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Identification of Inducible Genes during Mast Cell Differentiation

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Mast cells play an important role in allergic inflammation by releasing their bioactive mediators. The function of mast cells is enhanced by stimulation because of the induction of specific genes and their products. While many inducible genes have been elucidated, we speculated that a significant number of genes remain to be identified. Thus, we applied differential display (dd) PCR to establish a profile of the induced genes in bone marrow-derived mast cells (BMMCs) after they were co-cultured with 3T3 fibroblasts. To date, 150 cDNA fragments from the connective-type mast cells (CTMCs) were amplified. Among them, thirty cDNA fragments were reamplified for cloning and sequencing. The ddPCR strategy revealed that serine proteases were the most abundant genes among the sequenced clones induced during the maturation. Additionally, unknown genes from the co-culture of BMMCs with 3T3 fibroblasts were identified. We confirmed their induction in the CTMCs by Northern blot analysis and RT-PCR. Characterization of these induced genes during the maturation processes will provide insight into the functions of mast cells.

Key words: Bone marrow-derived mast cells (BMMC), Connective-type mast cells (CTMC), Differential display (dd) PCR, Inflammation

INTRODUCTION

Mast cells are one of the most important effector cells involved in the allergic response (Galli *et al.*, 1996). It is believed that the release of biologically active molecules, including histamine, serotonin, proteoglycans and serine proteases by mast cells represents a critical component of many important allergic reactions. Mast cells may also play an important role in the innate immunity against bacterial and parasitic infection (Malaviya *et al.*, 1996; Echtenacher *et al.*, 1996).

There are at least two phenotypically distinct subpopulations of mast cells found in rodents: one type is the connective-type mast cells (CTMCs) and the other type is the mucosal mast cells (MMCs) (Stevens *et al.*, 1989). One of the striking phenotypic differences between these two populations is the cells' responsiveness to polycationic non-immunological compounds, such as compound 48/80 and substance P. These compounds stimulate CTMCs, but not the MMCs (Swieter *et al.*, 1993). However, recent research has shown that mast cells alter their phenotypes depending on the current microenvironment in which they reside (Friend *et al.*, 1996). Nevertheless, mast cells belonging to the CTMC phenotype possess several remarkable features that are not shared with the MMCs or with the bone marrow-derived mast cells (BMMCs), which is an immature population of mast cells. Thus, only the CTMCs synthesize heparin, express CTMC specific serine proteases and contain high concentrations of granule histamine (Galli *et al.*, 1999; Wong *et al.*, 1999; Kitamura *et al.*, 1999).

The growth factors, which include interleukin-3 (IL-3), IL-4, IL-9, IL-10 and the tissue derived cytokine stem cell factor (SCF) have been shown to be important for the development of mast cells (Tsuji *et al.*, 1991; Lantz *et al.*, 1995; Beaven *et al.*, 1996). SCF is an essential factor in the development and function of mast cells. Mice that have a functional defect for SCF and *c-kit* exhibit severe deficiencies in both their CTMCs and MMCs (Nakano *et al.*, 1985; Tsuji *et al.*, 1990), and this indicates that the SCF/*c-kit* interaction regulates the development of mature mast cells. However, the SCF/*c-kit* interaction alone is not sufficient to allow mast cells to undergo full maturation. For this process, some other accessory factors may be required (Galli *et al.*, 1999; Kitamura *et al.*, 1999).

A co-culture of IL-3-dependent BMMCs with 3T3 fibroblasts in the presence of SCF results in the morphological and functional development towards a more mature

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CTMC-like phenotype (Levi-Schaffer *et al.*, 1986; Levi-Schaffer *et al.*, 1987; Dayton *et al.*, 1988; Ogasawara *et al.*, 1997). The BMMCs co-cultured with 3T3 fibroblasts (CTMCs) were able to produce more prostaglandin D_2 (PGD_2), and the cells obtained a marked sensitivity to polybasic secretagogues (Ogasawara *et al.*, 1997). The cDNA subtraction between BMMCs before and after co-culture with 3T3 fibroblasts was applied to identify the genes that were upregulated during the mast cell maturation (Kikuchi-Yanoshita *et al.*, 2003; Taketomi *et al.*, 2003).

To survey more of the inducible genes during mast cell maturation, we employed differential display (dd) PCR to identify a series of genes that are highly upregulated in BMMCs during co-culture with 3T3 fibroblasts in the presence of SCF.

MATERIALS AND METHODS

Generation of BMMC

Male BALB/c mice (Daehan Biolink, Eumsung, Korea) were sacrificed by cervical dislocation, the intact femurs and tibias were removed and then the bone marrow cells were harvested by repeated flushing of the marrow cavities with RPMI1640 (Invitrogen, Carlsbad, CA). The bone marrow cells were cultured for up to 10 weeks in the enriched medium (RPMI 1640 supplemented with 100 uints/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids and 10% heat-inactivated fetal calf serum) and 10% pokeweed mitogen-stimulated spleen-conditioned medium, which acted as a source of IL-3. The non-adherent cells were transferred to fresh culture plates every 2-4 days to remove the adherent macrophages and fibroblasts.

Co-culture of BMMCs with Swiss 3T3 fibroblasts

The BMMCs were washed once, suspended at 2×10^5 cells/mL in enriched medium supplemented with 100 ng/mL SCF and then seeded onto confluent Swiss 3T3 fibroblast monolayers grown in 100-mm dishes. The medium was changed every 2 days and the cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 days. The viability of the BMMCs was maintained at nearly 100% under these culture conditions.

Histochemical staining of mast cells

The mast cells that were obtained from bone marrow cells cultured with IL-3 or from bone marrow cells co-culture with 3T3 fibroblasts were cytospun. The slides were incubated for 5 min in a solution of a 0.5% alcian blue/0.3% acetic acid, and they were next washed with distilled water. After a brief drying, the slides were incubated for 5 min with a solution of 0.1% safranin/0.1% acetic acid, next washed with distilled water and then dried. The stained cells were examined using light microscopy.

Differential display PCR

The total RNA was prepared from BMMCs and CTMCs using Trizol (Invitrogen). Differential display (dd) PCR was performed using the Delta Differential Display system (Clontech, Palo Alto, CA) according to the manufacturer's instructions in the presence of [α^{35}S] dATP. The amplified radioactive products were resolved by electrophoresis on a denaturing urea 5% polyacrylamide gel. After overnight autoradiography, the differentially expressed bands were identified by a comparison of the products that were obtained when using the RNA from BMMCs and CTMCs. The products found to be differentially expressed in a reproducible fashion were reamplified and subcloned into pCR2.1-TOPO vectors using the TA cloning system (Invitrogen). The plasmids containing the amplified inserts were purified, the sequences of the insert were determined by automated dideoxy sequencing at Yeungnam University and they were compared to known sequences using the BLAST network of the National Center for Biotechnology Information. In addition, individual colonies containing the cDNA fragments of interest were prepared for use as hybridization probes.

Northern blot analysis and RT-PCR for confirmation of differential gene expression

Northern analysis was carried out according to the procedure of Sambrook et al (Sambrook *et al.*, 1989). Briefly, thirty μg of total RNA were electrophoretically separated on 1.4% denaturing agarose gel using a 1 × MOPS [3-(*N*-morpholino)-propanesulfonic acid] buffer, and the RNA was transferred to nylon membranes (Schleicher&Schuell, Keene, NH) in 25 mM phosphate buffer, pH 7.0, for 12 h. The membrane was crosslinked under an UV lamp. The amplified DNA fragments were used as probes. These DNA probes were synthesized and labeled with [^{32}P] dCTP as described by the procedures of the Prime-a-Gene® Labeling System (Promega, Madison, WI). Hybridization was performed with the labeled probes for 14 h at 42°C in hybridization buffer (50% formamide, 1.0 M NaCl, 10% (w/v) dextran sulfate, 1% SDS and 0.1 mg/mL denatured salmon sperm DNA). The membrane was washed twice in 2 × SSC, 0.1% SDS for 5 min at room temperature, once in 1 × SSC, 0.1% SDS for 10 min at room temperature, once in 0.1 × SSC, 0.1% SDS for 20 min at room temperature, and once in 0.1 × SSC, 0.1% SDS for 5 min at 50°C. The washed membrane was exposed for 24 h to X-ray film for autoradiography.

For RT-PCR, the total RNAs from BMMCs, CTMCs and 3T3 fibroblasts were prepared from BMMCs using Trizol

(Invitrogen). The first strand cDNA was generated from 1 μg of the total RNA using a RNA PCR kit (Takara, Kyoto, Japan), and it was then amplified. The PCR amplications were performed in a total 25 μL volume containing 5 μL cDNA, 1 mM dNTP, 2.5 mM $MgCl_2$, 12.5 pmol of primers and 0.6 U Taq DNA polymerase for 30 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 57-63°C for 30 s, and extention at 72°C for 30 s. The PCR samples were electrophoresed on 1.2% agarose gels, stained with 10 mg/mL ethidium bromide and then visualized with UV light. Actin specific primers were used as the positive comparative controls.

RESULTS AND DISCUSSION

Staining of BMMC and CTMC

The co-cultured BMMCs (CTMCs) have displayed several different features compared to those of the BMMCs, and these features including the production of PGD_2 at a level over that of leukotriene C_4 after cell activation and the cellular responses to the polybasic secretagogues (Galli *et al.*, 1999). The development of mast cells under the different culture conditions was also distinguished by the histochemical staining properties. The granules from the CTMCs differ from those of the BMMCs in that the CTMC granules stain for alcian$^+$/safranin$^+$, they contain high levels of histamine, and they have heparin proteoglycans rather than condroitin sulfate E proteoglcans. Four week old BMMCs contained granules that stained with alcian blue, but the granules did not stain with safranin as shown in Fig. 1. We also stained the co-cultured BMMCs with alcian blue followed by safranin staining. As predicted, the granules were safranin-stained,

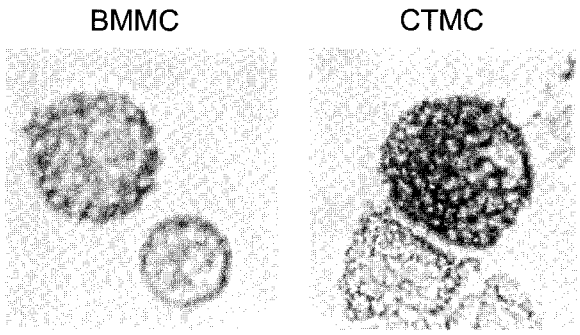

Fig. 1. Cytochemical staining of BMMC and co-cultured BMMC with 3T3 fibroblasts (CTMC). Cytospins of 4 weeks old BMMC and CTMC were stained for 5 min with a solution of 0.5% alcian blue/0.3% acetic acid. The alcian blue-stained cells were washed with distilled water, stained for 5 min with a solution of 0.1% safranin/0.1% acetic acid, washed, air-dried, and examined microscopically. Cell with safranin-positive is indicated by arrow.

and this proved that the CTMCs contained heparin-positive granules (Fig. 1).

Inducible genes in co-coultured BMMC

The differential display (dd) technique was originally described to detect differently expressed genes from cells lines (Liang *et al.*, 1992). In order to characterize a series of genes induced in the BMMCs during the co-culture, we investigated the changes in the expression of transcripts by the ddPCR method. We tested 90 combinations of arbitrary and oligo (dT) primers as described in the manufacturer's protocol. The expression profiles for each primer combination reaction revealed 1 or 2 different PCR fragments (Fig. 2). The differentially expressed bands that had a strong appearance in the CTMCs and were not

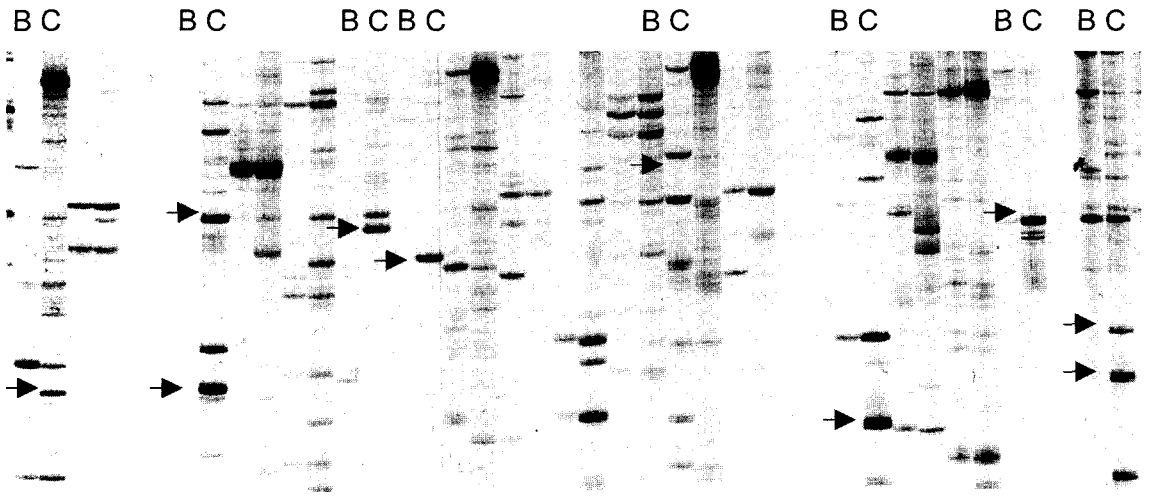

Fig. 2. Differential display of partial mRNA from BMMC (B) and CTMC (C). Three μL of each PCR reaction were loaded and electrophoresed on a 5% denaturing polyacrylamide gel. Following electrophoresis, the gels were dried and autoradiographed. The autoradiograph of [α^{35}S] dATP-labeled PCR products is shown and the arrows indicate the position of the amplified bands corresponding to cDNA of CTMC in the differential display. The Kodak X-OMAT film was exposed for one day at room temperature.

present in 3T3 fibroblasts were excised from the gel and then eluted for reamplification using the same primers.

Analysis of the authenticity of the primary expressed cDNA fragments from CTMCs

The ddPCR is a powerful and rapid method to isolate cDNA fragments of differentially expressed genes because it requires only small amounts of total cellular RNA. However, the drawback of this method is a high incidence of false positives. For that reason, verification of the differential gene expression after ddPCR is important to clarify the non-specific bands. In this study, we used 3T3 mRNA for ddPCR along with CTMC and BMMC mRNA because it was important to obtain CTMC mRNA that was free of both BMMC mRNA and 3T3 mRNA. A number of eluted gels were used for reamplication by employing the corresponding primer sets. The increased expression during co-culture was further verified by the slot hybridization of RNA to eliminate the false positive fragments by using the amplified DNA fragment as probes (data not shown). Of 150 fragment gels, one third was amplified after verification of the slot hybridization. Thirty cDNA fragments were subcloned into the pCR2.1 vector and transformed into *E. coli*. The cDNA fragments obtained from the bacterial colonies were then sequenced. These data indicate that 20% of the genes that were amplified by ddPCR were indeed differentially expressed.

The sequences of the identified gene fragments were compared with the released sequences using the BLAST sequence alignment program. The genes induced in the CTMCs by the ddPCR method were compared with those of the BMMCs co-cultured with 3T3 fibroblasts based on our previously published data (Kikuchi-Yanoshita *et al.*, 2003; Taketomi *et al.*, 2003) and we showed that they have a similar gene profile. Of the number of genes that were induced in BMMCs co-cultured with 3T3 fibroblasts, serine proteases were the most frequently found. Among them, mast cell-specific proteases such as MMCP-1 and MMCP-4 were found. Another frequently detected gene from ddPCR was granzyme B, which is an apoptosis-inducing protease secreted from cytotoxic T cells (Darmon *et al.*, 1995). The induced genes from the CTMCs that were detected by ddPCR, which were not presented in our previous data (Kikuchi-Yanoshita *et al.*, 2003; Taketomi *et al.*, 2003), are now listed in Table I.

We examined three unknown cDNA fragment clones and performed Northern blot analysis to confirm their induction in BMMCs during co-culture, and there was little or no expression noted in the 3T3 fibroblasts (Fig. 3), as tested for by Northern blot analysis. The expression levels of each clone were also examined by RT-PCR using the specific primers that were derived from the nucleotide sequences of each clone. The confirmed unknown genes were #8 (Accession number: NM_172450; hypothetical protein), #17 (Accession number: BC019462), and #35 (Accession number: AK089766; hypothetical protein). To date, we do not know the functions of those unknown genes induced in the CTMCs, and we are trying to isolate the full length of cDNA for further investigation.

In summary, we carried out a differential display to identify the different induced genes between the BMMCs and the CTMCs. Several unknown genes, which have not been reported previously in CTMC, were identified and subsequently confirmed by Northern blotting and RT-PCR. Further cloning is needed to clarify the biological function of the increased expression of these unknown genes during the mast cell maturation process. Although

Table I. cDNA fragments amplified from CTMC[a]

Calcium binding protein A6 (calcyclin)
Carboxypeptidase A metalloprotease (M14) family
Peroxiredoxin v-like pseudogene
Protein synthesis initiation factor 4A (elf4A) gene
Putative translation initiation factor
Tumor-associated membrane protein (TMP) gene
NADH dehydrogenal Fe-s protein 6
Mitochondrial DNA polymerase gamma
Ubiquinone flavoprotein 2
Ubiquitin fusion degradation protein 2 (ufd2) gene
Ubiquinon oxidoreductase

[a] Unknown genes from ddPCR fragments were not listed.

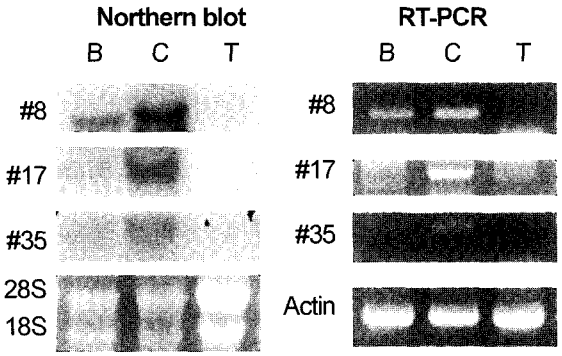

Fig. 3. Northern blot analysis and RT-PCR. Total RNAs (30 μg) from BMMC (B), CTMC (C), and 3T3 fibroblasts (T) were prepared and used for Northern blot to confirm differentially expressed bands using the reamplified PCR products as probes. The #8, #17, and #35 on the left indicates the cloned number with accession number NM_172450, BC019462, and AK089766, respectively. Three of the 30 ddPCR amplified bands were confirmed by Northern analysis, as shown. Total RNAs were reverse-transcribed into the complementary DNA, and PCR-amplified to detect the expression of confirmed three genes by RT-PCR using the clone specific primres. The equality of the Northern blot and RT reaction of isolated RNA was confirmed by ribosomal RNA and amplification of β-actin.

our results certainly do not include all the changes of gene expression that occur during mast cell maturation, the genes we have isolated may provide information for a better understanding of the differentiation and maturation of mast cells cell, and this is information that could be applicable to the treatment of allergic disease.

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REFERENCES

Beaven, M. A. and Baumgartner, R. A., Downstream signals initiated in mast cells by Fc epsilon RI and other receptors. *Curr. Opin. Immunol.,* 8, 766-772 (1996).

Darmon, A. J., Nicholson, D. W., and Bleackley, R. C., Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature,* 377, 446-448 (1995).

Dayton, E. T., Pharr, P., Ogawa, M., Serafin, W. E., Austen, K. F., Levi-Schaffer, F., and Stevens, R. L., 3T3 fibroblasts induce cloned interleukin 3-dependent mouse mast cells to resemble connective tissue mast cells in granular constituency. *Proc. Natl. Acad. Sci. U.S.A.,* 85, 569-572 (1988).

Echtenacher, B., Mannel, D. N., and Hultner, L., Critical protective role of mast cells in a model of acute septic peritonitis. *Nature,* 381, 75-77 (1996).

Friend, D. S., Ghildyal, N., Austen, K. F., Gurish, M. F., Matsumoto, R., and Stevens, R. L., Mast cells that reside at different locations in the jejunum of mice infected with Trichinella spiralis exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J. Cell Biol.,* 135, 279-290 (1996).

Galli, S. J., Tsai, M., and Lantz, C. S., In Signal transduction in mast cells and basophils(Eds, Razin, E., and Rivera, J.) Springer-Verlag, New York, NY, 11 (1999).

Galli, S. J. and Wershil, B. K., The two faces of the mast cell [news; comment]. *Nature,* 381, 21-22 (1996).

Kikuchi-Yanoshita, R., Taketomi, Y., Koga, K., Sugiki, T., Atsumi, Y., Saito, T., Ishii, S., Hisada, M., Suzuki-Nishimura, T., Uchida, M. K., Moon, T. C., Chang, H. W., Sawada, M., Inagaki, N., Nagai, H., Murakami, M., and Kudo, I., Induction of PYPAF1 during *in vitro* maturation of mouse mast cells. *J. Biochem. (Tokyo),* 134, 699-709 (2003).

Kitamura, Y., Morii, E., and Jippo, T., In Signal transduction in mast cells and basophils (Eds, Razin, E., and Rivera, J.) Springer-Verlag, New York, NY, 31 (1999).

Lantz, C. S. and Huff, T. F., Differential responsiveness of purified mouse c-kit+ mast cells and their progenitors to IL-3 and stem cell factor. *J. Immunol.,* 155, 4024-4029 (1995).

Levi-Schaffer, F., Austen, K. F., Gravallese, P. M., and Stevens, R. L., Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells. *Proc. Natl. Acad. Sci. U.S.A.,* 83, 6485-6488 (1986).

Levi-Schaffer, F., Dayton, E. T., Austen, K. F., Hein, A., Caulfield, J. P., Gravallese, P. M., Liu, F. T., and Stevens, R. L., Mouse bone marrow-derived mast cells cocultured with fibroblasts. Morphology and stimulation-induced release of histamine, leukotriene B4, leukotriene C4, and prostaglandin D2. *J. Immunol.,* 139, 3431-3441 (1987).

Liang, P. and Pardee, A. B., Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science,* 257, 967-971 (1992).

Malaviya, R., Ikeda, T., Ross, E., and Abraham, S. N., Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature,* 381, 77-80 (1996).

Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y., and Galli, S. J., Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/Wv mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells. *J. Exp. Med.,* 162, 1025-1043 (1985).

Ogasawara, T., Murakami, M., Suzuki-Nishimura, T., Uchida, M. K., and Kudo, I., Mouse bone marrow-derived mast cells undergo exocytosis, prostanoid generation, and cytokine expression in response to G protein-activating polybasic compounds after coculture with fibroblasts in the presence of c-kit ligand. *J. Immunol.,* 158, 393-404 (1997).

Sambrook, J., Fritsch, E., and Maniatis, T., Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY, (1989).

Stevens, R. L. and Austen, K. F., Recent advances in the cellular and molecular biology of mast cells. *Immunol. Today,* 10, 381-386 (1989).

Swieter, M., Midura, R. J., Nishikata, H., Oliver, C., Berenstein, E. H., Mergenhagen, S. E., Hascall, V. C., and Siraganian, R. P., Mouse 3T3 fibroblasts induce rat basophilic leukemia (RBL-2H3) cells to acquire responsiveness to compound 48/80. *J. Immunol.,* 150, 617-624 (1993).

Taketomi, Y., Sugiki, T., Saito, T., Ishii, S., Hisada, M., Suzuki-Nishimura, T., Uchida, M. K., Moon, T. C., Chang, H. W., Natori, Y., Miyazawa, S., Kikuchi-Yanoshita, R., Murakami, M., and Kudo, I., Identification of NDRG1 as an early inducible gene during *in vitro* maturation of cultured mast cells. *Biochem. Biophys. Res. Commun.,* 306, 339-346 (2003).

Tsuji, K., Nakahata, T., Takagi, M., Kobayashi, T., Ishiguro, A., Kikuchi, T., Naganuma, K., Koike, K., Miyajima, A., and Arai, K., *et al.*, Effects of interleukin-3 and interleukin-4 on the development of "connective tissue-type" mast cells: interleukin-3 supports their survival and interleukin-4 triggers and supports their proliferation synergistically with interleukin-

3. *Blood,* 75, 421-427 (1990).

Tsuji, K., Zsebo, K. M., and Ogawa, M., Murine mast cell colony formation supported by IL-3, IL-4, and recombinant rat stem cell factor, ligand for c-kit. *J. Cell Physiol.,* 148, 362-369 (1991).

Wong, G., Friend, D. S., and Stevens, R. L., In Signal transduction in mast cells and basophils (Eds, Razin, E., and Rivera, J.) Springer-Verlag, New York, NY, 39 (1999).