by day 3 with Zeb2-3, whereas the other single siRNAs had much less or little effect. There was enhancement or minimal inhibition when the cells were tested at earlier time points. The changes in CCL-4 release were minimal. On immunoblotting, changes in Zeb2 expression paralleled the phenotype changes; the greatest decrease in protein levels was with Zeb2-3 with more modest effects with Zeb2-4 or Zeb2-1 and less effects with Zeb2-2 (Fig. 3B–D). Notably, although the Zeb2 protein levels were low on day 1 posttransfection, the decrease in responses was progressively stronger over the 3 d with the most inhibition observed on day 3. The effectiveness of the different single siRNAs in decreasing protein expression and cellular responses validate a role of Zeb2 in regulating FceRI-mediated responses.

Knockdown of Zeb2 decreased FceRI-induced phosphorylations

Protein phosphorylation was then examined to understand the mechanism by which decrease in Zeb2 impaired FceRI-mediated degranulation and cytokine release (Fig. 4). Activation of Syk is critical for signaling in mast cells; a marker for this is the phosphorylation of the two adjacent tyrosines in the activation loop (24–28). There was some minor decrease in this phosphorylation of the activation loop tyrosines of Syk in the Zeb2 siRNA-treated cells (Fig. 4B). Downstream of Syk activation is the phosphorylation of several proteins including LAT and PLCγ. There was a dramatic decrease in phosphorylation of LAT-Tyr191, which is partly responsible for phosphoinositide 3 kinase/phospholipase translocation to the membrane (Fig. 4B). Activation of PLCγ is a crucial step in the cellular response pathway; there was decreased phosphorylation of PLCγ2 on Tyr1217 especially at the early time points after Ag stimulation indicating reduced activation. The activation of PLCγ is regulated, at least in part, by the activation of PI3K (29, 30). The phosphorylation of the serine/threonine protein kinase Akt, a PI3K substrate, was used as a

FIGURE 2. Zeb2 regulates FceRI- and ionophore-mediated cellular responses in primary mast cells. (A) Mouse BMMC were transfected with control nontargeting siRNA or the pool of four siRNAs specific for Zeb2. The Ag-induced release of β-hexosaminidase, TNF-α, IL-13, and CCL-4 were determined at days 1, 2, and 3 posttransfection (n = 2). The changes in the cellular responses are presented as a percentage of that in control-transfected cells. (B) Cell lysates from BMMC transfected with nontargeting siRNA (“C”) or the Zeb2-specific (“T”) were analyzed by immunoblotting with Abs to Zeb2 or actin. Fraction of protein still expressed in cells is shown under each lane. (C) Comparison of the IgE-Ag and ionomycin–PMA responses in siRNA-treated cells; changes in cellular responses are presented as a percentage of that in control transfected cells. *p < 0.1, **p < 0.01, ***p < 0.005.

FIGURE 3. Cellular response of BMMC treated with single and pooled siRNAs targeting Zeb2. (A) BMMC were treated with individual and pooled siRNAs specific for Zeb2 (indicated by 1 to 4 and P) or with control nontargeting siRNA. Release of β-hexosaminidase, TNF-α, IL-13, and CCL-4 was determined at days 1, 2, and 3 posttransfection. The results are expressed as a percentage of that in controls. Cell lysates were analyzed by immunoblotting for the expression of Zeb2 at day 1 (B), day 2 (C), and day 3 (D).
measure of the activation of the PI3K pathway. There was up to 80% decrease in the phosphorylation of Akt Ser473 after the Zeb2-3-specific siRNA treatment compared with the control non-targeting siRNA. This indicated a less active PI3K pathway after Zeb2 knockdown. Later events in the pathway of cell activation include the phosphorylation of MAPKs. Zeb2 knockdown decreased phosphorylation of both ERK and p38; these changes were more striking in p38 (Fig. 4A, Supplemental Fig. 2A). The results for all these phosphorylations were similar at days 2 and 3 posttransfection.

The decreased response in the Zeb2 siRNA-treated cells in degranulation and cytokine release with ionophore/PMA suggested that there were defects in the late steps of these pathways. However, these phosphorylation results indicate that there are multiple defects in signaling including early defects such as those that involve the PI3K, PLCγ, and LAT besides late events such as those in the MAPKs, which together can explain the changes in degranulation and release of cytokines. Zeb2 regulated the expression level of several proteins in mast cells

During these phosphorylation studies, it was noted that there was decreased expression of PLCγ2 in the Zeb2 siRNA-transfected cells (Fig. 4A). These observations raised the possibility that Zeb2 could, as a transcription repressor, regulate the expression of other signaling molecules. To begin to understand how Zeb2 controls signaling in mast cells, we investigated whether its knockdown induced changes in cell-surface FceRI expression. Compared to the controls or cells transfected with siRNA for Syk, there was decreased FceRI expression in BMMC treated with the most active single or the pool of Zeb2 siRNA (Fig. 5). The greatest decrease was with Zeb2-3 and the least with Zeb2-2, similar to the effect of these siRNAs on Zeb2 protein expression and cellular responses. Lower cell-surface FceRI expression would result in decreased Ag-induced clustering, and thus diminished cellular response. However, increasing the concentration of Ag did not reverse the reduced cellular response in Zeb2 siRNA-treated cells. These results suggest that the decreased receptor levels had a minor role in changes in degranulation or cytokine release.

As Zeb2 regulates critical cellular functions such as proliferation, differentiation, and survival (18, 31, 32), we investigated whether there were changes in cell cycle progression in the treated cells (Fig. 6). FACS analysis was used to determine the percentages of BMMC undergoing each of the three phases of the cell cycle, G1, S, and G2/M, after treatment with the most efficient individual siRNA (Zeb2-3) and the pooled mix (Zeb2-P). Treatment with Zeb2 siRNA resulted in alteration in cell cycle progression with the more easily detectable changes in the S or G2/M phase. In the treated cells, there was significant decrease in the number of cells in S phase, which was most prominent on day 2 (Fig. 6B). There was a similar reduction in treated cells in the G2/M phase (Fig. 6C). There were minor increases in cells in the G1
phase, however this was statistically significant only at one time point (Fig. 6A). These results indicate that Zeb2 is a positive regulator of G1–S and S–G2/M transitions in mast cells, but even in the treated cells most were still in the G1 phase. Therefore, there was only a minor shift in the cell cycle, which could not explain the dramatic change in cell responses.

Previous reports have shown that Zeb2 regulates the protein level of the adhesion molecule E-cadherin and the transcription factor Snai1 (33, 34). At 3 d after Zeb2 siRNA treatment, there was a decrease in the level of these two proteins (Fig. 7). We then tested for proteins involved in FcεRI-mediated signaling (Fig. 7A and Supplemental Fig. 3 show representative experiments, and Fig. 7B is the expression level of these proteins from multiple experiments). There were decreases in the level of several proteins that are required for signaling; these included the β subunit of FcεRI, the two isoforms of PLCγ, and Gab2, which are all important for FcεRI signaling. However, there were minimal or no changes in the level of some of the other proteins such as Btk and the p85 subunit of PI3K (Fig. 7). Among the inhibitory regulators, decreased expression of which would enhance signaling, there was reduction in the levels of SHIP-1 and PTEN. There was much less change in the expression of these proteins at earlier time points after transfection (Supplemental Fig. 4). As noted in Fig. 4, Zeb2 siRNA had no effect on the expression level of Syk, LAT, Akt, and the MAPKs. Therefore, in mast cells the knockdown of Zeb2 resulted in reduced expression of FcεRI and of several signaling molecules with decreased signal transduction as measured by reduced degranulation and release of cytokines indicating effects on early and late mast cell responses (Fig. 8).

**Discussion**

Zeb2 interacts with Smad proteins, which are involved in TGF-β signaling; a pathway important for cell growth and differentiation. The current experiments suggest a role for Zeb2 in mast cell differentiation and development, as knockdown of Zeb2 decreased the rate of cell cycle progression. This also reduced the expression of FcεRI, as well as that of several other downstream intracellular proteins. The current experiments suggest that Zeb2 is a positive regulator of G1–S and S–G2/M transitions in mast cells, but even in the treated cells most were still in the G1 phase. Therefore, there was only a minor shift in the cell cycle, which could not explain the dramatic change in cell responses.

**FIGURE 5.** Knockdown of Zeb2 decreased cell-surface FcεRI expression. The expression of FcεRI was determined in BMMC treated with nontargeting siRNA or the single and the pooled siRNAs specific for Zeb or Syk. The change in FcεRI expression was calculated from the mean fluorescence intensity as determined by FACS (n = 4) using the nontargeting siRNA-transfected cells as controls. *p < 0.1, **p < 0.01.

**FIGURE 6.** Decreased Zeb2 expression alters cell cycle progression. BMMC were transfected with nontargeting siRNA (“Control”) or the most efficient single siRNA (Zeb2-3) and the pooled siRNA specific for Zeb2 (“Target”). Changes in the cell cycle were monitored by FACS. Percent of cells in G1 phase (A), S phase (B), and G2/M phase (C) at days 1, 2, and 3 posttransfection is presented (n = 4). *p < 0.1, **p < 0.01, ***p < 0.005.

**FIGURE 7.** Zeb2 regulates the expression of several proteins in mast cells. BMMC were treated with nontargeting siRNA (“Control”) or the single siRNA specific for Zeb2 (“Zeb2-3”) and the pooled siRNAs (“Zeb2-P”). (A) Lysates from day 3 posttransfection were analyzed by immunoblotting with the indicated Abs. Similar results were in four to seven different experiments. (B) Densitometry data of the changes induced by decreased expression of Zeb2 as a percentage of controls; data are with both Zeb2-3 and Zeb2-pool siRNA from 6 to 15 determinations (mean ± SD). *p < 0.1, **p < 0.01, ***p < 0.005.
transducers. The decreased expression of Zeb2 affected multiple sites in the FceRI-induced signaling pathway with decreased phosphorylation of proteins at multiple sites in the pathway. This results in a dramatic decrease in degranulation and reduced cytokine release.

The Zeb2 protein has two zinc finger clusters each of which binds to the CACCT(G) sequence located in gene promoters (17, 18). Zeb2 functions as a repressor of transcription; this action may also depend on the binding of a corepressor, CtBP. A decrease in Zeb2 levels induced by siRNA treatment should therefore result in an increase in the level of a protein if the promoter region of its gene has a binding site for Zeb2. As we observed a decrease in expression of these signaling molecules, binding of Zeb2 to their promoter region cannot therefore be the mechanism for their decrease.

MicroRNAs (miRNAs) are endogenous, noncoding, ~22 nucleotide short RNAs that pair to microRNA response elements (MREs) in the mRNA of protein-coding genes and result in their suppression by either inhibiting translation and/or by inducing degradation of the target mRNAs (35). Most mRNAs have multiple MREs and therefore can be regulated by several miRNAs, and miRNAs are known to target many mRNA transcripts (36, 37). It was recently proposed that competitive endogenous RNAs sequester miRNAs to regulate mRNA transcripts containing common miRNA recognition elements (38). Notably, Zeb2 transcript was identified in a study to find such competitive inhibitor decoys for PTEN, and it was shown that siRNA-induced knockdown of Zeb2 results in a decrease in the expression of PTEN protein levels in a miRNA-dependent, protein coding-independent manner (39). Interrogation of the TargetScanMouse site for predicted miRNA targets (www.targetscan.org/mmu_50) showed that the proteins whose level changed in the current experiments have multiple targets for the same miRNA as Zeb2. Therefore, the disruption of the Zeb2 mRNA by treatment with siRNA would free these miRNAs to bind other mRNA leading to the repression of that message with a decrease in protein expression levels (35).

Zeb2 suppress the epithelial phenotype by regulating transcription especially of genes that are important for epithelial mesenchymal transitions. Zeb2 decreases the expression of E-cadherin, which is a component of the adherens junctions in epithelial cells and upregulates genes normally expressed in mesenchymal cells. It is also essential for hematopoietic cell differentiation (31). Zeb2 overexpression correlates with aggressive phenotype in various cancers where it may be involved in malignant transformation. In BMMC, we found that when Zeb2 expression was reduced, there was also decrease in the level of the transcription factor Snai1, which also regulates epithelial mesenchymal transitions. The decreased levels of both Zeb2 and Snai1 would result in changes in the expression of proteins that reflect less the mesenchymal phenotype and cause the BMMC to revert to a less differentiated phenotype.

Zeb2 knockdown resulted in decreased IgE–FceRI–induced signal transduction most likely due to changes in the expression of several signaling molecules. The response of mast cells is the result of a sequence of biochemical events that begins with the clustering of the IgE–FceRI complexes by a multivalent Ag. The FceRI on the cell surface binds IgE with high affinity through the \( \alpha \) subunit and transduces intracellular signals through the \( \beta_2 \) subunits. Therefore, strength of the signal depends on the number of surface receptors, the amount of IgE bound to the \( \alpha \) subunit, and the amount of Ag. We found that with low Zeb2 expression, there was decreased cell-surface expression of the receptor with a more dramatic reduction of the \( \beta \) subunit, which is important for signal transduction (Fig. 7). These changes would contribute to a decrease in the initiation step of the FceRI-induced signal transduction pathway.

The downstream progression of signaling from the receptor depends on several key molecules; among these is the protein tyrosine kinase Syk, which is essential for progression of signal toward both degranulation and cytokines synthesis (23, 26). Syk directly or indirectly controls the activity of other key substrates, such as PI3K and LAT. We found that Zeb2 knockdown induced changes in signal transduction from these molecules. Although the expressions of Syk, LAT, and the p85 subunit of PI3K were not changed, the extent of the FceRI-induced tyrosine phosphorylation and activation of these molecules was reduced. There was decreased activation of Syk as indicated by the level of phosphorylation of the activation loop tyrosines, which are important for downstream propagation of signals (27, 28). The reduction in the initiation of signals from FceRI could be the explanation for the decrease in the activation of Syk. Impaired signaling downstream of Syk was evident by the reduced phosphorylation of Tyr191 on LAT and Ser473 on Akt. Phosphorylated tyrosine sites on LAT bind Grb2, Gads/SLP-76, PLC\( \gamma \)1, and PI3K to propagate the signal further (40, 41). The reduced PI3K activation was apparent from the decreased phosphorylation of its substrate Akt, the phosphorylation of which reflects PI3K activity. There was also decreased expression of Gab2, a regulator of the PI3K pathway. Decreased Gab2 levels, combined with reduced activation of Syk, which is the major kinase that phosphorylates Gab2, could also contribute to the decreased PI3K activation (42). The decrease in PI3K activity would depress the membrane recruitment and activation of Btk and PLC\( \gamma \), which are essential through the generation of inositol 1,4,5-triphosphate and diacylglycerol for the increase in intracellular calcium and activation of protein kinase C (6, 43, 44). Further contributing to the decreased activation of this pathway was the reduced levels of both Btk and PLC\( \gamma \)2 in the Zeb2 siRNA-treated cells. Zeb2 knockdown therefore results in reduction in the early signaling events, which precede activation of protein kinase C, LAT, MAPks, NEAT, and NF-\( \kappa B \) (23, 45).

FceRI-mediated activation of the NFAT and NF-\( \kappa B \) transcription factors is pivotal for initiation of transduction of cytokines genes. Activation of these transcription factors depends on upstream signals that were clearly inhibited in the Zeb2 siRNA-treated cells. NFAT controls diverse cellular processes and transcription of ILs (IL-2, IL-3, IL-4, IL-5, IL-13), GM-CSF, IFN (IFN-\( \gamma \)), and TNF-\( \alpha \) (10, 46). NFAT also regulates the induction of other transcription factors and is also crucial for the FceRI activation-induced survival of mast cells (47, 48). Similarly NF-\( \kappa B \) regulates the transcription of proinflammatory cytokines (12). Therefore, the reduced FceRI-induced activation of NFAT and
NF-κB after Zeb2 knockdown resulted in the decreased release of cytokines. There were also changes in molecules that are known to negatively regulate signaling from FceRI. Knockdown of SHIP-1 and PTEN is known to enhance signaling by the PI3K pathway leading to increased degranulation and cytokine release. There was modest to considerable decrease in these two proteins in the Zeb2 siRNA-treated cells although there was inhibition of the mast cell responses. The reduced cellular responses indicate that the decrease in molecules essential for signaling are on balance more important than the changes in these two regulatory proteins.

In Zeb2 siRNA-treated cells, there was rapid reduction in the expression of the Zeb2 protein that was apparent within 24 h, whereas the phenotypic changes in degranulation and cytokine release were more prominent at later time points. In fact, at early time points after transfection, there was enhancement in the release of cytokines. There was also much greater inhibition of degranulation than cytokine release by reduced Zeb2 expression. As Zeb2 is known to regulate the transcription of other proteins, the results suggest that the phenotypic effects are due to changes in the expression of these other molecules (31, 49, 50). The difference in the extent of inhibition in degranulation compared with the cytokine response with decreased Zeb2 levels is likely due to different rates at which there are changes in the expression of signaling proteins.

Notably, when Zeb2 expression was decreased, the LPS-mediated NF-κB response was enhanced although FceRI-induced NF-κB activation was inhibited (Fig. 1). Therefore, not all the effects of Zeb2 siRNA treatment are inhibition of signaling pathways. The mechanism of this enhancement was not a focus of this study, but it is possible that it was due to changes in the PI3K pathway, which is known to play a role in signaling from TLRs (51–53). Alternatively, the enhanced NF-κB response may be related to the interaction of Zeb2 with Smad proteins, as the LPS-induced cytokine response is enhanced in mice that are deficient in Smad3 (54).

Stimulated mast cells release a wide range of cytokines and chemokines, many with inflammatory potential. Besides their direct effects, these products also recruit and activate other cells such as neutrophils and lymphocytes that influence the intensity of the inflammatory response and also regulate immune and autoimmune conditions (55). As Zeb2 knockdown results in a decrease in the release of these mediators, it is possible that control of Zeb2 may be another approach to regulate the inflammatory response. In summary, these studies show that Zeb2 regulates the expression of several molecules in mast cells that are involved in cell division and signal transduction. As a regulator of mast cell responses, Zeb2 may prove a possible target for pharmacological agents that would control the inflammatory response.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. Changes in the antigen-dose response of BMMC with decreased Zeb2 expression. BMMC were transfected with control non-targeting siRNA or the pool of four siRNA specific for Zeb2. The antigen-induced release of β-hexosaminidase, TNF-α, IL-13 and CCL-4 from both the scrambled siRNA (control) and Zeb2 treated cells were determined at day 1, 2 and 3 post-transfection. The maximum response in the control scrambled siRNA treated cells was set at 100% for each day and changes in the cellular responses with Zeb2 siRNA are presented as a percentage of that in control cells. One representative set of results is presented, similar results were observed in three experiments.
Supplemental Figure 2. Knockdown of Zeb2 decreases FcεRI-induced phosphorylation in mast cells. BMMC transfected with control scrambled siRNA or the pool of Zeb2 siRNA (“Treated”) were sensitized with IgE and then activated with antigen for the indicated times (minutes). Cell lysates were immunoblotted with the anti-phospho-antibodies, then stripped and probed with the specific antibodies. Densitometry data were corrected for differences in loading except for PLCγ2 where there was decreased expressed of this protein after treatment with siRNA specific for Zeb2. The different proteins as indicated are shown in (A) and (B). The results here are from day 3 (A) and day 2 (B) post-transfection. Similar results were in 3 separate experiments.
Supplemental Figure 3. *Zeb2* regulates the expression of several proteins in mast cells. BMMC were treated with non-targeting control siRNA (“C”) or the single and pooled siRNA specific for *Zeb2* (indicated by 1 to 4 and P). Lysates from day 3 post-transfection were analyzed by immunoblotting with the indicated antibodies.
Supplemental Figure 4. Time course of changes in the expression of several proteins in mast cells after Zeb2 siRNA transfection. BMMC were treated with non-targeting control siRNA, the single siRNA specific for Zeb2 (Zeb2-3) or the pooled siRNA’s (Zeb2-P). Lysates from each day post-transfection were analyzed by immunoblotting with the indicated antibodies; the data with siRNA for Zeb2 (Zeb2-3) and (Zeb2-P) were compared to cells treated with the controls. N=2-4 on day1; 6-15 for Day 2 and Day 3. The expression level is presented as a percentage (mean ± S.D.) of that in control siRNA treated cells.