

# Co-culture with podoplanin<sup>+</sup> cells protects leukemic blast cells with leukemia-associated antigens in the tumor microenvironment

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**Abstract.** Podoplanin<sup>+</sup> cells are indispensable in the tumor microenvironment. Increasing evidence suggests that podoplanin may support the growth and metastasis of solid tumors; however, to the best of our knowledge no studies have determined whether or not podoplanin serves a supportive role in acute myeloid leukemia (AML). The effects of co-culture with podoplanin<sup>+</sup> cells on the cellular activities of the leukemic cells, such as apoptosis and cell proliferation, in addition to the expression of podoplanin in leukemic cells, were investigated. Due to the fact that genetic abnormalities are the primary cause of leukemogenesis, the overexpression of the fibromyalgia-like tyrosine kinase-3 gene in colony forming units was also examined following cell sorting. Podoplanin<sup>+</sup> cells were found to play a protective role against apoptosis in leukemic cells and to promote cell proliferation. Tumor-associated antigens, including Wilms' tumor gene 1 and survivin, were increased when leukemic cells were co-cultured with podoplanin<sup>+</sup> cells. In combination, the present results also suggest that podoplanin<sup>+</sup> cells can function as stromal cells for blast cell retention in the AML tumor microenvironment.

## Introduction

Relapsed acute myeloid leukemia (AML) is considered to be the result of leukemic stem cell (LSC) survival following chemotherapy (1). AML is a heterogeneous clonal disorder, characterized by the accumulation of immature myeloblasts (2). Malignant cell proliferation is maintained by a

small fraction of LSCs, and similar to normal hematopoietic stem cells (HSCs), LSCs exhibit certain stem cell properties, including self-renewal, differentiation capacity and expression of cell surface phenotype CD34<sup>+</sup>CD38<sup>-</sup> markers (3). LSCs also predominantly produce colony-forming units (CFUs) *in vitro*, indicating their potential for full differentiation (4). The CFU assay is traditionally used for the detection of hematopoietic progenitor cells (HPCs) in the blood (5). Despite the differences in CFU formation between leukemic and normal progenitor cells, it remains unclear whether colonies are derived from normal HPCs or LSCs/HPCs expressing leukemia-associated genes.

Bone marrow (BM) microenvironments and stem/progenitor cells communicate in order to sustain drug resistance or differentiate into cell lineages; therefore understanding the stromal condition against leukemic cells expressing abnormal genes is required for the development of advanced therapeutic strategies to prevent relapse. Since 1863, when Rudolf Virchow highlighted the importance of the tumor microenvironment for cell growth (6), studies have supported the existence of an association between tumor cell fate and the microenvironment (6-8).

Podoplanin, a 38 kDa integral membrane mucoprotein, predominantly expressed in the lymphatic capillaries, has been identified to be involved in tumor progression, epithelial-to-mesenchymal transition and lymphatic function (9,10). Its expression has also been observed in intratumoral stromal cells, which can function as normal stromal cells (11,12). Previous studies have been demonstrated that podoplanin is a potent cancer-associated factor in the microenvironments of various tumor types (11,13,14). Podoplanin has been identified to be expressed in osteoblasts and osteocytes in normal bone tissue, and highly expressed in mesenchymal stromal cells, the main component of the BM microenvironment, under conditions of abundant vascular endothelial growth factor C (14,15). Despite the fact that the role of podoplanin in tumor development has been extensively studied (16-18), the role of podoplanin<sup>+</sup> cells as tumor microenvironmental factors in leukemia remains to be fully elucidated.

The present study examined the role of podoplanin<sup>+</sup> cells in leukemia, in addition to investigating its protective role

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against apoptosis in leukemic blasts, which are enriched by the fibromyalgia-like tyrosine kinase-3 (*FLT3*) gene. These present study aimed to provide insight into the role of podoplanin as a tumor microenvironmental factor, and contribute to the development of targeted therapies.

## Materials and methods

**Human primary cells and cell lines.** All experiments were approved by the Institutional Review Board of the Human Research at the Catholic University of Korea (Seoul, South Korea). A total of 12 AML blood samples were obtained from patients admitted to the Catholic Blood and Marrow Transplantation Center at Seoul St. Mary's Hospital (Seoul, South Korea). The patients were diagnosed with various subtypes of AML using the World Health Organization (WHO) classification system (19). A total of seven patients had AML not otherwise specified, three had AML with an inversion in chromosome 16, one had AML with myelodysplasia-related change, and one had acute promyelocytic leukemia. BM and peripheral blood (PB) samples were frozen in fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored in liquid nitrogen. BM- and PB-derived mononuclear cells (MNCs) were fractionated by density gradient centrifugation at  $1,220 \times g$  for 30 min at 4°C, using Ficoll-Paque™ (17-1440-03; GE Healthcare Life Sciences, Shanghai, China). The clinical characteristics and laboratory data of the patients with AML enrolled in the present study are listed in Table I. TIB152 human Jurkat cells (American Type Culture Collection, Manassas, VA, USA), were grown in RPMI medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. CellTrace™ carboxyfluorescein diacetate succinimidyl ester (CFSE; C34554; Invitrogen, Thermo Fisher Scientific, Inc.) with 5 μM in DMSO was used to stain the Jurkat cells.

**Magnetic-activated cell sorting and CFU assay.** Podoplanin<sup>+</sup> cells (BAF3670; R&D Systems, Inc., Minneapolis, MN, USA) were sorted and isolated from AML primary cells using magnetic beads (130-056-701; Miltenyi Biotec, Inc. Cambridge, MA, USA) in order to validate human clonogenic hematopoietic progenitor properties. Anti-biotin microbeads were used to isolate podoplanin (120-000-900; Miltenyi Biotec, Inc.). Sorted cells were cultured in methylcellulose media (H4434; STEMCELL Technologies, Inc., Vancouver, BC, Canada) for 7-10 days and colonies were counted using an inverted microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

**Jurkat proliferation assay.** CFSE-labeled Jurkat cells ( $2.5 \times 10^3$ ) were co-cultured with the sorted podoplanin<sup>+</sup> and podoplanin<sup>-</sup> cells ( $2.5 \times 10^3$ ) from BM-MNCs in RPMI medium supplemented with 1% FBS. After 24 h, the cells were stained with rabbit anti-human Ki67 antibody (cat no. ab15580; Abcam, Cambridge, UK) and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Cells positive for green fluorescent protein, CFSE-labeled Jurkat cells, Ki67 positive cells and DAPI-stained cells were counted under the inverted microscope.

**Flow cytometry.** Fluorescence activated cell sorting (FACS) staining and analysis was performed as previously described (20). Briefly, the cells were resuspended in 100 μl rinsing buffer and incubated with all antibodies at 4°C for 20 min. These included phycoerythrin (PE)-conjugated mouse anti-CD34 (1:20; cat no. 555822; BD Pharmingen, San Diego, CA, USA) and PEcy™ 5-conjugated mouse anti-CD38 (1:20; cat no. 555461; BD Pharmingen) antibodies, which were used to label leukemic stem cells (LSCs), and allophycocyanin (APC)-conjugated anti-human podoplanin polyclonal antibody (1:20; cat no. FAB3670A; R&D Systems, Inc.), which was used for the detection of podoplanin. Subsequently, the cells were incubated with PE-annexin V (cat no. 556421; BD Pharmingen) for 20 min at room temperature for the detection of apoptosis. Following washing with 1% bovine serum albumin in phosphate-buffered saline (PBS; Thermo Fisher Scientific, Inc.), the cells were analyzed using a FACSCalibur flow cytometer equipped with CellQuest software, version 3.0 (BD Biosciences, San Diego, CA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA isolation and DNA synthesis were performed as previously described (21). PCR reactions were performed in a 50 μl PCR reaction mixture (Promega Corporation, Madison, WI, USA) containing 100 ng of each primer, 1X Tris-ethylenediaminetetraacetic acid buffer, 100 ng template DNA, 2.5 units HQ Taq polymerase, and 2.5 mM deoxyribonucleotide triphosphate. PCR amplification was performed using a conventional thermocycler (Px2 Thermal Cycler; Thermo Fisher Scientific, Inc.) under the following cycling conditions: 94°C for 4 min; 30-36 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min; extension cycle was at 72°C for 7 min. The RT-qPCR products were separated on a 2.0% agarose gel (Sigma-Aldrich) at 12 V/cm using a Tris-acetic acid-ethylenediaminetetraacetic acid buffer, and were subsequently stained with ethidium bromide (Thermo Fisher Scientific, Inc.), and visualized and photographed under an ultra-violet transilluminator (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Information regarding the primer/probe sets (TaqMan; Biosearch Technologies, Inc., Novato, CA, USA) and the primers used in the present study is provided in Table II. The relative mRNA expression of target genes was calculated using the comparative C<sub>q</sub> method. All target gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in multiplexed reactions performed in triplicate. Differences in C<sub>q</sub> values were calculated for each target mRNA by subtracting the mean value of the GAPDH expression (relative expression =  $2^{-\Delta\Delta C_q}$ ) (22).

**Immunostaining.** Immunostaining was conducted as previously described (14). Briefly, using the cytospin method (4,23), cells were spun onto slides and fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10 min at 25°C. Following washing with PBS, the cells were blocked with 5% horse serum (Thermo Fisher Scientific, Inc.) and incubated with the primary antibodies overnight at 4°C, followed by incubation with the secondary antibody for 30 min at room temperature. The primary antibodies used were as follows: Biotinylated anti-podoplanin (cat no. BAF3670; R&D Systems, Inc.), rabbit

Table I. Clinical and laboratory features of patients with AML.

Patient	WHO subtype	Cell	Age (years)	Gender	WBC/mm <sup>3</sup> at diagnosis	Molecular defects	Cytogenetic anomalies
1	AML NOS	PB	19	M	32240	NEG	46, XY [20]
2	AML NOS	PB	64	F	127350	FLT3	46, XX [20]
3	AML with MRC	BM	65	M	260300	MRC	46, XY, del(5)(q11.2q15)[4]/46, XY[16]
4	APL	PB	41	M	43010	RARA	46, XY, t(15;17)(q22;q12)[20]
5	AML with inv(16)	PB	31	M	154500	CBFB	46, XY, t(9;22)(q34;q11.2), inv(16)(p13.1q22)[13]/47, idem, +17[15]/48, idem, +8, +17[2]
6	AML NOS	PB	54	F	227830	MLLT3	46, XX, t(9;11)(p22;q23)[20]
7	AML NOS	PB	41	M	248521	NPM1	46, XY [20]
8	AML with inv(16)	PB	45	M	42234	CBFB	46, XY, inv(16)(p13.1q22)[20]
9	AML NOS	PB	54	M	195104	NPM1	46, XY [20]
10	AML NOS	PB	36	F	240640	NPM1	46, XX [20]
11	AML with inv(16)	PB	46	M	108400	CBFB	46, XY, inv(16)(p13.1q22)[20]
12	AML NOS	PB	65	F	114510	NEG	46, XX [20]

WHO, World Health Organization; WBC, white blood cell; NOS, not otherwise specified; MRC, myelodysplasia-related change; APL, acute promyelocytic leukemia; inv(16), inversion in chromosome 16; PB, peripheral blood; BM, bone marrow; M, male; F, female; NEG, negative; FLT3, receptor-type tyrosine-protein kinase; RARA, retinoic acid receptor alpha; CBFB, core-binding factor subunit beta; MLLT3, myeloid/lymphoid or mixed-lineage leukemia protein translocated to 3; NPM1, nucleophosmin 1.

Table II. Primers and probes for reverse transcription-quantitative polymerase chain reaction.

Gene	Primers and probes (5'-3')
Mouse <i>GAPDH</i>	F: GGTGGTCTCCTCTGACTTCAACA R: GTGGTCGTTGAGGGCAATG P: CCACTCCTCCACCTTTGACGCTGG
Mouse <i>Wt1</i>	F: AGCTGTCGGTGGCACAGTTGTCA R: TGCCTGGGATGCTGGACTGTC P: ACCCCTCAAAGCGCCAGCTGGAGTTT
Mouse <i>survivin</i>	F: TCTGCTTTAAGGAATTGGAAGG R: CTCTGTCTGTCCAGTTTCAAG P: ACGGTTAGTTCTTCCATCTGCTTCTTGAC
Human <i>GAPDH</i>	F: GGTGGTCTCCTCTGACTTCAACA R: GTGGTCGTTGAGGGCAATG
Human <i>podoplanin</i>	F: CAGGTGCCGAAGATGATGTG R: TGTTGCCACCAGAGTTGTCA
Human <i>FLT3</i>	F: GCATGCCTGGTTCAAGAGAA R: TGCCAGGGTAAGGATTACA

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Wt1, Wilms' tumor gene 1; FLT3, Fms-like tyrosine kinase-3; F, forward; R, reverse; P, probe.

anti-CD34 (cat no. GWB-BBP214; GenWay Biotech, Inc., San Diego, CA, USA) and rabbit anti-Ki67. The Cy3 affinity-purified goat anti-IgG (cat no. NC9771594; Jackson Immuno-Research Laboratories, Inc., Inc., West Grove, PA, USA) secondary antibody was used. The cells were incubated with DAPI for 1 min at room temperature to stain the nuclei. Images were

captured using the Zeiss LSM 510 META confocal laser scanning microscope and LSM 510 Imaging software, version 3.2 (Carl Zeiss, Inc., Gottingen, Germany).

*Statistical analysis.* All results are presented as the mean  $\pm$  standard error. The comparison between groups was performed

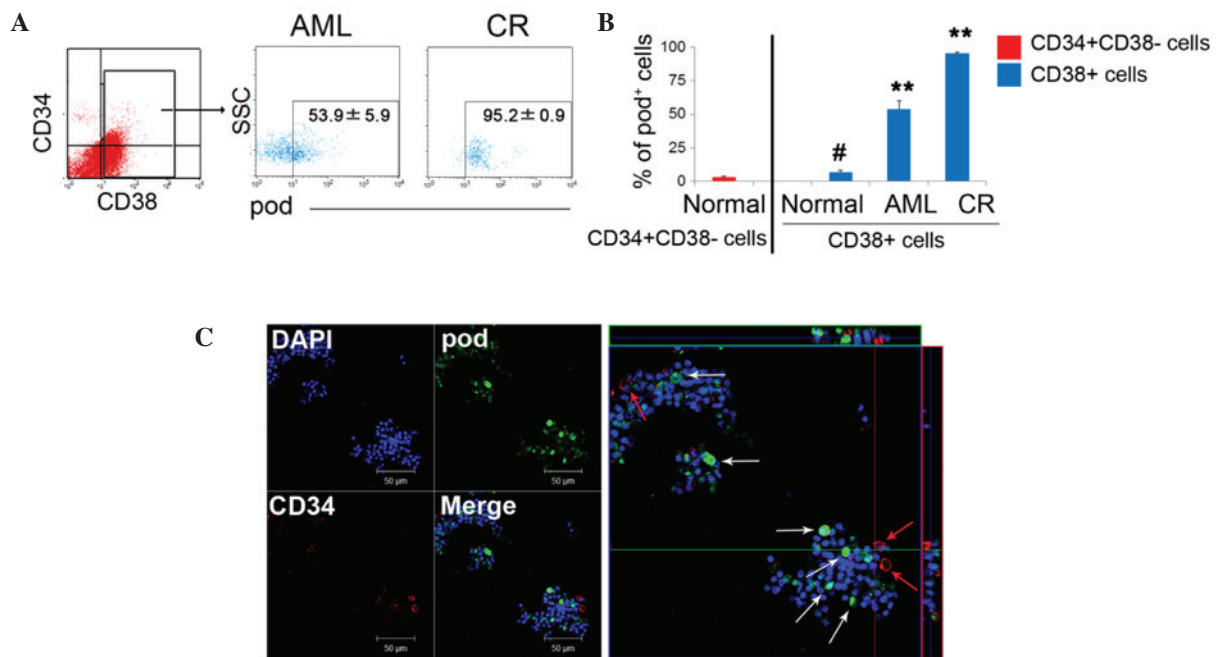


Figure 1. Expression of podoplanin in normal donors and patients with AML and those under CR. (A) Fluorescence activated cell sorting analysis revealed a high pod expression in CD38<sup>+</sup> differentiated cells. Additionally, AML and CR states led to increased podoplanin in CD38<sup>+</sup> cells. (B) Statistical analysis of pod in normal patients and patients with AML and CR. In normal conditions, the expression of pod was higher in CD38<sup>+</sup> cells than in CD34<sup>+</sup>CD38<sup>-</sup> leukemic stem cells. Data are presented as the mean ± standard error. \*\*P<0.01 and #P<0.05 vs. the CD34<sup>+</sup>CD38<sup>-</sup> cells (C) Leukemic cells were subjected to immunocytochemistry for CD34 (red) and pod (green) expression, and DAPI (blue) was used for nuclear staining. Red arrows indicate CD34<sup>+</sup> leukemic stem cells and white arrows depict pod<sup>+</sup> stromal cells. Scale bar, 50 μm. AML, acute myeloid leukemia; CR, complete remission; DAPI, 4',6-diamidino-2-phenylindole; pod, podoplanin.

using the Mann-Whitney U test. GraphPad Prism version 4 software (GraphPad Software, Inc, La Jolla, CA, USA) was used for the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

## Results

**High podoplanin expression on CD38<sup>+</sup> differentiated cells in leukemia.** To investigate the expression of podoplanin in leukemic cells, FACS analysis was performed in AML patient-derived cells. Under normal conditions, podoplanin is expressed in CD45<sup>-</sup> stromal cells, including osteocytes and osteoblasts; however, this protein is only expressed in CD45<sup>+</sup> hematopoietic cells under disease conditions (14,21,24). The results of the present study demonstrated that the expression of podoplanin was markedly higher in mature CD38<sup>+</sup> cells in complete remission (CR) than in those cells in the *de novo* AML state (AML, 53.9%; CR, 95.2%; Fig. 1A). Of note, under normal conditions, podoplanin<sup>+</sup> cells were significantly more frequent in mature CD38<sup>+</sup> cells (6.9%) than they were in CD34<sup>+</sup>CD38<sup>-</sup> HSCs (1.7%) (Fig. 1B). In CD38<sup>+</sup> differentiated cells, the expression of podoplanin was significantly and gradually increased during the complete remission (CR) state, compared with the AML and normal states. This suggests that podoplanin-sustaining cells are required for BM reconstruction or blast protection, and that most podoplanin<sup>+</sup> cells function as supportive cells rather than as LSCs. Due to the fact that CD38<sup>+</sup> cells consist of a number of immune cells such as T, B, and nature killer cells, most CD38<sup>+</sup> leukocytes that survive chemotherapy, may serve a role in blast communication in the tumor environment. A low frequency of CD34<sup>+</sup>

podoplanin<sup>+</sup> cells was also detected in flushed cells, whereas, podoplanin single positive cells exhibited a high frequency (Fig. 1C), again suggesting that podoplanin cells can potentially function as supportive cells rather than as LSCs.

**Enrichment of FLT3 in podoplanin<sup>-</sup>, however not podoplanin<sup>+</sup> cells and high CFU-colony forming efficiency of podoplanin<sup>-</sup> cells.** To further examine CFU potency, sorted cells were cultured in Matrigel gel supplemented with cytokines, and CFUs were observed after 10 days. Common myeloid progenitors were identified to be able to differentiate into two cell lineages: i) Granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM), which includes megakaryocytes and erythrocytes, and ii) granulocyte-macrophage (GM) cells, which represent myeloblasts. Fig. 2 presents the colonies formed, including GEMM, G, GM and M from podoplanin<sup>+</sup> or podoplanin<sup>-</sup> cells. The number of CFU-GM colonies detected in CD34<sup>+</sup> podoplanin<sup>-</sup> cells was significantly higher than that of other colonies (Fig. 2). Colonies produced from normal HSCs were characterized and enumerated by their distinct cell morphology. Similarly, leukemic-derived colonies were also rapidly formed by a progenitor population; however, leukemic-derived colonies with atypical morphologies in CD34<sup>+</sup> podoplanin<sup>-</sup> cells overwhelmingly produced abnormal HSCs. The majority of formed colonies were small and condensed (<0.4 mm), which is consistent with previous studies (4,25,26), suggesting a putative leukemic stem/progenitor cell function of podoplanin<sup>-</sup> cells. To examine whether these CFUs expressed leukemia-associated genes, and had a differential potency based on podoplanin expression, CD34<sup>+</sup> podoplanin<sup>+</sup> or CD34<sup>+</sup> podoplanin<sup>-</sup> cells were isolated using a microbead system. Sorted cells were



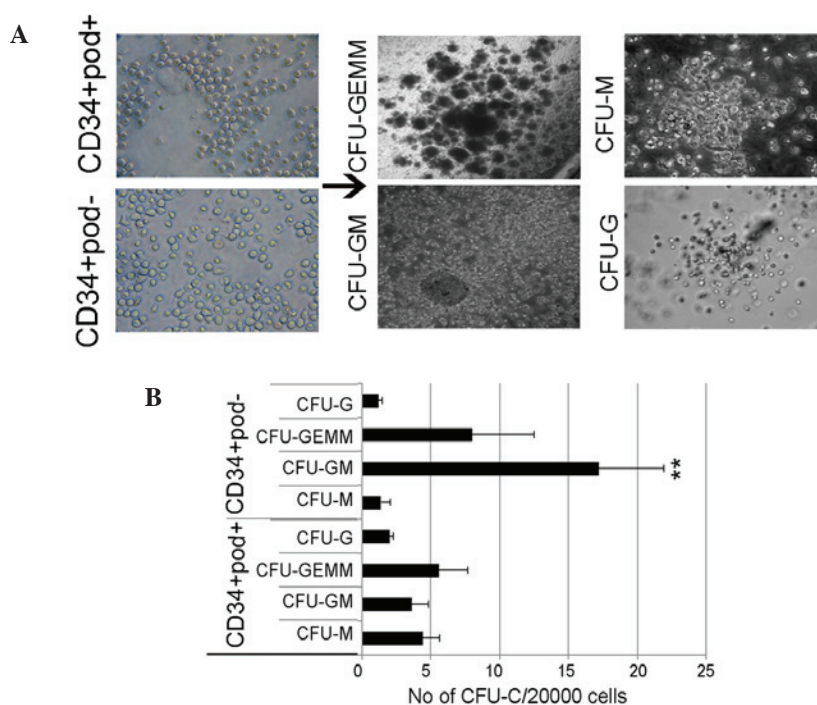


Figure 2. Leukemic-derived CFU-assay in CD34<sup>+</sup> podoplanin<sup>+</sup> or CD34<sup>+</sup> podoplanin<sup>-</sup> cells. (A) Morphologies of colonies. (B) Podoplanin<sup>-</sup> cells produced high numbers of CFUs, including CFU-GM and CFU-GEMM, compared with podoplanin<sup>+</sup> cells. Values are expressed as the mean  $\pm$  standard error. \*\*P<0.01 vs. CD34<sup>+</sup> podoplanin<sup>+</sup> cells. Scale bar, 100  $\mu$ m. CFU, colony forming unit; GM, granulocyte-macrophage; GEMM, granulocyte, erythrocyte, monocyte, megakaryocyte; G, granulocyte; M, macrophage.

immediately subjected to RT-qPCR to confirm the purity using podoplanin-specific primers, and the cells were then measured for *FLT3*, which is known to be overexpressed in patients with leukemia (27,28). The RT-qPCR data demonstrated that the podoplanin gene was exclusively expressed by the sorted podoplanin<sup>+</sup> cells, and that the *FLT3* gene was markedly increased in podoplanin<sup>-</sup> cells, however not in podoplanin<sup>+</sup> cells; however, the expression of these genes was similar in both podoplanin<sup>+</sup> and podoplanin<sup>-</sup> cells during differentiation (Fig. 3A). Sorted cells exhibited changeable expression of *FLT3* and *podoplanin* at the time of differentiation, implying that there is some flexibility in the expression of AML genes.

These results suggested that leukemic properties are enriched by podoplanin<sup>-</sup> rather than podoplanin<sup>+</sup> cells. *FLT3* acts as a molecular marker, and so it reflects a leukemic state (29,30); however, podoplanin<sup>+</sup> cells may not be directly representative of leukemic cells. It has been reported that translocation of the chromosome containing the core-binding factor subunit beta 1 (*CBFBI*) gene results in AML (31). The expression of *CBFBI* was restricted in podoplanin<sup>-</sup> cells regardless of further differentiation, suggesting that podoplanin<sup>+</sup> cells may function as stromal cells to podoplanin<sup>-</sup> cells (data not shown), which contain leukemic stem cells expressing *FLT3*. At a protein level, podoplanin is primarily sustained in differentiated CFUs, and simultaneously detected in podoplanin<sup>-</sup> cells (Fig. 3B), further suggesting its necessity in the maintenance of leukemic cells.

*Leukemic cells can promote proliferative and anti-apoptotic effects under co-culture with podoplanin<sup>+</sup> cells.* To investigate the function of podoplanin<sup>+</sup> cells as stromal cells, CFSE-stained Jurkat cells were cultured with podoplanin<sup>+</sup> or podoplanin<sup>-</sup> cells. After 24 h the Jurkat/podoplanin<sup>+</sup> co-cultured cells exhibited a

lower number of annexin-V<sup>+</sup> cells (2.29-fold), compared with the Jurkat/podoplanin<sup>-</sup> co-cultured cells (Fig. 4A), thus suggesting that podoplanin<sup>+</sup> cells can protect leukemic cells from apoptosis. Additionally, Jurkat cells proliferated rapidly during co-culture with podoplanin<sup>+</sup> cells. There was a significantly increased number of Ki67<sup>+</sup> green fluorescent protein<sup>+</sup> Jurkat cells during co-culture with podoplanin<sup>+</sup> cells (1.47-fold), compared with the results of co-culture with podoplanin<sup>-</sup> cells (Fig. 4B), suggesting the supportive role of podoplanin<sup>+</sup> cells in leukemic cell activity. These results raised the question of whether primary blasts are able to upregulate their leukemic-associated genes in podoplanin<sup>+</sup> stromal cell. Wilms' tumor gene 1 (*WT1*) and *survivin*, an apoptosis inhibitor encoded by *survivin* and expressed primarily in human blast cells, were selected for co-culture with podoplanin<sup>+</sup> or podoplanin<sup>-</sup> cells. Both genes are commonly regarded as leukemic-specific antigens and have been suggested to be upregulated under leukemic conditions (32). It was identified that the expression of *WT1* and *survivin* was significantly increased (27.4-fold and 6.2-fold, respectively) in the cells co-cultured with podoplanin<sup>+</sup> *in vitro* (Fig. 5), which supports a role of podoplanin<sup>+</sup> cells in the maintenance of leukemic cells.

## Discussion

Podoplanin was originally known as a protein marker for lymphatic endothelium (10). Previous studies have suggested a potential role of podoplanin in sustaining tumor cells in the tumor microenvironment (33,34). In addition, podoplanin<sup>+</sup> cells may function as stem/progenitor cells under lymphangiogenic or lymphavascuogenic conditions in BM-derived cells (21) and regulate tumor metastasis (35), suggesting a

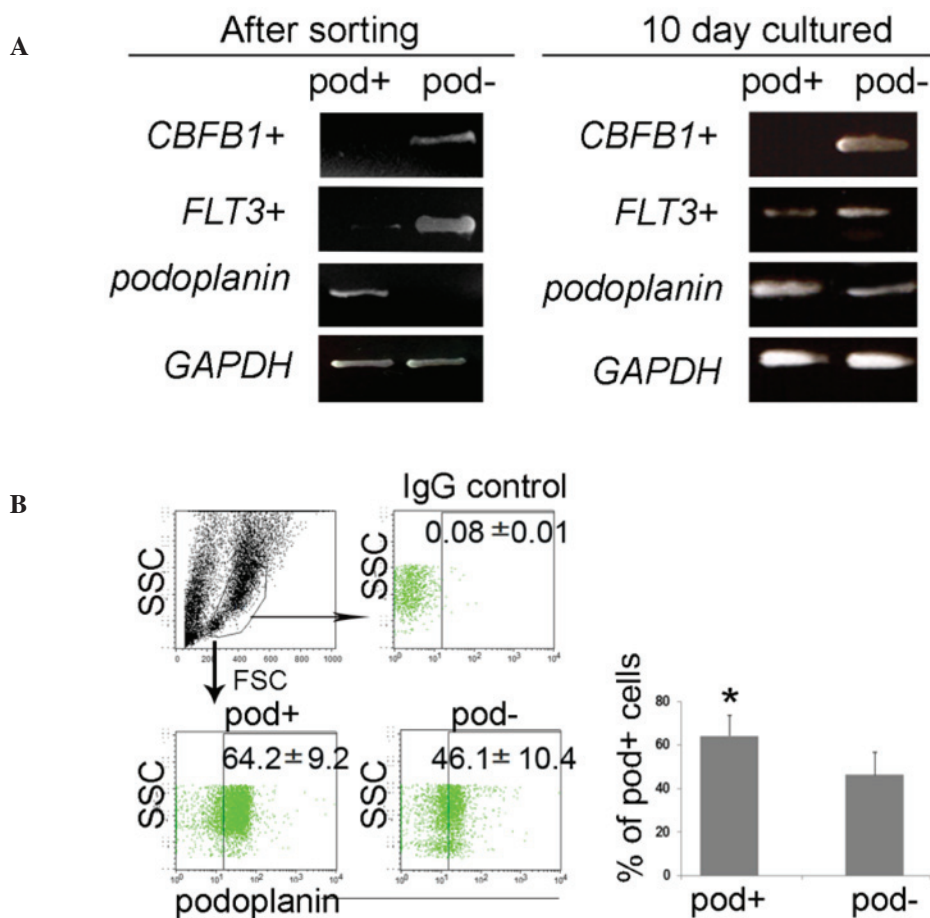


Figure 3. Enrichment of *FLT3* in sorted cells, and further differentiation from podoplanin<sup>+</sup> or podoplanin<sup>-</sup> cells. (A) Isolated podoplanin<sup>+</sup> and podoplanin<sup>-</sup> cells maintained high purity following magnetic-activated cell sorting, and *FLT3* was exclusively expressed in podoplanin<sup>-</sup> cells; however, their expression was altered by differentiation. (B) At the protein level, the podoplanin expression was also upregulated in the podoplanin<sup>+</sup> cell population, implying flexibility in leukemic status. Values are expressed as the mean ± standard error. \**P* < 0.05 vs. podoplanin<sup>-</sup> cells. *CBFB1*, core-binding factor subunit beta 1; *FLT3*, Fms-like tyrosine kinase-3; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; SSC, side scatter; FSC, forward scatter; pod, podoplanin.

multifactorial role of podoplanin in solid tumors. The role of podoplanin in leukemia, however, remains unclear. Previous studies reported that lymphangiogenic cytokines and markers, including podoplanin, are involved in leukemia, and in the BM microenvironment in particular (36,37).

Leukemic stem cells require stromal cells to survive chemotherapy (38). In numerous niches, stromal cells, including osteoblasts in normal BM, express podoplanin; this expression has been demonstrated to increase markedly under tumor conditions (24). In the present study, an increased level of podoplanin was observed in leukemic cells, which is consistent with previous studies of solid tumors (39-41). Of note, CD38<sup>+</sup> cells sustained a high podoplanin expression in the *de novo* AML and CR states following chemotherapy, and increased podoplanin is continuously required to maintain BM reconstruction or blast survival. The high expression of podoplanin in CD38<sup>+</sup> cells, including leukocytes, may be associated with the release of podoplanin-soluble mediators. Cross-linkage between podoplanin-soluble mediator defensive action and surviving leukemic stem cells should be investigated in order to assist the development of targeted AML therapy.

Previously, Kim *et al* (42) reported that osteopontin (OPN) production by tumor cells, however not by stromal cells, enhances the propagation of tumor initiating cells

in tumor environments, and that OPN silencing can delay tumor growth and extramedullary myelopoiesis. Like the diverse roles of OPN in tumor cells, the effects of podoplanin may alter depending on the environment; thus the present study investigated whether the inhibition of podoplanin was able to suppress leukemic blasts. A protective effect of podoplanin<sup>+</sup> cells against apoptosis in blasts was detected, and further studies are required to identify cell type from podoplanin<sup>+</sup> cells, which are associated with leukemic blasts. Stromal cell impairment leads to deficient hematopoiesis and chromosomal abnormalities, which may contribute to leukemogenesis (43,44), indicating the importance of micro-environment alteration in leukemia.

In the present study, leukemia-derived cells that express leukemia-related genes were markedly increased on podoplanin<sup>-</sup> CD34<sup>+</sup> cells. Podoplanin<sup>+</sup> cells, which contain stromal cells, partly expressed hematopoietic-associated genes during differentiation; however, the mechanism through which the switching of podoplanin expression occurs, and the way it evolves to the progression of leukemic cells, remains unknown. Stromal cells appear to serve a role in AML by preventing apoptosis (45). Boyerinas *et al* (7) suggested that dormant leukemic cells are heavily regulated by the BM niche. By contrast, Flach *et al* (46) and Schepers *et al* (47)

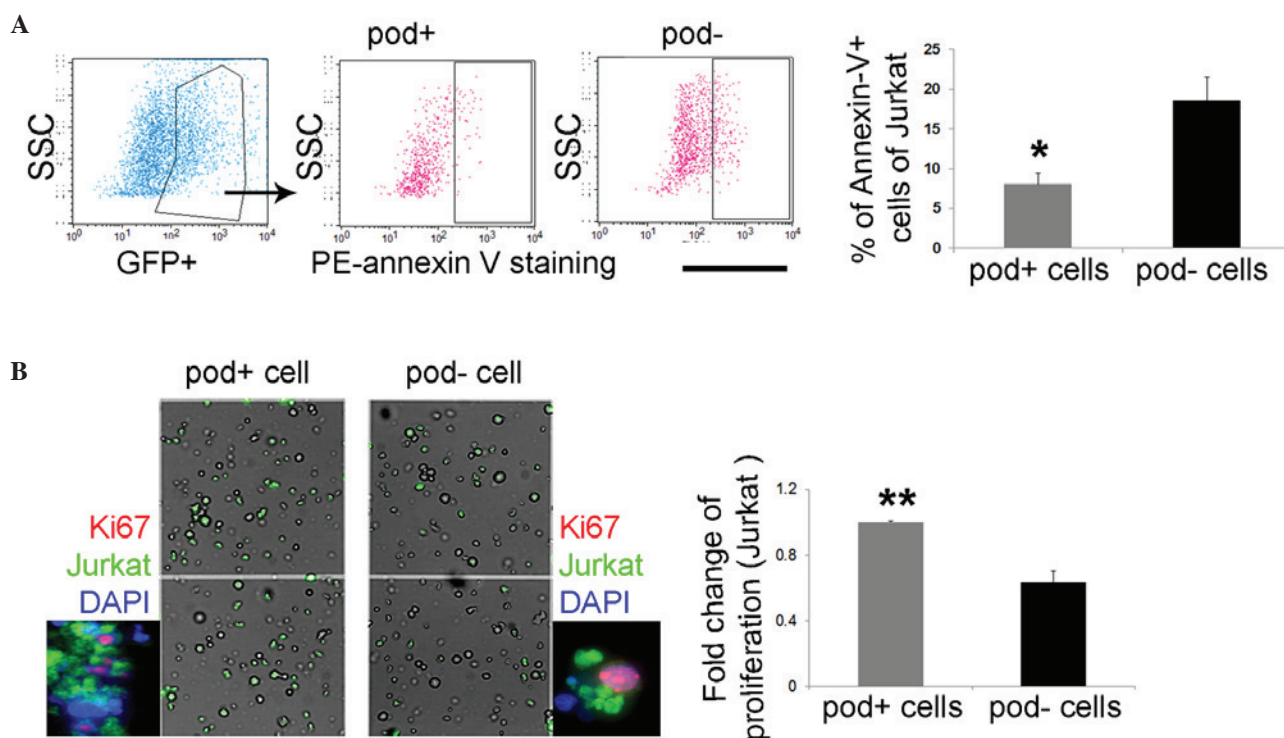


Figure 4. Pod<sup>+</sup> mediated protection against apoptosis and proliferation of blast cells. (A) Annexin-V<sup>+</sup> cells were reduced in GFP<sup>+</sup> Jurkat cells co-cultured with pod<sup>+</sup> cells. (B) Proliferation of Jurkat cells was significant with pod<sup>+</sup> cell co-culture. Ki67 (red) and GFP<sup>+</sup> Jurkat cells (green) were clearly detected in leukemic cells. Double positive cells were counted for proliferation. Values are expressed as the mean  $\pm$  standard error. \*\*P<0.01, \*P<0.05 vs. pod<sup>-</sup> cells. DAPI, 4',6-diamidino-2-phenylindole; pod, podoplanin. GFP, green fluorescent protein; SSC, side scatter; FSC, forward scatter; PE, phycoerythrin.

emphasized that DNA damage is responsible for the conversion of normal HSCs into malignant cells, and that LSC eventually leads to disruption of BM niches. Despite the controversy, understanding the association between LSCs and their surrounding environment is required for the treatment of AML.

Chemotherapy-resistant leukemic stem cells are typically observed in BM, and interact with stromal cells to promote blast retention (47-50). Since the development of leukemia leads to alterations in microenvironmental factors, including immune and stromal cells, these alterations may directly or indirectly affect leukemic cells in a reciprocal manner (7,45,47). In the present study, a marked reduction in blast cell apoptosis was observed following co-culture with podoplanin<sup>+</sup> cells, suggesting that blast cells rapidly promote cell proliferation, and have a protective role.

Further studies on syngeneic mouse models are required in order to gain insight into the function of podoplanin cells in leukemia, as well as to fully elucidate the functional properties of podoplanin<sup>+</sup> stromal cells in the presence of cytokines or trafficking leukemia-associated mutant genes. The observations of the present study indicated that podoplanin<sup>+</sup> cells in patients with leukemia are able to function as stromal cells, in order to protect against apoptosis and leukemic propagation with increased leukemic antigens.

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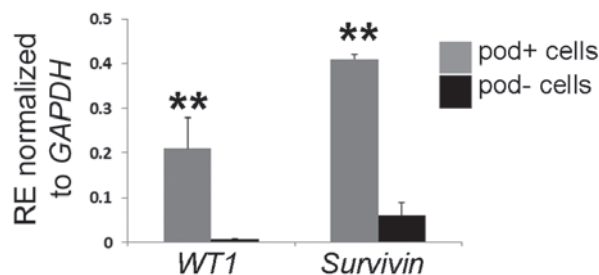


Figure 5. Increase in the leukemic antigens *WT1* and *survivin* following co-culture with pod<sup>+</sup> cells. *WT1* and *survivin* RNA expression levels were also increased in the pod<sup>+</sup> co-cultured cells, compared with pod<sup>-</sup> co-cultured cells. Values are expressed as the mean  $\pm$  standard error. \*\*P<0.01 vs. pod<sup>-</sup> cells. RE, RNA expression; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *WT1*, Wilms' tumor gene 1; pod, podoplanin.

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