 DOI: 10.1002/eji.201343838 Eur. J. Immunol. 2014. 44: 204–214

European Journal of Immunology

Histamine synthesis is required for granule maturation in murine mast cells

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Mast cells are the major sources of histamine, which is released in response to immunological stimulations. The synthesis of histamine is catalyzed by histidine decarboxylase (HDC). Previous studies have shown that *Hdc*$^{-/-}$ mast cells exhibit aberrant granule morphology with severely decreased granule content. Here, we investigated whether the histamine synthesized in mast cells regulates the granule maturation of murine mast cells. Several genes, including those encoding granule proteases and enzymes involved in heparin biosynthesis, were downregulated in *Hdc*$^{-/-}$ peritoneal mast cells. Impaired granule maturation was also found in *Hdc*$^{-/-}$ BM-derived cultured mast cells when they were cocultured with fibroblasts in the presence of c-kit ligand. Exogenous application of histamine and several H_4 receptor agonists restored the granule maturation of *Hdc*$^{-/-}$ cultured mast cells. However, the maturation of granules was largely normal in *Hrh4*$^{-/-}$ peritoneal mast cells. Depletion of cellular histamine with tetrabenazine, an inhibitor of vesicular monoamine transporter-2, did not affect granule maturation. In vivo experiments with mast cell deficient Kit^{W}/Kit$^{W\text{-}v}$ mice indicated that the expression of the *Hdc* gene in mast cells is required for granule maturation. These results suggest that histamine promotes granule maturation in mast cells and acts as an proinflammatory mediator.

Keywords: Cell differentiation · Histamine · Inflammation · Mast cell

See accompanying Commentary by Hallgren and Gurish

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Introduction

Histamine plays a critical role in various physiological responses, such as inflammation, gastric acid secretion, neurotransmission, and immune modulation, which are mediated by its specific receptors, the H_1, H_2, H_3, and H_4 subtypes [1–4]. Mast cells are the primary sources of tissue histamine and it is liberated from the cytoplasmic granules upon various stimuli including IgE-dependent Ag stimulation [5]. Histamine synthesis is mediated by L-histidine decarboxylase (HDC), which is initially synthesized as a 74 kDa precursor and then is in posttranslationally modified [6]. The 74 kDa HDC is mainly localized in the cytosol, while the processed 53 kDa form is distributed to the granules in mast cells and activated neutrophils [6, 7]. In a mouse mastocytoma P-815 cells, caspase-9-mediated cleavage results in enzymatic activation of HDC [8].

We have explored novel functions of histamine using a mouse strain lacking the *Hdc* gene. In the first report of *Hdc*$^{-/-}$ mice, we noticed that peritoneal mast cells (pMCs) and skin mast cells exhibited an aberrant morphology with severely decreased granule contents [9]. Although several studies indicated that mast cells express specific histamine receptors, it remains unknown how histamine affects the nature of mast cells. On the other hand, positive modulators for growth and differentiation of mast cells, such as IL-3 and c-kit ligand (Kitl), were found to induce a transient increase in histamine synthesis [10, 11], raising a possibility that histamine is involved in the process of mast cell maturation. We hypothesized that newly synthesized histamine may play a critical role in granule maturation of mast cells in an autocrine fashion. We, here, took two different approaches to investigate the mechanism underlying the histamine-mediated promotion of granule maturation of mast cells; an in vitro approach using a mast cell fibroblast coculture model and an in vivo one using the mast cell reconstitution system with mast cell deficient *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice. This study proposes a novel function of histamine in mast cell biology.

Results

Characterization of the pMCs in *Hdc*$^{-/-}$ mice

We previously reported that the cutaneous and pMCs of *Hdc*$^{-/-}$ mice demonstrate aberrant granules with severely decreased contents [9]. pMCs in *Hdc*$^{-/-}$ mice were poorly-stained with Safranin-O and acidic Toluidine blue, compared with those in WT mice (Fig. 1A). We found no significant differences in the number of total peritoneal cells and in the percentage of mast cells based on the May–Grünwald–Giemsa staining between WT and *Hdc*$^{-/-}$ mice (Fig. 1B and data not shown). Histamine was not detectable in *Hdc*$^{-/-}$ peritoneal cells (Fig. 1C). Enzymatic activities of granule proteases, such as chymase, tryptase, and mast cell carboxypeptidase A (MC-CPA), were found drastically decreased in *Hdc*$^{-/-}$ peritoneal cells (Fig. 1D–F); the decrease is compatible with the electron-lucent appearance of granules of

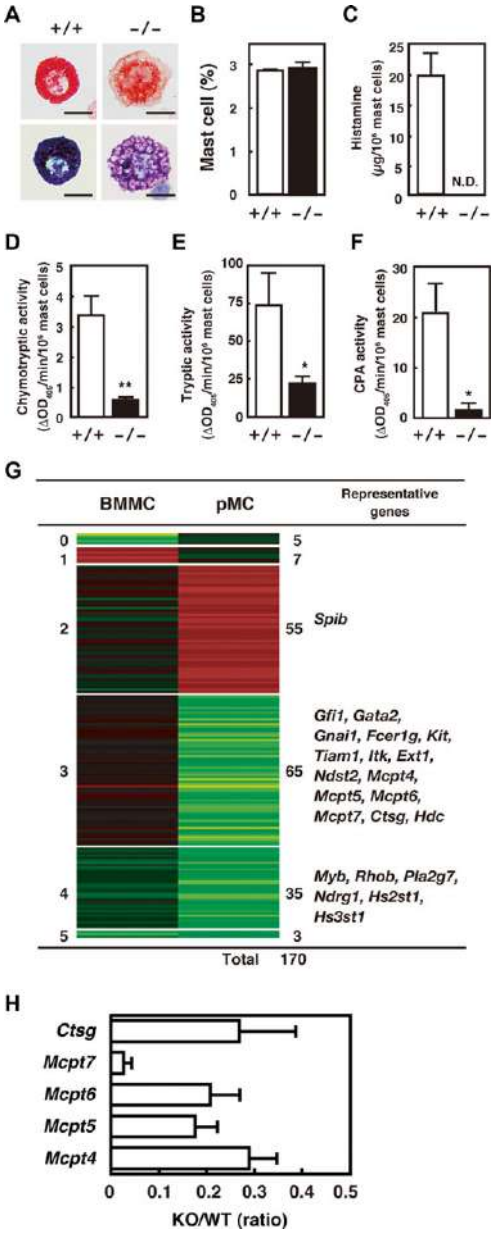

Figure 1. Characterization of *Hdc*$^{-/-}$ peritoneal mast cells (pMCs). (A) Peritoneal cells collected from WT (+/+) and *Hdc*$^{-/-}$ (−/−) mice were stained with Safranin-O (upper panels) or with acidic Toluidine blue (lower panels); bar = 10 μm. (B) Percentages of mast cells of WT (white) and *Hdc*$^{-/-}$ (black) mice are determined based on the May–Grünwald–Giemsa staining. (C) Histamine contents of peritoneal cells of WT (open column) and *Hdc*$^{-/-}$ mice were presented. N.D., not detectable. (D–F) Enzymatic activities of (D) chymase, (E) tryptase, and (F) MC-CPA in pMCs of WT (open columns) and *Hdc*$^{-/-}$ (closed columns) mice were measured. (G) Gene expression profiles in BMMCs or in pMCs were compared between WT and *Hdc*$^{-/-}$ mice. Downregulated genes in the *Hdc*$^{-/-}$ mast cells (BMMCs or pMCs), were represented by green columns, whereas upregulated genes were by red columns. The number of the genes classified into each category is indicated. This is a representative of three independent experiments performed. (H) Expression of granule protease genes, such as *Mcpt4*, *Mcpt5*, *Mcpt6*, *Mcpt7*, and *Ctsg*, in the purified pMCs was compared through quantitative RT-PCR analyses based on the expression of *Gapdh*. (A–H) The values are represented as the means + SEM and are representative of (A–F) 5 and (H) 3 independent experiments. The values of $^{*}p < 0.05$ and $^{**}p < 0.01$ are regarded as significant by Student's *t*-test.

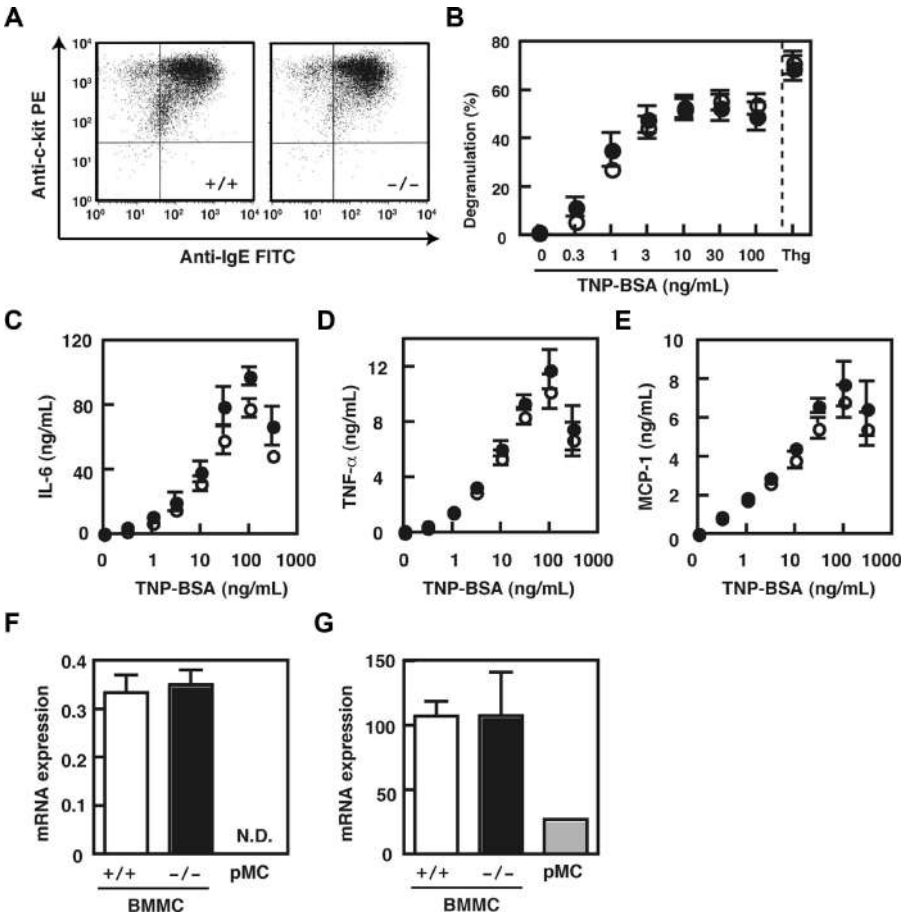

Figure 2. Characterization of *Hdc*$^{-/-}$ BMMCs. (A) Surface expression levels of c-kit and FcεRI in WT (+/+) and *Hdc*$^{-/-}$ (−/−) BMMCs were measured by flow cytometry. This is a representative of three experiments with similar results. (B) BMMCs prepared from WT (white circles) or *Hdc*$^{-/-}$ (black circles) mice were sensitized with anti-TNP IgE (clone IgE-3, 1 μg/mL) for 24 h and then stimulated with the indicated concentrations of TNP-BSA for 30 min. Degranulation of mast cells were determined by measuring β-hexosaminidase activity. Thapsigargin (Thg, 300 nM) was used as a positive control. (C–E) BMMCs sensitized with IgE as described above were stimulated with the indicated concentrations of TNP-BSA for 3 h. The levels of various cytokine (C, IL-6; D, TNF-α; and E, MCP-1) released into the medium were measured. (F and G) Expression levels of H_2 (F) and H_4 (G) receptors in BMMCs prepared from WT (+/+) and *Hdc*$^{-/-}$ (−/−) mice and pMCs from WT mice (pMC) were determined by quantitative RT-PCR analyses. The expression levels were normalized based on those of GAPDH and presented as the relative expression levels (H_2 receptor, the expression level in mouse stomach = 1.0, and H_4 receptor, the expression level in mouse spleen = 1.0). (A–G) The values are represented as the means + SEM and are representative of three independent experiments. N.D., not detectable.

Hdc$^{-/-}$ mast cells, since neutral proteases account for a large part of constituents of mast cell granules.

We then compared gene expression profiles in IL-3-dependent BM-derived cultured mast cells (BMMCs), and those in mature mast cells purified from the peritoneal cavity between WT and *Hdc*$^{-/-}$ mice (Fig. 1G); the former cells have been used as one of the common culture models of mast cells. The expression levels of 170 genes were changed greater than twofold between WT and *Hdc*$^{-/-}$ mast cells. Among them, the expression levels of 158 genes were changed between WT and *Hdc*$^{-/-}$ pMCs, whereas the expression levels of only 15 genes were changed between WT and *Hdc*$^{-/-}$ BMMCs. The genes encoding granule proteases, such as *Mcpt4*, *5*, *6*, *7*, and cathepsin G (*Ctsg*), and enzymes involved in heparin biosynthesis, such as *Ext1*, *Ndst2*, *Hs2st1*, and *Hs3st1*, were downregulated in *Hdc*$^{-/-}$ pMCs, in good agreement with impaired granule maturation observed in *Hdc*$^{-/-}$ pMCs. Because we could not detect any significant changes in mRNA expression of several granule proteases with semiquantitative RT-PCR analysis previously [9], quantitative RT-PCR analysis was performed in this study. Expression of granule protease genes, such as *Mcpt4*, *5*, *6*, *7*, and *Ctsg*, was found to be decreased (<30% of the WT) in *Hdc*$^{-/-}$ pMCs (Fig. 1H). These findings raised a possibility that histamine affects the function of master transcription factors that regulates the terminal differentiation of mast cells. Impaired expression of hematopoietic transcription factors, such as *Gfi1*, *Gata2*, and *Myb*, in *Hdc*$^{-/-}$ pMCs, makes it possible that they are involved in the process of granule maturation.

Characterization of *Hdc*$^{-/-}$ BMMCs

As described above, comparison of gene expression profiles (Fig. 1G) revealed that the absence of HDC has a lesser impact on BMMCs than on pMCs. Surface expression levels of c-kit and FcεRI were similar in WT and *Hdc*$^{-/-}$ BMMCs (Fig. 2A). The levels of degranulation and production of inflammatory cytokines, such as IL-6, TNF-α, and MCP-1, of *Hdc*$^{-/-}$ BMMCs upon IgE-mediated Ag stimulation were also comparable to those of WT BMMCs (Fig. 2B–E). Quantitative RT-PCR analyses revealed that BMMCs expressed both histamine H_2 and H_4 receptor subtypes at transcriptional levels whereas peritoneal mast cells did only the H_4 subtype (Fig. 2F and G). No significant changes were detected in mRNA levels of the H_2 and H_4 subtypes between WT and *Hdc*$^{-/-}$ BMMCs. H_1 and H_3 receptor mRNAs were under the detection levels in both BMMCs and peritoneal mast cells (data not shown).

Impaired granule maturation of *Hdc*$^{-/-}$ CTMC like cultured mast cells

We have recently established a culture model that can reproduce at least in part the process of maturation of connective tissue-type

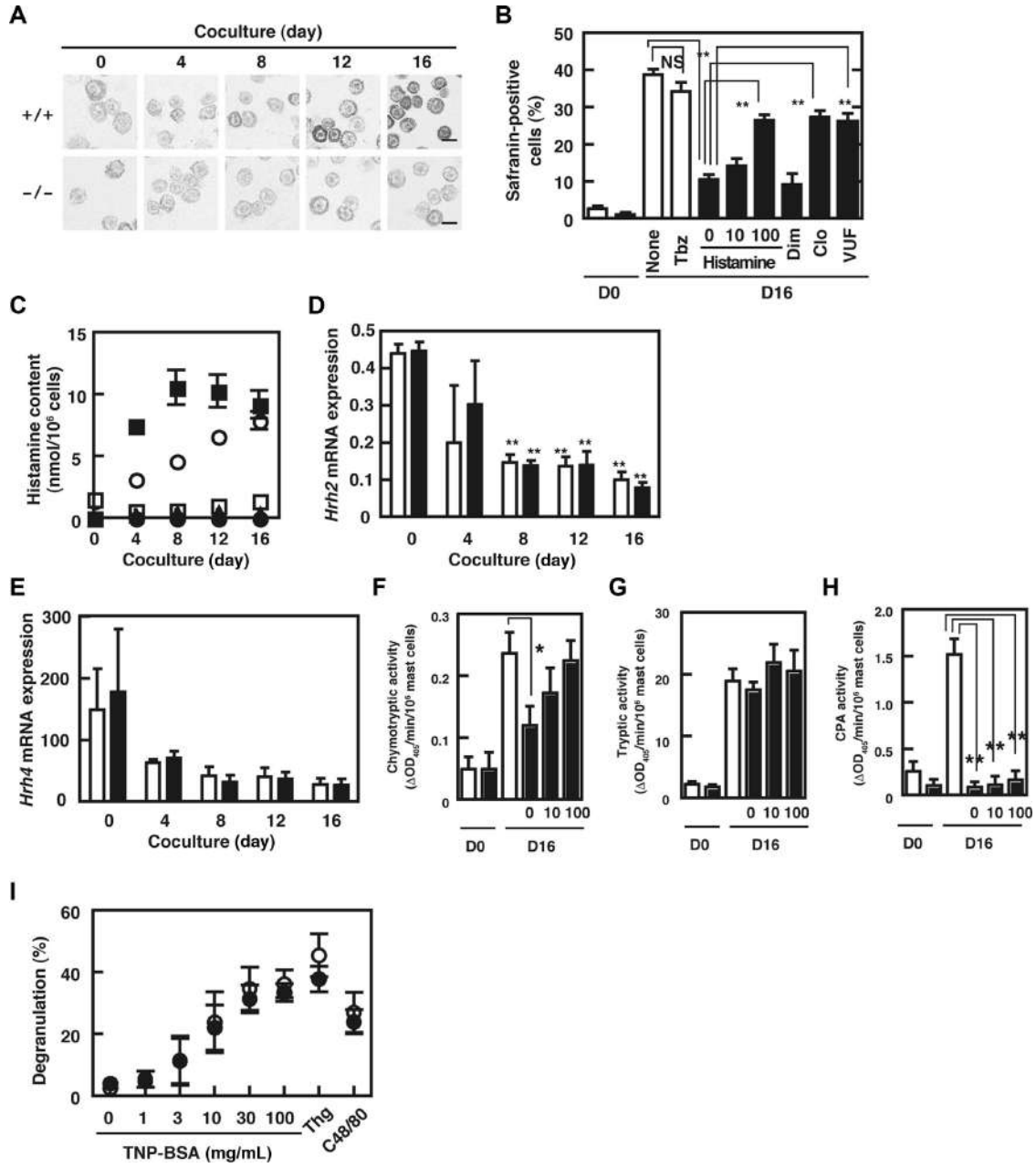

Figure 3. Restoration of granule maturation in *Hdc*$^{-/-}$ cultured mast cells by exogenous histamine. (A) BMMCs prepared from WT (+/+) and *Hdc*$^{-/-}$ (−/−) mice were cocultured with mitomycin C-treated Swiss 3T3 fibroblasts in the presence of 30 ng/mL Kitl. The cells were visualized by Alcian blue/Safranin-O staining at indicated time points; bar = 10 μm. (B) WT BMMCs (white columns) were cocultured as described above in the absence (none) or presence of 3 μM tetrabenazine (Tbz) for 16 days, while *Hdc*$^{-/-}$ BMMCs (black columns) were cocultured in the absence or presence of the indicated concentrations of histamine (histamine, 0, 10, 100 μM), 3 μM dimaprit (Dim), 3 μM clobenpropit (Clo), or 10 μM VUF8430 (VUF). The number of Safranin-positive cells were counted at day 0 (D0) and day 16 (D16). (C) WT BMMCs were cocultured as described above in the absence (white circles) or presence (white squares) of 3 μM tetrabenazine. *Hdc*$^{-/-}$ BMMCs were cocultured in the absence (black circles) or presence of histamine (10 μM, black triangles, and 100 μM, black squares). (D and E) Expression levels of H_2 (D) and H_4 (E) receptor mRNAs in cocultured BMMCs prepared from WT and *Hdc*$^{-/-}$ mice were determined by quantitative RT-PCR analyses. The expression levels were normalized based on those of GAPDH and represented as the relative expression levels (H_2 receptor, the expression level in mouse stomach = 1.0, and H_4 receptor, the expression level in mouse spleen = 1.0). (F–H) WT and *Hdc*$^{-/-}$ BMMCs were cocultured as described above in the absence or presence of histamine (10 μM, and 100 μM). Granule protease activities (F, chymotryptic activity; G, tryptic activity; and H, CPA activity) at day 0 (D0) and day 16 (D16) were measured. (I) The cocultured cells (day 16) prepared from WT (white circles) or *Hdc*$^{-/-}$ (black circles) BMMCs were sensitized with anti-TNP IgE (clone IgE-3, 1 μg/mL) for 6 h and then stimulated with the indicated concentrations of TNP-BSA for 30 min. Degranulation of mast cells was determined by measuring β-hexosaminidase activity. Thapsigargin (Thg, 300 nM) and compound 48/80 (C48/80, 10 μg/mL) were used as positive controls. The values are presented as the means ± SEM and are representative of (B, C, F–H) 5 and (D, E, I) three independent experiments. The values of $^{*}p < 0.05$ and $^{**}p < 0.01$ are regarded as significant by one-way ANOVA followed by Dunnett multiple comparison test (D–H) or Tukey–Kramer multiple comparison test (B).

mast cells (CTMCs); in this model, BMMCs are cocultured with mitomycin C-treated Swiss 3T3 fibroblasts in the presence of Kitl [12]. Induction of HDC during the coculture period raised a possibility that transiently induced histamine synthesis results in granule maturation of mast cells. The enzymatic activity of HDC was transiently but significantly increased in the early phase (day 0, 3.37 ± 0.646, and day 4, 20.7 ± 1.68 pmol/min/mg protein, $n = 5$). The number of Safranin-positive cells was increased during the coculture period in WT cells. However, such an increase was significantly impaired in *Hdc*$^{-/-}$ cells (Fig. 3A and B). Exogenously added histamine significantly augmented the number of Safranin-positive *Hdc*$^{-/-}$ cultured mast cells (Fig. 3B). Because histamine synthesis was undetectable in Swiss 3T3 fibroblasts and FBS used in this study contained only a trace amount of histamine (<50 nM), these results indicate that mast cell derived histamine is responsible for the increase in the number of Safranin-positive cells.

We then measured histamine contents in the cocultured mast cells. Although the amount of cellular histamine in *Hdc*$^{-/-}$ mast cells was under the detection level when cultured in medium containing 10 μM histamine, the relatively higher levels of histamine, which were comparable to those in WT mast cells, were detected in the *Hdc*$^{-/-}$ mast cells cultured in the presence of 100 μM histamine (Fig. 3C). These results are reminiscent of the previous studies that showed influx of extracellular histamine through organic cation transporter(s) (OCT) with low affinity and high capacity for histamine, such as OCT-3 [13, 14]. An increase in the cytosolic histamine might result in accumulation of histamine in the granules. It is conceivable that the lack of histamine may affect the electrostatic interactions among the contents of the granules, such as those between sulfated proteoglycans and granule proteases. Previous studies demonstrated colocalization of heparin and histamine in mast cell granules and characterized the electrostatic interaction between them [15]. However, tetrabenazine, which depleted histamine storage in cultured mast cells, failed to suppress the increase in the number of Safranin-positive cells during the coculture period, indicating that storage of histamine in granules is not essential for their maturation (Fig. 3B and C).

We then explored another possibility that histamine enhances granule maturation through acting on the surface receptors. The increase in the number of Safranin-positive cells induced by histamine was mimicked by clobenpropit, which is known to act as both an H_3 antagonist and an H_4 agonist, and by VUF8430, which is an H_3/H_4 dual agonist, but not in the presence of dimaprit, an H_2/H_4 dual agonist (Fig. 3B). Since BMMCs were found to express H_2 and H_4 receptors (Fig. 2F and G), we investigated the time course of mRNA expression of these receptors during the coculture period and found that expression levels of these receptors in *Hdc*$^{-/-}$ cultured mast cells were not significantly different from those in the WT cells (Fig. 3D and E). The different effects among the various H_4 agonists on the granule staining suggests the involvement of the H_2 receptor in histamine-mediated granule maturation in *Hdc*$^{-/-}$ mast cells [16–18]. However, no significant increase in cAMP levels were detected in BMMCs stimulated with histamine or dimaprit (data not shown), indicating that the H_2 receptor subtype plays no relevant roles.

Induction of chymotryptic activity during the coculture period was also impaired in *Hdc*$^{-/-}$ mast cells, while exogenously added histamine significantly restored the induction (Fig. 3F). In contrast to the impaired expression of tryptase in *Hdc*$^{-/-}$ pMCs, induction of tryptic activity during the coculture period was not affected by the absence of HDC (Fig. 3G). Impaired induction in *Hdc*$^{-/-}$ mast cells was also observed in MC-CPA activity but the induction of the enzymatic activity was not restored in the presence of exogenous histamine (Fig. 3H). These results suggest that this culture model should not reflect the endogenous action of histamine on the expression of tryptase and MC-CPA.

Although Safranin-positive population and granule protease activities, such as chymase and MC-CPA, were decreased in *Hdc*$^{-/-}$ cultured mast cells, degranulation induced by IgE-mediated Ag stimulation or by secretagogues such as thapsigargin and compound 48/80, remained unchanged (Fig. 3I), indicating that the absence of HDC should not affect the FcεRI signaling pathway or degranulation machinery in mast cells. Ag-induced production of proinflammatory cytokines, such as IL-6, TNF-α, and MCP-1, was severely downregulated to the undetectable level by the coculture in both the WT and *Hdc*$^{-/-}$ mast cells (data not shown).

Characterization of the histamine H_4 receptor expressed in mast cells

We then characterized the histamine receptors expressed in BMMCs. Because the H_4 subtype evokes Ca^{2+} mobilization by coupling with a trimeric G protein, G_i [19], we first measured changes in the cytosolic Ca^{2+} concentrations in response to histamine. When treated with histamine, both WT and *Hdc*$^{-/-}$ BMMCs clearly exhibited a dose-dependent increase in cytosolic Ca^{2+} concentrations (Fig. 4A). The histamine-induced Ca^{2+} mobilization was abolished by pretreatment of BMMCs with a specific H_4 antagonist, JNJ7777120, but not with an H_1 antagonist, pyrilamine, or with an H_2 antagonist, cimetidine (Fig. 4B), indicating that the H_4 subtype is responsible for the histamine-induced Ca^{2+} mobilization in BMMCs. Histamine receptor ligands with an H_4 agonistic activity, such as clobenpropit, VUF8430, and dimaprit, all evoked detectable Ca^{2+} mobilization in *Hdc*$^{-/-}$ BMMCs (Fig. 4C).

We then compared pMCs from *Hrh4*$^{-/-}$ mice with those from WT mice. However, we unexpectedly failed to find any significant differences in granule staining and in the number of pMCs between WT and *Hrh4*$^{-/-}$ mice (Fig. 5A and B). On the other hand, the histamine content in peritoneal cells of *Hrh4*$^{-/-}$ mice was slightly but significantly lower than that of WT mice (Fig. 5C). In *Hrh4*$^{-/-}$ pMCs, MC-CPA activity was significantly decreased compared with that in WT mice, although an enzymatic activity of chymase or tryptase was not changed (Fig. 5D–F). An increase in the number of Safranin-positive cells observed during the coculture period in the presence of the H_4 agonists (Fig. 3B) raised a possibility that the H_4 subtype might be involved in promotion

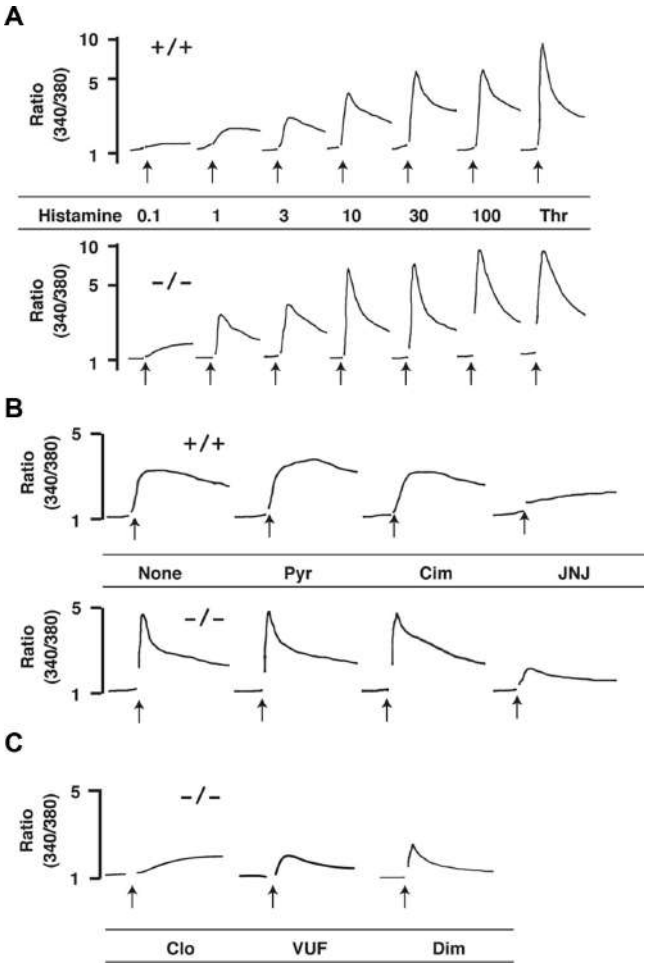

Figure 4. Ca^{2+} mobilization induced by histamine in BMMCs. (A) WT (+/+) and *Hdc*$^{-/-}$ (−/−) BMMCs were stimulated with the indicated concentrations of histamine (micromolar). Ca^{2+} mobilization induced by thrombin (Thr, 10 U/mL) was measured as a positive control. (B) WT and *Hdc*$^{-/-}$ BMMCs pretreated without (none) or with pyrilamine (Pyr, 3 μM), cimetidine (Cim, 3 μM), or JNJ7777120 (JNJ, 3 μM) for 45 min were stimulated with 3 μM histamine at the time point indicated by an arrow. (C) *Hdc*$^{-/-}$ BMMCs were stimulated with clobenpropit (Clo, 30 μM), VUF8430 (VUF, 30 μM), or dimaprit (Dim, 30 μM) at the time point indicated by an arrow.

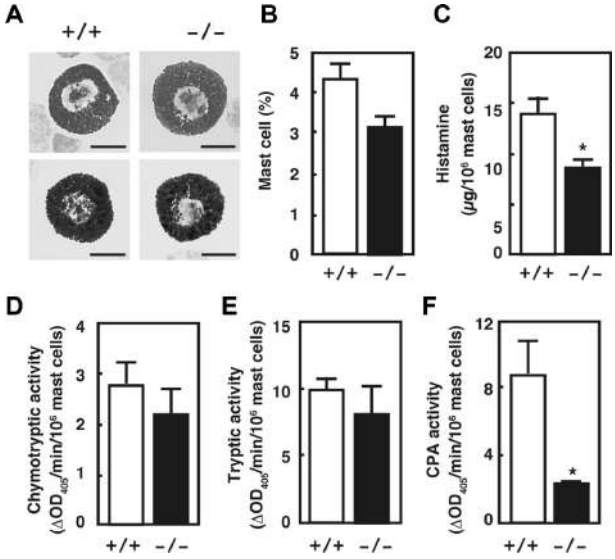

Figure 5. Characterization of *Hrh4*$^{-/-}$ pMCs. (A) Peritoneal cells collected from WT (+/+) and *Hrh4*$^{-/-}$ (−/−) mice were stained with Safranin-O (upper panels) or with acidic Toluidine blue (lower panels); bar = 10 μm. (B) Percentages of mast cells of WT (white column) and *Hdc*$^{-/-}$ (black column) mice are determined based on the May–Grünwald–Giemsa staining. (C) Histamine contents of peritoneal cells of WT and *Hrh4*$^{-/-}$ mice were measured. (D–F) Enzymatic activities of chymase, tryptase, and CPA in pMCs of WT (open columns) and *Hrh4*$^{-/-}$ (closed columns) mice were measured. (B–F) The values are represented as the means + SEM and are representative of five independent experiments. The value of $^*p < 0.05$ is regarded as significant by Student's *t*-test.

of the proteoglycan sulfation. However, unlike *Hdc*$^{-/-}$ pMCs (Fig. 1A), the granule staining was not impaired in *Hrh4*$^{-/-}$ mice (Fig. 5A), suggesting that the H_4 subtype may not be the primary target of histamine with respect to granule maturation. However, MC-CPA activity was decreased in both the *Hdc*$^{-/-}$ and *Hrh4*$^{-/-}$ pMCs as described above (Figs. 1F and 5F), indicating that the H_4 subtype is responsible, at least in part, for MC-CPA expression.

Impaired granule maturation of *Hdc*$^{-/-}$ BMMCs reconstituted in peritoneal cavity of *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice

Exogenous histamine-mediated restoration of Safranin-O staining in the coculture system strongly indicated that histamine can directly promote granule maturation of mast cells in an autocrine fashion. However, there remained a possibility that histamine produced by nonmast cells could promote the granule maturation in vivo. We therefore investigated the role of HDC expressed in mast cells in granule maturation using a reconstitution system, in which BMMCs were engrafted into the peritoneal cavity of mast cell deficient, *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice. Five weeks after the engraftment, the *Hdc*$^{-/-}$ mast cells were marginally stained, whereas the WT mast cells exhibited a similar staining pattern to that of pMCs in the mast cell sufficient control mice (Fig. 6A). The contrast in the staining property was much more pronounced 10 weeks after the engraftment. Differential counting of mast cells based on the Alcian blue/Safranin-O staining revealed an increase in the number of mast cells with abnormal staining properties in the peritoneal cavity engrafted with *Hdc*$^{-/-}$ BMMCs (Fig. 6B). The percentages of mast cells in the total peritoneal cells were not significantly different between the mice engrafted with WT and *Hdc*$^{-/-}$ BMMCs, in which no differences in the number of total cells were observed in the peritoneal cavity (Fig. 6C and data not shown), indicating that HDC is not implicated in colonization of the engrafted mast cells. The engraftment of WT BMMCs restored the histamine content in the peritoneal cells, whereas that of *Hdc*$^{-/-}$ BMMCs did not (Fig. 6D). Enzymatic activities of granule proteases, such as chymase, tryptase, and MC-CPA, were all significantly lower in the peritoneal cells of the mice engrafted with *Hdc*$^{-/-}$ BMMCs than in those with WT BMMCs (Fig. 6E–G). These findings are in agreement with the characteristics of the

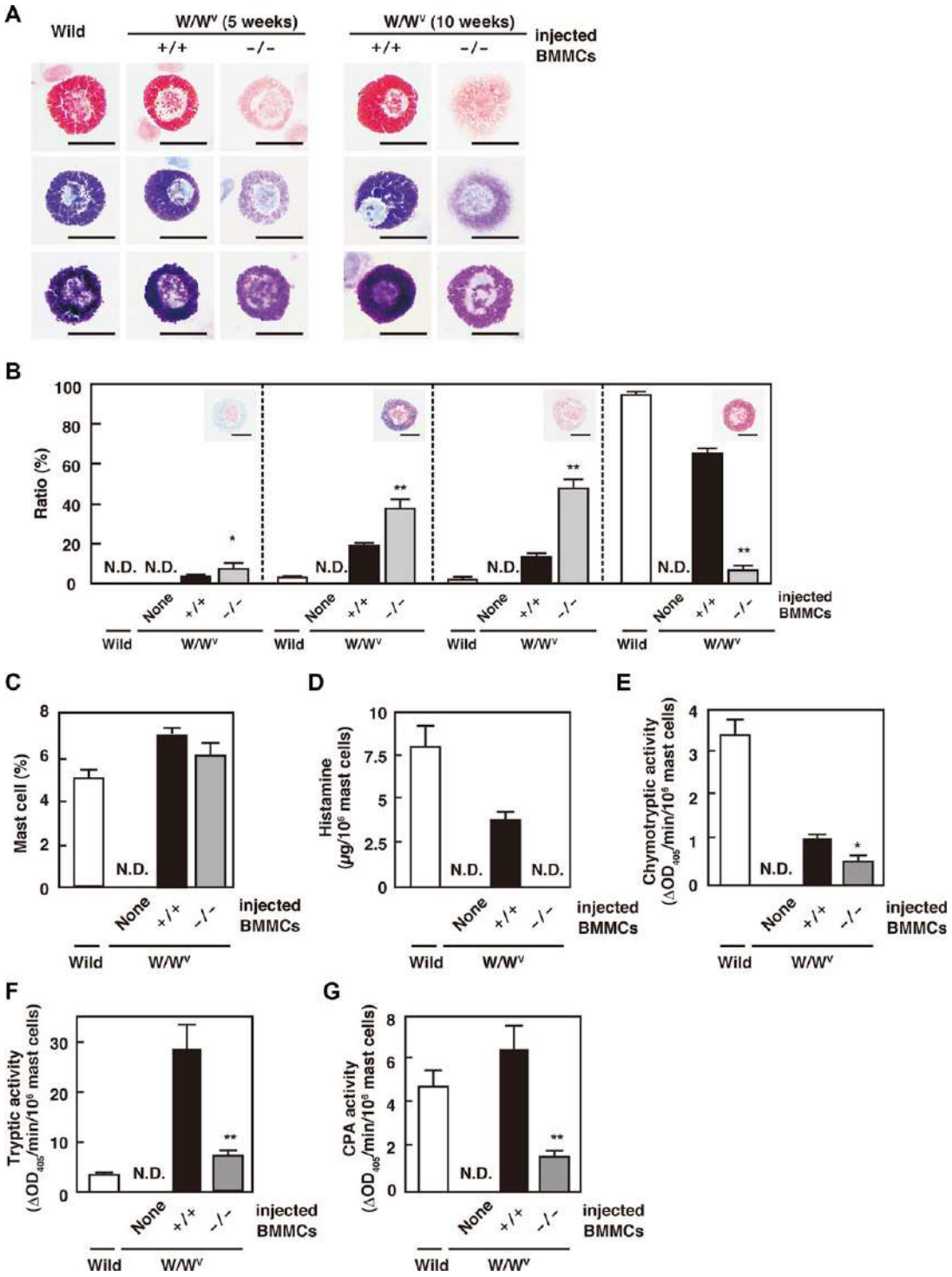

Figure 6. Impaired granule maturation in *Hdc*$^{-/-}$ mast cells reconstituted in the peritoneal cavity of mast cell deficient, *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice. BMMCs prepared from WT (+/+) or *Hdc*$^{-/-}$ (−/−) mice were engrafted into the peritoneal cavity of mast cell deficient, *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice (W/W^V). Peritoneal cells were collected from the *Kit*^{W}/*Kit*$^{W\text{-}v}$ mice 5 or 10 weeks after the engraftment or from the mast cell sufficient, *Kit*$^+$/*Kit*$^+$ mice (WT). (A) The peritoneal cells were collected and visualized by Safranin-O (top), acidic Toluidine blue (middle), or May–Grünwald–Giemsa (bottom) staining; bar = 10 μm. (B) The peritoneal cells were classified into four categories according to the staining property. Typical staining patterns are shown; bar = 10 μm. (C) Percentages of mast cells are determined based on the May–Grünwald–Giemsa staining. (D) Histamine contents in the peritoneal cells were measured. N.D., not detectable. (E–G) Enzymatic activities of chymase (E), tryptase (F), and MC-CPA (G) in the peritoneal cells were measured. (A–G) The values are represented as the means + SEM (WT, $n = 5$, W/W^V—none, $n = 3$, W/W^V—reconstituted, $n = 7$, each). The values of $^*p < 0.05$ and $^{**}p < 0.01$ are regarded as significant (versus W/W^V—+/+) by Student's *t*-test.

pMCs in *Hdc*$^{-/-}$ mice (Fig. 1) and suggest that HDC expression in mast cells plays a critical role in their granule maturation.

Discussion

In this study, we demonstrated that histamine synthesis in mast cells is required for their granule maturation. Although the previous study indicated the involvement of histamine in granule maturation of mast cells, it remained obscure how histamine should promote the maturation. Accumulating lines of evidence have suggested that newly synthesized histamine in the cytosol follows two fates; one is mediated by vesicular histamine transporters, including vesicular monoamine transporter-2, which contribute to granule storage of histamine, and the other is by surface OCTs, including OCT-3, which excrete excess amount of cytosolic histamine [13, 14, 20]. Granule maturation of mast cells, therefore, could be enhanced by histamine found in three different sources; namely, (i) granule histamine, (ii) extracellular histamine, and (iii) cytosolic histamine. It is not likely that granule histamine affects the granule maturation of mast cells, because depletion of granule histamine by treatment with tetrabenazine had no obvious effects on the granule staining. The functional H_4 receptor was found to express in murine mast cells and several kinds of the H_4 agonists mimicked the effects of histamine, indicating the positive roles of the H_4 receptor in the granule maturation. However, granule maturation of the *Hrh4*$^{-/-}$ pMCs was found to be largely intact, excluding the possibility that the H_4 subtype play critical roles in granule maturation. These findings highlight a possibility that cytosolic histamine is involved in granule maturation of mast cells. Schneider et al. reported that cytosolic histamine, which is incorporated into cells through OCT-3 when excess amount of histamine is present in the extracellular spaces, inhibits the induction of HDC and several cytokines in murine IL-3-dependent BM-derived basophils [13], although the exact intracellular targets of histamine remains unknown. Very recently, covalent modification of heterotrimeric and low molecular weight G proteins by histamine (histaminylation) has been detected in a mouse mastocytoma cell line, P-815 [21]. This modification is catalyzed by transglutaminase-2 (TGM2). Since histamine synthesis was transiently induced and *Tgm2* is upregulated during the coculture period in our system [12], it is possible that histaminylation of the cytosolic proteins by TGM-2, which can target and activate $G\alpha_{o1}$ and Cdc42, is involved in granule maturation of mast cells.

Aberrant granule formation has also been found in mast cells derived from mice lacking *Ndst2* or *Srgn* [22–24]; the former encodes an enzyme involved in heparan sulfate biosynthesis and the latter encodes a core protein of hematopoietic cell granule proteoglycans. However, mature mast cells of these gene targeting mice harbored a small number of large low-density granules, in contrast to the normal size and number of granules in *Hdc*$^{-/-}$ mature mast cells. *Ndst2*$^{-/-}$ and *Srgn*$^{-/-}$ mast cells shared several characteristics, such as decreased storage of granule proteases with *Hdc*$^{-/-}$ mast cells and it was concluded that major defects in granule maturation in *Ndst2*$^{-/-}$ and *Srgn*$^{-/-}$ cells should occur at the posttranslational levels; impaired accumulation of sulfated proteoglycans might lead to instability of the granule proteases in *Ndst2*$^{-/-}$ and *Srgn*$^{-/-}$ mast cells. Histamine contents in pMCs of *Hdst2*$^{-/-}$ and *Srgn*$^{-/-}$ mice were found to be significantly decreased [23,25], although it remains unknown whether expression of the *Hdc* gene is suppressed or granule storage of histamine is impaired in these cells due to the absence of sulfated proteoglycans. A decrease in histamine synthesis may also be involved in the aberrant granule maturation in *Hdst2*$^{-/-}$ and *Srgn*$^{-/-}$ mast cells. Very recently, serious failure in granule maturation of mast cells has also been found in *Pla2G3*$^{-/-}$ mice, in which significant decreases in histamine synthesis and storage were observed in mast cells [26]. The impaired granule maturation of *Pla2G3*$^{-/-}$ mast cells were also reproduced in the similar coculture system. Since *Hdc*$^{-/-}$ mast cells are deficient in expression of multiple granule proteases in addition to the absence of histamine, we should give a cautious interpretation to the phenotypes observed in *Hdc*$^{-/-}$ mice, as well as those in *Ndst2*$^{-/-}$ and *Srgn*$^{-/-}$ mice. Since accumulating evidence suggests that a series of granule proteases are also involved in various immune responses [27], the roles of histamine may be overestimated in the previous studies using *Hdc*$^{-/-}$ mice.

Several murine IgE clones have a potential to induce cytokine production in the absence of the Ag and promote survival and maturation of immature mast cells [28]. We previously reported that histamine synthesis is transiently induced in BMMCs by IgE in an Ag-independent manner [29]. Increased IgE levels, which are often observed during chronic allergic diseases, were found to upregulate the surface expression of FcεRI and to lead to exacerbation of the allergic diseases [30]. IgE-mediated exacerbation may be mediated, at least in part, by self-reinforced mast cells. Elevated histamine levels were also observed in various human chronic inflammatory diseases, such as atopic dermatitis, chronic urticaria, allergic asthma, multiple sclerosis, rheumatoid arthritis, and psoriasis [1]. Histamine may have a far greater influence on the vicious cycle observed in chronic inflammatory responses than expected. A strategy of inhibition of local histamine synthesis may have clinical potentials for chronic inflammatory diseases, which are characterized by accumulation of immature mast cells.

Materials and methods

Animals

Hdc$^{-/-}$ mice, which were originally generated with a mixed genetic background of 129/Sv and CD1 [9], were backcrossed to BALB/c strain or C57BL6 strain for 8–10 generations. *Hrh4*$^{-/-}$ mice were generated by Lexicon Genetics, Inc. (The Woods, TX) and were backcrossed to C57BL6 strain for 8–10 generations [19]. Specific pathogen-free, 8–12 week-old male and female Balb/c mice and 5-week-old male WBB6F1-*Kit*^{W}/*Kit*$^{W\text{-}v}$ mice were obtained from Japan SLC (Hamamatsu, Japan), and all mice were kept in a specific pathogen-free animal facility at Kyoto University

and Okayama University. This study was approved by the Committee on Animal Experiments of Kyoto University, Mukogawa Women's University, and Okayama University.

Materials

The following materials were commercially obtained from the sources indicated: Histodenz™, *p*-nitrophenyl-β-*D*-2-acetoamide-2-deoxyglucopyranoside, compound 48/80, and JNJ 7777120 from Sigma-Aldrich (St. Louis, MO), tetrabenazine, cimetidine, clobenpropit, dimaprit, pyrilamine, and VUF8430 from TOCRIS Bioscience (Bristol, UK), recombinant mouse IL-3 from R&D Systems (Minneapolis, MN), S-2586 and S-2288 from Cromogenix (Milano, Italy), M-2245 from Bachem (Bubendorf, Switzerland), and Fura-2/AM from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were commercial products of reagent grade.

Purification of pMCs

pMCs (>95% purity, confirmed by Alcian blue/Safranin-O and May–Grünwald–Giemsa staining) were purified from total peritoneal cells by a density gradient centrifugation with Histodenz™ as previously described [31]. Since the density of *Hdc*$^{-/-}$ pMCs was found to be lower than that of WT cells, 20% of Histodenz™ solution was used.

Degranulation and granule protease assay

Degranulation of mast cells was evaluated by measuring enzyme activity of a granule enzyme, β-hexosaminidase. Enzyme activity of β-hexosaminidase and three kinds of granule proteases were measured as described previously [12]. Total β-hexosaminidase activity in peritoneal cells and BMMCs was not significantly different between WT and *Hdc*$^{-/-}$ mice.

Measurement of histamine

The amount of histamine was determined by the fluorometrical method with *o*-phthalaldehyde [32]. Cultured and pMCs were twice washed in PBS and lysed in the PBS containing 0.5% Triton X-100 and 2 M NaCl to obtain the soluble fraction for histamine assay.

Flow cytometry

Flow cytometry analyses were performed with FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) equipped with the CELLQUEST software as previously described.

Measurement of cytokine release

Released cytokines (IL-6, TNF-α, and MCP-1) were measured by ELISA kits (OptEIA, BD Biosciences, San Diego, CA) according to the manufacturer's instructions, respectively.

Preparation of BMMCs and CTMC-like MCs

BMMCs were prepared as previously described [12]. Briefly, BM cells were cultured in the presence of 10 ng/mL IL-3 for 30–35 days to obtain mature BMMCs. Maturation of BMMCs were evaluated by the surface expression levels of FcεRI and c-kit and by granule maturation based on the acidic Toluidine blue staining. A CTMC culture model was originally established through coculture of BMMCs with Swiss 3T3 fibroblasts [33, 34]. We modified this culture method and obtained CTMC-like cultured mast cells using mitomycin C-treated Swiss 3T3 fibroblasts in the presence of Kitl (30 ng/mL) as previously described [12].

Oligonucleotide microarray

Total RNAs were labeled and hybridized to the MG_U74AV2 murine GeneChip (Affymetrix, Santa Clara, CA) according to the manufacturer's instruction. We used the RMA (robust multiarray analysis) expression measure that represents the log transform of (background corrected and normalized) intensities of the GeneChips. The RMA measures were computed using the R package program, which is freely available on the web site (http://www.bioconductor.org). We then removed all genes whose maximum minus minimum values were less than 2 (2-fold change), and selected 170 genes, which were differentially expressed between the wild and *Hdc*$^{-/-}$ mast cells. Using the k-means clustering algorithm, these genes were classified into six clusters on the basis of similarity of their expression profiles.

Quantitative RT-PCR analysis

Expression levels of granule proteases and histamine receptor mRNAs in the cultured and pMCs were analyzed by quantitative RT-PCR with DNase-treated total RNAs. PCR was performed using the specific primer pairs (forward, reverse) with Lightcycler ST 300 (Roche Diagnostics, Basel, Switzerland); *Hrh1* (H_1R), 5′-TCA CTC CAG GCC TCA CAT GAC-3′, 5′-CAA AGT TCT CAT CCC AAG TTT CCA-3′, *HrH2* (H_2R), 5′-CAG TCC TAA GCG ACC CGG TA-3′, 5′-GGC ACT GCT GGA TGT ATC TTG A-3′, *HrH3* (H_3R), 5′-ATG ACC GAT TCC TGT CAG TCA CTC-3′, 5′-TTC CGA ACA GCC CGT CTT G-3′, *HrH4* (H_4R), 5′-TGT GCA CCG CAT CTG TCT ACA A-3′, 5′-GAA TCA TCG GGC CAT TTA CCA A-3′, *Mcpt4* (MMCP-4), 5′-CCT TAC ATG GCC CAT CT-3′, 5′-CTT CCC CGG CTT GAT A-3′, *Mcpt5* (MMCP-5), 5′-AGA ACT ACC TGT CGG C-3′, 5′-GTC GTG GAC AAC CAA AT-3′, *Mcpt6* (MMCP-6), 5′-CTT TGA ACC GGA

TCG T-3′, 5′-CTC GTC ATT ATC AAT GTC GC-3′, *Mcpt7* (MMCP-7), 5′-AGC TAT GAC ACG AGA AGG-3′, 5′-GCT TAC GGA GCT GTA CT-3′, *Ctsg*, 5′-CTG TGT GGG AAA CCC GAG-3′, 5′-TCT TGG TGC AAA GCG TCT-3′, and *Gapdh*, forward, 5′- TGT GTC CGT CGT GGA TCT GA-3′, reverse, 5′-TTG CTG TTG AAG TCG CAG GAG-3′.

Measurement of cytosolic Ca^{2+} concentrations

Cytosolic Ca^{2+} concentration was measured as previously described using Fura2-AM (Dojindo Laboratories, Kumamoto, Japan) [29]. Fluorescent intensities were measured, at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrometer (Jasco, CAF-100, Tokyo, Japan).

Reconstitution of mast cells in peritoneal cavity of the mast cell deficient $Kit^{W}/Kit^{W\text{-}v}$ mice

BMMCs were injected into the peritoneal cavity (2×10^6 cells/cavity) of the 5-week-old male WBB6F1-$Kit^{W}/Kit^{W\text{-}v}$ mice. Peritoneal cells were collected 5 or 10 days after the engraftment and then investigated.

Statistical analysis

Data represent means ± SEM. Statistical significance for comparisons between groups was determined using Student's paired *t*-test or ANOVA. Additional comparisons were made with Dunnett multiple comparison test for comparison with the control groups or Tukey–Kramer multiple comparison test for all pairs of column comparison. The values of "*n*" mean the number of the individual trials with different animals.

Acknowledgments: This study was supported in part by a grant from Hyogo Science and Technology Association, Sankyo Foundation of Life Science, Takeda Science Foundation, and by JSPS KAKENHI (grant numbers 19590091, 21790100, and 23590077).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **BMMC**: IL-3-dependent BM-derived cultured mast cells · **CTMC**: connective tissue-type mast cells · ***Ctsg***: cathepsin G · **HDC**: histidine decarboxylase · **Kitl**: c-kit ligand · **MC-CPA**: mast cell carboxypeptidase A · **MCP-1**: monocyte chemotactic protein-1 · **OCT-3**: organic cation transporter · **pMC**: peritoneal mast cell · **TGM2**: transglutaminase-2

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See accompanying Commentary:
http://dx.doi.org/10.1002/eji.201344262

Received: 26/6/2013
Revised: 1/8/2013
Accepted: 29/8/2013
Accepted article online: 3/9/2013

