CD34$^+$ cells from AML with mutated *NPM1* harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice

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Acute myeloid leukemia (AML) with mutated *NPM1* shows distinctive biologic and clinical features, including absent/low CD34 expression, the significance of which remains unclear. Therefore, we analyzed CD34$^+$ cells from 41 *NPM1*-mutated AML. At flow cytometry, 31 of 41 samples contained less than 10% cells showing low intensity CD34 positivity and variable expression of CD38. Mutational analysis and/or Western blotting of purified CD34$^+$ cells from 17 patients revealed NPM1-mutated gene and/or protein in all. Immunohistochemistry of trephine bone marrow biopsies and/or flow cytometry proved CD34$^+$ leukemia cells from *NPM1*-mutated AML had aberrant nucleophosmin expression in cytoplasm. NPM1-mutated gene and/or protein was also confirmed in a CD34$^+$ subfraction exhibiting the phenotype (CD34$^+$/CD38$^-$/CD123$^+$/CD33$^+$/CD90$^-$) of leukemic stem cells. When transplanted into immunocompromised mice, CD34$^+$ cells generated a leukemia recapitulating, both morphologically and immunohistochemically (aberrant cytoplasmic nucleophosmin, CD34 negativity), the original patient's disease. These results indicate that the CD34$^+$ fraction in *NPM1*-mutated AML belongs to the leukemic clone and contains *NPM1*-mutated cells exhibiting properties typical of leukemia-initiating cells. CD34$^-$ cells from few cases (2/15) also showed significant leukemia-initiating cell potential in immunocompromised mice. This study provides further evidence that *NPM1* mutation is a founder genetic lesion and has potential implications for the cell-of-origin and targeted therapy of *NPM1*-mutated AML. (*Blood*. 2010;116(19): 3907-3922)

Introduction

Acute myeloid leukemia (AML), with mutated *nucleophosmin* (*NPM1*) and aberrant cytoplasmic expression of nucleophosmin (NPMc$^+$ AML),[1] accounts for approximately one-third of AML. Because of its distinctive molecular, clinical, and prognostic features,[2-5] AML with mutated *NPM1* was included as a new provisional entity in the 2008 World Health Organization classification of myeloid neoplasms.[6]

Its unique gene expression profile is characterized by up-regulation of most *HOX* genes and down-regulation of *CD34* and *CD133*.[7,8] Because *HOX* genes are involved in stem-cell phenotype maintenance,[9] gene expression profile findings strongly suggest that *NPM1*-mutated AML originates from an early hematopoietic progenitor. This view is also supported by immunohistochemistry with antibodies specific for NPM1 mutants and by mutational analysis of laser-microdissected bone marrow cells showing that *NPM1*-mutated AML frequently displays multilineage involvement,[10] with exclusion of lymphoid lineage.[11] Conversely, the observation that leukemic cells in most *NPM1*-mutated AML show down-regulation of CD34[1,7,8] raises questions as to whether the *NPM1* mutation occurs in a CD34$^-$ multipotent hemopoietic progenitor[12,13] or whether a minimal pool of CD34$^+$/CD38$^-$ *NPM1*-mutated progenitors exists.

CD34$^+$/CD38$^-$ cells usually contain the so-called leukemia-initiating cells (LICs) or leukemic stem cells (LSCs) that exhibit long-term repopulating potential and the ability to propagate and maintain the AML phenotype in immunocompromised mice.[14,15] Engraftment capability of AML cells has been also associated with prognosis.[16]

CD34$^+$/CD38$^-$ hematopoietic stem cells (HSCs) are thought to be the cell of origin of most AML cases. Indeed, these cells were found to carry the same genetic lesion as the more mature CD34$^+$/CD38$^+$ and CD34$^-$ leukemic populations in various cytogenetic AML subtypes, including those with inv(16), t(6;9), and +8,[17,18] but not in acute promyelocytic leukemia, which may derive from a more mature hemopoietic progenitor.[19,20]

Until the discovery of *NPM1* mutation,[1] the genetic and functional characterization of CD34$^+$/CD38$^-$ cells in AML with normal karyotype was difficult because of the lack of reliable molecular markers. Molecular and/or immunohistochemical detection of *NPM1* mutations allows tracking of the genetic lesion in leukemic cells at different hierarchical stages in approximately 60% of AML with normal cytogenetics.

Aims of this study were: (1) to search for NPM1-mutated gene and/or protein in CD34$^+$ cells (including CD38$^-$ and CD38$^+$ subsets) purified from *NPM1*-mutated AML patients; (2) to determine by immunohistochemistry and/or flow cytometry whether

these CD34^{+} cells carried aberrant cytoplasmic NPM (a distinctive functional feature of *NPM1*-mutated AML); (3) to investigate the capability of purified CD34^{+} and CD34^{−} cells from *NPM1*-mutated AML to engraft in immunocompromised mice and to evaluate the nature and topographic distribution of engrafted cells; and (4) to compare the morphologic, immunophenotypic, and molecular features of murine-engrafted and patients' primary AML cells.

We proved that the minor population of CD34^{+} hemopoietic progenitors in *NPM1*-mutated AML consistently carried the *NPM1* mutation, at least when CD34^{+} cells represented more than 1% of the bulk cell population. In most cases, CD34^{+}, but not CD34^{−}, cells generated in immunocompromised mice a leukemia recapitulating, both morphologically and immunohistochemically (aberrant cytoplasmic NPM1 and CD34 negativity), the original patient's disease. As previously reported by Taussig et al,[21] we also found that CD34^{−} cells from a few *NPM1*-mutated AML patients had significant LIC potential in immunocompromised mice.

Methods

Samples from AML patients

We studied 41 leukemia samples from 38 consecutive *NPM1*-mutated AML patients, including 3 cases evaluated at diagnosis and relapse (Table 1; supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Patients were from the Institutes of Hematology of the Universities of Perugia, Bari, and Catania (Italy), and Dresden (Germany). AML was defined as *NPM1*-mutated based on cytoplasmic expression of nucleophosmin at immunohistochemistry or flow cytometry, which is predictive of *NPM1* mutations.[22,23] Mutated NPM1 protein and/or gene was also confirmed by Western blotting (WB)[24] and/or mutational analysis.[1] The study was approved by the local ethical committees, and written informed consent for analysis of leukemic samples was obtained at each participating center.

Flow cytometric immunophenotyping

Immunophenotyping was performed using the following antibodies: peridinin chlorophyll protein complex-conjugated anti-CD45 (CD45-peridinin chlorophyll protein complex), fluorescein isothiocyanate (FITC)–conjugated anti-CD34 (CD34–FITC), phycoerythrin (PE)–conjugated, or allophyco-cyanin (APC)–conjugated anti-CD38 (CD38-PE, CD38-APC), CD33-APC, and CD123-PE (Becton Dickinson [BD] Biosciences) or phycoerythrin-Texas Red (ECD)–conjugated anti-CD45 (CD45-ECD), CD34–FITC, phyco-erythrin-cyanin 5 (PC5)–conjugated anti-CD38 (CD38-PC5), CD33-PE, phycoerythrin-cyanin 7 (PC7)–conjugated anti-CD19 (CD19-PC7), CD11b-FITC, CD90-FITC, and CD90-PC5 (Beckman Coulter). Cytoplasmic NPM1 in leukemic cells was detected by flow cytometry as previously described.[23] Analysis was performed on either a Cytomics FC500 cytometer equipped with the CXP analysis software 2.0 (Beckman Coulter) or a FACSCalibur or FACSAria flow cytometers using the CellQuest Pro analysis software 6.0 (BD Biosciences). Gates were drawn to exclude nonviable cells and debris.

MACS cell sorting

All leukemia samples were subjected to CD34^{+} cell selection by magnetic-activated cell sorting (MACS) technology, according to manufacturer's instruction (CD34 MicroBeads; Miltenyi Biotec). Both positive and negative cell fractions were analyzed for CD34^{+} cells percentage.

FACS

The CD34^{+} cells MACS-enriched cell fraction from 1 case and leukemic bulk cells from 2 additional cases were subjected to sorting for specific cell subpopulations by the cell sorter FACSAria (BD Biosciences) equipped with blue, red, and violet lasers. Dead cells were excluded by analyzing

forward scatter versus side scatter dot plots. Doublets were excluded by forward scatter-H versus forward scatter-A dot plots. Data compensation and analysis were obtained using the "logicle" display method.[25]

Detection of NPM1 mutated protein and gene

NPM1 mutant protein was detected on lysates from 1 to 2×10^6 cells by WB analysis with a rabbit polyclonal antibody specific for the mutated NPM1 protein.[10,24] Lysate from the human leukemic cell line OCI/AML3[26] was used as positive control for NPM1 mutant A protein expression. In selected cases, *NPM1* mutations were analyzed by either direct sequencing[8] or genomic DNA fragment analysis.[27]

Immunohistochemical studies

Immunohistochemistry was performed on human and mice paraffin-embedded samples fixed in B5 (Bio-Optica) for 2 hours; bone tissues were also decalcified in ethylenediaminetetraacetic acid (Osteodec; Bio-Optica) for 5 to 6 hours. Antigen retrieval was carried out by microwaving in 0.1mM ethylenediaminetetraacetic acid, pH 8.0.

Cytoplasmic nucleophosmin was revealed using a mouse anti-NPM monoclonal antibody (mAb).[1] Other antigens included: nucleolin (C23; mAb MS-3; Santa Cruz Biotechnology), myeloperoxidase (rabbit anti-myeloperoxidase antibody; Dako Denmark), macrophage-restricted CD68 (mouse mAb PG-M1 generated by B.F.), CD20 (mouse mAb; L26; Dako Denmark), CD3 (rabbit mAb, SP7; Thermo Scientific), CD45 and glycoph-orin (Dako Denmark). The antibody/antigen interaction was revealed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique.[1]

Double stainings for CD34/NPM and CD34/C23 were performed using a sequential immunoperoxidase/APAAP procedure.[10]

Leukemia-initiating ability of CD34^{+} versus CD34^{−} cells from *NPM1*-mutated AML in immunocompromised mice

Isolated CD34^{+} and CD34^{−} cells from *NPM1*-mutated AML were screened for their potential to engraft and generate leukemia in either nonobese diabetic/severe combined immunodeficient (NOD/SCID) or NOD/SCID/IL2rγ[null] (NOG)[28] mice. Mice were originally obtained from The Jackson Laboratory. Mouse colonies were maintained in the certified Animal Facility of University of Perugia, Perugia, Italy, in accordance with national guidelines. They were kept in microisolator cages and fed sterile food and acidified water, containing 100 μg/mL ciprofloxacin. Mice 6 to 10 weeks of age were subjected to 3.5 Gy γ-irradiation up to 24 hours before intrave-nous (tail vein) injection of cells. Mice were killed at 3 to 21 weeks after transplantation; bone marrow was removed from one of the femurs and tibias by flushing with phosphate-buffered saline and analyzed for engraft-ment using a specific anti-hCD45 mAb. Other bones, including vertebral bodies, were fixed/decalcified and processed for paraffin embedding. Positive marrow samples ($> 0.1\%$ hCD45^{+} cells) were further analyzed by immunophenotyping, immunohistochemistry, WB, and molecular analysis for NPM1-mutated protein and/or gene to confirm and characterize the type of engraftment. In some mice, spleen, liver, lung, and brain were also studied by immunohistochemistry for leukemic infiltration. Self-renewal capacity of cells recovered from primary recipients was assessed by serial transplantations in mice.

CFC assay

A total of 10 to 50×10^3 cells from the CD34^{+} or CD34^{−} fractions of patients 17, 32, and 34 were plated in triplicate in 1 mL of MethoCult GF H4434 (StemCell Technologies) in 35-mm tissue-culture dishes. Colony scoring was performed after 14 days of culture. Colonies were analyzed for *NPM1* mutation by genomic DNA fragment analysis.

Statistics

Leukemia-initiating cells frequency in CD34^{+} and CD34^{−} cells from *NPM1*-mutated AML was calculated in limiting-dilution experiments using the StemSoft's L-Calc software Version 1.1.1 (StemCell Technologies).

Table 1. Characteristics of samples from 21 patients with *NPM1*-mutated AML

Patient code	Disease status	FAB	IHC (NPM)	WB	Karyotype	FLT3 status	WBC/μL	Sample source	CD34,* % (of MNCs)	IHC (CD34), %	Post-MACS* purity (% CD34+)	CD34+/CD38−, % (gated on CD34+)	MFI* (CD34+/CD38−)
1	Diagnosis	M1	NPMc+	+	NA	NA	20 000	Pb	23	20-30	93	25	2.62
2	Diagnosis	M5	NPMc+	+	46XX; t(2; 17)(p22;q25)	FLT3-ITD	31 040	Pb	5	–	90.64	94	15
2R	Relapse	M5	NPMc+	+	46XX; t(2; 17)(p22;q25)	FLT3-ITD	67 080	Pb	34	–	99	98.4	18.5
4	Diagnosis	NC	NPMc+	+	Normal	wt	26 870	Pb	60	40-50	96.3	0.16	16
7	Relapse	M5b	NPMc+	NA	Normal	wt	30 170	Pb	7.4	–	94	15.6	14
8R	Relapse	M4	NPMc+	+	Normal	wt	127 700	Pb	1.9	–	77	33.3	1.7
10	Diagnosis	M2	NPMc+	+	Normal	FLT3-ITD	25 000	Pb	27	Rare	98.5	70.5	3.2
11	Relapse	M2	NPMc+	+	Normal	wt	66 790	Pb	11.8	–	96.2	70.1	5.1
12	Diagnosis	M1	NPMc+	+	NA	NA	116 000	Pb	21.1	Rare	96	75.7	2.8
15	Diagnosis	M4	NPMc+	NA	Normal	wt	28 730	Pb	1.5	–	70	37	7.47
17	Relapse	M4	NPMc+	+	Normal	FLT3-ITD	35 750	Pb	27	Rare	98.3	70	5.9
18	Diagnosis	M4	NPMc+	+	Normal	FLT3-ITD	62 440	Pb	8.7	–	95	92.5	4.5
19	Diagnosis	M4	NPMc+	+	Normal	wt	4070	BM	1.5	–	99.7§	39	5.2
22	Diagnosis	M1	NPMc+	+	Normal	wt	44 000	Pb	4.3	–	86.2||	11	2
22R	Relapse	M1	NPMc+	+	NA	wt	10 800	Pb	72	50-60	99.9	15	2.4
23	Relapse	M2	NPMc+	+	Normal	wt	19 780	Pb	1.7	Rare	18.5||	45.5	2
25	Diagnosis	M5b	NPMc+†	NA	NA	FLT3-ITD	48 920	BM	2.0	NA	99¶	20	8.3
26	Diagnosis	M2	NPMc+†	NA	46XX; 46XX; del(3)(p21)	FLT3-ITD	2600	BM	2.6	NA	99¶	2.2	7.0
30	Diagnosis	M4	NPMc+	+	47XX; +8	FLT3-D385	84 770	Pb	2.93	–	79	29.4	4.8 and 11.4#
31	Diagnosis	M4	NPMc+	+	Normal	wt	23 000	Pb	4.76	–	92.4	45	11
32	Diagnosis	M4	NPMc+	+	46XX; t(2;12)	FLT3-ITD	264 000	Pb	1.3	–	81/92.3§	88	6.2 and 12.4#
34	Diagnosis	M4	NA‡	+	NA	wt	150 000	Pb	5	NA	96.5	2.48	3.8 and 13.6#
35	Diagnosis	M2	NPMc+	+	Normal	FLT3-ITD	7600	Pb	22	–	96.4	68	4.8

Characteristics of other patients are shown in supplemental Table 1.

FAB indicates French-American-British classification; IHC, immunohistochemistry analysis; WB, Western blot with specific anti-NPM mutant antibodies[24]; WBC, white blood cell; MFI, mean fluorescence intensity; NC, not classified; NA, not available; wt, wild-type; Pb, peripheral blood; R, relapse; –, CD34+ cells not detectable at IHC; and BM, bone marrow.

*Analyzed on Cytomics FC500 cytometer equipped with the CXP analysis software (Beckman Coulter).

†Cytoplasmic NPM was detected by flow cytometry.[23]

‡Only studied by WB.

§After 2 purification steps.

||CD34+ MACS-enriched cell fraction used for FACS sorting.

¶CD34+ FACS sorted.

#Two distinct cell populations (CD34low and CD34bright).

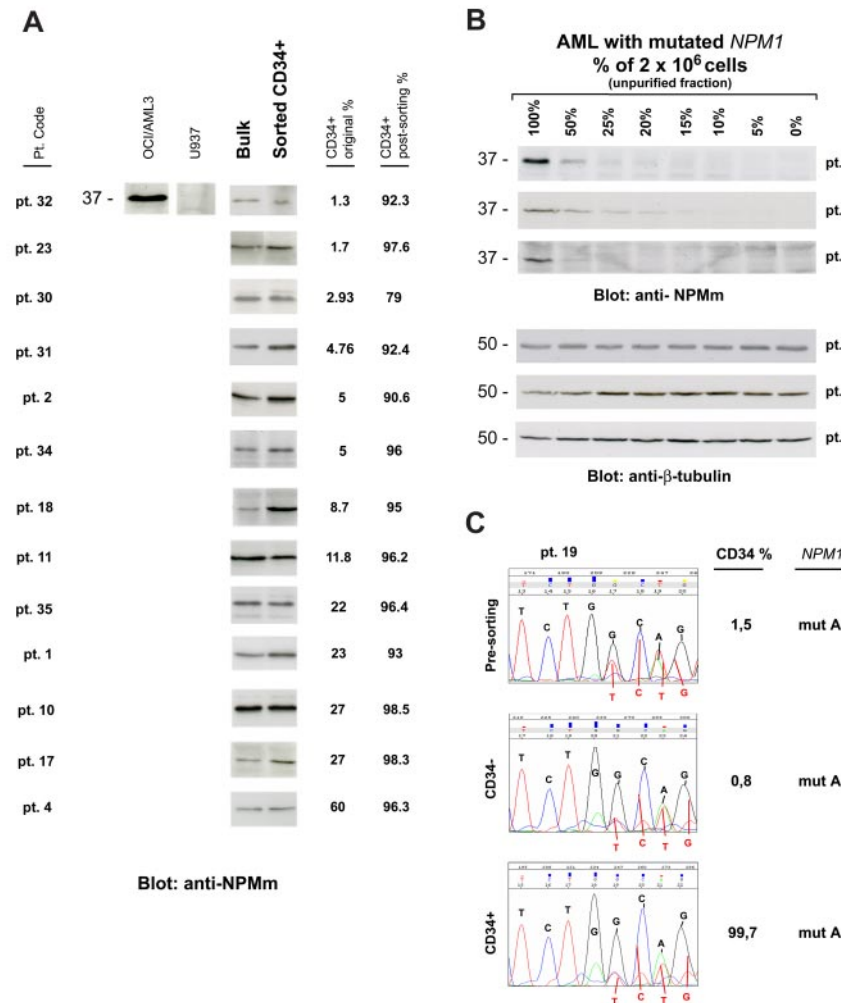**A**

Blot: anti-NPMm

Pt. Code			Bulk	Sorted CD34+	CD34+ original %	CD34+ post-sorting %
pt. 32	OCI/AML3	U937			1.3	92.3
pt. 23					1.7	97.6
pt. 30					2.93	79
pt. 31					4.76	92.4
pt. 2					5	90.6
pt. 34					5	96
pt. 18					8.7	95
pt. 11					11.8	96.2
pt. 35					22	96.4
pt. 1					23	93
pt. 10					27	98.5
pt. 17					27	98.3
pt. 4					60	96.3

B

AML with mutated *NPM1*
% of 2 x 10^6 cells
(unpurified fraction)

100% 50% 25% 20% 15% 10% 5% 0%

37 -   pt. 30
37 -   pt. 32
37 -   pt. 33

Blot: anti- NPMm

50 -   pt. 30
50 -   pt. 32
50 -   pt. 33

Blot: anti-β-tubulin

C

	pt. 19	CD34 %	NPM1
Pre-sorting		1,5	mut A
CD34-		0,8	mut A
CD34+		99,7	mut A

Figure 1. CD34$^+$ cells from AML with mutated *NPM1* express mutated NPM1 gene and protein. (A) WB analysis of CD34$^+$ cells isolated from 13 *NPM1*-mutated AML patient samples. Patient sample codes are indicated on the left (Table 1). Lysates from 1 to 2 × 10^6 cells of either leukemic bulk (Bulk) or CD34$^+$ MACS sorted (Sorted CD34$^+$) cell population were loaded and run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, transferred to polyvinylidene difluoride membrane, and probed with an anti-NPM mutant (anti-NPMm) rabbit polyclonal antibody. A specific band corresponding to NPM mutant protein is detected at 37-kDa molecular weight (MW) in both bulk and CD34$^+$ cell populations from patients. Columns on the right show percentage of CD34$^+$ cells in the original patient sample and in the CD34-sorted cell population. Lysates from human myeloid leukemic cell lines OCI/AML3 and U937 were used, respectively, as positive and negative control for NPM1 mutant protein expression. (B) AML patient sample dilution test for WB with anti-NPM mutant specific antibody. Unpurified cell fraction from 3 representative *NPM1*-mutated AML patient samples (patients 30, 32, and 33) was progressively diluted (100%, 50%, 25%, 20%, 15%, 10%, 5%, and 0%) with cells of AML with unmutated *NPM1*. Lysates from a total of 2 × 10^6 cells were loaded in each lane and checked for NPM1 mutant protein detection by WB (anti-NPMm, top panels). Progressive dilution of signal indicates that saturation for NPM1 mutant protein detection is not reached in our experimental conditions. Signal is not anymore detectable when *NPM1*-mutated AML sample is less than 15% to 10% of the original sample. Equal protein lysate loading was demonstrated by blotting the same membranes with an anti–β-tubulin monoclonal antibody (bottom panels). (C) Chromatograms of direct sequencing of the *NPM1* gene exon 12 (forward sequence reading) showing both wild-type sequence (coming from the wild-type allele) and TCTG insertion (type A mutation; coming from the mutated allele) in CD34$^+$ cells isolated from bone marrow of 1 patient with *NPM1*-mutated AML (patient 19; bottom panel) and in the relative controls (leukemic bulk and CD34$^-$ cell populations; top and middle panels, respectively). Percentage of CD34$^+$ cells in each sample is shown on the right. Pt. code indicates patient code; and mut A = *NPM1* gene mutation A.

Results

Immunophenotypic characterization and isolation of CD34$^+$ cells in *NPM1*-mutated AML

Flow cytometry of the 41 *NPM1*-mutated AML samples showed variable percentage of CD34$^+$ cells (range, 0.02%-75%) with 31 of 41 (75.6%) samples expressing less than 10% CD34$^+$ cells (Table 1; supplemental Table 1). Notably, 18 of 41 samples expressed less than 1% CD34$^+$ cells (supplemental Table 1). Mean fluorescence intensity for CD34 was low (range, 1.7-18.5) in most of samples (Table 1; supplemental Table 1).

Immunohistochemistry for CD34 was carried out in bone marrow trephines from 37 of 41 cases. No or rare CD34$^+$ cells (Table 1; supplemental Table 1) were detected in 34 of 37 samples, whereas in 3 cases (patients 1, 4, and 22R) the CD34$^+$ cells ranged between 20% and 60% (Table 1). The lower percentage of CD34$^+$ cells detectable by immunohistochemistry compared with flow cytometry is probably the result of the lower sensitivity of immunohistochemistry for detecting low-intensity CD34 expression.

CD34$^+$ cells were purified by MACS (39 of 41 samples) or FACS (2 of 41 samples; Table 1; supplemental Table 1). The MACS-sorting approach was chosen because it allows reliable recovery of cells expressing CD34 at different levels, including low-intensity CD34$^+$ cells, which are characteristically found in *NPM1*-mutated AML. Indeed, MACS-purified CD34$^+$ cells showed the same mean fluorescence intensity for CD34 as the CD34$^+$ cells in the original sample, with low, intermediate, or bright intensity cell populations similarly represented. The CD34$^+$ cell fraction also showed the expected heterogeneity in terms of CD38 expression (Table 1; supplemental Table 1). In particular, early CD34$^+$/CD38$^-$ hematopoietic progenitors were variously represented within the CD34$^+$ population (range, 0.16%-98.4%; Table 1; supplemental Table 1).

These results clearly indicate that the minor population of CD34$^+$ cells in *NPM1*-mutated AML usually express the CD34 molecule at low intensity and exhibit the expected heterogeneous phenotype of this cell fraction.

CD34$^+$ cells from *NPM1*-mutated AML express mutated NPM1 gene and protein

We then asked whether CD34$^+$ cells from *NPM1*-mutated AML patients harbor *NPM1* mutation and, thus, belong to the leukemic clone. CD34$^+$ sorted cells (at least 1 × 10^6 cells) from 13 of 41 samples (CD34$^+$ cells ranging from 1.3% to 60% of mononuclear cells [MNCs]) were suitable for WB with anti-NPM mutant specific antibodies (Figure 1A). In all of them, a specific 37-kDa molecular weight band, corresponding to NPM1 mutant protein, was detected (Figure 1A). This signal could not be the result of contaminating CD34$^-$ leukemic cells because in 12 of 13 samples CD34$^+$ cells accounted for more than 90% of the total analyzed population (2 × 10^6 cells), whereas our WB detection method is

not sensitive enough to reveal NPM1 mutant protein from cells, which accounts for less than or equal to 10% (Figure 1B). Moreover, unlike that observed in AML cell dilution assay (Figure 1B), the WB signal intensity was not decreased in the CD34^{+} purified cells compared with leukemic bulk (Figure 1A). Thus, it is conceivable that, even in the case with lower CD34^{+} cell purity (patient 30, 79% CD34^{+} cell purity; Table 1), positive signal could not be ascribed to contaminating CD34^{−} cells.

In 1 leukopenic patient (patient 19), only 0.1×10^6 CD34^{+} bone marrow cells (99.7% pure) could be recovered, which were not enough for WB analysis. In this case, *NPM1* mutation A was detected by direct gene sequencing in purified CD34^{+}, CD34^{−}, and presorting cell populations (Figure 1C). Again, detection of *NPM1* mutation in the CD34^{+} cells could not be the result of the minority of contaminating CD34^{−} cells (0.3%) because the latter was under the detection threshold ($\sim$ 25%) of our direct gene sequencing assay (supplemental Figure 1).

These findings clearly show that the CD34^{+} fraction from *NPM1*-mutated AML with CD34^{+} cells representing more than 1% of the bulk cell population is mutated both at gene and protein level, thus indicating it belongs to the leukemic clone.

The CD34^{+} fraction from *NPM1*-mutated AML contains CD34^{+}/CD38^{−} cells harboring NPM1 mutated gene and protein

Given the heterogeneity of CD34^{+} cells and to better track the *NPM1* mutation in the context of hematopoietic hierarchy of *NPM1*-mutated AML, we next investigated whether CD34^{+}/ CD38^{−} cells carry the genetic lesion. The CD34/CD38 flow cytometric staining patterns of the 5 cases investigated (Table 1: patients 2, 18, 22, 25, and 26) are shown in Figure 2A.

In 2 of 5 cases (Table 1: patients 2 and 18) whose MACS-sorted CD34^{+} cell fraction was represented almost exclusively by CD34^{+}/ CD38^{−} cells (purity of 94% and 92.5%, respectively), expression of mutated NPM1 protein was detected by WB (Figure 2B).

In the remaining 3 cases (patients 22, 25, and 26), the FACS-sorted CD34^{+}/CD38^{−} subpopulation (purity of 96%, 99%, and 99%, respectively) was *NPM1*-mutated by either direct gene sequencing (patient 22, harboring *NPM1* mutation 7[3]) or genomic DNA fragment analysis (patients 25 and 26). The finding of *NPM1* mutation in these samples could not be the result of the small number of contaminating CD34^{−} cells (range, 1%-4%) because this is below the detection threshold of our molecular assays (supplemental Figure 1). A representative case (patient 25) harboring the *NPM1* mutation in the CD34^{+}/CD38^{−} and CD34^{+}/CD38^{+} cell fractions (both 99% pure), as well as in the bulk leukemic population, is shown in Figure 2C-D. In this case, the CD34^{+}/ CD38^{−} cells also carried *FLT3*-ITD mutation (Figure 2Di), further confirming that they belong to the leukemic clone. *FLT3*-ITD showed the highest prevalence in the CD34^{+}/CD38^{−} cells (Figure 2Di), whereas, in the more differentiated cells (CD34^{+}/CD38^{+}), the *FLT3*-ITD mut/wt ratio was significantly lower (Figure 2Dii) and, in the bulk leukemic population, *FLT3*-ITD was only present in a small fraction of cells (Figure 2Diii).

In conclusion, our results clearly indicate that, in the 5 cases studied, the CD34^{+}/CD38^{−} subpopulation carried the mutated NPM1 gene and protein.

Immunophenotypic features of CD34^{+}/CD38^{−} cells from *NPM1*-mutated AML

We then asked whether CD34^{+}/CD38^{−} cells from *NPM1*-mutated AML displayed the immunophenotype (CD123^{+}/CD33^{+}/

CD90^{−})[29-31] typical of LSCs. Most or all of CD34^{+}/CD38^{−} cells were also CD123^{+} in 21 of 24 samples and CD33^{+} (generally at high intensity) in 14 of 15 samples (eg, patient 18 of Figure 2A; supplemental Table 2). CD90 was negative in all cases (data not shown). In 9 of 41 (21.9%) samples (Table 1; supplemental Table 1), a double CD34^{low} and CD34^{bright} cell population was observed (Figure 2E). Interestingly, CD34^{low} and CD34^{bright} cells within the CD34^{+}/CD38^{−} fractions showed a different phenotype (Figure 2E; supplemental Table 2). CD34^{low} were CD123^{+}/CD33^{+} (a phenotype resembling that of LSCs), whereas CD34^{bright} cells were negative for both antigens, probably representing residual circulating normal CD34^{+} HSCs. The latter have been previously described in some AML patients at diagnosis.[32]

Our results clearly indicate that CD34^{+}/CD38^{−} cells from *NPM1*-mutated AML show the genotypic and phenotypic features of LSCs (ie, NPM1-mutated gene/protein and expression of CD123 and CD33). In some cases, a variable fraction of CD34^{bright} cells was detected, possibly representing residual circulating normal HSCs.

CD34^{+} cells in *NPM1*-mutated AML show cytoplasmic expression of nucleophosmin

Then we asked whether CD34^{+} *NPM1*-mutated AML cells expressed aberrantly nucleophosmin in their cytoplasm, which is the most distinctive functional consequence of *NPM1* mutations.[33-36]

To address this issue, we double-stained for CD34 and NPM bone marrow paraffin sections from 5 *NPM1*-mutated AML containing a percentage of CD34^{+} cells ranging between 2% and 20%. In all cases, the CD34^{+} cells showed aberrant cytoplasmic positivity for nucleophosmin (NPMc^{+}) and nucleus-restricted positivity for another nucleolar protein (C23/nucleolin) that was used as control (Figure 3A-D). Double-stained CD34^{+}/NPMc^{+} cells were randomly scattered in bone marrow biopsies from patients, without predilection for areas close to bone trabeculae or marrow vessels. Expression of cytoplasmic NPM1 in CD34^{+} cells (both CD38^{−} and CD38^{+} subsets) was also found by flow cytometry (Figure 3E-F).

Thus, CD34^{+} leukemic cells (including CD34^{+}/CD38^{−} and CD34^{+}/CD38^{+} subpopulations) not only carry the mutated NPM1 gene and protein but also show aberrant nucleophosmin expression in cytoplasm, which is a distinguishing feature of AML with mutated *NPM1*.[33-36]

CD34^{+} cells from *NPM1*-mutated AML generate leukemia in immunocompromised mice

Isolated CD34^{+} cells from *NPM1*-mutated AML were screened for their potential to engraft and generate leukemia in immunocompromised mice. Engraftment was observed in 10 of 16 samples (Table 2; supplemental Tables 3-4), as documented by positivity for hCD45 (at flow cytometry and immunohistochemistry). The leukemic nature of hCD45^{+} engrafted cells was clearly demonstrated by the combined morphologic and immunohistochemical analysis of bone marrow sections that showed 3 main infiltration patterns: (1) sparse aggregates of human leukemic cells mainly adjacent to the bone (suggestive of initial marrow involvement, supplemental Figure 2); (2) leukemic infiltration of marrow areas near to and distant from the bone (the most common pattern, Figure 4); and (3) massive marrow infiltration by leukemic cells (supplemental Figure 3).

The leukemic nature of all hCD45^{+} engrafted cells was further supported in 7 of 10 cases where the morphology and immunophenotype of engrafted cells paralleled that of patient's primary AML
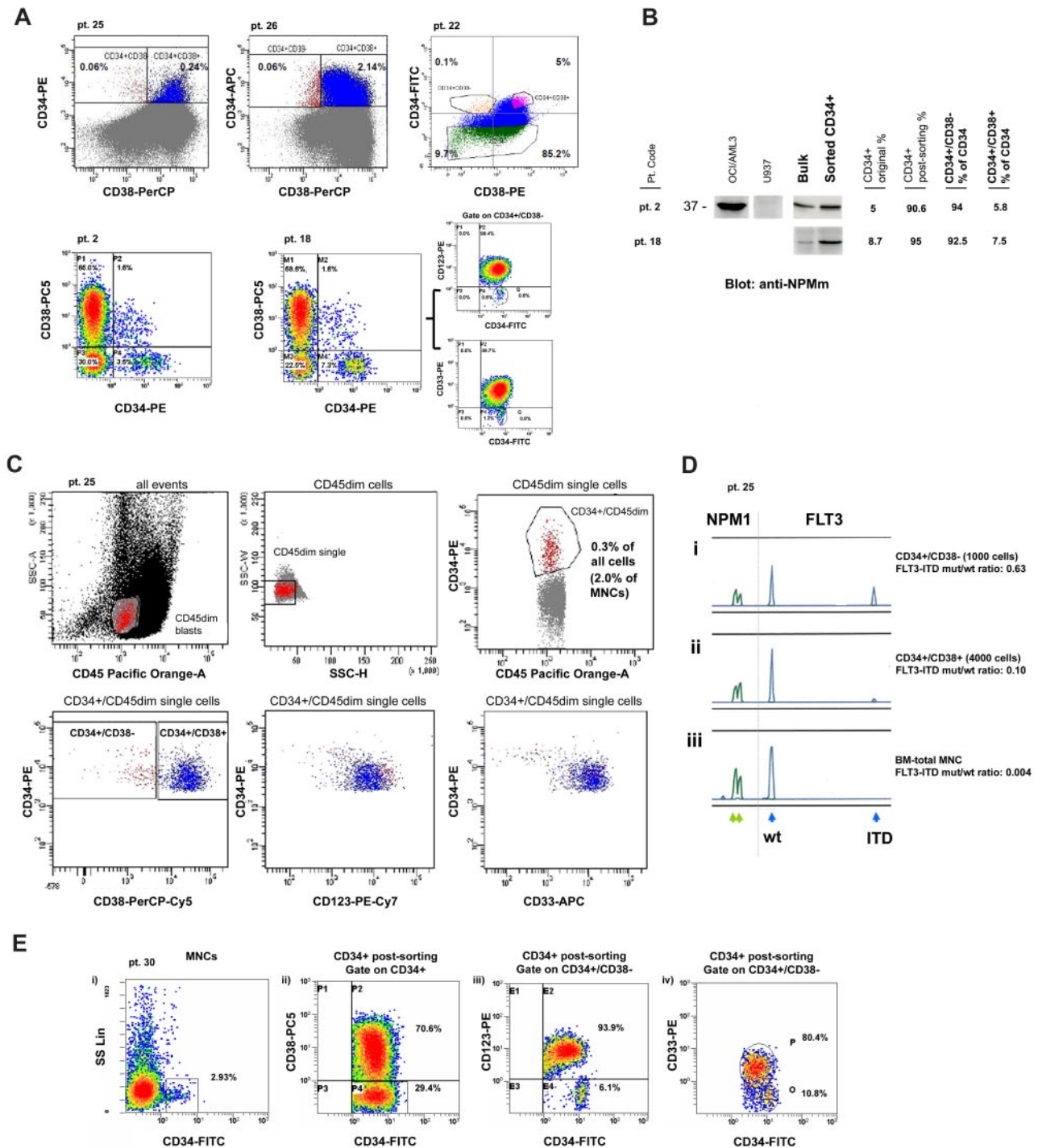
Figure 2. CD34⁺/CD38⁻ cells from AML with mutated *NPM1* express mutated NPM1 gene and protein and display immunophenotypic features of LSCs.
(A) CD34/CD38 flow cytometry staining patterns of peripheral blood MNCs (patients 2, 18, and 22) or whole bone marrow (patients 25 and 26) from the 5 *NPM1*-mutated AML patients studied for involvement of CD34⁺/CD38⁻ cells by *NPM1* mutation. Flow cytometric analysis was performed on either FACSAria (BD Biosciences; patients 22, 25, and 26) or Cytomics FC500 cytometer (Beckman Coulter; patients 2 and 18), as indicated in "Flow cytometric immunophenotyping." Concomitant expression of CD123 and CD33 on the CD34⁺/CD38⁻ cell population is shown in a representative example (patient 18, bottom right panels). Here, within the CD34⁺/CD38⁻ cell population, only a very small cell fraction (gate Q: ~ 0.8%) was negative for both CD123 and CD33 that could represent normal residual HSCs. (B) WB showing expression of NPM1 mutant protein in MACS-sorted CD34⁺ cells from 2 selected cases from Figure 1 (patients 2 and 18) where CD34⁺/CD38⁻ cells represented almost the whole CD34⁺ cell population. Here, percentages of CD34⁺/CD38⁻ and CD34⁺/CD38⁺ cells are shown. (C-D) CD34⁺/CD38⁻ and CD34⁺/CD38⁺ cells were FACS-sorted from bone marrow of a patient (patient 25) with AML with cytoplasmic NPM1 with 0.3% CD34⁺ cells. Expression of CD123 and CD33 antigens in the 2 cell subpopulations is shown (C bottom panels). Molecular analysis for *NPM1* and *FLT3*-ITD mutations was performed on genomic DNA by high-resolution fragment analysis (D). Results obtained on sorted CD34⁺/CD38⁻ (purity of 99%; i) and CD34⁺/CD38⁺ (purity of 99%; ii) cells were compared with the total bone marrow mononuclear cells (BM-MNC; iii). As shown in green, the typical double peak indicating the *NPM1* mutation (green arrows) was detectable at similar levels in both populations as well as in the MNCs. Peaks indicating *FLT3* gene status (wild-type [wt] and *FLT3*-ITD [ITD] mutation) are in blue. *FLT3*-ITD mutation/*FLT3*-wt ratio (mut/wt) is shown on the right. (E) Flow cytometric analysis of a representative *NPM1*-mutated AML patient sample (patient 30) showing an evident double-cell population with distinct immunophenotypic features within CD34⁺/CD38⁻ progenitor cells: (i) CD34⁺ cell percentage (2.93%) in the leukemic bulk from the patient's peripheral blood (MNCs); (ii) expression of CD38 on the purified CD34⁺ cells (CD34⁺ after sorting); and detection of 2 distinct cell subpopulations within CD34⁺/CD38⁻ cells: CD34^bright/CD38⁻/CD123⁻/CD33⁻ (iii) versus CD34^low/CD38⁻/CD123⁺/CD33⁺ (iv) cells.
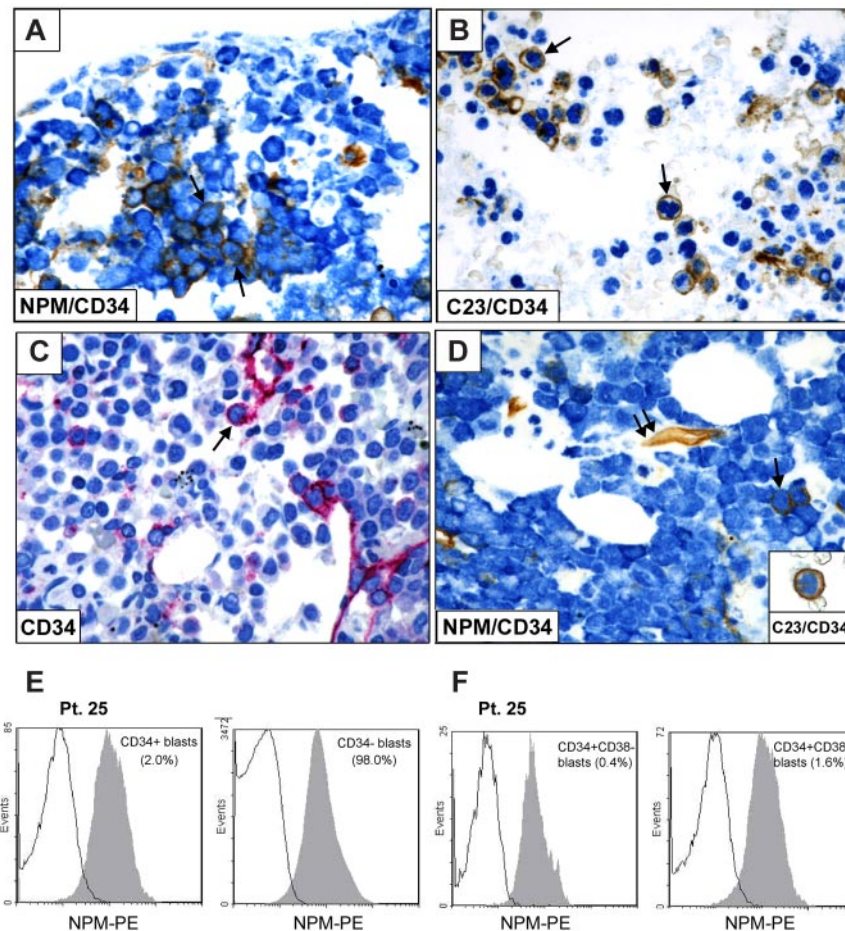
Figure 3. CD34+ cells in *NPM1*-mutated AML show cytoplasmic expression of nucleophosmin. (A-B) CD34+ leukemic cells (brown) from a patient with *NPM1*-mutated AML showing cytoplasmic expression of NPM (blue; A arrows) and nucleus-restricted positivity for C23/nucleolin (B arrows). (C-D) Another *NPM1*-mutated patient showing rare CD34+ cells (C) that double stain for CD34 (brown) and cytoplasmic NPM (blue; D single arrow); endothelium of a vessel express CD34 (D double arrow). CD34+ leukemic cell (brown) from the same case show nucleus-restricted positivity for C23/nucleolin (blue; D inset). (A-B,D) Sequential immunoperoxidase/APAAP staining; no counterstaining. (C) APAAP technique (hematoxylin counterstaining). (A-D) Paraffin sections from bone marrow biopsies. All images were collected using an Olympus B61 microscope and a UPlan Fl 100×/1.3 NA oil objective; Camedia 4040, Dp_soft Version 3.2; and Adobe Photoshop 7.0. (E-F) Flow cytometric detection of NPM-cytoplasmic expression (patient 25) in CD34+ and CD34− blasts (E), and after additional gating on CD34+/CD38− or CD34+/CD38+ cells, respectively (F).

(Table 2; supplemental Table 4). In these cases, all engrafted cells were myeloid (hCD45+/CD33+ and myeloperoxidase-positive; Figure 4B,E-F). In contrast, no hCD45+ cells coexpressing CD19 and/or CD20 (suggestive of normal engraftment) were detected in the great majority of evaluated mice (Table 2; supplemental Table 4). Conclusive evidence that all human engrafted cells belonged to the leukemic clone came from the immunohistochemical demonstration that they expressed cytoplasmic NPM/nuclear C23 (Figure 4C-D), the hallmark feature of human *NPM1*-mutated AML cells. Moreover, in 5 of 7 cases (patients 4, 10, 17, 22R, and 23), the WB and/or molecular analysis showed a mutated NPM1 protein and/or gene (Figure 4G; Table 2; supplemental Table 4). Although CD34+ cells from 1 patient (patient 17) did not show the ability to produce AML colonies in in vitro colony-forming cell (CFC) assay (data not shown), cells recovered from primary mice recipients demonstrated self-renewal capacity in serial transplantations (Table 3: patients 4, 10, 11, 17, and 22R).

Interestingly, in 3 of 10 cases (patients 30, 32, and 34), immunohistochemistry and flow cytometry showed either normal or mixed (normal plus leukemic) engraftment. The latter pattern of engraftment was characterized by areas of bone marrow infiltrated by human NPMc+ AML cells adjacent to areas of normal human

trilineage hematopoiesis (Table 2; Figure 5). Molecular analysis (performed on the whole cell population recovered by bone flushing) confirmed *NPM1*-mutation in all cases (Table 2). AML development was usually seen later than normal human hematopoietic engraftment (Table 2). Interestingly, flow cytometry of original MNCs from patients 30, 32, and 34 showed a relevant subpopulation of CD34bright/CD38−/CD123−/CD33− cells, which probably represent normal HSCs (Figure 2E; supplemental Table 2). Furthermore, in CFC assay, CD34+ cells from 2 of these cases (patients 32 and 34) produced mixed and erythroid colonies, which, because of germline *NPM1* gene (data not shown), are normal in origin.

Our findings clearly indicate that the small fraction of CD34+ cells from most *NPM1*-mutated AML cases generate a leukemia showing the same morphologic and immunohistochemical features (aberrant cytoplasmic NPM1) as the original patient's AML.

Patterns of engraftment of CD34− cells from *NPM1*-mutated AML in immunocompromised mice

The main aim of this study was to characterize the small fraction of CD34+ cells in *NPM1*-mutated AML. However, there is experimental evidence that, in some AML cases[37,38] (including

Table 2. Leukemic engraftment of CD34+ cells from 10 NPM1-mutated AML in immunocompromised mice

Patient code	% CD34+ in original sample	Purity of inoculum	No./mice	No. of cells/mice ×10^6	No. of mice with AML/ no. of mice evaluated	Time of evaluation, w	% hCD45 in mice BM	% CD34+ in mice (gated on hCD45)	% hCD45+/CD19+ (or CD20+)	WB (NPMm)	IHC (NPM)
4	60	96.3	1/NOD	5	1/1	4	27	50	-‡‡	+	c+
			3/NOD	2	2/3	8	60.4/80§	ND/55	-‡‡	+	c+
10	27	98.5	2/NOD	5	1/2	16	0.62	ND	ND	ND	ND
			4/NOD	2	3/4	16/16/20/20	12.8‖/9.7/0/17.4§	1.9/ND/—/2.4	-‡‡	+	c+
11	11.8	96.2	2/NOD	2	2/2	20	39.8§/19.8	1	-‡‡	ND	c+
13	0.8	90	1/NOD	2.5	1/1	19	32.3	CD34- ‡‡	-‡‡	ND	c+
22R*	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4
17*†	27	98.3	4/NOG	2	4/4	9/9/12/20	4.5/15.8/52‖/80§	ND/52/35/14	ND/0/3.2**/0	+	c+
			4/NOG	1	4/4	9/9/13/20	1.5/1.8/5.8/24.7	ND/ND/21/23.7	0	+	c+
23†	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4
CD34+	1.7	99	1/NOG	0.2	1/1	15	21	0.57	0	+	c+
			2/NOG	0.001	0/2	21	0	—	—	—	—
CD34-		99.9	1/NOG	0.2	0/1	15	0	—	—	—	—
			2/NOG	0.001	2/2	21	53.8‖/95.7§	0	0	+	c+
30†	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4
CD34+	2.93	79	4/NOG	1	2/4	7/12/18/18	28#/27.3#/33**††/47.3**††	13/15.2/6.9/10.8	25/85/22/52.6	-	c-/c+§§
CD34-		99.5	4/NOG	1	0/4	7/12/18/18	0	—	—	—	—
32†	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4
CD34+	1.3	92.3	3/NOG	0.5	2/3	12/18/20	8.2#/1.25**/1.54**	ND/0.42/1	81.5/15/1	ND	c-/c+‖‖
			3/NOG	0.2	1/3	3/6/18	80.8#/32.8#/59.6**††	1.2/2.57/0	0/36/0.2	-/+	c-/c+‖‖
			3/NOG	0.1	1/3	17/19/20	0.72#/0.6†/0.14#	0.1/2.7/0.3	50/5.2/0	—	c-/c+‖‖
			4/NOG	0.01	0/4	3/3/15/20	0	—	—	—	—
CD34-		98.8	3/NOG	0.5	0/3	13/18/20	0	—	—	—	—
			3/NOG	0.2	0/3	3/6/15/20	0	—	—	—	—
			4/NOG	0.1	0/4	12/15/18/20	0	—	—	—	—
			4/NOG	0.01	0/4	6/7/20/20	0	—	—	—	—
34*†‡	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4	sT4

BM indicates bone marrow; WB (NPMm), Western blot analysis with anti-NPM mutant specific antibody; IHC (NPM), immunohistochemical analysis for NPM1 subcellular localization on hCD45+ cells; —, not applicable; ND, not done; sT4, full details on the characteristics of the engraftment are provided in supplemental Table 4; NOD, NOD/SCID; and NOG, NOD/SCID/IL2Rγ^null.

*Experiments to calculate frequency of LIC were performed with these samples (see Table 5).

†Paired experiments using corresponding doses of CD34+ versus CD34- cells were performed with these samples (Table 4, details of patients 17 and 34).

‡Higher cell doses of CD34- cells gave rise to engraftment as pattern 4 (see "Patterns of engraftment of CD34- cells from NPM1-mutated AML in immunocompromised mice" for description; Table 4).

§Used for serial transplantation experiments.

‖FLT3-ITD+.

¶FLT3-ITD-.

#Normal engraftment.

**Mixed normal-leukemic engraftment.

††Positive for NPM1 mutation by genomic DNA fragment analysis.

‡‡Evaluated only at immunohistochemistry.

§§In the same mice areas of leukemia (NPMc+) and normal engraftment (NPMc-) were detected.

‖‖In the same mice foci of NPMc+, cells were detected within areas of normal hemopoiesis.

Figure 4. Leukemic engraftment of CD34⁺ cells from *NPM1*-mutated AML in immunodeficient mice. (A-D) Bone marrow paraffin sections from a NOG mouse, inoculated (12 weeks before) with 2 × 10⁶ CD34⁺ cells (purity 98.3%) isolated from 1 patient (patient 17) with *NPM1*-mutated AML, showing infiltration by human cells, which are positive at immunostaining with a specific anti–human CD45 antibody (A), display a myeloid phenotype (myeloperoxidase, MPO-positive; B) and aberrant cytoplasmic expression of nucleophosmin (NPM; C arrow). Double arrows in panel C indicate a normal osteoblast with nucleus-restricted NPM on the endosteal surface of bone. As expected, nucleolin/C23 was also restricted to the nucleus (D, arrow). (A-D) APAAP; hematoxylin counterstaining. Images were collected using an Olympus B61 microscope and a UPlanApo 20×/0.70 (A-B) and a UPlan Fl 100×/1.3 NA oil objective (C-D) Camedia 4040, Dp_soft Version 3.2; and Adobe Photoshop 7.0. (E) Flow cytometric analysis of murine bone marrow confirmed engraftment of human cells (hCD45, 52%) which are prevalently CD33⁺ (92.7%) and express the monocytic marker CD11b (18.6%). CD34/CD38 staining pattern of engrafted hCD45⁺ cells is also shown (E right panels). CD34⁺/CD38⁻ cells (gate on F4) were also CD123⁺ (E right bottom panel). (F) May-Grünwald-Giemsa staining of a cell cytospin preparation from murine bone marrow of the same case (patient 17) showing large-size leukemic cells with monocytoid appearance (cleaved nucleus, basophilic cytoplasm; arrow) admixed with cells of murine origin (double arrow indicate a normal murine polymorphonucleated cell). (G) WB analysis with anti-NPM mutant specific antibodies (anti-NPMm) of mouse bone marrow cells confirmed expression of NPM1 mutated protein in cells engrafted in mice (lane 2) as well as in the original AML patient sample (lane 3). Positive (OCI/AML3, lane 1) and negative (U937, lane 4) controls are shown.
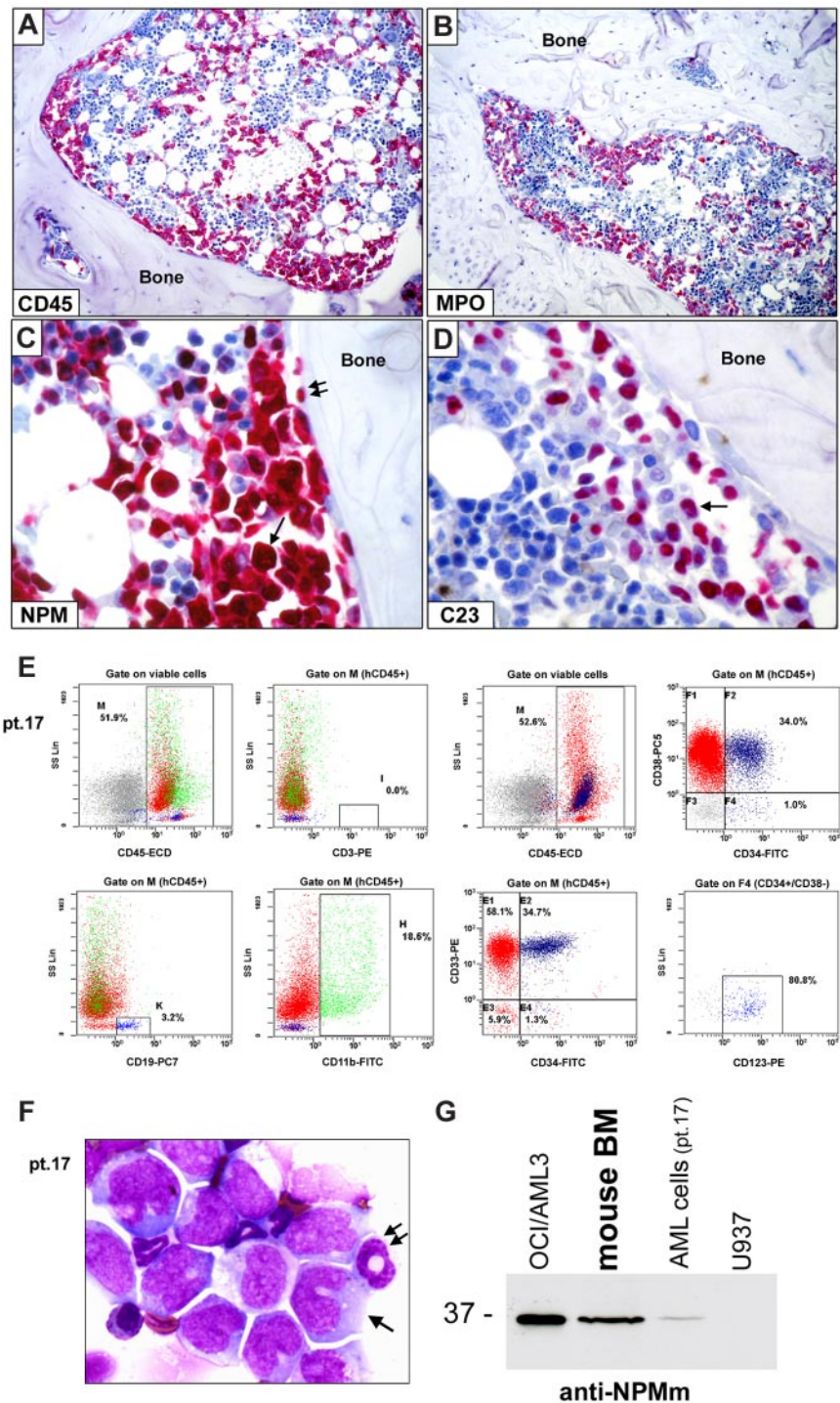
NPM1-mutated AML[21]), also the CD34⁻ population may contain the LICs. Therefore, we assessed the engraftment capability of CD34⁻ cells from 15 *NPM1*-mutated AMLs (Table 4; supplemental Table 5). Inoculation of these cells in immunocompromised mice led to 4 different patterns, which varied also according to the injected cell dose:

Pattern 1. CD34⁻ cells from 10 patients did not engraft in mice when inoculated at doses less than or equal to 1 × 10⁶ (supplemental Table 5). In contrast, CD34⁺ cells from 4 of these cases generated typical NPMc⁺ AML, when injected in mice at the same doses (Table 2: patients 17, 30, 32, and 34; supplemental Table 4).

Moreover, CD34⁻ cells from patients 17, 32, and 34 did not outgrow into AML colonies in CFC assays (data not shown).

Pattern 2. Engraftment of CD34⁻ as typical NPMc⁺ AML (similar to that observed injecting mice with purified CD34⁺ cells) was observed in 2 cases (not shown). In both of them, CD34⁻ cells were purified from *NPM1*-mutated AML at relapse (Table 4: patients 8R and 23). Self-renewal capability of CD34⁻ cells from these cases was demonstrated after transfer to secondary recipients (Table 3).

Pattern 3. Inoculation of CD34⁻ fraction (≥ 2 × 10⁶ cells) from 3 cases (Table 4: patients 22, 27, and 28) resulted into

Table 3. Summary of serial transplantations experiments

Patient code	Engrafted cell fraction in primary recipients*	No./mice	No. of cells/ mice, ×10⁶†	No. of mice with AML/ no. of mice evaluated	Time of evaluation, w	% hCD45 in mice BM‡
4	CD34⁺	4/NOD	2	4/4§	6	87/15/89/87.5
10	CD34⁺	1/NOD	0.6	1/1	18	94
11	CD34⁺	1/NOD	1.6	1/1	18	3
17‖	CD34⁺	2/NOG¶	0.125¶	1/2	25	0.12
17‖	CD34⁺	4/NOG#	0.125#	0/4	25	0
22R	CD34⁺	4/NOG	0.52	4/4	11	7.1/6/8.3/7
8R	CD34⁻ (pattern 2)	3/NOD	1	3/3	6.5	6.7/1.2/0.5
23	CD34⁻ (pattern 2)	3/NOG	4/4/2	3/3	12	60.5/79/69.5
22	CD34⁻ (pattern 3)	2/NOG	2.5	0/2	8	0
22	CD34⁻ (pattern 3)	2/NOG	1	0/2	12	0
27	CD34⁻ (pattern 3)	1/NOG	0.5	0/1	12	0

R indicates relapse.

*See Tables 2 and 4 for details on cases used for serial transplantations.

†hCD45⁺ cell equivalent.

‡Human cells were all hCD45⁺/CD33⁺ and expressed cytoplasmic NPM1 at immunohistochemical analysis.

§From these mice, we obtained engraftment up to quaternary recipients.

‖Human cells recovered from engrafted mice were sorted in CD34⁺ and CD34⁻ cell fractions and inoculated separately.

¶CD34⁺.

#CD34⁻.

marrow engraftment by hCD45⁺/hCD33⁺ cells (Figure 6A), which, in tissue sections, consisted of 2 populations (Figure 6B-D): (1) myeloperoxidase-positive cells located close to bone trabeculae that exhibited weak cytoplasmic NPM1 positivity (not shown); and (2) mature CD68⁺ histiocytes that were located in the central area of bone marrow. In all cases, detection of NPM1 mutant protein and gene (Figure 6 E-F) proved the leukemic nature of engrafted cells. These findings possibly reflect short-term engraftment by
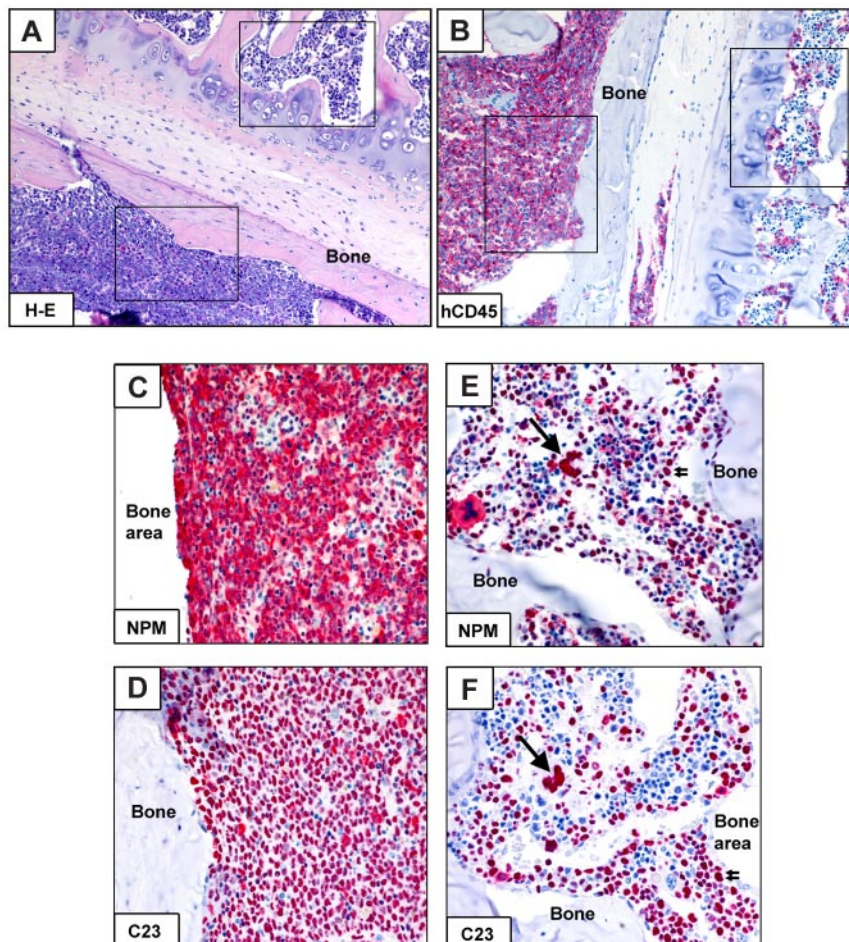

Figure 5. Mixed (normal and leukemic) engraftment of CD34⁺ cells from *NPM1*-mutated AML-immunodeficient mice. (A-F) Bone marrow paraffin sections from a vertebral body of an NOG mouse, inoculated (18 weeks before) with 1 × 10⁶ CD34⁺ cells (purity 79%) from patient 30 (Table 2). An area in the bottom part of panel A (square) is packed with leukemic cells expressing hCD45 (B left square), cytoplasmic NPM1 (C) and nucleus-restricted C23 (D). Another area from the same section shows involvement by normal human hemopoietic cells (square in the top part of panel A), expressing CD45 (B right square), nucleus-restricted NPM1 and C23 (E-F single and double arrows). (A) Hematoxylin-eosin. (B-F) APAAP, hematoxylin counterstaining. Images were collected using an Olympus B61 microscope and a UPlanApo 20×/0.70 (A-B), UPlanApo 40×/0.85 (C-F); Olympus E330-ADU1.2× camera; and Adobe Photoshop 7.0.

Table 4. Heterogeneous patterns of engraftment of CD34− cells from *NPM1*-mutated AML in immunocompromised mice

Patient code	% CD34+ in original sample	Purity of CD34− cells	No./type mice evaluated	No. of cells/ mice, × 10^6	No. of mice engrafted/ no. evaluated	Time of evaluation, w	% hCD45+ in mice BM*	% hCD45+/CD33+ (gated on hCD45)*	Pattern of engraftment†	WB (NPMm)	*NPM1* gene status‡
8R	1.9	98.5	2/NOD	2	2/2	16	36/73‖	97.5/99	2	+	ND
23	1.7	99	1/NOG	0.2	1/1	15	21	0.57	2	+	Mutated
21	0.02	99.99	2/NOG	2	2/2	6	29/7.5¶	13.8/95	4	ND	ND
22§	4.5	99.9	6/NOG	10	6/6	4/5/6/7/8/9	75/72/86‖/82/87#/94‖	82/85/93/90/89/97	3	+	Mutated
27	0.02	99.98	3/NOG	10	3/3	4/6/8	33/64‖/42	73/86/56	3	+	Mutated
28§	0.07	99.98	1/NOG	10	1/1	5	42	46	3	ND	Mutated
28§			3/NOG	2	3/3	6/9/13	4.3/12/5¶	30/35/82	3	ND	Mutated
29§	0.5	99.9	1/NOG	10	1/1	6	14¶	0.7	4	NA	NA
			4/NOG	4	4/4	5/6/7/14	16/ND/2.7/10.7¶	2.2/−/1.7/1	4	NA	NA
			2/NOG	2	2/2	7/14	1.6/16	4/2.2	4	NA	NA
34§	5	96	2/NOG	10	2/2	4	0.61/4.9	−/0.2	4	NA	NA
36	0.26	99.98	3/NOG	10	3/3	6	4.7/2.3/5.2¶	0.3/5.9/9.9	4	NA	NA
37§	0.2	99.8	4/NOG	10	3/4	5/7/8/11	1.8/3.2/0/1.65	95.8/98.7/−/96	4	NA	NA
38§	0.16	99.9	3/NOG	10	3/3	7	0.68/1.2/20¶	1.8/2.5/1	4	NA	NA

BM indicates bone marrow; NOD, NOD/SCID; NOG, NOD/SCID/IL2Rγ^{null}; WB (NPMm), Western blot analysis with anti-NPM mutant specific antibody; R, relapse; ND, not done; and NA, not applicable

*Evaluated by flow cytometry on cells recovered from femurs or tibias.

†See "Patterns of engraftment of CD34− cells from *NPM1*-mutated AML in immunocompromised mice" for description of patterns of engraftment.

‡Evaluated by genomic DNA fragment analysis.

§Lower cell doses did not engraft (supplemental Table 5).

‖Used for serial transplantation experiments.

¶Human cell infiltration of mice BM was much higher when evaluated at immunohistochemistry.
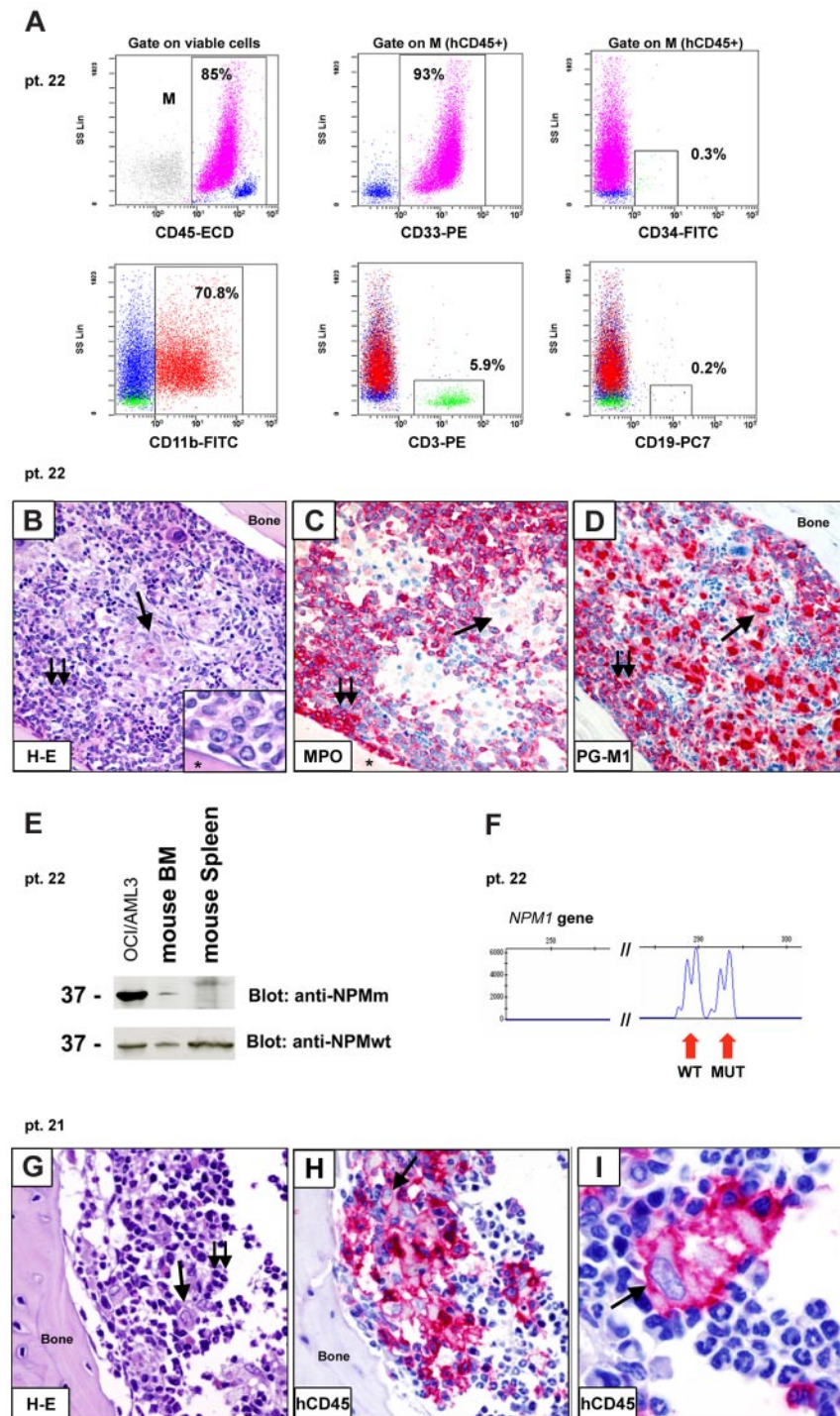
#*FLT3*-ITD-negative.

Figure 6. Patterns of engraftment of CD34⁻ cells from *NPM1*-mutated AML in immunodeficient mice. (A-F) Example of engraftment (pattern 3) in NOG mice inoculated with 10 × 10⁶ CD34⁻ cells (purity 99.9%) from patient 22. (A) Flow cytometric analysis of bone marrow (6 weeks after inoculum) showing engraftment of human myeloid (93% hCD45⁺/CD33⁺) cells, which appeared CD34⁻ (0.3% CD34⁺ cells) and CD11b⁺ (70.8% hCD45⁺/CD11b⁺). A small percentage of T cells (5.9% hCD45⁺/CD3⁺) was also detected. (B-D) Tibia paraffin sections showing massive marrow infiltration by a double population: (1) mononuclear cells close to bone trabeculae (B double arrows; inset, from a different section), which are MPO⁺ and PGM1(CD68)⁺ (C-D double arrows); (2) mature histiocytes located in the central area of bone marrow (B single arrow), which are MPO⁻ and PGM1⁺ (C-D single arrows). Asterisk in panel B inset and in panel C indicates bone area. (E-F) Leukemic origin of these cells was confirmed by WB with anti-NPM mutant (anti-NPMm) antibody (E) and genomic DNA fragment analysis showing double peaks (F). (G-I) Example of engraftment (pattern 4) in NOG mice inoculated 6 weeks before with 2 × 10⁶ CD34⁻ cells (purity 99.99%) isolated from patient 21. Tibia paraffin sections showing marrow infiltration by a mixed population of mature histiocytes (single arrow) and lymphocytes (double arrows). Immunostaining for hCD45 confirmed the human origin of these cells. (B,G) Hematoxylin and eosin. (C-D,H-I) APAAP; hematoxylin counterstaining. Images were collected using an Olympus B61 microscope and a UPlanApo 40×/0.85 (B-D,G-H) and a UPlan FI 100×/1.3 NA oil objective (B inset, I); Olympus E330-ADU1.2× camera; and Adobe Photoshop 7.0.

leukemic cells devoid of self-renewal potential that differentiated into mature elements. Indeed, serial transplantation experiments performed with cells from 2 of 3 cases (Table 3: patients 22 and 27) did not result in further engraftment in secondary recipients.

   Pattern 4. Engraftment of CD34⁻ cells as a mixed population of mature-looking human CD68⁺ histiocytes (Table 4; Figure 6G-I) and CD3⁺ lymphocytes (not shown) in the absence of cells with clear blastic appearance was observed in 6 cases inoculated with more than or equal to 2 × 10⁶ cells. The inability to recover enough histiocytes because of their high cohesivity precluded molecular studies. Thus, the nature (leukemic vs normal) of these cells remains uncertain.

Frequency of LICs in CD34⁺ and CD34⁻ cell fractions in *NPM1*-mutated AML

The presence and frequency of LICs in specific cell fractions were assessed by limiting-dilution transplantation assay in 3 cases (patients 17, 22R, and 34). Results are shown in Table 5.

CD34⁺ cells generate a CD34⁻ *NPM1*-mutated AML in immunocompromised mice

Because down-regulation of CD34 at both RNA[7,8] and protein[1] level is a unique characteristic of *NPM1*-mutated AML, we monitored CD34 expression after engraftment of purified hCD34⁺ leukemic cells in immunocompromised mice.

Table 5. LICs frequency in CD34+ and CD34− cell fractions of *NPM1*-mutated AML

Patient code	Fraction (purity)	Dose, ×10⁶							LIC frequency (95% CI)	LICs/10⁶ MNCs
		2	1	0.5	0.2	0.1	0.01	0.001		
17	CD34+ (98.3%)	—	—	—	3/4	4/4	3/4	0/2	1 in 50 400 (19 967–127 223)	5.4
17	CD34− (90%)	—	—	—	0/3	0/4	0/4	—	—	—
22R	CD34+ (99.9%)	3/3	2/2	—	—	4/4	0/4	—	1 in 41704 (13 820–125 855)	18
34	CD34+ (96.5%)	—	2/3	3/5	4/4	0/4	—	—	1 in 448 907 (214 463–939 640	0.11
34	CD34− (96%)	—	0/4	0/4	0/4	0/4	—	—	—	—

Mice used were NOD/SCID/IL2Rγ^null. Values indicate no. of mice with AML/no. of mice evaluated. Details on immunophenotypic and immunohistochemical analysis of engrafted mice are shown in supplemental Table 4. Time of evaluation (weeks): +17 (patient 17); +10 (patient 22R); +4 (patient 34).

R indicates relapse; and —, not done.

Notably, transplanted hCD34+ cells gave rise to an *NPM1*-mutated AML mainly consisting of CD34− cells. In 6 of 9 evaluable cases (patients 4, 13, 17, 23, 30, and 32, Table 2), a small pool of CD34+ cells similar to that observed in the original patient's sample was also detected. In 3 cases (patients 10, 11, and 22R, Table 2), the percentage of CD34+ leukemic cells (and the proportion of CD34+/CD38− and CD34+/CD38+ subsets) in engrafted tumors was even lower that that detected in the original patient's sample, probably reflecting the different influence of mice microenvironment in maintaining stem cell phenotype and pool size. As an example, the bone marrow sample from patient 10 (Table 2) at diagnosis contained 27% AML cells expressing CD34 at low intensity (Figure 7A), whereas only 1.9% of hCD45+/CD33+ leukemic cells engrafted in mice (16 weeks after the inoculum) expressed CD34 (Table 2; Figure 7B). Thus, most of mice leukemic bulk consisted of *NPM1*-mutated (Figure 7C), CD34− cells expressing cytoplasmic NPM1 (Figure 7D-F). Interestingly, in some mice, leukemic cells showing cytoplasmic (dot-like) CD34 were seen (supplemental Figure 4).

In patients 17 and 22R, we were able to track CD34 expression in engrafted leukemia at different time points. Interestingly, starting from CD34+ cells with a purity of 98.3% and 99.9%, respectively, we observed a progressive reduction of the percentage of CD34+ cells within the leukemic bulk with time (eg, patient 17, from 52% at 9 weeks to 35% at 12 weeks and 14% at 20 weeks; Figure 5G-I; Table 2) and outgrowth of leukemia from 15.8% to 52% and 80% at correspondent time points (Table 2).

Our findings suggest the CD34− phenotype of *NPM1*-mutated AML developing in mice may be the result of CD34 down-regulation.

Discussion

Most *NPM1*-mutated AML patients are CD34−.[1] Here, we demonstrate that the small fraction of CD34+ cells in *NPM1*-mutated AML expresses CD34 at low intensity, exhibits variable expression of CD38, carries the mutated NPM1 gene/protein, and shows aberrant NPM1 cytoplasmic expression.

As the mutated NPM1 gene and protein were detected in the CD34+/CD38− and CD34+/CD38+ subsets, they both belong to the leukemic clone. CD34+/CD38− cells from *NPM1*-mutated AML usually displayed LSC immunophenotypic features (ie, CD123 and CD33 expression[29,30] and absence of CD90[31]). The CD34+ cell fraction consistently engrafted in immunocompromised mice as AML exhibiting the same characteristics as the patient's primary leukemic cells (mutated/cytoplasmic NPM1 and

CD34 negativity), suggesting it contains the LICs (or LSCs), which characteristically recapitulate human AML in mice.[39] Moreover, as recently reported by Taussig et al,[21] we found that also CD34− cells from a few *NPM1*-mutated AML patients had significant LIC potential in immunocompromised mice.

Definitive evidence that CD34+ cells belong to the leukemic clone and contain the LICs mainly apply to *NPM1*-mutated AML cases containing more than 1% of CD34+ cells. In contrast, the nature of CD34+ cells from our *NPM1*-mutated AML patients, when they represented less than 1% of MNCs (supplemental Table 1), remains controversial. Indeed, the CD34+/CD38− cell subpopulation from these cases contained a variable percentage of CD34+ cells with normal immunophenotypic features (supplemental Table 2), a finding that is in keeping with previous reports.[32,40] In 3 of such patients (not shown), we detected NPM1-mutated protein by WB analysis of purified CD34+ cells, suggesting that the majority of cells may have belonged to the leukemic clone. However, definitive conclusions on this issue can only be drawn from the study of additional cases, possibly searching for the *NPM1* mutant by single-cell polymerase chain reaction analysis.

Our findings that in most cases CD34+, but not CD34−, cells generated *NPM1*-mutated AML in immunocompromised mice are consistent with previous observations in other AML subtypes where the capability to engraft was shown to be mainly related to the CD34+/CD38− fraction.[14] Moreover, engraftment of *NPM1*-mutated AML cells occurred preferentially within the bone marrow endosteal region, where primitive CD34+/CD38− AML cells have been described to home in NOG mice.[41] Interestingly, the bone marrow endosteal region was identified as the microenvironmental niche for human primary LSCs[42] which, during engraftment, appear to compete with normal hemopoietic stem cells for this area.[41] Finally, if the small pool of CD34+ cells in *NPM1*-mutated AML were of leukemic origin, a clonal outgrowth of them might be expected at relapse. Notably, in 3 *NPM1*-mutated AML patients studied at diagnosis and relapse, we found a 6.8-, 15.8-, and 16.7-fold increase in CD34+ cell percentage at relapse, respectively (Table 1; supplemental Table 1, patients 2, 8, and 22). Increases in CD34+ cells at relapse have been reported in other AML subtypes.[43]

In immunocompromised mice, CD34+ cells from *NPM1*-mutated AML usually generated a leukemic outgrowth similar to the original patient's disease (ie, mainly CD34− cells with few CD34+ leukemic cells). It appears improbable that this small CD34+ cell pool derived from contaminating CD34− leukemic cells, as described for normal hemopoietic stem cells.[44] In fact, flow cytometric studies at different time points showed progressive
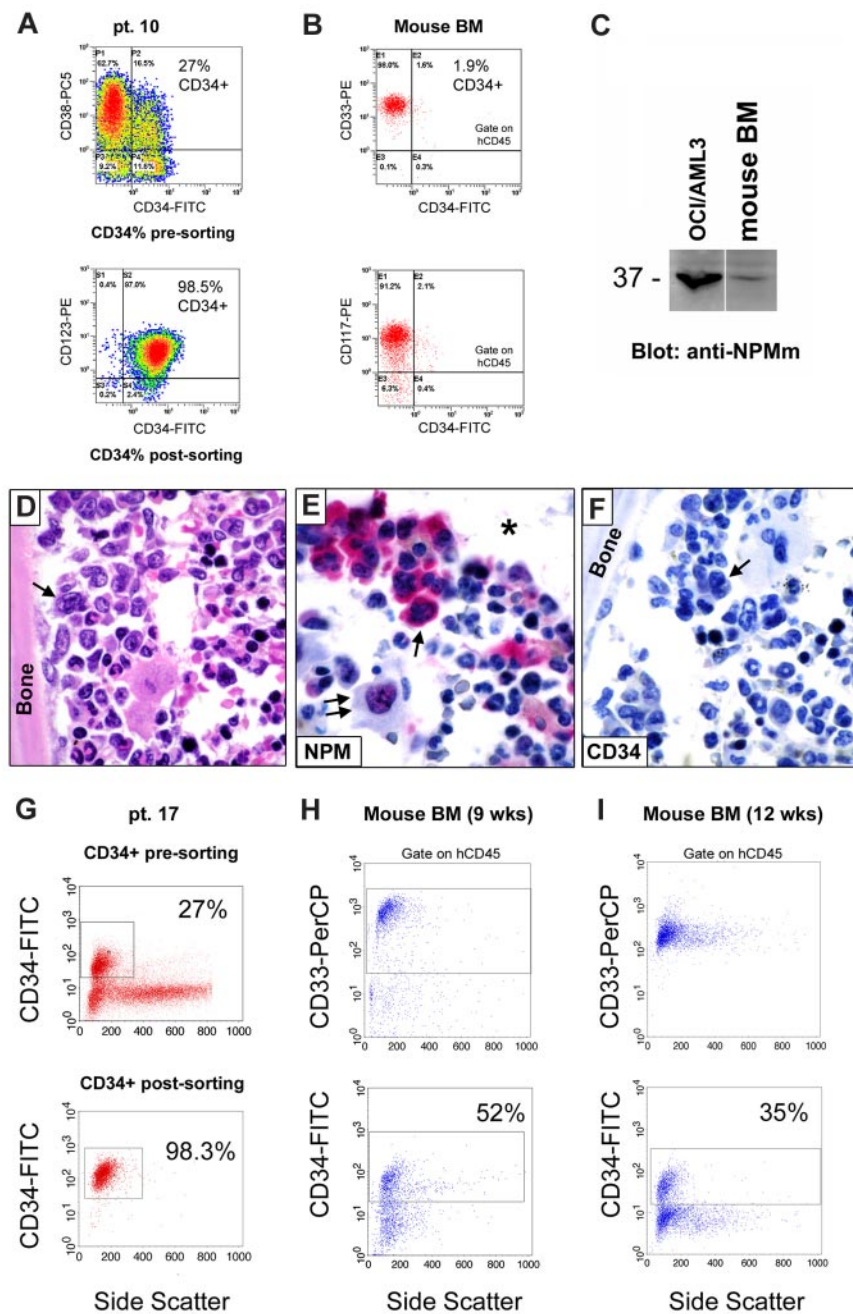
Figure 7. CD34+ cells from *NPM1*-mutated AML generate CD34− NPMc+ AML in mice. (A-F) Flow cytometry and immunohistochemistry analysis of CD34 antigen expression in leukemia developed in immunodeficient mice (NOD/SCID) inoculated with CD34+ cells (purity 98.5%) isolated from 1 patient (patient 10) with *NPM1*-mutated AML (27% CD34+ cells in the original sample; A). Flow cytometric analysis of mice bone marrow (BM; 16 weeks after the inoculum) showing engraftment of myeloid (CD33+ and CD117+) human cells, which appeared mainly CD34− (1.9% CD34+ cells; B). The leukemic nature of these cells was confirmed by WB with anti-NPM mutant antibody (C), as well as morphologic analysis (D hematoxylin and eosin) and expression of nucleophosmin (NPM) in the cytoplasm of human leukemic cells on bone marrow paraffin sections (E, arrow). As staining control, NPM was characteristically nucleus-restricted in normal murine cells (E, double arrows). The asterisk in (E) indicates an empty space originally filled by bone. CD34 immunostaining was negative in leukemic cells (F arrow). (E-F: APAAP; hematoxylin counterstaining). All images were collected using an Olympus B61 microscope and a UPlan Fl 100×/1.3 NA oil objective; Camedia 4040, Dp_soft Version 3.2; and Adobe Photoshop 7.0. A vertical line has been inserted in panel C to indicate a repositioned gel lane. (G-I) Serial flow cytometric evaluation of CD34 antigen expression in human leukemic cells grown in immunodeficient mice (NOD/SCID/IL2Rγ^null) killed at 9 (H) and 12 (I) weeks after injection of the same number of CD34+ cells (2 × 10^6, purity 98.3%) isolated from 1 patient (patient 17) with *NPM1*-mutated AML (27% CD34+ cells in the original sample; G).

CD34 down-regulation and retention of a small pool of CD34+ leukemic cells that were consistent with a derivation of the leukemic population from CD34+ engrafted cells. Moreover, immunohistochemistry frequently showed dot-like CD34 positivity in cytoplasm, which could reflect CD34 internalization and down-regulation. Further studies are required to clarify the molecular mechanisms of CD34 down-regulation in *NPM1*-mutated AML.

In normal hemopoiesis, a cell compartment devoid of lineage markers and CD34 antigen with SCID-repopulating ability was isolated,[13] where leukemic transformation could conceivably occur.[37,38] Recently, Taussig et al[21] reported that, in *NPM1*-mutated AML, the LICs were found only in the CD34− fraction or in both the CD34+ and CD34− subpopulations, suggesting that the phenotype of LICs is more heterogeneous than previously realized.

Our results that CD34− cells from a few *NPM1*-mutated AML patients showed significant LIC potential in immunocompromised mice is in keeping with the findings by Taussig et al.[21] Moreover, use in combination of flow cytometry, histology, and immunohistochemistry showed that leukemic engraftment of CD34− fraction was more heterogeneous than that observed with the CD34+ fraction, giving rise to different patterns. Rarely, CD34− cells generated an AML with the same morphologic and immunohistologic features (cytoplasmic NPM1) as in patients' bone marrow trephines and in mice injected with *NPM1*-mutated CD34+ cells. More frequently, CD34− cells engrafted as leukemia showing more differentiated morphologic and immunohistologic features. We hypothesize that the latter pattern might be ascribed to a limited proliferative ability of CD34− cells, which allows them to expand in mice, although with a more limited engraftment potential than CD34+ cells. Further studies are needed to address this issue.

In conclusion, in our series of patients, both CD34+ and, at lower extent, CD34− cells exhibited LIC activity. Whether LSCs

in *NPM1*-mutated AML originate from very early progenitors or committed myeloid precursors[45] remains to be elucidated. Our studies have biologic and potential clinical implications. The finding that CD34+/CD38− cells from *NPM1*-mutated AML may harbor the same genetic lesion as the CD34− tumor bulk population adds to the evidence that the *NPM1* mutation is a founder genetic lesion defining a new leukemia entity. This evidence includes: (1) specificity of *NPM1* mutation for AML among human tumors[1]; (2) mutual exclusion of *NPM1* mutation with other AML recurrent cytogenetic abnormalities[46]; (3) secondary nature of chromosomal aberrations in 15% of *NPM1*-mutated AML[47]; (4) association of *NPM1*-mutated AML with distinctive gene expression[7,8] and microRNA profiles[4]; and (5) results of whole genomic sequencing in AML with normal karyotype.[48,49]

As LSCs from *NPM1*-mutated AML strongly express CD33 and CD123, immunotherapy with CD33 and/or CD123[50] targeting drugs combined with chemotherapy is an attractive strategy. Development of novel therapeutic approaches is important because, although *NPM1*-mutated without *FLT3*-ITD is usually characterized by a favorable prognosis,[5] a significant number of patients with *NPM1*-mutated AML still die of their disease.

Acknowledgments

Authorship

Contribution: M.P.M. and B.F. had the original idea for the study, designed experiments, and wrote the paper; V.P. designed and performed experiments, analyzed data, and wrote the paper; C.T. and U.O. performed FACS experiments, flow cytometry, and molecular analyses, analyzed data, and contributed to the writing of the manuscript; F.M. performed mice experiments; E.B., I.G., D.C., and L.B. performed immunophenotypic analysis; M.G. and L.D.V. performed FACS experiments; F.F., M.D.I., and R.C. were responsible for molecular diagnostics and analysis; N.M., R.R., and L.G. processed patient samples and performed WB studies; A.L., R.P., and A.T. performed immunohistochemical studies; and M.D., G.S., F.D.R., and M.F.M. provided patient samples and clinical information and contributed to discussion of data.

Conflict-of-interest disclosure: B.F. applied for a patent on clinical use of NPM1 mutants. The remaining authors declare no competing financial interests.

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References

1. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med.* 2005; 352(3):254-266.

2. Falini B, Sportoletti P, Martelli MP. Acute myeloid leukemia with mutated NPM1: diagnosis prognosis and therapeutic perspectives. *Curr Opin Oncol.* 2009;21(6):573-581.

3. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. *Blood.* 2007;109(3):874-885.

4. Garzon R, Garofalo M, Martelli MP, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A.* 2008;105(10):3945-3950.

5. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med.* 2008; 358(18):1909-1918.

6. Arber D, Brunning RD, Le Beau MM, et al. Acute myeloid leukaemia with recurrent genetic abnormalities. In: Swerdlow SE, Campo E, Harris NL, et al, eds. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.* Lyon, France: International Agency for Research on Cancer; 2008:110-123.

7. Alcalay M, Tiacci E, Bergomas R, et al. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood.* 2005;106(3):899-902.

8. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their fa-

vorable prognostic significance. *Blood.* 2005; 106(12):3747-3754.

9. Magli MC, Barba P, Celetti A, De Vita G, Cillo C, Boncinelli E. Coordinate regulation of HOX genes in human hematopoietic cells. *Proc Natl Acad Sci U S A.* 1991;88(14):6348-6352.

10. Pasqualucci L, Liso A, Martelli MP, et al. Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: impact on WHO classification. *Blood.* 2006;108(13): 4146-4155.

11. Martelli MP, Manes N, Pettirossi V, et al. Absence of nucleophosmin leukaemic mutants in B and T cells from AML with NPM1 mutations: implications for the cell of origin of NPMc+ AML. *Leukemia.* 2008;22(1):195-198.

12. Engelhardt M, Lubbert M, Guo Y. CD34(+) or CD34(-): which is the more primitive? *Leukemia.* 2002;16(9):1603-1608.

13. Bhatia M, Bonnet D, Murdoch B, Gan OI, Dick JE. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med.* 1998;4(9):1038-1045.

14. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature.* 1994; 367(6464):645-648.

15. Warner JK, Wang JC, Hope KJ, Jin L, Dick JE. Concepts of human leukemic development. *Oncogene.* 2004;23(43):7164-7177.

16. Pearce DJ, Taussig D, Zibara K, et al. AML engraftment in the NOD/SCID assay reflects the outcome of AML: implications for our understanding of the heterogeneity of AML. *Blood.* 2006; 107(3):1166-1173.

17. Haase D, Feuring-Buske M, Konemann S, et al. Evidence for malignant transformation in acute myeloid leukemia at the level of early hematopoi-

etic stem cells by cytogenetic analysis of CD34+ subpopulations. *Blood.* 1995;86(8):2906-2912.

18. Mehrotra B, George TI, Kavanau K, et al. Cytogenetically aberrant cells in the stem cell compartment (CD34+lin−) in acute myeloid leukemia. *Blood.* 1995;86(3):1139-1147.

19. Turhan AG, Lemoine FM, Debert C, et al. Highly purified primitive hematopoietic stem cells are PML-RARA negative and generate nonclonal progenitors in acute promyelocytic leukemia. *Blood.* 1995;85(8):2154-2161.

20. Guibal FC, Alberich-Jorda M, Hirai H, et al. Identification of a myeloid committed progenitor as the cancer-initiating cell in acute promyelocytic leukemia. *Blood.* 2009;114(27):5415-5425.

21. Taussig DC, Vargaftig J, Miraki-Moud F, et al. Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood.* 2010; 115(10):1976-1984.

22. Falini B, Martelli MP, Bolli N, et al. Immunohistochemistry predicts nucleophosmin (NPM) mutations in acute myeloid leukemia. *Blood.* 2006; 108(6):1999-2005.

23. Oelschlaegel U, Koch S, Schaich M, et al. Rapid flow cytometric detection of aberrant cytoplasmic localization of nucleophosmin (NPMc) indicating mutant NPM1 gene in acute myeloid lekemia. *Leukemia.* 24(10):1813-1816.

24. Martelli MP, Manes N, Liso A, et al. A western blot assay for detecting mutant nucleophosmin (NPM1) proteins in acute myeloid leukaemia. *Leukemia.* 2008;22(12):2285-2288.

25. Parks DR, Roederer M, Moore WA. A new "Logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. *Cytometry A.* 2006;69(6):541-551.

26. Quentmeier H, Martelli MP, Dirks WG, et al. Cell

line OCI/AML3 bears exon-12 NPM gene muta-tion-A and cytoplasmic expression of nucleo-phosmin. *Leukemia.* 2005;19(10):1760-1767.

27. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood.* 2006;107(10):4011-4020.

28. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipi-ent mouse model for engraftment of human cells. *Blood.* 2002;100(9):3175-3182.

29. Hauswirth AW, Florian S, Printz D, et al. Expres-sion of the target receptor CD33 in CD34+/CD38−/CD123+ AML stem cells. *Eur J Clin Invest.* 2007;37(1):73-82.

30. Taussig DC, Pearce DJ, Simpson C, et al. Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood.* 2005;106(13):4086-4092.

31. Blair A, Hogge DE, Ailles LE, Lansdorp PM, Sutherland HJ. Lack of expression of Thy-1 (CD90) on acute myeloid leukemia cells with long-term proliferative ability in vitro and in vivo. *Blood.* 1997;89(9):3104-3112.

32. Pearce DJ, Taussig D, Simpson C, et al. Charac-terization of cells with a high aldehyde dehydro-genase activity from cord blood and acute my-eloid leukemia samples. *Stem Cells.* 2005;23(6):752-760.

33. Falini B, Bolli N, Shan J, et al. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleo-phosmin leukemic mutants in NPMc+ AML. *Blood.* 2006;107(11):4514-4523.

34. Bolli N, Nicoletti I, De Marco MF, et al. Born to be exported: COOH-terminal nuclear export signals of different strength ensure cytoplasmic accu-mulation of nucleophosmin leukemic mutants. *Cancer Res.* 2007;67(13):6230-6237.

35. Bolli N, De Marco MF, Martelli MP, et al. A dose-dependent tug of war involving the NPM1 leukae-mic mutant, nucleophosmin, and ARF. *Leukemia.* 2009;23(3):501-509.

36. Falini B, Bolli N, Liso A, et al. Altered nucleophos-min transport in acute myeloid leukaemia with mutated NPM1: molecular basis and clinical implications. *Leukemia.* 2009;23(10):1731-1743.

37. Terpstra W, Prins A, Ploemacher RE, et al. Long-term leukemia-initiating capacity of a CD34− subpopulation of acute myeloid leukemia. *Blood.* 1996;87(6):2187-2194.

38. Sutherland HJ, Blair A, Zapf RW. Character-ization of a hierarchy in human acute myeloid leukemia progenitor cells. *Blood.* 1996;87(11):4754-4761.

39. Bonnet D, Dick JE. Human acute myeloid leuke-mia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997;3(7):730-737.

40. van der Pol MA, Feller N, Roseboom M, et al. Assessment of the normal or leukemic nature of CD34+ cells in acute myeloid leukemia with low percentages of CD34 cells. *Haematologica.* 2003;88(9):983-993.

41. Ishikawa F, Yoshida S, Saito Y, et al. Chemo-therapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol.* 2007;25(11):1315-1321.

42. Lapidot T, Goichberg P, Lapid K, Avigdor A, Kollet O. The endosteum region keeps human leukemic stem cells alive. *Cell Stem Cell.* 2007;1(5):483-484.

43. Baer MR, Stewart CC, Dodge RK, et al. High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection (Cancer and Leu-kemia Group B Study 8361). *Blood.* 2001;97(11):3574-3580.

44. Nakamura Y, Ando K, Chargui J, et al. Ex vivo generation of CD34(+) cells from CD34(−) he-matopoietic cells. *Blood.* 1999;94(12):4053-4059.

45. Krivtsov AV, Twomey D, Feng Z, et al. Transfor-mation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature.* 2006;442(7104):818-822.

46. Falini B, Mecucci C, Saglio G, et al. NPM1 muta-tions and cytoplasmic nucleophosmin are mutu-ally exclusive of recurrent genetic abnormalities: a comparative analysis of 2562 patients with acute myeloid leukemia. *Haematologica.* 2008;93(3):439-442.

47. Haferlach C, Mecucci C, Schnittger S, et al. AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biologic, pathologic, immunophenotypic, and prognostic features. *Blood.* 2009;114(14):3024-3032.

48. Ley TJ, Mardis ER, Ding L, et al. DNA sequencing of a cytogenetically normal acute myeloid leukae-mia genome. *Nature.* 2008;456(7218):66-72.

49. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med.* 2009;361(11):1058-1066.

50. Jin L, Lee EM, Ramshaw HS, et al. Monoclonal antibody-mediated targeting of CD123, IL-3 re-ceptor alpha chain, eliminates human acute my-eloid leukemic stem cells. *Cell Stem Cell.* 2009;5(1):31-42.