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Expression profile analysis of aorta-gonad-mesonephros region-derived stromal cells reveals genes that regulate hematopoiesis

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ABSTRACT

The aorta-gonad-mesonephros (AGM) region is involved in the generation and maintenance of the first definitive hematopoietic stem cells (HSCs). A mouse AGM-derived cell line, AGM-S3, was shown to support the development of HSCs. To elucidate the molecular mechanisms regulating early hematopoiesis, we obtained subclones from AGM-S3, one of which was hematopoiesis supportive (S3-A9) and the other one of which was non-supportive (S3-A7), and we analyzed their gene expression profiles by gene chip analysis. In the present study, we found that Glypican-1 (GPC1) was highly expressed in the supportive subclone AGM-S3-A9. Over-expression of GPC1 in non-supportive cells led to the proliferation of progenitor cells in human cord blood when cocultured with the transfected-stromal cells. Thus, GPC1 may have an important role in the establishment of a microenvironment that supports early events in hematopoiesis.

Hematopoiesis is regulated by the close interaction between hematopoietic cells and the hematopoiesis supporting microenvironment in a hematopoietic organ, such as bone marrow. Stromal cells are major components of the microenvironment and they provide hematopoietic cells with factors that regulate differentiation and proliferation [1–3]. For example, recent studies have demonstrated that osteoblastic cells play important roles for HSC maintenance and Ang-1, a Tie2 ligand, plays crucial role in hematopoietic stem cell maintenance [4].

Heparan sulphate proteoglycans (HSPGs) are reported to be involved in the establishment of the microenvironmental niche and mediate the interaction between HSCs and stromal cells [5]. It is postulated that cell-surface molecules such as CD45, PECAM-1, and Thy-1, which are known heparin-binding proteins, cooperatively contribute to form the hematopoietic niche by presenting growth factors, and they further serve as co-receptors for the growth factors [6–8].

During the embryonic development of the mouse, the first definitive hematopoietic stem cells (HSCs) emerge in the aorta-gonad-mesonephros (AGM) region at E10.5 and thereafter shift to the fetal liver (FL) at E12.5 [9–11]. We previously reported the establishment of stromal cell lines from the AGM region of an E10.5 mouse embryo and one of the cell lines, AGM-S3, was shown to be capable of supporting hematopoiesis [12]. Moreover, E8 yolk sac cells, which normally have no repopulating ability, were shown to mature into long-term repopulating HSCs upon coculture with AGM-S3. This observation suggests that the microenvironment of the stromal cells, derived from the E10.5 AGM region, is important for the generation of long term repopulating-HSCs and that AGM-S3 cells can be used to elucidate the molecular mechanisms regulating early hematopoiesis.

Here, we established cell lines with varying hematopoietic supporting abilities from AGM-S3. Gene chip analysis of the established supportive and non-supportive cell lines revealed a large proportion of genes up-regulated in the supportive cell line which could be involved in cell–cell interactions. Through the forced expression of these candidate genes in the non-supportive subclone, we show that GPC1, a member of HSPG family, can confer hematopoiesis supporting activity to non-supportive stromal cells.

Materials and methods

Cell preparation. AGM-S3 was subcultured in MEMα medium (Invitrogen Corp., Carlsbad, CA) supplemented with inactive 10% fetal calf serum (FCS, HyClone Laboratories, Inc., UT). Subcloning

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was performed by sorting cells using a cell sorter (FACS Vantage; Becton Dickinson Biosciences, San Jose, CA) and plating them in a 96-well plate (BD Falcon, Bedford, MA) at an expected density of one cell per well. Human umbilical cord blood cells were obtained and manipulated with informed written consent in accordance with the Declaration of Helsinki and with permission from the institutional ethics committee of Kirin Pharma Co., Ltd. Mononuclear cells were separated by Ficoll density gradient centrifugation using Lymphoprep (Nycomed Pharma). Differentiated blood cells, which expressed differentiation antigens, CD2, CD11c, CD19, CD15, and CD41, and Glycophorin A, were removed using the Dynal MPC-1 magnetic separator (Dynal Biotech, Norway). $CD34^+$ or $CD34^+$ $CD38^-$ cells were recovered using a cell sorter after labeling with FITC-conjugated anti-CD34 and PC5-conjugated anti-CD38 antibodies (Immunotech). Bone marrow cells were collected from the femur of male C57BL/6-Ly5.1 pep mice (ages ranging from eight to ten weeks) and suspended in PBS and the hematopoietic stem cell fraction was obtained as described previously [13,14]. $CD34^-$, $Sca\text{-}1^+$, $c\text{-}Kit^+$, Lin^- cell population was separated using a cell sorter. GPC1 expression was detected using a rat-anti-mouse GPC1 monoclonal antibody and visualized using a PE- labeled anti-Rat IgG (BD Bioscience, CA). All studies using animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Discovery Research Laboratories of Kirin Pharma Co. Ltd.

Colony-forming assay. Coculture of human hematopoietic stem cells with stromal cells and methylcellulose clonal culture was performed using a modification of the technique described previously [12].

The analysis was performed with the addition of 10 ng/ml of human SCF, human IL-3, human IL-6, human G-CSF, human TPO, and EPO at 2 IU/ml to MethoCult H4230 (Stem Cell Technologies Inc., Vancouver, BC, Canada). The abbreviations used for the colony-forming assay are as follows: BFU-E, erythroid burst-forming units; CFU-GM, granulocyte-macrophage colony-forming units; CFU-E mix, mixed colony-forming units.

Transplantation assay. AGM-derived stromal cell lines (AGM-S3-A9 and AGM-S3-A7); OP9 cells (RCB1124, RIKEN Cell Development Bank); L929 cell (ATCC); or NIH3T3 cells (ATCC) were seeded in a 48-well culture dish (BD Falcon, MA) at 5×10^4 cells/well and cultured in MEMα medium (Invitrogen, CA) containing 10% FCS (HyClone, UT) for three days. A total of 30 pre-sorted mouse hematopoietic stem cells (derived from C57BL/6-Ly5.1) per well were added to the stromal cells ($n = 5$). After 7 days of coculture, the cells were trypsinized and recovered. The whole recovered cells (30 34^-KSL cells equivalent) were then mixed with 3000 Lin-negative bone marrow cells derived from C57BL/6-Ly5.2 mouse (Charles River) and introduced via the tail vein into a C57BL/6-Ly5.2 mouse (eight weeks age and male, Charles River) that had been irradiated with X-rays at 8.5 Gy. The peripheral blood cells were isolated and those derived from the C57BL/6-Ly5.1 mice were detected by FACS using previously described methods [14].

Gene chip expression analysis. Genome-wide gene expression was examined using the Mouse Genome MG-U74A, B, C Probe array (GeneChip, Affymetrix, Inc., USA). Assays were performed according to the manufacturer's protocol. Total RNA was isolated from each stromal cell lines with an RNeasy mini-kit (Qiagen, Chatsworth, CA). GeneChip software was used to determine the average difference (AD) in the levels of gene expression among genes on the array. The mean ADs for 3'-terminal probe sets corresponding to four constitutively expressed genes (β-actin, GAPDH, pyruvate carboxylase and transferrin receptor) were calculated, and hereafter, we use STD to refer to the mean AD of the control probe set. To normalize staining intensity among chips, the AD values for all genes on a given chip were divided by the ratio of the STD for each chip to the average STD for all chips. Normalized AD values less than 0.1 were set to 0.1. Then, the dataset was sorted by the ratio of the mean AD of each gene in a target group to that in a reference group in order to identify highly expressed genes within an experimental group.

Transfection into plat-E cells and retroviral infection of stromal cells. The PCR amplified GPC1 cDNAs were subcloned into the plasmid vector pMX/IRES/GFP (pMXIG) or pLRT. The pMXIG or pLRT-derived plasmid vectors were transfected into PLAT-E cells using FuGene6 (Roche Diagnostics GmbH, Mannheim, Germany) [15]. After a 48-h culture, the supernatants were collected and centrifuged at 6000g for 16 h at 4 °C to enrich virus particles. The stromal cell culture medium was replaced with virus-containing media. GFP positive pMXIG transfectants were sorted and used in the coculture assay. The GPC1-A7 cell populations were obtained by positive cell sorting in the presence of doxycycline (Dox) (500 μg/ml) and negative cell sorting in the absence of Dox.

Reverse transcriptase PCR. Total RNA was extracted from AGM-S3-A9, AGM-S3-A7, OP9 or NIH3T3 and subjected to PCR. Oligonucleotide primer sequences used were as follows: GPC1 forward, 5'-CC TGGCTTACCAAGGCTGTCA-3', and GPC1 reverse, 5'-GTGCTGGCAATA GCCTCTCTAAC-3'; GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3', and GAPDH reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

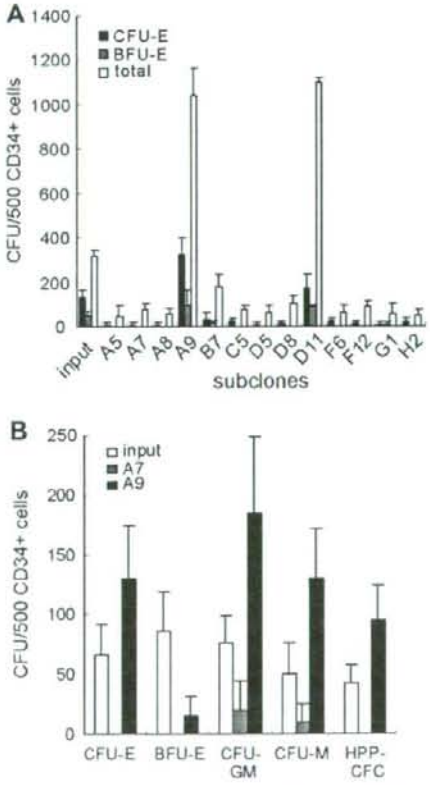

Fig. 1. Subcloning of AGM-S3 stromal cells. (A) Coculture of human CB $CD34^+$ cells with AGM-S3 derived subclones. Human CB $CD34^+$ cells (500 per well) were added to the stromal cells and cultured for two weeks. Clonogenic progenitors were evaluated by culturing a fraction of the cells after they were harvested. Colonies were counted at day 14. (B) Coculture of human CB $CD34^+$ cells with AGM-S3-A9 and AGM-S3-A7.

Results and discussion

Establishment of the subclones from AGM-S3 stromal cells

We previously reported the establishment and characteristics of the stromal cell line AGM-S3, which was generated from the AGM region of an E10.5 mouse embryo [12]. However, since the cell line might be unstable through repeated passages, we tried to obtain stable subclones. We isolated thirteen proliferative subclones from ninety six single-sorted AGM-S3 cells. According to the method for establishment of AGM-S3, we evaluated their hematopoiesis supporting ability by coculture with human hematopoietic progenitor cells (HPCs) (CB $CD34^+$ cells). The results of the clonal assays showed that eleven clones had no effect on hematopoiesis and the remaining two clones supported hematopoiesis (Fig. 1A). One of the non-supportive eleven, AGM-S3-A7, and one of the supportive two, AGM S3-A9, were more stable than the others.

After 14 days of coculture, the number of colony-forming cells (CFU-Cs) increased 3.4-fold in the coculture with AGM-S3-A9, whereas the effect of AGM-S3-A7 was very poor. In subsequent clonogenic experiments, AGM-S3-A9 supported proliferation of several different types of progenitor cells, including CFU-GM, BFU-E, and CFU-E mix (Fig. 1B). Taken together, these results indicate that AGM-S3-A9 has the potential to support human hematopoiesis, but AGM-S3-A7 does not.

To investigate the effect of the newly established cell lines on murine HSCs that have long-term repopulating ability (LTR-HSCs), we cocultured Lin^-, $c\text{-}Kit^+$, $Sca\text{-}1^+$, $CD34^-$ cells (34^- KSL cells) from the adult bone marrow with AGM-S3-A9 cells, AGM-S3-A7 cells, OP9, L929, or NIH3T3 cell lines. 34^-KSL cells cocultured with AGM-S3-A9 were able to repopulate lethally irradiated mice with high level of chimerism in the same way as OP9 cells that are known to support HSCs [16] (Fig. 2). However, HSCs cocultured with AGM-S3-A7, L929, or NIH3T3 cells could only rarely repopulate the recipient mice. Thus, the hematopoiesis supporting ability of AGM-S3-A9 cells or non-supporting ability of AGM-S3-A7 cells is relevant to human and mouse hematopoietic cells.

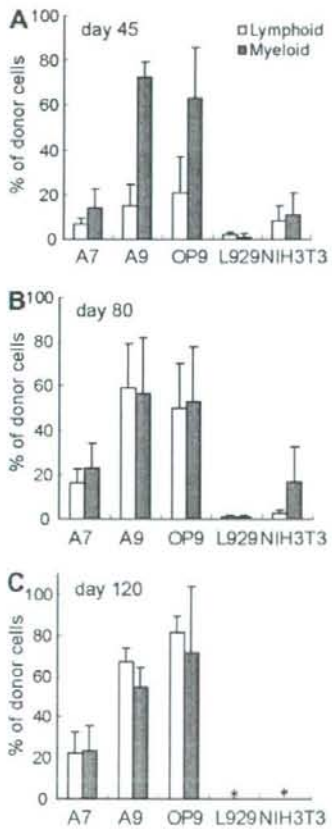

Fig. 2. Hematopoietic reconstitution via coculture of mouse BM 34-KSL cells with AGM-S3-A7, AGM-S3-A9, OP9, L929, or NIH3T3 cells. The cocultured 34-KSL cells were transplanted into a group of lethally irradiated mice ($n = 5$). The percentage of $Ly\text{-}5.1^+$ cells in lymphoid cells ($Thy\text{-}1^+$ and $B220^+$) and in myeloid cells ($Gr\text{-}1^+$ and $CD11c^+$) were determined from the peripheral blood of Ly-5.2 recipient mice at (A) 45, (B) 80 days, and (C) 120 Days post-transplantation. *, no mouse survived.

Comparative transcriptional profiling of AGM-S3-A9 and AGM-S3-A7 cells

We hypothesized that the genes that promote the support of hematopoiesis would be preferentially expressed in the supportive cell lines such as AGM-S3-A9 and OP9, whereas they would be less expressed in the non-supportive AGM-S3-A7. To investigate differentially regulated genes, we performed a gene chip analysis. In the present study, we focused on membrane proteins or membrane binding proteins, since conditioned medium from AGM-S3 cells showed a much reduced supportive ability as compared with coculture using whole cells [12].

As a result of the comparison between AGM-S3-A9, OP9, and AGM-S3-A7, we identified 216 up-regulated and 417 down-reg-

Table 1
Genes upregulated in AGM-s3-A9 or OP9 compared to AGM-s3-A7 cells.

Gene	GenBank Accession No.	Fold change A9/A7	Fold change OP9/A7
Cytokine-related genes			
Interleukin 1 receptor antagonist (Il1rn)	NM_031167	5.6	6.1
Stem cell factor (SCF)	NM_013598	3.5	2.9
Chemokine (C-X-C motif) ligand 12 (Cxcl12)	NM_021704	3.4	4.3
Growth arrest specific 6 (Gas6)	NM_019521	3.3	4.2
Chemokine (C-C motif) ligand 9 (Ccl9)	NM_011338	3.2	6.7
Wingless related MMTV integration site 10b (Wnt10b)	NM_011718	2.5	3.1
Fibroblast growth factor 10 (Fgf10)	NM_008002	2.3	4.6
Adipo Q (Adipoq)	NM_009605	2.3	19.1
Receptor-related genes			
G protein-coupled receptor 109A (Gpr109a)	NM_030701	11.4	30.3
Ephrin B1 (Efnb1)	NM_010110	3.4	3
PTK7 protein tyrosine kinase 7 (Ptk7)	NM_175168	2.9	2.1
Notch gene homolog 3 (Drosophila) (Notch3)	NM_008716	2.4	3
Extracellular matrix-related genes			
Procollagen, type XV (Col15a1)	NM_009928	23.5	9.4
Procollagen, type IV, alpha 1 (Col4a1)	NM_009931	5.7	2.7
Matrilin 4 (Matn4)	NM_013592	5.3	2.2
Glypican 6 (Gpc6)	NM_011821	5.3	4.5
A disintegrin and metallopeptidase domain 15 (ADAM15)	NM_009614	2.5	2.8
Glypican 1 (Gpc1)	NM_016696	2.4	2.3
Syndecan 3 (Sdc3)	NM_011520	2.1	3.7
Other			
R-spondin 3 homolog (Xenopus laevis) (Rspo3)	NM_028351	27.8	200.2
Intercellular adhesion molecule (Icam1)	NM_010493	8.8	24.7
Retinoic acid receptor responder (tazarotene induced) 2 (Rarres2)	NM_027852	5.8	3.7
Plexin domain containing 2 (Plxdc2)	NM_026162	2.3	2.5

ulated genes with at least a 2-fold increase or decrease in the average difference between the supportive and non supportive cell lines. The expression profile data were deposited in the GEO database (GSE11891). The list of genes that displayed the greatest degrees of difference and were predicted to be membrane proteins or secreted proteins is shown in Table 1. Several genes that have previously been identified as regulators of cell–cell interactions were found to be up-regulated in AGM-S3-A9, including intercellular adhesion molecule (ICAM), plexin domain containing 2 (Plxdc2), and several HSPGs. Those molecules might be involved in the interaction between HSCs and their stromal niche.

CXCL12 is also highly expressed in an AGM-derived stromal cell line established by another group [17]. CXCL12-CXCR4 signaling has been shown to be important for maintaining a pool of HSCs in the bone marrow [18] and to enhance engraftment of *in vitro* cultured HSCs to bone marrow [19]. However we observed no hematopoietic supportive activity of recombinant CXCL12 protein in AGM-S3-A7 cell coculture assays (data not shown).

Since stem cell factor (SCF), listed in Table 1, is well known as a hematopoiesis supportive factor [20,21], recombinant SCF was supplemented in the coculture of 34$^+$KSL and AGM-S3-A7 or AGM-S3-A9 and spleen colony-forming units (CFU-S) were measured. CFU-S were not altered by addition of SCF to the cocultures (Supplementary Fig. S1A). Furthermore, forced expression of SCF to AGM-S3-A7 cells did not result in an augmentation in the HSC maintenance ability of AGM-S3-A7 cells (Supplementary Fig. S1B). These findings are consistent with our previous report, in which we showed a neutralizing antibody against c-kit could reduce the number of HPCs observed in coculture with AGM-S3-A9, whereas long-term reconstitution ability was not affected by the antibody [22]. Taken together, the results suggest that SCF is required for the maintenance of HPCs and the proliferation of HSCs, but not the maintenance of HSCs.

Glypican-1 stimulates proliferation of human hematopoietic progenitor cells

Most of the up-regulated genes in the supportive cell lines could not alter the hematopoietic supporting ability of the non-supportive cells with respect to mouse CFU-S and mouse LTR-HSCs in experiments involving coculture with AGM-S3-A7 retrovirally infected with those genes. In all, seventy nine genes, including some genes listed in Table 1, were expressed and assayed for their effect on mouse or human hematopoiesis. We found that only when GPC1 was expressed in AGM-S3-A7 cells could these cells support human hematopoietic progenitor cells in a similar fashion

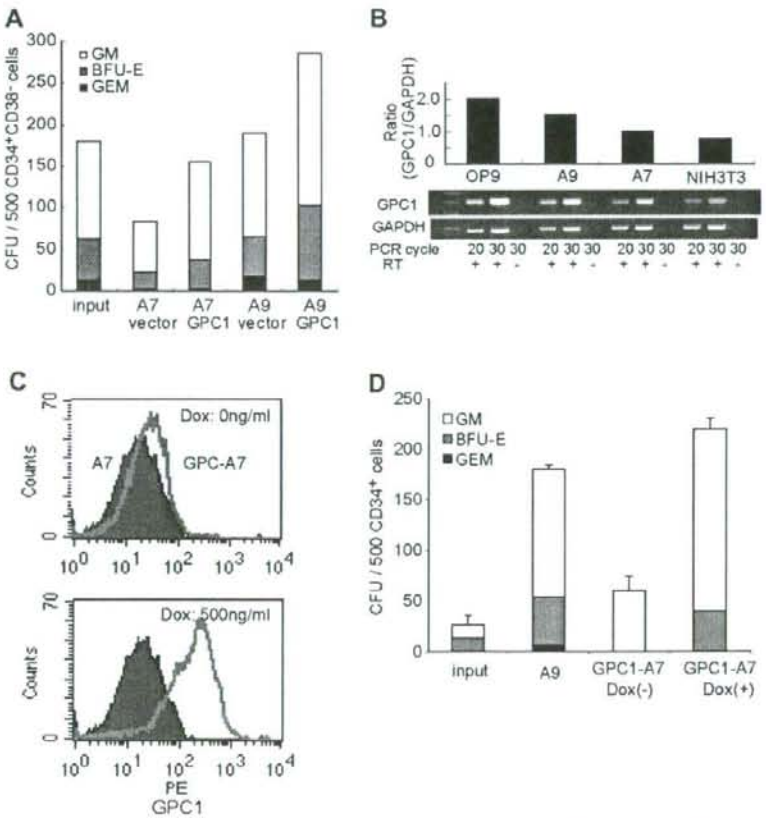

Fig. 3. Hematopoietic supportive activity of Glypican-1. (A) Coculture of Human CB CD34$^+$ CD38- cells with AGM-S3-A9 or AGM-S3-A7 cells that had been retrovirally infected with the GPC-1 expression vector. A total of about 500 human CB CD34$^+$ CD38- cells per well were added to the stromal cells and the cells were cocultured for two weeks. Colonies were counted at day 14. (B) RT-PCR analysis of GPC1 expression on AGM-S3-A9, AGM-S3-A7, OP9, and NIH3T3 cells. (C) Dox-dependent inducible expression of GPC1 protein in the cell population GPC1-A7 as detected by FACS analysis. (D) Coculture of Human CB CD34$^+$ cells with the cell population GPC1-A7. A total of about 500 human CB CD34$^+$ cells per well were cocultured with GPC1-A7 for two weeks in the presence or absence of Dox (500 ng/ml). Colonies were counted at day 14.

to S3-A9 cells (Fig. 3A). Furthermore, the forced expression of GPC1 in AGM-S3-A9 cells enhanced the supporting ability, which means that the supportive activity of AGM-S3-A9 by intrinsic expression of GPC1 is not saturated in those cells and compulsory expression of GPC1 could induce the cells to perform to their full potential. In support of the gene chip assay results, RT-PCR analysis confirmed that AGM-S3-A9 and OP9 cells expressed 1.5 to 2.0 fold more of GPC1 than AGM-S3-A7 (Fig. 3B).

To confirm the ability of GPC1 to support human hematopoiesis, we used the tetracycline (tet)-regulated gene expression system. We established AGM-S3-A7 cells expressing GPC1 (henceforth, GPC1-A7 cells) under the control of a tet-responsive element using the reverse tet-regulated retroviral vector pLRT [23]. The tet-responsive expression of GPC1 was verified by FACS analysis (Fig. 3C). In the absence of Dox, GPC1-A7 cells exhibited little supportive activity and were comparable to the parental AGM-S3-A7 cells. In contrast, in the presence of Dox, GPC1-A7 stimulated the proliferation of HPCs, including BFU-E, in the human $CD34^+$ cells to levels similar to that achieved with S3-A9 cells (Fig. 3D). These results suggest that an increase in the level of expression of GPC1 could restore the ability of AGM-S3-A7 cells to stimulate HPC proliferation. However, it remains unclear how GPC-1 could affect hematopoiesis. In accordance with the fact that GPC1 is known as a co-receptor that facilitates the interaction of growth factors, we found that bFGF and VEGF bind to a soluble form of recombinant human GPC1 protein *in vitro* (data not shown). In the context of those observations, GPC1 may help to coordinate the microenvironment of hematopoiesis by presenting growth factors.

Our experiments have characterized one gene, GPC1, that plays a role in the HSC supporting capacity of AGM-S3-A9. Also, our studies provide important clues to elucidate the molecular mechanisms of a HSC supportive niche in AGM. However, we could not reconstitute a hematopoiesis-supportive microenvironment by only using recombinant proteins without stromal cells. Defining the culture conditions required for expanding LTR-HSCs might be helpful for clinical use. Perhaps combinations of up-regulated genes in AGM-S3-A9 might support hematopoiesis, though further investigation is still needed.

Accession number

Microarray data are accessible through accession number GSE11891, the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.123.

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Direct Development of Functionally Mature Tryptase/Chymase Double-Positive Connective Tissue-Type Mast Cells from Primate Embryonic Stem Cells

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ABSTRACT

Conditions that influence the selective development or recruitment of connective tissue-type and mucosal-type mast cells (MCs) are not well understood. Here, we report that cynomolgus monkey embryonic stem (ES) cells cocultured with the murine aorta-gonad-mesonephros-derived stromal cell line AGM-S1 differentiated into cobblestone (CS)-like cells by day 10–15. When replated onto fresh AGM-S1 with the addition of stem cell factor, interleukin-6, and Flt3 ligand, these CS-like cells displayed robust growth and generated almost 100% tryptase/chymase double-positive MCs within 3 weeks. At all time points, the percentage of tryptase-positive cells did not exceed that of chymase-positive cells. These ES-derived MCs were CD45+/Kit+/CD31+/CD203c+/HLA-DR− and coexpressed a high-affinity IgE receptor on their surface, which was upregulated after IgE exposure. Electron microscopy showed that they contained many electron dense granules. Moreover, ES-derived MCs responded to stimulation by via IgE and substance P by releasing histamine. These results indicate that ES-derived MCs have the phenotype of functionally mature connective tissue-type MCs. The rapid maturation of ES-derived MCs suggests a unique embryonic pathway in primates for early development of connective tissue-type MCs, which may be independent from the developmental pathway of mucosal-type MCs. STEM CELLS *2008; 26:706–714*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Multipotential progenitor cells, capable of becoming mast cells (MCs), leave the bone marrow (BM) and enter the circulation, but they complete their differentiation into mature MCs only after arriving in peripheral tissues [1]. Mature MCs can be distinguished from other cell types by surface expression of the high-affinity IgE receptor (FcεRI), high levels of c-Kit, and characteristic secretory granules. In addition to histamine, MC granules contain many other neutral proteases, such as tryptase, chymase, carboxypeptidase and cathepsin G. Predicted biologic outcomes of tryptase might include anticoagulation, fibrosis and fibrolysis, kinin generation and destruction, cell surface protease activated receptor (PAR)-2 activation, enhancement of vasopermeability, angiogenesis, inflammation, and airway smooth muscle hyperreactivity. Chymase is a potent activator of angiotensin I, inactivates bradykinin and PAR-1 receptors, attacks the basement membrane of skin, processes type I procollagen, and stimulates mucus production from glandular cells. Biologically active c-Kit can be released from the cell surface by chymase. Although tryptase and chymase serve as the selective markers that distinguish MCs from other cell types and different MC subpopulations from one another, physiological functions for these enzymes in MCs are still not well-defined. In humans, two types of MCs have been identified based on their neutral protease compositions [2]. Connective tissue-type MCs (CT-MCs), predominantly located in normal skin and in intestinal submucosa, contain tryptase, chymase, MC carboxypeptidase, and cathepsin G in their secretory granules and are therefore named MC_{TC}. Mucosal-type MCs (M-MCs), the main type of MCs in normal alveolar wall and in small intestinal mucosa, contain tryptase in their secretory granules but lack the other proteases, and are named MC_T.

Conditions that influence the selective development or recruitment of CT-MCs and M-MCs are not well understood. Stem cell factor (SCF)-dependent in vitro-derived human MCs are considered as a model of M-MCs because they have a low percentage of chymase-expressing cells and display M-MC type immunopharmacological responses [3]. In these cultured MCs, cytokines such as interleukin (IL)-4, IL-6, and nerve growth factor reportedly induce chymase expression [4–6], but these

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chymase-positive cells do not release histamine in response to stimulation with substance P. Additional in vivo observations suggest that CT-MCs and M-MCs develop along distinct pathways. In humans with inherited combined immunodeficiency disease or with AIDS, marked and selective decreases in M-MC concentrations occur in the bowel, whereas the concentration and distribution of CT-MCs are unaffected [7]. This suggests that the appearance of M-MCs in tissues is dependent on functional T lymphocytes and that CT-MC development proceeds independently.

In murine fetal stages, MC precursors are highly concentrated in the yolk sac and fetal blood, suggesting that there exists a strong early embryonic wave of MC development [8, 9]. However, because of the impossibility of conducting experimental manipulations on human embryos, little is known about human MC development during the embryonic and fetal stages. Old World monkeys, such as the cynomolgus monkey (*Macaca fascicularis*), are widely used for medical research [10]. Recently, primate embryonic stem (ES) cell lines have been established, and hematopoietic differentiation from primate ES cells was induced successfully in vitro [11, 12]. Therefore, monkey ES cells may provide a useful model to elucidate early hematopoietic development in nonhuman primates as well as in human.

Here, we report the development of MCs from cynomolgus monkey ES cells by coculture with the murine aorta-gonad-mesonephros (AGM)-derived stromal cell line AGM-S1 with the addition of SCF, IL-6, and Flt3 ligand (FL). The primate ES-derived MCs contain abundant, metachromatically stained granules and were positive for both tryptase and chymase from early time points in their development. They express the high-affinity IgE receptor (FcεRI) on their surface, and the FcεRI is upregulated after exposure to IgE. Moreover, the ES-derived MCs responded to stimulation with IgE and substance P by releasing histamine. These results indicate that the primate ES-derived MCs have the phenotype of CT-MCs. The rapid maturation of ES-derived MCs suggests a unique embryonic pathway in primates for the early development of CT-MCs, which may be independent from the M-MC pathways.

MATERIALS AND METHODS

Cell Lines

The cynomolgus monkey ES cell line (CMK6), which had been transfected with an enhanced green fluorescent protein (GFP) driven by CAG promoter using Lipofectamine 2000 [13], was maintained as described [11]. The establishment of AGM-S1 cells and their potential to support human hematopoiesis had been reported elsewhere [14]. AGM-S1 cells within 10 passages were used throughout our experiments. AGM-S1 cells ($1–2 \times 10^5$) were cultured in 35-mm gelatin-coated dishes overnight to make a confluent feeder layer. On the next day, they were exposed to 15 Gy of radiation. The human MC line HMC-1 was cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). The procedures using primate ES cells, experimental animals, and human samples in this study were approved by the Internal Review Board of Kyoto University.

Coculture of Primate ES Cells with Murine AGM-S1 Cells

To induce hematopoietic differentiation, we used a primate ES cell and murine AGM-S1 cell coculture system that is similar to that in our recent report on human ES cell and murine fetal liver stromal cell coculture [15]. Briefly, approximately 20–30 undifferentiated primate ES cell colonies (each consisting of 500–1,000 cells) were physically picked up under a microscope using a fine glass rod, transferred in each well of six-well plate (diameter, 35 mm) that had been covered with irradiated confluent AGM-S1 cells, and cultured in α-MEM containing 15% FBS and no added cytokines. Culture medium was replaced every 3 days. At given time points, whole cells were collected with a 0.05% trypsin/EDTA solution (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) for further use.

Hematopoietic Colony Assay

Hematopoietic colony assay was done as described previously [16]. Briefly, 1 ml aliquots of a culture mixture containing primate ES/AGM-S1 coculture cells or ES-derived day 12–15 cultured cells, α-MEM, 1.2% methylcellulose (Shinetsu Chemical, Tokyo, http://www.shinetsu.co.jp), 30% FBS, 1% deionized fraction V bovine serum albumin (BSA), 10^{-4} M mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and a cocktail of cytokines with SCF, IL-6, IL-3, granulocyte-colony stimulating factor, thrombopoietin, and erythropoietin were plated in 35-mm suspension culture dishes (Nunc, Naperville, IL, http://www.nuncbrand.com). Colony types were determined on days 7–14 of the incubation by in situ observations using an inverted microscope, according to criteria established by Nakahata and Ogawa [17].

Harvest of Primate ES-Derived Cells and Secondary MC Cultures

Using a 10-μl microtip (Molecular BioProducts, San Diego, http://www.mbpinc.com/html/index.html), 5,000–10,000 primate ES-derived cobblestone (CS)-like cells were physically picked up on days 12–15 of the coculture and replated into a 35-mm culture well containing a freshly prepared, irradiated AGM-S1 feeder layer in medium containing 15% FBS with SCF, IL-6, and FL (secondary MC culture). Cytokines and their doses were as follows: 100 ng/ml SCF, 100 ng/ml IL-6 (both provided by Kirin Brewery, Maebashi, Japan, http://www.kirin.co.jp/english), and 20 ng/ml FL (R&D Systems Inc., Minneapolis, http://www.rndsystems.com). Half of the volume of culture medium was replaced with fresh medium twice a week.

Morphological Observations and Immunochemistry

From day 12 on, cells in secondary MC cultures were picked up and centrifuged onto glass slides every 3–6 days. Centrifuged preparations were then stained with May-Grünwald-Giemsa, acidic toluidine blue, Alcian Blue, and Safranin O solutions. For MC-specific tryptase and chymase assays, an alkaline phosphatase anti-alkaline phosphatase (APAAP) method (DakoCytomation, Glostrup, Denmark, http://www.dakocytomation.com) was used according to the manufacturer's instructions [16], and anti-human tryptase monoclonal antibody (mAb) (DakoCytomation) and anti-human chymase mAb (Chemicon, Temecula, CA, http://www.chemicon.com) were used. In some experiments, frozen cynomolgus monkey skin samples were stained by the same APAAP method to detect tryptase- and chymase-containing skin MCs.

Flow Cytometry

For immunological staining, cultured cells were preincubated with 10 μl of normal rabbit serum for 30 minutes to block Fc receptors on the cell surface. After a wash by phosphate-buffered saline (PBS) with 0.1% BSA, cells were stained for 30 minutes on ice with various mAbs conjugated with phycoerythrin (PE) or allophycocyanin (APC) or unconjugated mAbs. Samples stained with unconjugated mAbs were then incubated with PE- or APC-conjugated goat-derived anti-mouse mAbs for an additional 30 minutes. Stained cells were washed with BSA-containing PBS and analyzed using a FACSCalibur cytometry system (BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) with CellQuest software (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Propidium iodide-stained dead cells were gated out. mAbs against CD34, CD31, and HLA-DR (BD Biosciences), CD45 (DakoCytomation), c-Kit (Nichirei, Tokyo, http://www.nichirei.co.jp/english/index.html), FcεRI (CRA-1; eBioscience Inc., San Diego, http://www.ebioscience.com), and CD203c (Beckman Coulter, Miami, FL, http://www.beckmancoulter.com) were used. Isotopes of mouse

IgG (BD Biosciences) and goat IgG (DakoCytomation) were used as the negative controls.

Reverse Transcription-Polymerase Chain Reaction

To detect early development of the primate ES-derived hematopoiesis, total RNA was prepared for reverse transcription-polymerase chain reaction (RT-PCR) using TRIzol (Invitrogen) and then reverse-transcribed to cDNA using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen). The oligonucleotide primers were as follows: HPRT, forward (F), 5'-CTTGCGACCTTGACCATCTTTGGA-3', and reverse (R), 5'-GGCGTCGTGATTAGTGATGATGAACC-3'; OCT-4, F, 5'-CGTTCTCTTTGGAAAGGTGTT-3', and R, 5'-ACACTCGGACCACGTCTTTC-3'; c-kit, F, 5'-CAAGATTAGAAGCTGAAAACCT-3', and R, 5'-TCAAATCCATTGAGTACAATGC-3'; GATA1, F, 5'-GATCCCACAACTACATGGAAC-3', and R, 5'-ACAGTTGAGCAATGGGTACACC-3'; and CD34, F, 5'-TGCACCCTGTGTCTCAACATGG-3', and R, 5'-GCACAGCTGGAGGTCTTATTTTGC-3'. Primers for α-fetoprotein, Nestin, Brachyury, FLK-1, SCL, and GATA-2 have been published elsewhere [12].

Since the cynomolgus monkey-specific sequences were not available, we used the corresponding human or other Old World monkey genes to design our PCR primers for detection of MC-specific tryptase and chymase: glyceraldehyde-3-phosphate dehydrogenase, F, 5'-CGGGAAGCTTGTCATCAATGG-3', and R, 5'-GGCAGTGATGGCATGGACTG-3'; tryptase, F, 5'-GGAGCAGCACCTCTACTACC-3', and R, 5'-ATTCACCTTGCACACCAGGG-3'; and chymase, F, 5'-AAGGAGAAAGCCAGCCTGACC-3', and R, 5'-TCCGACCGTCCATAGGATACG-3'. PCR-amplified samples were sequenced by the dideoxynucleotide termination method with an automated sequencer (ABI3100; Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com).

Activation of MCs

For IgE stimulation, day 40 primate ES-derived MCs were cultured with the addition of 4 μg/ml IgE in the medium for 3 days. Cells were then harvested, washed, and stained with mAb against human FcεRI (CRA-1). In activation experiments, day 40 primate ES-derived MCs were washed and suspended in histamine-release buffer and preincubated for 5 minutes at 37°C. For stimulation, 5 μl of CRA-1 or control mouse IgG, or substance P (Sigma-Aldrich), was added to a 25-μl cell suspension (1×10^6 cells per milliliter) and incubated for an additional 15 minutes at 37°C. The reaction was stopped by adding 200 μl of ice-cold buffer. The cells were separated by centrifugation at 300*g* for 7 minutes at 4°C, and the supernatant was collected. The cell pellet was resuspended in 200 μl of buffer containing 0.5% Triton X-100 and 0.1% BSA and quick frozen in liquid nitrogen and thawed four times. After centrifugation at 12,000*g* for 15 minutes at 4°C, the soluble extract was collected. Histamine levels were measured by an enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter), and β-hexosaminidase activity was measured with pNA-β-D-glucosaminide in 0.1 M sodium citrate buffer, pH 4.5, as described previously [18].

Assay of Intracellular Histamine

The intracellular histamine concentration of cell lysates was measured using an ELISA histamine assay kit (Medical & Biological Laboratories, Nagoya, Japan, http://www.mbl.co.jp/e/index.html). Briefly, 500 ES-derived MCs were washed and pooled in 0.5 ml of PBS on days 40–50. The cell suspensions were then briefly frozen in liquid nitrogen and soon returned to a 37°C water bath to thaw. The freeze-thaw was repeated twice, and the cell lysates were then examined for histamine content according to the manufacturer's protocol. For comparison, histamine concentration of day 70 human cord blood (CB)-derived MCs were analyzed by the same method.

MC Culture from CB Progenitors

Human MC culture was performed as reported previously [19], with slight modifications. Briefly, human CB was obtained from a normal full-term delivery with informed consent. Mononuclear cells were separated by density-gradient centrifugation, and CD34+ cells were isolated using a CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). The separated cells were cultured with 5% FCS, 100 ng/ml SCF, and 50 ng/ml IL-6 in AIM-V medium (Invitrogen).

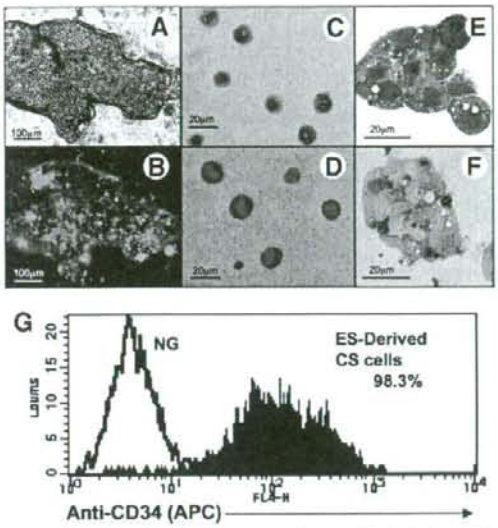

Figure 1. Cynomolgus monkey embryonic stem (ES)-derived cobblestone (CS)-like cells in coculture with AGM-S1 cells. **(A):** CS-like cells derived from ES cells in coculture with AGM-S1 cells on day 14 (original magnification, ×200). **(B):** Fluorescence micrograph of **(A)** showing that CS-like cells are derived from green fluorescent protein-positive ES cells (original magnification, ×200). **(C):** May-Grünwald-Giemsa staining of ES-derived day 14 CS-like cells (original magnification, ×400). **(D):** ES-derived CS-like cells were positive for CD34, as indicated by anti-human CD34 APAAP staining (original magnification, ×400). **(E):** May-Grünwald-Giemsa staining of undifferentiated ES cells. **(F):** Negative staining of CD34 by alkaline phosphatase anti-alkaline phosphatase (cells were only shown the counterstaining of undifferentiated ES cells by Carrazi's hematoxylin solution). **(G):** A fluorescence-activated cell sorting profile shows that ES-derived CS-like cells are almost all CD34 positive. NG shows the negative staining for CD34 on undifferentiated ES cells. Abbreviations: APC, allophycocyanin; NG, negative control.

Statistical Analysis

In some experiments, data are presented as the mean ± SD. Statistical significance was determined with the Student *t* test. *p* values less than .05 were considered significant.

RESULTS

Development of Multipotential Hematopoietic Progenitor Cells from Primate ES Cells

Primate ES cells, cocultured on a confluent monolayer of irradiated AGM-S1 cells [14] in α-MEM containing 15% FCS and no added cytokines, began to differentiate concomitantly with rapid proliferation at day 3–5. By days 8–10, some areas where the cells accumulated grew to form translucent sac-like structures containing bright, small, round cells that looked like congested CS-like cells (Fig. 1A). Because we used cynomolgus monkey ES cells that were transfected with GFP, fluorescent microscopy revealed that these CS-like cells were positive

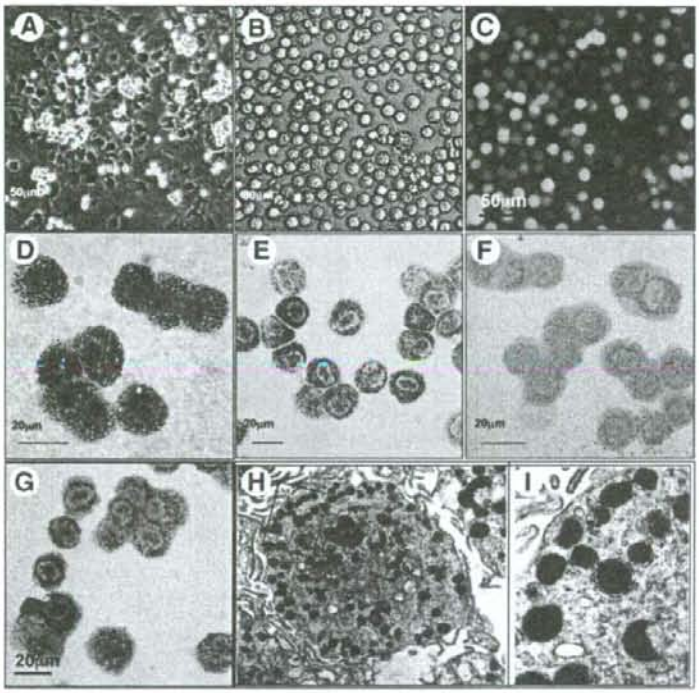

Figure 2. Phenotype of cynomolgus monkey embryonic stem (ES)-derived mast cells (MCs). **(A):** Phase-contrast dark cell clusters could be observed after 3 days when day 14 primary ES-derived cobblestone-like cells were replated on aorta-gonad-mesonephros-S1 cells in secondary MC cultures (original magnification, ×100). **(B):** On day 12, secondary MC culture produced a uniform cell population of round, small, granule-containing cells (original magnification, ×200). **(C):** Green fluorescent protein-expressing cells shown in **(B)** (original magnification, ×200). **(D):** On day 21 of secondary MC culture, ES-derived cells displayed a typical MC phenotype when stained by May-Grünwald-Giemsa solution (original magnification, ×400). **(E):** Cells from these cultures stained with toluidine blue (original magnification, ×400). **(F):** The same cultures stained with Alcian Blue (original magnification, ×400). **(G):** Safranin O-stained cells from the same cultures. **(H):** Electron micrograph of day 40 ES-derived MCs. Typical rough, dark granules; pseudopodia; highly developed mitochondria; and Golgi bodies could be observed. **(I):** An enlarged view of the box in **(H)**, showing granules with various electron densities.

for GFP, indicating that they were derived from the ES cells (Fig. 1B).

RT-PCR analysis revealed that along with downregulation of the undifferentiated ES marker OCT-4, various developmental markers of endoderm (such as the hepatocyte marker AFP), ectoderm (such as the neural stem cell marker Nestin), and mesoderm (such as the early mesoderm marker Brachyury), as well as hemoangiogenic markers critical for primitive and definitive hematopoiesis (such as FLK-1, SCL, c-kit, GATA-1, GATA-2, and CD34) were simultaneously expressed (supplemental online Fig. 1). These data indicate that AGM-S1 cells are able to support hematopoietic differentiation of the primate ES cells.

At day 14, the CS-like cells in the transparent sac-like areas had proliferated to the extent that we were able to pick them up by a simple physical method. Routinely, a single 35-mm culture well gave rise to $1–2 \times 10^4$ CS-like cells at days 10–15. These CS-like cells showed a homogeneous phenotype of mononucleated small cells, similar to undifferentiated hematopoietic stem/progenitor cells, as indicated by May-Grünwald-Giemsa staining (Fig. 1C). More than 95% of the primate ES-derived CS-like cells were CD34+ by immunochemical staining and fluorescence-activated cell sorting (FACS) analysis (Fig. 1D, 1G), whereas undifferentiated ES cells were negative for CD34 expression (Fig. 1E–1G). A clonal cell culture showed that hematopoietic activities were highly concentrated in these CS-like cells. ES-derived day 14 CS-like cells, when planted at 5,000 per assay, generated 234 ± 36 total hematopoietic colonies (including 24 ± 6 mixed colonies), which was 134-fold more than the total coculture cells at the same time point (supplemental online Table 1). These CS-like cells also generated CD144+/CD31+ endothelial-like cells when cultured by adding vascular endothelial growth factor (data not shown). Thus, the primate ES-derived CS-like cells may represent a highly concentrated fraction of hematopoietic and hemangioblastic progenitors.

MC Differentiation from ES-Derived CS-Like Cells

Physically harvested day 14 ES-derived CS-like cells were replated in a secondary coculture on newly irradiated AGM-S1 cells to induce MC differentiation (secondary MC culture) with a cytokine cocktail of SCF, IL-6, and FL. ES-derived phase-contrast dark cell clusters appeared within 24 hours (Fig. 2A). These cell clusters grew gradually during the first 5 days and showed a robust cell proliferation from day 7, with the phase-dark cells floating in the medium and becoming bright, round, small cells. These cells further proliferated during the following week and gradually developed granules in the cytoplasm (Fig. 2B). GFP expression demonstrated that these granule-containing cells were all derived from ES cells (Fig. 2C).

May-Grünwald-Giemsa staining of the cells at day 21 revealed that they gave rise to a uniform phenotype of rough basophilic granule-containing cells (Fig. 2D). The granules in these cells showed a characteristic metachromatic staining pattern when stained with acidic toluidine blue (Fig. 2E), and they were also positive for Alcian Blue and Safranin O (Fig. 2F, 2G). However, undifferentiated ES cells were never positively stained with these stains (supplemental online Fig. 2). Electron microscopic analysis of day 40 ES-derived cells from the secondary MC cultures revealed that the cells had many pseudopods and rough, dark granules of various densities (Fig. 2H, 2I). FACS analysis revealed a homogenous fraction in which the majority of the cells strongly expressed c-Kit (99.9%), CD45 (99.7%), and CD31 (99.8%), mildly expressed CD203c (72.2%), and were negative for HLA-DR (0.8%) (Fig. 3). Interestingly, more than half of these cells (51.3%) coexpressed FcεRI, a marker for functionally mature MCs. Thus, in view of

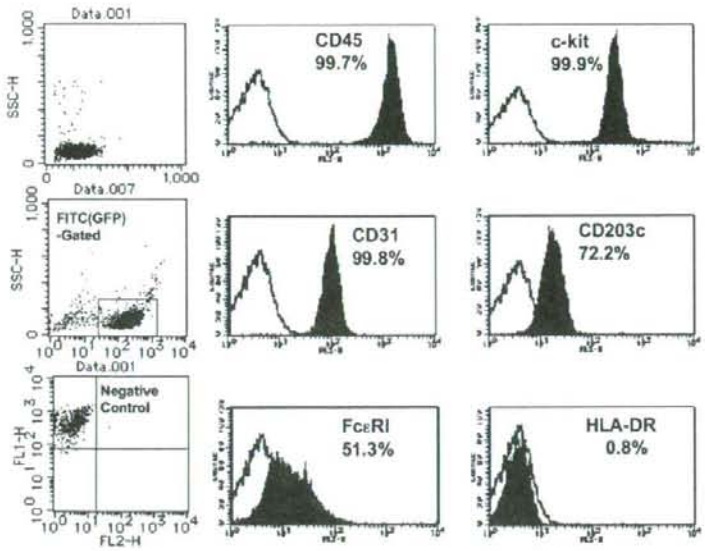

Figure 3. Fluorescence-activated cell sorting profiles of embryonic stem (ES)-derived day 21 mast cells (MCs). A homogeneous cell population of low side scatter and forward scatter could be seen (upper left panel), most of which expressed green fluorescent protein (middle left panel). The monkey ES-derived MCs were c-Kit+/CD45+/CD31+/CD203+/FcεRIdim+/HLA-DR−.

the unique staining pattern and surface phenotype of c-Kit+/CD45+/CD31+/CD203c+/HLA-DR−, these ES-derived basophilic granule-containing cells are MCs.

ES-derived MCs reached peak growth at approximately day 21, at which point there were 480-fold as many floating cells as initial CS-like cell input into the secondary MC culture (Fig. 4A). After day 21, cell proliferation rates gradually decreased. However, when 1×10^5 day 40 ES-derived MCs from secondary MC cultures were replated on fresh AGM-S1 feeder cells with a cytokine cocktail, the cells proliferated continuously to $1.47 \times 10^5 \pm 0.14 \times 10^5$ on day 50 ($n = 4$). Repeated, replating of 1×10^5 day 50 ES-derived MCs gave rise to $1.30 \times 10^5 \pm 0.11 \times 10^5$ cells ($n = 4$) after another 10 days in culture (Fig. 4B). Stable proliferation of ES-derived MCs was observed through day 80 in our experiments. Thus, these ES-derived granule-containing cells retain their proliferative potential, as demonstrated with MCs purified from human skin [20].

Cytokine Dependence of ES-Derived MCs

Since ES-derived CS-like cells were still heterogeneous, different cytokines might have pleiotropic effects by stimulating hematopoietic progenitors at early times in culture and MC progenitors at later times. To determine the factors critical for the development of ES-derived MCs, we compared various combinations of cytokines in the secondary MC culture. Figure 4C shows the proliferation of total cells by day 20 in secondary MC cultures from three independent experiments. Neither SCF nor FL alone supported cell proliferation by ES-derived CS-like cells. Combinations SCF+IL-6 and SCF+FL generated a low level of cell proliferation, with the former mixture supporting the MC phenotype (MC 88.6%), whereas the latter mainly stimulated macrophage growth along with a weak MC activity (MC 11.8%). This cytokine requirement was consistent with human MC development in vitro from CB. The combination FL+IL-6 did not support cell proliferation or development of MCs. However, in cultures treated with SCF, FL, and IL-6, ES-derived CS-like cells gave rise to a robust production of pure MCs (99.3%) (Fig. 4C). Further addition of other known MC-stimulating factors (such as IL-4, IL-9, IL-10, and IL-3) only gained ambiguous results for MC production, mostly paralleling with a promoted growth of macrophages and granulocytes (data not shown).

ES-Derived MCs Are Positive for both Tryptase and Chymase

With mAbs reactive to human MC specific tryptase and chymase, we found that approximately 10% of ES-derived MC cells stained double-positively for both proteases as early as day 12 in the secondary MC culture (Fig. 5A, 5B). To our surprise, the double-positive cells rapidly increased to 98%–99% at day 18 and 100% at day 21, along with robust cell proliferation. By adopting the previous criteria to determine the intensity of staining [4], we found that ES-derived MCs showed strongly positive staining for chymase from early in the culture period (Fig. 5B, 5D), whereas they stained only weakly for tryptase (Fig. 5A, 5C). At all time points of the secondary MC culture, the percentage of tryptase strongly positive cells did not exceed that of chymase strongly positive ones. These observations were quite different from in vitro-derived human MCs from BM, CB, or fetal liver, in which tryptase was always expressed earlier and more strongly than chymase [19, 21]. Because the mAbs we used to detect tryptase and chymase in monkey ES-derived MCs were specific to human antigens, we confirmed that both mAbs reacted with MCs in cynomolgus monkey skin (Fig. 5E, 5F). These data indicate that the weak reaction against tryptase in monkey ES-derived MCs is not due to low specific binding by the anti-human tryptase mAb.

To confirm the results of immunochemistry, we performed RT-PCR analysis, and clear and specific bands were developed from day 21 primate ES-derived MCs (Fig. 5G). Consistent with the immunochemistry results, the expression level of chymase mRNA in ES-derived MCs was much higher than that of tryptase, in contrast to human CB-derived MCs (cultured more than 8 weeks) and a human MC line, HMC-1, both of which show stronger expression of tryptase than chymase. Since the cynomolgus monkey tryptase and chymase sequences were not available, the homology of PCR-amplified genes was further confirmed by direct cDNA sequencing analysis. As a result, the homologies in the PCR-amplified region of the tryptase and

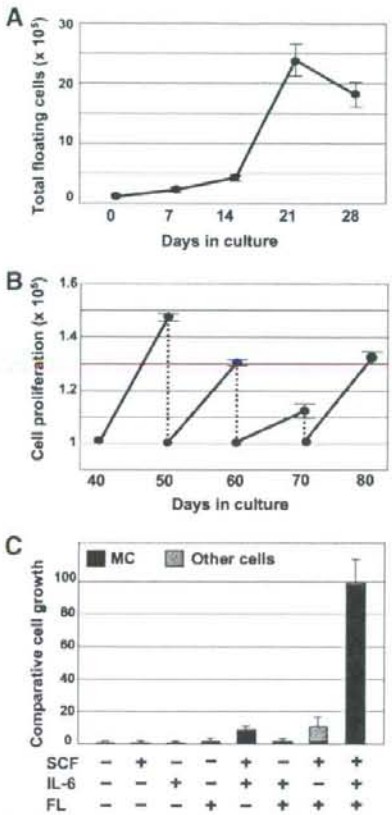

Figure 4. Proliferation, recruitment, and cytokine requirements of cynomolgus monkey embryonic stem (ES)-derived mast cells (MCs). **(A):** 5×10^3 ES-derived cobblestone (CS)-like cells were plated in a 35-mm culture dish in secondary MC culture. At given times, floating cells were quantified. A peak of 480-fold proliferation could be observed at day 21. **(B):** Continuous proliferation of ES-derived MCs starting from day 40 of secondary MC culture to day 80, with 10-day replating intervals, showing typical characteristic of MC recruitment. **(C):** 1×10^4 ES-derived CS-like cells were replated in a 35-mm culture dish in the secondary MC culture with various cytokine combinations. On day 20, the number of floating cells was quantified. Results are shown in converted values from three different experiments, normalized to the highest cell growth of the three-cytokine combination (stem cell factor + interleukin-6 + Flt3 ligand) as 100%. The MC phenotype was confirmed by May-Grünwald-Giemsa staining. Abbreviations: FL, Flt3-ligand; IL-6, interleukin-6; SCF, stem cell factor.

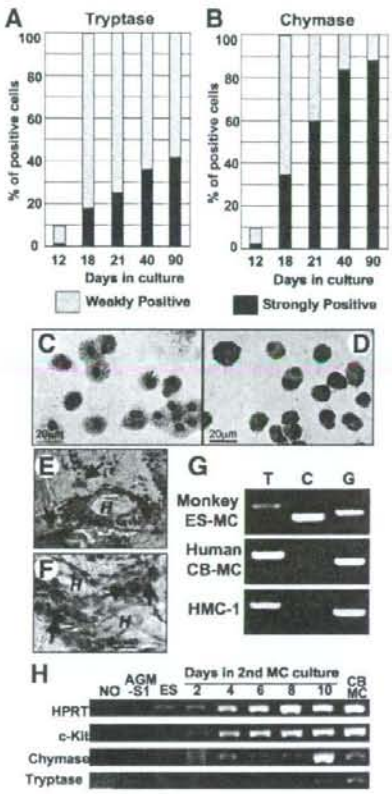

Figure 5. Differentiation of tryptase/chymase double-positive mast cells (MCs) from primate embryonic stem (ES)-derived cobblestone-like cells. **(A):** A time-course of ES-derived MC differentiation defined by staining with MC-specific tryptase-positive cells. Most of the tryptase-positive cells reacted weakly with the monoclonal antibody (mAb) that recognizes human tryptase. **(B):** The same time-course analysis as in **(A)**, showing a tendency for time dependently strong expression of chymase in ES-derived MCs. **(C):** Day 40 ES-derived MCs positively stained with an mAb against human tryptase; more than half of the cells showed weak to mild staining (tryptase strongly positive, 36%). **(D):** The same day 40 ES-derived MCs as in **(C)** stained strongly with an mAb to human chymase (chymase strongly positive, 83%). **(E):** Adult cynomolgus monkey skin section stained with the same tryptase mAb as in **(C)**, showing a strong positive reaction (arrows). **(F):** The same monkey skin sample stained with chymase mAb, showing an intensity similar to that of tryptase staining (arrows). **(G):** Reverse transcription-polymerase chain reaction analysis of tryptase and chymase gene expression on ES-derived MCs (day 21), human CB-derived MCs (more than 8 weeks), and a human MC line, HMC-1. Stronger expression of chymase relative to tryptase was observed in ES-derived MCs. **(H):** A time-course RT-PCR analysis of expression of tryptase and chymase in ES-derived MCs. Chymase mRNA expression appeared earlier (day 4) than tryptase expression (day 8) in the secondary MC culture. Abbreviations: AGM, aorta-gonad-mesonephros; C, chymase; CB, cord blood; ES, undifferentiated embryonic stem cells; G, glyceraldehyde-3-phosphate dehydrogenase; H, hair; NO, no transcripts; T, tryptase.

chymase genes between human and monkey were 86% and 98%, respectively (supplemental online Fig. 3). In a time course study, chymase mRNA could be detected as early as day 4 in the secondary MC culture, whereas detection of tryptase mRNA could only be confirmed at day 8 (Fig. 5H).

The Primate ES-Derived MCs Are Functionally Mature CT-MCs

Analysis of intracellular histamine content revealed that ES-derived MCs contained histamine at 7.4 ± 0.5 to 7.6 ± 0.6 pg/cell at day 40 and 10.1 ± 1.8 to 11.3 ± 1.6 pg/cell at day 50. Moreover, in two separate experiments, ES-derived day 50 MCs had a higher histamine content than human CB CD34+ cell-derived day 70 MCs (8.6 ± 1.2 to 9.4 ± 1.1 pg/cell), indicating full maturation of ES-derived MCs (Table 1).

FACS analysis revealed that by day 21 of the secondary MC culture, these ES-derived MCs expressed FcεRI on their surface