Protein Cell 2013, 4(3): 220–230
DOI 10.1007/s13238-013-2066-5

Protein & Cell

RESEARCH ARTICLE

Dynamic roles of angiopoietin-like proteins 1, 2, 3, 4, 6 and 7 in the survival and enhancement of *ex vivo* expansion of bone-marrow hematopoietic stem cells

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Received July 23, 2012 Accepted December 18, 2012

ABSTRACT

Recent advances in hematopoietic stem cells (HSCs) expansion by growth factors including angiopoietin-like proteins (Angptls) have opened up the possibility to use HSCs in regenerative medicine. However, the unavailability of true *in vitro* HSCs expansion by these growth factors has limited the understanding of the cellular and molecular mechanism of HSCs expansion. Here, we report the functional role of mouse Angptls 1, 2, 3, 4, 6 and 7 and growth factors SCF, TPO, IGF-2 and FGF-1 on purified mouse bone-marrow (BM) Lineage$^-$Sca-1$^+$(Lin$^-$Sca-1$^+$) HSCs. The recombinant retroviral transduced-CHO-S cells that secrete Angptls in serum-free medium were used alone or in combination with growth factors (SCF, TPO, IGF-2 and FGF-1). None of the Angptls stimulated HSC proliferation, enhanced or inhibited HSCs colony formation, but they did support the survival of HSCs. By contrast, any of the six Angptls together with saturating levels of growth factors dramatically stimulated a 3- to 4.5-fold net expansion of HSCs compared to stimulation with a combination of those growth factors alone. These findings lead to an understanding of the basic function of Angptls on signaling pathways for the survival as well as expansion of HSCs in the bone marrow niche.

KEYWORDS hematopoietic stem cells, angiopoietin-like proteins, growth factors, survival, *ex vivo* expansion, cell culture

INTRODUCTION

HSCs are rare cells within the hematopoietic hierarchy responsible for the permanent establishment of hematopoiesis (Bryder et al., 2006). HSCs have the unique capacity to differentiate into all mature hematopoietic lineages as well as to generate more HSCs by a mechanism referred to as self-renewal. This self-renewal feature is essential for their expansion throughout hematopoietic development, hematopoietic homeostasis, after bone marrow (BM) transplantation and/or in response to different physiologic stresses (Wilson et al., 2008; Essers et al., 2009). During the past decade, much progress has been made in providing a physical phenotype for this rare population of stem cells. Currently however, the only reliable clinical use for the most primitive stem cell compartment is long-term *in vivo* transplantation. Moreover, efforts to overcome the relative shortages of HSCs have led to technologies to expand the functionally defined HSCs *ex vivo*. HSCs can not only re-establish blood-forming cells after transplantation into immunodeficient recipients, but they can also produce artificial blood that would eliminate the reliance on blood donors as well as the risk of infectious disease transmission and blood-type rejection (Giarratana et al., 2005; Douay and Giarratana 2009). In regenerative medicine, HSCs are committed to generate immune cells for therapeutic use in cancer, gene therapy for a variety of genetic blood disorders and the restoration of diseased or damaged tissues to overcome the shortage of donated organs and the risk associated with their rejection (Weekx et al., 2000; Atala 2009; Cartier et al., 2009). Increasing demand for using expanded HSCs for these capabilities has been limited due to the lack of a detailed understanding of the factors that

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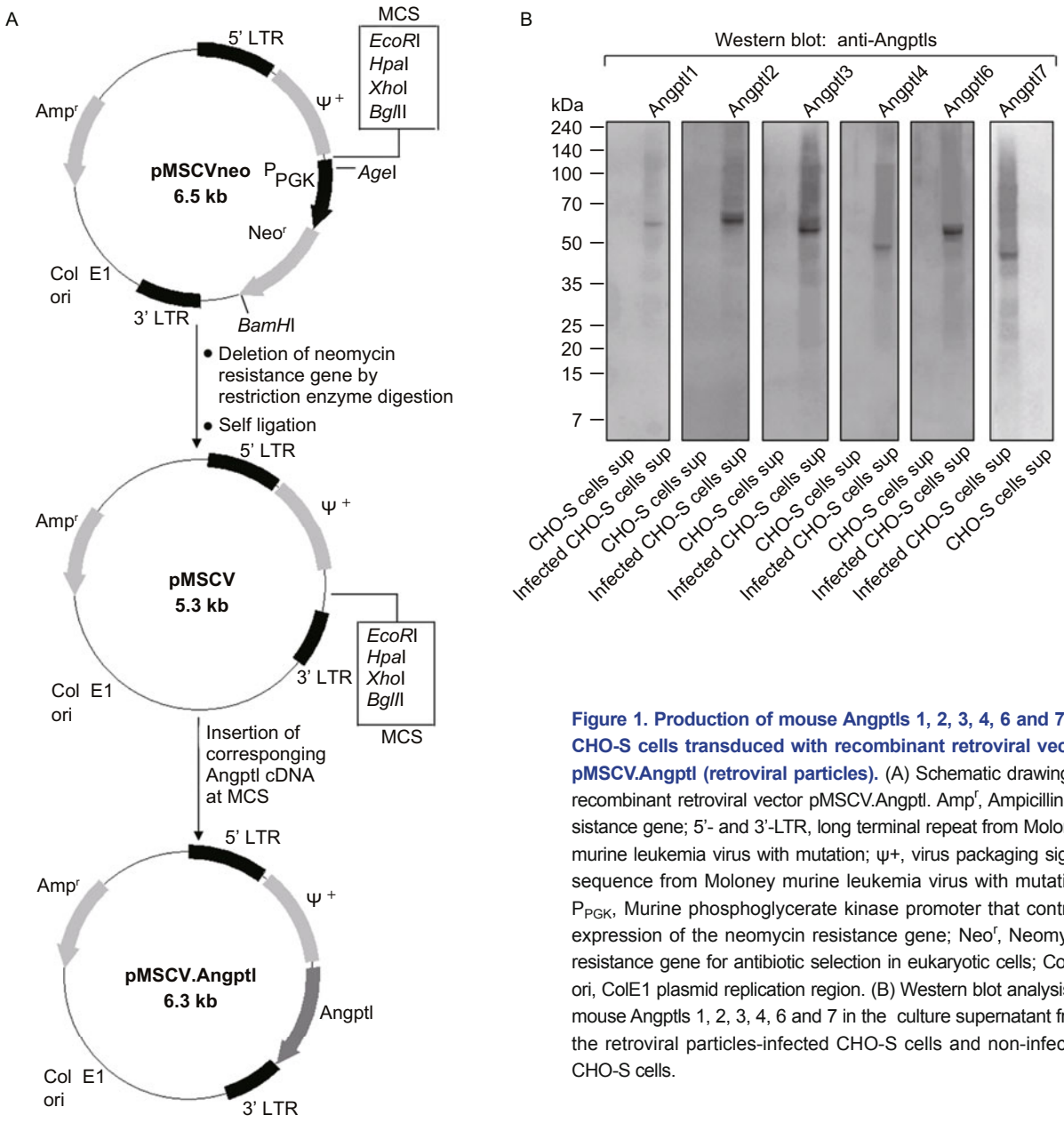

Figure 1. Production of mouse Angptls 1, 2, 3, 4, 6 and 7 by CHO-S cells transduced with recombinant retroviral vector pMSCV.Angptl (retroviral particles). (A) Schematic drawing of recombinant retroviral vector pMSCV.Angptl. Amp^r, Ampicillin resistance gene; 5'- and 3'-LTR, long terminal repeat from Moloney murine leukemia virus with mutation; ψ+, virus packaging signal sequence from Moloney murine leukemia virus with mutation; P_{PGK}, Murine phosphoglycerate kinase promoter that controls expression of the neomycin resistance gene; Neo^r, Neomycin resistance gene for antibiotic selection in eukaryotic cells; ColE1 ori, ColE1 plasmid replication region. (B) Western blot analysis of mouse Angptls 1, 2, 3, 4, 6 and 7 in the culture supernatant from the retroviral particles-infected CHO-S cells and non-infected CHO-S cells.

regulate symmetrical HSCs expansion as well as access to culture conditions that maintain HSCs in an uncommitted state (Araki et al., 2007).

Generally, the isolation of human and mouse HSCs for *ex vivo* expansion is based on cell surface markers. Sca-1 is the most common marker found on undifferentiated mouse HSCs *in vivo* as well as *in vitro*. The expression of Sca-1 decreases upon differentiation to other mature cell types. Clonogenic multipotent mouse HSCs are contained within the Sca-1$^+$ population of HSCs, which maintain the bone marrow stem cell pool throughout the life (Chatterjee et al., 2009). Several attempts have been made to expand mouse BM HSCs by a combination of different growth factors alone or with Angptls in a feeder-cell-free, serum-free culture (Huynh et al., 2008;Nishino et al., 2011), but there is no information available on the role of Angptls alone on adult mouse HSCs expansion. Zhang et al. (2006) reported that Angptls enhances the *ex vivo* expansion of long-term repopulating mouse HSCs. In their HSCs expansion efforts, they used several members of the Angptl family (Angptl2, Angptl3, Angptl5 and Angptl7) with a mixture of growth factors, such as SCF, TPO, IGF-2 and FGF-1, in serum-free medium. However, whether HSCs expansion is driven by Angptls alone or in combination with growth factors still remains elusive.

In this study, we show the functional role of mouse Angptls, expressed in CHO cells, on adult mouse BM Lin$^-$Sca-1$^+$ HSCs in se-

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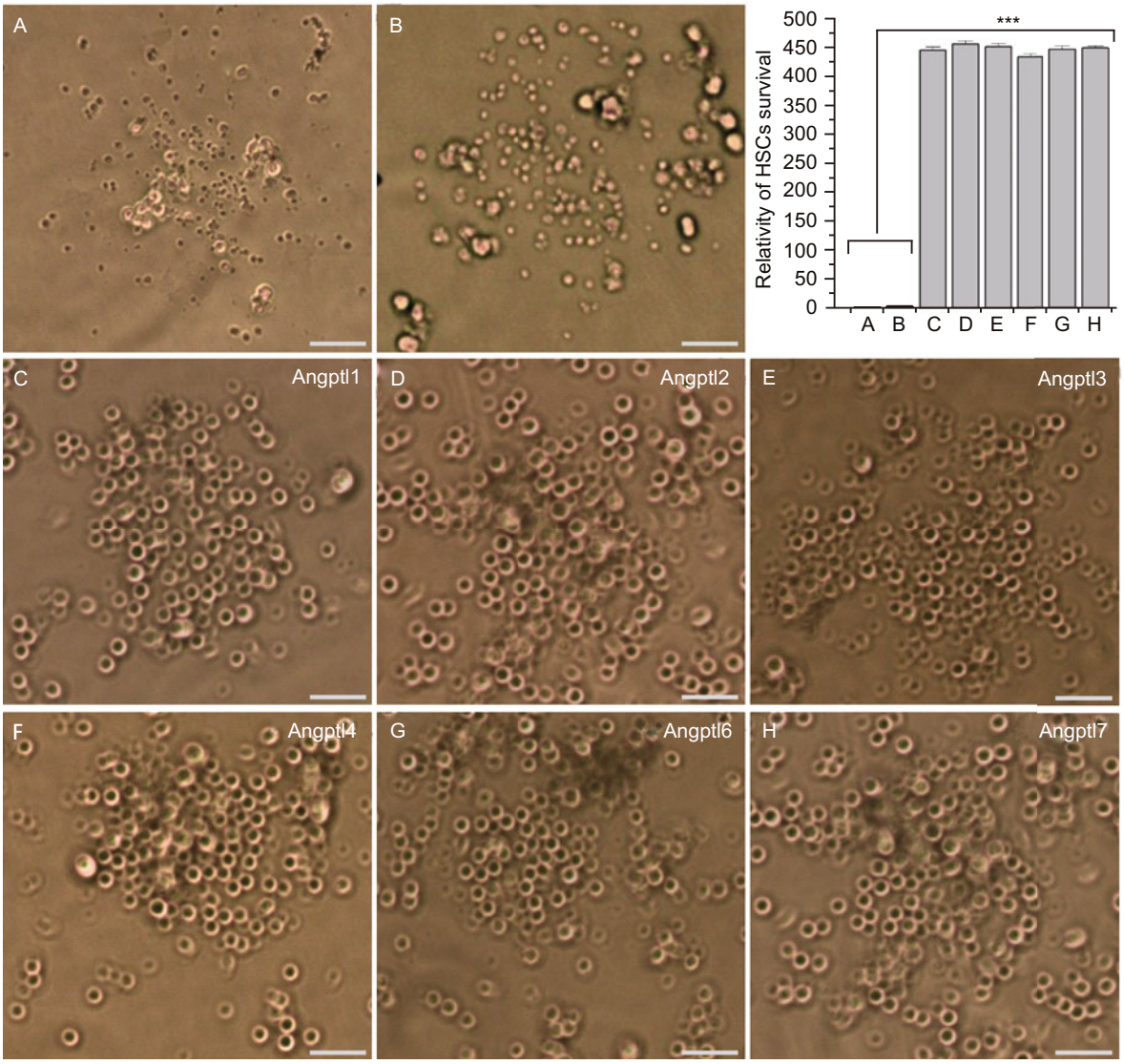

Figure 2. Mouse Angptls 1, 2, 3, 4, 6 and 7 supported the survival of mouse bone-marrow-isolated HSCs in culture. (A) HSCs were cultured in serum-free stem cell growth medium. (B)HSCs were cultured in serum-free stem cell growth medium with conditioned medium from CHO-S cell culture. (C–H) HSCs were cultured in serum-free stem cell growth medium in the presence of Angptl1 (C), Angptl2 (D), Angptl3 (E), Angptl4 (F), Angptl6 (G) or Angptl7 (H). Images of HSCs after 6 days of culture were captured by an inverted fluorescence microscope. Magnification: 100×. Scale bar: 100μm. Values are the mean ± STD of trypan blue dye excluded viable cells from 5 wells of a 96-well plate. ***$P < 0.001$, a culture of HSCs in serum-free stem cell growth medium fed with the respective Angptls compared with a culture of HSCs in serum-free stem cell growth medium or in serum-free stem cell growth medium with conditioned medium from CHO-S cell culture.

rum-free medium with or without a combination of growth factors.

RESULTS

Cells infected with recombinant retroviral particles secrete Angptls into culture supernatants

To produce Angptls by CHO-S cells in culture media, we constructed plasmids containing the entire coding sequence for mouse Angptls 1, 2, 3, 4, 6 and 7 without the neomycin resistance gene or any tag in the retroviral expression vector pMSCVneo (Fig. 1A). The recombinant retroviral particles were successfully produced by co-transfecting GP2-293 cells with recombinant retroviral vectors pMSCV.Angptl and pVSV-G. We then cultured recombinant retroviral particles-infected CHO-S cells and non-infected CHO-S cells as a control in serum-free CHO-S cell medium under the same experimental conditions. Next, the culture supernatant from fresh CHO-S cells or recombinant retroviral particles-infected CHO-S cells were

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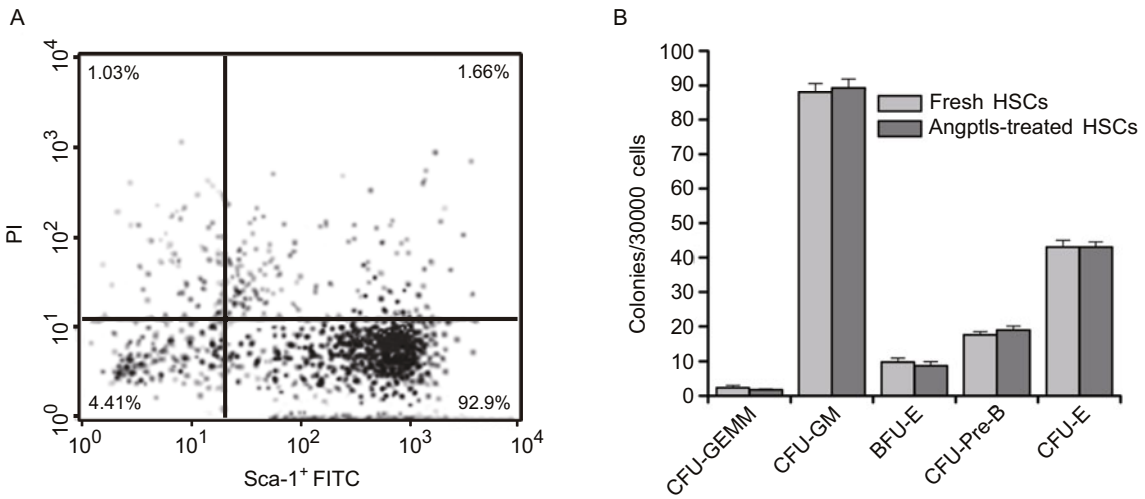

Figure 3. Angptls-supported-survival HSCs did not lose the Sca-1$^+$ stemness and clonal functionality. (A) Flow cytometry analysis of Angptls-supported-survival HSCs. Lin$^-$Sca-1$^+$ HSCs were cultured in presence of Angptls (described in methods) and then subjected to Flow cytometry analysis. (B) Similarly as freshly isolated Sca-1$^+$ HSCs, Angptls-supported-survival HSCs were able to form colony forming units granulocyte/erythroid/monocyte/ megakaryocyte progenitors (CFU-GEMM), granulocyte/monocyte progenitors (CFU-GM), erythroid (BFU-E, burst-forming unit progenitors), B lymphoid progenitors (CFU-Pre-B) and erythroid precursors (CFU-E). Sca-1$^+$ HSCs were cultured in presence of Angptls (described in methods) and then subjected to colony forming assays.

subjected to western blot analysis by using antibodies against Angptls. The culture supernatants from infected CHO-S cells contained secreted Angptls 1, 2, 3, 4, 6 and 7, that migrated to the expected ~56, ~57, ~52, ~45, ~51 and ~39 kDa size, respectively, while no protein was detected in the culture supernatant from non-infected CHO-S cells (Fig. 1B). These results indicate that the production of mouse Angptls by CHO-S cells infected with recombinant retroviral particles was successful. It should be noted that our recombinant retroviral particles-infected CHO-S cells is the first report of its kind. In fact, all Angptls contain a highly hydrophobic N-terminal signal peptide that directs the proteins towards secretion, an N-terminal coiled-coil domain, a linker region and a C-terminal fibrinogen-like domain (Ito et al., 2003; Oike et al., 2004).

Adult mouse bone-marrow is enriched with Sca-1$^+$ HSCs that maintains the bone marrow stem cell pool

Mouse HSCs are contained within the Sca-1$^+$ and lineage marker$^-$(CD34$^-$KSL) population of hematopoietic cells (Haylock et al., 2007; Christensen et al., 2001) and Sca-1 is the most common marker used to identify adult mouse HSCs (Spangrude et al., 1988; Okada et al., 1992). Therefore, we isolated and purified Lin$^-$Sca-1$^+$ HSCs from mouse bone marrow mono-nucleated cells by magnetic activated cell sorting (MACS). Sca-1 stemness was compared between freshly purified HSCs and *ex vivo*-expanded HSCs below. However, 93.18% of total bone marrow HSCs were Sca-1$^+$ demonstrated by FCM. We also performed a trypan blue dye exclusion assay to detect the viability of the sorted cells. The cell survival was at 96%~98%. These results indicate that the sorted Sca-1$^+$ HSCs had high purity and viability and they could be used for both *in vitro* and *in vivo* clonal analyses. Consistent with our results, it was reported that HSCs that are highly enriched for a population of Sca-1$^+$ and lineage marker$^-$(CD34$^-$KSL) cells among bone marrow cells of adult mice enables both *in vitro* and *in vivo* clonal analyses of HSCs (Osawa et al., 1996; Ema et al., 2000).

Several members of the Angptl family proteins support the survival of HSCs

Although several members of the Angptl family were known to stimulate the expansion of mouse HSCs *ex vivo* (Zhang et al., 2006, 2008), whether HSC expansion is driven by Angptl alone or in combination of growth factors is not clear. To answer this question, we cultured Sca-1$^+$ HSCs for 7 days in serum-free stem cell growth medium (Fig. 2A), serum-free stem cell growth medium with conditioned medium from CHO-S cells (Fig. 2B) or serum-free stem cell growth medium in the presence of Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or Angptl7 in conditioned medium from recombinant retroviral particles-infected CHO-S cells (Figs. 2C–H). Control cultures consisting of cells from serum-free stem cell growth medium showed no identifiable live cells (Fig. and Bar 2A) while control cultures consisting of cells from conditioned medium from CHO-S cells infected with recombinant retroviral particles (Fig. and Bar 2B) showed a few live cells detected by trypan blue dye exclusion. In contrast, cultures consisting of cells that were in the presence of Angptls exhibited viable (96%–98%) and healthy cells with the proper morphology, but the cell number did not increase (Figs. and Bars 2C–H). Moreover, there were no significant differences observed among the HSCs cultured in the presence of Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or

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Angptl7. These results indicate that Angptl protein alone plays a crucial role in HSCs survival but not expansion.

Angptls supported-survival-HSCs retain Sca-1$^+$ stemness and multipotentiality to differentiate in discrete colonies

In order to verify whether Angptls have direct effect on the surface marker protein Sca-1 on the HSCs, we cultured Sca-1$^+$ HSCs for 7 days in serum-free stem cell growth medium supplemented with Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or Angptl7 and subjected to flow cytometry analyses. As shown in Fig. 3A, 92.9% of the total cells were Sca-1$^+$ which is similar to freshly isolated HSCs-exposed surface marker Sca-1$^+$ (93.18%). This result indicates that Angptls enhance the survival of HSCs without altering or losing the surface marker Sca-1 stemness. We then verified the multi-potential activity of freshly isolated HSCs and Angptls-supported-survival-HSCs by *in vitro* colony forming unit (CFU) assays. Both the freshly isolated HSCs and Angptls-supported-survival-HSCs were able to form hematopoietic colonies (so-called CFUs or CFCs) represent a stage of hematopoietic differentiation between HSCs and more terminally differentiated progenitors. Interestingly, both the Angptls-supported-survival HSCs and freshly isolated HSCs formed similar numbers of granulocyte/erythroid/ monocyte/megakaryocyte progenitors (CFU-GEMM), granulocyte/monocyte progenitors (CFU-GM), erythroid (BFU-E, burst-forming unit progenitors), B lymphoid progenitors (CFU-Pre-B) and erythroid precursors (CFU-E) (Fig. 3B). Thus, Angptls-supported-survival HSCs had not greater multi-potential capacity to give rise to colonies. This result suggests that Angptls only support the survival of HSCs without affecting their functions as stem cells.

Several members of the Angptl family proteins stimulate *ex vivo* expansion of HSCs with reduced Sca-1 in presence of growth factors

Because Angptls alone were unable to stimulate HSC expansion, we cultured Sca-1$^+$ HSCs for 7 days in serum-free stem cell growth medium supplemented with growth factors alone (Fig. 4A) or supplemented with GFs as well as Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or Angptl7 in conditioned medium (Figs. 4B–G) from CHO-S cells infected with recombinant retroviral particles. As expected, GFs supplementation with or without any of the six Angptls stimulated *ex vivo* HSC expansion. The cell counting data showed a 2.5-, 2.9-, 2.8-, 2.01-, 2.7- or 2.75-fold net expansion of HSCs in culture in the presence of Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or Angptl7 together with the combination of growth factors respectively compared to the cells in the presence of growth factors (Figs. and Bars 4A–G). These results suggest that HSCs expansion depends on the presence of Angptls and clearly address the effects of Angptls on HSCs. Next, we tested the expression of surface marker protein Sca-1 on *ex vivo*-expanded HSCs by flow cytometry. As shown in Fig. 5, 33.02% of the total *ex vivo*-expanded HSCs were Sca-1$^+$ whereas 93.18% of the

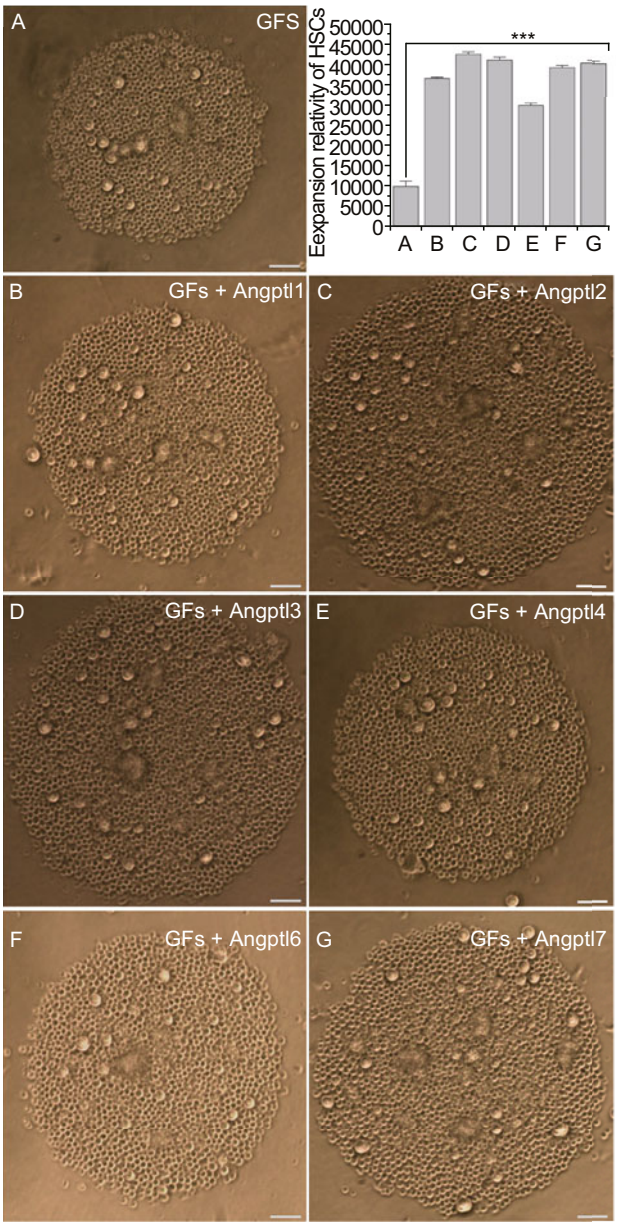

Figure 4. Mouse Angptls 1, 2, 3, 4, 6 and 7 in the presence of other growth factors stimulated the *ex vivo* expansion of mouse bone-marrow-isolated HSCs in culture. (A) HSCs were cultured in serum-free stem cell growth medium together with a combination of growth factors (heparin, SCF, TPO, IGF-2 and FGF-1). (B–G) HSCs were cultured in serum-free stem cell growth medium together with a combination of growth factors (heparin, SCF, TPO, IGF-2 and FGF-1) in the presence of Angptl1 (B), Angptl2 (C), Angptl3 (D), Angptl4 (E), Angptl6 (F) or Angptl7 (G). Images of HSCs after 6 days of culture were taken by an inverted fluorescence microscope. Magnification: 100×. Scale bar: 100 μm. GFs, combination of growth factors. Values are the mean ± STD of trypan blue dye excluded viable cells from 5 wells of a 96-well plate. ***$P < 0.001$, a culture of HSCs in stem cell growth medium together with the combination of growth factors in the presence of respective Angptls compared with a culture of HSCs in stem cell growth medium together with a combination of growth factors.

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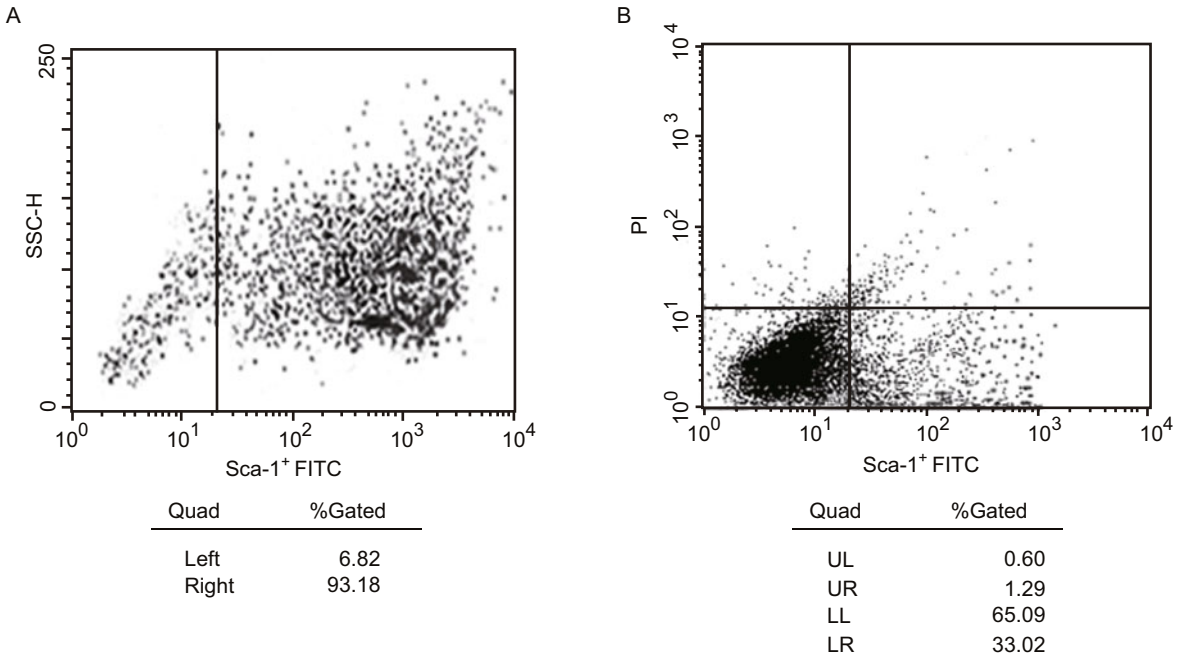

Figure 5. Flow cytometry analysis for Sca-1$^+$ HSCs before and after *ex vivo* expansion of HSCs. (A) Flow cytometry analysis for the determination of Sca-1$^+$ HSCs after MACS purification. (B) Flow cytometry analysis for the determination of Sca-1$^+$ HSCs after *ex vivo* expansion of HSCs in presence of Angptls and a combination of growth factors.

total freshly-isolated HSCs were Sca-1$^+$. This result indicates that the expression of surface marker protein Sca-1 is reduced by 2.82 folds on the *ex vivo*-expanded HSCs as compared to freshly isolated HSCs. However, the expression of surface marker protein Sca-1 is retained to a some extent even on *ex vivo*-expanded HSCs, which is also present on freshly isolated HSCs. Consistent with published reports, our result establishes that the LT-HSC activity of cultured HSCs is in the Sca-1$^+$ fraction similar to freshly isolated HSCs (Zhang and Lodish 2005).

DISCUSSION

The prevalence of hematological disorders and cancer is increasing worldwide, which has prompted researchers to investigate the growth factors that may function *in vitro* and *in vivo* to stimulate HSCs expansion. HSCs have received much attention not only for its relevance to hematological disorders and cancer but also for a promising cell target for gene therapy and a broad variety of other human diseases (Verma and Weitzman 2005). In the present study, we revealed a previously undefined function of several members of the Angptl family on mouse HSCs.

Researchers have shown that stromal cell lines alone or in combination with cytokines have a considerable effect on the self-renewal of mouse HSCs assayed after transplant (Krosl et al., 2003; Willert et al., 2003). Although the introduction of exogenous transcription factors can dramatically expand HSCs, this approach may have undesirable outcomes for recipients in the clinical settings (Varnum-Finney et al., 2000; Antonchuk et al., 2002; Reya et al., 2003). Angptl3-null mice were suboptimal recipients for engraftment of normal mouse bone marrow cells, which demonstrates the need for endogenous Angptl3 for optimal HSC engrafting capabilities (Zheng et al., 2011). Therefore, the angiopoietin-like family proteins were initially identified as a group of endogenous proteins produced in the fetal liver lineage positive cells that support HSCs (Huynh et al., 2008; Zhang et al., 2006). However, none of the previous studies have produced untagged Angptls to observe the effects of Angptl alone on HSCs. Here, we demonstrate that several Angptls (Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 and Angptl7), which share similar structures but not similar activities, have influence on the survival of HSCs rather than the proliferation or *ex vivo* expansion of HSCs upon culturing with Angptl alone. Consistent with other published reports, we also found that Angptls enhanced cytokine- or growth factor-dependent *ex vivo* expansion of HSCs (Zhang et al., 2006). Recently, Angptls 2, 3, 4 and 6 have all been detected in systemic circulation suggesting that some Angptls function similar to an endocrine *in vivo* (Shimizugawa et al., 2002; Ono et al., 2003). Thus, it may be possible for the Angptl family to activate signal transduction pathways that play a role in HSC survival and proliferation in the bone marrow niche. Recent evidence has implicated Angptls in activating signal transduction pathways that cannot be activated by SCF, TPO, or FGF-1 (Zhang et al., 2006). To demonstrate that Angptl molecules exert their effects directly on HSCs or HPCs, assays should be conducted on extremely pure HSC populations. Clinical applications and knowledge of the surface phenotype of HSCs or *ex vivo*-ex-

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panded HSCs will be critical to their purification and analysis. Among the surface proteins present on freshly isolated mouse BM HSCs are Sca-1, Kit, Mpl, CD38, Endoglin, Tie-2, CD31, 1, 4-9 and prion protein (PrP). These are also expressed on other types of BM cells and not all are conserved between species or during development (Zhang and Lodish 2005). Prion protein and Tie-2, which are present on freshly isolated HSCs, were not present on cultured HSCs (Zhang and Lodish 2005). We purified 93.18% of total mouse bone marrow HSCs based on the Sca-1 surface receptor. Similar to freshly isolated HSCs, *ex vivo* expanded HSCs in culture were positive for the cell surface marker Sca-1. None of the previous studies have used a cell population with such a high degree of purity. Finally, further investigation should be conducted to answer how and in what specific context the Angptl protein molecules functions at the receptor and intracellular level. The repression of the transcription factor Ikaros expression by Angptl3 has been reported (Zheng et al., 2011), but until the receptors for the different Angptl molecules are identified and characterized, we will not understand exactly how Angptl molecules manifest their receptor mediated effects within target cells.

This is the first specific demonstration that Angptl (Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 or Angptl7) alone supports mouse HSCs survival but not enhanced or inhibited HSC colony formation and in combination with other growth factors stimulate HSC proliferation or expansion in culture. Although the work described in this paper is confined to mouse HSCs, our identification of Angptls both as a survival and growth factor suggests that they might also be useful for both the survival and expansion of human bone marrow or cord blood HSCs. Our results on Angptls production, HSC purification, HSCs culture, HSC survival and HSC expansion by Angptls will be useful for a variety of applications including HSC transplantation, gene delivery and drug discovery.

MATERIALS AND METHODS

Antibodies and growth factors

The primary rabbit polyclonal anti-angptl1 antibody (Cat# 14709-1-AP) was purchased from Protein Tech Group Inc., Chicago, Illinois, USA. Mouse polyclonal anti-angptl2 antibody (Cat# AF1444), recombinant mouse SCF, recombinant mouse thrombopoietin (TPO) and recombinant mouse IGF-2 were purchased from R&D Systems Inc., Minneapolis, USA. Rabbit polyclonal anti-angptl3 (Cat# sc-68916), goat polyclonal anti-angptl4 (Cat# sc-32186) and goat polyclonal anti-angptl6 (Cat # sc-160958) primary antibodies were ordered from Santa Cruz Biotechnology Inc. The primary mouse polyclonal anti-angptl7 (Cat# ab70147) and the secondary horseradish peroxidase-conjugated donkey anti-goat IgG (H + L) (Cat # ab6885) antibodies were purchased from Abcam, Cambridge, UK. The secondary horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (W4021) and goat anti-rabbit IgG (H + L) (W4011) were purchased from Promega Corporation, Madison, USA. Heparin was purchased from Sigma-Aldrich, St. Louis, MO and human fibroblast growth factor (FGF-1) was purchased from Invitrogen, Carlsbad, CA, USA.

Animals

Eight weeks old C57BL/6 mice, were purchased (Japan SLC Inc., Hamamatsu, Japan) and housed in a specific-pathogen-free (SPF) environment until the next morning before starting the experiment for isolating hematopoietic stem cells (HSCs) in accordance with the Chonbuk National University Animal Care and Use Committee standards. Animal studies followed the protocols approved by the Animal Care and Use Committee of the Chonbuk National University Medical School, Jeonju, South Korea.

Cell lines culture and maintenance

The retroviral packaging cell line GP2-293 (Clontech, Palo Alto, CA, USA), which stably expresses gag and pol genes derived from MoMuLV, was cultured in high glucose Dulbecco's modified Eagle's medium ((DMEM), Hyclone, Logan, USA) supplemented with 10% fetal bovine serum ((FBS), Hyclone, Logan, UT) and 20 mmol/L HEPES (Welgene, South Korea) buffer. The cell line was sub-cultured twice a week by seeding at 4×10^4 cells/mL in a collagen coated T-75 flask (BD Falcon, Bedford, UK) at 37°C with an atmosphere of 5% CO_2 and 95% air. Cells from the fourth through sixth passages were used for all of the retroviral transfection experiments.

The FreeStyle CHO-S cell line (Invitrogen, Carlsbad, CA, USA) cell line was cultured in FreeStyle CHO Expression Medium (Invitrogen, Carlsbad, CA, USA) containing 8 mM of L-glutamine. The cell line was sub-cultured twice a week using a seeding density of 0.2–0.3 $\times 10^6$ cells/mL and they were grown at a density of 1×10^6 cells/mL in 125 mL shaker flasks (Corning Life Science, NY, USA) at 130 rpm (rotation diameter 16 mm) in an orbital shaker (Stuart, UK) at 37°C in a 8% CO_2 and 95% air atmosphere. The cells in continuous culture were sub-cultured for a minimum of 5 passages and centrifuged at 100 g for 5 min and resuspended in fresh medium to be used for transduction.

The cells that were frozen in cryovials were thawed rapidly in a 37°C water bath. For the GP2-293 cell line, following triturating, the entire content of the cryovial was mixed with fresh medium (ratio1:10), centrifuged at 100 g for 5 min and re-suspended in pre-warmed fresh medium. In the case of CHO-S cells, the entire content of the cryovial was transferred directly into the shaker flask containing 30 mL of pre-warmed fresh medium.

Construction of retroviral expression vector

Corresponding DNA sequences to mouse Angptls (Angptl1, Angptl2, Angptl3, Angptl4, Angptl6 and Angptl7) were cloned in-frame into the neomycin resistance gene-deleted retroviral vector pMSCVneo (Clontech, Palo Alto, CA, USA). Briefly, the retroviral vector pMSCVneo was digested with AgeI and BamHI and self-ligated to generate pMSCV in which the PGK promoter and neomycin resistance gene were deleted. The cDNA encoding mouse Angptl1, Angptl3, Angptl4, Angptl6 and Angptl7 were amplified by PCR from cDNA clones in the pCMV6 Entry (Myc/DDK tagged ORF) (OriGene, Rockville, MD, USA) using gene specific primers flanked by EcoRI and XhoI restriction sites for Angptls 1, 4, 6, 7 and EcoRI and HpaI restriction sites for Angptl3 (Table 1). The amplified PCR products were digested with the flanked restriction enzymes and subcloned into the EcoRI and XhoI sites of pMSCV for Angptls 1, 4, 6, 7 and into the EcoRI and HpaI sites of pMSCV for Angptl3. The cDNA encoding for mouse Angptl2 was excised from the

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Table 1. List of primers used to amplify corresponding ORF of angiopoietin-like proteins

	Gen Bank No.	Primer sequences	
Angptl1	NM_028333.2	Forward primer	5´-GAATTCATGAAGGCTTTTGTTTGG-3´
		Reverse primer	5´-CTCGAGTAAGTCAATAGGCTTGAT-3´
Angptl3	NM_013913.2	Forward primer	5´-GAATTCATGCACACAATTAAATTA-3´
		Reverse primer	5´-GTTAACTAAGGTGGTGGGCTGGAG-3´
Angptl4	NM_020581.1	Forward primer	5´-GAATTCATGCGCTGCGCTCCGACA-3´
		Reverse primer	5´-CTCGAGTAAAGAGGCTGCTGTAGC-3´
Angptl6	NM_145154.2	Forward primer	5´-GAATTCATGGGGACCGCCAGGCTA-3´
		Reverse primer	5´-CTCGAGTAACAAGCGCACAAGCCG-3´
Angptl7	NM_001039554.1	Forward primer	5´-GAATTCATGCTGAGGGAGACCTGG-3´
		Reverse primer	5´-CTCGAGTAAGGGCTTGAAGGCTTC-3´

pReceiver-M01.Angptl2 plasmid (NM_011923, GeneCopoeia, Rockville, MD, USA) by digesting with NotI/Klenow and EcoRI and was subcloned into the XhoI/Klenow and EcoRI sites of pMSCV. The resulting recombinant vector constructs were confirmed by colony PCR, restriction analysis and DNA sequencing. The respective constructs were propagated in *Escherichia coli* DH5α (Real Biotech Corporation, Taiwan) and purified with a NucleoBond(R) Xtra midi kit (Macherey-Nagel Gmbh & Co., Neumann-Neander-Str., Germany) according to the manufacturer's instructions.

Production of recombinant retroviral particles

For the production of recombinant retroviral particles, GP2-293 cells were co-transfected with recombinant retroviral vectors pMSCV.Angptl and pVSV-G (containing the viral envelope gene). In detail, GP2-293 cells were plated at a density of 4×10^6 cells in a 10 cm collagen-coated tissue culture dish (Biocoat, UK) containing 10 mL of growth medium. When the cells were at 80% confluence, the spent medium from the culture dish was replaced with 8 mL of pre-warmed transfection medium (Opti-MEMI, Invitrogen, Carlsbad, USA) and the cells were incubated for 1 h at 37°C in an atmosphere of 5% CO_2 and 95% air. During incubation, an equal amount (11 μg) of recombinant retroviral plasmid DNA and pVSV-G was mixed together in 1.46 mL of transfection media. Forty microliters of Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) was diluted in the 1.46 mL of transfection media and incubated for 5 min at room temperature. Immediately after incubation, the diluted lipofectamine was combined with the mixtures of DNA, mixed gently and incubated for 30 min at 37°C in a water bath to create lipoplexes. Following the removal of 3 mL of transfection medium from the culture dish, the lipoplexes were added drop wise onto the cells by shaking the dish back and forth. The culture dish was placed on a rocker (SLS4, Seoulin, Korea) at 10 rpm for 30 min at 37°C in an atmosphere of 5% CO_2 and 95% air. After rocking, the cells were incubated in steady state for another 5 h and then the transfection medium was replaced with fresh growth medium. The supernatant containing viral particles was collected at 48 h post-transfection, concentrated by Retro-X Concentrator (Clontech, CA, USA) according to manufacturer's instructions and stored at 80°C for the transduction of CHO-S cells.

Transduction of CHO-S cells by recombinant retroviral particles for the production of Angptls

Recombinant retroviral particle transduction was performed by a spinoculation method. CHO-S cells were seeded at a density of 5×10^4 cells/well in two 24-well plates (Corning Life Science, NY, USA) containing 50% fresh CHO expression medium and 50% conditioned medium from a 2-day CHO-S cell culture in a shaker flask along with or without 4 μg/mL of polybrene (Sigma-Aldrich, St. Louis, Mo). The plate containing polybrene was used for recombinant retroviral particle transduction and the plate without polybrene was used as a control. Both plates were placed on an orbital shaker at 140 rpm and incubated for 1 h at 37°C with an atmosphere of 8% CO_2 and 95% air. Following incubation, each well of the plate containing polybrene was fed at a 1:10 recombinant retroviral particles to cell volume ratio and both plates were spinoculated at 1000 g for 1 h at 30°C. After spinoculation, the plates were incubated for 12 h with shaking at 140 rpm in the 37°C incubator with 8% CO_2 and 95% air. The plates were subjected to second and third rounds of spinoculation performed after adding recombinant retroviral particles in the same well at the same ratio used in the first spinoculation. Both plates were cultured for 12 h after each spinoculation in similar conditions as after the first spinoculation. Thereafter, the whole cell suspension from 3 wells were aggregated and transferred to a single well from a 6-well plate containing fresh medium and grown further for 72 h with shaking at 130 rpm in the 37°C incubator with an atmosphere of 8% CO_2 and 95% air. After 72 h, a 50 μL aliquot of the cultured CHO-S cells were centrifuged at 14,000 g for 15 min at 4°C. The fresh supernatant was collected and subjected to western blot for the detection of Angptls.

Cell culture suspension processing

After Angptls detection in the suspension culture of CHO-S cells transduced with recombinant retroviral particles, the whole cell suspensions from both the transduced and control plates were collected separately, centrifuged at 14,000 g for 15 min at 4°C and the fresh supernatant was collected. The collected supernatant from CHO-S cells cultured in the 6-well plate was regarded as conditioned medium while the collected supernatant from recombinant retroviral particles-transduced-CHO-S cells cultured in the 6-well plate was regarded as Angptls. The

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concentration of Angptls in the conditioned medium was measured by BCA assay kit (Sigma-Aldrich, St. Louis, MO, USA).

Western blot

To investigate whether recombinant retroviral particles-transduced CHO-S cells secreted Angptls in culture medium, equal volume aliquots of conditioned medium and Angptls in conditioned medium were mixed with NuPAGE 4× LDS sample buffer (Invitrogen, Carlsbad, USA) and NuPAGE 10× sample reducing agent (Invitrogen, Carlsbad, USA) in a total reaction volume of 20 μL. Samples were boiled for 5 min at 70°C and 10 μL was loaded to a 4%–12% Bis-Tris gradient gel and subjected to electrophoresis by MES running buffer system (Invitrogen, Carlsbad, USA). After electrophoresis, the proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk (Bio-Rad Laboratories, Harcules, CA) in PBST-Triton X-100 [phosphate-buffered saline (PBS), 0.05% Tween-20 and 0.1% Triton X-100] for 3 h at room temperature. Thereafter, the membrane was washed briefly with PBST-Triton X-100 and incubated with the respective anti-Angptl primary antibodies for 3 h at room temperature. The membrane was washed 4 times for 10 min using PBST-Triton X-100 to remove unbound primary antibodies, incubated with the corresponding secondary antibodies for 1 h at room temperature and washed 4 times for 10 min using PBST-Triton X-100. The chemiluminescent substrate, Immun-Star™ Western CTM Kit (Bio-Rad Laboratories, Harcules, USA), was applied to the membrane and the images were captured with a Chemi-doc XRS camera equipped with a Bio-Rad Quantity One imaging system. The stock solutions of primary and secondary antibodies were diluted using an antibody dilution buffer (0.5% BSA in PBST-Triton X-100).

Isolation of bone marrow cells

Mice were euthanized and the femurs and tibias were removed. As previously described, the marrow cavities were flushed with MACS buffer (PBS with 0.5% BSA and 2 mmol/L EDTA) (Miltenyi Biotech, Auburn, CA, USA) using a 1 mL syringe with a 26-gauge needle under sterile conditions (Gilner et al., 2007). Single cell suspension was made by repeatedly pipeting up and down with a 10 mL serological pipette. Following passing the bone marrow cell suspension through a cell strainer (40 μmol/L, BD Falcon, USA) to remove debris, the strained cell suspension was washed 2 times with MACS buffer by centrifugation at 300 g for 10 min at 4°C.

Isolation and purification of hematopoietic stem cells (HSCs)

Before isolation of Lin$^-$Sca-1$^+$ HSCs, bone marrow cells were purified using standard Ficoll-Paque gradient centrifugation according to the manufacturer's instructions (Amersham Pharmacia, Uppsala, Sweden). Briefly, 20 mL of Ficoll-Paque gradient was pipetted into a 50 mL conical tube. The bone marrow cell suspension (26 mL) was carefully layered over the Ficoll-Paque gradient. Cell separation was carried out by centrifugation at 1000 g for 20 min at 18°C followed by aspirating most of the top layer supernatant without interfering with the interface white band. Cells at the white band were gently collected into a 50 mL conical tube and they were washed twice with MACS buffer by centrifugation at 600 g for 10 min at 18°C. The cell pellet containing the mononuclear fraction was ready for HSCS separation.

For the isolation of Lin$^-$ HSCs, the mononuclear cell pellet was resuspended in ice-cold MACS buffer and the cells were counted. Ten microliters of Biotin-Antibody Cocktail was added to every 10^7 cells in 40 μL of MACS buffer and incubated for 15 min at 4°C. Following incubation, the antibody-cell mixture was diluted by adding 30 μL of MACS buffer. The diluted mixture was then incubated with 20 μL of anti-Biotin MicroBeads for 20 min at 4°C. After washing the mixture with MACS buffer, the cell pellet was resuspended in 0.5 mL of room temperature degassed MACS buffer. The cell suspension was applied to a MS column pre-rinsed with 0.5 mL of room temperature, degassed MACS buffer followed by placing in the magnetic field of the MACS separator. Flow-through containing the Lin$^-$ cells was collected by centrifugation at 300 g for 10 min at 4°C.

For the isolation of Sca-1$^+$ HSCs, the Lin$^-$ cell pellet was resuspended in 90 μL of MACS buffer and treated with 10 μL of monoclonal anti-mouse Sca-1 antibody conjugated to FITC for 15 min at 4°C in the dark. After washing the mixture with MACS buffer by centrifugation at 300 g for 10 min at 4°C, the cell pellet was resuspended in 80 μL of MACS buffer. The antibody-cell conjugates were probed with 20 μL of anti-FITC microbeads for 20 min at 4°C. Following washing with MACS buffer, the cell pellet was resuspended in 0.5 mL of room temperature, degassed MACS buffer and the suspension was applied to a MS column pre-rinsed with 0.5 mL of room temperature, degassed MACS buffer followed by placing in the magnetic field of the MACS separator. The column was then washed 3 times with 0.5 mL of MACS buffer, separated from the magnetic field and immediately flushed out with 1 mL of room temperature, degassed MACS buffer. This fraction contained the Lin$^-$Sca-1$^+$ HSCs confirmed by flow cytometry (FCM) analysis.

Assay for the functional role of Angptls on HSCs

Five hundred bone marrow Lin$^-$Sca-1$^+$ HSCs isolated from 8-week-old C57BL/6 mice were plated in one well of a U-bottom 96-well plate (Corning Life Science, NY, USA) containing 160 μL of StemSpan serum-free medium (Stem Cell Technologies, Vancouver, Canada) with or without Angptls at a concentration of 250 ng/mL in conditioned medium from cultured CHO-S cells infected with recombinant retroviral particles or in the presence or absence of Angptls in conditioned medium supplemented with a combination of growth factors (10 μg/mL heparin, 10 μg/mL SCF, 20 μg/mL TPO, 20 μg/mL IGF-2 and 10 μg/mL of FGF-1). The plates were incubated in a humidified incubator at 37ºC in an atmosphere of 5% CO_2 and 95% air for the growth of HSCs. On day 7, cells from all conditional experiments were analyzed, counted by trypan blue dye exclusion, imaged with an inverted fluorescence microscope (TE2000-S, Nikon, Japan) and tested by FCM analysis.

Flow cytometry

To check whether the freshly purified HSCs, Angptls-supported-survival HSCs and *ex vivo*-expanded HSCs were Sca-1$^+$, we incubated 1 × 10^6 cells with monoclonal anti-mouse Sca-1 antibody conjugated to FITC for 30 min at 4°C in the dark and then washed twice in MACS buffer to wash off unbound antibodies. Propidium iodide (PI) at a concentration of 1 μg/mL was used to exclude dead cells. Samples were analyzed by BD FACS Callibur (Becton Dickenson) using CellQuest-pro software.

In vitro colony assays

To investigate whether freshly isolated Sca1$^+$ HSCs and Angptls-supported-survival HSCs were clonally functional, we collected

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freshly purified bone marrow Sca1$^+$ HSCs as a control and cultured Sca-1$^+$ HSCs for 7 days in serum-free stem cell growth medium supplemented with Angptl alone as a Angptls-supported-survival HSCs from the U-bottom 96-well plate. Thereafter, cells were centrifuged at 300 g for 5 min at 4°C. Following washing the cells two times with Iscove's modified Dulbecco's medium (IMDM) with 2% FBS, cells at 10x concentration per mL were resuspended in IMDM with 2% FBS to achieve the final concentration of 1 × 10^5 cells per 35 mm dish. Then 0.4 mL of cell suspension were added to 4 mL of methylcellulose medium M3434 for CFU-GEMM, CFU-GM, and BFU-E colony formation, M3630 for CFU-pre-B colony formation, or 3.6 mL of M3334 for CFU-E colony formation assays. Following vigorous mixing, triplicate culture of 1.1 mL of cell-methylcellulose medium mixture was plated into 35 mm culture dish. The culture dish was rotated to distribute the medium evenly, placed into a 100 mm petri dish carrying an uncovered 35 mm dish containing 3–4 mL of sterile water and incubated in a humidified incubator at 37°C in an atmosphere of 5% of CO_2 and 95% air for 7 days for the formation of colonies. Colonies were scored in the same dish using 60 mm Gridded Scoring Dishes under an inverted fluorescence microscope. For colony forming assays, all reagents were purchased from Stem Cell Technologies, Vancouver, BC, Canada.

Statistical analysis

All statistical analyses were performed using Origin 7 software. Data were reported as the mean ± STD and significance was calculated using one-way ANOVA followed by Bonferroni/Tukey multiple tests for individual means. *P* values less than 0.001 were considered statistically significant.

ACKNOWLEDGEMENTS

This research was supported by Technology Development Program for Agriculture and Forestry No. 610002-3, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

ABBREVIATIONS

Angptls, angiopoietin-like proteins; BM, bone-marrow; BSA, bovine serum albumin; FCM, flow cytometry; FGF-1, fibroblast growth factor 1; FITC, fluorescein isothiocyanate; GFs, growth factors; IGF-2, insulin-like growth factor 2; LDS, lithium dodecyl sulfate; HSCs, hematopoietic stem cells; NC, nitrocellulose; SCF, stem cell factor; TPO, thrombopoietin

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