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ORIGINAL ARTICLE

MN1 overexpression is an important step in the development of inv(16) AML

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The gene encoding the transcriptional co-activator MN1 is the target of the reciprocal chromosome translocation (12;22) (p13;q12) in some patients with acute myeloid leukemia (AML). In addition, expression array analysis showed that *MN1* was overexpressed in AML specified by inv(16), in some AML overexpressing ecotropic viral integration 1 site (EVI1) and in some AML without karyotypic abnormalities. Here we describe that mice receiving transplants of bone marrow (BM) overexpressing *MN1* rapidly developed myeloproliferative disease (MPD). This BM also generated myeloid cell lines in culture. By mimicking the situation in human inv(16) AML, forced coexpression of MN1 and Cbfβ–SMMHC rapidly caused AML in mice. These findings identify *MN1* as a highly effective hematopoietic oncogene and suggest that MN1 overexpression is an important cooperative event in human inv(16) AML.
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Introduction

The *MN1* gene was identified as the target of a unique-balanced t(4;22) in a patient with meningioma and was thought to be a prime candidate for the meningioma tumor-suppressor gene on chromosome 22 (meningioma 1; Lekanne Deprez, 1991[1]), but its relation to meningioma remains unresolved. *MN1* encodes a nuclear protein of 150 kDa, highly conserved among vertebrates and no homology to other proteins. Its amino acid (aa) sequence suggested a role in transcription,[2] which was confirmed by the observation that MN1 activated transcription of the Moloney sarcoma virus long terminal repeat (MSV-LTR) in transient transcription assays.[3] MN1 appeared to activate transcription of the LTR via direct repeat sequences (DR5) that bind RAR-RXR nuclear receptor dimers. MN1 interacts with RAR-RXR most probably via the protein intermediates p300 and RAC3 (also known as nuclear receptor co-activator 3, NCOA3).[4] RAC3 and MN1 are transcription co-activators[5,6] and coexpression of MN1 with p300 or RAC3 synergistically activated the transcriptional activity of RAR-RXR dimers in the presence of retinoic acid.[4] MN1's co-activation activity is not restricted to the RAR-RXR nuclear receptor, as MN1 expression inhibits proliferation of an osteoblast cell line via co-activation of the vitamin D receptor.[7]

MN1 is also the target of a balanced t(12;22) in myeloid leukemia,[3] in which its first exon is fused to *TEL,* a member of the family of ETS transcription factors.[8] Although TEL generally functions as a site-specific transcriptional repressor,[9–11] MN1-TEL moderately activates transcription of TEL-responsive reporters in transient transfection experiments, and the fusion protein has transforming activity in both NIH3T3 fibroblasts[3] and mouse bone marrow (BM).[12] The transforming activity in NIH3T3 cells critically depends on DNA binding via the ETS domain of TEL and on the presence of the N-terminal 500 aa of MN1.[3] In BM cells, the transforming activity also depends on the MN1 N-terminal 500 aa, but it is independent of DNA binding.[12]

Conditional *MN1-TEL* knock-in mice that expressed the gene under the control of *Aml1* regulatory sequences developed lymphoid or myeloid malignancies depending on the nature of the cooperating mutations.[13,14] This confirmed MN1-TEL's role as a *bona fide* hematopoietic oncogene.

Interestingly, the association of *MN1* with myeloid malignancy might go beyond *MN1*'s involvement in the t(12;22), as the gene was found to be overexpressed in inv(16)(p13;q22) acute myeloid leukemia (AML)[15,16] in some AMLs overexpressing the transcription factor ecotropic viral integration 1 site (EVI1)[16] and in some adult AMLs without karyotypic abnormalities.[17] In the latter case, overexpression of *MN1* was associated with a worse prognosis and a shorter survival rate.[17]

Inv(16) is the chromosomal hallmark of one of two core-binding factor (CBF) leukemias and encodes the CBFβ–smooth muscle myosin heavy chain (CBFβ–SMMHC) fusion protein.[18] CBF consists of a CBFβ/RUNX1 heterodimer that regulates genes associated with lymphoid and myeloid differentiation.[19] The RUNX1 subunit is the target of the recurrent t(8;21) in AML, giving rise to a RUNX1-ETO fusion protein. Both CBFβ–SMMHC and RUNX1-ETO have a dominant-negative effect on CBF function.[20,21] This was concluded from the observation that *Runx1* and *Cbfb* knockout mouse embryos and heterozygous *Runx1-ETO* or *Cbfb-MYH11* knock-in embryos all die at mid-gestation due to the inability to switch to definitive hematopoiesis[21–23] Mice chimeric for *Runx1-ETO* or *Cbfb-MYH11* show alterations in multilineage differentiation of hematopoietic cells in the BM, but do not spontaneously develop myeloid leukemia.[24,25] Consistent with the notion that leukemogenesis is a multistep process,[26] such mice only developed myeloid disease after treatment with the chemical carcinogen *N*-ethyl-*N*-nitrosourea (ENU).[24,25] Retroviral mutagenesis of *Cbfb-MYH11* chimeric knock-in mice identified the cooperating zinc finger genes *Plag1* and *PlagL2*,[27] which are also overexpressed in 20% of human AML samples with *PLAGL2* preferentially increased in inv(16) leukemia samples.[28]

Here we report that mice receiving transplants with BM overexpressing MN1 rapidly developed a fatal myeloproliferative disease (MPD), while mice receiving transplants with

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Cbfb-MYH11 chimeric BM overexpressing MN1 developed AML. In addition, quantitative real-time (QRT)–PCR analysis of AML samples confirmed *MN1* overexpression in inv(16) patient samples, but elevated expression was also found in other pediatric AML samples. Our data suggest that MN1 overexpression is an important secondary mutation in inv(16) AML, but its upregulation may also contribute to the development of other AML subtypes.

Materials and methods

Patient materials

All patients and patient materials used in this paper have been described previously.[15] Informed consent for the use of the leukemic cells for research was obtained from parents, guardians or patients (as age-appropriate) in accordance with the Declaration of Helsinki, and study approval was obtained from the SJCRH Institutional Review Board (IRB).

Plasmids and retrovirus production

MN1 cDNA[3] was cloned into the *Eco*RI site of MSCV-IRES-GFP and high-titer ecotropic virus (5×10^5–1×10^6 CFU/ml) was obtained as described.[29] As a control, we used MSCV-IRES-GFP virus without cDNA insert.

BM transplantation

For transplantation of MSCV-MN1-IRES-GFP-transduced wild-type BM into C57Bl/6 females, BM was harvested from femurs and tibiae of 8-week-old male C57BL/6 or C57BL/6/129svJ mice treated with 5-fluorouracil (5FU). 5FU injection, isolation of Lin^- cells, viral transduction and BM transplantation into lethally irradiated female recipients was performed as described previously.[29] For transplantation of inv(16) chimeric BM, heterozygous *Cbfb-MYH11*knock-in ES cells[20] were injected into C57BL/6 blastocysts and ten highly chimeric male mice were generated. After treatment with 5FU, BM of these mice was isolated, transduced with MSCV-MN1-IRES-GFP or MSCV-IRES-GFP retrovirus, yielding cell populations that were 30 and 60% GFP^+, respectively. The MSCV-MN1-IRES-GFP-transduced cells were transplanted into 12 lethally irradiated female C57BL/6 recipients and the MSCV-IRES-GFP control cells into six such recipients.

Immunofluorescence

Cytospin preparations of BM of two pediatric inv(16) patients from the St Jude Children's Research Hospital tumor bank and BM of a healthy donor were fixed with 4% paraformaldehyde for 4 min. Cells were permeabilized with 0.2% Triton X-100 and incubated with MN1 monoclonal antibody 2F2,[3] followed by incubation with a CY3-labeled goat-anti-mouse secondary antibody (1/400 dilution). MN1 staining of cytospin preparations of *MN1*-transduced BM-derived cell lines was performed following the same procedure. Fluorescent images of cytospin preparations were obtained using a BX-50 microscope (equipped with a UPlanFI $\times 40/0.75$ or $\times 100/1.30$ numeric aperture objectives, Olympus, Tokyo, Japan) with a SPOT camera and SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI, USA).

Cell cycle analysis by Fluorescence-activated cell sorting

Cell cycle analysis of *MN1*-transduced BM cell lines and vector-transduced control BM cells was performed as described previously.[29]

Secondary BM transplantation

Secondary BM transplantation of leukemic BM was performed as described previously.[12]

Hematopoietic progenitor assays

Sequential methylcellulose-based cultures (MC1–4) of BM cells was performed using MethoCult GFM3434 (StemCell Technologies, Vancouver, BC, Canada), containing mouse stem cell factor (SCF) (50 ng/ml), mouse interleukin (IL)-3 (10 ng/ml), human IL-6 (10 ng/ml) and human erythropoietin (3 units/ml) and were performed as described.[12]

Analysis of diseased mice and tissue preparation

All animal procedures were conducted in accordance with the US Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Collection of blood, green fluorescent protein (GFP) analysis of peripheral blood (PB), BM, spleen and suspensions, euthanasia of diseased animals, tissue collection, fixation, paraffin embedding, sectioning and histological staining and May-Grunwald–Giemsa staining of PB were performed as described.[12] Select tissues were also processed for immunohistochemical analysis with antibodies to hematopoietic phenotype markers: CD3 (Dako, Carpinteria, CA, USA), CD45R/B220 (PharMingen, San Diego, CA, USA), terminal deoxynucleotidyl transferase (TdT, Supertechs, Bethesda, MA, USA), myeloperoxidase (MPO, Dako, Carpinteria, CA, USA), TER 119 (PharMingen), GATA (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and GFP (Clontech, Palo Alto, CA, USA).

Cell surface markers

Marker analysis by fluorescent-activated cell sorting (FACS) of single-cell suspensions of BM and spleen was performed as described.[12] Cells were incubated with monoclonal antibodies (CD3c, CD4, CD8, CD11b/Mac1, CD19, CD34, B220, TER-119, Gr1, Sca1, c-Kit, Flt3, all from Pharmingen; anti-mouse IgM from Southern Biotechnology Associates, Birmingham, AL, USA) on ice for 30 min. Stained cells were analyzed using a BD Biosciences FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Sorting of mouse BM for stem cell and progenitor fractions

Following the methods of Akashi *et al.*,[30] different mouse BM progenitor populations representing the hematopoietic stem cell (HSC), the myeloid/erythroid progenitor (MEP), the common myeloid progenitor (CMP), the granulocyte/monocyte progenitor (GMP) and the common lymphoid progenitor (CLP) were isolated and their identity verified by reverse trancriptase (RT)–PCR analysis for the expression of signature genes.[30]

QRT–PCR

The RT reactions of patient BM RNA were done with 50 ng total RNA in 20 μl total volume using TaqMan Reverse Transcription

Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations.

Primers and probes for genes were chosen with the assistance of the computer program Primer Express (PE Applied Biosystems, version 2.0.0). We confirmed the gene specificity of the nucleotide sequences chosen for the primers using BLASTN searches. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. The primers and probes are shown in Table 1. Each probe was synthesized with the 5′-end reporter dye (FAM: 6-carboxy fluorescene phosphoramidite) and 3′-end BHQ1 dark quencher dye at St Jude's Hartwell Center for Bioinformatics and Biotechnology.

Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (PE Applied Biosystems). All PCR mixtures contained 2 μl cDNA (corresponding to 5 ng reverse transcribed total RNA), 1 × TaqMan Universal PCR Master Mix (PE Applied Biosystems), 1 × Eukaryotic 18S rRNA endogenous control, 300 nM of each primer and 200 nM probe in 30 μl reaction volume in 96-well plates. The thermal cycling conditions were as follows: after incubation at 50°C for 2 min and an initial denaturation step at 95°C for 10 min, 40 cycles were performed at 95°C for 15 s and 1 min at 60°C. Standard curves were obtained using cDNA generated with human or mouse total BM RNA (pooled human normal BM from eight male/female from BD Bioscience Clontech, Mountain View, CA, USA and pooled mouse BM from three male C57Bl/6/129svj mixed-background mice). Each PCR run included the six points of the standard curve (fivefold serially diluted human or mouse BM cDNA), a non-template control with water, a calibrator cDNA and the unknown cDNA samples. Baseline and threshold C_T value were analyzed manually with the ABI Prism SDS2.1 software. Values for each PCR product were normalized against 18S rRNA to compare expression in patient BM samples with that in human BM total RNA (BD Bioscience Clontech). Quantitative PCR assays were conducted in duplicate for each sample and a mean value was used to calculate mRNA levels.

Table 1 Primers

Human PLAGL2 *cDNA primers*
Forward: CACTGTGGCAAGGCTTTTGC
Reverse: GATGGTCCTTGCGGTGAAACAT
Probe: ATACAAGCTGTATAGGCACATGGCCACCC

Human EVI1 *cDNA primers*
Forward: AATGTGAAAACTGTGCCAAGGTT
Reverse: CCGACATGCTGAGAGCGAAT
Probe: TCACGGACCCTAGCAACCTTCAGCGGCA

Human MN1 *cDNA primers*
Forward: GAAGGCCAAACCCCAGAAC
Reverse: GATGCTGAGGCCTTGTTTGC
Probe: CCAACAGCAAAGAAGCCCACGACC

Human MN1 *exon 1 primers*
Forward: ATTGACCTGGACTCGCTGATG
Reverse: TGTCCACCAGGGCCTTGT
Probe: CAGCGCTGCCTGGTACATGCCC

Human GAPDH *exon 8 primers*
Forward: ACCACAGTCCATGCCATCACT
Reverse: CCATCACGCCACAGTTTCC
Probe: CCCAGAAGACTGTGGATGGCCCC

Mouse Mn1 *cDNA primers*
Forward outer/inner: TGGTGGAGATGAGGACAAGA
Reverse outer: CTTGGGGTCACCATCTGTG
Reverse inner: GTGGCTGAGGCCTTGTTGG
Probe: CCCAACAACAAAGAAGCCCATGACC

Mouse Hprt *cDNA primers*
Forward outer/inner: TTATCAGACTGAAGAGCTACT
Reverse outer: CTTAACCATTTTGGGGCTGT
Reverse inner: TTACCAGTGTCAATTATATCTTCAACAATC
Probe: TGAGAGATCATCTCCACCAATAACTTTTATGTCC

Q–PCR reactions to compare the copy number of the *MN1* gene in inv(16) patient BM DNA samples with that in normal BM DNA was performed using the TaqMan Reverse Transcription Reagent following the manufacturer's protocols. A dilution series of 1, 0.5, 0.25, 0.125, 0.025, 0.005, 0.001, 0.0002 μg genomic BM DNA of two different inv(16) patients (indicated in red in Figure 1) and a normal individual were subjected to one-step PCR with *MN1* first exon 1 and *GAPDH* exon 8 primers (Table 1), using 40 cycles of amplification (10 s 95°C, 1 min 60°C) after incubation of the samples for 10 min at 25°C, 30 min at 48°C and 10 min at 95°C. The *GAPDH* 5′ end reporter dye was tetrachlorofluorescein instead of FAM. Baseline and threshold C_T value were analyzed manually with the ABI Prism SDS2.1 software. Values for the *MN1* PCR product were normalized against that of the *GAPDH* product in inv(16) and normal BM DNA samples.

Analysis of *Mn1* expression in mouse HSCs and BM progenitor populations was carried out using a two-step real-time RT–PCR protocol. FACS sorted HSCs and BM progenitor fractions were pelleted by centrifugation and subsequently lysed in 800 μl of TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification Kit (Invitrogen Corporation) following the manufacturers protocol, however, with addition of 10 μg of RNase-free glycogen before binding to the column. Genomic DNA was degraded by addition of 1 unit of DNase I for 15 min at room temperature, followed by inactivation of DNase I by addition of 1 μl of 25 mM ethylenediaminetetraacetic acid and heating at 65°C for 10 min. First-strand cDNA synthesis was carried out on the total RNA isolate using SuperScript III First-Stand Synthesis SuperMix (Invitrogen Corporation), following the manufacturers instructions. Real-time analysis was carried out using a modified version of the multiplexed tandem PCR approach.[31] For first-round multiplexed amplification, 5 μl of the first-strand cDNA reaction was added to MN1 or HPRT outer primers (300 nM final of each), 200 nM dNTPs (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, GoTaq buffer (Promega) and 0.5 U GoTaq (Promega) in a total volume of 20 μl. PCR was carried out on a PTC-200 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) for 15 cycles using the following conditions: 95°C, 10 min for 1 cycle followed by 20 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. The resulting products were mixed and first-round primers, dNTPs and Taq, were removed using the QIAGEN mini elute PCR clean-up kit (QIAGEN Inc., Valencia, CA, USA). The real-time PCR consisted of 5 μl of an appropriate dilution of the first-round amplified cDNA mixed with 15 μl of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 300 nM each of the inner *Mn1* or *Hprt* forward and reverse primers and 200 nM probe in a final reaction volume of 30 μl. Real-time PCR was carried out in a 96-well plate on Bio-Rad iQ5 Multicolor Real-Time PCR Detection System using the following cycling conditions: one cycle each of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For all samples, the expression level of

Mn1 was normalized to *Hprt* and expressed relative to the level in total BM.

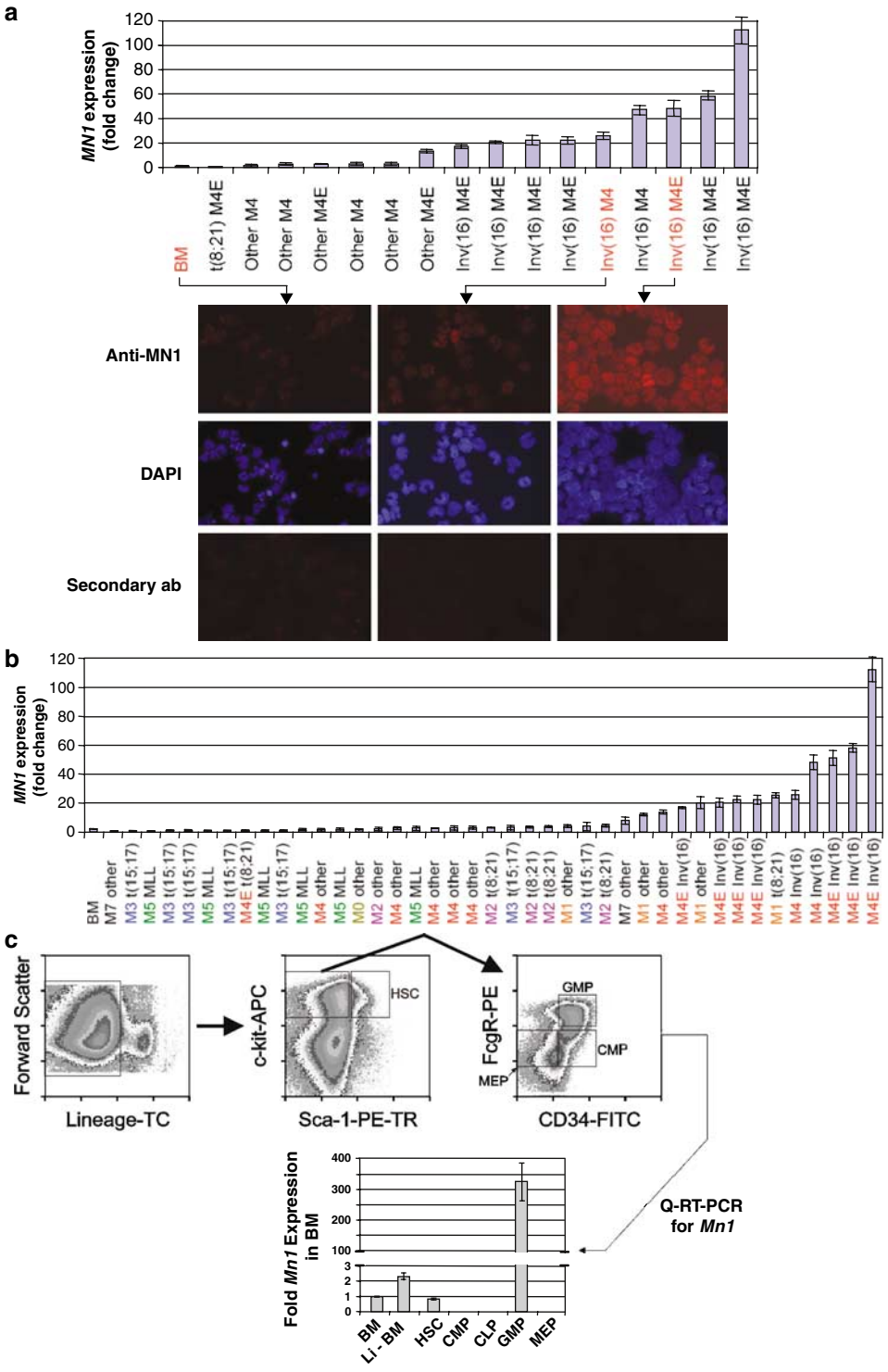
Retroviral integration sites in leukemia samples

To determine the number of MN1 retroviral integration sites, Southern blots containing tumor DNA digested with EcoRI were hybridized with a GFP probe. This detects hybrid DNA fragments containing murine stem cell virus (MSCV) sequences downstream of the viral 3′ *Eco*RI site (Figure 1a) to the first downstream *Eco*RI site in mouse genomic DNA.

Results

Inv(16) leukemia cells overexpress MN1

Expression profiling of pediatric and adult patients with AML[15,16] has shown that BM from patients with inv(16) AML,



who express the CBFβ–SMMHC fusion protein, expressed up to 10 times more *MN1* mRNA than normal BM. Inv(16) AML is often of the French–American–British classification[32] (FAB)-M4 subtype with eosinophilia. Also AML patients overexpressing EVI1 showed upregulated expression of *MN1*.[16] We therefore assessed the level of *MN1* expression in 41 pediatric AML BM samples in comparison with expression in normal BM using QRT–PCR. This group included: 1 acute undifferentiated leukemia (FAB-M0), 4 acute myeloblastic leukemias (FAB-M1; 1 with t(8;21)), 5 acute myeloblastic leukemias with maturation (FAB-M2; 4 with t(8;21), 1 other), 7 acute promyelocytic leukemias (FAB-M3; all t(15;17)), 16 acute myelomonocytic leukemias (FAB-M4; 7 inv(16) with eosinophilia, 2 inv(16) without eosinophilia, 1 t(8;21) with eosinophilia, 6 other M4)), 6 acute monocytic leukemias (FAB-M5, all MLL translocations) and 2 megakaryoblastic leukemias (FAB-M7). All 9 inv(16) samples, with or without eosinophilia (E), showed between 17- to 112-fold higher *MN1* expression than normal BM, whereas only one non-inv(16) FAB-M4 sample showed medium to high *MN1* expression (Figures 1a and b and Supplementary Figure 1A). The six other FAB-M4 samples showed no (1 t(8;21)M4E) or only moderate upregulation (1.7- to 2.9-fold; Figures 1a and b). Also the four FAB-M1 leukemia samples showed significant *MN1* upregulation (4.1- to 25.3-fold) (Figure 1b and Supplementary Figure 1B). Of the remaining 21 AML samples, 9 showed moderately increased expression (2.2- to 8-fold) of *MN1*, including all M2 samples, 4 of which carried the t(8;21), whereas 12 samples showed a lower than twofold increase in *MN1* expression (Figure 1b). The latter group contained five of six FAB-M5 samples harboring MLL translocations, five of seven t(15;17) FAB-M3 samples, one of two FAB-M7 samples and one FAB-M0 sample.

These results not only confirmed overexpression of *MN1* in inv(16) FAB-M4, but also indicated that the level of *MN1* overexpression substantially exceeded that determined by expression profiling.

Because *Plag1* and *PlagL2* overexpression was shown to cooperate with *Cbfb-MYH11* in a mouse model of inv(16) leukemia[27] and *PLAGL2* expression was preferentially upregulated in BM samples of inv(16) leukemia patients,[28] we tested whether there was a direct correlation between the levels of *MN1* and *PLAGL2* expression in our 9 inv(16) patient samples. QRT–PCR for *PLAGL2* mRNA showed that only 1 FAB-M4E sample showed a 4.4-fold higher expression of *PLAGL2* than control BM, whereas the other 8 samples showed equal or lower *PLAGL2* expression than control BM (Supplementary Figure 1A). This result suggested that upregulation of *MN1* and *PLAGL2* are not functionally linked and appear to be independent genetic events in inv(16) leukemia.

Because Valk and co-workers[16] also reported overexpression of *MN1* in leukemia samples with upregulated EVI1 expression, we repeated the QRT–PCR analysis of the same 41 AML samples for *EVI1* expression. Only five samples showed increased expression of this gene (Supplementary Figure 1C), and two of those (1 FAB-M7 and 1 FAB-M1) also showed upregulated expression of *MN1*. These data suggest that upregulation of *MN1* does occur in EVI1 leukemia, but is not an obligatory step.

Using an MN1 monoclonal antibody,[3] immunofluorescence detection of MN1 in the BM cells of 2 inv(16) patients and a healthy subject (fold overexpression of *MN1* mRNA patient1: patient2: normal BM = 50:25:1; Figure 1a) indeed confirmed increased speckled staining in the nucleus of tumor cells of patient1 and slightly increased staining in cells of patient2 (Figure 1a). The same cells incubated with secondary antibody alone showed no staining (Figure 1a).

To determine whether MN1 upregulation might be the result of gene amplification, we employed QRT–PCR of DNA of the same 2 inv(16) patient samples that we used for MN1 immunofluorescence to determine whether their *MN1* copy number was higher than that in normal BM DNA. However, no amplification could be detected (not shown).

Expression of mouse Mn1 *in selected mouse BM progenitor populations*

Because MN1 is upregulated in inv(16) leukemia, we wished to address which hematopoietic progenitor cells normally express this gene. To answer this question, we used mouse BM and followed the method of Akashi and co-workers[30] to sort populations representing the HSC, CMP, CLP, MEP and GMP (Figure 1c). We then determined the amount of *Mn1* mRNA present in these fractionated cell populations by using quantitative (Q)RT–PCR with whole mouse BM RNA as a control. This showed that *Mn1* mRNA was present at a similar level in whole BM and in the HSC fraction, while Lin^- BM cells were twofold enriched in *Mn1*-expressing progenitors. Analysis of the other fractions showed that this signal was derived from the GMP fraction in which *Mn1* expression is 300-fold higher than that in whole BM, whereas there was no *Mn1* expression in the CMP, CLP or MEP fractions. This result suggests a specific role for Mn1 in the GMP, which could involve expansion and/or differentiation of the progeny of this fraction and that forced overexpression of *MN1* causes this progenitor population to expand abnormally.

Figure 1 *MN1* expression in acute myeloid leukemia (AML) patient samples and mouse bone marrow (BM). (**a**) Upper panel: BM RNA of pediatric AML FAB-M4 patients (16 in total, of which 10 with eosinophilia (E), including 9 samples with inv(16), 1 with t(8;21) and 6 with other karyotypic abnormalities (other)) were analyzed for expression of MN1 by quantitative real-time (QRT)–PCR. Expression levels are depicted as fold expression of *MN1* in normal human BM. Results are the average of two experiments. The upper row of the nine fluorescence micrographs shows indirect immunofluorescence detection of MN1, using BM cytospin preparations stained with an MN1 monoclonal antibody of 2 inv(16) patients (indicated by red labels in the upper panel) and a healthy individual. The middle three micrographs show the 4′-6-diamidino-2-phenylindole (DAPI) nuclear staining of the corresponding samples in the upper row. The lower row shows control staining of the same cytospin preparations using secondary antibody only. (**b**) BM RNA of 41 pediatric patients with AML (1 FAB-M0 (olive green), 4 FAB-M1 (orange), 5 FAB-M2 (purple), 7 FAB-M3 (blue), 16 FAB-M4 (red), 6 FAB-M5 (green) and 2 FAB-M7 (black)) with the indicated chromosomal translocations or other karyotypic abnormalities (other) were analyzed for expression of *MN1* by QRT–PCR. Expression levels are depicted as fold expression of *MN1* in normal human BM. 'MLL' signifies samples with different chromosomal translocations involving the *MLL* gene. 'E' indicates eosinophilia. Results are the average of two experiments. (**c**) Mouse BM (FVB) was fluorescent-activated cell sorting (FACS) sorted into fractions representing the hematopoietic stem cells (HSC), the common myeloid progenitors (CMP), the common lymphoid progenitors (CLP), the granulocyte/macrophage progenitors (GMP) and the megakaryocyte/erythrocyte progenitors (MEP) using the forward scatter of differentiation lineage-negative cells, expressing or not of the cell surface markers c-Kit, Sca-1, Fcγ-receptor and CD34. RNA of each fraction was extracted and subjected to QRT–PCR to determine the level of *Mn1* expression in relation to *Hprt*. Expression levels are depicted as fold expression of *Mn1* in unfractionated mouse BM. Lin^- BM represents a sample containing all progenitors depleted of cells expression lineage markers.

Overexpression of MN1 in mouse BM stimulates outgrowth of myeloid cells and produces immortalized cell lines

We reported that MN1-TEL transforms NIH3T3 fibroblasts, an activity dependent on both the presence of MN1 N-terminal sequences and a functional TEL DNA-binding domain.[3] Paradoxically, the self-renewal activity of mouse BM cells was equally stimulated by MN1-TEL as by an MN1-TEL mutant with a nonfunctional ETS DNA-binding domain, as measured by colony-forming assays in semisolid medium.[12] To determine if MN1 alone also possessed this capacity, BM transduced with MSCV expressing MN1-IRES-GFP (30% GFP$^+$) (Figure 2a) was plated in methylcellulose and its colony-forming activity compared with that of BM transduced with MSCV-IRES-GFP. In the first methylcellulose assay (MC1), MSCV-MN1-IRES-GFP-transduced cells gave twice as many colonies as vector-transduced BM (Figure 2a). Upon serial replating (MC2, MC3, MC3), the number of MN1$^+$ BM and vector BM colonies dropped to equal numbers in the MC2, but the number of MN1$^+$ colonies increased drastically in the MC3 and MC4, whereas vector-transduced cells produced no colonies. FACS analysis of cells recovered from the MC3 culture revealed that $>95\%$ of cells were GFP$^+$ (not shown). This together with the observation that MN1-overexpressing

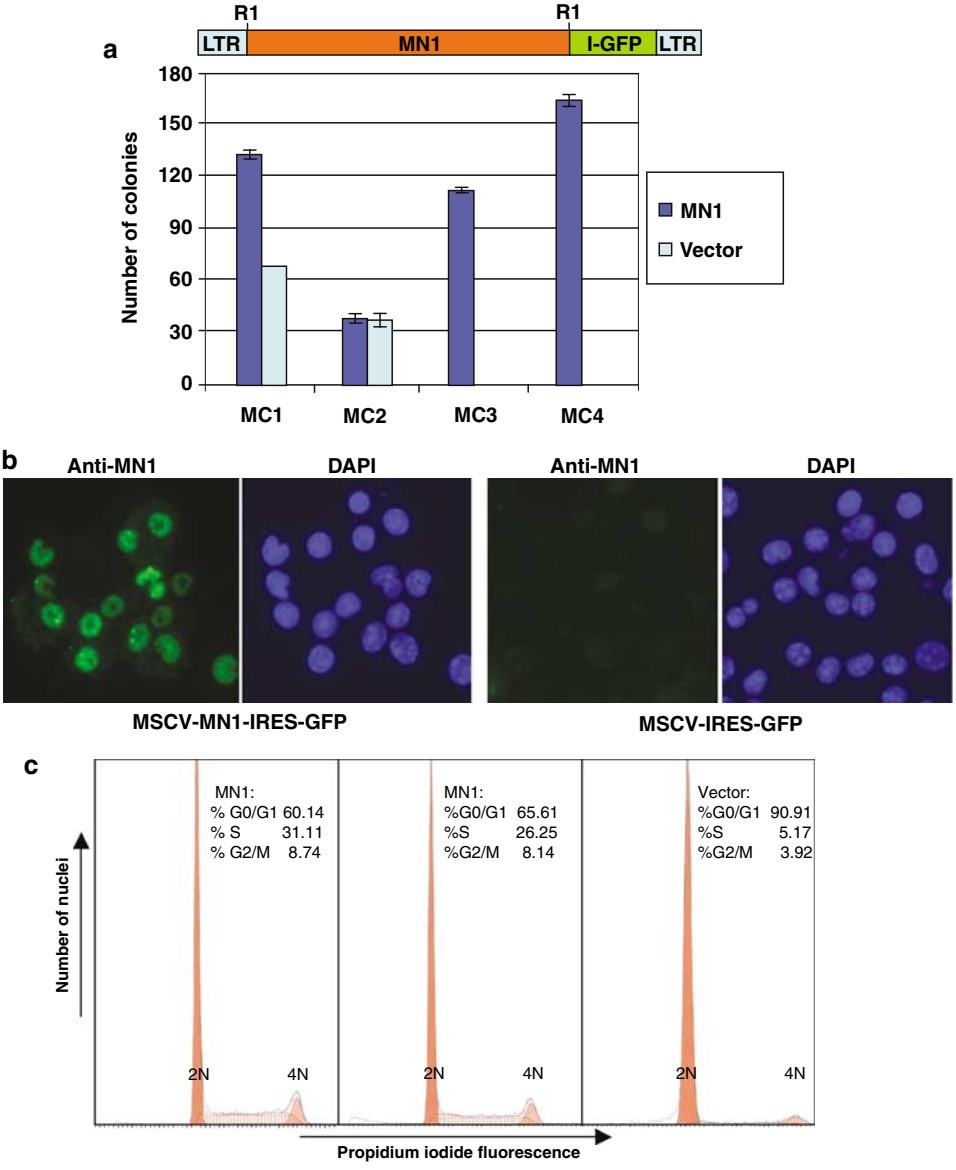


Figure 2 Mouse bone marrow (BM) transduced with MN1 retrovirus shows increased proliferation and generates cytokine-dependent cell lines. (**a**) The map at the top depicts the structure of the MSCV-MN1-IRES-GFP retroviral vector used to transduce mouse BM. The MN1 open reading frame (MN1) is fused to IRES-GFP (I-GFP), flanked by murine stem cell virus (MSCV) long terminal repeat (LTR) sequences. As control, we used the same vector not containing MN1 sequences. R1 indicates *EcoRI* sites flanking the insert. BM from C57Bl/6/129svJ mice, transduced with MSCV-MN1-IRES-GFP (MN1) or MSCV-IRES-GFP retrovirus (vector), were serially plated in methylcellulose semisolid medium (MC1 (20.000 BM cells plated), MC2 (1000 MC1 cells plated), MC3 (1000 MC2 cells plated), MC4 (1000 MC3 cells plated)) and at each step colonies were counted after 2 weeks of culture. Results are the average of two experiments. (**b**) Fluorescence micrograph of an MSCV-MN1-IRES-GFP-transduced cell line and MSCV-IRES-GFP-transduced BM stained with an MN1 antibody. (**c**) Flow cytometric analysis by fluorescent-activated cell sorting (FACS) of the DNA content of propidium iodide stained nuclei from two independent MSCV-MN1-IRES-GFP-transduced cell lines (MN1) and MSCV-IRES-GFP-transduced BM (vector), cultured for 2 months after transduction of the BM.

cells recovered from the MC1 grew rapidly in liquid culture, and were 98% GFP$^+$ within 4 weeks of culture, strongly suggested that MN1 overexpressing cells acquired a distinct growth advantage over that of non-transduced cells. Immunofluorescence analysis with an MN1 antibody showed mostly nuclear, punctate MN1 signals in all cells (Figure 2b), whereas vector-transduced cells showed only faint GFP fluorescence encoded by the retroviral vector. Cell surface marker analysis showed that the cells were c-Kit$^+$/Sca1$^+$/Mac1$^+$ with 10% of the cells also expressing Gr1 (not shown). Cells cultured this way were immortalized but their growth and survival was strictly dependent upon addition of the cytokines IL3 and SCF to the culture (not shown). Cell cycle analysis using flow cytometry of two independent *MN1* cell lines and vector-transduced BM, all cultured for 2 months after transduction, showed that the fraction of MN1-transduced cells in the S- and G_2/M-phase of the cell cycle was drastically increased compared to that of vector-transduced cells (Figure 2c). This showed that MN1 overexpression strongly stimulates cell cycle traverse.

Mice receiving transplants of BM transduced with MN1 *retrovirus rapidly develop MPD*

We next tested the effect of MN1 overexpression on mouse BM *in vivo*. Lethally irradiated C57Bl/6/129svJ mice ($n=14$) died of fulminant hematopoietic disease 5–8 weeks after receiving transplants of $3–5 \times 10^5$ MSCV-*MN1-IRES-GFP*-transduced (Figure 3a) C57Bl/6 Lin$^-$ BM cells (60% GFP$^+$), whereas mice receiving transplants with the same number of MSCV-*IRES-GFP* C57Bl/6 Lin$^-$ BM cells (60% GFP$^+$) ($n=5$) remained healthy (Figure 3a). The average white blood cell (WBC) count of diseased mice was 1.5×10^7/ml, and their PB contained large numbers of neutrophils, neutrophil precursors and some blast-

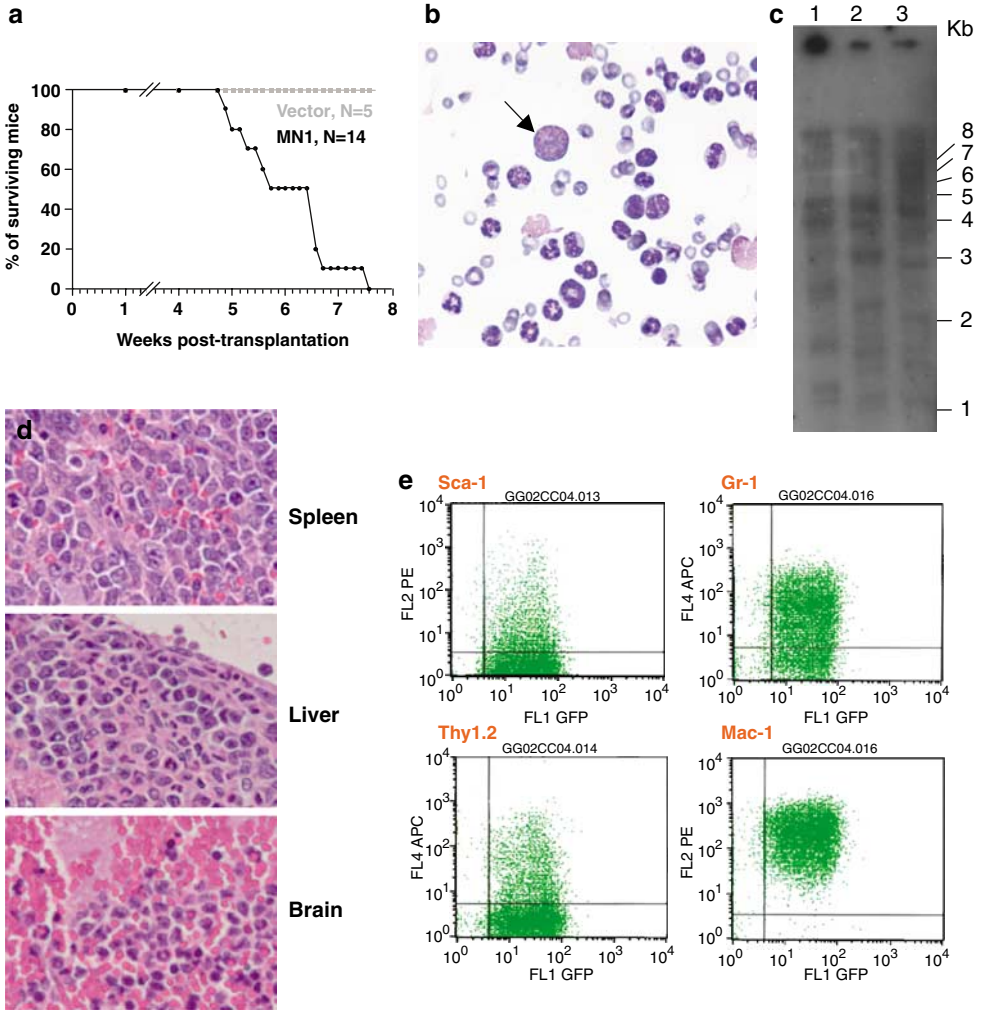


Figure 3 Mice receiving transplants of MSCV-MN1-IRES-GFP-transduced bone marrow (BM) rapidly develop myeloproliferative disease (MPD). (**a**) Survival curve of lethally irradiated C57Bl/6 mice receiving transplants of syngeneic BM transduced with MSCV-MN1-IRES-GFP (MN1, $n=14$) or MSCV-IRES-GFP (Vector, $n=5$) retrovirus. Mice receiving MN1-overexpressing BM died of hematopoietic disease between 34 and 52 days after transplantation, whereas vector-transplanted mice remained healthy. (**b**) Peripheral blood (PB) smear of a representative moribund MN1-BM-transplanted mouse (average WBC is 150×10^7/ml) showing large numbers of partially and fully differentiated neutrophils and some blast-like cells (arrow), showing the mice suffered of an MPD. (**c**) Southern blot of BM DNA of three MPD mice overexpressing MN1, digested with *Eco*RI and hybridized with a green fluorescent protein (GFP) probe. All three samples contain multiple retroviral integrations. (**d**) Malignant cells invaded the spleen and liver. All diseased mice showed brain hemorrhages, often causing sudden death. Secondary recipients receiving transplants of diseased BM rapidly (18–23 days) developed the same disease (not shown). (**e**) fluorescent-activated cell sorting (FACS) analysis of cell surface markers of MPD BM showing that cells were GFP$^+$/Gr1$^+$/Mac1$^+$ and some also expressed Sca-1 and/or Thy-1. Cells were negative for c-Kit.

like cells (Figure 3b) all expressing GFP (Figure 3e). Southern blotting of the BM DNA of three of the diseased mice probed with IRES-GFP showed multiple integrations of the MSCV-MN1-IRES-GFP retrovirus (Figure 3c), suggesting that the disease was oligoclonal. Malignant cells invaded the spleen, liver and brain (Figure 3d), and most of the mice died of brain hemorrhage that was probably caused by the high WBC. FACS analysis showed that BM and spleen were each composed of a single population of $Mac1^+/Gr1^+$ cells (Figure 3e). Together, these features suggested that the mice suffered from an MPD.

Transplantation of pooled 5×10^5 BM cells from two of the diseased MN1 mice into five sublethally irradiated secondary recipients recreated the same fulminant MPD 18–23 days later (not shown), suggesting the disease was cell autonomous. We conclude that *MN1* is a highly efficient oncogene that strongly promotes the growth of myelomonocytic cells, but does not substantially inhibit their differentiation.

Coexpression of Cbfβ–SMMHC and MN1 causes AML in mice

Because MN1 is consistently overexpressed in inv(16) AML, we tested whether its overexpression in BM of mice expressing Cbf*β*–SMMHC cooperates to cause AML. Chimeric mice generated with *Cbfb-MYH11*knock-in ES cells do not spontaneously develop hematopoietic disease, and hematopoietic cells expressing the Cbf*β*–SMMHC fusion protein fail to differentiate and remain in the BM.[24] We used *Cbfb-MYH11* ES cells to generate 10 highly chimeric mice (>90% chimerism of the coat color). Lin^- BM cells from these mice were transduced with *MN1-IRES-GFP* retrovirus (30% GFP^+) and transplanted into 12 lethally irradiated C56Bl/6 recipients. As a control, we transplanted chimeric BM transduced with control *IRES-GFP* retrovirus (45% GFP^+) into 6 irradiated recipients. Given that all our human inv(16) samples overexpressed *MN1*, it was possible that transcription of *MN1* is regulated by Cbf*β*–SMMHC. Therefore, we determined by QRT–PCR whether the level of endogenous *Mn1* mRNA in MSCV-IRES-GFP-transduced chimeric mouse BM was more abundant than in similarly transduced wild-type BM. As shown in Figure 4a *Mn1* expression in both types of BM was similar, opposing the possibility that Cbf*β*–SMMHC directly or indirectly upregulated *Mn1* expression in mouse BM.

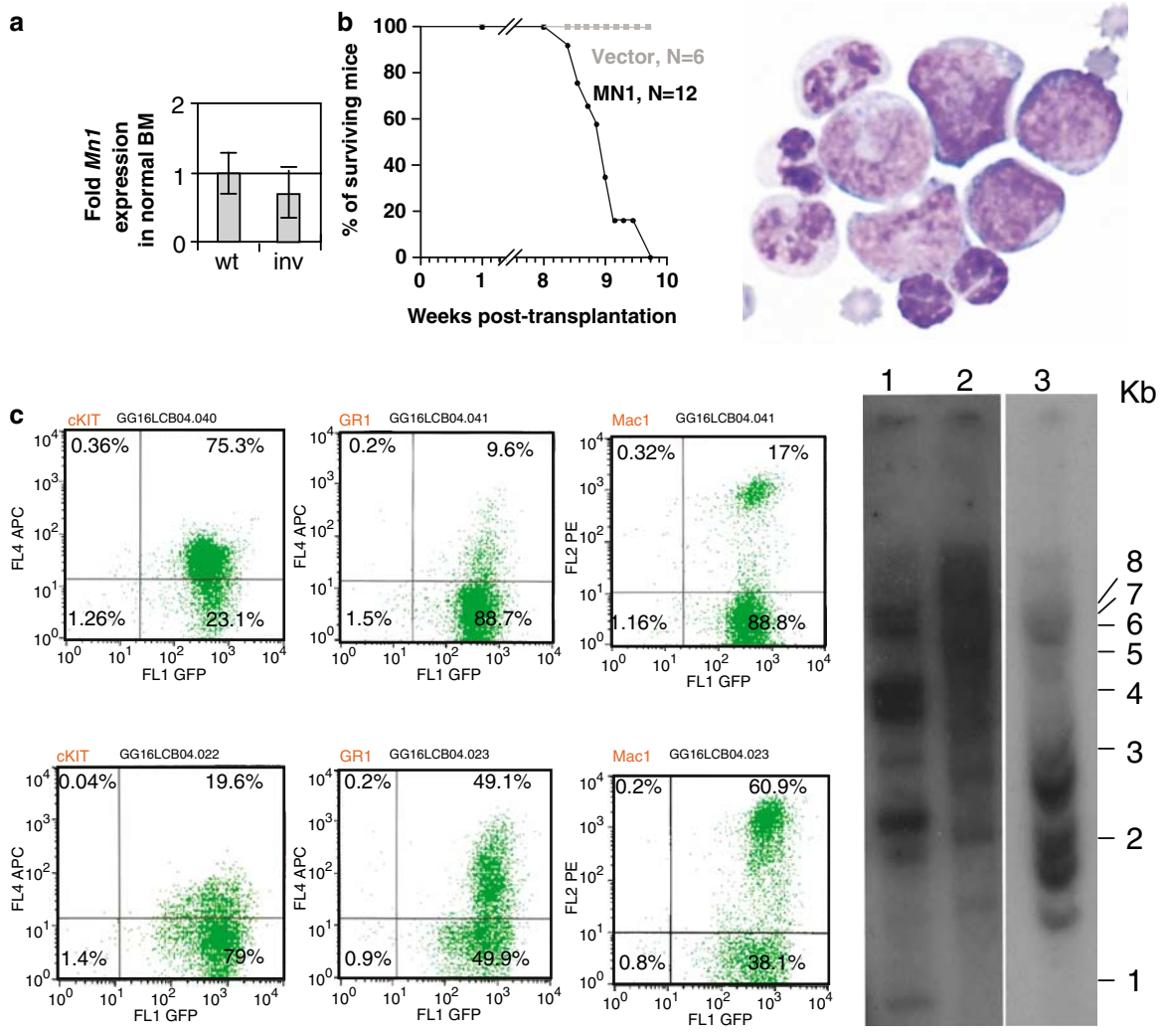
All 12 mice receiving inv(16)/MN1 transplants died of hematopoietic disease 58–68 days after transplantation, whereas the 6 mice given vector-transduced inv(16) transplants remained well (Figure 4b). Leukemic cells infiltrated the spleen and lymph nodes and effaced their normal architecture (not shown). In addition, myeloid cells infiltrated the liver, brain, uterus, lungs, stomach and heart (not shown). Southern blot analysis of BM DNA of three of these mice with a GFP probe showed multiple integrations (Figure 4c), suggesting the disease was oligoclonal (see discussion). The diseased mice could be divided into those whose PB contained a predominance of blast cells (Figure 4b), and those whose PB contained partly blast cells and partly more differentiated myeloid cells (neutrophils and neutrophil precursors). Cell surface marker analysis by FACS (Figure 4c) showed that BM cells of the former type mostly expressed no markers other than c-Kit, with a small percentage of cells expressing Mac1 and Gr1, whereas the BM of the latter mice contained fewer $c\text{-}Kit^+$ cells and more $Mac1^+$ and $Gr1^+$ cells. The expression of c-Kit in the MN1/Cbf*β*−SMMHC leukemic cells was in sharp contrast with the malignant cells of mice that received *MN1*-transduced BM, which expressed Mac1 and Gr1 but not c-Kit (Figure 3e). This result is similar to *Cbfb-MYH11*chimeric mice treated with ENU which also developed a $c\text{-}Kit^+$ AML.[24] GFP signal was present in 99% of all cells (Figure 4c). Immunofluorescense using an MN1 antibody and an antibody to the CBF*β*–SMMHC fusion break point[33] to double label cytospin preparations of BM from both types of mice showed that in mice with mostly blasts in the PB, most BM cells expressed both MN1 and CBF*β*–SMMHC, whereas mice with fewer blasts and more differentiated cells in the PB also contained cells in the BM expressing MN1 only (Figure 4d). This suggested that mice whose PB contains more differentiated cells have a mixed AML/MPD in which the AML arose from inv(16) cells overexpressing MN1 and the MPD from wild-type cells overexpressing MN1, whereas mice with mostly blast cells in the PB have inv(16)/MN1 AML. This result showed that compared to MN1 overexpression alone, the combined expression of MN1 and CBF*β*–SMMHC neither changed the latency nor the penetrance of the disease but had a clear effect on the phenotype of the disease. We next assessed whether the level of *MN1* expression in the BM of our MN1/Cbf*β*–SMMHC transplanted mice would be comparable with that in inv(16) patient BM. Using QRT–PCR with human *MN1* primers (our mouse *Mn1* primers do not amplify human *MN1* cDNA, not shown), we determined the amount of *MN1* mRNA in the leukemic BM of four Cbf*β*–SMMHC/MN1 transplanted mice, in BM of three inv(16) patients and in the inv(16) M4E cell line ME-1.[34] This showed that *MN1* expression in these 4 Cbf*β*–SMMHC/MN1 BM samples was 0.7- to 3.9-fold as abundant as in the patient sample with the highest *MN1* expression (Figures 1 and 5a). Thus, *MN1* expression in the BM of three of the mice was

Figure 4 Mice receiving transplants of chimeric *Cbfb-MYH11* bone marrow (BM) transduced with MSCV-MN1-IRES-GFP develop acute myeloid leukemia (AML). (**a**) RNA from normal mouse BM (wt) and chimeric inv(16) BM (inv), both transduced with MSCV-IRES-GFP virus was analyzed by quantitative real-time (QRT)–PCR for the expression of endogenous *Mn1*. The values are the average of two independent experiments. *Mn1* expression is not upregulated in inv(16) chimeric BM. (**b**) Survival curve showing that lethally irradiated C57Bl/6 mice receiving transplants of chimeric *Cbfb-MYH11* BM transduced with MSCV-MN1-IRES-GFP ($n=12$) developed hematopoietic disease 58–68 days after transplantation, while those receiving transplants of the same BM transduced with MSCV-IRES-GFP ($n=6$) retrovirus remained healthy. The blood smear to the right shows the peripheral blood (PB) of a mouse with AML with a preponderance of blast cells, but also showing neutrophil progenitors. (**c**) Left panel: fluorescent-activated cell sorting (FACS) cell surface marker analysis of the BM of a mouse with a preponderance of blast cells in the PB, with 75.3% of cells expressing c-Kit, 9.6% of cells expressing Gr1 and 17% Mac1 (upper three plots) and of the BM of a mouse with a lower number of blast cells in the PB showing 19.6% of cells expressing c-Kit and 49.1% of cells expressing Gr1 and 60.9% of cells expressing Mac1 (lower three plots). More than 98% of cells express green fluorescent protein (GFP). Right panel: Southern blot of BM DNA of three leukemic MN1/Cbf*β*–SMMHC-transplanted mice (1–3), digested with EcoRI and hybridized with a GFP probe. All three samples contain multiple retroviral integrations. Owing to DNA overloading of sample 3, a shorter exposure of this lane is shown. (**d**) Fluorescence micrographs of BM cytospin preparations of a diseased mouse containing mainly $c\text{-}Kit^+$ BM cells (left panel) and a mouse containing partial $c\text{-}Kit^+$ BM cells (right panel), double stained with antibodies specific for the core-binding factor-*β* encoding the smooth muscle myosin heavy chain (CBF*β*–SMMHC) fusion peptide (red) and MN1 (green). Most cells in the left panel are positive for both CBF*β*–SMMHC (cytoplasm) and MN1 signals (nucleus), whereas only part of the cells in the right panel are positive for both signals with the remainder of the cells only positive for the MN1 signal. The nuclei of the cells in the left panel were counter stained with 4′-6-diamidino-2-phenylindole (DAPI; blue).

considerably higher than in inv(16) patients, but in one mouse comparable expression also appeared sufficient to provoke disease.

Discussion

Myeloid leukemogenesis is a multistep process,[26] and it has been well documented that in AML, specified by recurrent chromosome translocations, cooperating mutations are essential for disease development.[13,24,27,35–37] In expression profiling experiments, the level of *MN1* mRNA was specifically elevated in BM samples of pediatric and adult patients with inv(16) AML.[15,16] *MN1* upregulation was also found in a subtype of AML associated with very poor prognosis,[38] which is defined by overexpression of the immortalizing[39] transcription factor EVI1.[16] Given that MN1 sequences confer oncogenic properties to the MN1-TEL fusion protein[3,12]



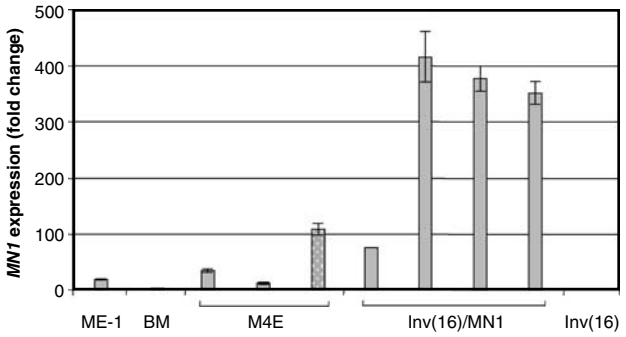


Figure 5 Comparison of *MN1* expression in bone marrow (BM) of inv(16) patients and diseased mice transplanted with Cbfβ–SMMHC/MN1-expressing BM. Quantitative real-time (QRT)–PCR with primers specific for human *MN1* was used to determine the level of *MN1* RNA expression in the human inv(16) cell line ME-1 (ME-1), in pooled BM of healthy individuals, in BM of three inv(16) patients (M4E) and in BM of four diseased Cbfβ–SMMHC/MN1-transplanted mice (inv(16)/MN1). As a negative control, we used BM of a mouse transplanted with vector-transduced Cbfβ–SMMHC chimeric BM (inv(16)). Expression levels are depicted as fold expression of *MN1* in normal human BM. *MN1* expression in BM of Cbfβ–SMMHC/MN1-transplanted mice was between 0.7- to 3.9-fold higher than in BM of the inv(16) patient with the highest level of *MN1* expression (speckled box M4E). Results are the average of two experiments.

the product of the t(12;22) in AML, it opened the possibility that overexpression of *MN1* in these AML subtypes actively contributed to the leukemic process. This suggestion was supported by the observation that retrovirus-mediated overexpression of MN1 in mouse BM boosted proliferation of myeloid cells, allowed the establishment of myeloid cell lines *in vitro* and caused rapid development of MPD after transplantation into lethally irradiated mice. Moreover, the MN1 effects were cell intrinsic as secondary recipients receiving transplants of BM of diseased primary recipients rapidly developed the same disease. Despite the rapid development of the primary MPD, Southern blot analysis of the malignant cells showed that the disease was oligoclonal rather than polyclonal, which implied that additional genetic changes must have occurred for the disease to develop. Currently we do not know the nature of these additional genetic changes.

Expression of endogenous *Mn1* is present in the sorted HSC but not in the CMP, CLP, MEP progenitor compartments. Given the profound proliferative effect of human MN1 overexpression on GMP-derived cells in mouse BM, we speculate that expansion of this compartment might be dependent on endogenous upregulation of *Mn1*. Therefore, forced expression using the *MN1* retroviral vector would lead to overexpansion. Why MN1 overexpression would specifically affect the proliferation of GMP-derived but not MEP-derived cells is currently unknown. *Mn1* knockout mice have defects in the development of membranous bones of the cranial skeleton,[40] but whether these mice also harbor hematopoietic defects is a question we are currently addressing.

We also do not know via which mechanism MN1 stimulates growth of myelomonocytic cells. We reported that in Hep3B cells, the protein is recruited to RAR/RXR dimers via the co-activators p300/CBP and RAC3[4] and stimulates the transcription activity of RAR/RXR in the presence of retinoic acid. It is possible that in myeloid cells, MN1 participates in a similar protein complex or in protein complexes with other transcription factors that recruit p300/CBP, such as MYB; RUNX1; GATA1, 2 and 3; C/EBP; PU.1 and MLL.[41] Growth stimulation of myeloid cells by MN1 is opposite to its reported effects in an osteoblast cell line,[7] in which MN1-mediated co-activation of the vitamin D receptor inhibited proliferation. Growth inhibition of several types of epithelial cells by TGF-β, an inhibitor of epithelial cell proliferation, is also associated with induction of MN1 expression.[42,43] This profound difference in response to MN1 upregulation can only be explained if MN1 can be recruited into different transcription factor complexes whose effects are cell type specific.

Previously we reported that BM of MN1-TEL knock-in mice showed increased self-renewal activity of myeloid progenitors[13] and produced myeloid cell lines *in vitro*. The same effects were observed with BM transduced with MN1-TEL retrovirus. In both scenarios, MN1-TEL cell lines displayed a more primitive phenotype (c-Kit$^+$/Sca1$^+$)[12,13] than the MN1 cell lines (c-Kit$^+$/Sca1$^+$/Mac1$^+$) reported here. We speculate that this difference is caused by the presence of TEL ETS domain in the fusion protein, which might recruit MN1-TEL to TEL recognition sites at promoter/enhancer areas of genes that inhibit differentiation.

As determined by QRT–PCR, *MN1* mRNA levels in inv(16) FAB-M4 samples were much greater than that determined by expression profiling. QRT–PCR analysis showed that *MN1* mRNA expression in our 9 inv(16) patient samples was on average 43.7-fold higher than in normal BM, while estimations using array analysis suggested an average 4.6-[15] or 9.2-fold[16] increase. This discrepancy leads to a distinct underestimation of MN1 expression using expression arrays. Indeed, inclusion of other AML subtypes in our QRT–PCR analysis showed substantial upregulation of MN1 in FAB-M1 samples (14.1-fold average) and moderate (two- to eightfold) upregulation in all M2-, 1 M7-, 2 M3- and 1 M5 sample(s), not reported in the study by Ross *et al*.[15] which included the very same patient samples. *MN1* expression was also moderately increased in all but two non-inv(16) FAB-M4 samples. QRT–PCR of a much larger group of AML samples will have to determine whether it will support our initial results. Given the small numbers of patients analyzed it was impossible to obtain a statistically significant correlation between levels of MN1 expression and the survival rates of inv(16) and FAB-M1 patients. Nonetheless, it was reported that *MN1* overexpression in adult patient samples with a normal karyotype correlated with a worse prognosis and a shorter survival rate.[17]

Immunofluorescence analysis with an MN1 monoclonal antibody of two of our inv(16) BM samples, expressing 50- and 25-fold more *MN1* mRNA than normal BM, showed that the MN1 signal was elevated in leukemic cells, but the difference was much more modest than expected from the increase in *MN1* mRNA levels. It is possible that not all *MN1* mRNA is translated in these AML cells, or alternatively that MN1 overexpression increases its protein turnover. To date all inv(16) patient samples analyzed showed upregulated *MN1* expression (Ross *et al*.[15] and Valk *et al*.[16]) strongly suggesting that overexpression of *MN1* is an obligatory step in the development of this disease.

An important question that remains to be answered is which molecular mechanism provokes *MN1* overexpression. Based on the observation that expression of the endogenous mouse *Mn1* gene was similar in Cbfβ–MYH11chimeric and normal BM (Figure 4a), we do not think that the gene is a direct transcriptional target of the CBF transcription factor. Given that the promoter regions of the mouse and human *MN1* genes show extensive sequence conservation, their transcriptional regulation

is likely to be similar. Also, *MN1* upregulation was reported in AML patients with a normal karyotype,[17] further indicating that other genetic changes than expression of CBF fusion proteins are responsible for *MN1* overexpression. Using PCR amplification of *MN1*, first exon sequences in the genomic DNA of the two patient samples that overexpressed MN1 protein (Figure 1a, indicated in red) and of normal BM samples revealed a diploid copy number in all three samples (not shown). Therefore, the increased MN1 expression in these two patients was not the result of *MN1* gene amplification.

Cbfβ–SMMHC chimeric knock-in mice do not develop myeloid leukemia,[24,28] whereas mice carrying a conditional *Cbfβ–SMMHC* knock-in gene do develop disease within 3–6 months after induction. This difference is most likely caused by the increased size of the Cbf*β*–SMMHC$^+$ preleukemic progenitor pool in the BM of the conditional knock-in mice.[44] Thus, the increased proliferative capacity of MN1 overexpressing cells may enlarge the pool of inv(16) cells enough to promote additional mutations allowing the leukemia to emerge. We favor this possibility because Southern blotting suggested that mouse inv(16)/MN1 leukemia is oligoclonal rather than polyclonal, although our analysis cannot exclude that the oligoclonality derives from the concomitant MN1-induced MPD in these transplanted mice. Irrespective of whether the MN1/inv(16) disease is monoclonal or oligoclonal, the finding that it is not polyclonal strongly suggests that additional mutations must occur during AML development in the transplanted mice. Known candidate genes are *Plag1* and *PlagL2*, which were identified as *Cbfb-MYH11*cooperating genes in mouse inv(16) AML,[28] and were found to be upregulated in human inv(16) AML.[16] We do not think *Plag1* or *PlagL2* are transcriptional targets of MN1 because the mRNA levels of these genes were not increased in QRT–PCR analysis of MN1 overexpressing mouse cell lines, MN1 MPD BM or inv(16)/MN1 BM. The same holds true for *PLAGL2* in inv(16) leukemia as we did not find any correlation between the levels of *PLAGL2* and *MN1* expression in our patient samples (Supplementary Figure 1C), of which only one showed increased expression of *PLAGL2*. We also investigated the mutation status of the *Npm* gene in BM of the three MN1 MPD mice shown in Figure 3c and the three inv(16)/MN1 AML mice shown in Figure 4c. Mutation in codons 288 or 290 of NPM causes relocalization of the protein from the nucleolus to the cytoplasm in 35% of AML patients,[45] which affects the p53 tumor suppressor pathway activity.[46] Sequencing of PCR-amplified *Npm* cDNA of these eight mice showed that the gene was not mutated (not shown).

Given the cooperation between MN1 overexpression and CBF*β*–SMMHC in inv(16) AML, interference with MN1 function might provide a novel therapeutic approach for this CBF leukemia. In addition, it will be interesting to determine whether MN1 overexpression similarly promotes leukemia development in a mouse model for t(8;21) AML, which also targets the Cbf transcription factor. On the other hand, *MN1* overexpression is much higher in inv(16) AML than in t(8;21) CBF leukemia, opening the possibility that the products of these two chromosome translocations function quite differently despite the fact that they target the same transcription factor complex.

Together our experiments suggest that the differentiation-inhibiting protein Cbf*β*–SMMHC and the proliferation-stimulating protein MN1 cooperate in the development of AML in this mouse model. Given that *MN1* expression is upregulated in all inv(16) patients investigated (Ross *et al.*[15] and Valk *et al.*[16]), it is reasonable to speculate that these two proteins play a similar cooperative role in human inv(16) AML.

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Supplementary information accompanies the paper on the Leukemia Web site (http://www.nature.com/leu)