Functional analysis of the NUP98-CCDC28A fusion protein

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Funding: this work was supported by grants from INSERM, Association pour la Recherche sur le Cancer (ARC) and Institut National du Cancer (INCa). AP was subsequently a recipient of ARC and Fondation pour la Recherche Médicale (FRM) fellowships. GS was a recipient of a FRM fellowship. CR was a recipient of a fellowship from the FRM and the Société Française d'Hématologie (SFH).

Manuscript received on May 17, 2011. Revised version arrived on July 1, 2011. Manuscript accepted on August 16, 2011.

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

The nucleoporin gene *NUP98* is rearranged in more than 27 chromosomal abnormalities observed in childhood and adult, *de novo* and therapy-related acute leukemias of myeloid and T-lymphoid origins, resulting in the creation of fusion genes and the expression of chimeric proteins. We report here the functional analysis of the NUP98-coiled-coil domain-containing protein 28A (NUP98-CCDC28A) fusion protein, expressed as the consequence of a recurrent t(6;11)(q24.1;p15.5) translocation.

Design and Methods

To gain insight into the function of the native *CCDC28A* gene, we collected information on any differential expression of *CCDC28A* among normal hematologic cell types and within subgroups of acute leukemia. To assess the *in vivo* effects of the *NUP98-CCDC28A* fusion, *NUP98-CCDC28A* or full length *CCDC28A* were retrovirally transduced into primary murine bone marrow cells and transduced cells were next transplanted into sub-lethally irradiated recipient mice.

Results

Our *in silico* analyses supported a contribution of *CCDC28A* to discrete stages of murine hematopoietic development. They also suggested selective enrichment of *CCDC28A* in the French-American-British M6 class of human acute leukemia. Primary murine hematopoietic progenitor cells transduced with *NUP98-CCDC28A* generated a fully penetrant and transplantable myeloproliferative neoplasm-like myeloid leukemia and induced selective expansion of granulocyte/macrophage progenitors in the bone marrow of transplanted recipients, showing that *NUP98-CCDC28A* promotes the proliferative capacity and self-renewal potential of myeloid progenitors. In addition, the transformation mediated by *NUP98-CCDC28A* was not associated with deregulation of the *Hoxa-Meis1* pathway, a feature shared by a diverse set of *NUP98* fusions.

Conclusions

Our results demonstrate that the recurrent *NUP98-CCDC28A* is an oncogene that induces a rapid and transplantable myeloid neoplasm in recipient mice. They also provide additional evidence for an alternative leukemogenic mechanism for *NUP98* oncogenes.

Key words: NUP98 fusions, T-ALL, mouse model.

Citation: Petit A, Ragu C, Soler G, Ottolenghi C, Schluth C, Radford-Weiss I, Schneider-Maunoury S, Callebaut I, Dastugue N, Drabkin HA, Bernard OA, Romana S, and Penard-Lacronique V. Functional analysis of the NUP98-CCDC28A fusion protein. Haematologica 2012;97(3):379-387. doi:10.3324/haematol.2011.047969

Introduction

NUP98 (11p15.4) encodes two proteins, NUP98 and NUP96, which are constituents of the nuclear pore complex. NUP98 is dynamically associated with the nuclear pore complex and mediates the nucleocytoplasmic trafficking of macromolecules. Additional NUP98 nuclear functions, linked to the control of euploidy[1] and transcription,[2] have been described. The early embryonic lethality associated with disruption of the *Nup98* gene in mice has precluded elucidation of this gene's functions in normal hematopoiesis.[3]

The *NUP98* gene lies at the breakpoint of chromosomal translocations responsible for the expression of hybrid genes in human hematologic malignant diseases. (reviewed in[4]). NUP98 fusion partners frequently encode homeodomain transcription factors, including both class I (*HOXA9*, *A11*, *A13*, *C11*, *C13*, *D11*, *D13*) and class II (*HHEX*, *PRRX1/PMX1* and *PRRX2/PMX2*) homeogenes. As a result, these chimeric proteins contain the NUP98 glycine-leucine-phenylalanine-glycine (GLFG)-repeats fused to the HOX DNA binding domain and act as aberrant transcription factors.[5-7] NUP98 partners may also encode chromatin structure regulators, such as HMGB3,[8] MLL,[9] NSD1, NSD3/WHSC1L1, SETBP1,[10] JARID1A/KDM5A/RBP2,[11] PHF23,[12] TOP1, TOP2, DDX10 and LEDGF/PSIP1/p75. Several of these are involved in the control of *HOX* expression during normal development.[13-16] In line with this, up-regulation of HOX and HOX co-activators (MEIS1, PBX1/3) encoding loci has been reported in humans and mice with malignant diseases induced by *NUP98* fusions.[17-21] Similarly *HOX* expression signatures have been described for *MLL* fusions (reviewed in [22]), indicating that activation of these developmentally critical loci underlies the leukemogenic activity of both *NUP98* and *MLL* fusions. However *NUP98* fusions may also activate alternative oncogenic pathways that do not include deregulation of the *HOXA/MEIS1-PBX* genes[8,9]

Here, we investigated the leukemogenic potential of the *NUP98-CCDC28A* fusion expressed as the consequence of a recurrent t(6;11) translocation in T-cell acute lymphoblastic leukemia (T-ALL; this study) and acute myeloid leukemia.[23]

Design and Methods

The protocol was approved by the Committee on the Ethics of Animal Experiments of the *Institut de Cancérologie Gustave Roussy* (SCEA, Villejuif, France).

Donor sample

The clinical and cytogenetic data of the patient studied have been reported elsewhere.[4]

Constructs

Hemagglutinin-tagged forms of human *NUP98-CCDC28A* and *CCDC28A* cDNA were cloned into the retroviral vector, murine stem cell virus (MSCV)-neo (Ozyme, Saint Quentin Yvelines, France) using polymerase chain reaction (PCR)-mediated techniques. The long (L)-isoform of human CCDC28A was cloned into a pCMV-hemagglutinin coding for a N-terminal HA tag (Ozyme). The short (S)-isoform of human CCDC28A was obtained by deleting the 5'-terminal codons 1-90 of the L-isoform by site-directed mutagenesis (Quickchange™, Ozyme). The hemagglutinin-tagged *NUP98-CCDC28A* and *CCDC28A* were cloned into an MSCV-IRES-eGFP retroviral vector for bone marrow transplantation assays.

Immunostaining

HeLa or Plat-E cells were transiently transfected with DNA constructs using Lipofectamine™ 2000 (Invitrogen SARL, Cergy Pontoise, France) according to the manufacturer's instructions. Twenty-four hours after transfection, samples were fixed and stained using a mouse antibody against gamma-tubulin (Sigma, L'Isle d'Abeau Chesenes, France) and a rat antibody against hemagglutinin (Eurogentec France SAS, Angers, France).

Bone marrow transplantation and animal analysis

Viral supernatants were obtained as described previously.[24] Briefly, 6- to 8-week-old C57BL/6 donor mice were injected with 5-FU 5 days prior to bone marrow collection and primary bone marrow cells were collected from femora and tibiae. Lineage-negative (Lin^-) cells were collected with the BD™ Mouse Hematopoietic Stem and Progenitor Cell Isolation Kit (Becton Dickinson France S.A.S, Le Pont-De-Claix, France) and cultured in Stemspan medium (StemCell Technologies Inc., Grenoble, France) supplemented with 10% fetal bovine serum (StemCell Technologies Inc.) in the presence of interleukin-3 (10 ng/mL), interleukin-6 (10 ng/mL), FLT3-ligand (100 ng/mL), stem cell factor (100 ng/mL), thrombopoietin (2 U/mL) and interleukin-11 (10 ng/mL) (all from PromoCell GmbH, Heidelberg, Germany). Bone marrow Lin^- cells were mixed with viral supernatants 48 h and 72 h after harvesting and spinfected for 90 min at 1000g. After the second spinfection, 5×10^4 to 1×10^5 cells were injected into the retro-orbital veins of sub-lethally irradiated (4.5 Gy) C57BL/6 recipients.

Cytological and histological analyses

Blood samples were obtained from the retro-orbital sinus using heparinized micro capillaries. Peripheral blood cells counts were automatically measured with an MS-9 (Melet Schloesing Technologies, Osny, France) calibrated for mouse blood. Morphological analysis was done on smears and cytospin preparations stained with May-Grünwald-Giemsa. Specimens of spleen, liver, lung and kidney were fixed in formol-containing solution before being embedded in paraffin. Hematoxylin-eosin stained sections of tissues were evaluated using conventional staining techniques.

Clonogenic progenitor assays

Ten thousand Lin^- bone marrow cells transduced with the retrovirus were plated in 35 mm Petri dishes in M3434 methylcellulose (StemCell Technologies Inc.) and scored on day 7.

Cell staining, antibodies and flow cytometry

Cells were stained using the antibodies c-Kit, Sca1, Mac1/CD11b, Gr1, B220, CD19, CD8, CD4, Ter119, CD41, CD71 (BD Biosciences), GPIbα/CD42b (Emfret Analytics Gmbh, Würzburg, Germany) and CD34 (eBiosciences, San Diego, CA, USA). Data were acquired with a CyAn™ ADP flow cytometer (Beckman Coulter France S.A.S., Roissy, France) and analyzed with FlowJo software.

RNA, reverse transcription, quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted using the RNAble reagent (Eurobio, Courtaboeuf, France). Reverse-transcription was carried out with 4 μg of RNA using random hexamers and MMLV Reverse Transcriptase (Invitrogen SARL) according to the manufacturer's

instructions. The primers for *NUP98-CCDC28A* fusion transcript were sense NUP98 (5'-GCCCCTGGATTTAATACTACGA-3') and antisense CCDC28A (5'-AGCGCCTTTGCCCTCTCC-3'). For the reciprocal *CCDC28A-NUP98* fusion transcript the primers were sense CCDC28A (5'-TGCGGCGGTGGCTTCT-GA-3') and antisense NUP98 (5'-AACCATAACCTTTCCGAC-CAAT-3'). Reverse transcriptase PCR products were cloned and sequenced.

Real-time PCR was performed in triplicate on an ABI PRISM 7000 Sequence Detection System (Applied BioSystem) using the TaqMan Universal PCR Master Mix (Applied BioSystem, Courtaboeuf, France) and the following probes: *HoxA3* (Mm01326402_m1), *HoxA5* (Mm00439362_m1), *HoxA7* (Mm00657963_m1), *HoxA9* (Mm00439364_m1) and *HoxA10* (Mm00433966_m1). The relative expression of these genes was normalized to the expression of *Abl* (Mm00802038_g1).

In silico *expression analysis*

We used the Oncomine v. 4.3 commands available on-line (www.oncomine.org) to compare *CCDC28A* expression levels between each individual French-American-British (FAB) subgroup of acute myeloid leukemia and all the others by t-test (the reporter probe was Affymetrix U133A: 209479_at). The datasets are as follows: GSE1159, 293 samples,[25] GSE12417, 405 samples,[26] GSE14468, 526 samples.[27]

Results

Fusion of NUP98 *to* CCDC28A

The t(6;11)(q24.1;p15.5) translocation has been described in a T-ALL sample[4] and in an acute megakaryoblastic leukemia.[23] Our molecular studies demonstrated an in-frame fusion between the 13th exon of *NUP98* and the second exon of *CCDC28A*, as reported by others[23] (Figure 1A). A reciprocal *CCDC28A-NUP98* fusion transcript was detected but is likely devoid of biological activity due to the lack of a predicted fusion protein.

The human CCDC28A *gene encodes for two putative protein isoforms*

Reverse transcriptase PCR analysis of a panel of cDNA from human tissues demonstrated ubiquitous expression of *CCDC28A* (also known as *C6orf80* and *MGC131913*) (*not shown*). *CCDC28A* coding sequences predicted a 274 amino-acid protein (e.g., Genbank accession NP_056254) whose last 184 amino acids are well conserved in all vertebrates. An internal start codon (methionine labeled with "#" in Figure 1C) may be used to translate this protein species. This region showed 93% amino acid identity with the murine protein (NP_659069) and possesses an approximately 100 amino acid-long predicted coiled-coil (CC) motif that is also observed in several of the NUP98 partner proteins.[4,28] In contrast, the first 90 N-terminal amino acids of the predicted human CCDC28A protein are poorly conserved across species, even though they share the characteristics of a globular domain (~1/3 strong hydrophobic amino acids). We, therefore, concluded that the human cDNA may code for two protein isoforms, one that spans 184 amino acids and is well conserved in evolution [the «short» (S)-isoform], and a larger one that would span 274 amino acids because of an extended N-terminus [«long» (L)-isoform]. The sequence of CCDC28A protein did not reveal obvious functional roles, and no well-characterized motifs were detectable apart from the CC domain.

In the human genome, *CCDC28A* is related to *CCDC28B* (*coiled-coil domain-containing protein 28B)/MGC1203*) located on 1p35.1 and the two proteins align unambiguously (50% amino acid identity; Figure 1B). CCDC28B bears no recognizable motifs and its functions are unknown, but it co-localizes with Bardet-Biedl syndrome proteins at peri-centriolar structures.[29] *MGC1203* mutations contribute epistatic alleles to Bardet-Biedl syndrome, an inherited oligogenic disease associated with basal bodies and cilia disorders.[29]

Misregulation of CCDC28A *is associated with a subset of human acute leukemias*

Because the *NUP98-CCDC28A* gene fusion was observed in both acute megakaryoblastic leukemia[23] and T-ALL (this study) samples, we investigated whether CCDC28A expression may be associated with specific subgroups of acute leukemia. Indeed, our analysis of microarray data showed that *CCDC28A* is more strongly expressed in T-ALL samples associated with *MLL* internal duplications than in other leukemias. The *CCDC28A* levels in T-ALL with *MLL* were significantly higher than in any other group ($P<0.01$, two-tailed z-test) although the difference with the group that included pediatric leukemias with normal karyotype or complex/incompletely characterized chromosomal rearrangements was barely significant (*Online Supplementary Figure S1A*, data from Ross *et al.*[30]). Our Oncomine analysis also showed selective enrichment for *CCDC28A* in the FAB-M6 class in three publicly available datasets: fold-ratios were 1.8,[25] 1.5[26] and 1.4,[27] and *P*-values were 0.040, 0.062, and 0.014, respectively (two-tailed t-test, M6 *versus* M0-M5) (*Online Supplementary Table S1*). One dataset[26] contained only leukemias with normal karyotype. This suggests a specific role for *CCDC28A* in leukemias involving the erythroid lineage. We found no association between *CCDC28A* expression levels and survival by Cox proportional hazards regression using the dataset including survival data for the patients.[26] To gain insight into the function of the native *CCDC28A* gene, we also collected information on any differential expression for murine *CCDC28A* among normal hematologic cell types in available microarray datasets from mice and found that *CCDC28A* was enriched in hematopoietic stem cells, common lymphoid progenitors and naive T- and NK cells compared to other progenitors or differentiated cell types (*Online Supplementary Figure S1B*), supporting a role for *CCDC28A* in hematopoietic development.

The NUP98-CCDC28A fusion protein has a predominant nuclear localization

We next analyzed the subcellular localization of the NUP98-CCDC28A fusion protein. The hemagglutinin-tagged NUP98-CCDC28A S- and L-isoforms of CCDC28A were investigated in transient transfection assays in murine NIH3T3 fibroblasts. Immunofluorescence showed that the fusion protein was expressed predominantly in the nucleus whereas S- and L-CCDC28A were located in both the cytoplasm and nucleus (Figure 1D). Co-staining with an anti-gamma tubulin antibody did not reveal a centrosome localization for CCDC28A in contrast to CCDC28B.[29]

The expression of NUP98-CCDC28A *enforces the proliferation of primary bone marrow cells*

To assess the *in vivo* effects of the *NUP98-CCDC28A* fusion, *NUP98-CCDC28A* or full length *CCDC28A* were

retrovirally transduced into primary bone marrow cells derived from C57Bl/6 mice using a MSCV. Unlike bone marrow-derived primary murine progenitors transduced with an empty MSCV vector or *CCDC28A*, progenitors transduced with *NUP98-CCD28A* showed serial replating activity in methylcellulose colony-forming assays (Figure 2A) and were able to be propagated for several months in liquid culture. Subsequent cultivation in medium supplemented with only serum yielded *NUP98-CCDC28A*-immortalized progenitors that proliferated in a cytokine-

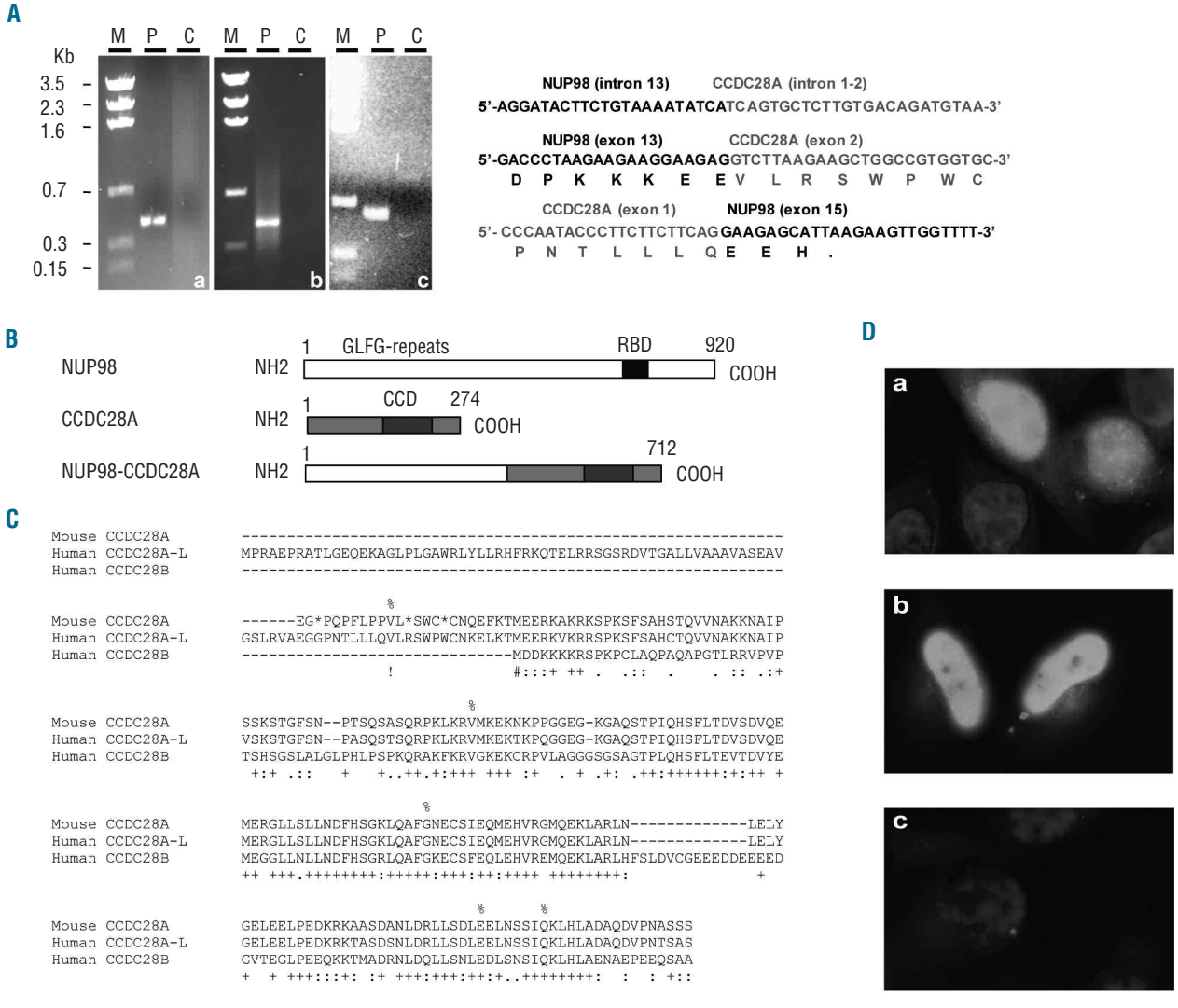


Figure 1. The t(6;11)(q24.1;p15.5) translocation fuses *NUP98* to *CCDC28A* and leads to the expression of a NUP98-CCDC28A protein localized to the nucleus. (A) Nucleotide and amino acid sequences around the *NUP98-CCDC28A* fusion junction. A specific PCR product of 503 bp was obtained using the DNA from the patient's sample (P) but not from control genomic DNA (C) (panel a); the fusion joins the nucleotide (Nt) 62503 of *NUP98* to the Nt 653 of *CCDC28A*. RT-PCR experiments performed on RNA extracted from the leukemic sample (P) show the amplification of two specific 444 bp and 612 bp products, corresponding respectively to the *NUP98-CCDC28A* (panel b) and reciprocal *CCDC28A-NUP98* (panel c) fusion transcripts. The nucleotide sequence of the *NUP98-CCDC28A* transcript shows an in-frame fusion, joining the Nt 1833 of *NUP98* to the Nt 384 of *CCDC28A*. The reciprocal *CCDC28A-NUP98* transcript joins the Nt 383 of *CCDC28A* to the Nt 2022 of *NUP98* but harbors a non-sense codon. M, molecular weight markers. (B) Schematic representation of the NUP98, CCDC28A and NUP98-CCDC28A human proteins. Identified domains [GLFG-repeats; RNA binding domain (RBD)] and the predicted coiled-coil domain (CCD) are indicated. The chimeric exon-exon boundary joins NUP98 to amino acid position 77 of the putative L-isoform, leading to a 712 amino acid-long fusion protein. (C) Clustal-W alignment of the predicted proteins for mouse CCDC28A (NP_659069), human CCDC28A (NP_056254) and for human CCDC28B (NP_077272). The coding potential of the mouse cDNA for *CCDC28A* is extended N-terminal of the first methionine, in order to show partial alignment for a short segment along with three in-frame stop codons (*). Human CCDC28A is labeled with an "-L" suffix to indicate the putative long isoform (see text). The symbols placed below the alignment are as follows: "!", first amino acid of the sequence from CCDC28A that is joined to NUP98 in leukemia; "#", first methionine for the "S-isoform" of human CCDC28A and for the two other proteins; "+", amino acid positions that are fully conserved in the three proteins; ":" and ".", amino acid positions with decreasing degrees of partial conservation. Above the alignment, the "%" symbols indicate exon-exon junctions for CCDC28A. In the murine CCDC28A protein, a short orthologous segment can be recognized upstream of the conserved methionine present in the human protein but contains in-frame stop codons. (D) Immunocytochemistry with an anti-hemagglutinin antibody detecting the nuclei of murine fibroblasts transiently transfected with constructs encoding the hemagglutinin-tagged NUP98-CCDC28A fusion protein (panel a) and S-isoform of CCDC28A (panel b); panel c is the negative control.

independent manner and exhibited myeloblast morphology and c-Kit expression. These results suggest that expression of *NUP98-CDC28A* enforces cellular proliferation and may also interfere with myeloid differentiation.

The expression of NUP98-CCDC28A *in a murine bone marrow transplantation model rapidly causes fatal myeloproliferative neoplasms*

Transduced primary bone marrow cells were next transplanted into sub-lethally irradiated recipient mice. In keeping with the results of *in vitro* experiments, *NUP98-CCDC28A* showed a strong transforming potential in mouse adoptive transfers since all animals that were transplanted with *NUP98-CCDC28A*-transduced cells (n=20) succumbed within 32 weeks after transplantation with an average post-transplant lifespan of 119 days (Figure 2B). The transforming potential of the retrovirally expressed *CCDC28A* was also evaluated but none of the engrafted mice developed leukemia (Figure 2B). Southern blot analyses performed on genomic DNA indicated the presence of the provirus in bone marrow samples of all transplanted mice and showed the monoclonal or oligoclonal nature of the *NUP98-CCDC28A*-induced proliferations in malignant samples (*Online Supplementary Figure S2A*).

Although the incidence of leukocytosis and neutrophilia varied among individual mice, *NUP98-CCDC28A* mice consistently showed a severe anemia and thrombocytopenia (Figure 2C) and an increase in immature/blasts myeloid cells in the bone marrow, spleen and peripheral blood when compared to control animals. Blood smears revealed the presence of circulating myeloid (granulocytic/monocytic) precursors as well as complete maturation of myeloid forms to segmented neutrophils (Figure 2D, panel b). Bone marrow cytology confirmed the presence of immature myeloid cells with minimal myeloid maturation and the disappearance of the erythroid compartment (Figure 2D, panel d). Upon necropsy, all *NUP98-CCDC28A* mice exhibited hepatosplenomegaly (Figure 2C). Histological analysis revealed severe disruption of spleen architecture when compared to that of *CCDC28A*- or MSCV-expressing mice (Figure 2D, panel e). Evidence of extramedullary hematopoiesis was observed in the liver (Figure 2D, panel f) and lung (Figure 2D, panel h), including perivascular infiltrations with myeloid cells. The percentage of immature forms/blasts in blood was less than 20%. Regarding the Bethesda classification proposals described by Kogan *et al.*,[31] we concluded that ectopic expression of *NUP98-CCDC28A* in hematopoietic stem cells and progenitors induced a myeloproliferative neoplasm-like myeloid leukemia. We also observed mouse lesions resembling myeloid leukemias with maturation, e.g., in which the neoplastic cells were moderately differentiated and neutrophilic (*not shown*). The fact that most *NUP98-CCDC28A*-induced myeloproliferative neoplasms did not evolve to acute myeloid leukemia suggests that *NUP98-CCDC28A* exerts a prominent effect on cellular growth and a weaker effect on differentiation.

To assess the malignant nature of the disease, we transplanted bone marrow cells from *NUP98-CCDC28A* primary recipients into sub-lethally irradiated wild-type secondary mice. All recipients (n=8) rapidly developed myeloid leukemias, which led to death at 7 weeks after transplantation (Figure 2B). Blood and bone marrow cytological analyses revealed overt myeloid leukemias with more than 20% of circulating blasts present in the blood and a massive invasion of the bone marrow (*not shown*). The transplantability and the rapid lethality in both primary and secondary recipients demonstrate the potent leukemogenic potential of NUP98-CCDC28A.

The bone marrow of NUP98-CCDC28A*-transduced mice is enriched in granulocyte/macrophage progenitors*

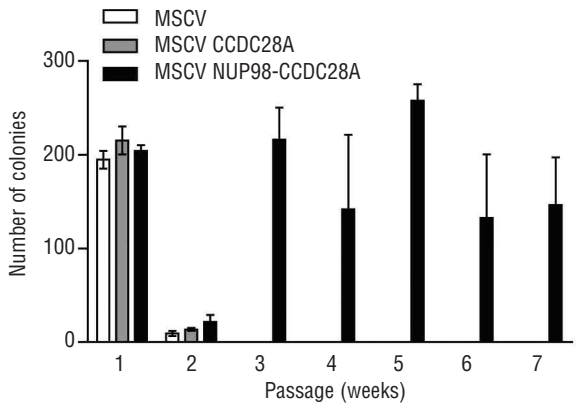
In line with cytological data, flow cytometric analysis of bone marrow cells from *NUP98-CCDC28A* moribund mice revealed a marked increase in the proportion of myeloid cells with monocytic and neutrophilic components when compared to MSCV-transduced counterparts (*Online Supplementary Figure S3*). Myeloid expansion was associated with lymphocytopenia and concomitant reduced erythropoieisis and enhanced megakaryopoiesis in the bone marrow (*Online Supplementary Figure S4*). The enhanced megakaryopoiesis correlated with an elevated number of mature megakaryoblasts observed by histological analyses of *NUP98-CCDC28A* mice spleens (Figure 2D, panel e). *NUP98-CCDC28A* leukemic mice also displayed significant infiltration of the spleen and thymus, with the cellular composition of these hematopoietic tissues reflecting those of the bone marrow (*not shown*).

To define the *NUP98-CCDC28A*-induced leukemias more precisely, FACS analyses were performed on bone marrow stem and progenitor cells phenotypically defined as $Lin^-Sca1^+c\text{-}kit^+$ and $Lin^-Sca1^-c\text{-}kit^+$ subsets, respectively. Analyses showed a selective expansion of a myeloid progenitor population enriched for myelo-monocytic progenitors (GMP)[32] while other progenitors (e.g. common myeloid progenitors and megakaryocytic/erythroid progenitors) were virtually absent (Figure 3). Interestingly, the prevalence of the GMP compartment in leukemic *NUP98-CCDC28A* bone marrow cells was reminiscent of that described for some *MLL* fusion-associated myeloid leukemias.[33] When compared to normal, the leukemic bone marrow populations showed a marked decrease in the frequency of the $Lin^-Sca1^+c\text{-}kit^+$ subset that encompasses multi-potent progenitors, and long-term and short-term hematopoietic stem cells (Figure 3). This indicates that *NUP98-CCDC28A* expression does not enforce the expansion of stem and primitive progenitor cells.

NUP98-CCDC28A *expression is not associated with strong* HoxA *and* Meis1 *expression*

We next addressed the question of *HoxA* expression in *NUP98-CCDC28A* neoplasms. Quantitative reverse transcriptase PCR experiments were performed on whole bone marrow cells isolated from *NUP98-CCDC28A*-transduced mice and compared to their *CCD28A*- and MSCV-transduced counterparts. Bone marrow cells from sick *NUP98-HoxA9*-transduced mice were used as a positive control, and cells transduced with an oncogenic form of the thrombopoietin receptor, MPL^{T487A},[34] were used as a negative control. We found weaker expression of *HoxA5*, *HoxA7* and *HoxA9* in the *NUP98-CCDC28A* bone marrow cells compared to their *NUP98-HoxA9* counterpart, whereas *HoxA10* showed similar levels. While *HoxA9* and *Meis1* were concomitantly misregulated in the *NUP98-HoxA9* samples, *NUP98-CCDC28A* bone marrow cells retained wild-type levels of *Meis1*, suggesting that the *NUP98-CCDC28A*-mediated transformation does not involve the canonical Hoxa-Meis1 pathway. We did not observe a concomitant up-regulated expression of the Pbx1 Hox

A



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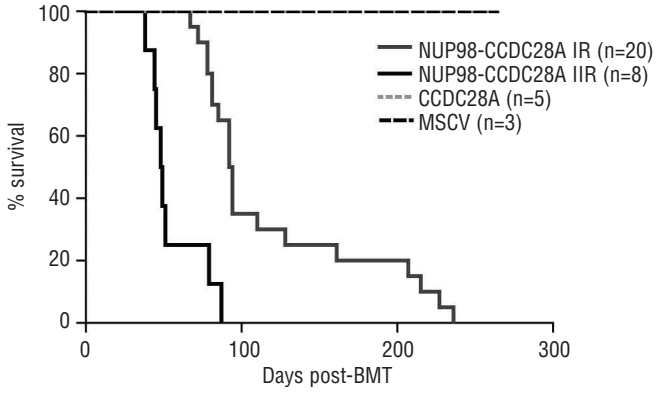

C

	NUP98-CCDC28A IR (n=20)	NUP98-CCDC28A IIR (n=8)	CCDC28A (n=5)	MSCV (n=3)
GFP (%)	80±8	93±6	2±4	10±9
WBC (x10^9/L)	67±49	110±72	7±3	8±2
Hemoglobin (g/dL)	6±2	7±3	13±2	13±1
Platelets (x10^9/L)	60±39	184±170	635±149	755±354
Spleen weight (mg)	783±238	585±91	150±75	90±13

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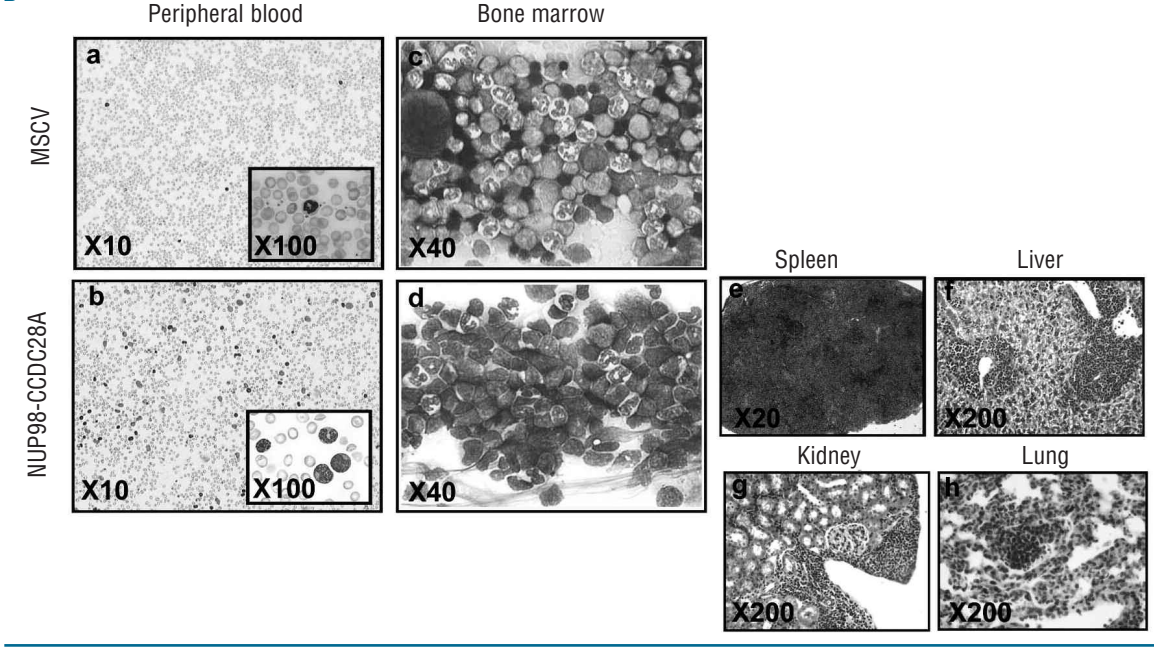

Figure 2. *NUP98-CCDC28A* expression induces a myeloproliferative neoplasm-like myeloid leukemia. (A) Clonogenic progenitor assays. Expression of *NUP98-CCDC28A* in primary bone marrow progenitors resulted in their enhanced proliferation *in vitro*. Colonies were scored every week and replated in secondary cultures. The mean number of colonies per round of replating of three independent replicates is indicated. The error bars indicate standard deviation (SD). (B) Kaplan-Meier survival curve of mice transplanted with bone marrow progenitors transduced with *NUP98-CCDC28A* (n=20), *CCDC28A* (n=5) or the vector alone (n=3). Primary *NUP98-CCDC28A* recipients (indicated as NUP98-CCDC28A IR) showed a 100% death rate at day 236 (7.8 months). Animals transduced with the *CCDC28A* or the MSCV vector alone were sacrificed for end-point analysis without evidence of disease. *NUP98-CCDC28A* secondary recipients (NUP98-CCDC28A IIR, n=8) succumbed between days 38 and 87 post-transplant. (C) Blood counts of primary *NUP98-CCDC28A*-transduced recipients (NUP98-CCDC28A IR) showed hyperleukocytosis, severe anemia and thrombocytopenia. These abnormalities were also observed in second recipients (NUP98-CCDC28A IIR) while *CCDC28A*- and MSCV-transduced mice showed normal blood count parameters. Spleen weights from primary transplanted mice are indicated. Values shown are mean ± SD. (D) Blood cytology and tissue histology of representative *NUP98-CCDC28A* and MSCV mice. Peripheral blood smears show anemia, thrombocytopenia and hyperleukocytosis for primary NUP98-CCDC28A animals (panel b) when compared to MSCV animals (panel a). In the former, maturation of myeloid forms to segmented neutrophils was observed (May-Grünwald-Giemsa staning, x100). Cytological analysis of bone marrow cells, evaluated on May-Grünwald-Giemsa staining of cytospin preparations, shows an over-representation of mature myeloid cells in *NUP98-CCDC28A*-engrafted mice (panel d) when compared to control animals (panel c) (May-Grünwald-Giemsa staining, x40). Histological analysis of the spleen (panel e), liver (panel f), kidney (panel g) and lung (panel h) of primary mice transplanted with bone marrow progenitors transduced with *NUP98-CCDC28A*, shows accumulation of myeloid precursors and destruction of normal organ architecture (hematoxylin and eosin, x20 and x200).

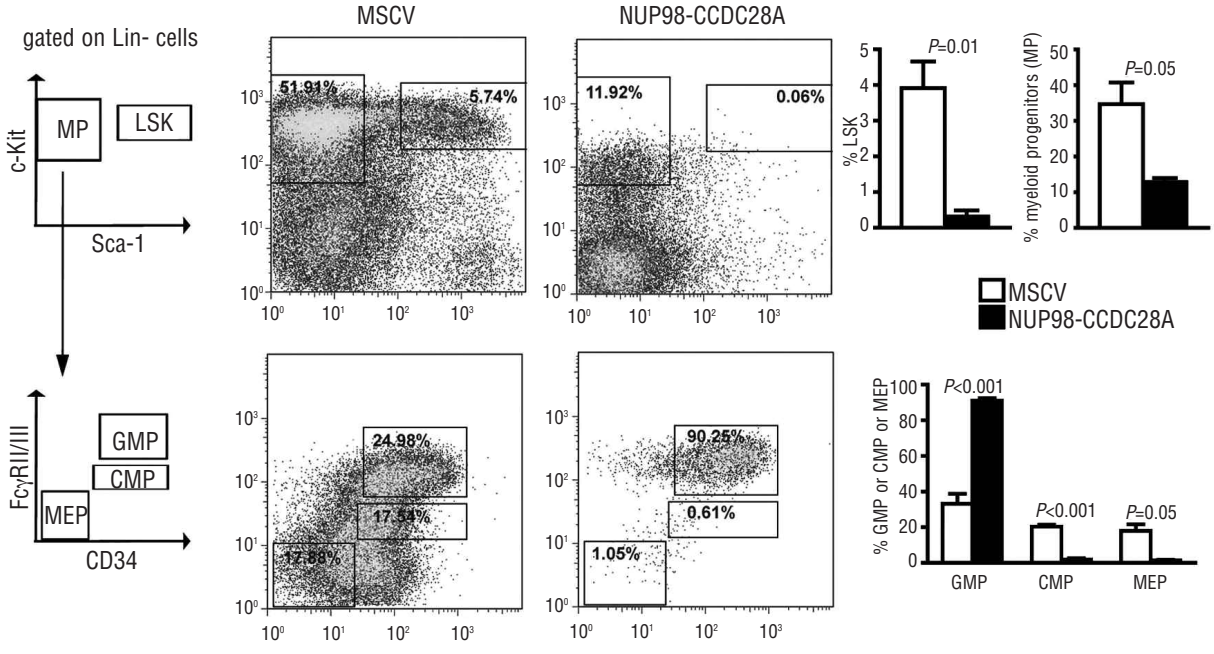

Figure 3. Leukemic cells from *NUP98-CCDC28A* mice are enriched in granulocytic-monocytic progenitors. Representative FACS profile of immature progenitors immunophenically defined as LSK (Lin$^-$Sca1$^+$c-Kit$^+$) and myeloid progenitors (MP, Lin$^-$Sca1$^-$c-Kit$^+$) in the bone marrow of *NUP98-CCDC28A*-engrafted mice. FACS analysis of Lin$^-$ cells shows the distribution of MP in the bone marrow of leukemic animals and specific expansion of a population immunophenotypically defined as granulocytic-monocytic progenitors (GMP), at the expense of the common myeloid progenitors (CMP) and megakaryocyte-erythroid progenitors (MEP) populations. A profile of bone marrow cells from mice transplanted with control-transduced progenitors shows typical GMP, CMP and MEP populations. A major reduction in LSK cells is observed in the bone marrow of *NUP98-CCDC28A*-engrafted mice. Histograms show the percentages of indicated cells in the bone marrow from leukemic and control mice (right panels). Values shown are mean ± standard error of the mean (SEM) (n=5 mice per group, Mann Whitney test).

cofactor, and *Pbx3* expression was only slightly increased in *NUP98-CCDC28A* samples. Collectively, these results indicate that *NUP98-CCDC28A* did not strongly affect the expression of *Hox* genes in hematopoietic cells, and that HoxA and Meis1 are not critical downstream mediators of the *NUP98-CCDC28A*-mediated oncogenic program. As *NUP98-CCDC28A*-expressing human blast cells did not show up-regulation of these single genes (*data not shown*), we infer that *NUP98-CCDC28A*-transformation is unlikely to involve global *HOX* gene up-regulation.

Discussion

We have confirmed that *CCDC28A* is a recurrent chromosomal translocation partner of *NUP98* in acute leukemia. In addition to the t(6;11) translocation studied here, five *NUP98* fusions have been reported in T-ALL (*NUP98-ADD3*, *NUP98-IQCG*, *NUP98-RAP1GDS1*, *NUP98-SETBP1* and *NUP98-LNP1*) whose contribution to the leukemogenic process is still unknown. Except for CCDC28B, the native CCDC28A protein has no recognizable similarity to other proteins or functional domains, and no function has so far been assigned to the coiled-coil domain, leaving the biological function of CCDC28A undetermined. The pattern of expression of the gene within hematopoietic lineages does, however, suggest that it contributes to discrete stages of hematopoietic development. We showed here that enforced *NUP98-CCDC28A* expression promoted the proliferative capacity and self-renewal potential of murine hematopoietic progenitors and rapidly induced fatal myeloproliferative neoplasms and defects in the differentiation of the erythro-megakaryocytic lineage. Our *in silico* analyses also suggested *CCDC28A* misregulation in human myeloid leukemias, specifically those of the FAB-M6 subgroup, suggesting that *CCDC28A* expression could be critical for normal myeloerythroid progenitor cell function. Although the leukemogenic mechanism remains unknown, NUP98-CCDC28A retains the NUP98 GLFG-repeats able to associate with core binding protein and/or p300 and has a nuclear localization that suggests possible transactivation activity. Several mechanisms may cooperate in dysregulated transcription, as NUP98 fusions also interfere with nucleocytoplasmic trafficking. Indeed, both NUP98-HoxA9 and NUP98-DDX10 impair the nuclear export of critical transcriptional regulators, leading to their aberrant nuclear retention and enhanced transcription from responsive promoters.[35] The functional significance of deregulated expression of *Hox* genes has been suspected in the oncogenic processes of some[17-21] but not all[8,9] NUP98 fusion proteins. Although the expression of *HoxA* genes was sustained in *NUP98-CCDC28A*- expressing leukemic cells, this may be related to the enrichment for immature myeloid cells in these populations compared to controls. Indeed, much higher transcript levels were measured in the *NUP98-HOXA9* samples compared to *NUP98-*

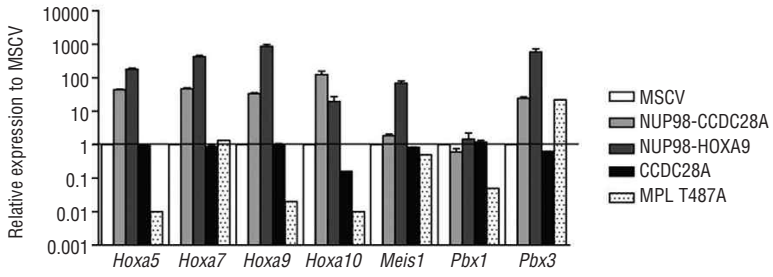

Figure 4. NUP98-CCDC28A leukemic cells do not overexpress *HoxA* genes. Real-time reverse transcriptase-PCR analysis of transcript levels of *HoxA5*, *HoxA7*, *HoxA9*, *HoxA10*, *Meis1*, *Pbx1* and *Pbx3* genes in the bone marrow cells from primary NUP98-CCDC28A-engrafted animals and their CCDC28A- and MSCV-transduced conterparts. Accumulation of transcript is quantified in primary recipients compared to *NUP98-HOXA9* and *MPL^{T487A}* recipients, respectively used as positive and negative controls of dysregulated expression of *HoxA* genes. Expression levels are normalized to *Gapdh* and results are expressed relative to the level of each gene in MSCV-engrafted mice (set at 1) (n=3 per genotype). Values shown are mean ± SD from two independent experiments.

CCDC28A, and the expression level of *Meis1* of the latter was close to controls. This suggests that strongly misregulated expression of *HoxA/Meis* is not a prevailing event in NUP98 fusion oncogenesis. The model reported here will help to dissect gene pathways involved in myeloid transformation. Additional models will be needed to investigate the role of *NUP98-CCDC28A* in lymphoid transformation.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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