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Journal of Cellular Biochemistry 112:1277–1285 (2011)

Journal of Cellular Biochemistry

Expression of CD34 in Hematopoietic Cancer Cell Lines Reflects Tightly Regulated Stem/Progenitor-Like State

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ABSTRACT

Hematopoietic cancer stem cells preserve cellular hierarchy in a manner similar to normal stem cells, yet the underlying regulatory mechanisms are poorly understood. It is known that both normal and malignant stem/progenitor cells express CD34. Here, we demonstrate that several cell lines (HL-60, U266) derived from hematopoietic malignancies contain not only $CD34^-$ but also $CD34^+$ subpopulations. The $CD34^+$ cells displayed a stem/progenitor-like phenotype since, in contrast to $CD34^-$ cells, they frequently underwent cellular division and rapidly formed colonies in methylcellulose-based medium. Strikingly, a constant fraction of the $CD34^+$ and $CD34^-$ cell subpopulations, when separated, rapidly switched their phenotype. Consequently, both separated fractions could generate tumors in immunocompromised NOD/LtSz-scid/scid mice. Cultures in vitro showed that the proportion of $CD34^+$ stem/progenitor-like cells in the population was decreased by cell–cell contact and increased by soluble factors secreted by the cells. Using cytokine arrays, we identified some of these factors, notably thymopoietin that was able to increase the proportion of $CD34^+$ cells and overall colony-forming capacity in tested cell lines. This action of thymopoietin was conserved in mononuclear cells from bone marrow. Therefore, we propose that hematopoietic cancer cell lines containing subpopulations of $CD34^+$ cells can provide an in vitro model for studies of cancer stem/progenitor cells. J. Cell. Biochem. 112: 1277–1285, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CANCER STEM CELLS; PROGENITORS; HEMATOPOIESIS; CD34 PROTEIN; THYMOPOIETIN

In humans, hematopoietic stem/progenitor cells express CD34, a cell-surface protein, that is gradually lost during differentiation [reviewed in Krause et al., 1996]. Quiescent $CD34^-$ stem cells [Osawa et al., 1996; Bhatia et al., 1998; Wang et al., 2003] give rise to actively cycling $CD34^+$ stem/progenitor cells, which in turn give rise to differentiated $CD34^-$ cells of various blood lineages [Zanjani et al., 1998; Nakamura et al., 1999; Sato et al., 1999; Wang et al., 2003; Dooley and Oppenlander, 2004]. It has been suggested that hematopoietic stem cells that become malignant and are at the origin of malignant clones preserve cellular hierarchy in a manner similar to normal cells [Sutherland et al., 1996; Bonnet and Dick, 1997]. The regulation of this hierarchy is not fully understood, mainly because bone marrow of patients contains both normal and malignant hematopoietic stem cells that cannot currently be distinguished by simple staining of cell surface markers. This problem could be overcome by the use of models such as cancer cell lines. Despite their acquired ability to grow in vitro, cancer cell lines exhibit genetic and molecular features of the primary cancers from which they were derived. Moreover, if we assume that cell lines can contain stem cells, they could only contain cancer stem cells, as their normal equivalents differentiate rapidly in culture conditions and stop proliferating.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ligue Nationale Contre le Cancer, Equipes Labellisées, (Comité du Septentrion); Grant sponsor: Institut National du Cancer (Program: IMMUDORM); Grant sponsor: Institut de Recherche sur le Cancer de Lille (K. Kuranda); Grant sponsor: Région Nord-Pas de Calais.

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Received 4 January 2011; Accepted 6 January 2011 • DOI 10.1002/jcb.23026 • © 2011 Wiley-Liss, Inc.
Published online 18 January 2011 in Wiley Online Library (wileyonlinelibrary.com).

Existing hematopoietic cancer cell lines can be divided into two categories, those that express CD34 (CD34-positive cell lines) and those that lack this protein at the cell surface (CD34-negative cell lines). However, we have recently showed that several CD34-negative cell lines derived from multiple myeloma contain a subpopulation of $CD34^+$ cells [Kuranda et al., 2010]. Here, we demonstrated that a $CD34^+$ subpopulation also exists in cell lines of other tumor types. This offered an opportunity to study the properties of a population that contain at the same time both $CD34^+$ and $CD34^-$ cells. We showed that expression of CD34 was reversible and indicated stem/progenitor-like state of cells in the tested cell lines. Furthermore, we revealed how the proportion of $CD34^+$ cells in whole population is regulated. Based on these results, we identified a novel function of thymopoietin as a modulator of CD34 expression in bone marrow cells.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

Hematopoietic cell lines were obtained from the European Collection of Cell Cultures (ECACC). U266, RPMI8226, KARPAS417, HL-60, K-562, Kasumi 1 and primary cells were cultivated in standard RPMI1640 GlutaMax medium (Gibco) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, MEM-Amino Acid Solution (PAN-Biotech), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were maintained at a standard concentration of 0.5×10^6 cells/mL. For cytokine assays, cells were cultivated for 24 h without FBS. In primary cell cultures, FBS was replaced with 10% BIT Serum Substitute (Stem Cells Technologies). Primary cells were obtained from bone marrow aspirates from healthy donors that gave informed consent in accordance with the Declaration of Helsinki. Experiments were approved by the Institutional Review Board at *Tumorotheque du Centre Hospitalier et Universitaire de Lille, Centre de Biologie*, Lille, France.

IMMUNOPHENOTYPING AND CELL SORTING

Prior to staining with antibodies, human or murine cells were treated with FcR blocking reagent (Miltenyi Biotec and BD Pharmingen, respectively). Cells were analyzed on an Epics XL-4 flow cytometer with Expo32 software (Beckman Coulter). Dead or apoptotic cells were excluded from analysis using morphology (SS/FS) and Violet LIVE/DEAD® Fixable Dead Cell Stain Kits (Invitrogen). We used human-specific monoclonal antibodies for all experiments: anti-CD34:FITC or anti-CD34:PE (clone AC136) from Miltenyi Biotec; anti-CD34:PE (clone 8G12) and anti-CD138:PC5 from Becton Dickinson; anti-CD34:PC5 (clone QBEnd10) and anti-CD45:ECD from Beckman Coulter. Primary cells were stained with anti-CD34:APC, anti-CD38:PECy7 and anti-CD45:PerCP from Becton Dickinson. Cells were sorted on an EPICS XL3-MCL cell sorter (Beckman) equipped with EXPO 2 WINCYCLE software for acquisition and analysis.

COMPARATIVE GENOME HYBRIDIZATION (CGH) ARRAYS

Genomic DNA was isolated (Qiagen) from $CD34^+$ or $CD34^-$ cells from U266 and HL-60 cell lines, then double-digested with the restriction enzymes AluI and RsaI and labeled by random priming with Cy5-dUTP or Cy3-dUTP. The labeled DNA was hybridized onto 44 K chips using the Agilent Human Genome Microarray Kit (Agilent Technologies) according to the manufacturer's recommendations. Raw data were processed and graphically visualized with Agilent CGH Analytics software (v.3.5.14) and treated by the WACA algorithm [Lepretre et al., 2010] using DNA sequence information from the UCSC Human Genome Browser database (Human Genome build 18). Data sets were reviewed for common copy number polymorphisms compared to the Genomic Variants Database (http://www.projects.tcag.ca/variation/).

XENOGRAFTS

A total of 5×10^4 HL-60 $CD34^+$ or $CD34^-$ cells were injected intraperitoneally into six-week-old male NOD/LtSz-scid/scid (NOD/SCID) mice (Charles Rivers Laboratories). All animals were handled in sterile conditions and maintained in microisolators. After 16–18 weeks, mice were sacrificed (CO_2 asphyxiation). The tumor mass was extracted and immediately analyzed for the presence of human cells by flow cytometry. The remaining cells were placed in standard cultures to follow changes in the percentage of $CD34^+$ cells. Experiments were performed in compliance with the guidelines of the JPARC/INSERM U837 animal facility and were approved by the Institutional Animal Care and Use Committee of the University of Lille.

CLONOGENIC ASSAYS IN SEMI-SOLID MEDIUM

$CD34^+$, $CD34^-$ or non-sorted U266 and HL-60 cells were suspended in Human Methylcellulose-Enriched Medium (Iscove's Modified Dulbecco's medium, 25% FBS, 2% BSA, 2 mM L-glutamine, β-mercaptoethanol 5×10^{-5} M, 50 ng/mL SCF, 20 ng/mL G-CSF, 20 ng/mL GM-CSF, 20 ng/mL IL-3, 20 ng/mL IL-6, 3 IU/mL Epo) (R&D Systems) at a concentration of 1,600 cells/mL. The number of colonies obtained was counted after 7 days. Where indicated, thymopentin (TP-5; Bachem) was added to the medium. Bone marrow mononuclear cells were cultivated under identical conditions (10,000 cells/mL). Colonies were scored after 12 days.

CELL CYCLE ANALYSIS

U266 and HL-60 cells were labeled with anti-CD34:PE, fixed with 1% paraformaldehyde and permeabilized with 70% ethanol. Cellular RNA was digested with 0.1 mg/mL RNase A (Roche Applied Science), and DNA was stained with 40 μg/mL propidium iodide (IP) (BD Pharmingen). Subsequently, the DNA content, indicated by the retention of IP in cells, was assessed in $CD34^+$ and $CD34^-$ cells using flow cytometry.

PROLIFERATION ASSAY

FACS-separated $CD34^+$, $CD34^-$, and non-sorted cells were seeded at a concentration of 0.2×10^6 cells/mL in 96-well plates in triplicate. The number of viable cells was evaluated every day using cytometer (Thoma's cell) and staining with trypan blue.

CELL–CELL CONTACT ASSAYS

(I) Non-sorted cells were seeded at concentrations ranging from 3 to 0.01×10^6 cells/mL in 24-well plates. After 24 h of growth, equal numbers of cells were analyzed for $CD34^+$ cell populations

using flow cytometry (Fig. 4A). (II) Various numbers of non-sorted cells were seeded in the upper compartments of trans-well plates, which allowed free diffusion of soluble factors to the lower compartment of the 24-well plate. The percentage of $CD34^+$ cells placed in the lower compartment (initial concentration of 0.05×10^6 cells/mL) was measured after 24 h of growth (Fig. 4B).

CULTURES WITH PRE-CONDITIONED MEDIUM

So-called "pre-conditioned" medium was defined as the cell-free supernatant of highly concentrated cell cultures (3×10^6 cells/mL) after 24 h of growth. Pre-conditioned medium containing secreted factors was mixed with fresh medium in various proportions to constitute 10%, 20%, 30%, 40%, or 50% of total volume. Non-sorted cells were seeded in the prepared media at an initial concentration of 0.5×10^6 cells/mL. The percentage of $CD34^+$ cells was measured after 24 h of growth.

CYTOKINE SECRETION

Cytokine arrays. FACS-separated $CD34^+$ and $CD34^-$ cells were cultured for 24 h in medium without FBS. The presence of 507 chosen proteins in cell-free supernatants from these cultures was assessed using a RayBio® Biotin Label-Based Human Antibody Array I (RayBiotech Inc.) according to the manufacturer's protocol. Briefly, proteins were biotinylated and incubated with antibody arrays followed by additional incubation with streptavidin coupled to AlexaFluor and extensive washing. Arrays were scanned with an Innoscan 700 (Innopsys™), and the obtained signals were transformed into numerical values with MicroVigene© software (Vers. 2.9.7.2). After normalization and removal of background noise, the ratio of secreted cytokines between $CD34^+$ and $CD34^-$ subpopulations was calculated. Experiments were performed twice in triplicate.

Dot-blots. $CD34^+$ cells were isolated from bone marrow mononuclear cells after Ficoll centrifugation (CD34 MicroBeads, Miltenyi Biotec). Enriched (~90% purity) populations of $CD34^+$, U266, or HL-60 cells were cultured without FBS (1×10^6 cells/mL) for 24 h. Cell-free supernatants were dialyzed for 30 min on filter membranes (0.025 μm, Millipore) and spotted at five different concentrations on nitrocellulose membranes (Sigma–Aldrich). Fresh medium was processed in the same way to serve as a background control. A standard curve was prepared using five concentrations of human recombinant thymopoietin I/II (29–41) (Bachem). The membranes were incubated overnight at room temperature with the primary anti-human thymopoietin antibody (R&D Systems). Alkaline phosphatase-conjugated anti-goat IgG was used as a secondary antibody. Signals were visualized with BCIP/NBT solution (Sigma), scanned and quantified using Gel Analyst Software (Clara Vision). Signal values from recombinant thymopoietin dilutions were used as a concentration curve to allow subsequent estimation of cytokine yield in samples.

RESULTS

HEMATOPOIETIC CELL LINES CONTAIN SMALL SUBPOPULATIONS OF CD34+ CELLS

We previously showed that certain myeloma cell lines (U266, KARPAS417, RPMI8226) contain a small subpopulation of cells expressing CD34, a stem/progenitor cell marker [Kuranda et al., 2010]. Here we showed that this subpopulation was also present in leukemic cell lines of myeloid origin (HL-60, K-562) using flow cytometry analysis (Fig. 1A). The fact that the existence of these subpopulations has been overlooked until now could be explained by the relatively low mean fluorescence intensity of CD34 staining compared to the CD34-positive Kasumi-1 or KG-1a cell lines. To exclude contamination of cell cultures with another CD34-positive cell line or the possibility that $CD34^+$ cells belong to a subclone, we performed comparative genomic hybridization (CGH) arrays, which allow visualization of gene copy-number variants and can be read as a "signature" of clonal populations. We tested two cell lines of different tumor origins, U266 lymphoid and HL-60 myeloid cells. $CD34^+$ and $CD34^-$ cells were separated using fluorescence activated cell sorting (FACS). Segmentation in DNA analytics using the ADM2 algorithm with a 3 point 0.14 filter [Lepretre et al., 2010] led to the discovery of 46 and 91 aberrant segments in U266 and HL-60, respectively (GEO accession number: GSE21923). In both cell lines, $CD34^+$ cells had a genomic profile identical to that of $CD34^-$ cells, which proved that they belonged to the same clonal population (Fig. 1B).

CD34 PHENOTYPE IS REVERSIBLE BOTH IN VITRO AND IN VIVO

We separated HL-60 $CD34^+$ and $CD34^-$ subpopulations by FACS and then placed each of them in standard culture conditions (0.5×10^6 cells/mL) or injected them into NOD/SCID mice. After 24 h of growth in vitro, 46% of the separated $CD34^-$ cells became $CD34^+$, while 34% of $CD34^+$ cells lost the CD34 antigen on the cell surface (Fig. 2). Five days later, both separated populations progressively regained the original ~5% level of $CD34^+$ cells typical for this cell line. Of six mice injected with 5×10^4 $CD34^+$ cells, only one formed a tumor that was palpable 16 weeks post-injection. Similarly, among six mice injected with $CD34^-$ cells, a tumor was detected in one mouse 18 weeks post-injection. Phenotype analysis of the tumor cells showed that they contained both $CD34^-$ and $CD34^+$ cells and that the proportion of the $CD34^+$ cells was 4.5-fold higher than in the cell line grown in vitro (Fig. 2). When tumor cells were placed in standard culture conditions, the proportion of $CD34^+$ cells reached 5% after 1 week of growth. These experiments showed that CD34 phenotype was reversible and that both $CD34^+$ and $CD34^-$ subpopulations could regenerate the total population in vitro and in vivo. Moreover, these results suggested that the optimal proportion of $CD34^+$ cells in the total population might depend on growth conditions.

CD34+ CELLS HAVE A PROLIFERATIVE ADVANTAGE OVER CD34− CELLS

Normal $CD34^+$ cells isolated from human bone marrow are able to form colonies in vitro in semi-solid medium. To assess whether this property extends to the $CD34^+$ cells in the cell lines, we investigated

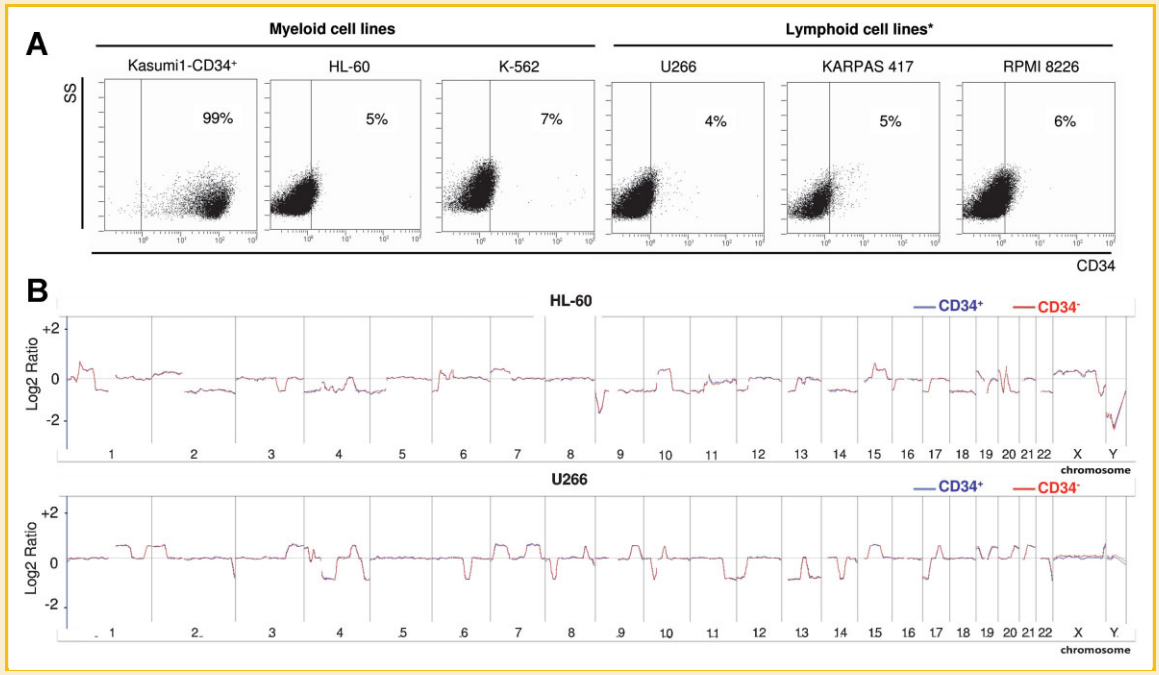

Fig. 1. Clonal CD34$^+$ subpopulations in hematopoietic cell lines. A: Flow cytometry (FC) analysis of cells stained with anti-CD34:FITC antibody. The percentage of CD34$^+$ cells was calculated based on the isotype control. Data represent one typical experiment that was performed three times with consistent results. *Lymphoid cell lines derived from multiple myeloma were described previously in [Kuranda et al., 2010]. B: CGH arrays showed identical genomic signatures for the CD34$^+$ and CD34$^-$ subpopulations from two cell lines of distinct tumor origins (qc metrics 0.3623 ± 0.0197, $P = 0.0476$ for SD; and 0.1403 ± 0.0128, $P = 0.0029$ for DLRS).

the clonogenic potential of HL-60 and U266 cells in methylcellulose-based medium (Fig. 3A,B). In both cell lines, only CD34$^+$ cells formed colonies in methylcellulose (U266, $41 \pm 2.9\%$; HL-60, $23 \pm 3.1\%$). Concordantly, the percentage of cells able to form colonies in total populations (U266, $5.1 \pm 0.9\%$; HL-60, $4.6 \pm 1.1\%$) corresponded to the percentage of CD34$^+$ cells in these cell lines. Analysis of propidium iodide staining showed that, in contrast to the CD34$^-$ cells, the majority of CD34$^+$ cells were actively

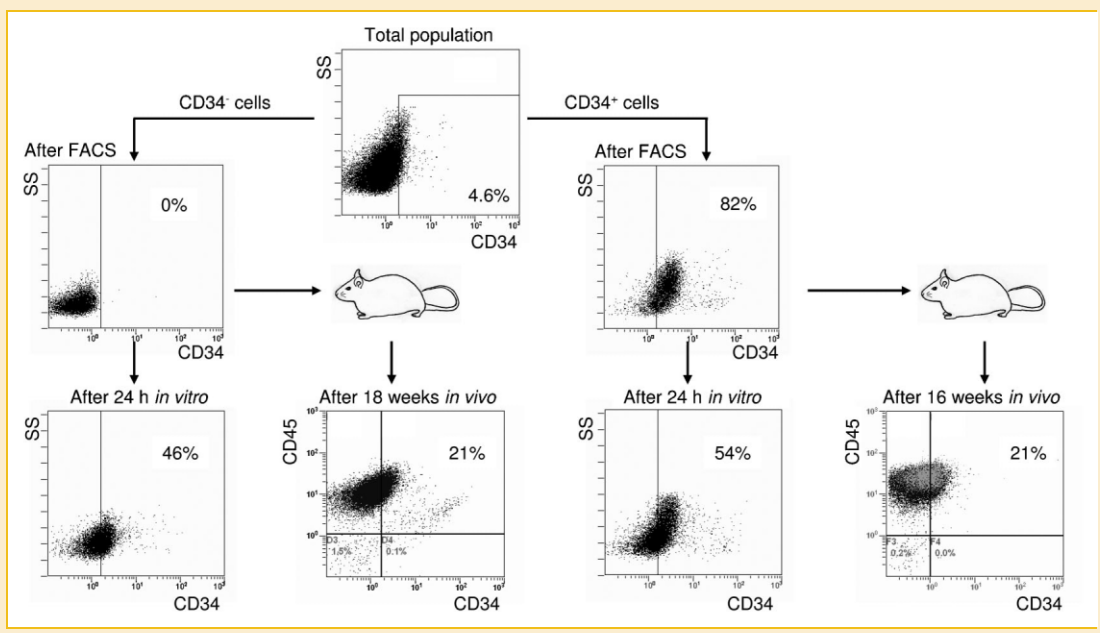

Fig. 2. The CD34$^{+/-}$ phenotype is reversible in vitro and in vivo. HL-60 CD34$^+$ and CD34$^-$ cells were separated by FACS and maintained in standard cultures or were injected into NOD/SCID mice. The percentage of CD34$^+$ cells from cell cultures and tumors was measured by flow cytometry analysis. Data from cell cultures represent one typical experiment that was performed three times with consistent results.

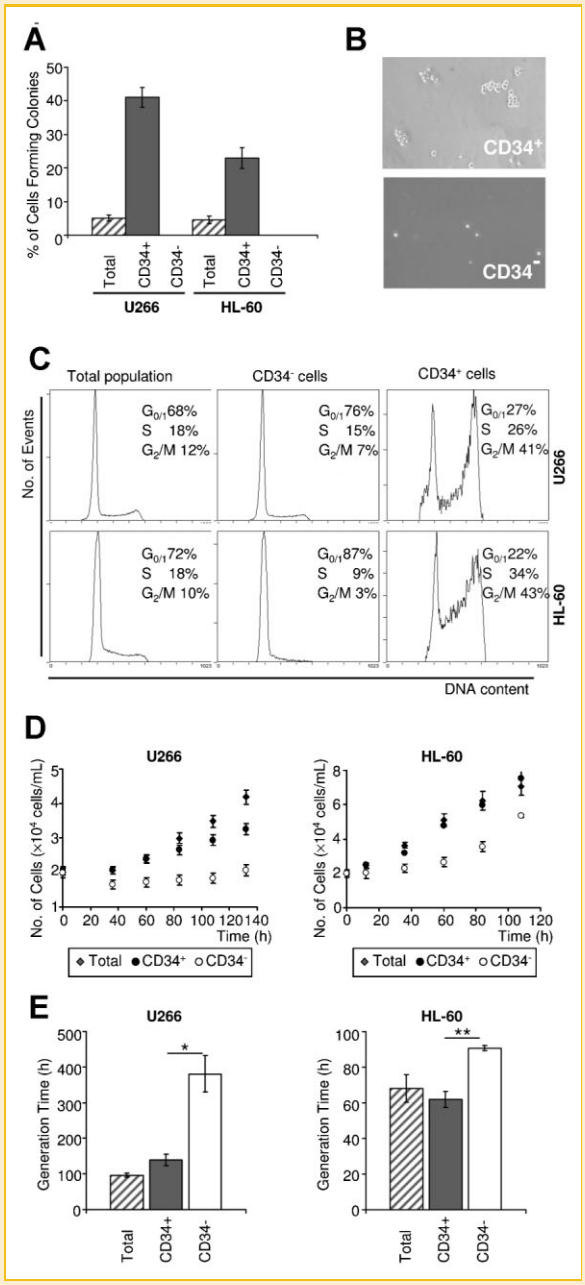

Fig. 3. $CD34^+$ cells have a proliferative advantage over $CD34^-$ cells. A: The colony forming potential of the total population and the $CD34^+$ and $CD34^-$ subpopulations was measured as a percentage of cells able to form colonies in methylcellulose. The results are representative of three independent experiments performed in duplicate ±SD. B: Picture of colonies formed by sorted $CD34^+$ and $CD34^-$ cells after 7 days of growth in methylcellulose. C: Cell cycle analysis using propidium iodide-staining of cellular DNA. Percentages of cells in particular phases of the cycle are given in each panel. Data shown are representative of at least three experiments with consistent results. D: In vitro proliferation assay performed on total populations and separated $CD34^+$ and $CD34^-$ subpopulations. The results are representative of three independent experiments performed in triplicate ±SD. E: Generation time based on in vitro proliferation assays in panel D. $^*P < 0.05$; $^{**}P < 0.03$; Student's *t*-test.

dividing (Fig. 3C). Only 24% (±5%) of $CD34^+$ cells were in $G_{0/1}$ phase compared to 80% (±10%) of the $CD34^-$ cells. As these results suggested that the $CD34^+$ subpopulation proliferated more efficiently, we placed separated subpopulations in standard culture conditions and observed their growth over several days (Fig. 3D). The growth of the $CD34^-$ subpopulation was triggered with a delay of 5 days in U266 cells and of 2 days in HL-60 cells. Moreover, in both cell lines, the $CD34^+$ subpopulation generation time was shorter when compared to $CD34^-$ cells (U266, 2.7-fold, $P < 0.05$; HL-60, 1.5-fold, $P < 0.03$; Fig. 3E). Thus, like normal $CD34^+$ progenitors, $CD34^+$ subpopulations from cell lines contain short-term colony-forming cells and proliferate more robustly when compared to $CD34^-$ cells.

THE PROPORTION OF THE CD34+ CELLS IS REGULATED BY SECRETED FACTORS AND CELL–CELL CONTACT

When cell motility is limited, pronounced dilution of cell cultures notably decreases physical contact between cells. Based on this supposition, we examined the effect of culture concentration on the percentage of the $CD34^+$ cells present in the total population (Fig. 4A). We performed this assay on U266 cells, which stay motionless at the bottom of culture wells, and on HL-60 cells, which migrate to some extent. Interestingly, the higher the culture dilution, the higher the percentage of $CD34^+$ cells; this effect was more pronounced in U266 (5.5-fold increase, $P < 0.004$) cultures, with reduced motility, than in HL-60 cultures (3.7-fold increase, $P < 0.05$). At this point, we could not exclude the possibility that when diluting the cultures, we also diluted some factors potentially secreted by tested cells (growth factors, metabolites) that could maintain the $CD34^+$ subpopulation at low levels. For this reason, we grew cells in trans-wells (Fig. 4B). The upper compartments contained various numbers of cells that served as a source of secreted factors. The cultures in the lower compartments were highly diluted (0.05×10^6 cells/mL), keeping cell–cell interactions low and the $CD34^+$ percentage high. After 24 h of growth, the percentage of the $CD34^+$ cells grown in the lower compartment remained elevated, suggesting that secreted factors were unable to decrease the number of $CD34^+$ cells and that physical contact between cells was necessary to keep it at a low level.

Next, we verified whether the factors secreted by cells could affect the basal quantity of the $CD34^+$ cells grown at the standard concentration (0.5×10^6 cells/mL) (Fig. 4C). To this end, we cultured highly concentrated cells (3×10^6 cells/mL) for 24 h and removed them from the medium by centrifugation. So-called "pre-conditioned" medium was mixed in various proportions with fresh medium and used for further 24-h cultures at the standard cell concentration. We observed progressively increasing proportions of $CD34^+$ cells in cultures containing 10–30% pre-conditioned medium (U266, 2.5-fold increase, $P < 0.03$; HL-60, 3.5-fold increase, $P < 0.05$). Higher volumes of pre-conditioned medium did not trigger this effect, probably due to nutrient insufficiency and acidification of the entire milieu.

These results demonstrated that the proportion of $CD34^+$ cells in the population is regulated by both secreted factors and physical interactions between cells.

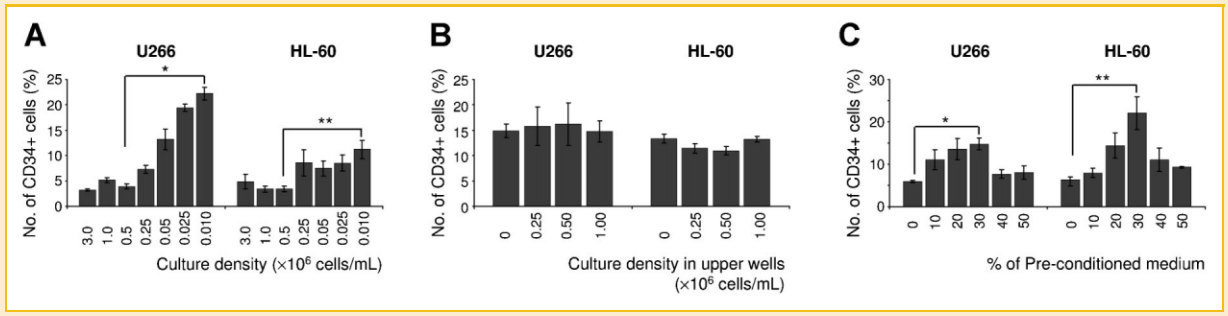

Fig. 4. Cell–cell contact and secreted factors regulate the proportion of $CD34^+$ cells in the total cell population. A: The percentage of $CD34^+$ cells in cultures with various cell concentrations (FC analysis). The results are representative of four independent experiments ±SD. $^*P < 0.004$; $^{**}P < 0.05$; Student's *t*-test. B: The percentage of $CD34^+$ cells in trans-well cultures (FC analysis). The initial concentration of cells in the lower compartment was 0.05×10^6 cells/mL. The concentration of cells in the upper compartment is indicated in the figure. The results are representative of six independent experiments ±SD. C: The percentage of $CD34^+$ cells in cultures with various proportions of fresh and pre-conditioned media (FC analysis). The results are representative of four independent experiments ±SD. $^*P < 0.03$; $^{**}P < 0.05$; Student's *t*-test.

CYTOKINES SECRETED BY CD34+ AND CD34− SUBPOPULATIONS

The above results led us to the assumption that hematopoietic cells might secrete some cytokines able to increase the percentage of the $CD34^+$ cells in the total population. To identify them, we performed cytokine arrays on the culture media after 24 h of growth. We found 8 proteins secreted by U266 and 16 proteins secreted by HL-60 that were produced at different rates by separated $CD34^+$ and $CD34^-$ subpopulations (Table I). The majority of these proteins were

TABLE I. Cytokines Differentially Secreted by Sorted $CD34^+$ and $CD34^-$ Subpopulations*

Protein	Ratio of secretion ($CD34^+/CD34^-$ cells) U266	Ratio of secretion ($CD34^+/CD34^-$ cells) HL-60	Functional category	Description
Thymopoietin	**4.26**	**6.34**		Polypeptide hormone involved in the induction of Thy-1 (CD90) in thymus
Thrombospondin-4	**2.35**	**3.69**	Cell-to-cell interactions	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions
Thrombospondin-1	**0.49**	1.02		Extracellular matrix protein; its expression is elevated in multiple myeloma, it can also induce apoptosis in leukemic cells
Slpi	7.61	**3.13**	Remodeling of extracellular matrix	Secretory leukocyte protease inhibitor
Timp4	0.73	**2.18**		Tissue inhibitor of metalloproteinase 4
Mmp20	0.83	**1.67**		Matrix metalloproteinase 20
Mmp11	**0.52**	1.00		Matrix metalloproteinase 11
Progranulin	1.44	**2.00**	Cellular proliferation	Secreted cysteine-rich granulins precursor; can promote or inhibit cell growth
TSLP	1.21	**1.71**		Thymic stromal lymphopoietin; can stimulate lymphoid and myeloid cell proliferation
FGF-18	1.21	**0.35**		Member of the fibroblast growth factor family that stimulates proliferation in a number of tissues
Neuritin	**0.56**	0.77		Protein that might be involved in neurite outgrowth
LRP-6	**0.56**	0.79		Low density lipoprotein-related protein 6; regulates cell differentiation and proliferation of many cancer types through its interaction with Wnt/β-catenin
CD40/TNFRSF5	**1.91**	1.40	TNF (tumor necrosis factor) signaling	B cell surface antigen; binding with CD40L triggers IL-6 secretion in multiple myeloma cells
CD30 ligand/TNFSF8	0.56	**3.35**		Cytokine expressed on B cells but also on acute myeloid leukemia blasts
CD30/TNFRSF8	0.85	**2.35**		Normally found on activated B and T cells; activates NF-κB
CD27/TNFRSF7	1.41	**1.76**		Receptor for CD70; may play a role in activated T cell survival
TROY/TNFRSF19	0.99	**0.49**		Can be expressed on myeloid and lymphoid cells and activate JNK and NF-κB
CLC	1.50	**2.12**	Others	Lysophospholipase; expressed in eosinophils and basophils, may be associated with inflammation and some myeloid leukemias
CSK	0.74	**2.01**		c-src tyrosine kinase; mainly cytoplasmic but also present in lipid rafts
MIF	0.94	**1.71**		Macrophage migration inhibitory factor; involved in cell-mediated immunity and inflammation
IL-28A	0.88	**1.71**		Immunomodulatory cytokine induced by viral infections
MCP-1/CCL2	**0.47**	0.95		Monocyte chemoattractant protein 1; augments monocyte anti-tumor activity

*Statistically significant (>average + 2 SD) results are in bold.

involved in cellular proliferation. Notably, several members of the tumor necrosis factor (TNF) superfamily were found, which can activate NF-κB. In agreement with the role of CD34 in cell–cell interactions, we observed differences in the secretion of 6 proteins (Thbs1, Thbs4, Mmp20, Mmp11, Timp4, and Slpi), including thrombospondins and metalloproteinases, that make up important parts of the cellular interface. Despite the distinct tumor origins of U266 and HL-60 cells, we found 2 cytokines that were over secreted by $CD34^+$ cells from both lines, i.e., thymopoietin and thrombospondin-4. We focused on thymopoietin and confirmed that U266 and HL-60 cells secrete this cytokine into the growth medium using dot-blot and specific antibodies against human thymopoietin. During 24 h of growth, 10^6 non-separated U266 or Hl-60 cells secreted 0.15 ng (±0.05 ng) and 0.14 ng (±0.01 ng) of thymopoietin per mL, respectively.

THYMOPOIETIN INCREASES THE PROPORTION OF CD34+ CELLS AND THE CLONOGENIC POTENTIAL OF THE TOTAL POPULATION

To investigate the function of thymopoietin, we used a synthetic pentapeptide, thymopentin (TP-5), that corresponds to the active site of thymopoietin and has been employed in the clinic as its substitute [Goldstein et al., 1979; Solmajer, 1990]. First, we added various concentrations of TP-5 to cultures of U266 and HL-60 cells. After 24 h of growth, the percentage of $CD34^+$ cells increased to 22% (±2.5%) in U266 cells and to 37% (±14%) in HL-60 cells (Fig. 5A). Consequently, the addition of TP-5 to the cultures on methylcellulose resulted in increased clonogenic potential of both tested cell lines (U266, 1.5-fold, $P < 0.02$; HL-60, 2.6-fold, $P < 0.001$) (Fig. 5B). A dot-blot assay performed on bone marrow samples confirmed thymopoietin secretion by normal $CD34^+$ cells (0.6 ng ±0.57 thymopoietin/mL) and $CD34^+$ cells from bone marrow of MDS (myelodysplastic syndrome) patient (Fig. S1). Therefore, we investigated its effects on primary cells cultivated in vitro. In three out of four tested samples, TP-5 increased the percentage of $CD34^+$ cells in mononuclear cell cultures (1.78-fold ±1) (Fig. 5C). This increase was followed by an increase in the capacity to generate mature cells on methylcellulose (Fig. 5D).

DISCUSSION

In this study, we demonstrated the existence of a $CD34^+$ subpopulation in several hematopoietic cell lines of distinct tumor origins. We found that what seemed to be at first an aberrant expression of the stem/progenitor cell marker in the partially differentiated [Kuranda et al., 2010] cancer cell lines reflected tightly regulated stem/progenitor-like state of cells (Fig. S2). In a manner similar to normal stem/progenitor cells [Sato et al., 1999; Dao et al., 2003; Dooley and Oppenlander, 2004], $CD34^+$ cells from cell lines could regenerate the total population in vitro and in immunocompromised mice. Importantly, both $CD34^+$ and $CD34^-$ cells were able to switch rapidly their phenotype. Similar behavior has been reported for stem/progenitor cells isolated from mobilized peripheral blood [Dooley and Oppenlander, 2004]. It has been shown that two days after separation, a part of each cellular fraction ($CD34^+CD38^-Lin^-$ and $CD34^-CD38^-Lin^-$) switched its CD34 phenotype, and growth initiation was delayed in $CD34^-$ cells. Although numerous groups have reported the presence of stem-like cells in cell lines derived from solid tumors, evidence of such cells in hematopoietic cell lines is lacking. Presence of stem-like cells has been shown in head and neck squamous cell carcinoma, neuroblastoma, bone sarcoma, glioma, breast cancer, and melanoma [for review see Kondo, 2007]. In general, these stem-like cells were characterized by higher resistance to chemotherapeutic agents, higher tumorigenicity in mice and slower cell cycle progression [Fillmore and Kuperwasser, 2008]. They could be distinguished from other cells by their unique features, such as exclusion of the DNA-binding dye Hoechst 33342 (up to 2% of the total population) [Kondo et al., 2004; Fillmore and Kuperwasser, 2008; Murase et al., 2009] and/or expression of *CD133* (up to 10% of the total population) [Harper et al., 2007; Dou et al., 2009]. Although we were unable to detect the presence of the ABCG2 transporter, responsible for Hoechst exclusion, or the expression of CD133 antigen (Fig. S3), we cannot exclude that quiescent $CD34^-$ stem-like subpopulation exists in the tested cell lines. However, as $CD34^+$ cells proliferated faster than $CD34^-$ cells and rapidly generated colonies in methylcellulose-based medium, they were unlikely to represent quiescent cancer stem cells.

In steady culture conditions, the percentage of $CD34^+$ progenitor-like cells was maintained at the same stable level. However, when the concentration of cells or/and secreted factors in the culture changed, the percentage of $CD34^+$ cells changed accordingly. Apparently, the progenitor-like state of cancer cells could be easily affected by certain cytokines and cell–cell interactions. Similarly, in the whole organism under normal conditions, the numbers of stem cells, progenitors, and differentiated cells remain relatively stable. However, certain conditions (e.g., blood loss) stimulate the proliferation and self-renewal of stem cells and progenitor expansion [Cheshier et al., 2007]. In our experiments, cultures enriched in $CD34^+$ cells or with a high overall cell concentration rapidly lost CD34 protein expression at the cell surface. It seems plausible that in these conditions, $CD34^+$ cells were rapidly converted into $CD34^-$ cells. Conversely, extreme dilution rapidly increased the proportion of $CD34^+$ cells. These results suggest that the balance of $CD34^+$ and $CD34^-$ cells is influenced by environmental settings and that, in general, the expression of stem cell markers in cancer cell lines should be interpreted carefully, taking culture conditions into account.

Furthermore, our experiments showed that separated $CD34^+$ and $CD34^-$ cells secrete different amounts of various proteins. In agreement with other observations, such as the proliferative advantage of $CD34^+$ over $CD34^-$ cells, these proteins are involved mainly in cell proliferation and cell–cell interactions. Thus, we speculated that both populations regulated each other through these secreted proteins, yet this remained to be demonstrated. To verify this hypothesis, we investigated the effect of thymopoietin, which is secreted by the $CD34^+$ subpopulation in both U266 and HL-60 cells. The short, active form of thymopoietin, thymopentin (TP-5), has been shown to have immunomodulatory effects in the treatment of AIDS [Singh et al., 1998], rheumatoid arthritis [Sundal and Bertelletti, 1994] and cancer [Botturi et al., 1993; Trinci et al., 1995; Cascinelli et al., 1998]. Interestingly, TP-5 has also been used to

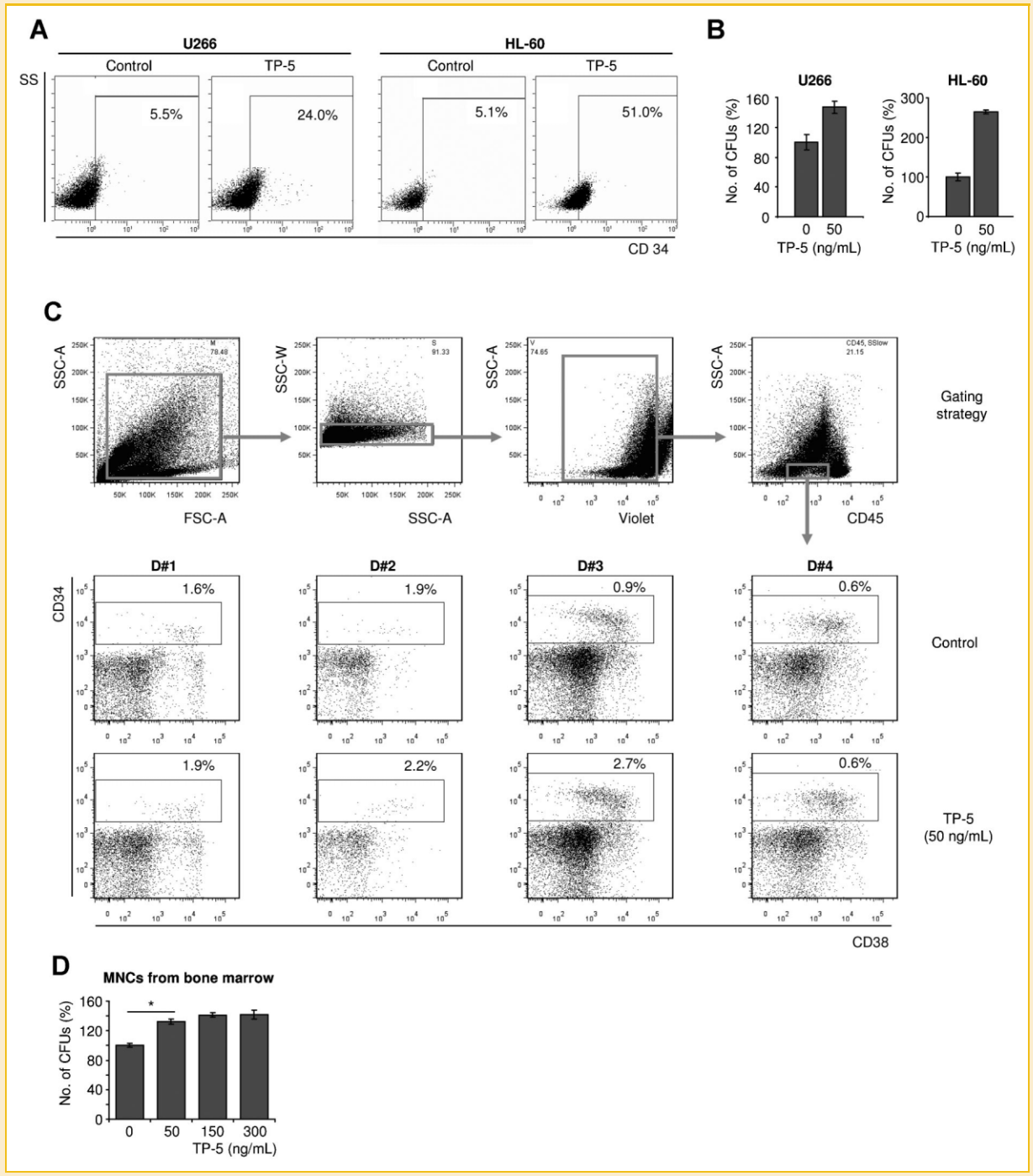

Fig. 5. TP-5 increases the percentage of $CD34^+$ cells and the clonogenic potential of the total cell population. A: After 24 h of growth with TP-5 (50 ng/mL), the percentage of $CD34^+$ cells increased to 22% (±2.5%) in U266 cells and to 37% (±14%) in HL-60 cells, as measured by FC analysis. Data are representative of four experiments with consistent results. B: The effect of TP-5 (50 ng/mL) on the clonogenic potential of the U266 and HL-60 total cell populations. Colonies were counted after 7 days. The results are representative of three independent experiments performed in duplicate (±SD). U266, $P < 0.02$; HL-60, $P < 0.001$; Student's *t*-test. C: The effect of TP-5 (50 ng/mL) on the percentage of $CD34^+$ cells in mononuclear cells (MNCs) isolated from the bone marrow of four healthy donors (D) after 24 h of culture as measured by FC analysis. The average 1.8-fold ±1 increase of the $CD34^+$ cells percentage was observed. D: The effect of TP-5 on the clonogenic potential of MNCs from bone marrow. Colonies were scored after 12 days. The results are representative of four independent experiments performed in duplicate (±SD). $^*P < 0.004$; paired Student's *t*-test.

restore normal hematopoiesis in some patients with myelodysplastic syndromes [Latagliata et al., 1991]. Our experiments showed that TP-5 increased the percentage of $CD34^+$ cells and colony formation in cell lines, which suggests that thymopoietin can be a part of an autoregulatory loop. Notably, experiments performed on mononuclear cells isolated from human bone marrow showed that the capacity of TP-5 to increase the $CD34^+$ cell percentage is not restricted to cancer cell lines. While further experiments are

necessary to elucidate TP-5's mechanism of action, our results reveal this compound as a new modulator of CD34 expression.

Collectively, these experiments showed that several hematopoietic cell lines contain relevant numbers of cells that express CD34, a stem/progenitor cell marker. However, these cells do not display characteristics of cancer stem-like cells previously identified in cell lines derived from solid tumors. Instead, CD34 expression delineates a group of cells in a stem/progenitor-like state that is reversible and may be modified by cell–cell interactions and secreted factors. One of these factors, thymopoietin, increases the proportion of $CD34^+$ cells and the clonogenic potential in cell lines as well as in bone marrow cells, indicating its new potential utility as a therapeutic agent. Altogether, these results demonstrate the common features of bone marrow $CD34^+$ stem/progenitor cells and $CD34^+$ cells in cancer cell lines. Thus, we propose that described cell lines could serve as an in vitro model to study properties of cancer stem/progenitor cells.

AUTHOR CONTRIBUTIONS

Klaudia Kuranda: Manuscript writing, conception and design, data analysis and interpretation, collection, and assembly of data; Céline Berthon: Collection and assembly of data; Nathalie Jouy: Cell sorting (FACS); Renata Polakowska: Data interpretation; Frédéric Leprêtre: CGH data analysis and interpretation; Bruno Quesnel: Conception and design, final approval of manuscript.

ACKNOWLEDGMENTS

We extend our greatest thanks the CGH engineers, Céline Villenet and Sabine Quief, for their relevant work.

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