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

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ABSTRACT

Acute myeloid leukemia (AML) with mutated NPM1 shows distinctive biological 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 <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+ sub-fraction 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 the CD34+ fraction in NPM1-mutated AML belongs to the leukemic clone and contains NPM1-mutated cells exhibiting properties typical of leukemia-initiating cells (LICs). CD34- cells from few cases (2/15) also showed significant LIC 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.
INTRODUCTION

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

Its unique gene expression profile (GEP) is characterized by upregulation of most *HOX* genes and downregulation of CD34 and CD133.\(^7\)\(^,\)\(^8\) Since *HOX* genes are involved in stem-cell phenotype maintenance\(^9\), GEP findings strongly suggest *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 downregulation 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 to prognosis.\(^16\)

CD34+/CD38- hematopoietic stem cells 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\textsuperscript{17,18}, but not in acute promyelocytic leukemia which may derive from a more mature hemopoietic progenitor.\textsuperscript{19,20}

Until the discovery of \textit{NPM1} mutation\textsuperscript{1}, the genetic and functional characterization of CD34+/CD38- cells in AML with normal karyotype was difficult due to the lack of reliable molecular markers. Molecular and/or immunohistochemical detection of \textit{NPM1} mutations allow to track the genetic lesion in leukemic cells at different hierarchical stages in about 60\% of AML with normal cytogenetics.

Aims of this study were: \textit{i}) to search for NPM1 mutated gene and/or protein in CD34+ cells (including CD38- and CD38+ subsets) purified from \textit{NPM1}-mutated AML patients; \textit{ii}) to determine by immunohistochemistry and/or flow cytometry whether these CD34+ cells carried aberrant cytoplasmic NPM (a distinctive functional feature of \textit{NPM1}-mutated AML); \textit{iii}) to investigate the capability of purified CD34+ and CD34- cells from \textit{NPM1}-mutated AML to engraft in immunocompromised mice and to evaluate the nature and topographical distribution of engrafted cells; and \textit{iv}) to compare the morphological, immunophenotypic and molecular features of murine-engrafted and patients’ primary AML cells.

We proved that the minor population of CD34+ hemopoietic progenitors in \textit{NPM1}-mutated AML consistently carried the \textit{NPM1} mutation, at least when CD34+ cells represented >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.\textsuperscript{21}, we also found that CD34- cells from a few \textit{NPM1}-mutated AML patients had significant LIC potential in immunocompromised mice.
MATERIALS AND 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). Patients were from the Institutes of Hematology of 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.\textsuperscript{22,23} Mutated NPM1 protein and/or gene was also confirmed by Western blotting (WB)\textsuperscript{24} and/or mutational analysis.\textsuperscript{1} The study was approved by the University of Perugia ethical committees and written informed consent for analysis of leukemic samples was obtained at each participating center in accordance with the Declaration of Helsinki.

Flow cytometric immunophenotyping

Immunophenotyping was performed using the following antibodies: peridinin chlorophyll protein complex (PerCP)–conjugated anti-CD45 (CD45-PerCP), fluorescein isothiocyanate (FITC)–conjugated anti-CD34 (CD34-FITC), phycoerythrin (PE)–conjugated or allophycocyanin (APC)–conjugated anti-CD38 (CD38-PE, CD38-APC), CD33-APC and CD123-PE (Becton Dickinson, San Jose, CA, USA) or phycoerythrin-Texas Red (ECD)–conjugated anti-CD45 (CD45-ECD), CD34-FITC, phycoerythrin-cyanin 5 (PC5)–conjugated anti-CD45 (CD45-PC5), CD33-PE, phycoerythrin-cyanin 7 (PC7)–conjugated anti-CD19 (CD19-PC7), CD11b-FITC, CD90-FITC, CD90-PC5 (Beckman Coulter Inc., Fullerton, CA, USA). Cytoplasmic NPM1 in leukemic cells was detected by flow cytometry as previously described.\textsuperscript{23} Analysis was performed on either a Cytomics FC500 cytometer equipped with the CXP analysis software (Beckman Coulter Inc.) or a fluorescence
activated cell sorting (FACS)-Calibur or FACSAria flow cytometers using the CellQuest Pro analysis software (Becton Dickinson). Gates were drawn to exclude nonviable cells and debris.

**MACS cell sorting**

All leukemia samples were subjected to CD34+ cell selection by MACS cell sorting technology, according to manufacturer’s instruction (CD34 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany). Both positive and negative cell fractions were analyzed for CD34+ cells percentage.

**FACS cell sorting**

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 (Becton Dickinson) equipped with blue, red, and violet lasers. Dead cells were excluded by analyzing forward scatter (FSC) vs side scatter (SSC) dot plots. Doublets were excluded by FSC-H vs FSC-A dot plots. Data compensation and analysis were obtained using the “logicle” display method.

**Detection of NPM1 mutated protein and gene**

NPM1 mutant protein was detected on lysates from 1-2 x 10^6 cells by WB analysis with a rabbit polyclonal antibody specific for the mutated NPM1 protein. Lysate from the human leukemic cell line OCI/AML3 was used as positive control for NPM1 mutant A protein expression. In selected cases, NPM1 mutations were analyzed by either direct sequencing or genomic DNA fragments analysis.
Immunohistochemical studies

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

Cytoplasmic nucleophosmin was revealed using a mouse anti-NPM monoclonal antibody (mAb).\textsuperscript{1} Other antigens included: nucleolin (C23) (mAb MS-3, Santa Cruz Biotechnology, Santa Cruz, CA); myeloperoxidase (rabbit anti-myeloperoxidase antibody, Dakocytomation, Glostrup, Denmark); macrophage-restricted CD68 (mouse mAb PG-M1 generated by B.F.); CD20 (mouse mAb, L26, Dakocytomation); CD3 (rabbit mAb, SP7, ThermoScientific, Fremont, CA); CD45 and glycophorin (Dakocytomation). The antibody:antigen interaction was revealed by the immuno-alkaline phosphatase (APAAP) technique.\textsuperscript{1}

Double stainings for CD34/NPM and CD34/C23 were performed using a sequential immunoperoxidase/alkaline phosphatase (APAAP) procedure.\textsuperscript{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 non-obese diabetic/severe combined immunodeficient (NOD/SCID) or NOD/SCID/IL2rγnull (NOG)\textsuperscript{28} mice. Mice were originally obtained from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME). Mouse colonies were maintained in the certified Animal Facility of University of Perugia, Perugia, Italy, in accordance with national guidelines. They were kept in microisolators cages and fed sterile food and acidified water, containing 100 μg/ml of ciprofloxacin. Mice aged 6-10 weeks were irradiated at 3.5 Gy γ-irradiation up to 24 hours before intravenous (tail vein)
injection of cells. Mice were sacrificed at 3 to 21 weeks post-transplant, bone marrow was removed from one of femurs and tibias by flushing with phosphate-buffer-saline and analyzed for engraftment 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, 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.

**Colony-Forming Cell (CFC) assay**

Ten to 50 x 10^3 cells from the CD34+ or CD34- fractions of pts. 17, 32, and 34 were plated in triplicate in 1 ml of MethoCult GF H4434 (StemCell Technologies, Vancouver, BC, Canada) in 35 mm tissue culture dishes. Colonies scoring was performed after 14 days of culture. Colonies were analyzed for \textit{NPM1} mutation by genomic DNA fragment analysis.

**Statistics**

Leukemia-initiating cells frequency in CD34+ and CD34- cells from \textit{NPM1}-mutated AML was calculated in limiting-dilution experiments using the StemSoft’s L-Calc software (StemCell Technologies).
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 <10% CD34+ cells (Table 1; supplemental Table 1). Notably, 18 of 41 samples expressed <1% CD34+ cells (supplemental Table 1). Mean fluorescence intensity (MFI) 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 whilst in 3 cases (pts. 1, 4 and 22R) the CD34+ cells ranged between 20 and 60% (Table 1). The lower percentage of CD34+ cells detectable by immunohistochemistry as compared to flow cytometry is likely due to the lower sensitivity of immunohistochemistry for detecting low intensity CD34 expression.

CD34+ cells were purified by MACS (39/41 samples) or FACS (2/41 samples) (Table 1; supplemental Table 1). MACS-sorting approach was chosen since allows to reliably recover 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 MFI for CD34 as the CD34+ cells in the original sample, with either 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 x 10^6 cells) from 13 of 41 samples (CD34+ cells ranging from 1.3 to 60% of 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 due to contaminating CD34- leukemic cells since in 12 of 13 samples CD34+ cells accounted for >90% of the total analyzed population (2 x 10^6 cells) whilst our WB detection method is not sensitive enough to reveal NPM1 mutant protein from cells that accounts for ≤10% (Figure 1B). Moreover, unlike that observed in AML cells dilution assay (Figure 1B), the WB signal intensity was not decreased in the CD34+ purified cells as compared to leukemic bulk (Figure 1A). Thus, it is conceivable that even in the case with lower CD34+ cell purity (pt. 30, 79% CD34+ cell purity) (Table 1), positive signal could not be ascribed to contaminating CD34- cells.

In 1 leukopenic patient (pt. 19), only 0.1 x 10^6 CD34+ bone marrow cells (99.7% pure) could be recovered, that were not enough for WB analysis. In this case, NPM1 mutation A was detected by direct gene sequencing in purified CD34+, CD34- and pre-sorting cell populations (Figure 1C). Again, detection of NPM1 mutation in the CD34+ cells could not be due to the minority of contaminating CD34- cells (0.3%), since the latter was under the detection threshold (about 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 >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, in order 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: pts. 2, 18, 22, 25, 26) are shown in Figure 2A.

In 2 of 5 cases (Table 1: pts. 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 (pts. 22, 25, 26) the FACS-sorted CD34+/CD38-subpopulation (purity of 96%, 99% and 99%, respectively) was NPM1-mutated by either direct gene sequencing (pt. 22, harboring NPM1 mutation 79) or genomic DNA fragment analysis (pts. 25 and 26). The finding of NPM1 mutation in these samples could not be due to the small number of contaminating CD34- cells (range 1%-4%) since this is below the detection threshold of our molecular assays (Supplemental Figure 1). A representative case (pt. 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 depicted in Figure 2C and D. In this case, the CD34+/CD38- cells also carried FLT3-ITD mutation (Figure 2C, panel a), further confirming they belong to the leukemic clone. FLT3-ITD showed the highest prevalence in the CD34+/CD38- cells (Figure 2C, panel a), whereas, in the more differentiated cells (CD34+/CD38+), the FLT3-ITD mut/wt ratio was significantly lower.
(Figure 2C, panel b) and, in the bulk leukemic population, FLT3-ITD was only present in a small fraction of cells (Figure 2C, panel c).

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-) 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 (e.g., pt. 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), whilst CD34 bright cells were negative for both antigens, likely representing residual circulating normal CD34+ hematopoietic stem cells (HSCs). The latter have been previously described in some AML patients at diagnosis.

Our results clearly indicate that CD34+/CD38- cells from NPM1-mutated AML show the genotypic and phenotypic features of LSCs (i.e., NPM1 mutated gene/protein and expression of CD123 and CD33). In some cases, a variable fraction of CD34 bright cells were 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.
To address this issue, we double-stained for CD34 and NPM bone marrow paraffin sections from 5 \textit{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 3 A-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 3 E, 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 \textit{NPM1}.\textsuperscript{33-36}

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

Isolated CD34+ cells from \textit{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 and 4), as documented by positivity for hCD45 (at flow cytometry and immunohistochemistry). The leukemic nature of hCD45+ engrafted cells was strongly suggested by the combined morphological and immunohistochemical analysis of bone marrow sections that showed 3 main infiltration patterns: i) sparse aggregates of human leukemic cells mainly adjacent to the bone (suggestive of initial marrow involvement) (supplemental Figure 2); ii) leukemic infiltration of marrow areas near to and distant from the bone (the most common pattern) (Figure 4); and iii) massive marrow infiltration by leukemic cells (supplemental Figure 3).
The leukemic nature of all hCD45+ engrafted cells was supported in 7 of 10 cases where the morphology and immunophenotype of engrafted cells paralleled that of patient’s primary AML (Table 2; supplemental Table 4). In these cases, all engrafted cells were myeloid (hCD45+/CD33+ and myeloperoxidase-positive) (Figure 4 B, E, F). In contrast, no hCD45+ cells co-expressing 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 and D), the hallmark feature of human NPM1-mutated AML cells. Moreover, in 5 of 7 cases (pts. 4, 10, 17, 22R, 23), the WB and/or molecular analysis showed NPM1 mutant protein and/or gene expression (Figure 4G; Table 2 and supplemental Table 4). Although CD34+ cells from one patient (pt. 17) did not show ability to produce AML colonies in in vitro CFCs assay (data not shown), cells recovered from primary mice recipients demonstrated self-renewal capacity in serial transplantations (Table 3: pts. 4, 10, 11, 17, 22R).

Interestingly, in 3 of 10 cases (pts. 30, 32, 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 tri-lineage 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 pts. 30, 32 and 34 showed a relevant subpopulation of CD34bright/CD38-/CD123-/CD33- cells which are likely to represent normal HSCs (Figure 2E; supplemental Table 2). Furthermore, in CFCs assay, CD34+ cells from 2 of these
cases (pts. 32, 34) produced mixed and erythroid colonies which, because of germ-line $NPM1$ gene (data not shown), are normal in origin.

Our findings clearly indicate the small fraction of CD34+ cells from most $NPM1$-mutated AML cases generate a leukemia showing the same morphological 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**

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\textsuperscript{37,38} (including $NPM1$-mutated AML\textsuperscript{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 $\leq 1 \times 10^6$ (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: pts. 17, 30, 32, 34; supplemental Table 4). Moreover, CD34- cells from pts. 17, 32 and 34 did not outgrow into AML colonies in CFCs 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: pts. 8R, 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 x 10⁶ cells) from 3 cases (Table 4: pts. 22, 27, 28) resulted into marrow engraftment by hCD45+/hCD33+ cells (Figure 6A) which, in tissue sections, consisted of 2 populations (Figure 6 B-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 leukemic cells devoid of self-renewal potential that differentiated into mature elements. Indeed, serial transplantations experiments performed with cells from 2 of 3 cases (Table 3: pts. 22, 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 6 G-I) and CD3+ lymphocytes (not shown) in the absence of cells with clear blastic appearance was observed in 6 cases inoculated with ≥ 2 x 10⁶ cells. 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 was assessed by limiting-dilution transplantation assay in 3 cases (pts. 17, 22R, 34). Results are shown in Table 5.
CD34+ cells generate a CD34- NPM1-mutated AML in immunocompromised mice

Since downregulation of CD34 at both RNA and protein level is a unique characteristic of NPM1-mutated AML, we monitored CD34 expression after engraftment of purified hCD34+ leukemic cells in immunocompromised mice.

Notably, transplanted hCD34+ cells gave rise to an NPM1-mutated AML mainly consisting of CD34- cells. In 6 of 9 evaluable cases (Table 2: pts. 4, 13, 17, 23, 30, 32), a small pool of CD34+ cells similar to that observed in the original patient’s sample was also detected. In 3 cases (Table 2: pts 10, 11, 22R) 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 pt. 10 (Table 2) at diagnosis contained 27% AML cells expressing CD34 at low intensity (Figure 7A), whilst only 1.9% 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 7, D-F). Interestingly, in some mice leukemic cells showing cytoplasmic (dot-like) CD34 were seen (Supplemental Figure 4).

In pts. 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 (e.g. pt 17, from 52% at 9 weeks to 35% at 12 weeks and 14% at 20 weeks) (Figure 5 G-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 due to CD34 downregulation.
DISCUSSION

Most NPM1-mutated AML patients are CD34-negative.\textsuperscript{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, i.e. CD123 and CD33 expression\textsuperscript{29,30} and absence of CD90.\textsuperscript{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.\textsuperscript{39} Moreover, as recently reported by Taussig et al.\textsuperscript{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 >1% of CD34+ cells. In contrast, the nature of CD34+ cells from our NPM1-mutated AML patients, when they represented <1% of MNCs (supplemental Table 1), remains controversial. In fact, 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.\textsuperscript{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 PCR analysis.
The extensive control experiments we carried out in this study clearly demonstrate that our finding of NPM1-mutated gene and protein in CD34+ cell from NPM1-mutated AML is not due to contaminating CD34- cells.

Our findings that in most cases CD34+ but not CD34- cells generated NPM1-mutated AML in immunocompromised mice is consistent with previous observations in other AML subtypes where capability to engraft was shown to be mainly related to the CD34+/CD38- fraction. Moreover, engraftment of NPM1-mutated AML cells occurred preferentially in proximity to bone-marrow osteoblast-rich areas, where primitive CD34+/CD38- AML cells have been described to home in NOG mice. Interestingly, the bone marrow endosteal region was recently identified as the microenvironmental niche for human primary LSCs which, during engraftment, appear to compete with normal hemopoietic stem cells for this area. 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, pts. 2, 8, 22). Increases in CD34+ cells at relapse have been reported in other AML subtypes.

In immunocompromised mice, CD34+ cells from NPM1-mutated AML usually generated a leukemic outgrowth similar to the original patient’s disease, i.e. 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. Flow cytometry studies at different time points showing progressive CD34 downregulation and retention of a small pool of CD34+ leukemic cells 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 downregulation. Further studies are required to clarify the molecular mechanisms of CD34 downregulation in NPM1-mutated AML.

In normal hemopoiesis, a cell compartment devoid of lineage markers and CD34 antigen with SCID-repopulating ability was isolated\textsuperscript{13}, where leukemic transformation could conceivably occur.\textsuperscript{37,38} Recently, Taussig et al.\textsuperscript{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 the phenotype of LICs is more heterogeneous than previously realized.

Our results that CD34- cells from some NPM1-mutated AML patients showed significant LIC potential in immunocompromised mice is in keeping with the findings by Taussig et al.\textsuperscript{21} 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 immunohistological 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 morphological and immunohistological features. We hypothesize 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\textsuperscript{45} remains to be elucidated. Our studies have biological and potential clinical implications. Our 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: i) specificity of NPM1 mutation for AML among human tumors\textsuperscript{1}; ii) mutual exclusion of NPM1 mutation with other AML recurrent cytogenetic abnormalities\textsuperscript{46}; iii) secondary nature of chromosomal aberrations in 15\% of NPM1-mutated AML\textsuperscript{47}; iv) association of NPM1-mutated AML with distinctive gene expression\textsuperscript{7, 8} and microRNA profiles\textsuperscript{4}; and v) results of whole genomic sequencing in AML with normal karyotype.\textsuperscript{48, 49}

As LSCs from NPM1-mutated AML strongly express CD33 and CD123, immunotherapy with CD33 and/or CD123\textsuperscript{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 favourable prognosis\textsuperscript{5}, a significant number of patients with NPM1-mutated AML still succumb to their disease.
ACKNOWLEDGEMENTS

This work was supported by grants from Associazione Italiana Ricerca Cancro (AIRC), Fondazione Cassa di Risparmio di Perugia (Grants n. 2007.0099.020 and 2008.020.058) and Fondazione Cassa di Risparmio di Spoleto. We are grateful to Dr Geraldine A. Boyd for editing the paper and thank Claudia Tibidò for secretarial assistance. We would like also to thank Luca De Carolis, Chiara Balucani, Tiziana Zei, Roberta Iacucci, and Federica Cecchetti for their precious technical help and the personnel of the Animal Facility of University of Perugia for their assistance.

AUTHORSHIP CONTRIBUTIONS AND DISCLOSURE OF CONFLICTS OF INTEREST

M.P.M. and V.P. equally contributed to this work. M.P.M. and B.F. had the original idea of 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 sorting experiments and flow cytometry and molecular analyses, analyzed data and contributed to write the manuscript. F.M. contributed to perform mice experiments; E.B., I.G., D.C., and L.B. performed immunophenotypic analysis; M.G. and L.D.V. performed FACS cell sorting 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. M.D., G.S., F.D.R. and M.F.M. provided patients samples and clinical information and contributed to discussion of data. B.F. applied for a patent on the clinical use of NPM1 mutants. The other authors have nothing to disclose.
REFERENCES


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


Table 1. Characteristics of samples from 21 patients with NPM1-mutated AML\(^a\)

<table>
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<tr>
<th>Pt. Code</th>
<th>Disease status</th>
<th>FAB (NPM)</th>
<th>IHC (NPM)</th>
<th>WB</th>
<th>Karyotype</th>
<th>FLT3 status</th>
<th>WBC/(\mu l)</th>
<th>Sample source</th>
<th>CD34% # (of MNCs)</th>
<th>IHC (CD34)</th>
<th>Post-MACS # purity (%CD34+)</th>
<th>CD34+/CD38- % (gate on CD34+)</th>
<th>MFI # (CD34+/CD38-)</th>
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<tr>
<td>1</td>
<td>Diagnosis M1</td>
<td>NPMc+</td>
<td>+</td>
<td>n.a.</td>
<td>n.a.</td>
<td>FLT3-IDT</td>
<td>20000</td>
<td>Pb</td>
<td>23</td>
<td>20-30%</td>
<td>93</td>
<td>25</td>
<td>2.62</td>
</tr>
<tr>
<td>2</td>
<td>Diagnosis M5</td>
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<td>+</td>
<td>46XX; t(2;17)(p22;q25)</td>
<td>FLT3-IDT</td>
<td>31040</td>
<td>Pb</td>
<td>5</td>
<td>neg</td>
<td>90.64</td>
<td>94</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>2R</td>
<td>Relapse M5</td>
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<td>+</td>
<td>46XX; t(2;17)(p22;q25)</td>
<td>FLT3-IDT</td>
<td>67080</td>
<td>Pb</td>
<td>34</td>
<td>neg</td>
<td>99</td>
<td>98.4</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Diagnosis N.C.</td>
<td>NPMc+</td>
<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>26870</td>
<td>Pb</td>
<td>60</td>
<td>40-50%</td>
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<td>0.16</td>
<td>16</td>
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<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>30170</td>
<td>Pb</td>
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<td>neg</td>
<td>94</td>
<td>15.6</td>
<td>14</td>
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<td>NPMc+</td>
<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>127700</td>
<td>Pb</td>
<td>1.9</td>
<td>neg</td>
<td>77</td>
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<td>1.7</td>
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<td>n.a.</td>
<td>FLT3-IDT</td>
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<td>Pb</td>
<td>27</td>
<td>rare</td>
<td>98.5</td>
<td>70.5</td>
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<td></td>
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<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>66790</td>
<td>Pb</td>
<td>11.8</td>
<td>neg</td>
<td>96.2</td>
<td>70.1</td>
<td>5.1</td>
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<td>96</td>
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<td>wt</td>
<td>FLT3-IDT</td>
<td>28730</td>
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<td>70</td>
<td>37</td>
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<td>+</td>
<td>Normal</td>
<td>FLT3-IDT</td>
<td>35750</td>
<td>Pb</td>
<td>27</td>
<td>rare</td>
<td>98.3</td>
<td>70</td>
<td>5.9</td>
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<td>+</td>
<td>Normal</td>
<td>FLT3-IDT</td>
<td>62440</td>
<td>Pb</td>
<td>8.7</td>
<td>neg</td>
<td>95</td>
<td>92.5</td>
<td>4.5</td>
<td></td>
</tr>
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<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>4070</td>
<td>BM</td>
<td>1.5</td>
<td>neg</td>
<td>99.7*</td>
<td>39</td>
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<td>+</td>
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<td>wt</td>
<td>FLT3-IDT</td>
<td>44000</td>
<td>Pb</td>
<td>4.3</td>
<td>neg</td>
<td>86.2**</td>
<td>11</td>
<td>2</td>
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<td>+</td>
<td>n.a.</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>10800</td>
<td>Pb</td>
<td>72</td>
<td>50-60%</td>
<td>99.9</td>
<td>15</td>
<td>2.4</td>
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<td>23</td>
<td>Relapse M2</td>
<td>NPMc+</td>
<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>19780</td>
<td>Pb</td>
<td>1.7</td>
<td>rare</td>
<td>18.5**</td>
<td>45.5</td>
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<td>25</td>
<td>Diagnosis M5b</td>
<td>NPMc+(^a)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>FLT3-IDT</td>
<td>48820</td>
<td>BM</td>
<td>2.0</td>
<td>n.a.</td>
<td>99***</td>
<td>20</td>
<td>8.3</td>
<td></td>
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<td>26</td>
<td>Diagnosis M2</td>
<td>NPMc+(^b)</td>
<td>46XX; 46XX; del(3)(p21)</td>
<td>FLT3-IDT</td>
<td>2600</td>
<td>BM</td>
<td>2.6</td>
<td>n.a.</td>
<td>99***</td>
<td>2.2</td>
<td>7.0</td>
<td></td>
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<td>30</td>
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<td>NPMc+</td>
<td>+</td>
<td>47XX; t(12;21)</td>
<td>FLT3-D385</td>
<td>84770</td>
<td>Pb</td>
<td>2.93</td>
<td>neg</td>
<td>79</td>
<td>29.4</td>
<td>4.8 and 11.4</td>
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<td>31</td>
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<td>NPMc+</td>
<td>+</td>
<td>Normal</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>23000</td>
<td>Pb</td>
<td>4.76</td>
<td>neg</td>
<td>92.4</td>
<td>45</td>
<td>11</td>
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<td>32</td>
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<td>+</td>
<td>46XX; t(2;12)</td>
<td>FLT3-IDT</td>
<td>264000</td>
<td>Pb</td>
<td>1.3</td>
<td>neg</td>
<td>81/92.3*</td>
<td>88</td>
<td>6.2 and 12.4</td>
<td></td>
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<td>34</td>
<td>Diagnosis M4</td>
<td>N.a.(^c)</td>
<td>+</td>
<td>n.a.</td>
<td>wt</td>
<td>FLT3-IDT</td>
<td>150000</td>
<td>Pb</td>
<td>5</td>
<td>n.a.</td>
<td>96.5</td>
<td>2.48</td>
<td>3.8 and 13.6</td>
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<td>+</td>
<td>Normal</td>
<td>FLT3-IDT</td>
<td>7600</td>
<td>Pb</td>
<td>22</td>
<td>neg</td>
<td>96.4</td>
<td>68</td>
<td>4.8</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Characteristics of other patients are shown in supplemental Table 1; Pt.=Patient; R=Relapse; FAB=French-American-British classification; IHC=Immunohistochemistry analysis; WB=Western Blot with specific anti-NPM mutant antibodies; WBC=White blood cell; MNCs=Mononuclear cells; MFI=Mean fluorescence intensity; N.C.=not classified; n.a.=not available; wt=wild-type; Pb=Peripheral blood; BM=Bone marrow; neg=negative; \(^b\) only studied by Western Blot; \(^c\) Cytoplasmic NPM was detected by flow cytometry; \(^d\) Analyzed on Cytomics FC500 cytometer equipped with the CXP analysis software (Beckman Coulter Inc.); \(^e\) after 2 purification step; \(^f\) CD34+ MACS-enriched cell fraction used for FACS sorting; \(^g\) CD34+ FACS sorted; \(^h\) Two distinct cell populations (CD34\(^{low}\) and CD34\(^{bright}\)).
Table 2. Leukemic engraftment of CD34+ cells from 10 NPM1-mutated AML in immunocompromised mice

<table>
<thead>
<tr>
<th>Pt. Code</th>
<th>% CD34+ in original sample</th>
<th>Purity of inoculum</th>
<th>N°/mice</th>
<th>N° cells/mice (x 10^6)</th>
<th>N° mice with AML/n° mice evaluated</th>
<th>Time of evaluation (weeks)</th>
<th>% hCD45 in mice BM</th>
<th>% CD34+ in mice (gate on hCD45)</th>
<th>% hCD45+/CD19+ (or CD20+)</th>
<th>WB (NPMm)</th>
<th>IHC (NPM)</th>
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<tbody>
<tr>
<td>4</td>
<td>60</td>
<td>96.3</td>
<td>1 / NOD</td>
<td>5</td>
<td>1/1</td>
<td>4</td>
<td>27</td>
<td>50</td>
<td>neg^</td>
<td>+</td>
<td>c+</td>
</tr>
<tr>
<td>10</td>
<td>27</td>
<td>98.5</td>
<td>2 / NOD</td>
<td>5</td>
<td>1/2</td>
<td>16</td>
<td>0.62</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
<td>11.8</td>
<td>96.2</td>
<td>2 / NOD</td>
<td>2</td>
<td>2/2</td>
<td>20</td>
<td>39.8^^ / 19.8</td>
<td>1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>c+</td>
</tr>
<tr>
<td>13</td>
<td>0.8</td>
<td>90</td>
<td>1 / NOD</td>
<td>2.5</td>
<td>1/1</td>
<td>19</td>
<td>32.3</td>
<td>CD34^-</td>
<td>neg^</td>
<td>n.d.</td>
<td>c+</td>
</tr>
</tbody>
</table>

^a^ 22R

^b^ 17

CD34+

1.7 | 99 | 1 / NOG | 0.2 | 1/1 | 15 | 21 | 0.57 | 0 | + | c+ |

2 / NOG | 0.001 | 0/2 | 21 | 0 | -- | -- | n.a. | n.a. |

CD34-

99.9 | 1 / NOG | 0.2 | 0/1 | 15 | 0 | -- | -- | n.a. | n.a. |

2 / NOG | 0.001 | 2/2 | 21 | 53.8^ / 95.7^^ | 0 | 0 | + | c+ |

^b^ 30

CD34+

2.93 | 79 | 4 / NOG | 1 | 2/4 | 7 / 12 / 18 / 18 | 28^ / 27.3^ / 33^t / 47.3^t | 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 | -- | -- | n.a. | n.a. |

^b^ 32

CD34+

1.3 | 92.3 | 3 / NOG | 0.5 | 2/3 | 12 / 18 / 20 | 8.2^ / 1.25^ / 1.54^ | n.d. / 0.42 / 1 | 81.5 / 15 / 1 | n.d. | c-/ c+ § |

3 / NOG | 0.2 | 1/3 | 3 / 6 / 18 | 80.8^ / 32.8^ / 59.6^t | 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 | n.a. | c-/ c+ § |

4 / NOG | 0.01 | 0/4 | 3 / 3 / 15 / 20 | 0 | -- | -- | n.a. | n.a. |
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<th>3 / NOG</th>
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<th>0/3</th>
<th>13 / 18 / 20</th>
<th>0</th>
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<th>--</th>
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<td>0/3</td>
<td>3 / 6 / 15 / 20</td>
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<td>--</td>
<td>--</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
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<td>0.1</td>
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<td>12 / 15 / 18 / 20</td>
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<td>0/4</td>
<td>6 / 7 / 20 / 20</td>
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<td>--</td>
<td>n.a.</td>
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* Experiments to calculate frequency of LIC were performed with these samples (see Table 5); †† Full details on the characteristics of the engraftment are provided in supplemental Table 4; Paired experiments using correspondent doses of CD34+ versus CD34- cells were performed with these samples (see Table 5 for details of pts. 17 and 34); †† Higher cell doses of CD34- cells gave rise to engraftment as pattern 4 (see ‘Results’ section for description and Table 4); Pt. = patient code; BM = Bone marrow; WB (NPMm) = Western blot analysis with anti-NPM mutant specific antibody; IHC (NPM) = immunohistochcmical analysis for NPM1 subcellular localization on hCD45 positive cells; NOD = NOD/SCID; NOG = NOD/SCID/IL2Rγnull; * Evaluated only at immunohistochemistry; ^^ Used for serial transplantation experiments; † Normal engraftment; ‡ Mixed normal-leukemic engraftment; § Positive for NPM1 mutation by genomic DNA fragments analysis; ¶ 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; * FLT3-ITD-negative; ** FLT3-ITD-positive.
Table 3. Summary of serial transplantations experiments.

| Pt. Code | Engrafted cell fraction in primary recipients | N°/Mice | N° cells/mice (x 10^5) | N° mice with AML/n° mice evaluated | Time of evaluation (weeks) | % 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 | ^aCD34- | 3 / NOD | 1 | 3/3 | 6.5 | 6.7 / 1.2 / 0.5
| 23 | ^aCD34- | 3 / NOG | 4 / 4 / 2 | 3/3 | 12 | 60.5 / 79 / 69.5
| 22 | ^bCD34- | 2 / NOG | 2.5 | 0/2 | 8 | 0
| 22 | ^bCD34- | 2 / NOG | 1 | 0/2 | 12 | 0
| 27 | ^bCD34- | 1 / NOG | 0.5 | 0/1 | 12 | 0

*See Tables 2 and 4 for details on cases used for serial transplantations;
^hCD45-positive 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;
Pattern 2 and Pattern 3 of engraftment in primary recipients (see Table 4);
Pt.=Patient; NOD=NOD/SCID; NOG=NOD/SCID/IL2Rγnull; AML=acute myeloid leukemia; BM=bone marrow

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

| Pt. Code | % CD34+ in original sample | Purity of CD34- cells | N°/type mice evaluated | N° cells/mice (x 10^6) | N° mice engrafted/ N° evaluated | Time of evaluation (weeks) | % hCD45+ in mice BM\# | % hCD45+/CD33+ (gate on hCD45) | Pattern of engraftment † | WB (NPMm) | NPM1 gene status ||
|----------|------------------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 8R       | 1.9              | 98.5             | 2 / NOD         | 2               | 2/2             | 16              | 36 / 73\^[5]   | 97.5 / 99       | 2               | +              | n.d.            |
| 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               | n.d.           | n.d.            |
| 27       | 0.02             | 99.98            | 3 / NOG         | 10              | 3/3             | 4 / 6 / 8        | 33 / 64\^[\[5] / 42 | 73 / 86 / 56   | 3               | n.d.           | Mutated         |
| 28\^[\[6] | 0.07             | 99.98            | 1 / NOG         | 10              | 1/1             | 5               | 42             | 46             | 3               | n.d.           | Mutated         |
| 29\^[\[6] | 0.5              | 99.9             | 1 / NOG         | 10              | 1/1             | 6               | 14 §           | 0.7            | 4               | n.a.           | n.a.            |
| 34\^[\[6] | 5                | 96               | 2 / NOG         | 10              | 2/2             | 4               | 0.61 / 4.9     | -- / 0.2        | 4               | n.a.           | n.a.            |
| 36       | 0.26             | 99.98            | 3 / NOG         | 10              | 3/3             | 6               | 4.7 / 2.3 / 5.2 § | 0.3 / 5.9 / 9.9 | 4               | n.a.           | n.a.            |
| 37\^[\[6] | 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               | n.a.           | n.a.            |
| 38\^[\[6] | 0.16             | 99.9             | 3 / NOG         | 10              | 3/3             | 7               | 0.68 / 1.2 / 20 § | 1.8 / 2.5 / 1   | 4               | n.a.           | n.a.            |

† Lower cell doses did not engraft (supplemental Table 5 for details); †† see ‘Results’ section for description of patterns of engraftment; ** Evaluated by flow cytometry on cells recovered from femurs or tibias; || Evaluated by genomic DNA fragments analysis; § Human cells infiltration of mice BM was much higher when evaluated at immunohistochemistry; ^ Used for serial transplantation experiments; FLT3-ITD-negative; **FLT3-ITD-positive; Pt.=patient code; BM=Bone marrow; NOD=NOD/SCID; NOG=NOD/SCID/IL2Rγnull; WB (NPMm)=Western blot analysis with anti-NPM mutant specific antibody; n.d.=not done; n.a.=not applicable.
Table 5. LICs frequency in CD34+ and CD34- cell fractions of NPM1-mutated AML.

<table>
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<th>LICs/10^6 MNCs</th>
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<td>1 in 50400 (19967-127223)</td>
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<td>CD34+ (99.9%)</td>
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<td>0/3</td>
<td>-</td>
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<td>CD34- (90%)</td>
<td>0.2</td>
<td>0/4</td>
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<td>CD34+ (99.9%)</td>
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<td>CD34- (90%)</td>
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<td>CD34+ (99.9%)</td>
<td>0.001</td>
<td>0/4</td>
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LICs=leukemia-initiating cells; Pt.=Patient; Dose=number of cells inoculated per mice; C.I.=Confident Intervals; MNCs=mononuclear cells. Mice used were NOD/SCID/IL2Rγnull; Numbers indicate n° mice with AML/n° mice evaluated; Details on immunophenotypic and immunohistochemical analysis of engrafted mice are shown in supplemental Table 4; Time of evaluation (weeks): +17 (pt. 17); +10 (pt. 22R); +4 (pt. 34).
**FIGURE LEGENDS**

**Figure 1.** CD34+ cells from AML with mutated *NPM1* express mutated NPM1 gene and protein. A) Western blot analysis of CD34+ cells isolated from 13 *NPM1*-mutated AML patient samples. Patient sample codes are indicated on the left (refer to Table 1). Lysates from 1 to 2 x 10^6 cells of either leukemic bulk (Bulk) or CD34+ MACS sorted (Sorted CD34+) cell population were loaded and run on 10% SDS-PAGE gel, transferred to PVDF 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 Western blot with anti-NPM mutant specific antibody. Unpurified cell fraction from 3 representative *NPM1*-mutated AML patient samples (pts. 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 x 10^6 cells were loaded in each lane and checked for NPM1 mutant protein detection by WB (anti-NPMm, upper 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 15-10% of the original sample. Equal protein lysate loading was demonstrated by blotting the same membranes with an anti-β-tubulin monoclonal antibody (lower 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 one patient with *NPM1*-
mutated AML (pt. 19) (lower panel) and in the relative controls (leukemic bulk and CD34- cell populations) (upper and middle panels, respectively). Percentage of CD34+ cells in each sample is shown on the right. Pt. code=Patient code; n.d.=not determined; mut A= NPM1 gene mutation A.

**Figure 2. CD34+/CD38- cells from AML with mutated NPM1 express mutated NPM1 gene and protein, and display immunophenotypic features of leukemic stem cells (LSCs).** A) CD34/CD38 flow cytometry staining patterns of peripheral blood MNCs (pts. 2, 18, 22) or whole bone marrow (pts. 25, 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 (Becton Dickinson) (pts. 22, 25 and 26) or Cytomics FC500 cytometer (Beckman Coulter Inc.) (pts. 2 and 18), as indicated in ‘Materials and methods’ section. Concomitant expression of CD123 and CD33 on the CD34+/CD38- cell population is shown in a representative example (pt. 18, lower right panels). Here, within the CD34+/CD38- cell population, only a very small cell fraction (gate Q: about 0.8%) was negative for both CD123 and CD33 that could represent normal residual HSCs. B) Western blotting showing expression of NPM1 mutant protein in MACS-sorted CD34+ cells from two selected cases from Figure 1 (pt. 2 and 18) where CD34+/CD38- cells represented almost the whole CD34+ cell population. Here, percentage 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 (pt. 25) with AML with cytoplasmic NPM1 with 0.3% CD34+ cells. Expression of CD123 and CD33 antigens in the two cell subpopulations is shown (C, lower 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%) (panel a) and CD34+/CD38+ (purity of 99%) (panel b) cells were compared to the total bone marrow mononuclear cells.
As shown in green, the typical double peak indicating the \textit{NPM1} mutation (green arrows) was detectable at similar levels in both populations as well as in the MNC. Peaks indicating \textit{FLT3} gene status (wild-type (wt) and \textit{FLT3}-ITD (ITD) mutation) are in blue. \textit{FLT3}-ITD mutation/\textit{FLT3}-wt ratio (mut/wt) is shown on the right. E) Flow cytometric analysis of a representative \textit{NPM1}-mutated AML patient sample (pt. 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 patient’s peripheral blood (MNCs); ii) Expression of CD38 on the purified CD34+ cells (CD34+ post-sorting); iii) and iv) Detection of two distinct cell subpopulations within CD34+/CD38- cells: CD34\textsuperscript{bright}/CD38-/CD123-/CD33- versus CD34\textsuperscript{low}/CD38-/CD123+/CD33+ cells.

\textbf{Figure 3. CD34+ cells in \textit{NPM1}-mutated AML show cytoplasmic expression of nucleophosmin.}

A,B) CD34+ leukemic cells (brown) from a patient with \textit{NPM1}-mutated AML showing cytoplasmic expression of NPM (blue) (A, arrows) and nucleus-restricted positivity for C23/nucleolin (B, arrows). C,D) Another \textit{NPM1}-mutated patient showing rare CD34+ cells (C; APAAP technique) 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 100x/1.3 NA oil objective; Camedia 4040, Dpソフト Version 3.2; and Adobe Photoshop 7.0. E,F) Flow cytometric detection of NPM-cytoplasmic expression (pt. 25) in CD34+ and...
CD34- blasts (E), and after additional gating on CD34+/CD38- or CD34+/CD38+ cells, respectively (F).

**Figure 4. Leukemic engraftment of CD34+ cells from NPM1-mutated AML in immunodeficient mice.** A-D) Bone marrow paraffin sections from a NOD/SCID/IL2Rγnull (NOG) mouse, inoculated (12 weeks before) with 2 x 10^6 CD34+ cells (purity 98.3%) isolated from one patient (pt. 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 (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 20x/0.70 [A,B] and a UPlan FI 100x/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 (pt. 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) Western blot 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.

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 a NOG mouse, inoculated (18 weeks before) with 1 x 10^6 CD34+ cells (purity 79%) from pt. 30 (Table 2). An area in the lower part of panel A (square) is packed with leukemic cells expressing hCD45 (panel B, left square), cytoplasmic NPM1 (panel C) and nucleus-restricted C23 (panel D). Another area from the same section shows involvement by normal human hemopoietic cells (square in the upper part of panel A), expressing CD45 (panel B, right square), nucleus-restricted NPM1 and C23 (panels E and F, single and double arrows). A: hematoxylin-eosin. B-F: APAAP, hematoxylin counterstaining. Images were collected using an Olympus B61 microscope and a UPlanApo 20X/0.70 [A-B], UPlanApo 40x/0.85 [C-F]; Olympus E330-ADU1.2x camera; and Adobe Photoshop 7.0.

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 x 10^6 CD34- cells (purity 99.9%) from pt. 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 (panel B, double arrows; inset, from a different section) which are MPO+ and PGM1(CD68)+ (panels C and D, double arrows); 2) mature histiocytes located in the central area of bone marrow (panel B, single arrow)
which are MPO-negative and PGM1+ (panels C and D, single arrows). (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 x 10^6 CD34- cells (purity 99.99%) isolated from pt. 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 and G: hematoxylin-eosin. C,D,H,I: APAAP; hematoxylin counterstaining. Images were collected using an Olympus B61 microscope and a UPlanApo 40x/0.85 [B,C,D,G,H] and a UPlan Fl 100x/1.3 NA oil objective [inset in B, and I]; Olympus E330-ADU1.2x camera; and Adobe Photoshop 7.0.

**Figure 7.** CD34+ cells from *NPM1*-mutated AML generate CD34-negative 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 one patient (pt. 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). Leukemic nature of these cells was confirmed by Western blotting with anti-NPM mutant antibody (C), as well as morphological analysis (D; hematoxylin-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 FI 100x/1.3 NA oil objective; Camedia 4040, Dp_soft Version 3.2; and Adobe Photoshop 7.0. A vertical line has been inserted in (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/IL2Rγnull) sacrificed at 9 (H) and 12 (I) weeks after injection of the same number of CD34+ cells (2 X 10⁶, purity 98.3%) isolated from one patient (pt. 17) with NPM1-mutated AML (27% CD34+ cells in the original sample) (G).
### Figure 1

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**Blot: anti- NPMm**

#### B

**AML with mutated NPM1**

% of $2 \times 10^6$ cells (unpurified fraction)

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**Blot: anti- β-tubulin**

#### C

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Figure 4

A

CD45

Bone

B

MPO

Bone

C

NPM

Bone

D

C23

Bone

E

Gate on viable cells

CD45-ECD

SS Lin

Gate on M (hCD45+)

CD3-PE

SS Lin

F

G

OCI/AML3

mouse BM

AML cells (pt. 17)

UB37

37 -

anti-NPMM
CD34+ cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice

Maria Paola Martelli, Valentina Pettirossi, Christian Thiede, Elisabetta Bonifacio, Federica Mezzasoma, Debora Cecchini, Roberta Pacini, Alessia Tabarrini, Raffaella Ciurnelli, Ilaria Gionfriddo, Nicla Manes, Roberta Rossi, Linda Giunchi, Uta Oelschlägel, Lorenzo Brunetti, Marica Gemi, Mario Delia, Giorgina Specchia, Arcangelo Liso, Mauro Di Ianni, Francesco Di Raimondo, Franca Falzetti, Luigi del Vecchio, Massimo F. Martelli and Brunangelo Falini

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