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Immune suppressor factor confers stromal cell line with enhanced supporting activity for hematopoietic stem cells

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Abstract

Immune suppressor factor (ISF) is a subunit of the vacuolar ATPase proton pump. We earlier identified a short form of ISF (ShIF) as a stroma-derived factor that supports cytokine-independent growth of mutant Ba/F3 cells. Here, we report that ISF/ShIF supports self-renewal and expansion of primary hematopoietic stem cells (HSCs). Co-culture of murine bone marrow cells with a stromal cell line overexpressing ISF or ShIF (MS10/ISF or MS10/ShIF) not only enhanced their colony-forming activity and the numbers of long-term culture initiating cells, but also maintained the competitive repopulating activity of HSC. This stem cell supporting activity depended on the proton-transfer function of ISF/ShIF. Gene expression analysis of ISF/ShIF-transfected cell lines revealed down-regulation of secreted frizzled-related protein-1 and tissue inhibitor of metalloproteinase-3, and the restoration of their expressions in MS10/ISF cells partially reversed its enhanced LTC-IC supporting activity to a normal level. These results suggest that ISF/ShIF confers stromal cells with enhanced supporting activities for HSCs by modulating Wnt-activity and the extracellular matrix.

Keywords: Immune suppressor factor; ISF; ShIF; Hematopoietic stem cell; HSC; Stromal cell; Co-culture

Self-renewal, proliferation, and differentiation of hematopoietic stem cells (HSCs) are governed by a variety of environmental cues mainly supplied by the stem cell niche, a microenvironment where HSCs reside in the bone marrow [1]. Such cues comprise signals from various cytokines, growth factors, and cell surface molecules that are expressed in the stem cell niche. However, the precise nature of the signaling networks between HSC and stem cell niche is complex and poorly understood.

It has been widely accepted that the direct contact between HSC and bone marrow stroma is critical for maintaining HSC in a immature state, since attempts to maintain or expand HSCs in vitro by a defined combination of cytokines have been largely unsuccessful [2] and it could only be achieved by co-cultivation on bone marrow stromal cells [3]. Along this line, many researchers have tried to identify cell surface molecules of stromal cells that are essential for HSC-support [4]. We earlier reported the short form of immune suppressor factor (ISF), ShIF, as a stroma-derived factor that is capable of supporting a mutant subline of Ba/F3 cells which became stroma-dependent by chemical mutagenesis [5]. ISF was first cloned as a

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suppressor of the T-cell response [6], but later proved to be the subunit of vacuolar ATPase proton pumps, an energy-dependent proton transport complex critical for a wide variety of biological functions in vivo [7]. We found that a stromal cell line overexpressing ShIF could support interleukin-3 (IL-3)-independent growth of mutant Ba/F3 and suggested the possibility that ShIF could also support the growth of primary hematopoietic cells [5].

Vacuolar H^+ ATPases (V-ATPases) are multi-subunit proton pumps that are highly conserved throughout organisms and are critical for acidification of various intracellular compartments such as lysosomes, endosomes, secretory vesicles, and clathrin-coated vesicles [8]. Intracellularly localized V-ATPases are essential for receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, and protein processing and degradation. In addition to intracellular compartments, V-ATPases also localize in the plasma membrane of certain type of cells such as renal cells, osteoclasts, and macrophages, where they are critical for secretion of proton ions, bone resorption, and control of cytoplasmic pH. Vacuolar ATPase is composed of two functional sectors, V_1 and V_0. V_1 sector, which is composed of eight different subunits, contains catalytic sites for ATP. V_0 sector is an integral complex that forms a pore for transporting proton ions. V_0 sector consists of five different subunits (a, c, c′, c″, and d) and subunit 'a' is the product of four isoforms, a1–a4. ISF corresponds to the a2 isoform and is expressed ubiquitously in various tissues including heart, brain, liver, and kidney. Although the physiological roles of a3 and a4 subunits are evident in osteoclasts [9] and renal intercalated cells [10], respectively, the role of the a2 subunit has remained unknown.

Here, we demonstrate that ISF or ShIF can enhance supportive capacity of stromal cells for primary HSC in vitro. Bone marrow stromal cell lines overexpressing ISF or ShIF showed an enhanced supportive capacity for HSC by clonogenic assays, long-term culture-initiating cell (LTC-IC), and long-term reconstitution assays. Proton transport activity of ISF/ShIF was essential for these effects and decreased expression of secreted frizzled-related protein (SFRP)-1 and tissue inhibitor of metalloproteinase-3 (TIMP-3) seemed to be the critical downstream target of ISF. These findings should lead to develop novel strategies for HSC expansion using stromal cells in vitro.

Materials and methods

Cell lines and culture. MS10, PA6, OP9, AGM-S3, and PLAT-E cells were maintained in αMEM/10% fetal bovine serum (FBS, Sigma), DMEM (F-12)/10% FBS, αMEM/20% FBS, αMEM/10% FBS, and DMEM/10% FBS, respectively. MS10 and PA6 cells expressing ISF or ShIF are described previously [5]. MS10 cells expressing ISF and TIMP-3 or SFRP-1 were established by infecting MS10/ISF cells with pMXs-IG/TIMP-3 or SFRP-1 retrovirus and sorting GFP-positive cells by FACS. All mediums were supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Flow cytomertry. Antibodies used in flow cytometry were anti-Sca-1-FITC, anti-c-Kit-PE, anti-Gr-1-PE, anti-CD11b-PE, anti-B220-PE, anti-CD3-PE, and anti-CD45.1-FITC, and all were purchased from Pharmingen. Depletion of lineage marker-positive cells was done using MACS Lineage depletion kit (Miltenyi Biotec) according to the manufacturer's protocol.

Co-culture of bone marrow cells and hematopoietic stem cell/progenitor assay. Bone marrow cells were harvested from 8- to 12-week-old C57BL/6 mice by flushing out of femurs and tibias. MS10 or PA6 cells expressing ISF or ShIF were seeded onto a 24-well dish and subconfluent layers were mitotically inactivated by 1500 rads of irradiation. Bone marrow cells (1×10^4) or 100 c-$Kit^+Sca\text{-}1^+lineage^-$ (KSL) cells were plated in a well and cultured for 7 days in αMEM/10% FBS without cytokines. Cells were harvested and subjected to colony assays using MethoCult3434 (Stem Cell Technologies) or bone marrow reconstitution assays, as described below. In the case of bulk LTC-IC assay, cells were co-cultured for 5 weeks and subjected to colony assays using MethoCult3434. For bone marrow reconstitution assay, we isolated KSL cells from C57BL/6-Ly5.1 mice (Sankyo Laboratory) by FACS sorting using FACS Vantage (Beckton–Dickinson) and performed co-cultivation on stromal cells for 7 days. Cells were collected by trypsinization and transplanted into lethally irradiated (950 rads) recipient mice (C57BL/6-Ly5.2) with 2×10^5 of bone marrow cells (Ly5.2) as a competitor. Contribution of Ly5.1 cells for peripheral blood was analyzed after 5, 10, and 20 weeks of transplantation by FACS. All animal experiments were reviewed and approved by Institutional Review Board.

Cobblestone-like area forming cell (CAFC) assay. Bone marrow cells (1×10^4/well) were plated on mitotically inactivated MS10 cells expressing ISF or ShIF in 24-well plates. Three to four days later, numbers of cobblestone-like area were counted under a microscope.

cDNA, plasmids, and retrovirus production. Murine TIMP-3 and SFRP-1 cDNA was obtained by PCR with *Pfu* polymerase (Stratagene) using bone marrow cDNA as a template. Amplified cDNAs were subcloned into pMXs-IG [11]. Integrity of the amplified sequence was confirmed by DNA sequencing. Retrovirus was produced as described [12]. Briefly, retrovirus vector was transiently transfected into PLAT-E cells using Fugene (Roche diagnostics), and retrovirus supernatant was collected after 48 h of transfection. Retrovirus infection was carried out in the presence of 10 μg/ml of Polybrene (Sigma).

Gene expression profiling by microarray. Total RNA was extracted from MS10 cells expressing vector alone, ISF or ShIF by Trizol (Invitrogen). cRNA was generated and hybridized against the Affymetrix HG_U95Av2 oligonucleotide arrays according to the manufacturer's protocol. The arrays were scanned using a Hewlett–Packard confocal laser scanner and analyzed using MicroArray Suite 5.0 (Affymetrix), GeneSpring 4.0 (Silicon Genetics, Redwood City, CA).

Northern blot. Total RNA was extracted from 1×10^7 cells by TRIzol (Gibco-Invitrogen) according to the manufacturer's protocol. The RNA samples (20 μg/lane) were separated on a formaldehyde-denaturing 1.0% agarose gel and transferred onto Hybond N+ membrane (Amersham). Full-length cDNA of ISF, TIMP-3, and SFRP-1 was used as probes. All probes were labeled using a Rediprime kit (Amersham). Hybridizations with ^{32}P-labeled probes were carried out in ExpressHyb buffer (Clontech) according to the manufacturer's protocol. The membranes were washed in 2× SSC, 0.1% SDS washing buffer for 30 min at room temperature with several buffer changes, followed by washing twice in 0.1× SSC, 0.1% SDS for 15 min at 42 °C. The membranes were exposed on XAR films (KODAK) at −80 °C for 1–5 days.

RT-PCR. Total RNA was reverse-transcribed by Superscript II reverse transcriptase (Gibco-Invitrogen) using a random hexamer. The amount of cDNA was normalized by GAPDH. Semi-quantitative RT-PCR was run for 25–35 cycles by Ex *Taq* polymerase (Takara) using normalized cDNAs as templates. Appropriate PCR cycles were chosen to obtain linear phase of amplification. The primers used were: GAPDH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′; SFRP-1 forward 5′-CTA CGT GAG CTT CCA GTC CG-3′, reverse 5′-TGG AGG ACA CAC GGT TGT AC-3′; and TIMP-3 forward 5′-TAC CAT GAC TCC CTG GCT TG-3′, reverse 5′-TGC AAT TGC AAC CCA GGT GG-3′.

Results

ISF and ShIF enhance clonogenic activity of primary bone marrow cells

To determine if ISF/ShIF expands the progenitor pool of primary bone marrow cells, we first examined their clonogenic activity by co-culturing them on stromal cell lines (MS10 or PA6) overexpressing ISF or ShIF. Murine bone marrow cells were cultured on ISF/ShIF-expressing bone marrow stromal cell line (PA6/ISF and PA6/ShIF) without cytokines for seven days. At day seven, cells were harvested by trypsinization and subjected to colony assay. As shown in Table 1, total colony numbers were significantly increased about 1.2- to 2.0-fold in PA6/ISF or PA6/ShIF compared with findings with freshly prepared cells (''before culture'' in Table 1). These differences were even greater (3- to 5-fold) when compared with cells co-cultured on control stroma (PA6/mock). Similar results were obtained with MS10 overexpressing ISF/ShIF (data not shown). Notably, the colonies formed by the cells cultured on MS10 or PA6 overexpressing ISF/ShIF were markedly larger in size compared with the cells cultured on mock control (Figs. 1A and B). We performed the same experiment with a highly purified HSC fraction, c-Kit^{+}Sca-1^{+} Lineage negative (KSL) cells, and obtained the same result (data not shown). These results strongly indicate that ISF/ShIF aids to maintain the immature fraction of clonogenic progenitors.

Flow cytometric analysis revealed that the fraction of c-Kit^{+}Sca-1^{+} cells was significantly increased when whole bone marrow cells were cultured on MS10/ISF or MS10/ShIF (Fig. 1C), again confirming that stromal cells expressing ISF/ShIF maintain an immature fraction of bone marrow cells.

To further examine the effect of ISF/ShIF on more primitive hematopoietic progenitors, we did CAFC and LTC-IC assays, which were shown to highly correlate the frequency of primitive hematopoietic cells. CAFC activities increased about 2-fold when cells were cultured on MS10/ISF or ShIF (Fig. 1D). Similarly, co-cultivation of bone marrow cells on MS10/ISF and MS10/ShIF resulted in an approximately 8- to 10-fold increase of LTC-IC activities (Fig. 1E).

Table 1
Colony formation of bone marrow cells cultured on PA6-expressing ISF/ShIF

	GM	Mk	B	EM	Mix	Total
Before culture	64 ± 8	0	0	2 ± 1	6 ± 3	74 ± 4
Mock	28 ± 4	0	0	0	0	28 ± 4
ISF	142 ± 8	0	0	0	0	142 ± 8
ShIF	86 ± 16	0	0	0	0	86 ± 16

Numbers are day 7-colonies formed from 2×10^4 unfractionated bone marrow cells. ''Before culture'' denotes colonies from freshly prepared bone marrow cells. GM, CFU-granulocyte/macrophage, Mk, CFU-megakaryocyte; B, BFU-E; EM, CFU-erythrocyte/megakaryocyte; Mix, CFU-mix.

These results suggest that ISF/ShIF overexpression in stromal cells confers an enhanced maintenance capacity for primitive hematopoietic progenitors.

ISF/ShIF maintains hematopoietic stem cells in vitro

To determine if HSCs with a long-term repopulating potential are maintained on the ISF/ShIF-expressing stroma, we assessed the long-term repopulating activity of co-cultured cells by competitive repopulation assay in mice. We purified KSL cells from Ly5.1 mice by FACS sorting and cultured them on stromal cells overexpressing ISF/ShIF or AGM-S3. After cultivation with no cytokines for 7 days, cells were harvested and transplanted into C57BL/6 mice (Ly5.2) with 2×10^5 of Ly5.2 bone marrow cells as a competitor. Cells cultured on parental stromal cells engrafted only 17% of mice (Table 2). Surprisingly however, cells cultured on ISF- or ShIF-expressing stroma showed a high repopulating frequency (75% and 82%, respectively), nearly comparable to those cultured on AGM-S3 cells, a well-known cell line for its potent activity to maintain HSCs [13]. Peripheral blood contribution of cells cultured on ISF- or ShIF-expressing stroma remained high (5–16%) from 5 to 20 week post-transplant, whereas cells from mock culture did not show any significant contribution (less than 1%) at these time points (Fig. 2A). Cells co-cultured on ISF/ShIF-expressing stroma repopulated all hematopoietic lineages including myeloid, B and T cells (Fig. 2B). These results suggest that HSCs with long-term repopulating potential can be effectively expanded by co-culturing with ISF/ShIF-expressing stroma with no exogenously added cytokines.

Proton transport activity of ISF is required to maintain stem cell activity

Given that ISF/ShIF was able to maintain HSCs in vitro, we next determined if the proton pump function of ISF is required for stem cell supporting activity. It was reported that substitution of Arginine-735 for Alanine in 'a' subunit leads to a complete loss of proton transport and ATPase activity in yeasts, while leaving intact assembly of the V-ATPase complex [14]. Therefore, we introduced the corresponding R755A mutation in ISF and ShIF (Fig. 3), and assessed their effects using the co-culture system described above. The bone marrow cells were co-cultured on MS10 cells expressing ISF, ShIF or their R755A mutants for 7 days and were subjected to colony assay. Total colony numbers increased about 2- to 3-fold in ISF and ShIF, and they were clearly decreased in R755A mutant of ISF (Table 3). Slight, but reproducible, reduction was also seen in mutant ShIF. To analyze the effect of R755A mutation in more detail, we classified colonies into HPP-CFC (more than 1 mm in diameter) and LPP-CFC (less than 1 mm in diameter), representing more immature and mature progenitors, respectively. HPP-CFC and LPP-CFC were

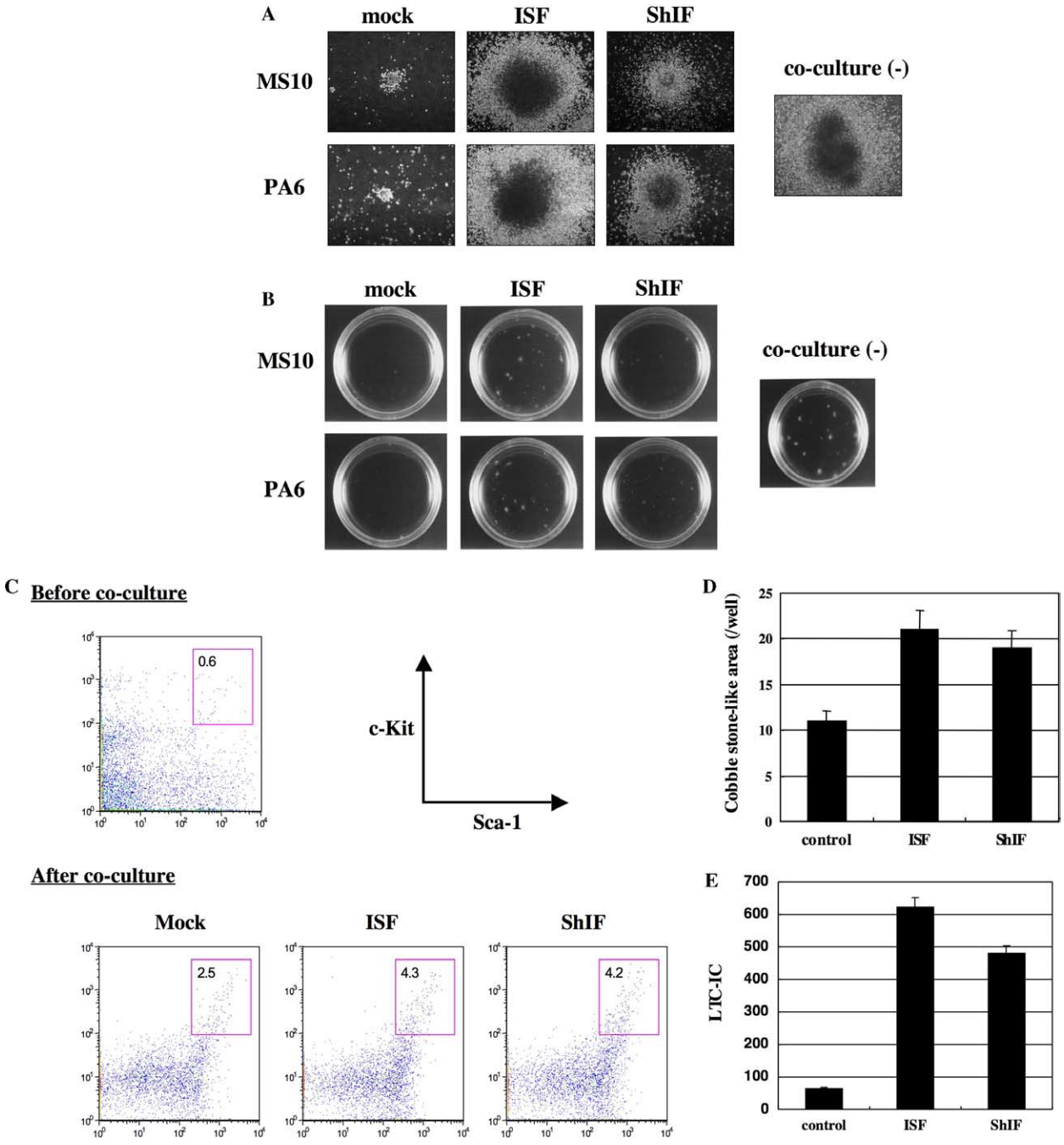

Fig. 1. Co-culture of bone marrow cells on stroma-expressing ISF/ShIF. (A,B) Primary murine bone marrow cells were cultured on MS10 or PA6 cells expressing ISF/ShIF for seven days and then subjected to colony assays. "Co-culture (-)" means colonies derived from freshly prepared cells. (A) Pictures of representative colonies from three independent experiments. Original magnification: 40×. (B) Pictures of the entire plates. (C) FACS analysis of cells from co-culture. Bone marrow mononuclear cells were cultured on MS10 cells expressing vector alone (mock), ISF or ShIF for seven days and then subjected to flow cytometry. Cells were stained with anti-Sca-1-FITC and c-Kit-PE antibodies and analyzed using a FACS Calibur. Numbers in the boxes are the percentages of each fraction. (D) Cobblestone-like area forming cell assay. Bone marrow cells (1×10^4/well) were plated in a 24-well plate and the numbers of cobblestone-like area were counted after 4 days of culture. (E) Bulk LTC-IC assays. Bone marrow mononuclear cells (2×10^5) were cultured on MS10/mock, MS10/ISF, and MS10/ShIF cells for five weeks and then subjected to colony assays to assess LTC-IC activities. Number of colonies generated from each culture is plotted. Data are means ± SD of three independent experiments.

equally increased by ISF and ShIF compared with mock control. Interestingly however, reduction of the colony numbers in R755A mutant was evident in HPP-CFC, but not in LPP-CFC. These data suggest that proton transport activity of ISF or ShIF is essential for maintaining clonogenic progenitors in vitro, and this effect is more evident on the immature rather than the mature progenitor fraction.

Table 2
Reconstitution of mice transplanted with KSL cells cultured on MS10-expressing ISF/ShIF or AGM-S3

	Frequency of reconstitution
Mock	17% (2/12)
ISF	75% (9/12)
ShIF	82% (9/11)
AGM-S3	91% (10/11)

100 KSL cells were co-cultured on MS10-expressing ISF/ShIF or AGM-S3 for seven days and subjected to competitive repopulation assay as described in Materials and methods. Frequencies of reconstitution at five weeks post-transplant are shown. Reconstitution equal to or more than 1% of donor cells is considered to be a significant engraftment. Numbers of reconstituted/transplanted mice are shown in parentheses.

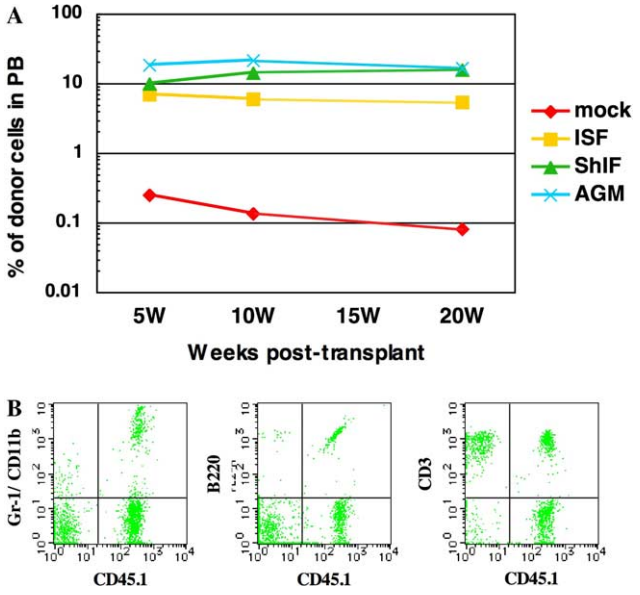

Fig. 2. Competitive repopulation assay. (A) One hundred KSL cells from Ly5.1 mice were co-cultured on MS10/mock, MS10/ISF, MS10/ShIF or AGM-S3 cells for 7 days and transplanted with 2×10^5 competitor cells into Ly5.2 recipient mice. Percentages of peripheral blood contribution by Ly5.1 donor cells at 5, 10, and 20-week post-transplant are shown. AGM; co-culture with AGM-S3. (B) FACS profile of peripheral blood leukocytes from mice transplanted with KSL cells cultured on MS10/ShIF after 20 weeks of transplantation. Cells were stained with anti-Gr-1-PE, CD11b-PE, B220-PE, CD3-PE or CD45.1 (Ly5.1)-FITC.

Fig. 3. Schematic views of ISF/ShIF mutants. Signal sequence and transmembrane domains are shown in black and gray, respectively.

Table 3
Colony forming assay of bone marrow cells cultured on MS10-expressing ISF/ShIF and their mutants

	HPP-CFC	LPP-CFC	Total
Pre co-culture	25 ± 1	30 ± 4	55 ± 5
MS10/mock	0 ± 0	43 ± 2	43 ± 2
MS10/ISF	18 ± 2	86 ± 7	104 ± 9
MS10/ShIF	16 ± 6	163 ± 27	179 ± 33
MS10/ISF (R755A)	2 ± 1	66 ± 12	68 ± 13
MS10/ShIF (R755A)	6 ± 1	151 ± 1	157 ± 14

Numbers are colonies formed from 2×10^4 unfractionated bone marrow cells. HPP-CFC, a colony larger than 1 mm in diameter. LPP-CFC, a colony smaller than or equal to 1 mm in diameter.

Gene expression profile of ISF/ShIF-expressing stromal cells

To elucidate the molecular mechanism of enhanced stem cell maintenance by ISF/ShIF, we first tried to identify the putative receptors for ISF/ShIF predicted to exist on HSCs or progenitors. However, our repeated attempts utilizing the expression cloning strategy failed to identify such proteins (data not shown). Therefore, we hypothesized that expression of ISF/ShIF modulated physiological pathways in stromal cells for the support of HSCs, instead of acting directly through putative receptors on HSC. To acquire clues as to such pathways, we analyzed gene expression profiles of MS10 and MS10 expressing ISF/ShIF using Affymetrix oligonucleotide arrays. Supplementary Table 1 summarizes the data of the representative experiment. In 19 genes down-regulated in MS10/ISF or MS10/ShIF, tissue inhibitor of metalloproteinase 3 (TIMP-3) and secreted frizzled-related protein-1 (SFRP-1) drew our particular attention. TIMP-3 is an endogenous inhibitor for metalloproteinases (MMPs), critical regulators for extracellular matrix turnover and tissue remodeling. In addition to MMP-inhibitory activities, TIMP-3 also displays anti-angiogenic effect by inhibiting endothelial proliferation. These effects are mediated through direct inhibition of vascular endothelial growth factor (VEGF) signaling by binding directly to the VEGF receptor 2 [15]. SFRP-1 is a secreted antagonist for Wnt, a well-known self-renewal factor for HSCs [16]. A ligand-binding site of Frizzled (Fz), a receptor for Wnt, shares high homology with the N-terminal cysteine-rich domain (CRD) of SFRP-1, and SFRP-1 is considered to antagonize Wnt signaling by either competitively inhibiting Wnt binding to Fz or forming an inactive receptor complex by associating with Fz.

We first checked whether expressions of TIMP-3 and SFRP-1 were indeed down-regulated in cells expressing ISF/ShIF, using Northern blots and semi-quantitative RT-PCR. As shown in Fig. 4A and B, the expression levels of TIMP-3 and SFRP-1 were profoundly decreased in MS10/ISF and MS10/ShIF. Interestingly however, these down-regulations were not seen in cells expressing proton-pump mutants (R755A mutants) for ISF and ShIF.

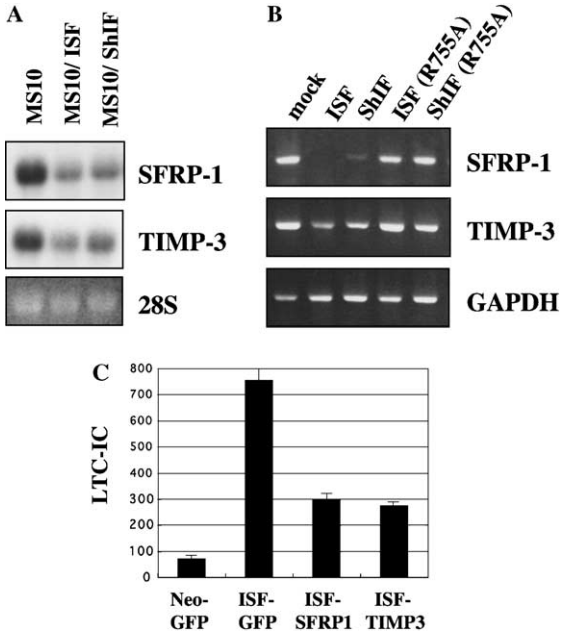

Fig. 4. Down-regulation of SFRP-1 and TIMP-3 is critical for HSC-supportive activity. (A) MS10 cells expressing vector alone, ISF and ShIF were analyzed for expressions of SFRP-1 and TIMP-3 by Northern blots. (B) Expressions of SFRP-1 and TIMP-3 in MS10 expressing ISF/ShIF and their mutants were analyzed by semi-quantitative RT-PCR. RNA quantities of each sample were normalized according to GAPDH. (C) Bulk LTC-IC assays. Bone marrow mononuclear cells (2×10^5) were cultured on MS10/mock and MS10/ISF cells overexpressing vector alone, SFRP-1 or TIMP-3 for five weeks and then subjected to colony assays to assess LTC-IC activities. Number of colonies generated from each culture is plotted. Data are means ± SD of three independent experiments.

These data indicate that enforced expression of ISF/ShIF in stromal cells leads to down-regulation of TIMP-3 and SFRP-1 mRNA in a proton-transport activity-dependent manner.

Restoring TIMP-3 or SFRP-1 expression partially reverses HSC-supporting activity in stromal cells expressing ISF

Since down-regulation of TIMP-3 and SFRP-1 mRNA occurred in parallel with enhanced HSC-supporting activity in stromal cells expressing ISF/ShIF and vice versa, we hypothesized that TIMP-3 and SFRP-1 might be their key downstream targets critical for enhanced HSC maintenance. To prove this hypothesis, we restored TIMP-3 or SFRP-1 expression in MS10/ISF and examined effects on their HSC-maintenance ability in LTC-IC assays. As shown in Fig. 4C, LTC-IC activity of bone marrow cells co-cultured on MS10/ISF was about 10-fold higher than that of the mock control. Interestingly however, the cells cultured on MS10/ISF stably expressing TIMP-3 or SFRP-1 showed reduced LTC-IC activities, which are only 3- to 4-fold higher than the mock control. These data suggest that TIMP-3 and SFRP-1 are at least partially involved in high HSC maintenance ability mediated by ISF/ShIF overexpression in stromal cells.

Discussion

We reported that ISF or ShIF was the key stroma-derived factor that supported stroma-dependent, IL-3-independent growth of mutant Ba/F3 cells [5]. In the present work, we demonstrated that overexpression of ISF/ShIF supported not only mutant Ba/F3 cells, but also murine primary HSC. Co-cultivation of HSC on stromal cells expressing ISF/ShIF efficiently maintained their clonogenic and long-term reconstitution activity, and this was at least in part mediated by the decreased expression of TIMP-3 and SFRP-1. Although the system we used here is artificial, these findings might be helpful to develop efficient HSC expanding strategies in vitro.

The bone marrow stroma was thought to be the critical microenvironment for nurturing HSC [1,17]. This idea was derived from the fact that immature hematopoietic cells could not be cultured ex vivo for a long term without bone marrow stromal cells [3,18]. Thus, researchers sought to identify soluble factors or surface molecules that support HSC from primary bone marrow stromal cells or cell lines, using a variety of approaches such as subtractive hybridization of mRNAs comparing supportive and non-supportive cell lines, and signal sequence trap. From such efforts, we and others have identified several stroma-derived proteins that support HSC. We earlier cloned mKirre, an immunoglobulin-like motif containing protein that is specifically expressed in bone marrow stroma and brain, and showed that it was essential for maintaining HSC on OP9 cells [19]. Down-regulation of mKirre expression by siRNA in OP9 cells markedly reduced LTC-IC activity generated from HSC co-cultivation. Moore et al. [4] cloned delta-like (Dlk) from fetal liver stromal cell line using a subtractive hybridization approach. They showed that overexpression of Dlk in non-supportive stroma significantly enhanced its HSC-supporting activity. ISF, which we reported here, is another stroma-derived factor that can support HSC. It is generally assumed that the molecular requirements of stroma-dependent maintenance of HSC in vivo are not simple and involve multiple membranous or soluble factors. Therefore, the critical question to be asked is whether these factors indeed play an essential role in vivo. None of the studies above have shown the physiological importance of these molecules in vivo, and hence, future studies must be aimed for revealing the precise role of these molecules in hematopoiesis using in vivo model, such as gene-deficient animals.

Vacuolar ATPases are essential in the various aspects of cellular functions, such as secretion of proton ions, bone remodeling, and control of cytoplasmic pH [8]. They are also important for acidification of intracellular compartments such as lysosomes, endosomes, and secretory vesicles. Biological importance of V-ATPases has been highlighted by the fact that their genetic defects lead to a variety of diseases such as renal tubular acidosis and osteopetrosis, defects in acidification of urine, and bone resorption, respectively [9,10]. Despite all this evidence, it has not been clear if ISF

plays a role in HSC-homeostasis in vivo. In the present work, we noted the novel role of ISF in stroma-dependent maintenance of HSC in vitro, and these results raised the possibility that ISF might play a critical role in HSC physiology in vivo. Although this is an intriguing possibility, several lines of evidence suggest that this is not the case. Our preliminary analysis indicates that expression of ISF is not regulated in murine bone marrow cells in response to cytotoxic stimuli, such as 5-FU. In addition, ISF is ubiquitously expressed in a variety of tissues, and its expression in various stromal cell lines does not necessarily correlate with their supportive capability for HSC (unpublished data) indicating that ISF is not the sole deterministic factor for HSC-supportive capacity in stromal cells. Moreover, the enhanced HSC-supportive capacity of stromal cells required overexpression of ISF at supra-physiological levels (10–20 times higher than primary bone marrow stromal cells) in our experiments (data not shown). Taken together, while we still do not rule out the possibility that ISF plays a role in HSC physiology in vivo, we rather think that the HSC-supporting activity of ISF takes effect only in our artificial overexpression system in vitro.

Although ISF itself does not seem to be directly involved in HSC homeostasis in vivo, our data suggest that ISF might modulate the physiological pathways for HSC regulation by its overexpression, which would give us some clues to uncover the molecular mechanism for HSC regulation in vivo. We speculate that TIMP-3 and SFRP-1 are the most likely candidates for such physiological pathways in vivo based on the following: (1) Expressions of TIMP-3 and SFRP-1 are both down-regulated in ISF/ShIF-expressing stroma, and levels of TIMP-3 or SFRP-1 mRNA correlate with HSC-supporting activity. (2) Restoration of TIMP-3 or SFRP-1 expressions in ISF/ShIF-expressing stroma partially reverses HSC-supporting activity of the stromal cells. (3) SFRP-1 is reported to be an antagonist for Wnt, a well-known self-renewal factor for HSC [16,20]. (4) TIMP-3 inhibits VEGF-signaling by binding with VEGFR2 [15], which is specifically expressed in HSC and endothelial cells [21]. Regardless, further studies including analysis of gene-deficient animals will be required to elucidate the precise role of ISF, TIMP-3, and SFRP-1 in HSC physiology in vivo.

A precise molecular mechanism as to how ISF/ShIF modulates gene expression profiles of stromal cells remains unclear. It is of note that gene expression profiles of MS10 expressing either ISF or ShIF showed a striking resemblance: any genes up- or down-regulated by ISF were similarly regulated by ShIF, strongly indicating that ISF and ShIF affect gene expression by a similar mechanism such as increased proton pump activity. Our data clearly show that proton transport activity of ISF is absolutely required for the enhanced HSC-supporting activity of ISF/ShIF, and it is probable that ISF/ShIF overexpression facilitates assembly of V-ATPase complex, thereby augmenting overall proton-transport activity of the cell. However, the mechanism as to how enhanced proton-pump function leads to an altered gene expression profiles and eventually an enhanced HSC-supporting activity is still unclear. One possibility is that enhanced proton transfer activity affects pH homeostasis of cytoplasm, endosomes or lysozomes, thereby modulating protein processing and eventually expression of genes that are involved in HSC-support. In this regard, it is interesting to note that ISF mainly localizes in the endoplasmic reticulum (ER), but little on the plasma membrane in stromal cells (unpublished data). On the other hand, V-ATPase was reported to suppress NF-κB activation and eventually its target gene expression in macrophages and fibroblasts, probably by affecting cytoplasmic pH [22]. Taken together, we speculate that overexpression of ISF/ShIF facilitates V-ATPase complex assembly in ER, which in turn leads to a modulation of protein processing (i.e., cytokines, cytokine receptors or extracellular matrix proteins) or transcription factor function (i.e., NF-κB) by affecting the cellular pH environment. Further studies will be required to elucidate how ISF overexpression endows stromal cells with enhanced HSC-maintenance potential in vivo.

It is surprising that ISF/ShIF-expressing MS10 or PA6 was capable of maintaining clonogenic and long-term reconstitution activity of HSCs without any added cytokines to the extent comparable to highly supportive cell lines, such as AGM-S3 (Table 2). Since ISF is not highly expressed in AGM-S3 or OP9 (data not shown), it is reasonable to speculate that ISF can also increase HSC-supporting activity of these cell lines. Moreover, we expect that the supplementation of various cytokines in the co-culture can accentuate the HSC-supportive capacity to an even higher level, since enhancement of HSC-supportive activity by ISF/ShIF could be achieved without adding exogenous cytokines or modulation of cellular cytokine secretion. These possibilities might open up an avenue to a generation of stromal cell lines and the culture system with a super-supportive capacity for HSC, which would be extremely useful in regenerative medicine such as HSC expansion in clinical settings.

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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.2005.11.146.

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