The integrin α9β1 on hematopoietic stem and progenitor cells: involvement in cell adhesion, proliferation and differentiation

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Acknowledgments: we are grateful to Diane Blaurock (Center for Regenerative Biology and Medicine (ZRM), University of Tübingen) for critically reading the manuscript. We thank Dr. Dean Sheppard (Lung Biology Center, San Francisco General Hospital, California, USA) for the initial supply of the anti-α9 integrin antibody. We are indebted to Drs. Torsten Kluba and Falk Mittag (Department of Orthopedic Surgery, University of Tübingen) for their assistance in obtaining the bone specimens.

Funding: this work was supported by a grant from the Landesstiftung Baden-Württemberg gGmbH (grant N. P-LS-AS/HSPA8-13).

Manuscript received on January 15, 2009. Revised version arrived on June 2, 2009. Manuscript accepted on June 3, 2009.

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ABSTRACT

Background

Hematopoietic stem and progenitor cells can interact with their microenvironment via integrins which are adhesion receptors consisting of α and β subunits. Current knowledge suggests that the integrin subunits α4 and α6 expressed on hematopoietic stem and progenitor cells have distinct roles in retaining stem cells in the bone marrow. The aim of our study was to gain insight into the expression and functions of the integrin subunits α7-α11 within the endosteal stem cell niche.

Design and Methods

Human osteoblasts isolated from trabecular bone and hematopoietic stem and progenitor cells purified from umbilical cord blood or bone marrow aspirates were analyzed for the expression of integrin α7-α11 chains by reverse transcriptase polymerase chain reaction. The involvement of the integrin α9β1 in hematopoietic stem and progenitor cell adhesion, proliferation and differentiation was analyzed in functional assays.

Results

Transcripts for all investigated integrin chains were found in primary osteoblasts. Highly purified hematopoietic stem and progenitor cells, however, expressed only transcripts encoding integrin subunits α7 and α9. Flow cytometric analysis verified extracellular expression of the integrin α9β1 on hematopoietic stem and progenitor cells. Cell-cell adhesion assays with osteoblasts and dye-labeled CD34$^+$ hematopoietic stem and progenitor cells in the presence of function-blocking antibodies revealed a role of integrin α9 in hematopoietic stem and progenitor cell adhesion to osteoblasts. Furthermore, the addition of anti-integrin α9 antibodies significantly inhibited proliferation and *in vitro* differentiation of CD34$^+$ hematopoietic stem and progenitor cells.

Conclusions

The integrin α9β1 has been identified as a new member of the integrin β1-subfamily expressed on human hematopoietic stem and progenitor cells. The functional studies strongly suggest that integrin α9β1 contributes to adhesion and differentiation of hematopoietic stem and progenitor cells in the endosteal stem cell niche.

Key words: cell adhesion, bone marrow microenvironment, hematopoietic stem and progenitor cells, osteoblasts.

Citation: Schreiber TD, Steinl C, Essl M, Abele H, Geiger K, Müller CA, Aicher WK, and Klein G. The integrin α9β1 on hematopoietic stem and progenitor cells: involvement in cell adhesion, proliferation and differentiation. Haematologica 2009; 94:1493-1501.
doi:10.3324/haematol.2009.006072

Introduction

Within the bone marrow, hematopoietic stem cells reside in microenvironments called the stem cell 'niches' which control the hallmark of stem cells, their self-renewal, and shelter them from several external influences.[1,2] Two different niches have been identified in the bone marrow, namely the endosteal stem cell niche at the endosteum of bone[3] and, more recently, the vascular niche in close proximity to the blood vessels.[4,5] The major cellular component of the endosteal stem cell niche is the osteoblast, which also synthesizes and secretes the specialized extracellular matrix of the niche.[6] Hematopoietic stem cells can interact with the cellular and non-cellular components of the niche via integrins, which are cell surface receptors for both extracellular matrix molecules and membrane-bound adhesion molecules.[7]

The integrins are heterodimeric transmembrane molecules consisting of non-covalently linked α and β chains.[8] Integrins can be subdivided into subfamilies on the basis of their β chains, the β1-subfamily being the largest with twelve members (α1-α11 and α_v).[9] Although the different β1 integrin receptors perform common functions such as cell attachment and cell survival and can share identical ligands, each member nevertheless seems to be highly specific, since mice carrying gene deletions of the different integrin α chains show different and often non-overlapping phenotypes.[10-12]

In the human hematopoietic system, the integrin chains α4, α5 and α6 are expressed by hematopoietic stem and progenitor cells (HSPC), whereas the α1-α3 chains have not been detected on these cells.[13-15] Both α4 and α6 integrins are functionally involved in stem cell homing.[16] Whether the integrin α7-α11 chains are also prominently expressed on the cell surface of human HSPC has not been studied in detail. The present study now provides evidence that the integrin α9β1 is also a cellular receptor directly involved in hematopoietic stem cell functions.

The integrin α9β1 is strongly expressed by airway epithelial cells, and α9-integrin-chain-deficient mice die shortly after birth due to respiratory failure.[17,18] Strong expression of α9β1 integrin is also found on hepatocytes and on different types of muscle cells.[17] In the hematopoietic system, α9β1 integrin is highly expressed on neutrophils. There it plays a critical role not only in neutrophil migration but also during development of the granulocytes.[19,20] Several ligands from both the extracellular matrix and the cell surface have been identified for α9β1, including tenascin-C, osteopontin, vascular cell adhesion molecule-1 (VCAM-1) and several membrane-bound proteins of the ADAM (a disintegrin and metalloprotease) family.[19,21-23] Interestingly, human osteoblasts synthesize all these α9β1 ligands[6] (*T. Schreiber, C. Steinl and G. Klein, unpublished observations*).

In the present study we analyzed different functional aspects of integrin α9β1 on purified human $CD34^+$ and $CD133^+$ cells (HSPC) which mainly represent an enriched cell population of progenitor cells, but also contain hematopoietic stem cells. Cell-cell and cell-matrix adhesive interactions were studied with HSPC and osteoblasts or with isolated extracellular matrix components and cell adhesion molecules in the presence of function-blocking antibodies against α9β1 integrin. An influence of α9β1 on HSPC proliferation and differentiation was determined in short-term proliferation assays and in colony formation assays, respectively. All these studies provided strong evidence that the integrin α9β1 plays a crucial role in the development of hematopoietic stem cells in their niche.

Design and Methods

Isolation of hematopoietic stem and progenitors cells and primary osteoblasts

Human $CD34^+$ and $CD133^+$ HSPC were isolated from umbilical cord blood or bone marrow aspirates which were obtained from the Department of Gynecology and Obstetrics (University Clinic Tübingen, UKT) according to the guidelines of the local ethics committee. Umbilical cord blood and bone marrow mononuclear cells were prepared using Percoll (1.077 g/mL) density gradient separation. The mononuclear cell populations were magnetically labeled with anti-CD34- or anti-CD133-conjugated microbeads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). For magnetic enrichment the automated magnetic activated cell separation unit (AutoMACS, Miltenyi Biotec) was used in the special double-positive separation mode (PosselD2).

Bone waste from endoprosthesis surgery was used to isolate human primary osteoblasts. Bone waste was obtained after written consent from the patients and approval by the local ethics committee of the Department of Orthopedic Surgery (UKT). The bones were mechanically minced and incubated in 1500 U/mL collagenase type XI (Sigma, Taufkirchen, Germany) and 2.4 U/mL dispase (Roche, Mannheim, Germany) for 1 h at 37°C with gentle agitation. The supernatant was discarded. After washing with phosphate-buffered saline (PBS), the bone fragments were incubated with collagenase for 3 h at 37°C with gentle agitation and subjected to two more washing steps with PBS. The isolated cells were then transferred together with the bone fragments into cell culture flasks. The cells were cultured in Dulbecco's modified Eagle's medium (4.5 g/L glucose, L-Gln) supplemented with 20% heat-inactivated fetal calf serum, 2% minimum essential medium vitamin solution, 1% fungizone (Invitrogen, Karlsruhe, Germany), 50 μg/mL ascorbic acid and 1.4 mM β-glycerophosphate (Sigma) based on the recently published protocol for osteogenic medium.[24] Osteoblasts were routinely tested for osteogenic marker genes such as osteopontin, osteocalcin, *RUNX2*, bone sialoprotein, collagen type I α 2, osteonectin and alkaline phosphatase using quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Protein expression of osteopontin, alkaline phosphatase and RUNX2 was evaluated by immunofluorescence staining. Cells were used in between passage one to five with the maximum being five passages.

Extracellular matrix proteins and antibodies

His-tagged human recombinant osteopontin and VCAM-1 were purchased from R&D Systems (Wiesbaden, Germany). Purified human tenascin-C was obtained from Millipore (Schwalbach, Germany). Mono-

Table 1. Primer sequences for the human α7–α11 integrin chains.

Integrin α chain	Primer sequence 5' → 3'	Nucleotide position	PCR product	GenBank accession numbers
Integrin α7	For: ATC AAG AGC TTC GGC TAC TCC	1467-1487	358 bp	NM_002206.1
	Rev: GCT TGG GTT CTT CCA GGT TAC	1824-1804		
Integrin α8	For: GTG TGT TCC TGA CTT GAA GCT G	1938-1959	491 bp	XM_167711.2
	Rev: CCT CTT CTG GTT CCC AGT TAT G	2428-2407		
Integrin α9	For: GTC TCC AAC CTC CTT TGT ATA TGG	2382-2405	383 bp	NM_002207.1
	Rev: AGT TAC AGT GTG CTG TTA GGC AAG	2764-2741		
Integrin α10	For: ATC AGT GTG GTT CAG AGG GAC T	2051-2072	331 bp	NM_003637.3
	Rev: GCC CTG GCT TTG TAG TAT TGT C	2381-2360		
Integrin α11	For: CCT TCT TTG GCT ACA CAG TGC	203-223	382 bp	NM_001004439.1
	Rev: ACG ATG TCC ATG TAG GTC TGG	584-564		

clonal antibodies against the human integrin α9 chain (clone Y9A2), the integrin β1 chain (CD29; clone 4B4) and VCAM-1 (clone BBIG-V1) were purchased from AbD Serotec (Düsseldorf, Germany), Beckman Coulter (Krefeld, Germany), and R&D Systems, respectively. The antibody W6/32.HL, which recognizes an epitope of the heavy chain of MHC class I antigens, was used as a control antibody.[25]

Reverse-transcriptase polymerase chain reaction analysis

Total RNA from highly purified CD34$^+$ or CD133$^+$ HSPC and from primary human osteoblasts was isolated according to the manufacturer's protocol using the RNeasy total RNA kit (Qiagen, Hilden, Germany). Further purification was achieved by DNaseI digestion (Qiagen). For RT-PCR analysis, 1 μg total RNA was used for first-strand cDNA synthesis using oligo-dT primers and the SuperScript III First-Strand synthesis system (Invitrogen). The PCR assay was performed using AmpliTaq DNA polymerase (Roche Applied Biosystems). cDNA quality was checked by PCR of the housekeeping gene β-actin. Primer pairs for the integrin α7-α11 chains (Table 1) were selected according to the sequences published in the GenBank database. After denaturation of cDNA at 95°C for 5 min, temperature cycling (35 cycles) was performed as follows: a denaturation step at 95°C for 45 seconds, a 40-second annealing step at 56°C and a 60-second elongation step at 72°C. Temperature cycling was concluded with a final elongation step for 10 min at 72°C. Products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining under ultraviolet light.

Flow cytometry analysis and isolation of lineage-negative cells

Expression of integrin chains on human HSPC was studied by dual color flow cytometry analysis. Lineage-negative cells were purified from leukocytes of umbilical cord blood or bone marrow aspirates with the Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's instructions. Lineage-negative cells were labeled with the individual antibodies as described recently.[26] Briefly, for each staining 1×10^5 to 5×10^5 cells were incubated for 20 min with monoclonal antibodies against the integrin α9 or β1 chains. The cells were washed and bound antibodies were detected by fluoroscein isothiocyanate-conjugated goat anti-mouse IgG secondary antibodies (Dianova, Hamburg, Germany). CD133-allophycocyanin antibodies (clone 293C3, Miltenyi Biotec) were applied after an additional blocking step with mouse serum. Appropriate background staining was performed with the corresponding isotype control antibodies. The antibody W6/32.HL was used as a positive control. As a negative control, the cells were labeled with the antibody W6/32.HK, an inactive variant of W6/32.HL.[25] Data were acquired with an LSR II (BD Biosciences, Heidelberg, Germany) flow cytometer and analyzed with FlowJo 7.2.5 (TreeStar, Ashland, OR, USA).

Cell adhesion assays

Cell adhesion to extracellular matrix proteins or to the recombinant cell adhesion molecule VCAM-1 was carried out as described previously.[27] Briefly, the extracellular matrix molecules or VCAM-1 were immobilized on plastic dishes by air-drying at room temperature before 1.0 μL of the appropriate solution (tenascin-C, osteopontin or VCAM-1) was spotted onto the dishes. Unless otherwise stated, 1.0 μg was used for each spot. Non-specific binding of MACS-sorted CD34$^+$ cells to the plastic dishes was prevented by pre-incubation with 1% bovine serum albumin/PBS. Then the CD34$^+$ cells were allowed to attach for 1 h in serum-free medium (supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM $MnCl_2$). Non-adherent cells were removed by gently rinsing the dishes with pre-warmed PBS. Specific cell adhesion was evaluated under a Zeiss Axiovert microscope. Photographs of representative fields were taken.

For the quantitative determination of adhesive cell-cell interactions, primary osteoblasts were seeded in 48-well plates and grown for 72 h to confluency. Then, 1×10^6 MACS-isolated CD34$^+$ progenitor cells were incubated with 2 μg of the fluorescent dye BCECF-AM (2', 7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, Sigma-Aldrich) for 15 min at 37°C. The labeled CD34$^+$ progenitor cells were washed twice with serum-

free RPMI containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 50 μM $MnCl_2$ and pre-incubated for 30 min at 37°C with 20 μg/mL function-blocking antibodies. The confluent monolayer of primary osteoblasts was washed twice with PBS, and 1×10^5 $CD34^+$ cells were allowed to attach to the osteoblasts for 1 h in the presence of the function-blocking antibodies. $CD34^+$ progenitor cells without antibody treatment were used for control experiments. After washing with PBS containing $CaCl_2$, $MgCl_2$ and $MnCl_2$, the fluorescence of attached $CD34^+$ progenitor cells was measured with the Fluoroskan Ascent (Thermo Scientific, Dreieich, Germany). The percentage of adherent $CD34^+$ cells was calculated as follows:

$$\frac{\text{fluorescence from experimental sample - fluorescence from negative control sample}}{\text{fluorescence of the positive control sample}} \times 100 = \%\text{cell adhesion}$$

The cell-cell adhesion assays were carried out in triplicate. After quantification, $CD34^+$ cells attached to primary osteoblasts were fixed and stained with 0.1% crystal violet to visualize cell adhesion of the $CD34^+$ cells under a Zeiss Axiovert light microscope. Photographs of representative fields were taken.

Cell proliferation assay

Ten thousand purified $CD34^+$ progenitor cells were incubated with antibodies against the integrin α9 chain, the W6/32.HL control antibody or PBS in serum-free expansion medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) containing the recombinant human cytokines Flt-3 ligand, stem cell factor (100 ng/mL each), interleukin-3, and interleukin-6 (20 ng/mL each) for 1 to 4 days in a 96-well plate at 37°C. For kinetic studies, cells were harvested after 24, 48, 72 or 96 h. Thereafter, cell proliferation was measured with the CyQuant Cell Proliferation Assay (Invitrogen) according to the manufacturer's instructions. Briefly, cells were centrifuged and the medium was removed. After being frozen overnight at –70°C, the lysed cells were thawed at room temperature, and 200 μL lysis buffer containing the DNA-binding CyQuant dye were added to each well. Fluorescence was measured after 5 min at 485-nm excitation wavelength and 538-nm emission wavelength with the Fluoroskan Ascent reader (Thermo Scientific). All assays were performed in duplicate.

Colony-forming assay

To analyze the influence of integrin α9β1 on the proliferation and differentiation of hematopoietic progenitor cells, colony-forming assays were performed in the presence of the monoclonal antibody Y9A2. The MACS-isolated $CD34^+$ cells (1.6×10^5) were pre-incubated with 5 μg of anti-integrin α9 or W6/32.HL control antibodies in 100 μL Iscove's modified Dulbecco's medium supplemented with 2% fetal bovine serum (Stem Cell Technologies) for 30 min at room temperature. As a positive control, $CD34^+$ cells were incubated without the addition of antibodies. Cell suspensions were added to 1 mL methylcellulose medium containing recombinant stem cell factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3, and erythropoietin (MethoCult H4434, Stem Cell Technologies). Cells plated in triplicate on 35-mm Petri dishes were cultured in a fully humidified atmosphere with 5% CO_2 at 37°C for 14 days. Cell aggregates containing more than 50 cells were scored as single colonies using an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany). Burst-forming unit erythrocyte (BFU-E) and colony-forming unit-granulocyte/macrophage (CFU-GM) colonies were counted on the basis of morphological criteria.

Statistics

Data are presented as means ± standard error (SE) unless otherwise stated. Differences between groups were analyzed using Student's *t* test. Differences for which the *p* value was less than 0.05 were considered statistically significant.

Results

Expression of integrin α9β1 on human hematopoietic stem and progenitor cells

To study the expression of the integrin subunits α7-α11, which has not been described so far in the context of the hematopoietic stem cell niche, we performed RT-PCR analyses with RNA isolated from purified $CD34^+$ and $CD133^+$ umbilical cord blood cells and from human primary osteoblasts. We used $CD133^+$ cells in addition to $CD34^+$ cells because the CD133 antigen defines an earlier stem and progenitor cell population than that defined by CD34. The mRNA of the integrin α7 and α9 chains could be detected in samples of isolated $CD34^+$ and $CD133^+$ cells (Figure 1A, B). In contrast, mRNA of all investigated integrin α chains could be found in human primary osteoblasts (Figure 1C).

The expression of the integrin α9 subunit on hematopoietic cells was analyzed in more detail by flow cytometry. To obtain untouched cells, we purified umbilical cord blood or bone marrow cells for lineage-negative cells. We found that 99% of the lineage-negative bone marrow cells that expressed the HSPC marker CD133 also expressed the integrin α9 subunit on their surface (Figure 2A). Similarly, lineage-negative $CD133^+$ cells isolated from umbilical cord blood were 99% positive for integrin α9 (Figure 2B). Analogously, 99.7% of all lineage-negative cells also expressed the integrin β1 chain which is the only known β subunit linked to the α9 chain. Similar data were obtained for $CD34^+$ HSPC (*data not shown*).

Involvement of integrin α9β1 in hematopoietic stem and progenitor cell adhesion

Integrin α9β1 is known to mediate cell-cell and cell-matrix interactions. To investigate these events in a simplified model of the hematopoietic stem cell niche, we performed cell adhesion assays using human primary osteoblasts and purified $CD34^+$ HSPC. Adhesion of the hematopoietic cells to osteoblasts was analyzed either visually or by labeling HSPC with the fluorescent substrate BCECF-AM, followed by a spectrofluorometric read-out. Figure 3A shows an example of how HSPC attach to primary osteoblasts in the presence or absence of antibodies. Pre-incubation of HSPC with anti-integrin α9

antibodies led to a significant decrease in the number of cells bound to osteoblasts. This effect was even more pronounced when anti-integrin β1 antibodies were used (Figure 3A). A quantification of the effect of the anti-integrin α9 antibody revealed a 60% decrease in binding in comparison to that occurring in the presence of the control antibody W6/32.HL directed against MHC-class I molecules (Figure 3B). The control antibody did not lead to a statistically significant reduction in cell binding compared to the control without antibody treatment (Figure 3B). These results suggest a potential role for integrin α9β1 in the control of HSPC adhesion within the hematopoietic stem cell niche. To define the binding partner(s) for integrin α9 in cell adhesion between osteoblasts and HSPC we used recombinant or purified integrin α9 substrates, which are known to be expressed by human osteoblasts. HSPC were able to bind to recombinant VCAM-1 and purified tenascin-C (Figure 4). However, cell binding to VCAM-1 or tenascin-C was not inhibited by anti-integrin α9 antibodies or the control antibodies W6/32.HL, suggesting the involvement of additional receptors besides integrin α9 for VCAM-1 and tenascin-C binding. Astonishingly, we observed no cell binding of CD34⁺ HSPC to either recombinant osteopontin (Figure 4), which is also a specific ligand for α9β1 integrin, or to the thrombin-digested osteopontin (*data not shown*).

Integrin α9β1-mediated inhibition of hematopoietic stem and progenitor cell proliferation and differentiation

Besides being involved in cell adhesion, integrins can also influence cell proliferation and differentiation, both crucial events in the fate of HSPC. Cell proliferation was investigated by culturing HSPC for 4 days in serum-free expansion medium containing stem-cell factor, Flt-3 ligand, interleukin-3 and interleukin-6. Without antibodies, HSPC proliferated effectively in this medium (Figure 5A). Incubation of the cells with anti-integrin α9β1 antibodies led to an almost complete stop in cell proliferation. No

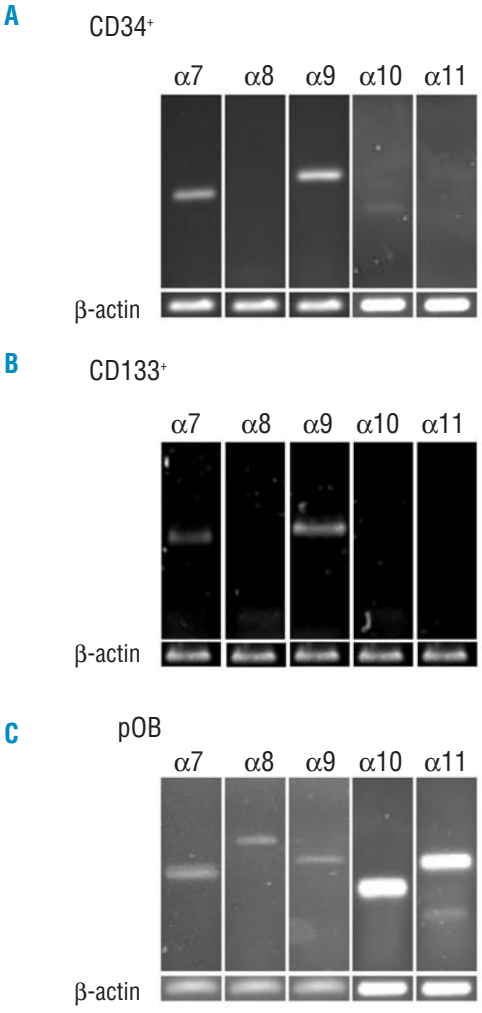

Figure 1. RT-PCR analysis of integrin α7-α11 mRNA expression in human CD34⁺ cells, CD133⁺ cells and human primary osteoblasts. RNA of isolated cells was reverse-transcribed in cDNA and amplified with the indicated primers. β-actin was amplified as a positive control. Human CD34⁺ cells (A) and CD133⁺ cells (B), isolated from umbilical cord blood, expressed the integrin α subunits 7 and 9, whereas human primary osteoblasts (pOB) expressed all investigated integrin subunits α7 - α11 (C).

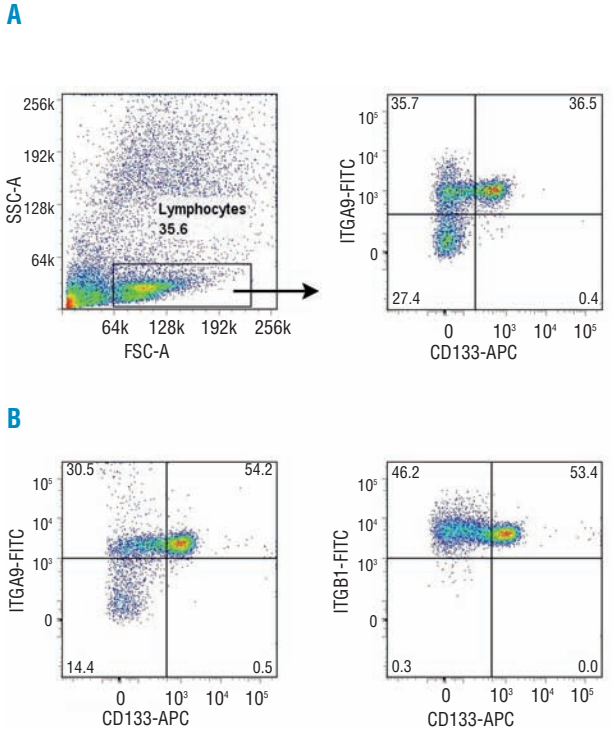

Figure 2. Expression of integrin α9β1 on human bone marrow and umbilical cord blood CD133⁺ cells. Bone marrow mononuclear cells or umbilical cord blood cells were depleted of cells expressing lineage markers. Lineage-negative cells were labeled with anti-integrin α9 antibodies and antibodies against the stem and progenitor cell marker CD133 and analyzed by flow cytometry. (A) The lymphocyte-containing fraction of lineage-negative cells isolated from human bone marrow was selected for further analysis (left); 36.5% of these selected cells expressed both CD133 and the integrin α9 chain, whereas only 0.4% expressed CD133 but not integrin α9, indicating that >98.5% of the CD133⁺ cells in the lymphocyte-containing fraction expressed the integrin α9 chain (right). (B) An analysis similar to that shown for bone marrow cells was also performed for human umbilical cord blood cells; 99% of the CD133⁺ cells of the lymphocyte-containing fraction expressed the integrin α9 (left), and analogously, 99.7% also expressed the integrin β1 chain (right).

effect was observed after the addition of the MHC class I-recognizing control antibodies, W6/32.HL, to the culture medium. To analyze the temporal sequence of the observed inhibition, HSPC proliferation was measured on each day of the 4-day period. Throughout the culture period, incubation of cells with W6/32.HL antibodies did not lead to a statistically significant difference compared to the proliferation in the PBS control group (Figure 5B). However, incubation of cells with anti-integrin α9 antibodies almost completely eliminated cell proliferation, with this reaching statistical significance on days 3 and 4. Taken together, these results imply that integrin α9-mediated signaling is important for HSPC proliferation.

As proliferation and differentiation of HSPC can be interconnected processes, we evaluated the differentiation capacity of stem and progenitor cells in the presence of

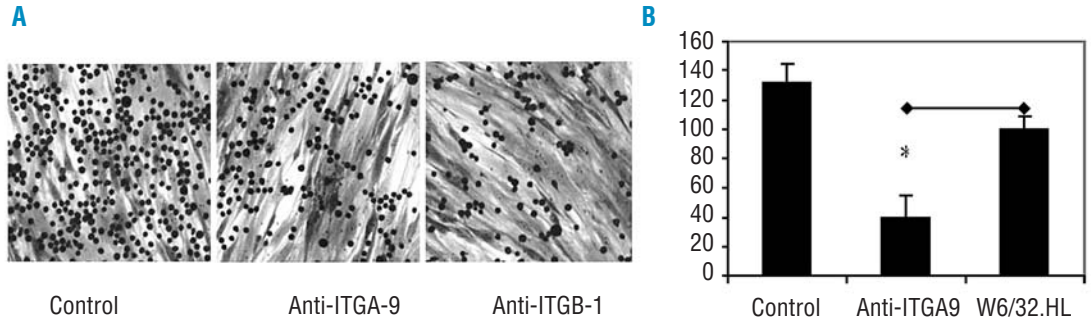

Figure 3. Influence of integrin α9β1 on HSPC cell adhesion to primary osteoblasts. MACS-isolated CD34⁺ HSPC were allowed to adhere to a confluent layer of isolated primary osteoblasts. (A) Without addition of an antibody, the CD34⁺ cells (small dark balls) adhered strongly to the osteoblasts, which can be recognized as the cell layer underlying the CD34⁺ cells. Addition of the monoclonal antibodies against the integrin α9 chain or the integrin β1 chain drastically reduced the number of adherent CD34⁺ cells (crystal violet stain, Zeiss Axiovert, magnification x100). (B) Quantification with BCECF-labeled CD34⁺ cells revealed that adhesion of these cells was significantly ($p<0.05$) reduced to 40±15% of that seen in the experiment with the control antibody W6/32.HL, which binds to MHC class I molecules on the cell surface. No significant difference was observed between the control experiment without antibody treatment (control) and the treatment with the antibody W6/32.HL. The individual experiments were performed in triplicate, and means of four independent experiments are shown with standard error bars.

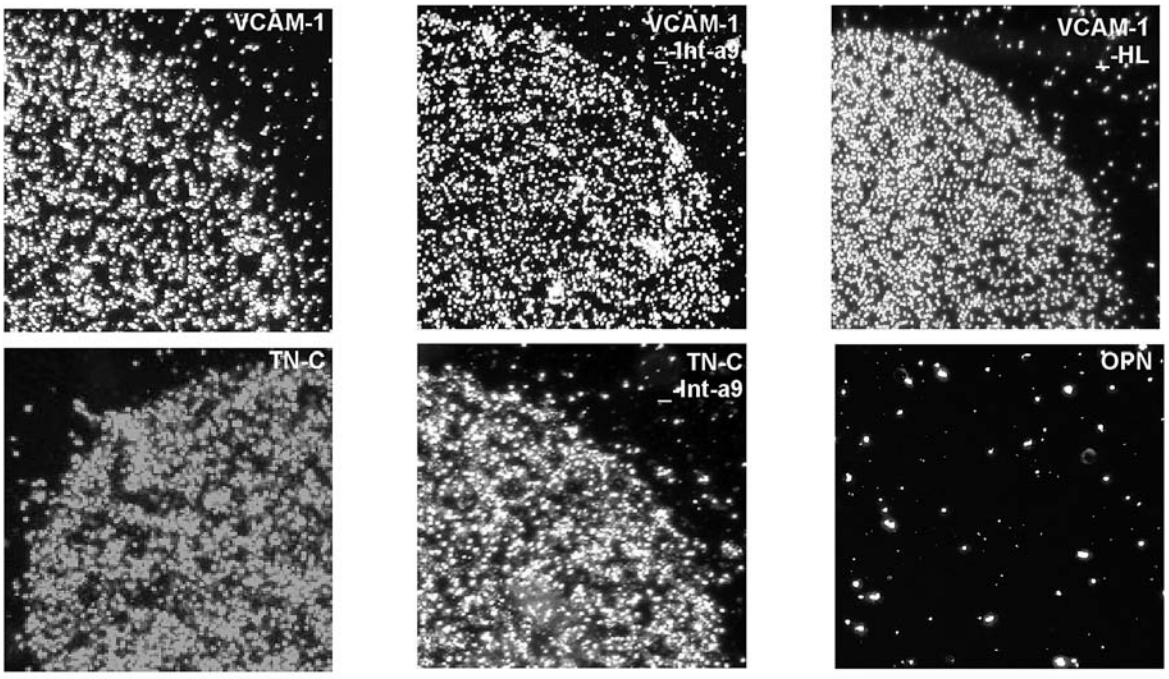

Figure 4. Cell attachment of human CD34⁺ HSPC to VCAM-1 and the extracellular matrix molecules tenascin-C and osteopontin. MACS-isolated CD34⁺ cells were allowed to attach to immobilized vascular cell adhesion molecule (VCAM-1), tenascin-C (TN-C) and osteopontin (OPN). Strong attachment was observed for VCAM-1 and tenascin-C, but not for osteopontin (dark field illumination, Zeiss Axiovert, magnification x60). Cell adhesion to VCAM-1 and tenascin-C was not inhibited by anti-α9-integrin antibodies (VCAM-1, α-Int-α9 and TN-C, α-Int-α9) or by the control antibody W6/32.HL (α-HL).

anti-integrin α9 antibodies with colony-forming assays. After 14 days in culture in a semi-solid medium with interleukin-3, stem cell factor, GM-CSF and erythropoietin, isolated CD34⁺ umbilical cord blood cells incubated with 5 μg anti-integrin α9 antibodies developed significantly fewer colonies than did the control groups (Figure 6). Interestingly, the effect on colonies of erythroid progenitors (BFU-E) was slightly greater than that on colonies derived from granulocyte-macrophage progenitors (CFU-GM). Cells that were incubated with W6/32.HL antibodies developed normally, indicating that an antibody non-specific effect on colony inhibition can be excluded. These results suggest an important influence of integrin α9 on HSPC development.

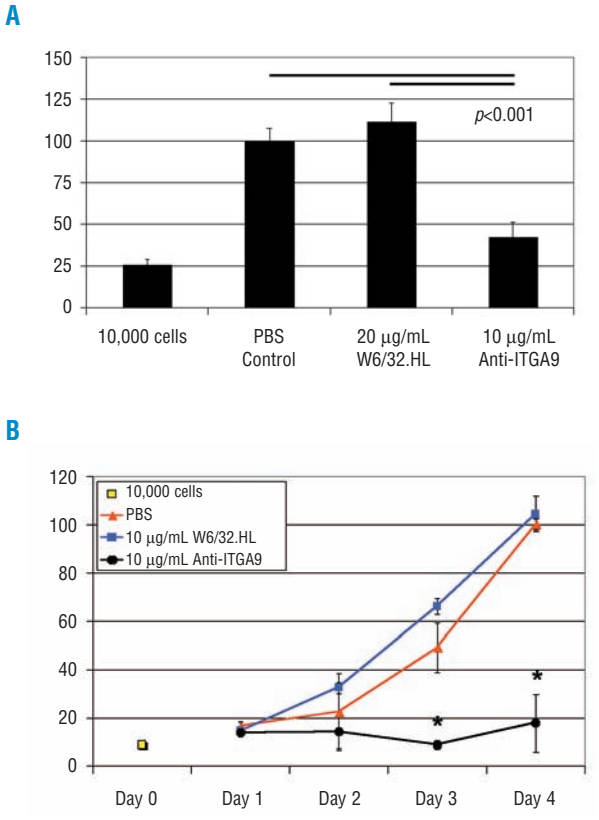

Figure 5. Inhibition of cell proliferation of human CD34⁺ cells by the anti-integrin-α9 antibody Y9A2. (A) Isolated human CD34⁺ HSPC ($1x10^4$) were cultured for 4 days with the indicated antibodies in serum-free expansion medium including 20 ng/mL each of interleukin-6 and as well as 100 ng/mL each of stem cell factor and Flt3L. Cell proliferation was measured with the CyQuant Cell Proliferation Assay by fluorescence read-out. Addition of anti-integrin α9 antibodies (anti-ITGA9) reduced cell proliferation significantly compared to the addition of W6/32.HL antibodies or the condition without antibody treatment (PBS control). Means with standard error bars are shown. n independent experiments were performed in duplicate. n=4 for PBS and anti-ITGA9, n=3 for W6/32.HL. (B) Time course of HSPC proliferation in the presence of 10 μg/mL anti-integrin α9 antibody (Y9A2). Isolated human CD34⁺ HSPC ($1x10^4$) were cultured for 4 days as described above. Cell proliferation was analyzed each day with the CyQuant Cell Proliferation Assay. Up to day 4, CD34⁺ cells incubated in the presence of W6/32.HL antibodies or without antibodies proliferated approximately five times better than cells cultured with anti-integrin α9 antibodies, which only doubled in number compared to the input. One representative experiment out of three independent experiments is shown. Means of duplicates with standard deviation are shown. *=$p<0.05$. RFU: relative fluorescence units.

Discussion

The integrins α4β1 and α6β1 are known to play fundamental roles in hematopoietic stem cell migration, homing and engraftment.[28,29] Our current study demonstrates that another member of the integrin β1-subfamily, the integrin α9β1, is also very important for HSPC biology. The integrin α9β1 can be detected on almost all early CD133⁺ HSPC. Adhesive interactions of HSPC with osteoblasts, the major cell type of the endosteal stem cell niche, are at least partially dependent on α9β1, although the ligand(s) responsible have not yet been identified. Furthermore, α9β1 is not only involved in adhesive interactions, but also in signal transduction in HSPC. Short-term proliferation and differentiation of CD34⁺ HSPC are drastically impaired by a function-blocking antibody against α9β1, indicating that this integrin has a crucial role in the maintenance of the hematopoietic stem cell pool.

Recent findings by Jaatinen and co-workers,[30] who detected integrin α9 chain transcripts in a microarray analysis of human CD133⁺ cord blood cells, are consistent with the results of our own RT-PCR expression study of α9 on CD34⁺ and CD133⁺ HSPC. The present flow cytometry analysis of CD133⁺ cells isolated from bone marrow aspirates or from umbilical cord blood revealed that both the HSPC in the bone marrow microenvironment and the circulating HSPC in umbilical cord blood express the integrin α9β1. Thus, a down-regulation of α9β1 on HSPC does not seem to be necessary for the egress of stem and progenitor cells from the bone marrow

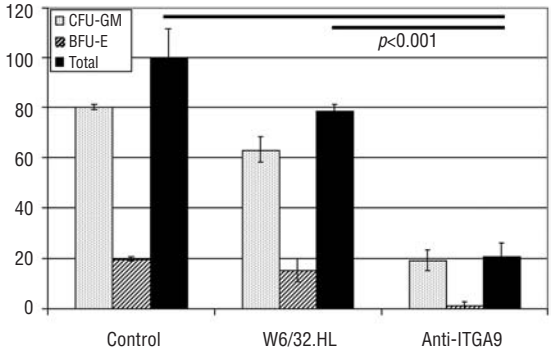

Figure 6. Colony formation assay of human CD34⁺ cells in the presence of 5 μg anti-integrin α9 antibody (Y9A2). Isolated human CD34⁺ HSPC ($1.6x10^3$) were incubated for 14 days in a methylcellulose-containing culture medium also containing recombinant cytokines and growth factors. Addition of 5 μg anti-integrin α9 antibodies (anti-ITGA9) drastically ($p<0.001$) diminished colony formation of progenitor cells in comparison to that in control cultures with 5 μg MHC class I-recognizing W6/32.HL antibodies or without antibodies (control). Total colony formation in the control experiments was set at 100%. The results of three independent experiments are shown as means with standard error bars. The mean absolute numbers of colonies formed in the three control experiments with cells from different donors were 76, 66 and 34 colonies; the mean numbers in the presence of anti-ITGA9 antibodies were 9, 15 and 10 colonies, respectively.

into the circulation.

Several adhesive ligands, including tenascin-C, osteopontin and VCAM-1, which are all synthesized by human osteoblasts[31-33] (*T. Schreiber, C. Steinl and G. Klein, unpublished data*), have been identified for the cell adhesion receptor α9β1 integrin. The α9β1$^+$ HSPC adhered strongly to VCAM-1 and tenascin-C in an *in vitro* cell adhesion assay, but, surprisingly, these interactions could not be blocked by the anti-integrin α9β1 antibody Y9A2. These results do not necessarily rule out an adhesive functional role for α9β1 on HSPC, but suggest that other adhesive receptors expressed on HSPC, such as α4β1, can compensate for the functional inactivation of α9β1. Interestingly, α9β1$^+$ HSPC did not bind to recombinant osteopontin in the *in vitro* adhesion assay. Even thrombin-cleaved osteopontin, which exposes an NH_2-terminal fragment normally recognized by α9β1 *in vitro*,[22] was not an adhesive ligand for isolated HSPC. Although the function-blocking antibody Y9A2 did not interfere with binding of HSPC to VCAM-1 or tenascin-C, the antibody efficiently blocked cell-cell interactions between HSPC and primary osteoblasts. Since human osteoblasts synthesize and express VCAM-1 and tenascin-C,[31,33] other ligands for α9β1 on HSPC such as ADAM proteases, thrombospondin-1 or fibulin-5 might be responsible for the observed α9β1-mediated adhesion.[23,34,35] On the other hand, α9β1 is also expressed by human osteoblasts and could, therefore, interact with adhesive ligands present on HSPC.

It has been shown that α9β1 has a permissive role in a granulocyte colony-stimulating factor (G-CSF)-receptor-mediated signaling pathway during granulocyte development.[20] This is also reflected by the fact that α9-deficient mice exhibit defective granulopoiesis with impaired differentiation of bone marrow precursors into mature granulocytes.[20] On early HSPC an α9-mediated G-CSF signaling pathway cannot be involved, since HSPC do not express the G-CSF receptor.[36] Signal transduction mediated by the α9β1 integrin, however, must also occur on HSPC because the antibody Y9A2 efficiently blocked HSPC proliferation and differentiation. The mechanism by which the antibody inhibits both HSPC responses is still unresolved. It is possible that the antibody directly blocks the signaling event, or it may mimic the natural ligand(s). Osteopontin is just such a natural hematopoietic stem cell niche component which can regulate the number of primitive HSPC by suppressing HSPC proliferation.[37,38] Although human CD34$^+$-isolated HSPC did not attach to osteopontin, they may nevertheless interact with this extracellular matrix component via α9β1 to control HSPC numbers. This hypothesis is supported by kinetic studies with CD34$^+$ cells in the presence of the antibody Y9A2 which showed a more or less equal number of HSPC, but no decrease, during the 4-day incubation period.

In the cell differentiation assay with CD34$^+$ cells the antibody Y9A2 not only affected the formation of CFU-GM as expected, but also the formation of erythroid colonies (BFU-E). In α9-deficient mice, however, an obvious defect in the development of erythrocytes has not been reported.[20]

In summary, we have identified α9β1 integrin as a new member of the integrin β1-subfamily which is strongly involved in human hematopoietic stem cell development in the bone marrow stem cell niche. It remains to be shown whether the integrin α9, whose cDNA sequence is more than 40% identical to the integrin α4 sequence, is redundant with α4β1 in stem cell mobilization since both integrins share common ligands such as VCAM-1 and fibronectin.[19] For HSPC proliferation, however, the integrin α9β1 seems to play an independent and unique role.

Authorship and Disclosures

TDS: collection and/or assembly of data, data analysis and interpretation; manuscript writing. CS: collection and/or assembly of data, data analysis and interpretation. ME: collection and/or assembly of data, data analysis and interpretation. HA: provision of study material. KG: collection and/or assembly of data, data analysis and interpretation. CAM: data analysis and interpretation. WKA: provision of study material or patients, data analysis and interpretation. GK: conception and design of the study, data analysis and interpretation, manuscript writing. The authors report no potential conflicts of interest.

References

1. Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 2006;6:93-106.
2. Jones DL, Wagers AJ. No place like home: anatomy and function of the stem cell niche. Nat Rev Mol Cell Biol 2008;9:11-21.
3. Yin T, Li L. The stem cell niches in bone. J Clin Invest 2006;116:1195-201.
4. Kopp HG, Avecilla ST, Hooper AT, Rafii S. The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology (Bethesda) 2005;20:349-56.
5. Kiel MJ, Morrison SJ. Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 2008;8:290-301.
6. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005;105:2631-9.
7. Chan JY, Watt SM. Adhesion receptors on haematopoietic progenitor cells. Br J Haematol 2001;112:541-57.
8. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11-25.
9. van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res 2001;305:285-98.
10. Hynes RO, Bader BL. Targeted mutations in integrins and their ligands: their implications for vascular biology. Thromb Haemost 1997;78:83-7.
11. Sheppard D. In vivo functions of integrins: lessons from null mutations in mice. Matrix Biol 2000;19:203-9.
12. Bouvard D, Brakebusch C, Gustafsson E, Aszodi A, Bengtsson T, Berna A, et al. Functional consequences of integrin gene mutations in mice. Circ Res 2001;89:211-23.
13. Liesveld JL, Winslow JM, Frediani KE, Ryan DH, Abboud CN. Expression of integrins and examination of their adhesive function in normal and leukemic hematopoietic cells. Blood 1993;81:112-21.
14. Reuss-Borst MA, Klein G, Waller HD, Müller CA. Differential expres-

sion of adhesion molecules in acute leukemia. Leukemia 1995;9:869-74.
15. Gu Y-C, Kortesmaa J, Tryggvason K, Persson J, Ekblom P, Jacobsen S-E, et al. Laminin isoform-specific promotion of adhesion and migration of human bone marrow progenitor cells. Blood 2003;101:877-85.
16. Qian H, Tryggvason K, Jacobsen SE, Ekblom M. Contribution of α 6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with α 4 integrins. Blood 2006;107:3503-10.
17. Palmer EL, Ruegg C, Ferrando R, Pytela R, Sheppard D. Sequence and tissue distribution of the integrin α 9 subunit, a novel partner of β 1 that is widely distributed in epithelia and muscle. J Cell Biol 1993;123:1289-97.
18. Huang XZ, Wu JF, Ferrando R, Lee JH, Wang YL, Farese RV Jr, et al. Fatal bilateral chylothorax in mice lacking the integrin α9β1. Mol Cell Biol 2000;20:5208-15.
19. Taooka Y, Chen J, Yednock T, Sheppard D. The integrin α9β1 mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. J Cell Biol 1999;145:413-20.
20. Chen C, Huang X, Atakilit A, Zhu QS, Corey SJ, Sheppard D. The integrin α9β1 contributes to granulopoiesis by enhancing granulocyte colony-stimulating factor receptor signaling. Immunity 2006; 25:895-906.
21. Yokosaki Y, Palmer EL, Prieto AL, Crossin KL, Bourdon MA, Pytela R, et al. The integrin α9β1 mediates cell attachment to a non-RGD site in the third fibronectin type III repeat of tenascin. J Biol Chem 1994; 269: 26691-6.
22. Smith LL, Cheung H-K, Ling LE, Chen J, Sheppard D, Pytela R, et al. Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by α9β1 integrin. J Biol Chem 1996;271: 28485-91.
23. Bridges LC, Bowditch RD. ADAM-integrin interactions: potential integrin regulated ectodomain shedding activity. Curr Pharm Design 2005; 11:837-47.
24. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
25. Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, et al. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. Cell 1978;14:9-20.
26. Armeanu S, Bühring HJ, Reuss-Borst M, Müller CA, Klein G. E-cadherin is functionally involved in the maturation of the erythroid lineage. J Cell Biol 1995;131:243-9.
27. Klein G, Müller CA, Tillet E, Chu ML, Timpl R. Collagen type VI in the human bone marrow microenvironment: a strong cytoadhesive component. Blood 1995;86:1740-8.
28. Papayannopoulou T. Mechanisms of stem-/progenitor-cell mobilization: the anti-VLA-4 paradigm. Semin Hematol 2000;37(1 Suppl 2):11-8.
29. Qian H, Georges-Labouesse E, Nystrom A, Domogatskaya A, Tryggvason K, Jacobsen SEW, et al. Distinct roles of integrins α 6 and α 4 in homing of fetal liver hematopoietic stem and progenitor cells. Blood 2007;110:2399-407.
30. Jaatinen T, Hemmoranta H, Hautaniemi S, Niemi J, Nicorici D, Laine J, et al. Global gene expression profile of human cord blood-derived CD133+ cells. Stem Cells 2006;24: 631-41.
31. Mackie EJ, Tucker RP. Tenascin in bone morphogenesis: expression by osteoblasts and cell type-specific expression of splice variants. J Cell Sci 1992;103:765-71.
32. Mark MP, Prince CW, Oosawa T, Gay S, Bronckers AL, Butler WT. Immunohistochemical demonstration of a 44-KD phosphoprotein in developing rat bones. J Histochem Cytochem 1987;35:707-15.
33. Tanaka Y, Mine S, Hanagiri T, Hiraga T, Morimoto I, Figdor CG, et al. Constitutive up-regulation of integrin-mediated adhesion of tumor-infiltrating lymphocytes to osteoblasts and bone marrow-derived stromal cells. Cancer Res 1998;58: 4138-45.
34. Hergeth SP, Aicher WK, Essl M, Schreiber TD, Sasaki T, Klein G. Characterization and functional analysis of osteoblast-derived fibulins in the human hematopoietic stem cell niche. Exp Hematol 2008; 36:1022-34.
35. Staniszewska I, Zaveri S, Valle LD, Oliva I, Rothman VL, Croul SE, et al. Interaction of α9β1 integrin with thrombospondin-1 promotes angiogenesis. Circ Res 2007;100: 1308-16.
36. Liu F, Poursine-Laurent J, Link DC. Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. Blood 2000;95:3025-31.
37. Stier S, Ko Y, Forkert R, Lutz C, Neuhaus T, Grunewald E, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 2005;201:1781-91.
38. Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 2005;106:1232-9.