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

Kenji Nagao [a], Takayuki Ohta [b], Atsushi Hinohara [b], Tomoyuki Tahara [b], Tetsuya Hagiwara [a], Yoshitake Maeda [a], Takashi Yoneya [b], Yoshiaki Sohma [b], Toshio Heike [c], Tatsutoshi Nakahata [c], Yoshimasa Inagaki [b,*], Mitsuo Nishikawa [b,1]

[a] *Frontier Laboratory, Kirin Pharma CO., LTD., 3 Miyahara, Takasaki, Gunma 370-1295, Japan*
[b] *Discovery Research Laboratories, Kirin Pharma CO., LTD., 3 Miyahara, Takasaki, Gunma 370-1295, Japan*
[c] *Department of Pediatrics, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

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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

* Corresponding author. Fax: +81 27 346 1971.
E-mail address: yoshimasa.inagaki@kyowa-kirin.co.jp (Y. Inagaki).
[1] Present address: Interprotein Corporation, SIC-1-202, 5-4-21 Nishihashimoto, Sagamihara, Kanagawa, 229-1131, Japan.

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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 6000*g* 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′.

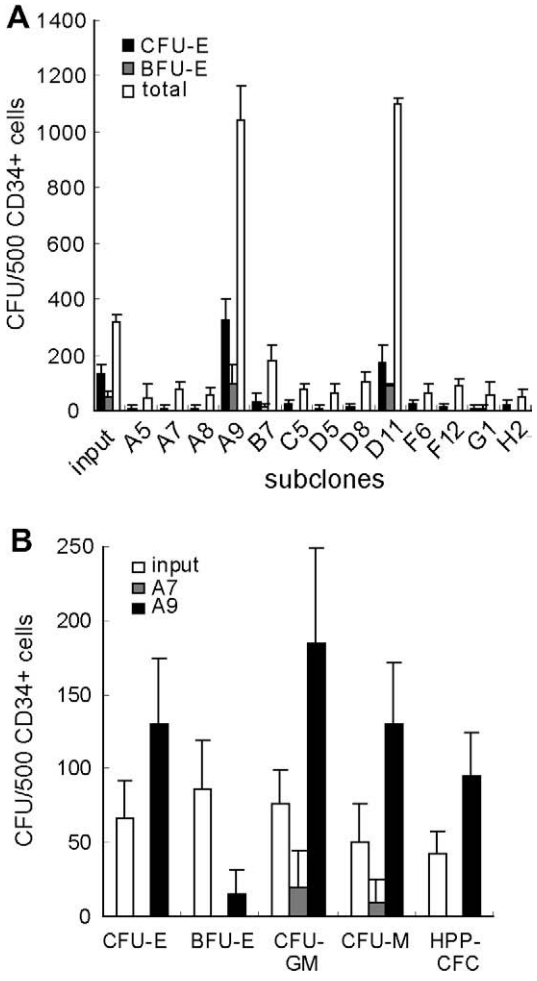

Fig. 1. Subcloning of AGM-S3 stromal cells. (A) Coculture of human CB $CD34^+$ cells with AGM-S3 derived subclones. Human CB $CD34^+$ cells (500 per well) were added to the stromal cells and cultured for two weeks. Clonogenic progenitors were evaluated by culturing a fraction of the cells after they 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-Kit^+, Sca-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.

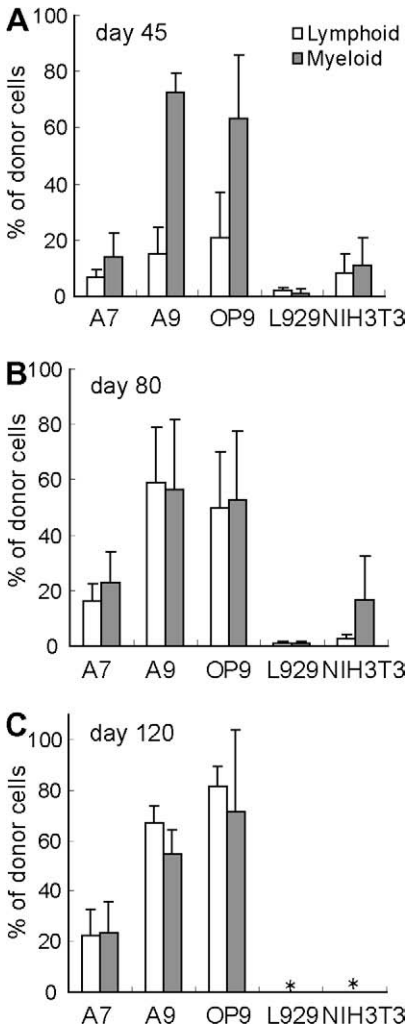

Fig. 2. Hematopoietic reconstitution via coculture of mouse BM 34-KSL cells with AGM-S3-A7, AGM-S3-A9, OP9, L929, or NIH3T3 cells. The cocultured 34-KSL cells were transplanted into a group of lethally irradiated mice ($n=5$). The percentage of Ly-5.1^+ cells in lymphoid cells (Thy-1^+ and $B220^+$) and in myeloid cells (Gr-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 (Il1m)	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

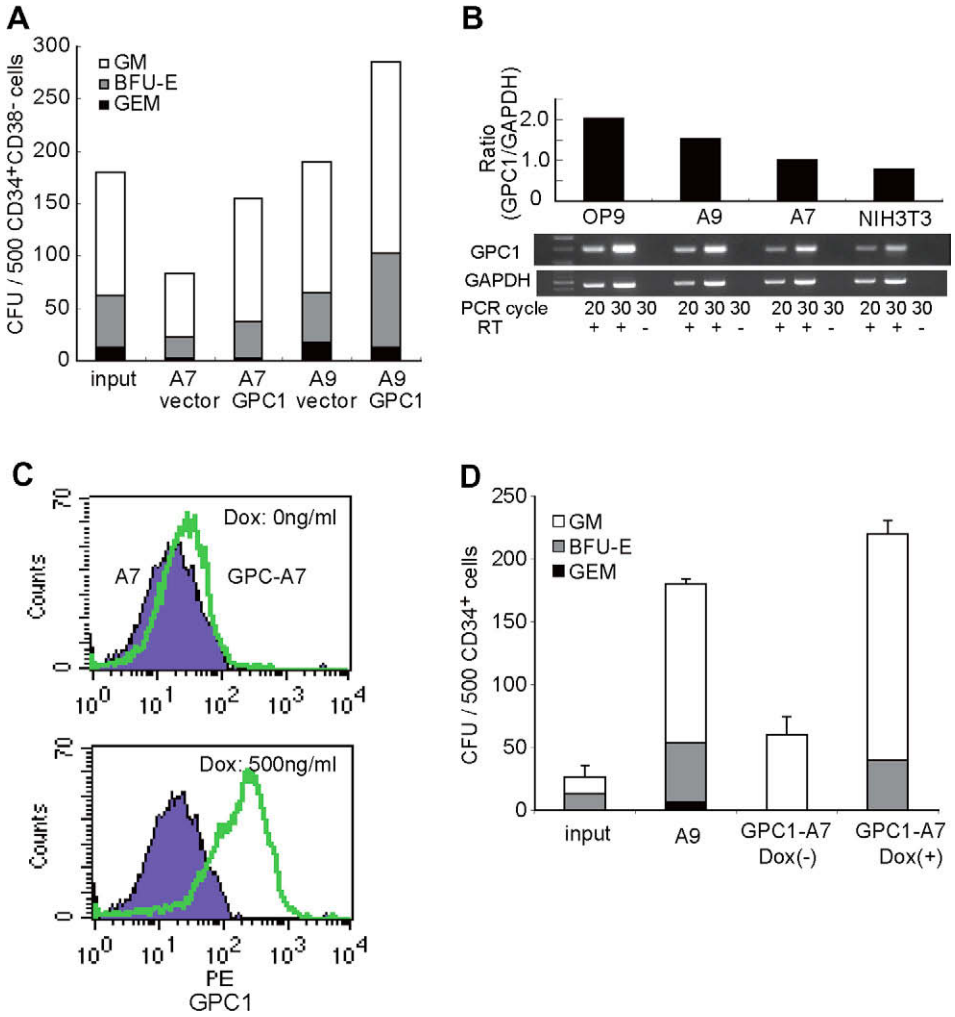

Fig. 3. Hematopoietic supportive activity of Glypican-1. (A) Coculture of Human CB CD34$^+$ CD38- cells with AGM-S3-A9 or AGM-S3-A7 cells that had been retrovirally infected with the GPC-1 expression vector. A total of about 500 human CB CD34$^+$ CD38- cells per well were added to the stromal cells and 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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